Endometrial Responses to Hormone Replacement Therapy

Thesis submitted for the degree of

Doctor of Philosophy

University of Leicester

by

Marwan A. Habiba

MB BCh, MSc, MRCOG

Department of Obstetrics and Gynæcology

University of Leicester

1998

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Dedication

to

my parents &
my wife

Acknowledgements

This research was carried in the Department of Obstetrics and Gynæcology, University of Leicester between 1992-1998.

I am grateful for the help and support of my supervisors Mr F Al-Azzawi and Dr S C Bell and the successive heads of the Department, Professor J MacVicar and Professor D J Taylor. I am grateful to Dr R James, and Mrs A Larkins from the Department of surgery for their help and patience during the generation of the monoclonal antibodies. I am also grateful for the excellent technical support of Miss J Royal, and Mrs S Spurling. I wish to thank Mrs J Jackson for her administrative support and Mrs B Phipps for her help with the clinical study.

Abbreviations

α_2PEG	Pregnancy-associated endometrial α_2-globulin
AP-1	Transcription factor activating protein-1
Average-BS	Average bleeding score
bv	Blood vessel
cHRT	Cyclical continuous combined hormone replacement therapy
dH_2O	Distilled water
EB	Early bleeder(s)
ECM	Extracellular matrix
ER	Œstrogen receptor
ERT	Œstrogen replacement therapy
EV	Œstradiol valerate
FL	Follicular phase
FMP	Final menstrual period
H&E	Hæmatoxylin and Eosin
HCG	Human chorionic gonadotropin
HDL	High density lipoprotein
hpf	High power field
HRT	Hormone replacement therapy
IHC	Immunohistochemistry
ISH	In-situ-hybridisation
IVF	In-vitro-fertilisation
IVF-ET	In-vitro-fertilisation & embryo transfer
LB	Late bleeder(s)
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LNG	Levonorgestrel
LP	Luteal phase
lpf	Low power field
MMP(s)	Matrix metalloproteinase(s)
MPA	Medroxyprogesterone acetate
NB	Non-bleeder(s)
NCS	Nuclear (nucleolar) channel system
OCP	Oral contraceptive pill
PA(s)	Plasminogen activator(s)
PAI-1	Plasminogen activator inhibitor-1
PI	Proliferation index
PLP	Pseudoluteal phase (progestogen phase of cHRT)
PR	Progesterone receptor
RT	Room temperature
SD	Standard deviation
TF	Tissue factor
TIMP(s)	Tissue inhibitor(s) of metalloproteinase
T/score	Total score
Total-BS	Total bleeding score
tPA	Tissue type plasminogen activator
uPA	Urokinase type plasminogen activator

Contents

Addenda

Habiba et al., Human Reproduction 1996,11(3);503-508.
Habiba et al., Human Reproduction 1998,13(6);1674-1682.

Chapter 1

General Introduction

The menopause which signals the end of the female reproductive life is associated with a gradual decline in health. This is most manifest in the increased risk of osteoporosis and of heart disease. The menopause is also a period of major social adjustment during which the woman experiences many psychological and psychosomatic symptoms, some of which have been linked to œstrogen deficiency. Many of the symptoms associated with the menopause respond to treatment by exogenous sex steroids, which are also effective in reducing the rate of the degenerative effects on the bone and blood vessels.

Taken together, the overall mortality rates are lower for women as compared to men with an obvious increase after the menopause, and this rising mortality rate has been interpreted to indicate an adverse health impact of the menopause *per se* (1).

1.1. Definitions

The following recommended definitions (2) will be used in this thesis:
- **Menopause:** is the permanent cessation of menstruation resulting from loss of ovarian follicular activity.
- **Perimenopause** (climacteric): is the period immediately prior to the menopause (when the endocrinological, biological and clinical features of approaching menopause commence), and the first year after menopause.
- **Menopause transition:** the period immediately prior to the menopause (when the endocrinological, biological and clinical features of approaching menopause commence) and till the final menstrual period (FMP).
- **Premenopause:** the whole reproductive life prior to the FMP.
- **Postmenopause:** commences after the FMP.

1.2. The biological significance of the menopause

The age at which natural menopuse occurs in women world-wide is between 45-55 years, and a woman who reaches that age will have a life expectancy

between 27-32 years (1, 3). Women past the menopause are faced with the age concerns of the elderly. Three of these are of particular importance:

1- Age related metabolic changes (as atherosclerosis, osteoporosis and diabetes).
2- Cardiovascular and cerebrovascular disease.
3- Malignancies.

The chronological association between the increased incidence of these conditions and the cessation of ovarian activity, has stimulated interest in a possible causative link. Although reliable statistics relating to overall and cause specific mortality is only available from developed countries, evidence suggest that the same general patterns apply elsewhere. Statistics from the UK and USA (Figure 1.1. & 1.2.) demonstrate the gradual increase in mortality rates in association with age and the higher rate of increased mortality after the age of menopause (4). The overall life expectancy of females is longer than that of males (Figure 1.3. & 1.4.), which suggests a biological advantage for females (5). The rise in cause specific mortality in females after the menopause suggests that this biological advantage may be, at least in part, related to factors that are operational in the premenopausal state, the most prominent of which are the functioning ovaries and their main steroid product: œstradiol. However, it is notable that despite the loss of œstrogen at the menopause, women retain a lower mortality rate compared to men.

1.3. The Menopause and ageing in the female

Advancing age is associated with adverse changes in many physiological functions, increased morbidity and mortality, and reduced reproductive capacity in both men and women. But contrary to the case in males, reproductive capacity in females is completely lost at menopause. As age advances women experience more anovulatory cycles, reduced fecundity, and a higher incidence of spontaneous abortion and congenital anomalies in the offspring, both of which are attributed to chromosomal anomalies in oocytes. It was suggested that reproductive capacity is a biological marker of ageing (6), and this was supported by the study of mortality in Seventh-Day Adventists (7), which demonstrated a positive link between the age at natural menopause and life expectancy. It was hypothesised that the menopause being consequent to depletion of ovarian follicles, is an index of ovarian ageing

which in turn is an index of an ageing body (7). In support of this, is the positive correlation between the duration of premenopause and longevity (Figure 1.5.) (8). This occurred despite the negative correlation between the age at menopause and the duration of postmenopausal life (8). Thus the relation between œstrogen deficiency and longevity is not unambiguously established.

1.4. The health risks in the postmenopause

1.4.1 Metabolic changes

The most important metabolic changes at the time of the menopause and postmenopause, are the effects on the bone resulting in osteoporosis, and on the vasculature leading to the development of atherosclerosis. Other degenerative conditions e.g. urogenital ageing will also manifest.

1.4.1.1. Osteoporosis

Is define as 'a disease characterised by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk' (9).

Bone mass reaches its peak in both sexes by the late teenage years (10), and this peak bone mass is equivalent in men and women after correction for body weight (11). After reaching this peak, bone loss commences in both sexes at an equivalent rate of 0.5-1% per year (12), till the first 5-10 years postmenopause when the rate of bone loss in women accelerates resulting in a loss of about 15% of the average bone density (12). The rate of bone loss subsequently reduces to parallel that in men, and continues albeit at a lower rate. The cause of the adverse effect on bone mass in postmenopausal women is subject for debate (13), but the majority attribute it to the loss of the protective effect of œstrogen (14).

1.4.1.2. Cardiovascular and Cerebrovascular disease

Data from industrial countries show that women of all ages, have a lower age specified cardiovascular and cerebrovascular mortality rates when compared to men. This apparent advantage diminishes with advancing ages to equal the rate in men by the age of 75 years. The increased rate of coronary heart

disease in postmenopausal women with advancing age compared to the rate in premenopausal women, has been taken as an indicator of a protective effect of œstrogen (15). Bilateral oophorectomy has also been shown to increase the risk of coronary heart disease (16).

1.4.1.3. Urogenital ageing

The female reproductive and lower urinary tracts contain œstrogen receptors (17), and both tracts are known to undergo atrophy upon withdrawal of œstrogen (18). The vaginal mucosa becomes thinner (19), and exfoliative cytology of the vagina demonstrates a predominance of basal and parabasal cells. The uterus and the cervix involute to about half the size in the premenopausal women (20). These atrophic changes manifest clinically with symptoms of dyspareunia, and may be contributory to the increased complaints of urgency, dysuria, nocturia and urinary stress incontinence reported by 25-50% of postmenopausal women (21).

1.4.2. Malignant tumours

Age specifies cancer mortality rate is higher in women in the early and mid reproductive years than it is in men. As is the case in men, the overall cancer mortality rate increases with age, but because of the higher rate of rise in men, a reversal takes place around the age of the menopause, when older men experience a higher mortality rate than women. This male/female disadvantage continues to widen into old age as exemplified in the statistics from the UK and USA (Figure 1.6.) (4). The female disadvantage during reproductive life is largely accounted for by the high rates of breast and cervical cancer (Figure 1.7.) (4).

1.4.3. Symptoms associated with the peri- and postmenopause

The menopause is not a universal experience of uncomfortable symptoms. On the contrary, it is uneventful for the majority of women (22). For symptomatic women the menopause is said to be characterised by thermoregulatory disturbances which manifest as hot flushes and night sweats, and the association was confirmed in prospective European and North American studies (23, 24). These symptoms may be responsible for the increased frequency of insomnia. The incidence of hot flushes, however, varies widely from 0% in Mayan women, 14.7% in Japanese women, 54% in South East

England and 80% in Holland (22, 25, 26). It is, however, difficult to explain these racial differences based on endocrinology, as women with a low incidence of flushes, have a similar endocrine profile (high FSH, low œstrone and œstradiol) and experience similar bone demineralisation as those with a high incidence of flushes (25).

A range of other psychological and psychosomatic symptoms, as headaches, depression, mood swings, etc. have been related to the menopause but none of these associations is specific (22).

1.5. Hormone replacement therapy (HRT)

Œstrogen levels fall gradually in the years preceding the menopause, with a sharp drop after the FMP (27). Œstrogen administration, coined 'œstrogen replacement therapy' (ERT), or combined œstrogen and progestogen replacement therapy (HRT), is designed to increase œstrogen levels in postmenopausal women aiming to prevent or to treat clinical features associated with the menopause.

Two main aspects distinguish HRT from other therapeutics; firstly, HRT is directed to healthy women with the aim of compensating for the effects of a biological process represented by the cessation of ovarian activity. Second, HRT is targeted at the whole population of postmenopausal women and is projected as having 'public health' objectives and thus commonly subjected to economic cost-benefit analyses (28, 29). This view of HRT assumes that the cessation of œstrogen production by the ovaries at the time of the menopause is a biological 'deficiency' in that it, *per se,* predisposes postmenopausal women to health risks. This view also assumes that this 'biological deficiency' can be compensated for by administering exogenous œstrogens in order to mimic the effect of ovarian œstrogen.

The use of the main ovarian endocrine product (œstrogen) has increased gradually over the past forty years. Initially used for treating menopausal symptoms and atrophic changes, the current indications include the treatment of menopausal symptoms as well as the prevention of osteoporosis (30, 31). There is also evidence that unopposed œstrogen therapy is protective against ischaemic heart disease and strokes (30, 32). It is estimated that 15% of women in Britain are currently using HRT (33). In the USA, where up to a

third of an upper middle class community aged between 50-65 are currently using HRT (34), the figure is even higher. This major expansion has been largely based on epidemiological studies that demonstrated a beneficial effects of œstrogen on bone mineral density and cardiovascular risk markers.

Although all the beneficial effects of HRT are linked to the œstrogenic component, unopposed œstrogen is suitable only for women who had a hysterectomy. In women with an intact uterus, progestogens are added to replacement regimens in order to avoid the increased incidence of endometrial carcinoma which was noticed with the use of unopposed œstrogen regimens (35). The most commonly used regimens contain continuous œstrogen and cyclical progestogens based on a 28 day cycle, but cyclic regimens based on longer cycles (bimonthly, trimonthly or quarterly) have been introduced. Continuous combined regimens containing a daily dose of œstrogen and progestogen, have recently been used as an alternative for those women who wish to avoid withdrawal bleeding.

1.6. Benefits of HRT

1.6.1. Cardiovascular disease

Evidence from epidemiological studies on the use of œstrogen replacement therapy support the hypothesis that postmenopausal œstrogen use is protective against cardiovascular disease, possibly by a factor of 50% (30), and the beneficial effect remains after adjusting for known cardiac risk factors (15). Studies have also identified possible mechanisms for a beneficial effect. These include lowering low density lipoprotein cholesterol (LDL) levels, increasing high-density lipoprotein cholesterol (HDL) levels (36-38), and arteriolar smooth muscle relaxation resulting in improving blood flow (39-41), and possibly mediated the production of vasodilators (42). Other proposed mechanisms include a favourable effect on insulin resistance (43) and on homocysteine metabolism (44). The known adverse effects of progestogens on the lipid profile (36) and on arterial dilatation and blood flow (45), have raised many questions about the magnitude of any possible cardioprotective effects of combined therapy, although the balance seems to be favourable (15, 30).

1.6.2. Osteoporosis

A cause and effect relationship is said to be established between œstrogen deficiency and osteoporosis (46). In all published studies, œstrogen has been shown to prevent postmenopausal bone loss and to reduce the risk of fractures (47, 48). C19 progestogens, such as norethisterone, also spare bone, and this effect may be additive (49). The effect of adding C21 progestogens is less clear but not adverse (50).

1.6.3. Menopausal symptoms

Double blind studies have demonstrated that hot flushes are significantly improved by œstrogen treatment (1, 51).

1.6.4. Urogenital Effects

Œstrogens are effective in reversing the atrophic changes in the urogenital tract which result from hormone deficiency (52).

1.7. Risks associated with the use of HRT

1.7.1. Side effects of treatment

Both œstrogenic and progestogenic side effects are noted with the use of HRT. Examples of the former include nausea, breast swelling and tenderness and increased vaginal discharge. Progestogenic side effects include bloating, weight gain, and symptoms of premenstrual tension (53).

1.7.2. Breast cancer

Long term use of HRT is associated with an increased risk of breast cancer, although estimates of the magnitude of this risk varied in different studies (54).

1.7.3. Thromboembolism

The risk of developing venous thromboembolic disease is increased by a factor of 2-4 in women who are current users of HRT (55-57).

1.8. Monitoring HRT

Little is known about the factors that any affect the efficacy of HRT. The paucity of knowledge may be partly attributable to the absence of an overall objective measurement of such efficacy, and the general assumption that œstrogen intake above the calculated minimum dose (2mg of œstradiol or 0.625mg conjugated equine œstrogen orally; or 50μg transdermally daily; or 50mg by implant every 6 months), is effective for bone protection in all women. Bone protection can be monitored over time by repeated bone densitometric measurements, but based on the assumption of uniformity of response above the minimum dose, monitoring is not routinely advised (58). However, this assumption may be inaccurate, as there is evidence that about 2-5% and 12-15% of women will continue to lose bone density from the spine and the proximal femur respectively, despite receiving doses of œstrogen that are thought to be effective bone conservers (52, 59)

Cardioprotection is even more problematic, as the changes induced by HRT in the levels of recognised cardiovascular risk markers vary widely. Furthermore, there is disagreement on the relative importance of each of these markers. The assessment of the relative efficacy of different regimens and of the same regimen on different individuals is therefore difficult (60), and the minimum dose of HRT required for cardiovascular protection remains unknown (61).

The choice of therapy has, so far, relied on dose adjustment in response to reported symptoms and/or side effects of a 'standard' œstrogen dose. But there is no data to show a correlation between serum œstrogen concentration and symptom control, or between serum œstrogen levels and bone response (58, 61). Furthermore the range of plasma œstradiol levels achieved with any dose of œstrogen may vary considerably between women (62). The lack of correlation between serum levels and clinical response, and the aforementioned assumption of uniformity of biological response are taken as evidence against the use of hormone levels as a guide (63), and in favour of symptom guided therapy. This method is not helpful in asymptomatic women, and may be misleading. It can be hypothesised that cardiovascular and bone protection may be - similar to symptomatic response - variable between individuals. As these three responses have not been found to correlate, symptomatic response cannot be taken as an index for adequate

cardiovascular or bone protection, this is particularly so if one takes into account the high response rate of symptoms to placebo (64).

One may reason that factors known to affect the efficacy of oral contraceptives may similarly influence HRT. These include drugs that affect liver function or decrease the ability of the body to absorb hormones as rifampin, phenytoin, carbamazepine, ampicillin or tetracycline or those that increases the level of œstrogen as vitamin C. Also, gastrointestinal factors as diarrhoea and vomiting or conditions like coeliac disease may affect intestinal absorption. Skin reaction may affect the absorption of transdermal preparations. But as most of these factors are short term or transient, they may not be significant in relation to the desired long term effects of HRT.

1.9. Factors affecting the acceptability of combined HRT

The effect of HRT on cardioprotection and preservation of bone mass can only be achieved after long term therapy, which is hindered by the low continuation rate (65). Factors that affect continuation of therapy include motivation, the presence and severity of symptoms of the menopause or of side effects.

1.9.1. Systemic factors

Many of the well recognised side effects of œstrogen and progestogen therapy are dose related, and some are regarded as 'physiological' effects of these hormones. Œstrogen related side effects include nausea, vomiting, cholestatic jaundice, bloatedness, fluid retention resulting in œdema, weight gain, breast tenderness, abdominal and leg pain, headache, migraine, depression, and dizziness. Progestogen related side effects include œdema and weight gain, skin pigmentation, chloasma, pruritus, premenstrual-like symptoms, change in libido and appetite, fatigue, loss of scalp hair, asthma, seizures, migraine, headache and depression (66). Of these, the most commonly reported causes for discontinuing therapy are breast tenderness, fluid retention, nausea and headache (67).

1.9.2. Local target factors

The addition of progestogens in hormone replacement regimens introduces monthly bleeding which many women regard as inconvenient. Withdrawal bleeding and irregular bleeding are the most common causes for non-continuance with therapy (65, 67, 68).

1.10. HRT and endometrial bleeding

The organ that exhibits the most obvious changes in response to œstrogen and progestogen is the endometrium.

1.10.1. The endometrium as a site of action of HRT

The first histological description of the cyclic changes in the endometrium, now recognised to reflect ovarian steroid action, was made by Hitchmann and Adler in 1908 (69). The histological changes which occur during the menstrual cycle are more pronounced than those occurring at any other site in the body, and have enabled the classification of the normal endometrium according to the duration of exposure to these steroids: endometrial dating (70). The endometrium also responds to administered hormones, e.g. the oral contraceptive pills (OCP), although the histological feature differ from those of the physiological cycle (69). Under the influence of œstrogen, the endometrium exhibits proliferative changes, and œstrogen primed endometrium reflects the influence of progestogen as secretory changes (71). It is also recognised that endometrial morphology reflects the relative potency of œstrogen and progestogen in combined preparations (72). Endometrial histological changes were suggested as a means for recommending preferable combinations of OCPs with improved 'hormonal balance' (73).

1.10.2 Endometrial bleeding as a physiological indicator of HRT action

Although formal evidence of a causal link is lacking, and no mechanism has been established at the molecular level, clinical evidence have linked unopposed œstrogen replacement therapy with an increased risk of endometrial hyperplasia and cancer (74-77). To guard against these developments, the current practice is to add a minimum of 10 days of progesterone to every 28 day treatment cycle (78), and the present evidence

suggested that this is effective in preventing both hyperplasia and carcinoma (77).

The addition of a cyclical progestin introduces a monthly bleed which simulates the menstrual cycle. This has been the focus of some studies, but only in relation to endometrial protection against cancer, and little research has been directed to the characteristics of this monthly bleed or to the factors that might influence it. Research into bleeding patterns was conducted with the objective of avoiding the need for endometrial biopsy. One study linked the bleeding pattern to the dose of hormones in HRT(79), and reported that the onset of bleeding on or beyond day 11 of the progestogen addition is an index of adequate progestogen dosage, and that the onset of bleeding on or before day 10 is an index of inadequate progestogen, but this was not confirmed (80).

This contrasts to the natural cycle where the relation between the ovarian and the endometrial cycle is well established. It is recognised that disturbance of the ovarian cycle will be reflected by abnormalities of menstruation and that endometrial histology reflects these disturbances e.g. anovulation, luteal phase defect, or persistent corpus luteum (81). Thus both endometrial biopsies and the bleeding patterns can be used for the evaluation of the endometrial and ovarian cycles, not only in relation to carcinoma but also for the diagnosis of functional disorders.

1.11. The histology of the endometrium

1.11.1. The menstrual cycle

The endometrium responds to ovarian hormones produced at puberty by proliferation and increase in thickness. The initial few cycles after menarche are often anovulatory, but the endometrium responds to progesterone as soon as ovulation is established. Subsequently the menstrual cycle follows the ovarian cycle. While adequate reproductive function is compatible with a range of physiological changes, the sequential endometrial morphological patterns are remarkably consistent (82). This consistency allows endometrial dating with a high degree of accuracy (70). The menstrual cycle can be divided into three phases; the proliferative, the secretory and the menstrual phase.

1.11.1.1. The proliferative phase

The wide variability in the duration of this phase (range=10-20 days, average=14 days) renders determining the exact day of the cycle impossible (69). For descriptive purposes, the proliferative phase is commonly divided into three stages; early (day 4-7), mid (day 8-10) and late (day 11-14) (considering the first day of bleeding as day 1). All stages are characterised by œstrogen induced proliferation reaching the maximum at day 8-10. In the early proliferative phase, the glands are narrow and tubular but gradually become more tortuous and distended in the late proliferative phase. The glandular epithelium proliferates and may appear pseudostratified and the whole endometrium increases in thickness from 1 to 10 millimetres. Surface and glandular epithelial cells become tall columnar and have sharply defined upper borders and sparse, short, and blunt microvilli.

During the proliferative phase, the stromal cells have little cytoplasm, and characteristic stromal œdema appears in the mid-proliferative phase in the functionalis layer (82).

- **The early proliferative phase** (day 4-7). At the beginning of this phase the thin basalis is fully epithelialised. The glands are sparse, narrow, and straight. The stroma is formed by loose spindle cells with dense chromatin and scanty cytoplasm. The superficial epithelium remains flat and the glandular epithelium is low columnar, with small oval nuclei and dense chromatin and no apparent nucleoli.

- **The midproliferative phase** (day 8-10). The endometrium increases in thickness due to proliferation and stromal œdema. The glands become tortuous and their lining epithelium becomes tall columnar with large oval nuclei, dense chromatin and apparent nucleoli. The stromal cells remain unchanged, spindle shaped with scanty cytoplasm. Interstitial œdema separates the stromal cells, which remain attached to a reticulum network. Mitosis is abundant in both glandular and stromal cells.

- **The late proliferative phase** (day 11-14). During this phase, stromal œdema regresses and this increases glandular tortuousity. Glandular epithelium continues to proliferate and forms into a pseudostratified layer. The nuclei enlarge but remain fusiform with multiple nucleoli, and the

cytoplasm becomes abundant. The epithelial cells acquire few basal glycogen granules at the end of this stage. With the regression of stromal œdema, the stromal cells appear compact, enlarge and acquire prominent nucleoli.

1.11.1.2. The secretory phase

This phase has a constant length (14±1 days), determined by the life span of the corpus luteum (83, 84), and is characterised by the accumulation of glycogen and glycoproteins in epithelial cells. Early signs of secretory activity begin in the late proliferative phase shortly before ovulation and may be triggered by progesterone derived from the adrenal cortex or from non-dominant follicles. Basal secretory granules increase in size after ovulation and displace the nuclei upwards. This being most pronounced at 3-4 days after ovulation. Subsequently secretory granules become displaced towards the lumen and the nuclei return to a basal position. Luminal secretions appear from the fifth or sixth postovulatory day, and apocrine secretions accumulate at the luminal borders which by the seventh postovulatory day, appear bosselated. Mitotic activity is inversely related to cellular differentiation and later becomes undetectable. There is little change in the stromal appearance during this phase.

The glands in the functionalis begin to involute with the demise of the corpus luteum. The luminal secretions inspissate or disappear and the distended glands collapse to produce the characteristic saw-toothed appearance. The epithelium degenerates and the luminal borders of the cells appear ragged. Apoptosis can be detected in the premenstrual or menstrual phases.

This involution contrasts with the progressive changes which continue in the stroma and the blood vessels. At about nine or ten days after ovulation, the spiral arteries and arterioles become more prominent, and areas of decidualization surround the blood vessels by day 11-12. The nuclei of stromal cells become larger and vesicular and the cells accumulate glycogen. Stromal œdema increases in the deep functionalis. Subluminal decidualization increases progressively in depth from day 12 postovulation to form a coalescent layer.

1.11.1.3. The menstrual phase

The glands in the functionalis show advanced degeneration by the time of menstruation. Break up commences in the decidualized compacta and stromal cells clump together and become more basophilic. The functionalis disintegrates and has lakes of proteinaceous fluid and focal haemorrhages. The first day of bleeding is characterised by haemorrhage in the superficial stroma and by the second day, only remnants of glands and scattered stromal cells remain amid fresh blood and polymorphonuclear leukocytes. A regenerative phase follows and lasts for 2-3 days, by the end of which the whole surface is reepithelialised. This occurs by both migration and proliferation of glandular epithelium from the remaining glandular stumps and from the tubal recesses and the isthmus.

1.11.2. Dating of the endometrium during the secretory phase

As mentioned previously, the predictability of endometrial changes under the influence of progesterone during the secretory phase of the natural cycle, allows accurate identification of date of the cycle (70).

1.11.3. Changes in the leukocyte population

Leukocytes are present within the endometrial stroma and between epithelial cells. The leukocyte cell population are under the influence of steroids, and form between 10-15% of stromal cells, rising to 20-25% premenstrually (85).

1.11.4. Effect on vasculature

In the uterus, arcuate arteries give off radial arteries that continue into the endometrium after giving off the basal artery which supplies the basalis. This basal artery does not respond to hormonal stimuli, whilst changes have been noted in the spiral artery and in the precapillaries (86). Changes in vasculature may have a pivotal role in menstruation, and it has been hypothesised that intense vasoconstriction of the spiral arterioles prior to menstruation results in ischaemic necrosis, which leads to tissue shedding once blood flow resumes (87).

Under the influence of steroid hormones, passing from the early proliferative into the late secretory phase, the arterioles increase in length by about five fold, with a noticeable increase in coiling (88).

Microvascular density does not vary with the stage of the normal cycle (89-92), but is increased with continuous progestogen therapy (90). There is also evidence that œstrogen antagonises the effect of progestogen and restores to normal the endometrial vascular density (90).

Vasculature has also been linked to bleeding on steroids. Abnormal proliferation of endothelial cells (93), dilatation and thrombosis of venules (94), inadequate development of spiral arteries (69), and an increase in plasmolemmal vesicles (95), have all been reported in endometrial tissue under the influence of OCP, and were thought to account for the increased incidence of irregular bleeding.

1.11.5. Ultrastructural changes

During the proliferative phase, the endometrium exhibits the ultrastructural evidence of the increased protein synthesis which accompany cellular proliferation, e.g. increase in free and bound ribosomes, mitochondria, Golgi and primary lysosomes both in the glands and the stroma.

Studies using endometrial biopsies dated from the LH surge have shown that the structural changes in the glandular epithelium during the first 6 days of the secretory phase are precisely regulated with small interindividual variation (Table 1.8.) (96-98).

In the luteal phase, ribosomes increase in number and the rough endoplasmic reticulum develops. The nuclear (nucleolar) channel system (NCS) which may be produced by infolding of the nuclear membrane is characteristic of the postovulatory uterine epithelium in the human, and is detected as seven sets of three-branched tubules connected to the nuclear membrane. This system is detected between days 18-25 of the cycle, and is maximally developed between days 19-21 (99). The NCS is ovulation related and is absent in users of progesterone IUDs or the Minipill. The NCS is found in the endometrium after œstrogen and progesterone treatment in IVF cycles, where the ultrastructure of the endometrium has been shown to be a sensitive indicator of the œstrogen/progesterone balance (100) and of infertility (101).

The development of the NCS system is not a prerequisite for secretory activity, and is a function of the type of progesterone used and of the adequacy of the initial œstrogenic priming. Progesterone, chlormadinone, and

medroxyprogesterone acetate, but not 19-nortestosterone derivatives, could produce this NCS (99), although 19-nortestosterone derivatives do induce giant mitochondria and the glycogen deposits (102).

1.11.6. The endometrium in the postmenopause

The endometrium, deprived of cyclical œstrogen and progesterone, involutes acquiring the histological features of either cystic or simple atrophy, or of inactive endometrium. The mucosa becomes thinner (0.4mm) and the gland diameters narrower (0.1mm). Many endometrial biopsies taken after the menopause have cystically dilated glands lined by inactive and flattened epithelium. These are surrounded by stroma made of spindle cells in a myxomatous ground substance. This histological form, is more commonly localised to form a polyp, and less commonly diffuse. The diffuse form may develop directly from premenopausal proliferative endometrium, especially if the last cycles were anovulatory (69), or as a consequence of ageing in a previously simple atrophic pattern (103). It is possible that with advancing age more glands become cystic and filled with proteinaceous material, and that the stroma becomes less cellular (82). Evidence suggest that both patterns may develop independently (104).

The inactive endometrium is thin and is made of sparse, small glands, set in a dense stroma with spindle shaped cells. These glands resemble those of the proliferative phase but are devoid of mitoses, and the supportive stroma is more compact (105). The surface epithelium is cuboidal or flattened. Mitosis are only occasionally seen in the glands and the stroma, and this continues for several years till the endometrium becomes atrophic (82). The dividing line between the inactive and the atrophic endometrium is, however, somewhat vague. Simple atrophic pattern proceeds progressively and may take several years (103). Here, the glands are sparse and narrow and are lined by low epithelium with inactive nuclei, and the stroma is dense and fibrous with no spiral arterioles.

1.12. Histological endometrial changes linked to steroid action

The endometrial histological changes (**1.11.**) develop in response to the cyclic hormones produced by the ovaries. The normal menstrual cycle can thus be described with reference to either the ovarian or the uterine cycle depending on the organ under investigation. The ovarian cycle has two phases: the

follicular phase during which follicles are recruited and subsequently develop with one single follicle reaching maturation and releasing its ovum before the rest. Follicle rupture at ovulation heralds the beginning of the luteal phase which commences from the time of ovulation till the onset of menstruation.

1.12.1. Hormonal changes during the normal ovarian cycle

Levels of ovarian steroids are low at the beginning of the follicular phase subsequent to the demise of the corpus luteum. The low level of ovarian steroids releases the negative feedback effect that œstrogen exerts on the pituitary. Consequently, the level of follicle stimulating hormone (FSH) starts to rise. The rising FSH level, recruits the next wave of ovarian follicles into growth, and these secrete increasing levels of œstrogens (mainly œstradiol). The rising levels of œstrogens exert a negative feedback on the pituitary FSH secretion, and inhibit further recruitment. The rising levels of œstrogens also exert a positive feedback effect on another pituitary hormone: luteinizing hormone (LH). The increasing LH release reaches a critical point when a sharp LH surge is triggered. This LH surge is, in turn, the trigger for ovulation which takes place 24-36 hours later. At ovulation the dominant follicle ruptures releasing the ovum. Subsequently, the follicle undergoes a process of luteinization and starts to secrete progesterone. Progesterone secretion increases gradually till the mid-luteal phase and reaches a plateau before it drops rapidly with the demise of the corpus luteum. Œstrogen levels reach a peak prior to ovulation, and drop in the few days following ovulation, and start to rise again to reach a second peak in the mid-luteal phase, they subsequently decline with the demise of the corpus luteum. The demise of the corpus luteum in the mid-luteal phase results in a drop in the levels of œstrogen and progesterone, this drop continues into the early follicular phase of the following cycle, when the levels of œstrogen start to rise (106).

1.12.2. Effect of hormones on the endometrium

1.12.2.1. The endometrium without hormones

There are two physiological conditions where the endometrium is not under the effect of ovarian steroids: before puberty, and after the FMP. Before puberty, the endometrium is at most 0.4mm thick, and contains short sparse glands embedded in a delicate stroma of spindle cells, and the glandular and surface epithelium is thin. The histological appearance of the postmenopausal

endometrium has been describe previously (**1.11.6**). During both these stages, the endometrium is dormant and shows no cyclic changes, but remains able to respond to administered hormones.

1.12.2.2. Effect of œstrogen

Various ovarian steroids as well as other chemical compounds have œstrogenic potency. The ovarian hormone with highest affinity to œstrogen receptor being œstradiol. Synthetic derivatives of œstradiol and other source œstrogens as the equine œstrogen premarin; are also suitable for oral administration, and are, therefore, used in therapeutics.

The endometrium responds to ovarian œstrogens produced by the developing follicles by proliferation of both the glandular and the stromal elements. The histological features have been discussed previously (**1.11.1.**). This follicular phase continues till the effect of œstrogen is antagonised by progesterone. If ovulation does not occur and the endometrium remains under unopposed œstrogenic stimulation, it grows in thickness, and may develop the histological features of hyperplasia. Over a prolonged period of time, this may develop into endometrial adenocarcinoma (35, 107). Cessation of unopposed œstrogen leads to an œstrogen withdrawal bleed after a period of amenorrhoea. The mechanism(s) responsible for this are not clearly understood, but are thought to be different from those operating in the normal menstrual cycle. It has been postulated that prolonged unopposed œstrogen stimulation leads to the development of abnormal vasculature and abnormal ground substance and that withdrawal of œstrogen results in vascular stasis, thrombosis and necrosis. It is assumed that it is this process of necrosis that is responsible for the irregular and often heavy bleeding that accompanies anovulation (81). Administering œstrogen during the secretory phase results in the development of stromal œdema and delayed secretory transformation of the glands and the stroma (108).

1.12.2.3. Effect of progesterone and progestogens

The effect of progestogens depends on many factors including the exact type of progestogen, its dosage, the duration of treatment, and on prior exposure to œstrogen. Progestogens belong to one of two groups: 17α-hydroxyprogesterone or 19-nortestosterone derivatives. The former have less androgenic effects than the later. The potency of different progestogens,

which varies between compounds, is measured by their ability to induce basal glycogen granules in proliferative endometrium (the transformation dose), or by their ability to delay the onset of menstruation (delay of menstruation test). The former test is believed to be more accurate than the latter, which may be attributed to the finding that neither œstrogen nor androgen produce or alter the effect of progestogens on inducing endometrial glycogen granules (109).

Generally, progestogens inhibit growth, induce secretory changes in the glandular epithelium, induce stromal decidualization, and increase leukocyte infiltration. Continuous progestogen stimulation induces glandular atrophy and focal stromal necrosis. Progestogens also induce an increase in the total number and the proportion of the different types of leukocytes in the endometrium. Derivatives of 19-nortestosterone have an enhanced effect on the stroma, thus producing more profound decidualization and subsequently more extreme atrophy than 17α-hydroxyprogesterone derivatives (69).

The effect of exogenously administered progestogen (alone) in oral contraception (minipill) on the endometrium, will be influenced by the effect of the pill on inhibiting ovulation. This aspects of pill use, is absent in postmenopausal women whose ovaries have ceased to ovulate. But, in general, the endometrium under the influence of the mini-pill may exhibit one of four main patterns: a histological appearance corresponding to the day of the cycle, a pattern with irregular secretion, a predecidual reaction with atrophic glands, or a pattern of complete atrophy with stromal fibrosis and inactive glands (110).

The administration of progesterone alone during the proliferative phase results in the appearance of secretory changes and inhibition of mitoses. Stopping treatment induces withdrawal bleeding, whilst continuing treatment with a low dose progesterone results in breakthrough bleeding. If treatment is continued in a somewhat larger dose, the endometrium exhibits reduction in the glandular epithelium, gland diameter and gland density, but features of the late secretory phase do not develop. The glandular epithelium appears pseudostratified (111, 112). With prolongation of treatment, focal stromal œdema and predecidualization appears. This treatment also affects endometrial vessels causing a reduction in the number of arteries and an increase in the number of veins and dilated venules in the functionalis (88).

If treatment is continued in higher doses, the secretory changes are abolished and the endometrium enters a stage of arrested proliferation. This progresses to atrophy of the glands and stromal decidualization. Continued therapy results in a state of total stromal atrophy (69).

The effect of progestogens on the endometrium is dependent on local bioavailability rather than on serum steroid levels (113). There does not appear to be any simple relationship between the plasma hormone levels and morphometric indices, but it has been shown that endometrial glands are a sensitive indicator for gauging the effect of steroids which is dependent on both the dose and the duration of therapy (112).

1.12.2.4. The effect of combined hormones

The development of secretory endometrial changes is dependent not only on the adequacy of the progestogenic challenge, but also on adequate œstrogenic priming. One theory is that there is a certain threshold of development before the endometrium can respond to progesterone (96). For a certain level of endometrial development there seems to be a level above which further progestogenic stimulation cannot lead to enhanced secretory changes (100, 114).

It has been demonstrated since 1933, that menstrual like bleeding can be induced in amenorrhoeic women by the administration of exogenous gonadal steroids (115). Good and Moyer, using the *Macaca mulatta* model, demonstrated that simulating the normal menstrual cycle is dependent on an optimal dose of œstrogen and progesterone (72). Alterations in the administered dosage result in (Figure 1.9.) (72):

1- A suboptimal dose of œstrogen and a suboptimal dose of progestogen results in glandular and stromal underdevelopment.
2- A suboptimal dose of œstrogen and a high dose of progestogen results in underdeveloped glands and a predecidualized stroma.
3- A high dose of œstrogen with a suboptimal dose of progestogen results in excessive glandular proliferation, dilatation and pseudostratification of the epithelium while the stroma remains underdeveloped.
4- A high dose of œstrogen and of progestogen result in involution of the glands and stromal predecidualization.

5- Shortening or prolongation of the length of the proliferative phase had no influence on endometrial histology in biopsies taken during the luteal phase.
6- The absolute amount is more important for an optimum response than the ratio between the two hormones.

The need for an optimal œstrogen/progestogen balance demonstrated in this study confirms the findings of the earlier, more limited observational study on oophorectomised women (116).

1.12.2.5. Modulation of leukocytic infiltration by steroids

There is evidence that sex steroids affect the function and number of endometrial lymphoid cells. The mechanisms underlying this effect are not completely understood, but it may be mediated through œstrogen receptor (ER) which has been localised on endometrial lymphoid cells (117). ER^+ cells are mainly localised in lymphoid aggregates and are rare amongst the scattered cells in the stroma or amongst the intraepithelial lymphocytes. T cells are believed to be the primary target cells for œstrogen action (117), but a role for œstrogen in regulating the number of macrophages has also been demonstrated (118).

T lymphocytes in lymphoid aggregates express œstrogen receptor (117) and it has been shown that œstrogen administration leads to an increase in the number of both macrophages and T lymphocytes of the helper/inducer phenotype in the rat (119). A role of progesterone in the regulation of T lymphocyte population cannot be excluded, for although progesterone receptors (PR) were not detected in lymphoid cells in the human endometrium (117), PR is expressed on peripheral blood lymphocytes (120).

1.12.3. Effect of endogenous and exogenous hormones

The hormonal changes of the normal menstrual cycle and the cyclical endometrial changes have been described previously (**1.12., 1.11.**). Menstruation usually coincides with the fall of œstrogen and progesterone consequent to the demise of the corpus luteum, although it can occur without very low hormone levels (71).

1.12.3.1. Contraceptive steroids and the endometrium

The most commonly prescribed OCP are combination pills containing a daily dose of an œstrogenic and a progestogenic compound for 21 days, although some of the older preparations contained sequential hormones. A 'withdrawal' bleeding attributable to withdrawal of combined œstrogen and progestogen, occurs during the seven days 'treatment free' period. The bleeding is, however, clinically remarkably similar to that occurring in the natural cycle. The distinction that is to be made between the OCP and HRT is that the OCP is neither designed or required to mimic the physiological state on the endometrium. On the contrary it is intended to prevent a physiological state: pregnancy. OCP achieve this through various mechanisms including the development of endometrium hostile to the embryo (121), and which differs morphologically (69, 73) and biochemically (71) from the normal endometrium.

Histological differences have been described according to the type of progestogen used (122). Combination preparations shorten the proliferative phase, resulting in the premature appearance of secretory changes. The glands remain underdeveloped and uncoiled. The glands and the stroma appear dysynchronous in the same field and from area to area. Glands vary from the atrophic to the cystically dilated, the lining epithelium spans the range from the low cuboidal to the tall columnar. Glycogen granules are irregular in size and distribution, with very few if any luminal secretions. Intracellular organelles are poorly developed. There is patchy stromal œdema, premature decidualization and dense leukocytic infiltration. The spiral arteries are poorly developed, but there develops a network of small or dilated capillaries in the subepithelial layer, and areas of focal necrosis appear in the stroma. During bleeding, the endometrium does not shed normally or may not shed at all. Accurate dating is impossible. Basal vacuoles usually appear on the 7th to 8th day of the cycle and reaches maximum by the 13-15th day. Focal decidualization begins on the 20th day. Prolonged treatment may result in the glands disappearing, or be lined by flattened endothelial like cells. If the œstrogenic influence predominates this may lead to the development of hyperplasia, but a predominant progestogenic influence leads to the dominance of stromal predecidualization (73).

Sequential oral contraceptive pills, result in prolongation of the proliferative phase. The secretory changes are more uniform but underdeveloped (123), the endometrium remains thin despite stromal œdema. There is no predecidualization and only rare endometrial granulated lymphocytes (73),

except in regimens with a long (10 days) progestin phase (124). Prolonged treatment with sequential agents may lead to hyperplasia but rarely to atrophy. Differences were also noted in the activity of alkaline and acid phosphatase, succinic and lactic dehydrogenase and carbonic anhydrase when compared to the normal endometrium (71, 125).

1.12.3.2. Assisted conception in the pre-menopausal endometrium

In Vitro Fertilisation and Embryo Transfer (IVF-ET) using the patient' s own ova has highlighted the importance of implantation failure (126). The higher success rate achieved in programmes using donor oocytes, was attributable to either better oocyte quality or to improved uterine receptivity (127, 128). But shared oocyte programmes provided further evidence for the significance of uterine receptivity (128, 129), and this was supported by evidence from IVF cycles in older women. These demonstrated that the age related decline in fertility is caused by ageing oocytes and that the uterus in older women can be induced into a receptive state comparable to that in younger women (130).

Uterine receptivity is achieved using hormonal regimens based on an incremental œstrogen to mimic the proliferative phase of the cycle with the addition of progesterone (the natural hormone) to a fully developed proliferative cycle. Continuation of œstrogen stimulation after the addition of progesterone may not be crucial (126), but ovarian androgens or other unidentified factors may play a part, at least in that they may impair receptivity in situations characterised by their increased production (126). Implantation in humans is limited to a narrow 'implantation window' between days 16-19 of a 28-day cycle (131), and as evidenced by impairment in regimens using clomiphene citrate or hyper-stimulation programmes, is dependent on a fine hormonal balance. The effect of disturbance of this balance may be demonstrated morphologically or using biochemical markers (132).

1.12.3.3. Assisted conception following ovarian failure

Achieving a pregnancy after ovarian failure whether this was timely or premature, and whether it occurred spontaneously or iatrogenically is dependent on donor oocytes. Initially confined to young women with premature ovarian failure, currently, more women past the natural reproductive age are undergoing such pregnancies.

Fecundity in women is known to decline with age and is almost totally lost by the age of 45 (130). However, it has been demonstrated that the postmenopausal uterus can still achieve a high pregnancy (39.02%) and delivery (21.6%) rate. Furthermore, the implantation rate per embryo is comparable in women aged <40 and those aged 40 or more (13.5% and 12.2% respectively), (133). There is, however, no data on endometrial histology in the group of older women undergoing oocyte donation.

1.12.3.4. HRT regimens and the postmenopausal endometrium

Data on the endometrial effects of HRT is scarce, except in relation to endometrial safety and the prevention of hyperplasia and cancer. Endometrial hyperplasia (134), and an increase by 3-25 fold in endometrial cancer risk (74-76), have been linked to ERT.

The addition of progestogens to œstrogens is advocated in order to reduce the risk of hyperplasia and cancer. In order to be effective, some argue that, progestogens need to be added for a minimum of 13 days (78, 135). Meta-analysis of all studies combined suggests that the relative risk for developing endometrial cancer in women on combined HRT is 0.8 (CI 0.6-1.2), although cohort studies suggested a different relative risk of 0.4 (CI 0.2-0.6) compared to the 1.8 (CI 1.1-3.1) calculated from case controlled studies (136).

1.12.4. Mechanism of steroid action

1.12.4.1. Mechanism of œstrogen action

Œstrogen exerts its effect on the endometrium through interaction with its specific receptors located in the nucleus. The œstrogen receptor (ER) is a member of the steroid hormone receptor superfamily. The protein has five regions (A-E) and a small *C*-terminal F region of unknown function (Figure 1.10.). ER activation requires the binding to œstrogen which is accompanied by dissociation of the heat-shock protein 90 (hsp 90) from the E region and dimerization of the receptor. This enables the complex to interact with œstrogen response elements to activate transcription of target genes, for example the progesterone receptor (PR) (137).

The action of œstrogen on the endometrium is complex. It has been demonstrated that the mitogenic effect of œstrogen on uterine epithelium is dependent on the presence of adjacent stroma. On the other hand, œstrogen can induce epithelial cells, in isolation from stromal cells, to produce specific proteins such as the progesterone receptor. The mechanisms that may mediate the mitogenic effect of œstrogen are not clear, but may involve paracrine, stromal mediated effects, organismal mediated effects, or the inhibition of circulating inhibitory factors (138). Œstrogen induces in target cells, a paracrine response which influences closely opposed neighbouring cells (139) and which may involve epidermal growth factor (EGF) and/or other protooncogenes such as c-*fos*, c-*myc*, N-*myc*, *erb* B, and c-ras^{Ha} (140).

1.12.4.2. Alterations in ER levels during the menstrual cycle

The level of ER in the endometrium rises gradually to reach its peak in the late follicular phase, and drops gradually during the luteal phase. The rate of drop is higher in the epithelium compared to the stroma (141, 142).

1.12.4.3. Mechanism of progesterone action

Progesterone and other progestogens bind to progesterone receptor (PR). Progesterone reaches its receptor, located inside the nucleus by passive diffusion (141), and progesterone-receptor complex modulates protein synthesis.

Progesterone antagonises the effects of œstrogen on the endometrium through inhibiting synthesis of ER especially in the epithelium (143, 144). It decreases ER binding to its nuclear binding site (145), and increases œstradiol inactivation by induction of the 17β-hydroxysteroid dehydrogenase (146). The anti-œstrogenic effect of progesterone may also be partly mediated by stromal signals. The exact mechanisms involved in this paracrine effect are not known but a likely mediator is transforming growth factor-β (TGF-β) (147).

PR has 2 subunits, α and β. The β subunit has receptor sites for progesterone and for chromatin. This binding to chromatin exposes a DNA-binding region on the α subunit and it is the binding of the α subunit to DNA that initiates transcription (92). The human PR exists in two isoforms PR_A (768 amino acids, 94kDa) and PR_B (933 amino acids, 114kDa). These result from translation of two different mRNA populations and are synthesised through

initiation of transcription from different sites of the same gene. Initiation of transcription is controlled by two different œstrogen induced promoters (148). The role of each of these forms is unclear although there is evidence that they interact differently with target genes (149), PR_B being predominantly a transcription activator whilst PR_A is a transcription inhibitor. It is thus possible that alterations in PR_A/PR_B balance is a mechanism for modulation of progestogen action.

1.12.4.4. Alterations in PR level during the menstrual cycle

The action of progesterone on the endometrium will depend on the level of PR which fluctuates during the menstrual cycle. Induction of PR requires the presence of œstrogen and is inhibited by progesterone (150). In the glandular epithelium, PR levels are low during the early- and mid- follicular phase and increase markedly in the late-follicular and the early-luteal phase, it then drops markedly during the mid-luteal phase till it completely disappears in the late luteal phase. PR levels in the stroma follow a similar pattern except that the decline during the mid luteal phase does not occur and the receptor level remains stable (141, 142). PR is present in the muscular layer in the spiral arterioles (151), but not in endometrial lymphoid tissue (117).

1.12.5. Menstruation

Menstruation occurs in humans and some subhuman primates. The only non-primates which menstruate are the bat (*Glossophaga soricina*) and the elephant shrew (*Elephantus myuras jamesoni*), and an experimental model was produced in the mouse (152).

Although most authors agree that the superficial two-thirds of the endometrium is lost at menstruation, an alternative view is that some areas of the zona spongiosa may be retained with only little tissue loss. These divergent views have been attributed to non-uniform loss, where most shed tissue being from the body and the fundus but little from other sites.

At the start of menstruation, the glands of the functionalis are mostly effete, the compacta begins to break up, the shed stromal cells agglutinate and become more basophilic. The functionalis, disintegrates and has lakes of

proteinaceous fluid and focal haemorrhages containing platelet masses between the sheets of collapsed glands. Polymorphs appear in the stoma.

The mechanism of onset of menstruation is not completely understood, but may involve collapse and spasm of arterioles (87) possibly secondary to increased capillary fenestration (153), or involve lysosomal action (154).

It is not clear whether endometrial glands contribute to the process of menstruation, but they have been shown to produce vasoactive substances as endothelins and nitric oxide (155). The role of apoptosis has also been argued (156). Increased vascular permeability under the influence of vascular endothelial growth factor (VEGF) (157), and increased tortuousity and vasoconstriction followed by vasodilatation precede menstruation (87). Mediators of the vascular events include vasoconstrictors such as endothelin-1 and prostaglandin $F_{2\alpha}$ and vasodilators like prostaglandin E_2, prostacyclin and nitric oxide (158).

1.13. Assessment of steroid action upon the endometrium

Monitoring endometrial response both in the physiological and the hormonally induced cycle relies on assessment of biopsy material, and on physiological response. Imaging methods used in clinical practice for measuring endometrial thickness have limited utility in this respect. Measurement of blood levels of endometrial-derived products, or of these products in luminal secretions are alternatives that have a promising potential but which are, as yet, less developed.

1.13.1. Physiological response: the bleeding pattern

The bleeding pattern has been used to monitor physiological cycles and cycles treated with oral contraceptive pills (71). The onset of bleeding may reflect the histology of the endometrium under HRT (79), but this has not been confirmed (80). However, there are very few studies on the bleeding patterns in women using HRT (159, 160) which, therefore, remains incompletely understood.

1.13.2. Assessment using endometrial biopsy

1.13.2.1. Methods for obtaining a biopsy

Obtaining a tissue sample from the endometrium is the traditional method for assessing endometrial functional states, and various techniques have been employed for this purpose. As endometrial lesions may be localised, it is favoured by the pathologists if the whole of the endometrium could be obtained (69), but in practice this is difficult to achieve (161-163). Furthermore, for the demonstration of the functional state of the endometrium, full curettage is unnecessary as it has been demonstrated that the endometrium usually develops homogeneously, bar the less responsive isthmus (164). Full curettage may also be undesirable if a repeat biopsy is required later in the same cycle (69), or for clinical reasons (165). "Curettage" of the endometrial surface using spoons of various sizes and shapes (usually under general anaesthesia) is the traditional method. But in order to avoid the need for a general anaesthetic, office biopsy techniques were introduced. These utilise thin cannulae to obtain a sample of the endometrium (e.g. Sharmann curette). These thin cannulae may be combined with syringe (166, 167), or with motorised (e.g. Vabra) (35) suction. The more recent modifications as the vabra curette and the pipelle sampler utilise thinner disposable cannulae to ensure sterility and to guard against infection. The criticism that has been directed to these devices is that they do not sample the whole of the endometrium (163) which, as discussed previously, does not threaten their utility except in cases where localised lesions are present, and despite several reports testifying to their accuracy in the diagnosis of cancer, some cases may be missed (162).

To overcome this criticism, hysteroscopy was introduced. Using the hysteroscope the whole endometrial cavity can be seen, but the device can only detect macroscopic not microscopic lesions. The same criticism applies for imaging techniques as X-ray hysterosalpingography, Saline Infusion Sonohysterography (SIS), and to ultrasound.

Endometrial cytological techniques include attempts to brush, wash, or aspirate the endometrial cavity, or to use cervical smears. Endometrial cytology, initially advocated to exclude malignant lesions, has a large failure rate (27% for lavage techniques), and is now largely abandoned (168, 169).

All biopsy techniques have a failure rate which may be either due to operator error, or inherent in the method used. Biopsy failure may be due to missed

localised lesions, or due to the small size of the sample obtained. This is particularly the case with the thin atrophic endometrium. The failure rate in obtaining endometrial biopsy by Dilatation and Curettage under general anaesthesia may be up to 42% (170). The endometrium was not assessable in 27.1% of cases using the pipelle sampler (80), and the vabra curettage failed to produce a specimen in 18% of cases (171). These figures cast doubt on the reliability of the methods currently widely employed for endometrial assessment (172). Worthy of mention is the difficulty encountered in postmenopausal women which is exemplified by the finding in a series of 1521 women, where a satisfactory specimen was obtained from only 13% of cases (104).

In research, these techniques have other inherent deficiencies with respect to adequate assessment of the endometrium. First: they are all blind biopsies which cannot be directed to any particular area of interest (173). Second, the biopsies obtained cannot be oriented and examined vertically (69, 132) in order to examine the whole thickness.

1.13.2.2. Methods for tissue assessment

The tissue obtained can be assessed histologically using light or electron microscopy, and after staining with chemical, histochemical, immunohistochemical or *in situ* Hybridisation techniques. The advantage of these techniques over biochemical examination of tissue homogenates is their ability to localise the agents under study within the section. Tissue homogenising, however, may be more reliable for quantitative studies.

Histology

Histology remains the basic method for assessment and for differentiating normal from abnormal or malignant endometrium. However, many structural differences induced by hormones may not be detectable by light microscopy and are better demonstrated using ultrastructural or image analysis techniques (114). Furthermore histological examination suffers from being subjective (98) which limits its utility in research.

Ultrastructure

The role of ultrastructural studies is the demonstration of those features that cannot be assessed using light microscopy. The technique is not suitable for examination of large areas of heterogeneous structure, and is subjective unless combined with morphometric assessment.

Immunohistochemistry

Immunohistochemistry (IHC) has been used for the detection of various components in the endometrium including the luminal and glandular epithelium, the stoma, the vasculature and the stromal leukocytes (174). IHC permits tissue localisation and facilitates comparative studies. However, it is hampered by the lack of knowledge of the specific markers of function, and of the function of many of the known molecules.

Morphometry

Morphometry allows objective and quantitative assessment, and thus overcomes the criticism directed to histological assessment (96, 97, 174, 175). Automated image analysers have been used in endometrial assessment, and the technique has a high consistency (98).

The gland density is constant (20), and does not vary significantly during the cycle (96). Other quantifiable structural changes include the number of mitosis, epithelial cell height, glandular lumen area, the number of glandular mitosis, basal vacuoles, glandular diameter, and the volume density of the glandular lumen (96).

1.13.2.3. Isolation of proteins from endometrial explants

Physiological and pathological events in the endometrium, e.g. implantation and carcinogenesis, may be mediated by, or reflected in changes in the composition of endometrial secretions. Isolation of proteins secreted by endometrial explants in culture medium, or of intracellular proteins, has been used to study endometrial protein production. Tissues may be cultured intact (176) or after enzymatic digestion to isolate the glands from the stroma (177). Identification of newly produced proteins could be achieved by radio-labelling or by electrophoresis. The limitation of this technique is that tissues may alter some of their physiological properties when grown in culture, and that some of the proteins found in the culture supernatant may be subject to

unidirectional secretion into the vascular compartment in vivo, and may not be normal constituents of the intrauterine environment.

1.13.3. Assessment using peripheral blood samples

Monitoring endometrial response to hormones by detecting serum endometrial proteins, provides a less invasive method. The protein most extensively studied is α_2PEG (Placental Protein 14, PP14), which has been suggested as an indicator of adequate endometrial response to progestogens (178-180). Serum α_2PEG rises premenstrually (181, 182), and in the progestogenic phase of combined HRT (183, 184), but has not been shown to correlate with endometrial histology (160, 185), which may be partly explained by the presence of alternative sources for the protein.

Insulin like growth factor binding protein-1 (IGFBP-1, PP12, α_1-PEG) is another major protein synthesised by the decidualized but not the proliferative endometrium (186). The synthesis and secretion of IGFBP-1 by the endometrium is related to progesterone-dependent differentiation (187). IGFBP-1 is detected in serum, but the concentrations are related to glucose homeostasis which reflects the major contribution from extrauterine sources (188).

1.13.4. Assessment using uterine luminal washings

The technique involves passing a thin cannula inside the uterus in order to obtain a sample of uterine luminal fluid after flushing the cavity with fluid (189). Identification of endometrial specific proteins using this technique is difficult as the vast majority of detected proteins are transudates from serum proteins (190). There is currently no known specific endometrial marker(s) that can reflect each physiological state (e.g. receptivity for implantation, corpus luteum degeneration and menstruation), which limits the utility of the technique.

1.14. Aim and plan of investigation

Whilst beneficial effects of HRT are linked to the use of œstrogen, adverse effects do occur, the most prominent of which are the effects of progestogens on the lipid profile. The balance of these effects on postmenopausal women is

incompletely understood. The current investigation is designed to examine the effects of a cyclical continuous combined HRT (cHRT) regimen on the endometrium in postmenopausal women in as far as these reflect the balanced potency of the compounds used. The objective is to establish the baseline and to utilise it as a model to ascertain steroid action elsewhere in the body. The endometrial effect is thus studied not only for its own interest and implications but also, as an index of the biological effects of cHRT on the woman as a whole.

The biological effects of sex steroids in general, are usually referred to in terms of œstrogenic, progestogenic, or androgenic potency. The œstrogenic and the progestogenic effects are particularly relevant in relation to the compounds used in cHRT and in relation to the cyclical changes in the endometrium, and will be the subject of the current investigation.

This investigation will attempt to ascertain the utility of known markers of steroid effect on the cHRT treated endometrium. But because of the paucity of recognised functional markers, will also attempt to identify new markers that reflect hormonal influence.

The overall objective is to identify features of endometrial differentiation that may link to potential differences in the action of endogenous and exogenous hormones on other body systems. This may lead to the generation of hypotheses and guidelines for the development of more 'physiological' cHRT regimens.

The investigation proposes to:

1- Study a group of women treated by a unified cHRT regimen in a clinically defined and standardised manner, in order to avoid any variability consequent to different treatment regimens, and also to avoid confounding variables.

2- Endometrial response will be monitored using the bleeding patterns and biopsy material at the end of the progestogenic phase. Timing was chosen in order to ascertain the full impact of both hormones.

3- Biopsy material from the functionalis, which is the most responsive layer to steroids, will be studied both histologically and using immunohistochemistry in order to appreciate the endometrial changes induces by cHRT.

4- Establish a detailed histological description of cHRT treated endometrium.

5- Compare cHRT treated endometrium with biopsy material obtained from women with natural cycles in order to appreciate the differences and the similarities. The underlying hypothesis is that optimal 'physiological' hormone replacement would produce endometrium that is similar to that of the natural cycle.

6- Markers of hormonal effect will be utilised in order to assess the developmental stage of each component of the endometrium; glands, stroma, vasculature, and leukocytes. The findings will be related to the functional state (bleeding) and to the hormonal influences.

7- Endeavour to generate new monoclonal antibodies against cell membrane antigens that are modulated through the cycle, and ascertain the utility of these in the study of cHRT treated endometrium.

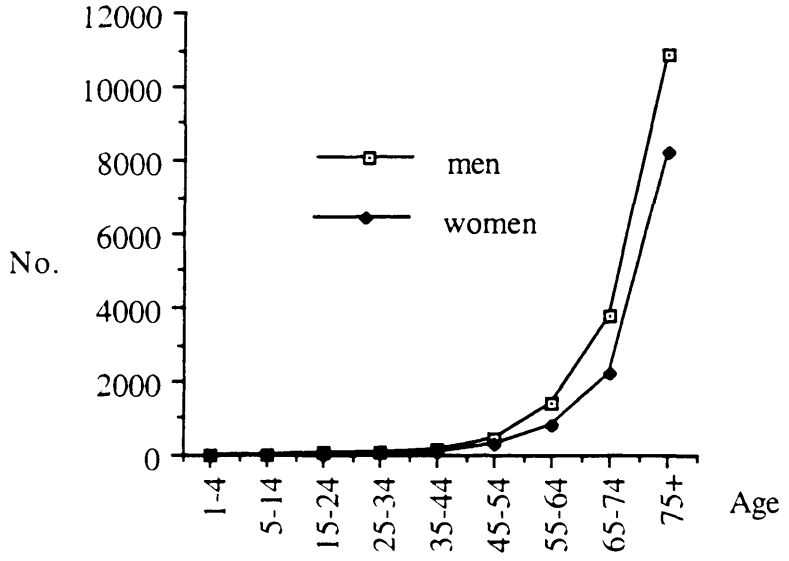
Proposed method of investigation

The endometrium is composed of several cell types, and cell-cell interactions do occur. An *in vivo* model is, therefore, more appropriate for the study of the balanced effect of the preparation used. It is also a more appropriate model for the study of the effects of systemically administered compounds which are known to be metabolised elsewhere in the body, as well as locally within the uterus.

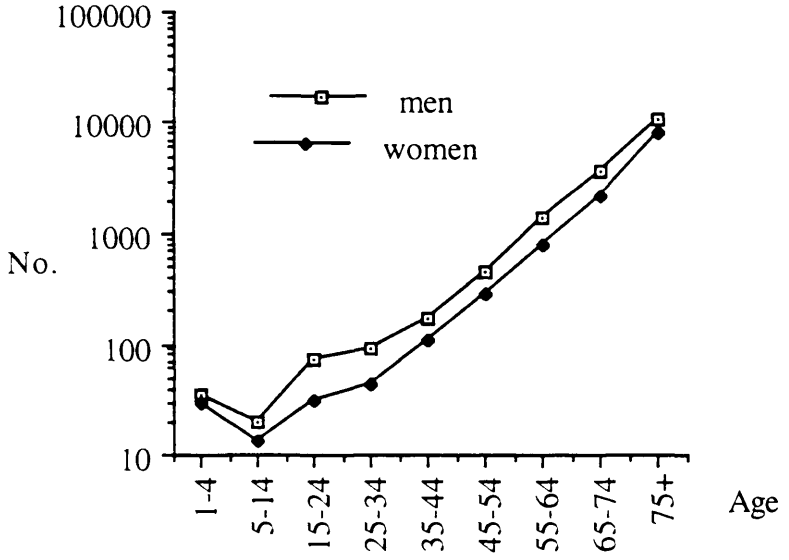
Biochemical methods if applied to the endometrium, although may be more accurate in estimating the levels of agents under investigation, do not enable the exact localisation of these agents to each cell type. Biochemical methods do not allow the accurate comparison of antigen expression in different cell types, or the study of tissue architecture, which remains the mainstay for endometrial studies. It was therefore considered more appropriate to employ histological and immunohistochemical - qualitative, semi-quantitative, and quantitative - methods, as well as *in situ* Hybridisation (ISH) in this study. This will employ specific monoclonal antibodies and probes. Assessment will be performed using light microscopy and image analysis.

A major limitation of these techniques is the effect of any minor variation of the tissue sections and of the experimental conditions (191), these will therefore be rigorously controlled.

In order to generate monoclonal antibodies, a cell membrane preparation will be obtained from normal human endometrium and this will be used to immunise mice. The developed antibodies will be tested in tissue sections obtained from the normal endometrium for their localisation according the cell type and cycle phase. Specific antibodies that show fluctuation during the cycle will be identified and developed, those that do not will be discarded.



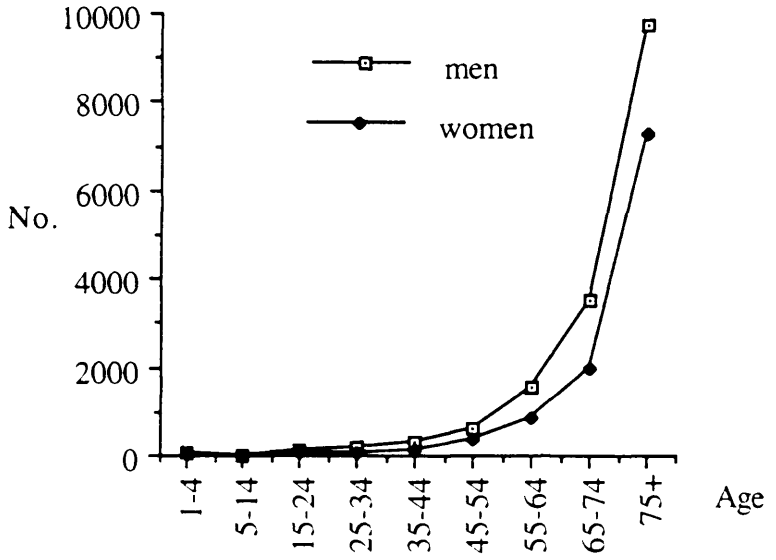
A. Linear scale



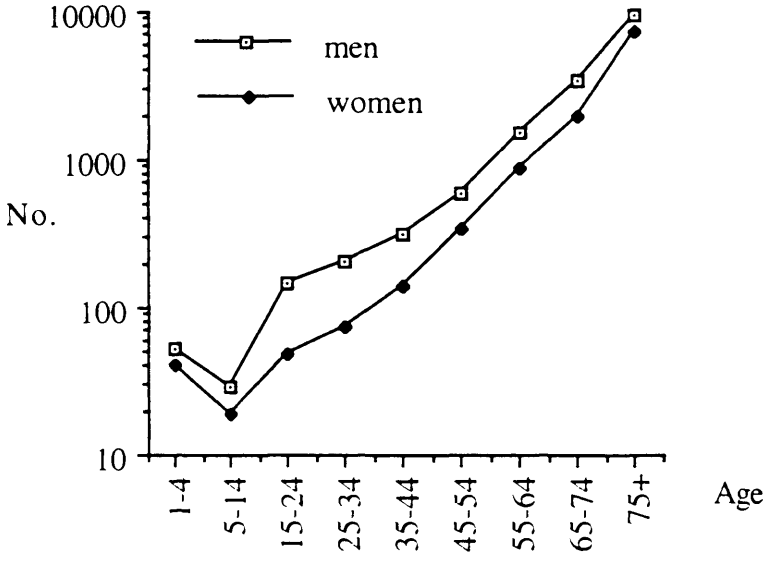
B. Logarithmic scale

Figure (1.1.)

The mortality rates in men and women in the UK in 1992 (**A**: linear, **B**: logarithmic scale), demonstrating a gradual increase in mortality with advancing age and the sharp increase between age 45-55 in both men and women. The rate for women remains below that for men at all ages (data from OPCS, 1992).



A: Linear scale



B: Logarithmic scale

Figure (1.2.)

The mortality rates in men and women in the USA in 1992 (**A**: linear, **B**: logarithmic scale). This demonstrates a gradual increase in mortality with advancing age and a sharp increase between age 45-55 in both men and women. The rate for women remains below that for men at all ages (Data from WHO, 1994).

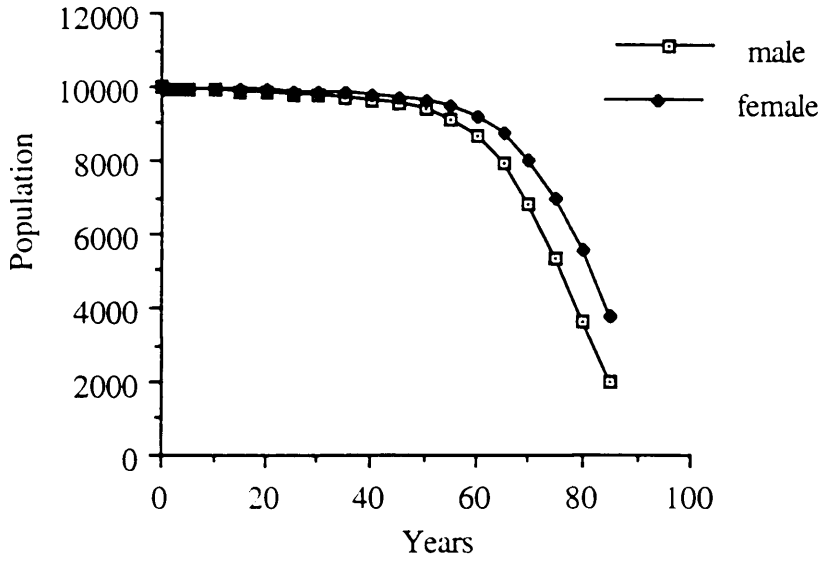


Figure (1.3.)

The population remaining alive out of every 10000 new-born in the UK with the passage of time, demonstrating the biological advantage for women (Data from OPCS, 1992).

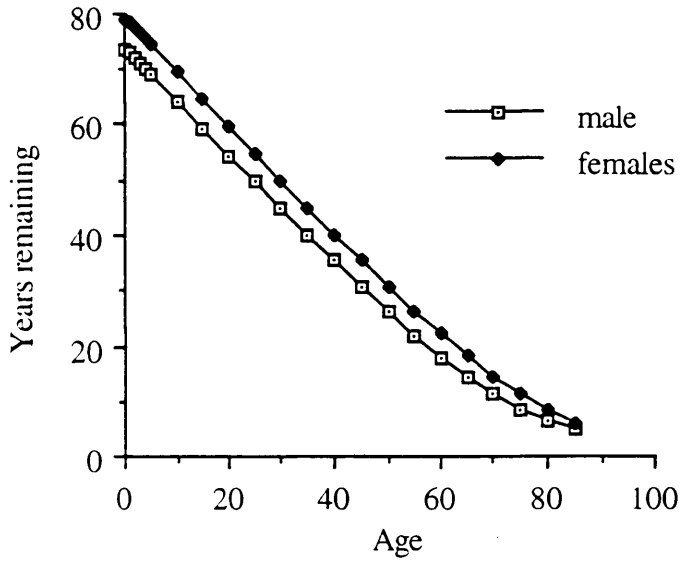


Figure (1.4.)

The number of years of life remaining for men and women at each age in the UK, demonstrates the longer life expectancy in women at each age group (Data from OPCS, 1992).

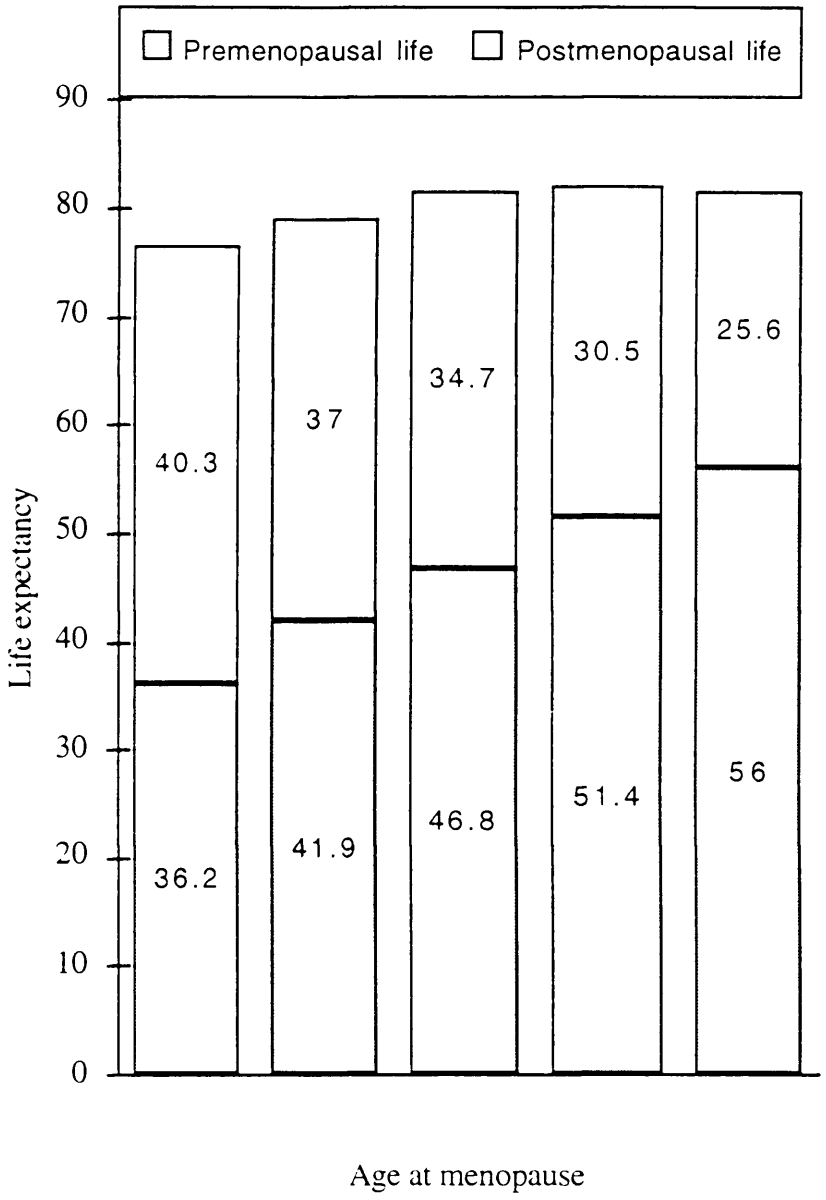
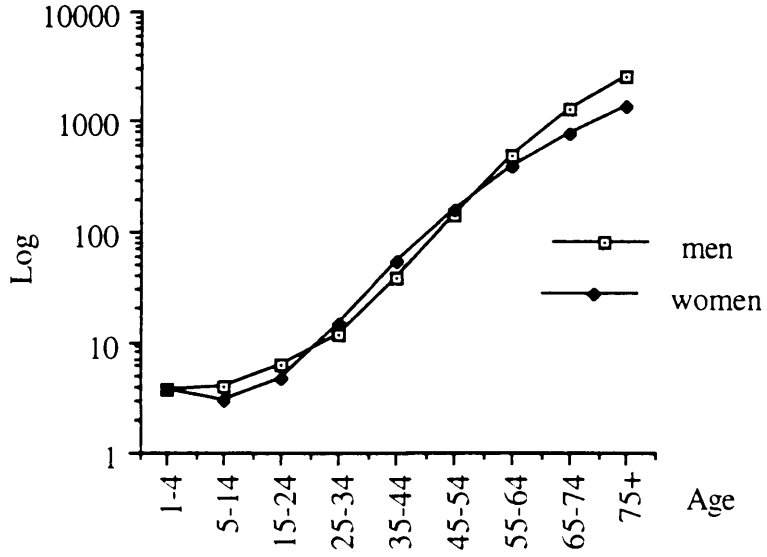
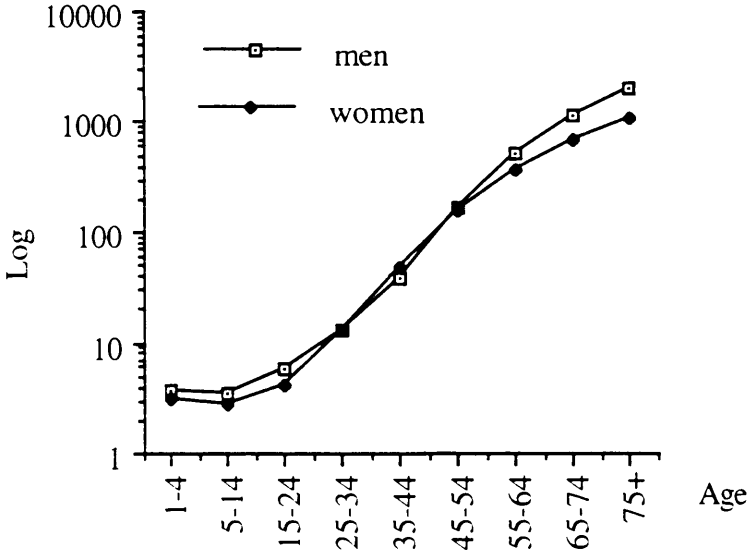


Figure (1.5.)

The relation between the premenopause, postmenopause and life expectancy. Despite a shorter postmenopause, women who had a longer premenopause had, in general, longer longevity. The exception being those whose menopause was beyond 55 years of age, and who had a slightly shorter life span (Date from Snowdon, 1990).



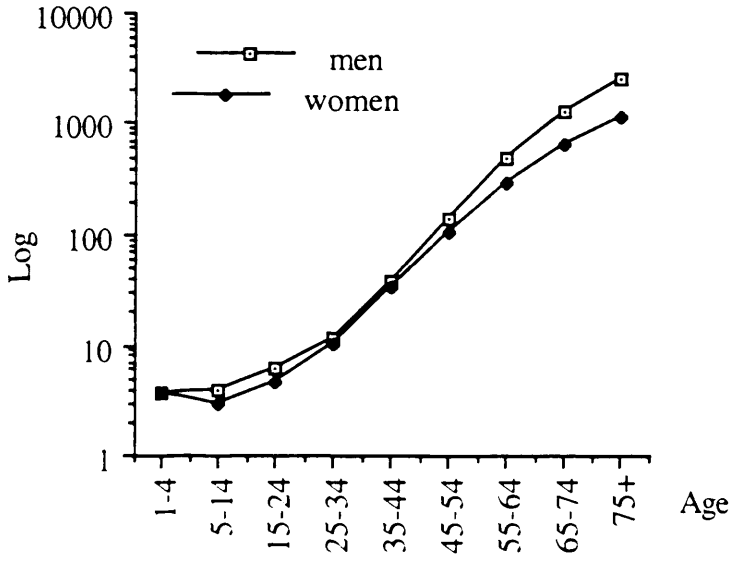
A: UK



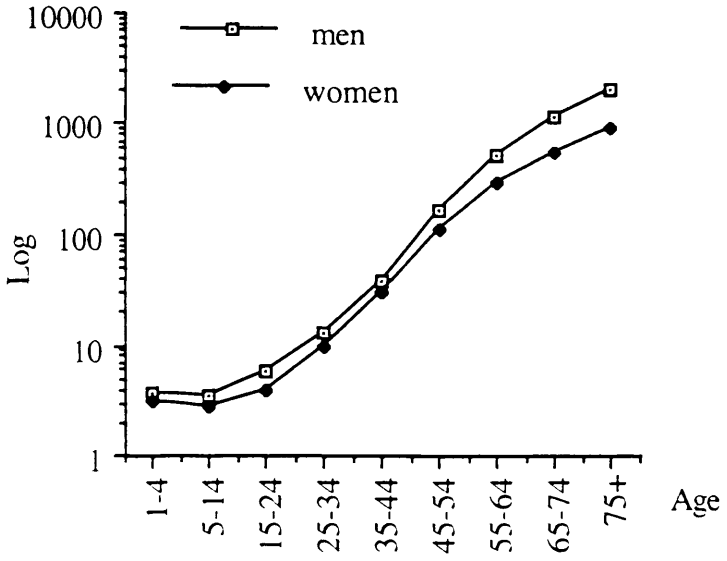
B : USA

Figure (1.6.)

Death rates in men and women due to malignant tumours (per 100000 population), in (**A**) the UK, and (**B**) the USA. The rates are lower in women compared to men except during reproductive life where this is offset by the incidence of breast and cervical cancer (Data from OPCS, 1992; WHO, 1994).



A: UK



B : USA

Figure (1.7.)

Death rates due to malignant tumours excluding breast cancer mortality (per 100000 population), in (**A**) the UK, and (**B**) the USA. This demonstrates the female advantage over males at all age groups (Data from OPCS, 1992; WHO, 1994).

LH day	Nucleus	Glycogen	Cytoplasm
LH+2	large nucleus, little hyper-chromatin, prominent nucleoli	little	polarised, free ribosomes, normal mitochondria, poorly developed apical Golgi parallel to membrane
LH+3	occasional nuclear channel	large basal	more polarised, variable cell morphology. Giant mitochondria, intimately associated with rough endoplasmic reticulum
LH+4	prominent nuclear channels	massive	very homogenous, polarised, supranuclear Giant mitochondria, Golgi parallel to long axis of the cell
LH+5	prominent nucleolus & nuclear channel	moving to apex & lumen	more variable appearance, dilated endoplasmic reticulum, supranuclear dilated Golgi and clear cytoplasmic vesicles
LH+6	rim of hetero-chromatin, NCS		fewer clear areas in basal cytoplasm. Apex full with Golgi and vesicles. Apical smooth endoplasmic reticulum. Lateral cell membrane interdigitate extensively
LH+7	fewer NCS		fewer giant mitochondria
LH+8	NCS uncommon, increased hetero-chromatin	lost secretory activity	stop secretory activity, loss of polarity. Rare giant mitochondria. Most prominent lateral interdigitations

Table (1.8.)

Ultrastructural changes in the endometrium from day 2-8 after the LH surge. Characteristic features include the appearance of the NCS, and the giant mitochondria.

NCS: Nuclear channel system

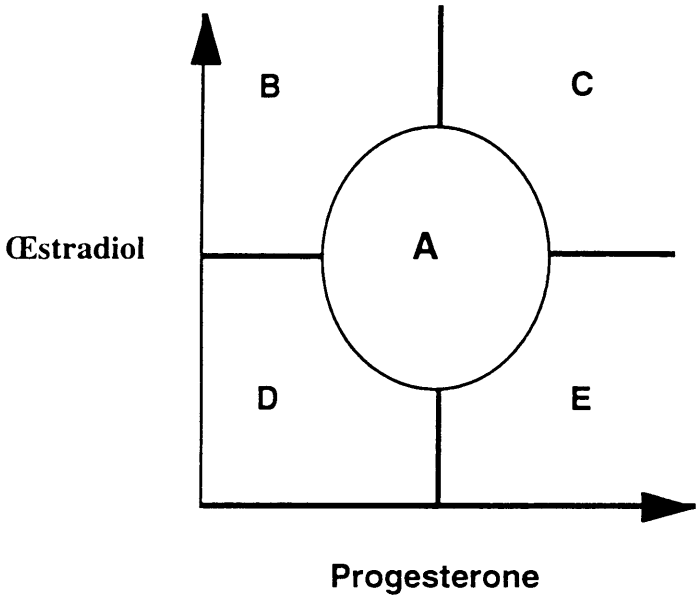


Figure (1.9.)

The relation between endometrial histological features and the dose of administered œstradiol and progesterone (modified from Good & Moyer, 1968).

A- Secretory endometrium like that of the normal cycle
B- Glands proliferated and dilated; epithelium pseudostratified, stroma underdeveloped
C- Progesterone predominant: glands involuting; stroma predecidual
D- Glands and stroma underdeveloped
E- Glands underdeveloped; stroma predecidual

Œstrogen receptor

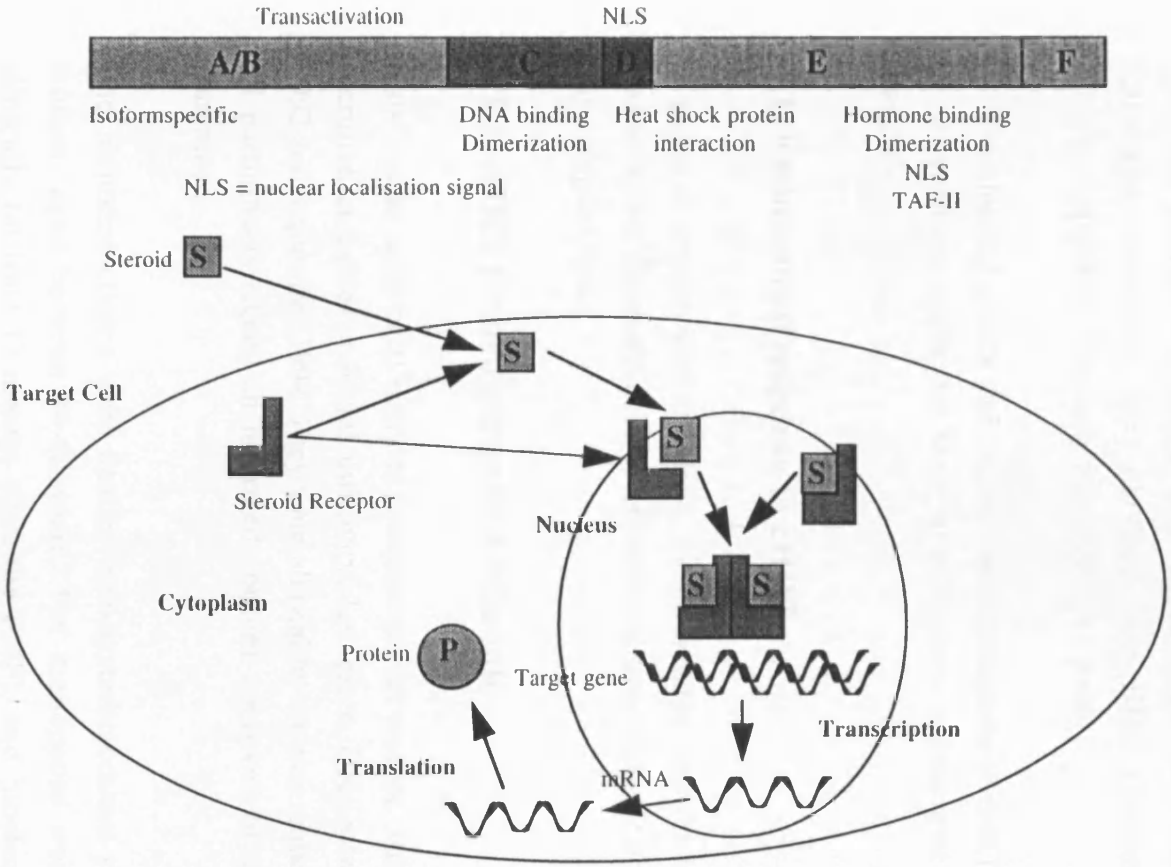


Figure (1.10.)

The structure of œstrogen receptor (ER). The steroid receptor and signal pathway. The A/B region contains isoform specific sequences and a constitutively active transactivation domain (TAF-I). The C region contains the DNA binding and dimerization domain. The D region contains the nuclear localisation signal. The D and E regions are required for interaction with Heat Shock Proteins. The E domain is involved in hormone binding, nuclear translocalisation of receptor, hormone dependent transactivation and receptor dimerization. Œstrogen but not progesterone receptor, contain the F region. Upon binding to receptors, the receptor translocates into the nucleus and dimerizes. Following this the receptors bind to the hormone response elements within the target gene, resulting in transcription activation or repression, and its interaction with protein kinases (Modified from Park-Sarge & Mayo, 1994) (192) .

Chapter 2

Materials and Methods

Here the sources of material and the methods used will be reviewed. Laboratory chemicals were obtained from BDH (Dorset, UK), unless otherwise specified. The study comprised two parts:

1- The bleeding pattern and the endometrial response to cHRT.
2- Monoclonal antibodies were raised against endometrial cell membrane antigens.

2.1. Endometrial response to cHRT

A group of women received cHRT for six months, and the bleeding pattern was analysed. Endometrial biopsies were compared to those obtained from the physiological cycle.

2.1.1. cHRT treated group and schedule

This was an open label study of postmenopausal women. All of whom were recruited at Leicester Royal Infirmary, Leicester, UK, between December 1992 and September 1994. They were all healthy women requesting HRT, and all participants signed an informed consent approved by the local ethics committee.

The inclusion criteria were healthy non-hysterectomised postmenopausal women, aged between 44-62 years. The menopause was defined both clinically (at least 12 months since the FMP), and biochemically (serum œstradiol <100 pmol/l and serum FSH >40 iu/l). None had received oral or topical œstrogen, progestogen or androgen within the previous 3 months, and because of the long half life of œstradiol implants, women who had ever received these were excluded. Other exclusion criteria were the presence of contraindications to HRT, e.g. known or suspected hormone sensitive tumours, or liver disease. Drugs known to influence the metabolic clearance of steroids by the liver were contraindicated. All women underwent routine investigations for liver, renal and thyroid function and those women who had

evidence of abnormalities were excluded. All women had a normal mammogram and a normal cervical smear before commencing therapy. A pelvic examination was performed at the beginning of the study, women with abnormalities on vaginal examination were excluded.

The normality of the endometrium was confirmed by histological assessment of an endometrial pipelle biopsy (Laboratoire CCD, Paris) prior to commencing therapy. Women were to be excluded if this biopsy could not be obtained because of administrative or technical reasons (e.g. cervical stenosis) or if the baseline biopsy showed evidence of endometrial hyperplasia or malignancy.

Participants adhered to a rigid schedule, The first clinic consultation being a pre-study assessment for inclusion, 2-3 weeks prior to commencement of the 168 days of active medication. During the treatment period women attended clinic every 56 days. At the final visit an endometrial biopsy was performed.

Women filled in Diary Cards to document the intake of study medication and to record the days of bleeding and its severity. The card design was simplified in order to minimise the chances of inaccuracy (Figure 2.1.). Bleeding was graded as: no bleeding=0, spotting=1, normal=2, or heavy=3. The total bleeding score (Total-BS) was represented by the summation of the daily scores, and was divided by the duration of bleeding to obtain the average bleeding score (Average-BS).

The objective was to evaluate 100 women who completed 6 months of treatment. There were 33 screen failures (Table 2.2.), and 28 withdrawals (Table 2.3.), 103 women completed the six months on therapy (Table 2.4.). Is it significant that only 5 (3.8%) stopped treatment because of heavy periods, a sixth patient stopped treatment as she decided that she did not wish to have any periods, although her periods were average.

The study endometrial biopsy was planned for the final clinic visit that was scheduled for days 27 to 29 of the sixth cycle (Table 2.5., 2.6.).

Medication

All women were prescribed a regimen of 2mg œstradiol valerate daily with the addition of 1mg norethisterone from day 17 through to day 28 of each cycle (Solvay Pharma, GmbH). No additional steroids were allowed.

2.1.2. Control group

LH-dated natural cycle biopsies (n=30) were obtained by dilatation and curettage from healthy fertile women with regular cycles at the time of scheduled tubal sterilisation. They were all given a urine ovulation detection kit (Clearplan, Unipath, UK) and instructed to its use. Sterilisation and dilatation and curettage were scheduled for the luteal phase. None of the women was using hormonal contraception or an intrauterine device, or had any pregnancies for the previous 6 months. Their mean age was 34.7years (range=26-39), and mean parity 3.2 (range=2-6). All women gave written consent to the procedure that was approved by the local ethics committee. The biopsies were obtained as follows:

- Early luteal phase day 2-6 (n =10).
- Mid luteal phase day 7-11 (n =10).
- Late luteal phase day 12-14 (n =10).

On histological examination, none of the biopsies showed features of hyperplasia or malignancy.

2.1.3. Sampling technique

Endometrial samples were obtained either with a pipelle or at dilatation and curettage. In all cases attempts were made to obtain the sample high from within the endometrial cavity avoiding the isthmus.

2.1.4. Tissue processing and preparation of sections

2.1.4.1. Cryostat sections

Although frozen sections have the advantage of better preservation of antigenicity for IHC detection, tissue preparation using cryostat fixation yielded sections with poorly preserved architecture. This was similar to the experience of others (193), and was therefore abandoned in favour of formalin fixed paraffin embedded preparations.

2.1.4.2. Formalin fixed paraffin-embedded preparations

Formalin fixation followed by paraffin embedding using standard methodology, yielded sections with excellent architectural preservation (193). Once obtained endometrial tissue was fixed in 10% buffered neutral formalin solution and were subsequently paraffin wax embedded. Paraffin embedding of formalin fixed tissue was processed automatically, through dehydration with grades of ethyl alcohol, followed by several washes in chloroform for clearing, followed by wash in xylol prior to embedding in wax (Ralwax 1). Sections (4μm thick) were cut using the Leica RM2035 microtome and suspended on Silane (3-Aminopropyl-Triethoxy-Silane, Sigma, Dorset, UK) coated slides, and dried overnight in a 37°C heated oven.

2.1.5. Staining techniques

2.1.5.1. Histological and histochemical staining

Hematoxylin and Eosin (194)**:**

Sections were dewaxed in 3 steps in xylene, rehydrated through 99%, 90%, 70% ethyl alcohol, (each step for 5 minutes), and washed in distilled water (dH_2O) for 5 minutes. They were then stained in Harris hematoxylin (Sigma) for 5 minutes and washed in running water. Following that, they were stained in Eosin (TAAB Laboratories, Berkshire, UK) and again washed in running water. The dehydration steps were then run in reverse order to the dehydration steps above. Sections were covered by no. '0' glass coverslips using DPX mountant (containing distrene 80, dibutyl phthalate and xylene).

Phloxine-Tartrazine method (Lendrum) (195):

This technique was initially used to identify endometrial granulated lymphocytes, but as staining of paraffin sections was not consistent, it was abandoned in favour of IHC.

2.1.5.2. Immunohistochemistry

Appendix (2) summarises the primary and secondary antibodies used in this work, and Appendix (3) lists other reagents used in IHC. All were stored as

per supplier's recommendations, antibodies that withstand freezing were stored in small aliquots at -20°C, and none were subjected to repeated freezing/thawing cycles. All steps were performed in a humidity chamber unless otherwise specified.

2.1.5.2.1. Method for single labelling

IHC followed the standard protocols using the Avidin-Biotin complex immunolabelling method (193), with modifications. The following steps were performed (solutions are referred to in Appendix 1):

1- Sections were dewaxed in the fume hood in three steps of xylene, and rehydrated in grades of ethyl alcohol (99%, 90%, and 70%), (each step for 3 minutes).
2- They were washed in dH_2O for 3 minutes.
3- Sections were pretreated using enzyme digestion or heat treatment, if necessary (**2.1.5.2.3.**).
4- If DAB substrate was to be used, endogenous peroxidase was blocked by incubating in freshly prepared 6% hydrogen peroxide (H_2O_2) for 10 minutes.
5- Sections were washed in running tap water for 5 minutes.
6- They were then transferred to TBS-BSA buffer for 5 minutes.
7- Slides were wiped around the tissue and covered in 100µl of Normal Rabbit Serum (NRS), (Dako, Bucks, UK), diluted to 1:10 in TBS (if the primary antibody was of rabbit origin swine serum was used instead), then incubated in a humidity chamber for 20 minutes at RT.
8- Excess NRS was removed and sections were incubated with the primary antibody at the required dilution and for the optimum time.
9- Slides were washed in TBS-BSA for 20 minutes and then incubated with the secondary antibody (100µl of biotinylated antibody from a heterologous species) diluted at 1:150 in TBS-BSA. Incubation was for 30 minutes at RT.
10- These were washed in TBS-BSA for 20 minutes.
11- Slides were incubated with a freshly prepared solution of Vectastain ABC alkaline phosphatase® or Vectastain ABC peroxidase® (Vector Laboratories, CA, USA) in PBS at room temperature for 30 minutes.
12- Sections were washed in PBS for 30 minutes and solution changed at 15 minutes interval.
13- Sections were incubated with alkaline phosphatase substrate (Fast Red TR/Naphthol AS-MX®, Sigma), or peroxidase substrate (DAB substrate,

Vector Laboratory) for approximately 5-10 minutes, or until colour fully develops.

14- Slides were washed in running tap water for 5 minutes.

15- Counter staining was seldom used, but if performed, Mayer's hematoxylin was used for 30 seconds, followed by a wash in Scotts tap water.

16- Sections were dried and mounted. For alkaline phosphatase substrate Apathy's aqueous mountant (Raymond A Lamb, London, UK) was used, and for DAB substrate sections were dehydrated in grades of alcohol, cleared in xylene and mounted using XAM or DPX mountant.

2.1.5.2.2. Method for double labelling

Double labelling for CD45 and Mib1 (Basic technique):

The following methodology was used for double labelling for CD45 and Mib1:

1- Sections were dewaxed and re-hydrated to distilled water as for single labelling.

2- They were subsequently pre-treated in 0.4% Pepsin HCl solution at 37°C for 20 minutes.

3- Sections were washed in running tap water for 5 minutes.

4- Sections were transferred in slide racks to TBS-BSA for 5 minutes.

5- Slides were wiped around the sections and the sections were covered with 100µl of Normal Rabbit Serum (NRS) diluted 1:10 in TBS, and incubated at RT for 10 minutes.

6- The excess NRS was wiped off, and the primary antibody against CD45 diluted to 1:150 in TBS was applied to cover the sections.

7- Slides were incubated overnight at 4°C.

8- Slides were washed in TBS-BSA for 20 minutes.

9- Slides were dried around the sections and 100µl of the secondary antibody (rabbit anti-mouse linked to alkaline phosphatase), was applied to cover the sections at a dilution of 1:100 in TBS. Sections were incubated for 30 minutes.

10- Slides were washed in TBS-BSA for 20 minutes.

11- Alkaline phosphatase substrate (Fast Red TR/Naphthol AS-MX®, Sigma) was applied to cover the sections until the colour fully develops as judged microscopically (approximately 5 minutes).

12- Sections were washed in running tap water for 5 minutes.

13- The slides were microwave treated in 6M Citrate Buffer (**2.1.5.2.3.**), for 30 minutes, whilst checking every 10 minutes to ensure that the buffer level covers the slides which were not allowed to dry out at any time. After cooling in the buffer, sections were transferred to tap water and rinsed.
14- Sections were transferred to TBS-BSA for 5 minutes.
15- Slides were wiped around each section and 100µl of NRS (Dako), diluted 1:10 in TBS, was applied to cover the sections. They were left at RT for 10 minutes.
16- Excess serum was removed and the Mib1 antibody, diluted to 1:50 in TBS, was applied to cover the sections.
17- Sections were left to incubate overnight at RT.
18- Slides were washed in TBS-BSA for 20 minutes.
19- Slides were wiped around the sections and 100µl Rabbit anti-mouse antibody applied at a concentration of 1:150 in TBS, and incubated for 30 minutes.
20- Vectastain ABC*elite*® peroxidase solution (Vector Laboratories) was reconstituted in PBS, 30 minutes before use. In 5ml of PBS, 2 drops of solution A was mixed, and then 2 drops solution B was added and mixed.
21- Slides were washed in TBS-BSA for 20 minutes.
22- 100µl ABC solution was applied to cover the sections and was left for 30 minutes.
23- Sections were washed in PBS for 30 minutes, and the solution was changed after 15 minutes.
24- Vector SG®, blue/grey peroxidase substrate (Vector Laboratories) was applied for approximately 5 minutes, or until the colour fully develops.
25- Sections were washed in running tap water for 5 minutes and mounted using Apathy's aqueous mountant (Raymond A Lamb).

Double labelling for CD3 and Mib1

The following modifications of the basic technique were introduces:

a- Step (2): Pepsin pretreatment was used for 40 minutes.
b- Step (5): Normal swine serum was used (100µl) at 1:10 dilution.
c- Step (6): The primary antibody anti-CD3 (Dako) was used at a concentration of 1:50.
d- Step (9): For the secondary antibody, 100µl of biotinylated F(ab)2 fragmented swine anti-rabbit antibody (Dako) was substituted at a concentration of 1:150 (for 30 minutes at RT). Sections

were subsequently washed for 20 minutes in TBS-BSA and incubated in a solution of Vectastatin ABC® alkaline phosphatase (Vector Laboratories) in PBS at RT for 30 minutes.

e- Step (15): Normal swine serum was used.

Double labelling for CD56 and Mib1

The following changes to the basic technique were used:

a- Step (2) Was replaced by microwave treatment as in step 13.

b- Step (6) The monoclonal anti-CD56 antibody (Neomarkers, USA) was used at a dilution of 1/50 instead of anti-CD45 antibody.

c- Step (13) Was omitted.

2.1.5.2.3. Modifications of the standard technique

The main modifications involved either enzymatic or heat pre-treatment. Alkaline phosphatase or peroxidase enzymes linked to avidine can be used interchangeably except for double labelling.

Pepsin pre-treatment

Enzyme pre-treatment of formalin fixed tissue unmasks many antigenic sites which allows bind to the primary antibody (193). In general the commonly used enzymes are pepsin and trypsin. Pepsin was used successfully in this work at a concentration of 0.4%. The duration of enzymatic treatment depends on the specific antigen and antibody (Pepsin solution: Appendix 1).

Microwave pre-treatment

Antigens were unmasked by microwave heating the sections whilst immersed in an antigen unmasking agent (196), alternatively pressure autoclaving may be used. The microwave technique was used in this work. Sections were transferred to plastic slide racks and immersed in 6M citrate buffer (Appendix 1), and heated in a 750 Watt Microwave (Techolec, T250T, UK) at full power for 20-40 minutes. The level of the buffer was checked every 10 minutes to

ensure that it covers the slides, dH_2O was used to replace evaporated water as needed.

2.1.5.2.4. Controls for IHC

Specificity of immunolocalisation was confirmed by:

1- The antibodies employed are known to be highly specific.
2- The pattern of staining followed the expected pattern both in the endometrium and using positive control tissue.
3- The use of positive and negative controls, where appropriate.
4- Negative control experiments omitting the primary antibody to control for background staining.
5- Negative control experiments replacing the primary antibody with IgG (Sigma) against different species.

2.1.5.3. TdT-mediated dUTP-biotin nick end labelling (TUNEL)

TUNEL identifies DNA fragments in cells undergoing programmed cell death (Apoptosis), and was performed as follows (197):

1- Sections are dewaxed in the usual way and then washed in distilled water (dH_2O) for 3 minutes.
2- Sections were treated with Proteinase K, 20units/mg (Boehringer Mannheim, GmbH), at a concentration of 20ug/ml, for 1 hour at RT.
3- The enzyme was rinsed in sterile dH_2O at 4°C for 10 minutes, the dH_2O renewed after 5 minutes.
4- Sections were soaked in 0.4% paraformaldehyde solution (Appendix 1), at 4°C for 20 minutes.
5- Sections were rinsed in dH_2O at RT for 5 minutes.
6- Sections were washed in TdT buffer (Appendix 1), at RT for 5 minutes.
7- The labelling solution was made up by adding 1µl Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate, alkali-stable (DIG-11-dUTP), and 4µl of the enzyme Nucleoside-triphosphate: DNA deoxynucleotidylexo-transferase (Boehringer Mannheim) to 500µl of TdT buffer (Boehringer Mannheim). Sections were covered, each with 50µl of the labelling solution, and a coverslip was applied to the sections to prevent drying whilst they were incubated at 37°C for 2 hours in a humidity chamber.
8- Slides were racked and rinsed twice (5 minutes each) in dH_2O at RT.

9- Sections were incubated for 10 minutes at RT, in a blocking solution made of 3% BSA (Sigma) in TBS, with Triton X-100 (Sigma) added at the concentration of 100µl per 100ml.

10- This was followed by incubation with anti-digoxigenin-AP fab fragmented antibody (Boehringer Mannheim), at a concentration of 1:600 in blocking solution at RT.

11- Sections were washed in TBS for 10 minutes at RT, and then rinsed in dH_2O at RT.

13- Sections were rinsed in substrate buffer (0.1M Tris HCL, pH 9.5) for 5 minutes at RT.

14- Alkaline phosphatase substrate kit (BCIP/NBT, Vector Laboratories) was used for colour detection, Slides were incubated with substrate at RT until colour developed (10-60 minutes). The reaction was monitored under microscopic guidance .

15- Sections were washed in tap water for 5 minutes, and mounted using Apathy's aqueous mountant (Raymond A Lamb).

Alternative technique for TUNEL

1- Sections were dewaxed and brought to water through grades of alcohol as for immunohistochemistry.

4- Sections were treated by Proteinase K (20units/mg) (Boehringer Mannheim) at a concentration of 20µg/ml for 20 minutes at RT.

5- The reaction was then stopped by washing for 2 minutes in dH_2O.

6- Sections were immersed in TdT buffer for 10 minutes at RT, The buffer was made up by adding 40µl of dH_2O and 10µl TdT buffer and 2µl of cobalt chloride, per section.

7- Sections were incubated at RT for 90 minute, covered with a solution made of Terminal deoxynucleotid Transferase (Tdt, Nucleoside-triphosphate: DNA deoxynucleotidylexo-transferase; Boehringer Mannheim) and Biotin-16-dUTP (Biotin-16-2'-deoxy-Uridine-5'-triphosphate; Boehringer Mannheim) in TdT buffer, (1µl of transferase and 1µl of dUTP added to 100µl of TdT buffer).

8- Sections were transferred to TB buffer (300mM sodium chloride, 30 mM sodium citrate, Boehringer Mannheim) for 15 minutes at RT.

9- Sections were incubated with a freshly prepared solution of Vectastain ABC® alkaline phosphatase (Vector Laboratories) for 15 minutes at RT.

10- Sections were washed in TBS buffer and the alkaline phosphatase substrate (Fast Red TR/Naphthol As-MX®, Sigma) was added for 15 minutes at RT.

12- The reaction was terminated by washing in dH_2O.
13- Sections were mounted with coverslips using Apathy's mountant (Raymond A Lamb).

in situ End Labelling (ISEL)

ISEL is a comparable technique which differs in that it utilises the Klenow fragment of DNA polymerase I, rather than terminal transferase for 5' to 3' DNA labelling (198).

2.1.5.4. *in situ* Hybridisation (ISH)

ISH was used for labelling with the histone oligonucleotide probe (199) (NCL-Histone-U, Novo Castra Laboratories, Newcastle upon Tyne, UK). All solutions and equipment for the stages up to hybridisation were treated with H_2O_2 and DEPC to remove nucleases, and all procedures were carried out wearing gloves, (solutions: Appendix 1):

1- Sections were dewaxed in a series of xylene and alcohol (as for immunohistochemistry) and rehydrated in DEPC (diethyl pyrocarbonate) water.
2- They were immersed in 2x Standard Saline Citrate solution (SSC), at 70°C for 10 minutes, and washed for 5 minutes in DEPC water at RT.
3- Proteinase K (Boehringer Mannheim) was applied to sections at a concentration of 5ug/ml, (diluted in 0.05M Tris-HCl, pH 7.6, DEPC). These were incubated at 37°C for 1hour.
4- The reaction was stopped by washing for 10 minutes at 4°C, in two changes of DEPC water.
5- Slides were transferred to 0.4% paraformaldehyde solution and incubated at 4°C for 20 minutes.
6- Slides were washed for 5 minutes at RT in DEPC water, wiped around the sections and covered in 100µl of pre-hybridisation solution and incubated at 37°C for 1hour
7- 50µl of the probe was added onto the sections, which were covered and left overnight at 37°C.
8- Sections were washed twice for 10minutes each in a solution of 2xSSC/30% Formamide at 37°C, and transferred to blocking solution (Appendix 1) for 10 minutes at RT.

9 -100µl of anti-digoxigenin alkaline phosphatase, Fab fragments antibody (Boehringer Mannheim) was applied (1/600 dilution in blocking solution) to cover the section and incubated for 30 minutes at RT.
10- Sections were washed in two changes of TBS over 10 minutes, then rinsed in sterile dH_2O. Following this ,they were transferred to Tris HCl buffer pH 9.5 for 5 minutes.
11- The alkaline phosphatase substrate kit (BCIP/NBT, Vector Laboratories) was added to cover the sections, which were then covered with a coverslip and incubated in the dark. The reaction was monitored microscopically at intervals. Overnight incubation was necessary.
12- Sections were washed in running tap water for 5 minutes and mounted using Aqua mount.

3' Labelling of oligonucleotide probes with digoxigenin

This method was used to label the Europath Histone probe, NCL-HISTONE-U (Novocastra Laboratories, Newcastle-Upon-Tyne, UK), that was designed to hybridise to the mRNA transcripts of human histone genes H2b, H3 and H4.

2.5µg of the probe was used in the following preparation:

1- The dried probe was reconstituted in 50µl of sterile ultra-pure water, ensuring thorough contact with the probe and left to stand at RT for 1hour.
2- To the eppendorf containing the reconstituted probe, the following were added in order (reagents from Boehringer Mannheim):

- Reconstituted probe	50µl
- Sterile pure water to a final volume of	100µl
- 10mM manganese chloride	10µl
- Digoxigenin -11-dUTP	10nm
- Terminal deoxynucleotidly-transferase (Tdt enzyme)	50U (2µl)
- TdT buffer	20µl

3- Solutions were mixed and then spun down in a microfuge followed by incubation for 2 hours at 37°C.
4- The enzyme activity was stopped by adding 5µl 0.5M EDTA.

5- The remaining reactants were removed by passing the solution through a spun column, and label incorporation was demonstrated by staining test strips.
6- The final concentration was calculated (assuming 100% recovery of the probe) from the equation:

Final concentration in ng/µl=2500 / final volume in µl

Preparation and development of test strips

1- Diluent was prepared by mixing:

- 20xSSC	300µl
- 10mg/ml sonicated and denatured salmon sperm DNA	20µl
- Ultra-pure water	680µl

2- 1µl of probe was used to make a series of dilutions of : 1ng/µl, 100pg/µl, 10pg/µl, 1pg/µl, 0.1pg/µl, and 0.0pg/µl.
3- 1µl of each of the above dilutions was added on a strip of nitro-cellulose (8x1 cm). These strips were sandwiched between postlip paper and baked at 80°C for 2 hours.
4- The strips were rehydrated by floating them on dH_2O at 37°C and then transferred to pre-warmed blocking solution (Appendix 1) and incubated at 37°C for 20 minutes.
5- Strips were baked again between postlip paper at 80°C for 2 hours.
6- Before use, the strips were rehydrated in dH_2O at 37°C, followed by incubation in blocking serum for 10 minutes at RT. They are now ready for use following the same stages as for ISH.

Preparation and use of spun columns

1- A 2ml plastic syringe was used, and its barrel was first filled with sterile polyallomer wool.
2- The syringe was filled with a slurry of Sephadex G50 (Sigma), pre-swollen in 1xTE buffer (Appendix 1), pH 8.0. The syringe was refilled till full.
3- The syringe was placed into a sterile 15ml centrifuge tube, and centrifuged at 1600g for 5 minutes. Sephadex was refilled until the packed volume was approximately 1.8ml.
4- 100µl of 1xTE buffer was added and the column recentrifuged, and the process repeated till the volume recovered is approximately 100µl.

5- A sterile eppendorf without a lid was placed under the column and the probe solution added to the top of the column and centrifuged for 5 minutes at 1600g.
6- The eluent was transferred to a fresh tube and measured.
7- The concentration was calculated after measuring the recovered volume and assuming a 100% recovery rate.

2.1.6. Examination of sections

Sections were examined qualitatively, semiquatitatively, and quantitatively.

2.1.6.1. Light microscopy and Image Capture System

The system was based on:

1- The microscope: Axioplan® (Carl Zeiss, Herfordshire, UK).
2- A single chip colour video camera: Sony DXC-151P, connected to the Sony CMA-151P camera adapter, and which transmits the image to the Apple Macintosh® computer (Centris 650) via a RasterOps 24STV graphics display board (Rasterops Corporation, Santa Clara, CA), and using the NIH Image™ software (version 1.51, National Institute of Health, USA). A scale slide (Graticule Ltd, UK) was used for translating linear measurements into μm scale. Captured images were processed (contrast enhancement, filtering, smoothing, etc.), and used to retrieve data.

2.1.6.2. Standardisation for image analysis

This was done through standardisation of tissue processing, the thickness of sections and the staining protocol. After optimising the protocol, staining for each antibody was performed in single batches, or by duplication of two sections between large divided batches. These were used for quality controls, and the experiment was repeated if the inter-assay coefficient of variation was >6%.

Standardisation of the image analysis was performed by maintaining the same settings of illumination, contrast, brightness and filters for each antibody, and the same cut off thresholding for each experiment. Based on previous work (200) control sections were repeatedly examined during the measurement

session, and the process was repeated if the intra-assay coefficient of variation was >6%.

2.1.7. Statistical analysis

The sample size for the number of women enrolled was calculated in order to obtain an estimate of the mean length of the luteal phase in postmenopausal women on cHRT. An internal pilot study including the first 15 women estimated the mean and SD to be 15.33 ± 2.537 days.
Based on this, the estimated sample size (n) needed to estimate the population mean (x), within a 95% confidence interval of 1 day can be calculated as follows:

95% confidence interval : x +0.5 to x -0.5 days
But : 95% CI=mean ± 2 SEM
∴ Standard error of the mean (SEM)=0.25 days
But : $SEM^2=\sigma^2/n$
∴ n=2.53 x 2.53 / 0.25 x 0.25=102.4

Therefore 103 women are needed in order for the estimate of the mean length of the luteal phase to be within a 95% CI of 1 day (201).

As the standard deviation of the test parameters was unknown, the number of fields randomly selected from each section (n=17) was calculated in order to satisfy a one sample t-test, with a power 1-β=0.90, and a 2-sided α=0.01 to detect the population mean with a standardised difference d_t =1 (202). The number of sections to be examined was calculated in order to satisfy a two-sided t-test with a power 1-β=0.90, a two sided α=0.05 to detect a 20% difference between the means. A pilot calculation gave the estimated d_t =1.5.

Statistical computations was done by repeat measurements analysis of variance, two sided unpaired t-test, and the Mann-Whitney test using the Statview™ version 4.01, and the SuperAnova™ version 1.11 programmes (Abacus Concepts, California, USA), for the Apple Macintosh® computer. Results are shown as (mean±SD).

2.2. Generation of Monoclonal antibodies

The methodology used was based on that previously published (203) and was performed with collaboration with Dr. R. James, Department of Surgery and the Animal House, Leicester University.

2.2.1. Immunisation protocol

1- (MF1 x Balb/C)F1 mice were pre-bled from the tail to obtain control serum.
2- They were injected subcutaneously at the base of the tail, with 25µg of the membrane protein prepared with Hunter's TiterMax adjuvant (Sigma). (2.2.1.1.).
3- After three weeks, mice were injected intraperitoneally with 25µg membrane protein (without adjuvant) dissolved in 200µl PBS.
4- Serum was obtained from tail bleed after 7 days, and tested for response.
5- If good response: proceeded as in 6 below. If inadequate response, 2 more injections (2 weeks apart) as in 3 above, were repeated.
6- 12 weeks after the initial immunisation, a boost with 10µg of antigen in 200µl of PBS was injected intraperitoneally.
7- 3 days later, the animals were culled, and a blood sample and the spleen were removed.

2.2.1.1. Preparation of the Antigen-Adjuvant mixture

Membrane preparation

This was prepared using a modified protocol (204, 205) as follows:

Curettings from endometrial tissue were homogenised in phosphate-buffered saline, pH 7.4 (PBS) using an ultra Turrax homogeniser at 0°C at a low speed. Homogenised tissue was centrifuged at 400*g*, at 4°C for 10 minutes. The supernatant was further centrifuged at 15000*g*, 4°C for 45 minutes. The supernatant was again centrifuged at 70000*g* , at 4°C for 45 minutes and the resulting pellet was resuspended in buffer and referred to as membrane preparation. The protein concentration was checked using the standard Bradford assay (Appendix 4) .

Mixing the Adjuvant

1- Antigen was concentrated to 100µg/100µl in PBS buffer.

2- 100µg of antigen was added to 125µl of adjuvant to make 150µl.

3- This was hardened by mixing using a needle and syringe, and used for immunisation.

2.2.1.2. Fusion Protocol

Splenocytes were fused to the NSO Mouse Myeloma cell line which is a non-Ig-secreting, non-light chain-synthesising subclone of NSI (P3-NSI/1-Ag4-1). These cells are resistant to 10µM azaguanine and die in the presence of Hypoxanthine-Azaserine or Hypoxanthine-Azaguanine-Thymidine medium. Cells are maintained in culture at 3-9 x 10^5 cells/ml, in 5% CO_2 at 37°C.

Material

- Washing medium (WM): 50:50 DMEM (Dulbecco's modified Eagle medium, Sigma): RPMI-1640 (Sigma) with Pen/Strep, L- Glut, Hepes, Na pyruvate.
- Final suspension medium: As above with 15% FCS (Fœtal Calf Serum, Sigma) and 1x HA
- HA: Hypoxanthine Azaserine (x50), (Sigma).
- Polyethylene glycol (PEG), (50% PEG 1500 v/w in 75 mM Hepes, pH 8.0, Boehringer Mannheim).

Method

1- NSO mouse myeloma cells were set up in tissue culture flask (T80), (Nunclon™, Nalge Nunc International, Denmark), 2-3 days before fusion, and kept in log growth phase.

2- On the day of fusion:

a- Water bath was set to 41°C.

b- 10ml WM and PEG were warmed.

c- The removed spleen was teased apart using fine sterile forceps, and pushed through a 106µ sterile mesh using a syringe plunger handle. Cells were washed through using WM.

d- Cell were centrifuged at 400*g* (1300rpm) for 10 minutes.

e- Cells were re-suspend in 20ml WM.

f- Lymphocytes were counted after staining with trypan blue (usually 100-200x10^6 cells), and left on ice.

g- NSO cells were suspended in culture and counted after staining with trypan blue.

h- Cells were re-suspended in WM after centrifugation at 400*g* for 10 minutes.

j- Splenic cells and myeloma cells were mixed in a ratio of 4:1 in a 50 ml tube and then spun at 400*g* for 10 minutes.

k- The pellet was isolated and placed in an insulated beaker of water at 41°C.

l- 800µl PEG was added over 1 minute, and stirred with a 1ml pipette for 1 minute.

m- While stirring at 41°C, the following volumes of warm WM were added:

1ml over 1 min
1ml over 1 min
2ml over 1 min
6ml over 2 min

n- Cells were spun down at 400*g* for 10 minutes.

o- Cells were re-suspended in final suspension medium to give $2x10^6$ splenic cells per well. Peritoneal macrophages from (MF1 x Balb/C)F1 mice were added making up the volume to 2ml per well.

p- Cells were plated in 24 well plates (P24, sterile tissue culture treated polysterene multi-well plates, Sigma) and incubated at 37°C.

q- After 24-48 hours, wells were fed by replacing 1ml of medium with 1ml of fresh final suspension medium.

r- Thereafter, cells were fed 3 times/week.

2.2.1.3. Limiting dilution cloning and monoclonal production

Hybridoma cells were harvested from P24 or T25 in log growth phase and re-suspended. They were subsequently counted after staining with trypan blue, which also established viability. Depending on the number of cells, dilutions were calculated to give 10-30 cells per ml. Cells were subsequently re-plated out at 100µl per well, i.e. 1-3 cells per well in P96 (48 wells per dilution), in medium containing 15% FCS.

Each dilution was plated out at 100µl per well with mouse macrophage feeder layer [Macrophages obtained from (MF1 x Balb/C)F1 mice]. After 4-5 days colonies were clearly visible, and the wells were scored for single and double clones by microscopy on day 5-6. Each well was then fed with 100µl of fresh medium containing 15% FCS. On day 10-14, when the medium was just beginning to turn yellow, 100µl of supernatant was harvested into a new

sterile P96 from all wells containing single clones, and was tested as previously.

Positive single clones were transferred from P96 to P24 in 0.5-1ml fresh medium containing 15% FCS. Original P96 wells were re-fed with medium containing 10% FCS.

Cells were grown and expanded as necessary from P24 to T25 and the supernatant was re-tested. Cells from positive clones were stored in liquid nitrogen, and the supernatant was stored in sterile tubes at 4°C.

2.2.1.4. Freezing and thawing

For freezing, cells were pelleted by centrifugation, and the pellet was resuspended in 0.5ml of medium (9 parts FCS, 1 part dimethyl sulfoxide, Sigma) at 4°C. The suspension was transferred in a freezing vial to -70°C for 24 hours before storing in liquid nitrogen. Thawing was carried out in a 37°C bath, then 10ml of cold 10% FCS-DMEM was added and the cells pelleted by centrifugation and resuspended in fresh medium.

2.2.1.5. Large scale production of monoclonal antibody

Before induction of ascites, mice were inoculated intraperitoneally with 0.5ml of pristane (2,6,10,14-tetramethyl-pentadecane, Sigma). 4 weeks later, about 10^7 cells were suspended in 0.5ml of medium and injected intraperitoneally. Accumulating ascites was removed by repeat "tapping".

2.2.2. Testing for antibody production and specificity

Between day 11-14, when cells cover >40% of the bottom of each P24 well, and when the medium is just beginning to turn yellow, 1ml sample of the supernatant was harvested from each well and tested for specific antibody, and wells were re-fed using 1ml of medium with 15% FCS with added HA. Testing was performed using positive and negative controls from mouse serum, and tissue culture medium respectively, and results were obtained within 48 hours.

Antibody producing wells were duplicated, and the original well was fed on HA medium, and the duplicate well was fed on the same medium without the addition of HA. Cells were grown and expanded into T25, and cultures were maintained at 2-9x10^6 cells per ml and, as soon as possible after re-testing, the supernatant positive wells were cloned. Two or three ampoules of cells were frozen in liquid nitrogen for storage.

Testing was performed by IHC on formalin fixed paraffin embedded tissue sections that was collected from proliferative and secretory endometrium from uteri removed for non-endometrial pathology. IHC followed the standard protocol (**2.1.5.2.1.**).

2.2.3. Characterisation of the antigen

Partial characterisation was performed by IHC examination of antigen expression in the endometrium in the menstrual cycle, and also the expression in other body tissues. The molecular weight was determined by Gel electrophoresis and Western blotting.

2.2.3.1. SDS - Polyacrylamide Gel Electrophoresis (SDS - PAGE)

A 10% Gel was prepared as follows:

1- The vertical glass plates of the apparatus (Minitube cell, BioRad Laboratories Ltd, Hertfordshire, UK) were cleaned and assembled.
2- Reagents were added in following order and volumes:

30% Acrylamide/0.8% bisacrylamide in deionized water (Ultrapure, Protogel, National Diagnostics, Atlanta, Georgia, USA)	6.7ml
2.0 M Tris (pH 8.8)	3.7ml
Water	9.6ml
10% SDS (Sodium dodecylsulfate)*	200µl
10% Ammonium Persulfate*	134µl
TEMED*	14µl

*Reagents from Sigma.

3- Reagents were mixed and swirled and rapidly poured into the gap between the glass plates. The solution was overlaid with distilled water.

4- The gel was left to polymerise (about 30 minutes), the distilled water layer was removed. The stacking gel (6%) was prepared by mixing the following:

30% Acrylamide/0.8% bisacrylamide	1.65ml
1.0 M Tris (pH 6.8)	1.25ml
Water	6.85ml
10% SDS*	100µl
10% Ammonium Persulfate*	100µl
TEMED*	10µl

*Reagents from Sigma.

5- Reagents were mixed and swirled, and rapidly poured onto the surface of the resolving gel, and a clean Teflon comb inserted into the solution.

6- The samples were prepared by dilution 1:1 in SDS gel-loading buffer (Appendix 1) and then heating to 100°C for 3 minutes to denature the protein.

7- After complete polymerisation, the comb was removed and the gel adjusted and mounted in the electrophoresis apparatus. Tris-glycine electrophoresis buffer (Appendix 1) was added to the top and bottom reservoirs avoiding air bubbles.

8- 25µl of each sample was loaded into the wells.

9- The first left hand side well was loaded with the same volume of molecular weight standards [Fluorescent High Molecular Weight Standard (MW 20,100-205,000), F3526 Sigma]. Calibrated for:

Molecular Weight:	20,100
	29,000
	39,800
	66,000
	116,000
	205,000

10- The electrophoresis apparatus was connected across a 60 mV current (BioRad Model 3000xi) till the dye moves through the resolving gel to reach about 1cm from the bottom (about 3 hours).

2.2.3.2. Western blotting

Was performed as follows:

1- A piece of nitrocellulose filter (Hybond C, Amersham, Buckinghamshire, UK) matching the size of the gel was soaked in deionized water, and six

corresponding pieces of Whatman 3MM paper (Whatman Ltd, UK) were soaked in transfer buffer (Appendix 1).

2- The SDS-PAGE gel was removed from the electrophoresis apparatus, briefly washed in deionized water, and placed on top of the nitrocellulose filter, and both were sandwiched between the 6 pieces of Whatman paper, and all were placed between the electrodes of the transfer apparatus (SDS-Page towards the cathode).

3- Electricity was connected, and the current was applied at 11mV for 24 hours.

4- The nitrocellulose membrane was removed.

2.2.3.3. Immunodetection

1- The removed nitrocellulose membrane (**2.2.3.2.**) was washed twice in deionized water and incubated for 1hour in blocking buffer (5% BSA in TBS + 0.5% Triton-X100) at 37°C, with gentle agitation.

2- The membrane was washed in washing buffer (0.5% Triton-X100 in TBS) for 20 minutes at 37°C, the procedure was repeated again twice (10 minutes each) after renewing the buffer.

3- The monoclonal antibody was added at a concentration of (1/500) in 15ml of TBS with 1% BSA, and incubated for 1hour at 37°C with gentle agitation.

4- Washing was repeated as in 2.

5- Secondary antibody, biotinylated $F(ab')_2$ fragmented sheep anti-mouse IgG (B4765, Sigma) was added at 1:2000 diluted in 15ml TBS with 1% BSA, and incubated for 1hour at 37°C with gentle agitation.

6- Washing was repeated as in 2.

7- The membrane was incubated in tertiary antibody (monoclonal anti-sheep/goat peroxidase linked, Sigma) diluted to 1:3000 in approximately 15ml TBS with 1% BSA, for 1hour at 37°C with gentle agitation.

8- Washing was repeated as in 2 using distilled water.

9- The membrane was developed and photographed using ECL reagent as per manufacturers instructions (RPN2105 Amersham).

10- The MW standards were used to identify the molecular weight(s) of the developed bands.

PATIENT DIARY

Center Number	Patient Number	Patient Initials
[illegible]	960	ZSC

Please fill in this diary daily. Bring it with you when you next see the doctor.

Please fill in the date and mark which tablet you took (x)

If you missed one tablet please mark with 0.

Please mark your bleedings as:

0 = no bleeding
1 = spotting or slight
2 = normal or moderate
3 = heavy

(K.240 5000/92)

at NW

Date	11/..	12/..	13/..	14/..	15/..	16/..	17/..	18/..	19/..	20/..
Day of cycle	1	2	3	4	5	6	7	8	9	10
Tablet taken	✓	✓	✓	✓	.	✓	✓	.	.	..
Tablet missed										
Bleeding	0	0	1	2	2	1	0	0	0	0

Date	21/..	22/..	23/..	24/..	25/..	26/..	27/..	28/..	29/..	30/..
Day of cycle	11	12	13	14	15	16	17	18	19	20
Tablet taken	✓	✓	✓	✓	-	✓				
Tablet missed							✓	✓	.	.
Bleeding	0	0	0	0	0	0	0	0	0	0

Date	31/..	1/11	2/..	3/..	4/..	5/..	6/11	7/11
Day of cycle	21	22	23	24	25	26	27	28
Tablet taken								
Tablet missed	✓	.	✓	✓	..	✓	.	.
Bleeding	0	0	0	0	0	0	0	0

NEXT VISIT (DATE): 8.11.94 at 345

Patient Diary checked by the Investigator

Signature [signature] Date 8/11/94

Figure (2.1.)

Diary cards used in this study were simplified to ensure compliance and reduce chances of error, data was verified when women attended their regular check-up.

Criteria	No. of women
High œstradiol (>30pg)	**9**
Changed her mind re participating	**6**
Age below or above specified	**3**
Lost to follow up	**3**
Administrative	**1**
11 months postmenopausal	**1**
Entry biopsy not performed	**1**
Contraindication to cHRT	**5**
Elevated liver enzymes	**2**
Developed thrombophlebitis	**1**
Bowel cancer	**1**
Total	**33**

Table (2.2.)

Reason for not being enrolled into the study following screening.

Primary reason for withdrawal	Total	%
Period related	6	**4.5%**
Heavy bleeding	*5*	
Not want periods	*1*	
Side effects	11	**8.3%**
Fluid retention	*3*	
Lack of efficacy poor symptom control	*3*	
Skin rash whilst on therapy	*1*	
Breast tenderness	*1*	
Depression	*1*	
Dysmenorrhea	*1*	
Headaches	*1*	
Administrative	8	**6.1%**
Declined end biopsy	*3*	
Poor compliance	*3*	
Lost to follow up	*1*	
Changed address unable to attend	*1*	
Other	3	**2.2%**
Breast cancer	*1*	
Superficial thrombophlebitis	*1*	
Widespread cancer	*1*	
Total	**28**	**21.3%**

Table (2.3.)

The primary reason for withdrawal after enrolment into the study (percentage calculated from total number enrolled). Only 4.5% withdrew because of bleeding related problems.

Feature	Mean
Age	53.4 years (range 44.2-61.5)
Parity	2.3 (range 0-5)
Weight	67.9 kg (range 48-96)
Height	160 cm (range 144-177)
BMI (kg/m^2)	26.3 (SD=4.3)
Duration of postmenopause	40 months[1] (range 12-240)

Table (2.4.)

Characteristics of the 103 women who completed 6 months on cHRT.
[1] Median

Endometrial histology	Pre-enrolment	Final biopsy	Timing of final biopsy			
			>day 27	Day 27-29	Day 30-32	Other
Insufficient	20	8	1	6[1]	1	0
Atrophic	54	1	0	0	0	1[2]
Inactive	23	4	0	4[3]	0	0
Secretory	0	89	9	75	2	3[4]
Proliferative	6	1	1[5]	0	0	0
Total	**103**	**103**	**11**	**85**	**3**	**4**

[1] Two of these also exhibited endocervical epithelium
[2] Stopped treatment prior to biopsy
[3] Non bleeders
[4] Within 2 days of day 28 but cannot be exactly identified
[5] Taken on day 13 of the cycle

Table (2.5.)

Histological classification of the biopsies obtained at the beginning and at the end of the study. At the end of the study there was only one proliferative endometrial sample, and this was take during the œstrogenic phase. All adequate samples that were optimally timed exhibited secretory endometrium. There were no cases of endometrial hyperplasia or cancer.

Bleeding Pattern Classification	No.	Timing of biopsy in relation to day of bleeding in biopsy cycle	No.	Histological assessment	No.
All Biopsies (n=103)					
Early Bleeders	49 (*)	After onset	32	Insufficient	6
				Secretory	26
		Before onset	15	Insufficient	1
				Secretory	14
Late Bleeders	50	After onset	4	Insufficient	0
				Secretory	4
		Before onset	43	Insufficient	1
				Secretory	42
		Not known	3	Secretory	3
Non Bleeders	4	Not applicable	4	Inactive	4
Day 27-29 Biopsies (n=85)					
Early Bleeders	39	After onset	28	Insufficient	5
				Secretory	23
		Before onset	11	Insufficient	1
				Secretory	10
Late Bleeders	42	After onset	3	Insufficient	0
				Secretory	3
		Before onset	39	Insufficient	0
				Secretory	39
Non Bleeders	4	Not applicable	4	Inactive	4

(*) Includes one biopsy performed on day 13 of cycle (proliferative endometrial histology) and one patient who terminated treatment prior to biopsy (atrophic endometrial histology).

Table (2.6.)

The histological classification of the endometrium from women on cHRT in relation to the bleeding pattern. Of the early bleeders, 32 biopsies were obtained after the onset of bleeding and of these, all the 26 samples that were sufficient for histological examination exhibited secretory features. Of the biopsies taken between day 27-29 in the early bleeders group (n=39), 11 were obtained before the onset of bleeding, 10 of which were secretory. In the late bleeders group, 39 biopsies were obtained before the onset of bleeding, of these 33 were secretory.

Chapter 3

Endometrial bleeding pattern

3.1. Introduction

Hormone "withdrawal" bleeding characterises cyclical combined HRT regimens (cHRT). In order to establish the value of bleeding as a marker of the adequacy of the hormone regimen, the bleeding pattern on cHRT was examined and linked to endometrial histology, and to the patients' demographic characteristics. The bleeding pattern was also analysed with reference to the pattern experienced by women in the natural cycle.

3.1.1. The bleeding pattern in the normal menstrual cycle

The variability in the length of the normal menstrual cycle is well recognised (Table 3.1.). A large study (206) on 30655 cycles recorded by 2316 women reported an overall mean & SD of 29.1±7.46 days. Cycles between 15-45 days long accounted for 95% of these and had a mean & SD of 28.1±3.95 days. The highest variability in cycle length was for women <25 years old, and the lowest between the ages 35-39, and only 13% of women had a range of cycle length of less than six days. 12.7% of women with spontaneous cycles have 'irregular' cycles (defined as those in whom no median cycle length could be defined over one year of observation), and a maximum variation of more than 14 days occurs in 30% of 'regularly' menstruating women (207). Many factors including weight, physical activity, and stress influence cycle length (208). The reported wide variability persists even after anovulatory cycles and early miscarriages are taken into account (209).

3.1.2. The duration of the luteal phase

The wide variability in the length of the menstrual cycle is not matched by variability in the length of the luteal phase, which is consistently close to 14 days (mean & SD, 14.13±1.41 days) (210). This is dictated by the life span of the corpus luteum (7 days), and by the length of time from the onset of corpus luteum demise till the onset of menstruation. The small range of variability here, indicates that the pre-determinants of menstruation in the physiological

cycle are precisely controlled, although the range of intra-individual variability remains unknown.

Very few factors are known to affect the duration of the luteal phase. Strenuous exercise, possibly confounded with weight loss (211), and young and old age (between 18-24 and 45-50 years) (210), are associated with a short luteal phase. This contrasts with the overall increase in cycle length associated with these factors (208, 212). Thus, the wide range of variability in the natural cycle is mostly accounted for by variability in the duration of the follicular phase.

3.1.3. Bleeding on oral contraceptive pills

The hormone withdrawal bleeding cycles induced by high dose (50µg ethinyl œstradiol/day) oral contraceptive pills are more regular than those observed in natural cycles, and are also characterised by being lighter and by a low occurrence of breakthrough bleeding (213). Cycles in users of low dose preparations (30-35µg ethinyl œstradiol/day), are less well controlled, and may be missing in 0.6-8% of women (213). Cycle control is also influenced by the type of progestogen although the distinction is not as clearly defined (214).

3.1.4. Bleeding on cHRT

It is not possible from available data to fully explain the reported discrepancy in cycle length within a regimen (160) or between regimens (159). These differences may be attributable to the different œstrogenic or progestogenic components, or to both, however, research focused on the latter. Little information is available, but one study reported that women taking 'stronger' progestogens start to bleed later compared to those receiving 'weaker' progestogens (79). Cyclic medroxyprogesterone acetate (MPA) added to œstradiol valerate (EV) results in shorter cycles compared to desogestrel and EV(184). Also, withdrawal bleeding started after 3.1±2.2 days of the end of 250 µg levonorgestrel (LNG), and 0.4±3.2 days after the end of 10mg MPA, when each was added for the last 10 out of 21 days of 2mg EV (215).

The intra-individual variability which causes low compliance (**1.9.2**), cannot be explained based on available evidence. A higher incidence of proliferative endometrium in women with short cycles, was reported in one study (79), but

not others (80, 184). Findings that have been reported include proliferative endometrium in women with regular cycles (79), and the absence of histological differences between the groups that had long or short cycles (184, 215), these are difficult to explain.

3.1.5. The duration of bleeding

There is very little research into the variations in the duration of menstrual flow in natural cycles, and only few studies on the duration of bleeding on oral contraceptives (216). The average duration of bleeding in the natural cycle is about 5 days, with 90% of cycles falling within the range of 2-7 days (217). Geographical (218, 219) and age related (220) variability have been reported. The duration of bleeding is also influenced by body weight and by other factors that influence the length of the cycle (208, 221). No work has addressed the duration of cHRT induced bleeding.

3.2. Aim: Bleeding as a marker of endometrial function

Bleeding represents the clinical manifestation of steroid action on the endometrium. Steroids also affect endometrial histology, and - at least in the physiological state - these two actions are linked. Evidence of this link in postmenopausal women treated with cHRT, is strong, but may not be uniform. The question raised is whether the same, or different, histological features are linked to bleeding in cHRT as those that precede menstruation in the physiological cycle.

3.3. Material and methods

3.3.1. Study population and regimen

The study population was described previously **(2.1.1).** All women received a cHRT **(2.1.1.)**, and kept daily records of bleeding and of the intake of tablets **(2.1.1)**. The final assessment visit was planned to be during day 27 or 29 of cycle 6, when an endometrial biopsy was performed.

3.3.2. Bleeding episodes: records and description

The following definitions were used:

- **Spotting**: any vaginal blood loss not requiring sanitary protection.
- **Bleeding**: any vaginal blood loss requiring the use of such protection as pads or tampons.
- **A period**: is at least two consecutive days during which blood loss (bleeding or spotting) was entered on the calendar record and that was bounded by more than one bleeding/spotting-free day.
- **Breakthrough bleeding**: any bleeding or spotting episode that occurred between consecutive periods and that was separated from the period days by more than one bleeding/spotting-free day.

For the purpose of this analysis, the day of commencement of the œstrogen was taken as the fixed point of reference. Thus a cycle starts on the day of commencement of œstrogen till the day of onset of bleeding, and the length of the progestogenic phase was calculated from the day of commencement of progestogen till the day of onset of bleeding.

3.3.3. Analysis of bleeding

In order to study the behaviour of each woman, data will be analysed by taking 'each woman' as the unit of analysis (222), considering the number of women with a particular cycle length or bleeding duration. Data will also be analysed using the 'cycle' as the unit, presenting data as the percentage of cycles with a given length, duration etc. This approach is favoured to the alternative of using the reference period methods (223), which do not yield themselves to longitudinal study of cyclicity.

3.3.4. Sample size

The sample size and statistical analysis was referred to previously (**2.1.7.**).

3.4. Results

3.4.1. Patient description

The characteristics of the 103 women who completed the six months on cHRT has been detailed previously (**2.1.1.** and Table 2.4.).

The characteristics of bleeding divided the women into three groups. Four patients did not experience any bleeding and were termed 'non-bleeders' (NB), 49 women had a mean cycle length of <29 days, termed 'early bleeders' (EB) and 50 women bled on or after day 29, termed 'late bleeders' (LB) (**3.4.2.**). Their demographic characteristics are detailed in Table (3.2.). There was no statistically significant difference between early bleeders, and late bleeders, in respect to the duration of the menopause, weight, height, BMI, systolic or diastolic blood pressures or in the number of cHRT preparations they used in the past.

3.4.2. The bleeding pattern

The last cycle for all patients was excluded in order to avoid over representing short cycles. There were 475 completed cycles, data was missing in 8 cycles and in 32 instances there was no bleeding, 20 of which were from 4 women. The frequency distribution of the duration of the luteal phase in all individuals is demonstrated in Figure (3.3.) The mean length of the luteal phase in all cycles was 12.7±2.62 days and the median was 13 days (range=1-23). Out of these 475 cycles, 171 (36%) commenced before day 13, and 304 (64%) commenced on or after day 13.

The frequency distribution of the duration of bleeding in all individuals is demonstrated in Figure (3.4.). The mean duration of bleeding in all cycles was 5.9±2.33 days and the median was 5 days (range=1-23). There was a negative correlation between cycle length and the duration of bleeding (Figure 3.5., Figure 3.6.). A mean onset of bleeding on day 29 or later was associated with less cycle to cycle variability (Figure 3.7., Figure 3.8.). The mean cycle length for those who bled before day 29 was 27.0±2.3 days, and for those who bled on or after day 29 was 30.4±1.3 days. The significant difference between the cycle lengths in the two groups was consistent when all the cycles were considered ($p<0.0001$, F value 161.7, repeat measurements ANOVA) (Figure 3.9.). Of the women who had a mean cycle length of <29 days (n=49), only 6 had a SD of <1. The mean cycle length in these 6 patients was 24, 24.8, 26, 26.6, 26.6, and 27 days. Thirty patients in this group had a SD of <2 days. Of the women who had a mean cycle length of 29 days or more (n=50), 39 had a SD of <1 day, 45 had a SD of <2 days, and 5 had a SD of >2 days. The mean cycle length of these 5 patients was 29, 29.2, 29.6, 29.8, and 31.4 days. Twenty nine out of this group had a range of variability between the shortest and the longest cycle of 0 or 1 days, and 10 had a range of two days, and 4 had

a range of 3 days. Seven out of the 49 women who bled early, had a range of variability of 2 or less days, and 11 had a range of 3 days.

EB exhibited a significantly longer duration of bleeding ($p<0.0001$, F value 19.702) and had a higher TotalBS than LB ($p<0.0001$, F value=22.3). These differences were consistent throughout the treatment cycles (Figure 3.9.). However, there was no significant difference in the AverageBS or in the incidence of spotting (3 in each group).

There was a significant difference between the standard deviations of the cycle lengths (a measure of cycle variability) of EB and LB (mean±SD 1.996 ±1.061 and 0.865±0.861 respectively, $p<0.0001$).

There were 26 women who smoked, 4 of whom did not bleed, 18 were EB and 4 were LB. Amongst the smokers, the average number of cigarettes smoked per day by EB was 16.4, by LB was 9.25 and by NB was 18. Excluding the non-bleeders, the mean cycle length for all smokers, (27.7±2.045 days), was shorter than for non-smokers (28.9±2.136 days), the difference was statistically significant ($p=0.0219$, Fisher exact test). EB as a group, smoked significantly more cigarettes than LB ($p<0.0002$). NB (n=4) smoked an even higher mean number of cigarettes per day, and the difference between NB and LB was statistically significant ($p<0.003$, Student *t*-test).

3.5. Discussion

Women who bled whilst using the same cHRT regimen responded in two distinct bleeding patterns : the first is the group of "late bleeders" who bleed at or later than day 29 of the cycle, their cycles were less variable, as demonstrated by the narrow standard deviation and by the small cycle to cycle variability. This group also bled for a shorter duration and had a lower TotalBS per cycle. The second group of "early bleeders" had cycles shorter than 29 days. These women exhibited a wide range of variability in cycle length, a longer duration of bleeding and a larger amount of blood loss. The two patterns of response point to differences in the mechanisms of cycle control. Whilst the first group is more reminiscent of menstrual bleeding in the physiological cycle, there is no obvious analogy to early bleeders, who bleed early and for a longer duration.

The wide range of biological variables that affect and contribute to the observed cycle variability in the natural cycle, are not operational in cHRT induced bleeding. Therefore, one cannot extrapolate a range of normal variability from natural cycles that could be applicable to cHRT induced bleeding. Indeed, it could be argued that cHRT induced cycles, in as far as they are drug regulated, should be under tighter control, and should be subject to a narrower range of variability both between individuals and within cycles of the same individual.

This study demonstrated that a shorter duration of bleeding characterises longer cycles, which may be a reflection of better hormonal balance, and contrasts with the more prolonged bleeding associated with short cycles. In contrast to studies on natural cycles, there were no significant differences in factors of weight, height, BMI, age or parity between EB and LB, which indicated that factors affecting cycle control in the two states are different.

The statistically significant difference in smoking habits, between LB and EB, and between LB and NB (albeit a small group) is striking. Women who smoke were shown to have an earlier menopause (224, 225). The lower incidence of breast cancer, and of endometrial cancer (226, 227), and the higher risk of osteoporosis (228) in smokers, have been attributed to antiœstrogenic properties of cigarette smoking and may be a reflection of a lower level of circulating œstrogen (229). The "œstradiol-2-hydroxylation pathway", which irreversibly metabolises œstradiol to inert catechol œstrogens, has been demonstrated to be higher in smokers as compared to non-smokers and has been proposed as a possible underlying factor for the hypo-œstrogenic state (230). Altered œstrogen metabolism has also been demonstrated in postmenopausal smokers receiving HRT (231). The correlation between cycle characteristics and the smoking habits may reflect the significance of the œstrogenic priming of the endometrium in relation to the day of onset of bleeding in cHRT regimens. Relative hyperandrogenism has been proposed as an alternative mechanism underlying the antiœstrogenic properties of smoking (232). The influence of this, if any, on the bleeding pattern is unknown.

The lower than expected drop-out rate attributable to bleeding in this study was fortunate, as it reduced the effect of selection bias. This allowed a unique opportunity to investigate the endometrial response in a model that combined menstrual-like control with early bleeding. This response appeared to be patient specific and may be a reflection of a critical hormonal balance.

A key observation is that patients using the same cHRT regimen had a different response to treatment, and the study suggests a different end organ response, and/or possibly different effective hormone levels.

Study	Age group	Mean cycle length (days)	SD of cycle length (days)
Treloar et al.(212)	30	29.3	3.2
Chiazze et al.(206)	25-29	29.4	6.5
Vollman (233)	30	29.6	5.9

Table (3.1.)

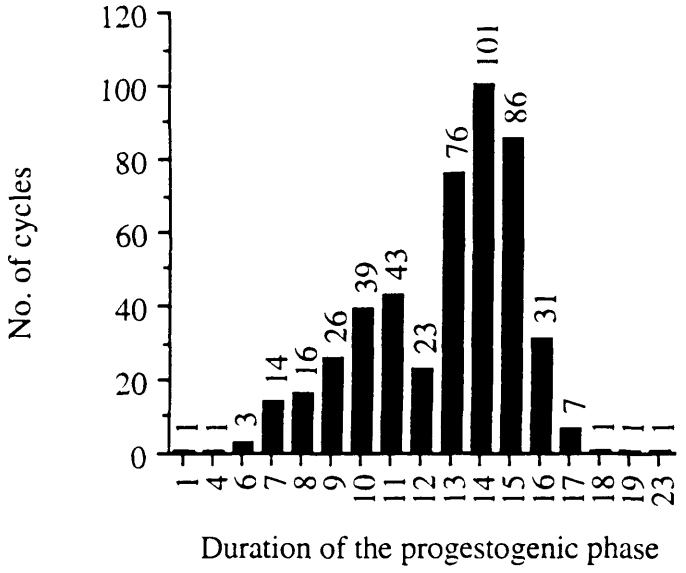
The mean and SD of cycle length in young women, quoted in different studies. The normal menstrual cycle is characterised by a wide variability in cycle length as demonstrated by the wide standard deviation, although the overall mean approaches the 'typical' 28 day cycle.

	Study population groups Mean (SD)			
Characteristic	Late-bleeder (n=50)	Early-bleeder (n=49)	Non-bleeder (n=4)	*P*
Age	54.3 (44.2-61.5[1])	52.7 (46.4-60[1])	52.8 (51-56.3[1])	
Weight (kg)	67.9 (10.5)	68.5 (13.6)	60.7 (10.2)	0.81
Height (cm)	159.2 (6.8)	161.4 (6.32)	160.2 (7.6)	0.2
BMI	26.7 (3.8)	26.2 (4.8)	23.5 (2.2)	0.55
Systolic BP	135.9 (17.1)	132.9 (17.3)	105 (17.3)	0.39
Diastolic BP	79.7 (8.8)	80.2 (9.4)	69 (8.1)	0.8
Pulse	72.5 (9.0)	71.4 (7.6)	64.5 (5.2)	0.51
Pervious cHRT [2]	0.9 (0-3[1])	0.9 (0-3[1])	1.2 (0-2[1])	
Months since LMP	57.8 (16-160[1])	52.3 (8-240[1])	45.7 (18-60[1])	
No. of cigarettes /day	0.74 (3.2)	6 (9.2)	13.7 (12.5)	0.0002
Duration of bleeding (days)	5.03 (1.57)	6.53 (3.0)	N/A	
Total bleeding score[3]	7.89 (3.11)	11.05 (4.6)	N/A	
Average bleeding score[4]	1.6 (0.94)	1.58 (0.33)	N/A	

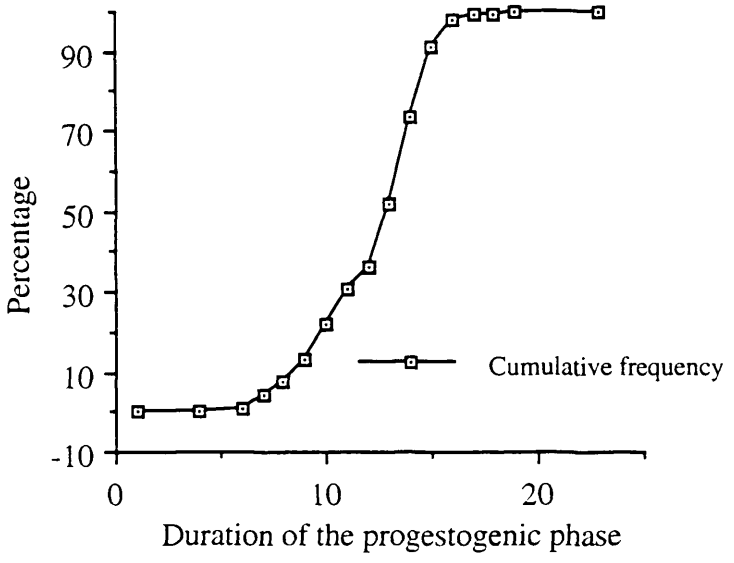
Table (3.2.)

Demographic and cycle characteristics of patients who had short cycles i.e. <29 days (early-bleeders), long cycles ≥29 days (late-bleeders), and the non-bleeders who experienced no bleeding on this regimen. The only statistically significant difference between the groups was their smoking habits.

[1] Range.
[2] Number of preparations used in the past.
[3] The sum of the bleeding score per bleeding episode.
[4] The bleeding score per episode divided by the duration of bleeding.
P value is the difference between early and late bleeders.
N/A not applicable.



A



B

Figure (3.3.)

The frequency distribution of the duration of the progestogenic phase in all cycles where bleeding occurred over the 6 months on cHRT, excluding the sixth cycle (**A**), and the cumulative frequency of the duration of that phase. The data suggest the presence of a subgroup with shorter cycles.

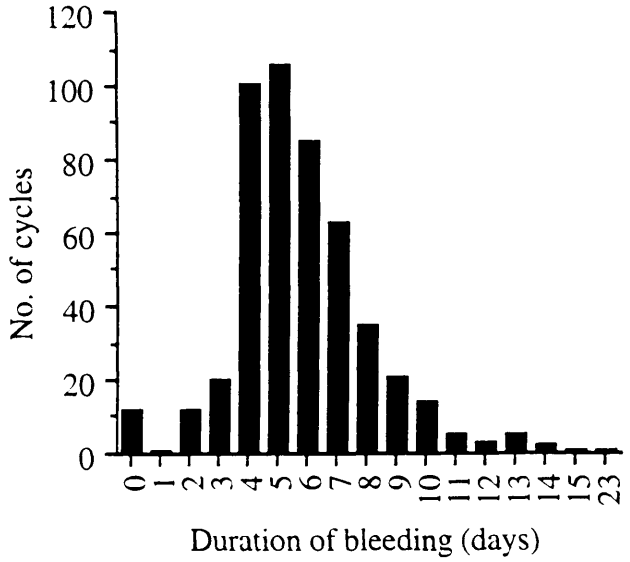


Figure (3.4.)

The frequency distribution of the duration of bleeding in the first five cycles of all except the non-bleeders. 74.5% of women bleed for between 4-8 days.

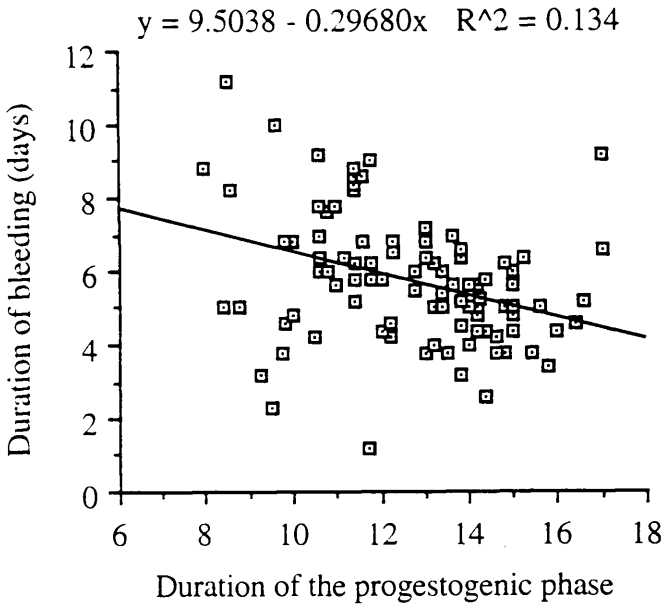
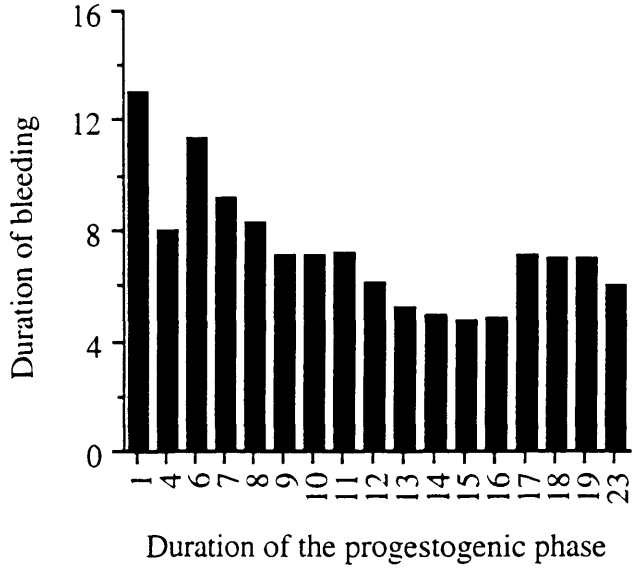
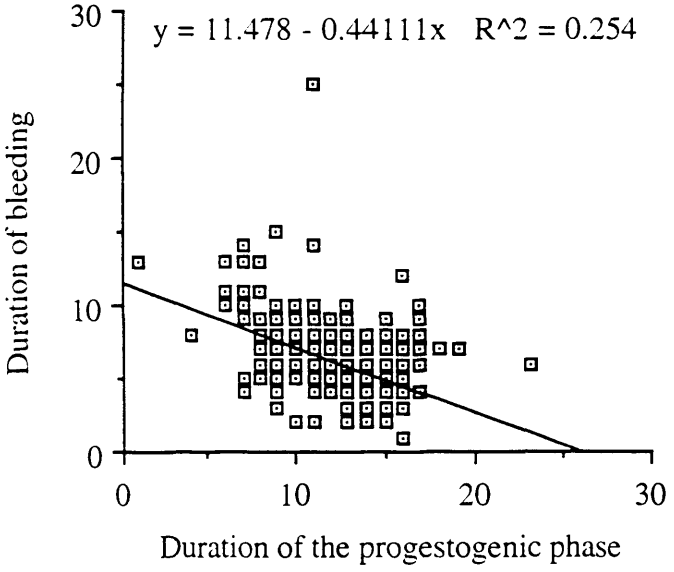


Figure (3.5.)

The correlation between the duration of the progestogenic phase and the duration of bleeding for each woman. Women whose bleeding commenced later had shorter duration of bleeding.



A



B

Figure (3.6.)

The relation between the length of the progestogenic phase and the duration of bleeding for all cycles examined.
A- Early onset of bleeding is associated with longer duration of blood loss.
B- The correlation between the duration of the progestogenic phase and the duration of bleeding in each cycle.

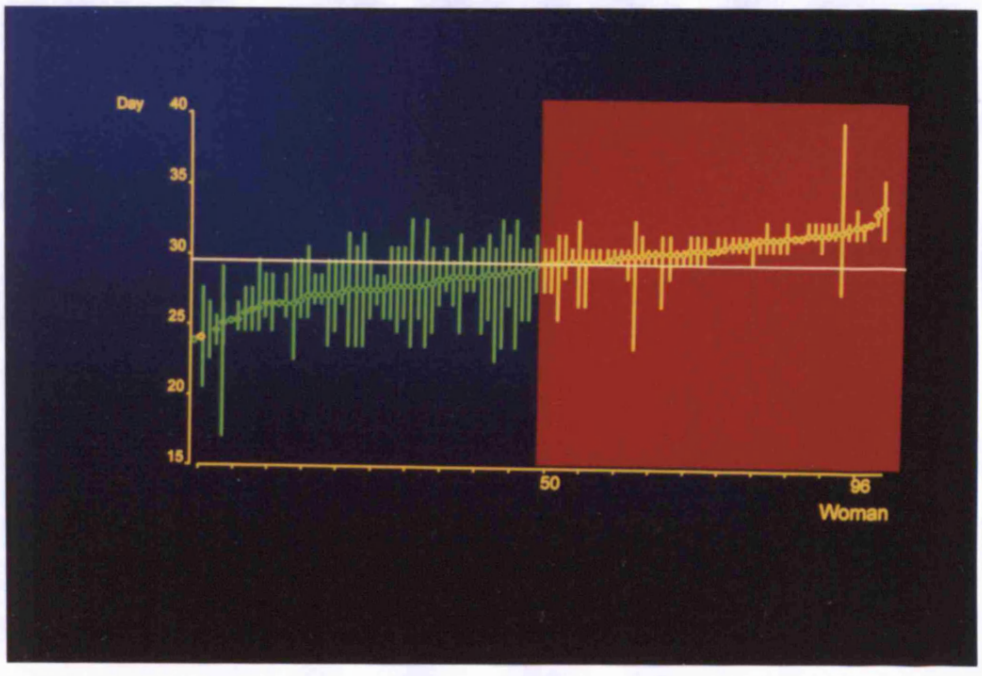


Figure (3.7.)

The relation between the mean cycle length for each woman and her individual range of cycle lengths. A mean cycle length of 29 days divides these women into two groups: those who had shorter cycles (<29 days) and who had larger variability, and those who had longer cycles (≥29 days) and who had smaller variability.

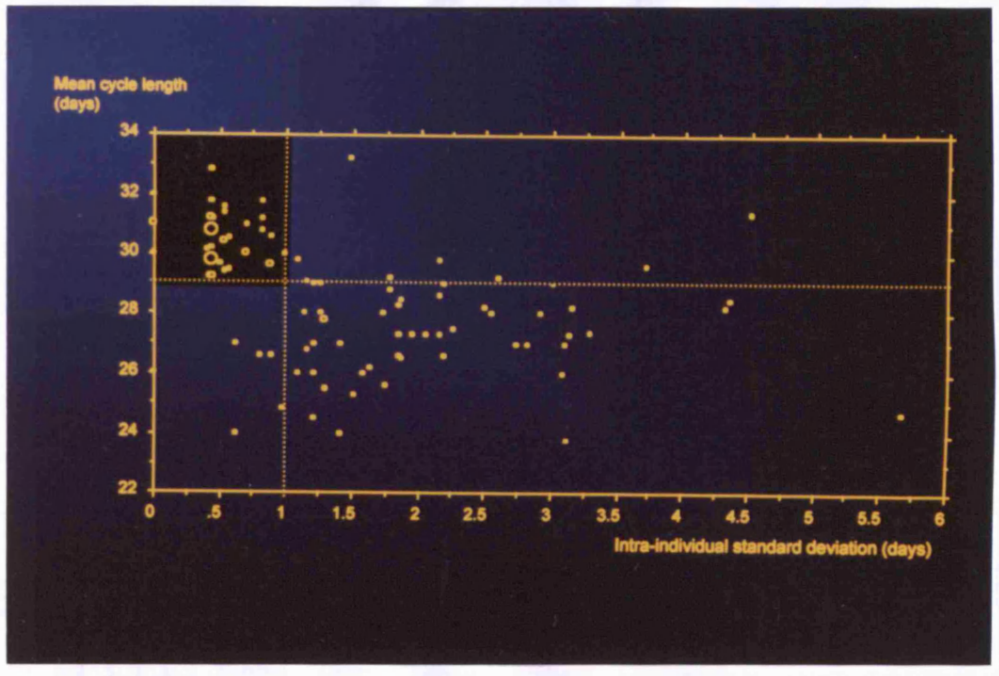


Figure (3.8.)

The relation between the mean cycle length and the individual standard deviation. Women who had long cycles (≥29 days) had smaller variability in cycle length compared to those who had short cycles (<29 days).

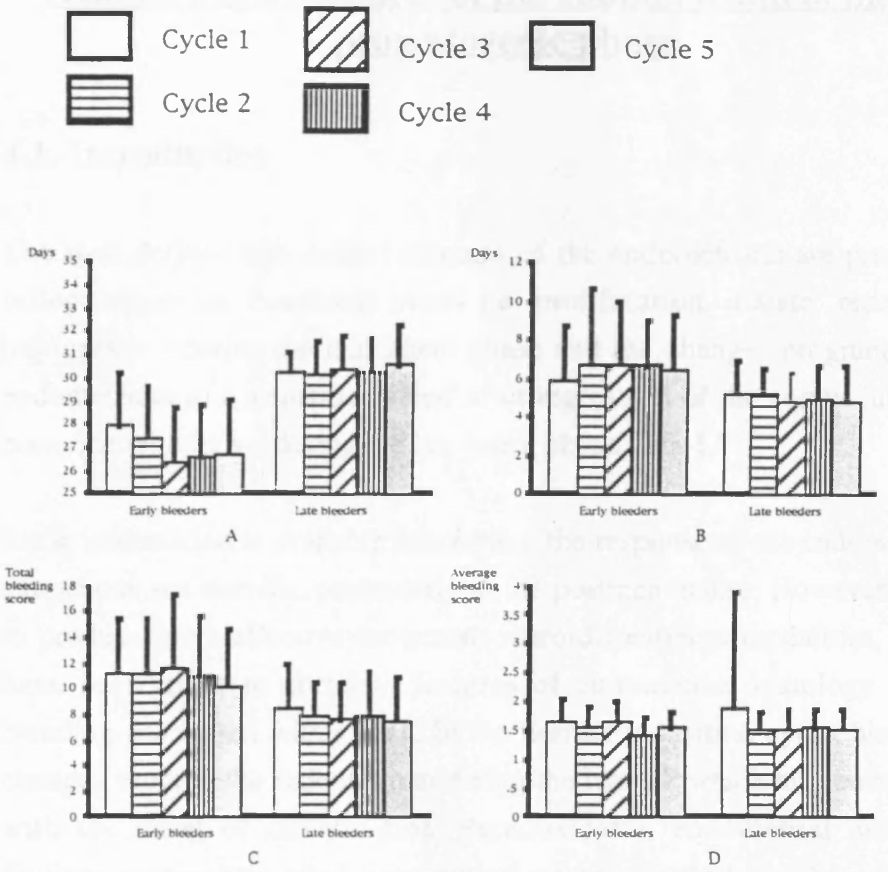


Figure (3.9.)

The relation between: (**A**) cycle length, (**B**) the duration of bleeding, (**C**) the TotalBS, and (**D**) the AverageBS in each treatment cycle for both EB and LB. The differences between EB and LB was consistent throughout the treatment cycles, and there were no significant differences within each group from cycles 1-5.

Chapter 4

Histological assessment of the endometrium in the late progestogenic phase

4.1. Introduction

The well defined histological changes of the endometrium are presumed to reflect sequential functional states i.e. proliferation, a state 'receptive' to implantation during the mid-luteal phase and the changes programming the endometrium to a menstrual 'bleed' after regression of the corpus luteum and hormone withdrawal during the late luteal phase (late-LP).

Little information is available concerning the response of the endometrium to exogenous sex steroids, particularly in the postmenopause. However, in order to produce more effective exogenous steroid treatment modalities, attempts have been made to correlate features of endometrial histology with the bleeding associated with cHRT. In the normal menstrual cycle, histological changes occur in the endometrium during the late-LP which may be associated with the onset of menstruation. Paradoxically, endometrial histological findings spanning the whole spectrum of atrophic, inactive, proliferative, early and late secretory and hyperplasia have been reported in women during the late progestogenic phase whilst on cHRT and exhibiting apparently normal bleeding patterns (79, 80). It is possible that histological changes in late-LP of normal menstrual cycle are not necessarily linked with bleeding, or that the mechanisms of bleeding associated with exogenous steroids in the dose ranges used in cHRT is different from those of the menstrual cycle.

Chapter (3) explored the bleeding pattern in women on a cHRT regimen (Reference 371). Here, the endometrial biopsies taken from the same group during the late progestogenic phase will be examined, and compared to biopsies obtained during the LP of the normal cycle.

4.1.1. Histology of the normal endometrium and under cHRT

Has been described previously (**1.11., 1.12.3.4.**).

4.2. Aim of the study

The aim of this work is to determine whether features could be identified that are predictive of subsequent endometrial behaviour (i.e. bleeding), and to ascertain whether histological differences may underlie the different clinical response. If differences were to be identified, they will be useful for monitoring women's response to cHRT. The study will also clarify the role of endometrial morphology in the assessing the response to cHRT, and may thus have implications on the utility of the criteria traditionally used in endometrial assessment when applied to cHRT treated endometrium.

4.3. Material and Methods

4.3.1. Study population and regimen

The characteristics of study and control group, and the schedules used have been described previously (**2.1.1., 2.1.2.**).

Five groups were compared:

1- Late bleeders: The first ten biopsies from the LB group and from whom the biopsies were obtained between day 27-29 and before the onset of bleed in the sixth treatment cycle.
2- Early bleeders: The ten biopsies from the patients in the EB group from whom the biopsies were obtained between day 27-29 and from whom the biopsies were obtained before the onset of bleed in the sixth treatment cycle.
3- Early luteal phase biopsies from day 2-6 of the LP of the physiological cycle (n =10).
4- Mid luteal phase biopsies from day 7-11 of the LP of the physiological cycle (n =10).
5- Late luteal phase biopsies from day 12-14 of the LP of the physiological cycle (n =10).

4.3.2. Endometrial assessment

The biopsies were assessed qualitatively using the standard criteria (234), and quantitatively/semi-quantitatively.

4.3.2.1. Feature analysis (Quantitative assessment)

Biopsies were examined to assess each histological feature, many of which are used in the classical descriptions for endometrial dating (70). The following features were examined using image analysis, as described previously (**2.1.6.**):

1- Epithelial height was measured and, the percentage of the luminal and of the glandular epithelium lined by each type of epithelium (cuboidal, low columnar, columnar, and pseudostratified columnar) and the percentage showing apical or basal vacuoles was estimated in 17 random hpf(x400), and the mean for each section was calculated.
2- The glandular diameter of 17 randomly selected glands was measured per section (x200) and the measurements converted to metric scale using a measurement grid.
3- The proportion of tubular and convoluted glands was calculated in a random selection of 20 glands per section.
4- The glandular density was counted in 17 random hpf(x400) per section.
5- The proportion of glands (out of 20 glands) containing luminal secretions was counted and the amount of secretion was graded using a semi-quantitative scale as minimal=1, moderate=2, and abundant=3. The amount of secretion was multiplied by the proportion of glands that was positive to obtain a 'Secretion Score'.
6- The percentage of glands containing glandular invaginations (telescoping) was counted in the whole section.
7- The glandular apical margins were described as either smooth or irregular and the percentage exhibiting each feature was estimated in 20 glands using high power microscopy (x400).
8- The stromal cellular density was calculated by counting the number of nuclei in 17 random hpf (x1000) per section under oil immersion.
9- The presence or absence of decidualization and whether this feature is present in the upper 1/3 of the stroma, is diffuse or whether it is only present in a peri-vascular location was described.
10- The number of leukocytes per section was assessed semi-quantitatively on a grade of: minimal/few=1, average=2, and heavy infiltrate=3.

4.3.3. Statistical analysis and sample size

Have been addressed previously (**2.1.7.**)

4.4. Results

4.4.1. Histological assessment

Of the 103 women who completed six months on treatment, 89 had secretory endometrium on final biopsy, and 75 of these were taken between day 27-29 of the treatment cycle. The four biopsies from non-bleeders were taken between day 27-29 and all had inactive endometrium. Of the 85 biopsies taken between day 27-29, 39 were from the early bleeders (EB), 42 were from the late bleeders (LB), and 4 were from non bleeders (NB) (Table 2.5, 2.6.). Biopsy material obtained prior to enrolment in 20 of the 103 patients who completed the study was insufficient for histological assessment and the endometrial specimens were assessed as either atrophic or inactive in 54 and 23 cases respectively, and only 6 were assessed as proliferative, and there were no secretory endometrial specimens. Of the biopsies obtained at the end of the study, 85 (82.5%) were obtained during days 27-29 of the biopsy cycle. The end biopsy material was insufficient for assessment in only 8 cases (2 of these included endocervical epithelium), and no cases exhibited hyperplastic or neoplastic changes. In 89 (86.4%) of patients who completed the study a secretory histology was recorded. Of the patients whose biopsies were taken on days 27-29 (n=85), all those who exhibited bleeding during the study period (n=81), and from whom sufficient endometrial tissue was obtained (n=75), had secretory endometrium.

Seventy five out of the 85 biopsies which were taken between days 27-29 exhibited secretory endometrium. Of these 33 were EB and 42 were LB. The pre-study biopsies of these 75 women exhibited atrophic endometrium in 41 cases, inactive endometrium in 15 cases and proliferative endometrium in 3 cases and was insufficient in 16 cases. Thus, in all (100%) these patients, the secretory endometrial histology was induced in response to cHRT. The pre-study endometrium in the four patients who did not experience withdrawal bleeding exhibited atrophic endometrium in one case and inactive endometrium in 3 cases.

4.4.2. Qualitative assessment

Although the majority of biopsies were classified as 'secretory' using the standard criteria, sections from cHRT treated women, which were classified as

such, were characterised by wide variability as regards the uniformity of the 'secretory' features. This was in contrast to the more uniform features exhibited by sections of secretory endometrial specimens obtained from the early-LP, mid-LP, and late-LP of the physiological cycle (Figure 4.1.-4.4.).

The glands featured variable development, some of which were well developed tubular or convoluted but others were of narrow and variable calibre. The epithelial lining exhibited a variety of secretory changes which ranged from glands with abundant supra-nuclear vacuolation to exhausted secretory changes, although these characteristics were poorly developed in the majority of cases. Most glands were lined with inactive or cubo-columnar epithelium. Few glands exhibited subnuclear vacuolation. Apoptotic bodies were sometimes seen. Glandular telescoping was noted in a large proportion (40%) of sections. In some cases glands appeared to be surrounded by an intense leukocytic infiltrate (Figure 4.4. a-i.).

The stroma featured small round or spindle cells, and although sometimes it exhibited well developed decidualization, this was often variable and sometimes absent. Stromal œdema or breakdown was apparent in some of the specimens. Vasculature was of narrow calibre sometimes with thin muscular wall but on occasions this was well developed. Dilated capillaries were often noted. Area to area dysynchrony as well as gland to stromal dysynchrony were seen to some extent in all sections examined. The presence of leukocytes in some sections was extensive but in others was difficult to distinguish (Figure 4.4. a-i.).

Because of these discrepancies and in order to distinguish it from the luteal phase, the late progestogenic phase of cHRT treated endometrium was coined the late '*pseudo*luteal' phase (late-PLP).

4.4.3. Quantitative and semi-quantitative assessment

The result of the quantitative and semi-quantitative analysis is shown in Tables (4.5.-4.10.).

Comparing the late-PLP between the EB and LB, showed no significant differences (Table 4.5.). Physiological cycle endometrium exhibited the expected cyclical changes (Table 4.6.). Since LB in whom biopsies were obtained before the onset of bleeding in the biopsy cycle and whose biopsies

exhibited 'secretory endometrium' represent the equivalent of the late-LP endometrium of the natural cycle, comparison was made between these two groups (Table 4.7.). The features seen in the endometrial epithelium, the glands and the stroma showed some differences. The luminal epithelium contained more cuboidal and pseudostratified and less low columnar and columnar cells. Glandular epithelial cells were also predominantly shorter, but contained more apical vacuoles. The glands were less numerous, and the total glandular area was smaller in cHRT treated endometrium. Individual glands were somewhat smaller on cHRT, but the difference was not statistically significant. The glands were also more tubular, less convoluted, and contained less luminal secretions, and the apical margins of the glandular cells were smoother than those found in the natural cycle. The stroma was less cellular than in the natural cycle, but there was no statistically significant difference in the degree of stromal œdema, haemorrhage or decidualization. Leukocytic infiltrate was significantly higher in the late-PLP.

Comparison between the LB in the late-PLP and the early-LP, mid-LP and late-LP (Table 4.7.,4.8.,4.9.), demonstrated that the late-PLP endometrium shared some features with the early-LP, e.g. the characteristics of the apical margins of the glandular epithelial cells, the incidence of pseudostratification in the luminal epithelium, and of low columnar cells in the glandular epithelium, and the gland number. Some features were shared with the mid-LP, e.g. the incidence of apical vacuoles in the glandular and luminal epithelium. On the other hand, the high incidence of cuboidal cells, and the low incidence of low columnar and columnar cells were unique to cHRT treated endometrium. The stroma under the influence of cHRT contained some of the features of the mid-LP, but mostly exhibited features of the late-LP. EB exhibited the same differences when compared to the late-LP, as the LB (Table 4.10.).

4.5. Discussion

Histological features induced by exogenous hormones acting on the endometrium are dependent on their type and dose. In relation to OCP these features are dissimilar to the physiological cycle, to the extent that histological dating criteria applicable to the normal cycle cannot be used. This study leads to a similar conclusion in relation to the late-PLP.

All adequate biopsies obtained between day 27-29, from women experiencing regular cycles, were secretory as assessed by the standard histological criteria, and in all cases where diagnosable biopsies were obtained, the endometrium exhibited a histological response to cHRT except in those four patients who did not bleed. The presence of endocervical epithelium in two samples suggests that these were obtained from the juxta-cervical and non-responsive isthmic portion, rather than absent hormonal response, and argues against the possibility of endometrium that does not respond histologically yet exhibits cyclical bleeding as implied in the previous publications (79, 80).

Previous attempts to determine the relation between endometrial histology and the bleeding pattern on cHRT reached divergent conclusions. A proliferative endometrial biopsy on day 6 of the progestogenic phase was reported to indicate bleeding on or before day 10, and a secretory endometrial biopsy correlated with late bleeding on or after day 11 (79), but a recent study found no correlation between histology on day 10.8 of the progestogenic phase and the occurrence of bleeding before or after day 11 (80). In the present study, endometrial biopsies obtained on day 11-13 of the PLP (cycle day 27-29) exhibited secretory features by the standard criteria, irrespective of cycle length, which supports the lack of correlation. However, it also reveals that histological assessment according to the classical definition may be inappropriate when applied to cHRT.

The absence of statistically significant differences between biopsies from EB and LB who had not bled by the time of the biopsy, demonstrates that the difference between the groups is in the statistical probability of short or long cycles, rather than a difference between 'early' and 'late' bleeders *per se*, or in end organ response. This difference may thus be related to extra-uterine factors e.g. steroid uptake and metabolism, which may also exhibit temporal variation. Since cycle variability translates to unpredictable bleeding and low compliance, identifying these factors has significant clinical implications. This similarity indicates that biopsies from both groups be taken together as representative of 'long cycles' on cHRT, which will be followed in the next chapters.

The endometrium from the LP, exhibited high homogeneity which reflects a uniform hormone effect, it also demonstrated the anticipated variations consistent with the postovulatory day. This contrasts with the cHRT endometria from the PLP which exhibited morphological heterogeneity within

each section, and which may represent poor control of endometrial proliferation and differentiation.

Histological features of late-PLP endometrium differed significantly from those noted in late-LP. The glandular component exhibited a mixture of features some of which are similar to the early-LP, mid-LP or late-LP, whilst others were unique and not normally found during the LP. The stroma exhibited relatively more advanced features. This supports the qualitative impression of glandular to stromal dysynchrony with cHRT, and suggests that there may be a disproportion between the doses and/or the effect of the œstrogen and the progestogen in this regimen (72). Comparing the histology from the group who experienced regular withdrawal bleeding whilst on cHRT to the endometrium of the late-LP, demonstrated that the main similarities were in the features of the stroma. This suggests that one or more of the stromal features may be important determinants of bleeding. It is interesting that endometrial samples obtained after the onset of bleeding on cHRT exhibited secretory features. This may be explained by patchy endometrial maturation/shedding, which, in turn, may be related to the fact that hormone administration in this regimen was continued to complete the 12 days of combined treatment irrespective of the occurrence of bleeding, and that the vast majority of these biopsies were obtained within 1-2 days after the onset of bleeding.

This study demonstrated the dissociation between the classical histological features and the occurrence of bleeding. This dissociation between morphology and function raises important questions concerning the utility of traditional histological classification in the assessment of the different states of the endometrium under cHRT. This conclusion is supported by the previously described discrepancy between endometrial histological features and immunohistochemical markers (235, 236). It also indicates the need to examine known correlates of function, and to discover new parameters. For, it is only if such correlates are known and measured can we adequately monitor current preparations and design new ones.

The hypothesis is thus advanced that functional markers may be more predictive of endometrial behaviour. In the following investigation, known functional markers in the different endometrial compartment - the glandular epithelium, the stroma, and the vasculature - will be examined in relation to endometrial behaviour and bleeding.

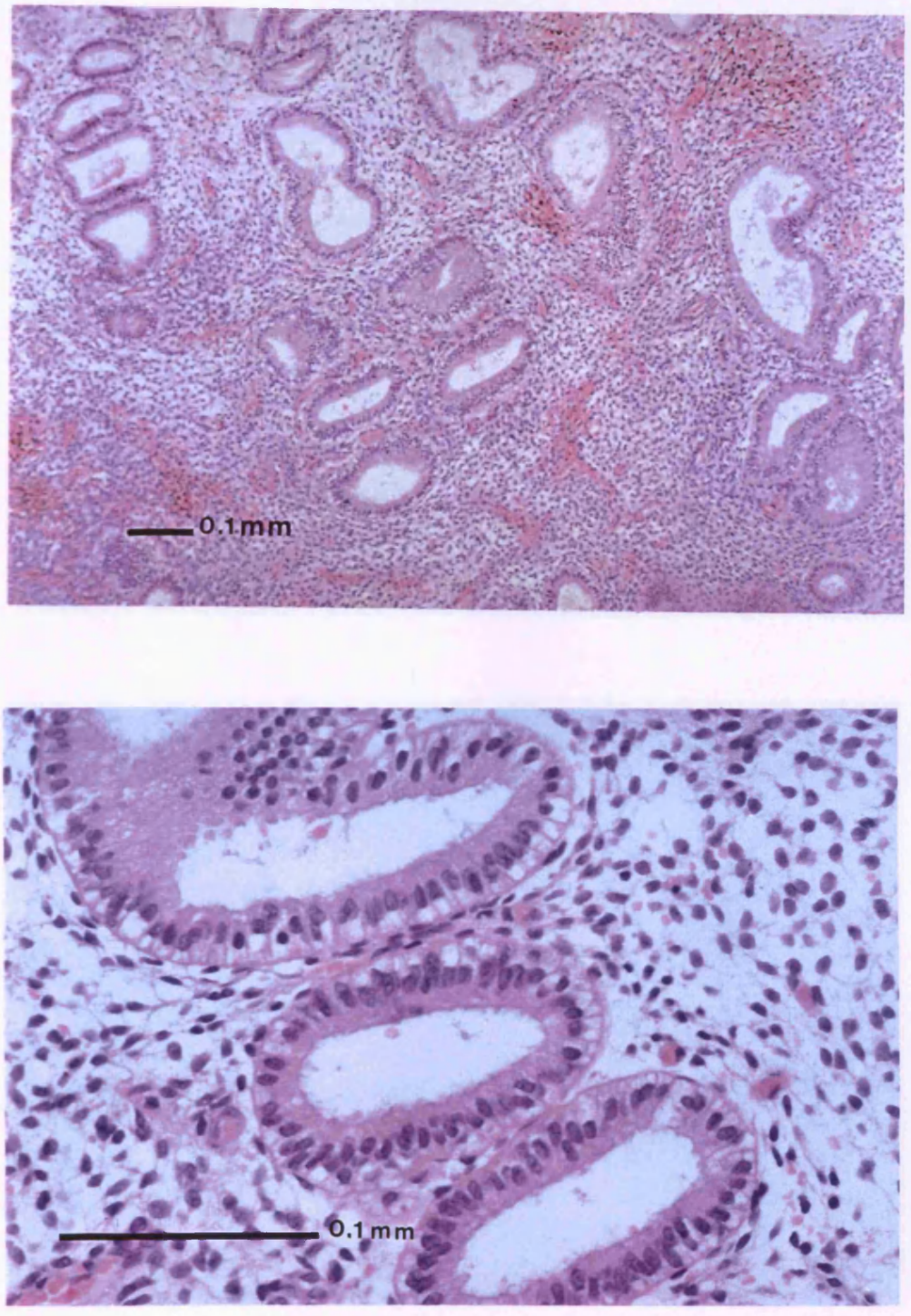


Figure (4.1.)

Hematoxylin-eosin stained section from the early-LP endometrium (low and high power), exhibiting uniform secretory features consistent with the phase of the cycle. Glands are tortuous and of narrow calibre. The stroma is abundant and loose with focal œdema (O). The glandular epithelium is formed of columnar cells that contain basal subnuclear vacuoles (arrow) which displace the nuclei from their basal position, (scale=0.1mm).

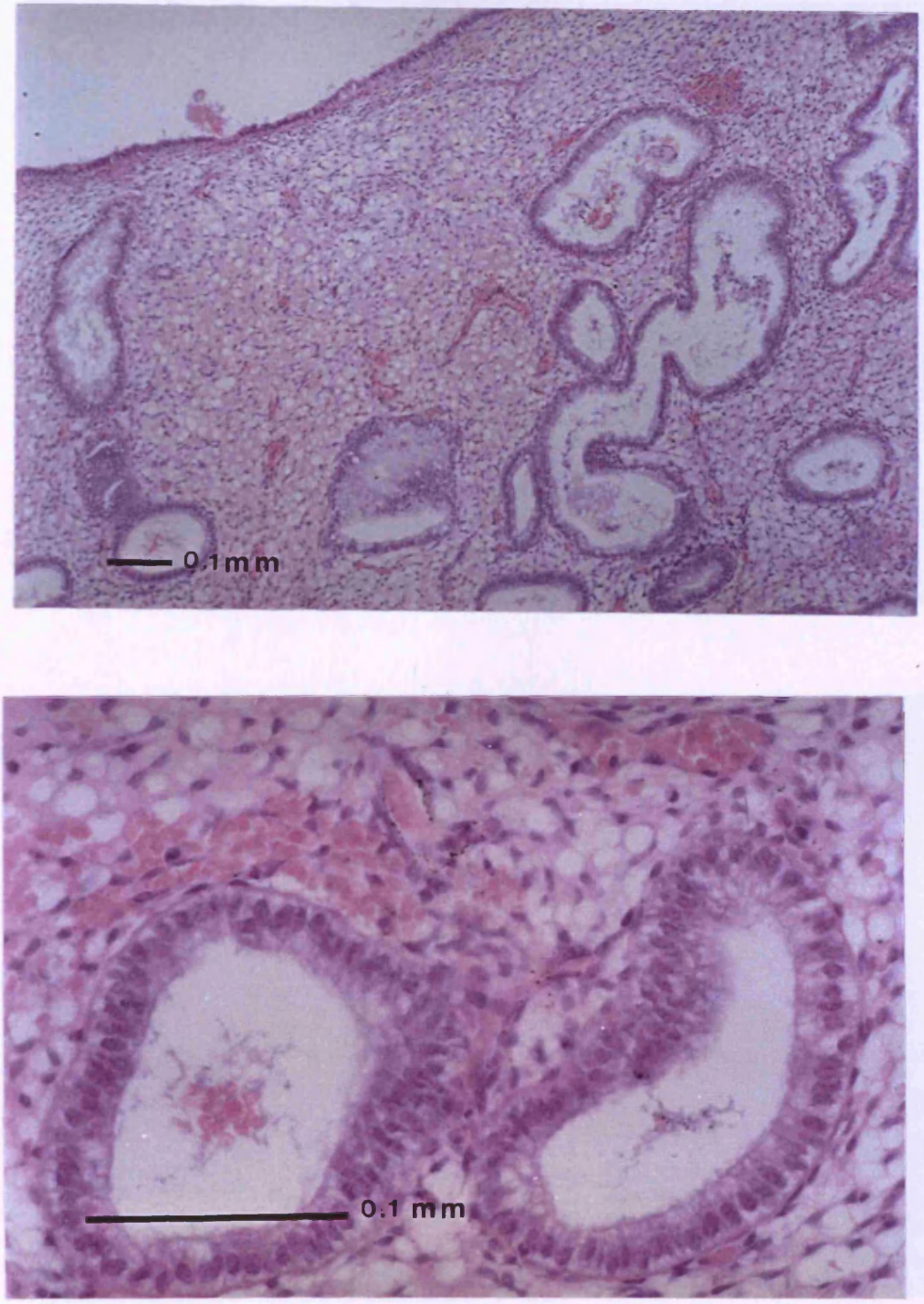


Figure (4.2.)

Hematoxylin-eosin stained section from the mid-LP endometrium (low and high power), exhibiting uniform features. The secretory vesicles shift towards the lumen displacing the nuclei towards the base of the columnar epithelial cells. The stroma is loose and exhibits the maximal degree of stromal œdema.

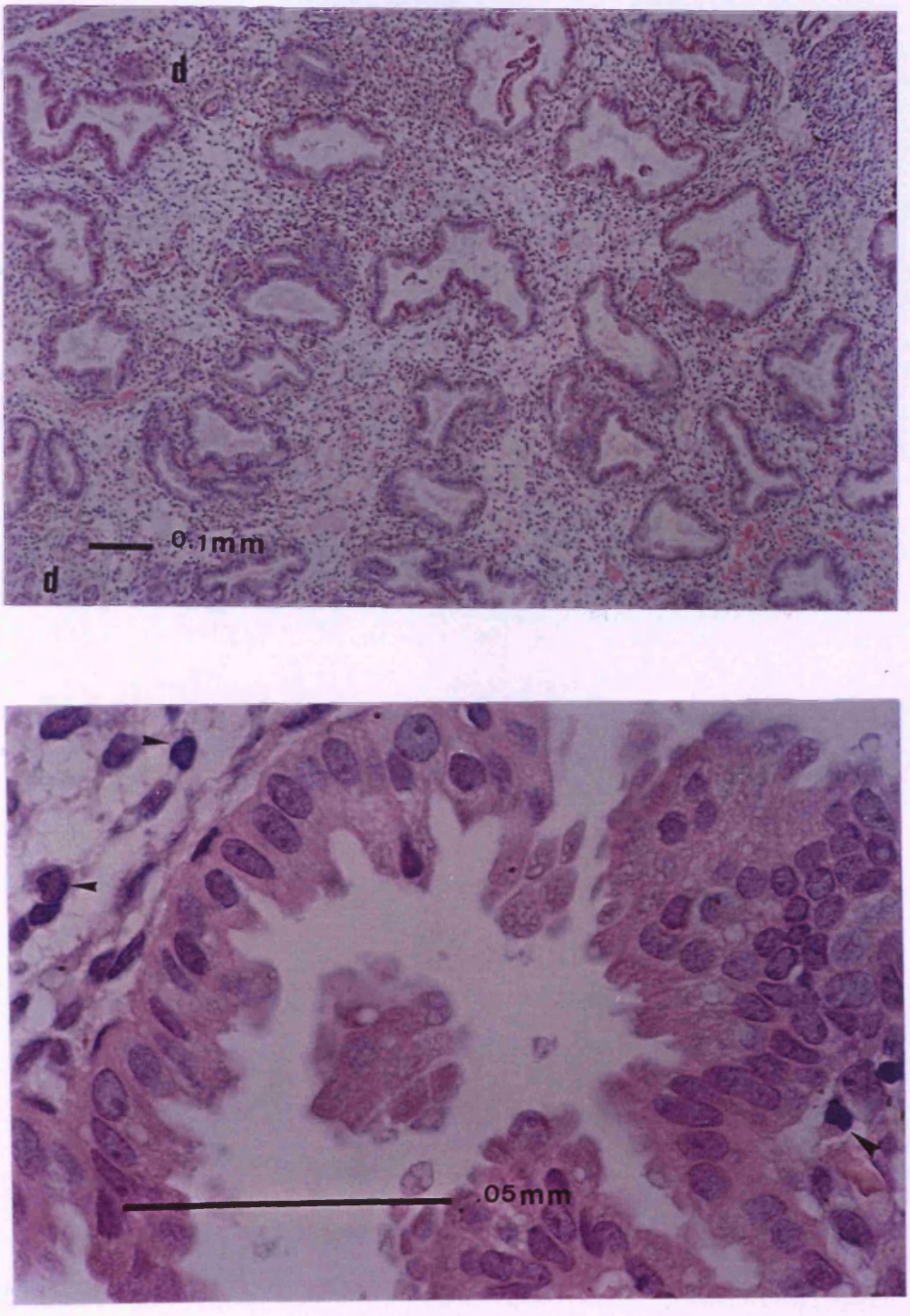


Figure (4.3.)

Hematoxylin-eosin stained section from the late-LP endometrium (low and high power), exhibiting exhausted glands. The glands are numerous and exhibit a saw toothed appearance. Secretory glands have a large basal nuclei with fine chromatin, cells lose their glycogen into the lumen. The stromal œdema regresses, and early predecidual reaction (d) appears. There is an increased leukocytic infiltrate (arrows).

Figure (4.4. a-i)

Hematoxylin-eosin stained sections from the late-progestogenic phase of cHRT endometrium (low and high power), exhibiting wide variability as regards the uniformity of the secretory features. Gland development is variable, a few are well developed tubular or convoluted but most are of narrow calibre. The glands are less convoluted, narrow, and less frequent with a reduced gland/stroma ratio. The majority of glands exhibit low columnar or cuboidal epithelium with minimal or low secretory activity, but a few have abundant supra-nuclear vacuolation or exhausted secretory changes. The stromal is dense with minimal œdema, decidualization is patchy, and there is excessive leukocytic infiltration

Some of the glands exhibit complex telescoping, and it is possible that this feature be a reflection of steroid imbalance (237).

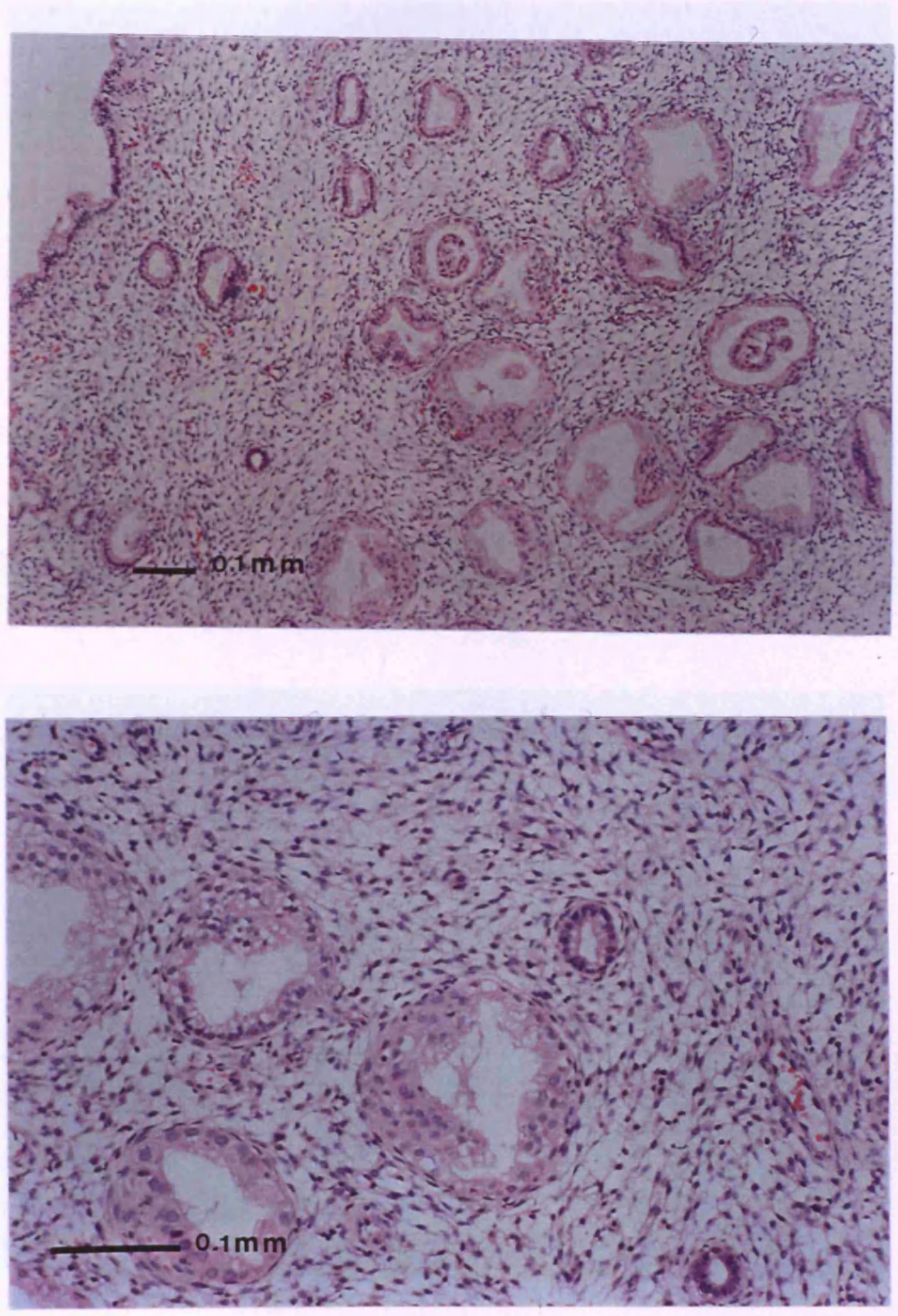


Figure (4.4. a)

Variable size glands, lined by cuboidal or low columnar epithelium, under cHRT. (Low and high power).

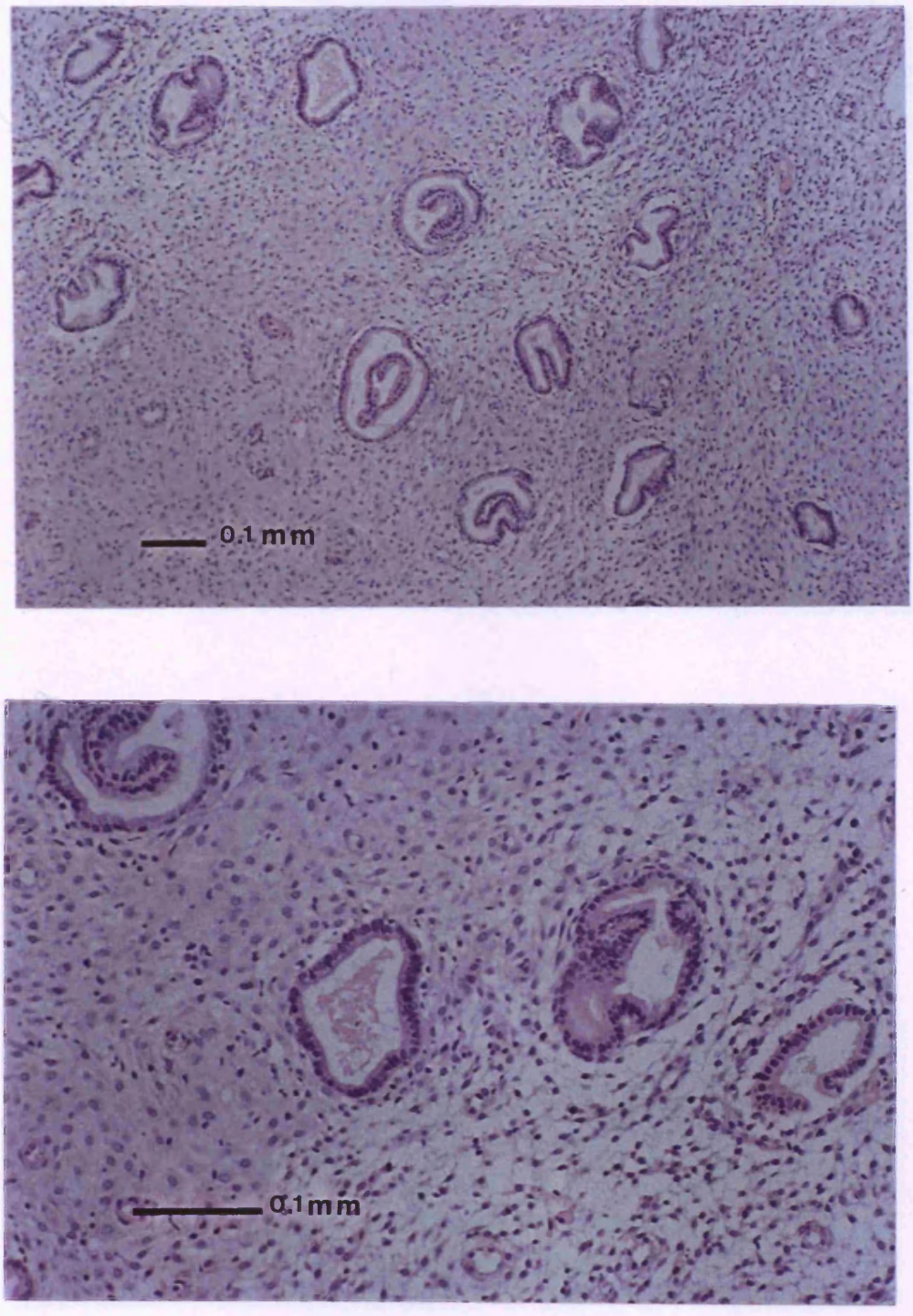
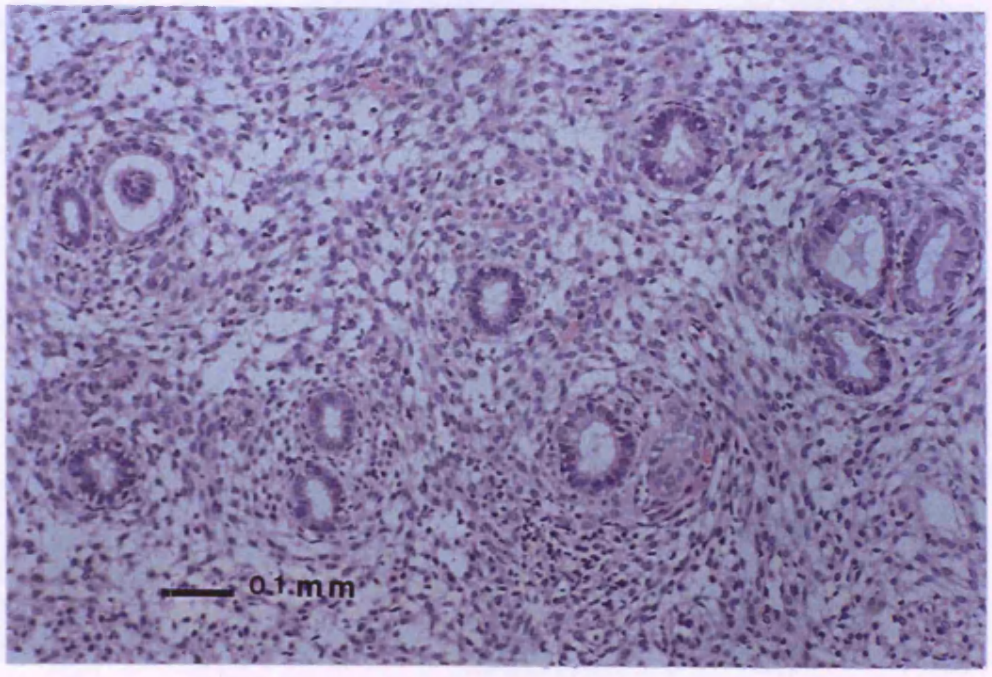


Figure (4.4. b)

Poorly developed glands with low cuboidal epithelium and low secretory activity, surrounded by decidualized stroma. Reduced gland/stroma ratio.



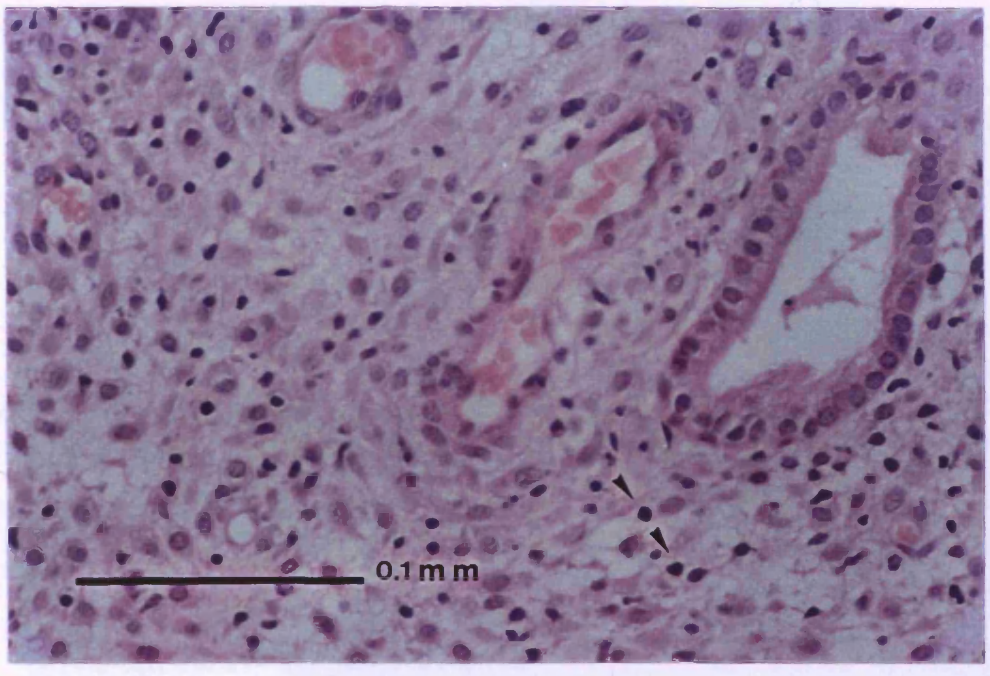


Figure (4.4. c)

Glands exhibiting cuboidal epithelium, poor secretory activity, and narrow diameter, surrounded by dense stroma and heavy leukocytic infiltrate (arrow).

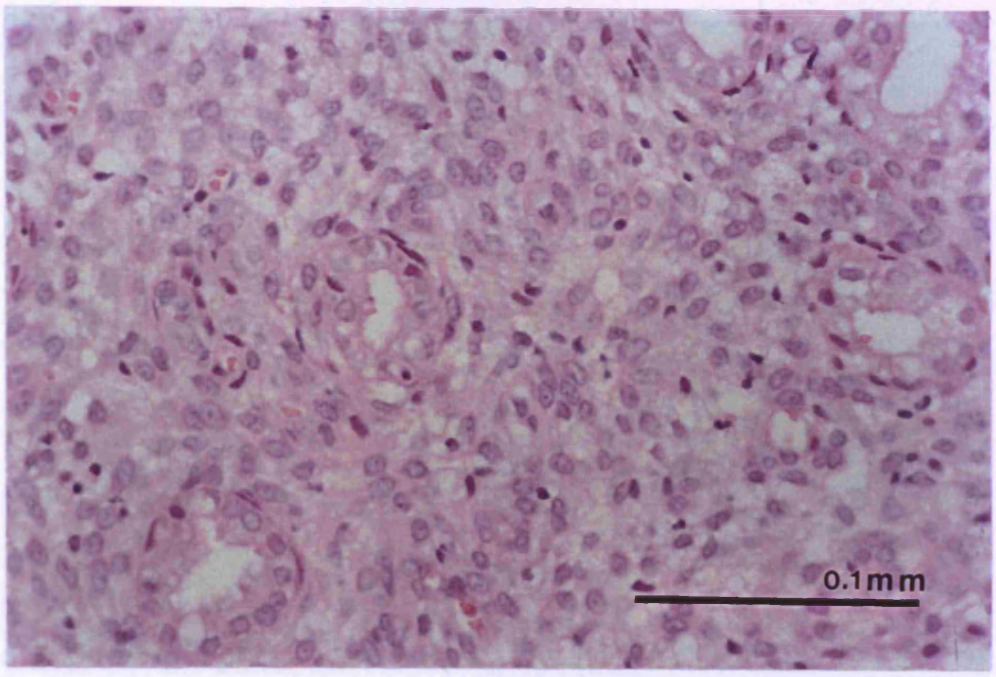


Figure (4.4. d)

Stromal decidualization and surrounding poorly developed glands.

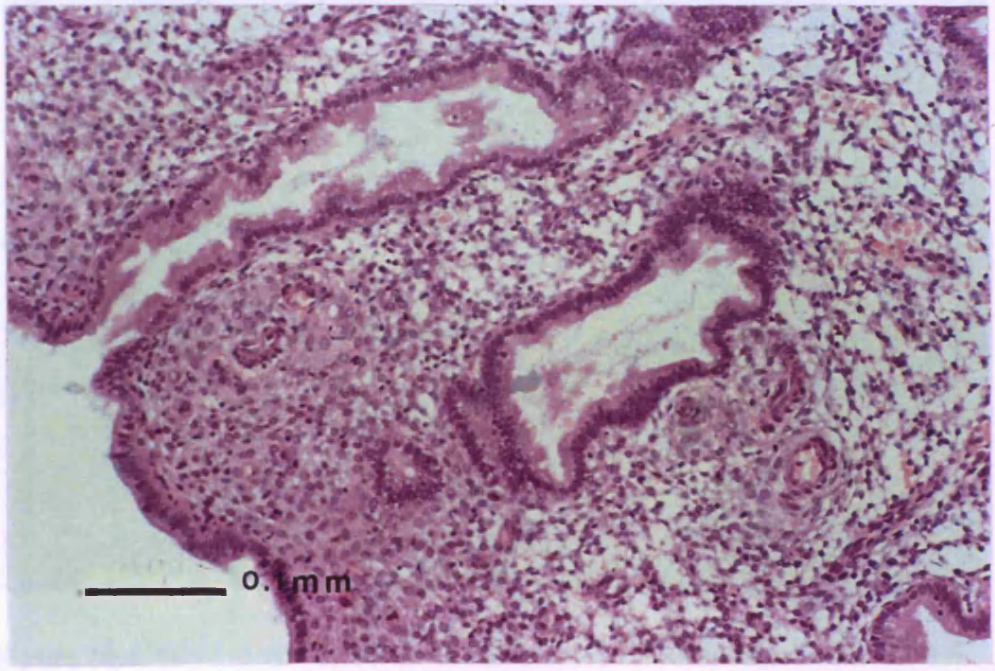
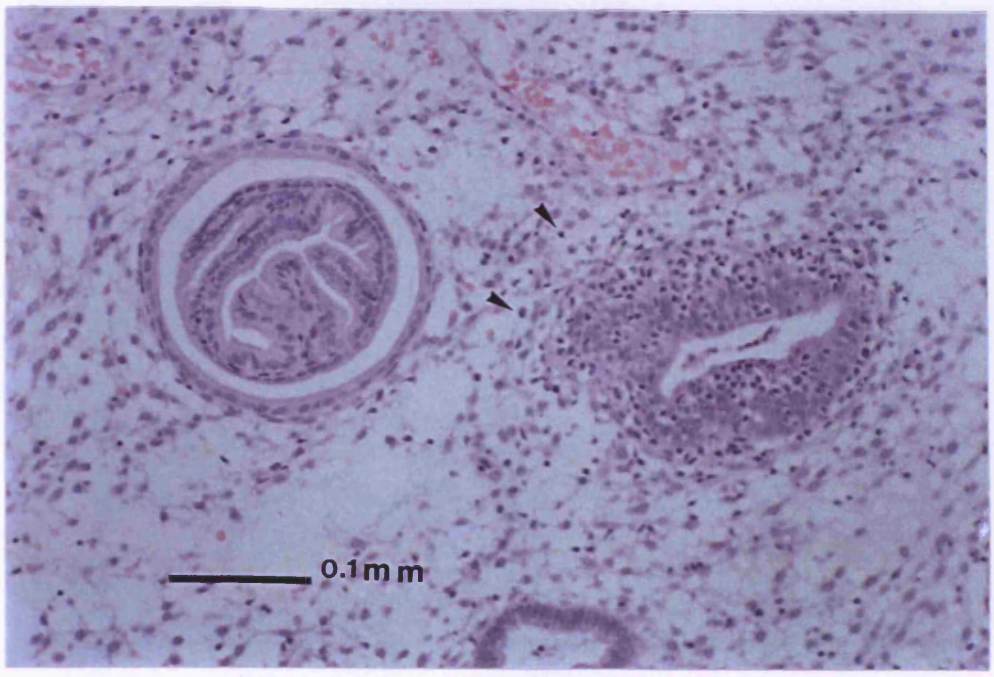


Figure (4.4. e)

Subepithelial and perivascular decidualization and leukocytic infiltration of the stroma. The glands exhibit secretory features but are less convoluted compared to the late-LP endometrium.



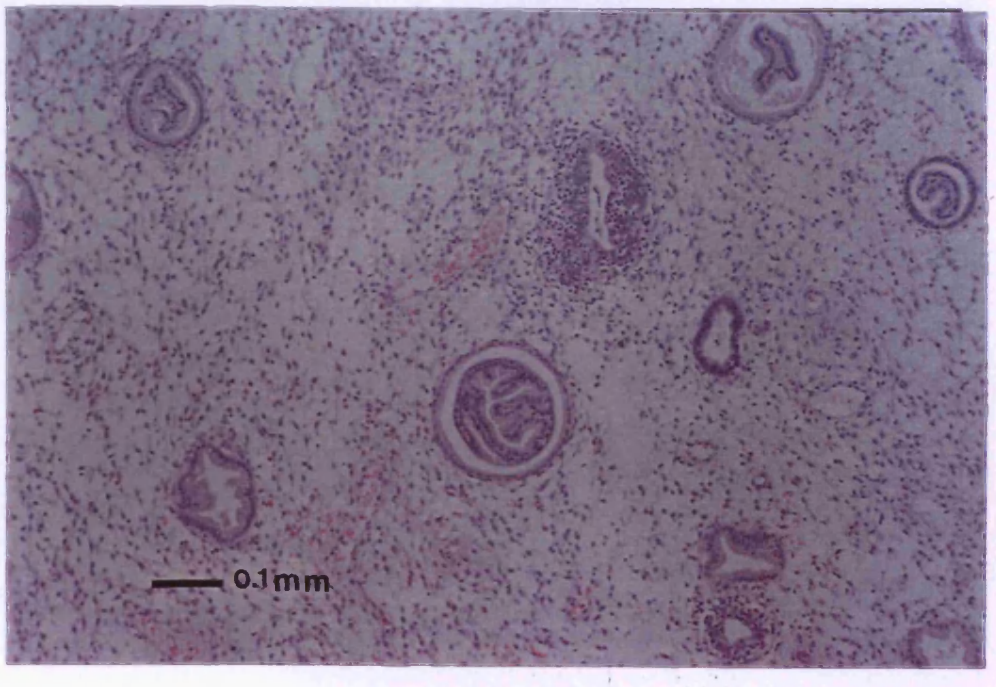
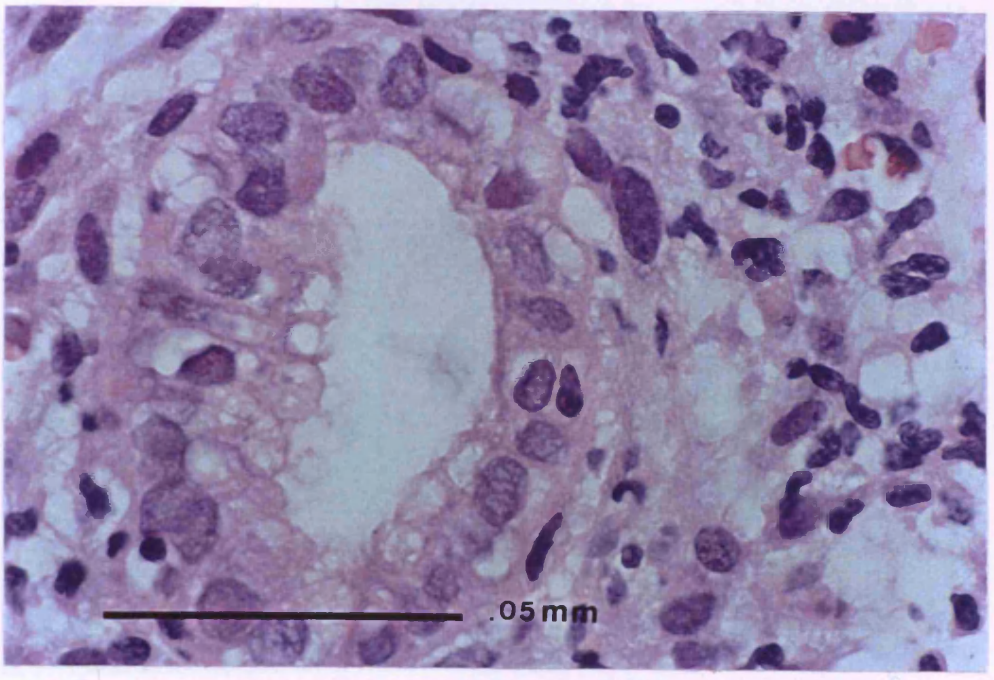


Figure (4.4. f)

Poorly developed glands lined by cuboidal epithelium with poor secretory features. The stroma exhibits patchy œdema and dense leukocytic infiltrate (arrows). Complex gland telescoping is also seen.



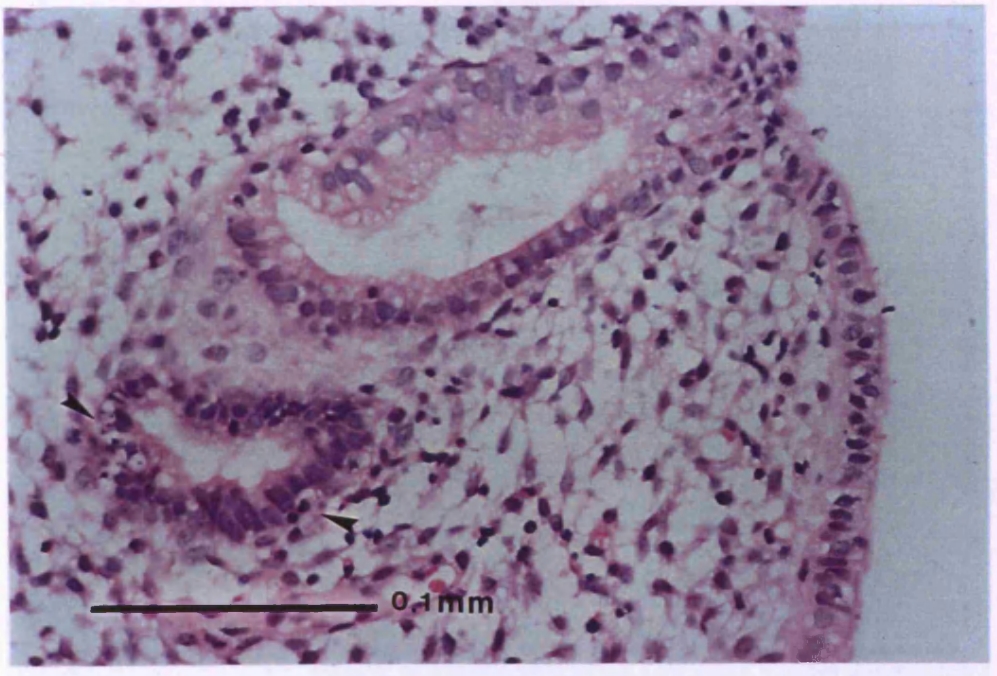


Figure (4.4. g)

Variable secretory features, some subnuclear as well as supranuclear vacuoles. These appear variable even within the same gland. Some glands exhibit apoptosis (arrow).

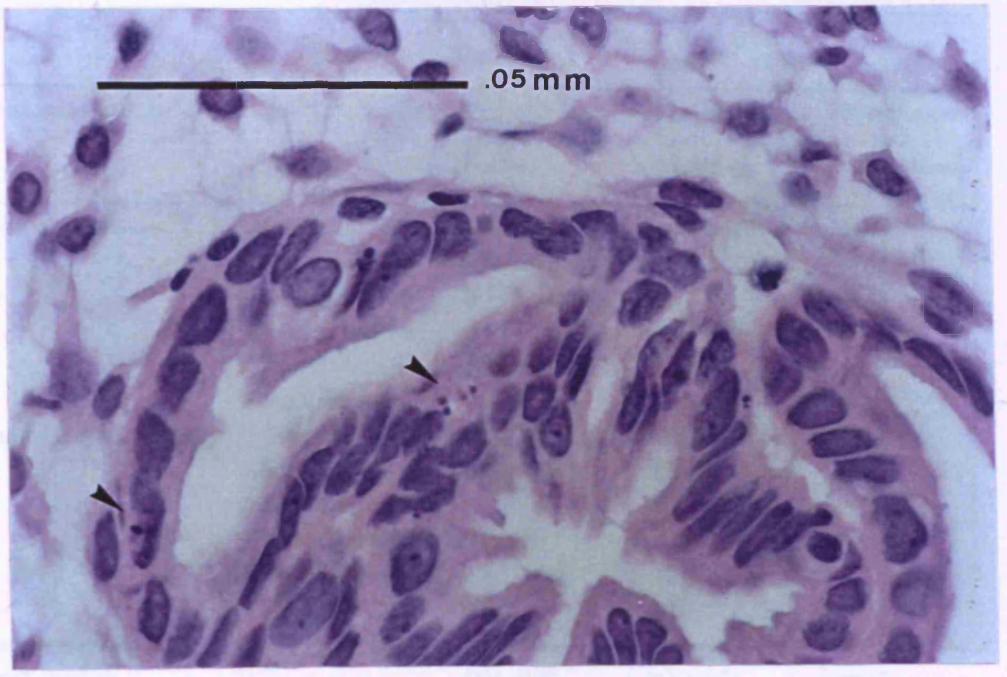


Figure (4.4. h)

High power magnification of apoptosis in the glandular epithelium.

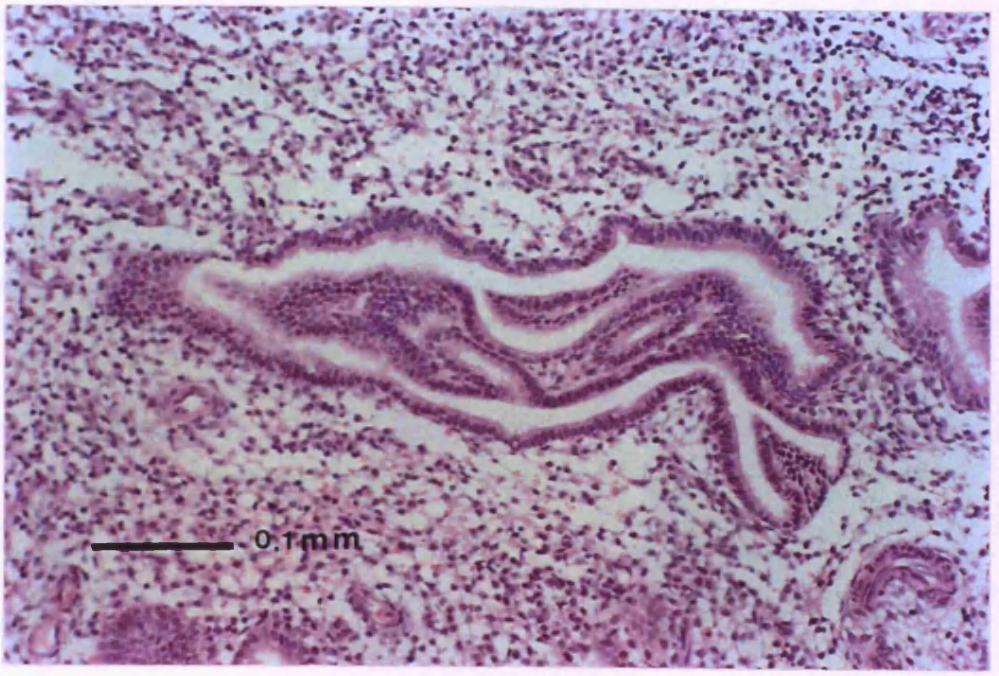


Figure (4.4. i)

Complex telescopic gland with multiple folds in the epithelium. Some of the glands exhibit complex telescoping, and although these are often referred to as artefacts, the degree of complexity they exhibit during the late-progestogenic phase is remarkable. It is possible that this feature is a reflection of steroid imbalance (237)

		Women group		
Histological feature		Early bleeders (n=10) Median (range)	Late bleeders (n=10) Median (range)	Significance level, P value Mann-Whitney test
Luminal epithelium	% Cuboidal	50 (20-90)	50 (20-90)	0.7
	% Low columnar	45 (10-70)	45 (10-70)	>0.99
	% Columnar	0	0	
	% Pseudostratified	2.5 (0-10)	2.5 (0-20)	0.6
	% Apical vacuole	2.5 (0-20)	5 (0-30)	0.3
	% Basal vacuole	0 (0-5)	0 (0-5)	>0.99
Glandular epithelium	% Cuboidal	60 (20-80)	40 (30-70)	0.2
	% Low columnar	40 (15-80)	55 (30-70)	0.3
	% Columnar	0	0	
	% Pseudostratified	0 (0-5)	0 (0-10)	0.5
	% Apical vacuole	20 (10-50)	40 (5-50)	0.2
	% Basal vacuole	0 (0-5)	0 (0-20)	0.7
Area per gland (mm2)		*0.0058 (0.0015)	*0.007(0.0018)	**0.15
Gland number /(x400hpf)		*3.6 (0.8)	*3.5(1.2)	**0.7
Total gland area (mm2)/(x400hpf)		*0.0229 (0.0086)	*0.026(0.0098)	**0.46
Gland shape	% Tubular	25 (0-75)	25 (0-100)	0.7
	% Convoluted	75 (25-100)	75 (0-100)	0.7
Luminal secretion	% Positive	25 (0-100)	25 (0-25)	0.3
	Secretion score	25 (0-100)	25 (0-25)	0.3
Glandular invaginations (telescoping)	Percentage	0 (0-15)	0 (0-20)	0.8
Apical margin	% Smooth	75 (25-100)	50 (50-100)	0.16
	% Irregular	25 (0-75)	37.5 (0-50)	0.17
Stromal cellular density	Cells/17 (x1000hpf)	*26.5 (3.9)	*23 (5.3)	**0.12
Stromal haemorrhage	% Area	20 (5-30)	15 (5-20)	0.1
Stromal œdema	% Area	5 (0-20)	7.5 (0-40)	0.9
Decidualization	% Subepithelial	20 (0-50)	20 (0-70)	0.6
	% Uniform	0	0	
	% Patchy	20 (0-50)	20 (0-50)	0.8
Leukocytic infiltrate		3(1-3)	3(1-3)	0.9

* Mean (SD)
** Two sided unpaired *t*-test

Table (4.5.)

Quantitative and semiquantitative comparison between the biopsies obtained before the onset of bleeding from both the EB and the LB. There are no statistically significant differences between the two groups.

		Physiological cycle		
Histological feature		Early luteal (n=10) Median (range)	Mid luteal (n=10) Median (range)	Late luteal (n=10) Median (range)
Luminal epithelium	% Cuboidal	5 (0-40)	20 (0-30)	10 (0-40)
	% Low columnar	80 (60-90)	70 (30-90)	80 (60-100)
	% Columnar	5 (0-20)	10 (0-70)	5 (0-20)
	% Pseudostratified	0 (0-10)	0	0
	% Apical vacuole	0 (0-50)	0 (0-5)	5 (0-20)
	% Basal vacuole	10 (0-50)	0 (0-10)	0
Glandular epithelium	% Cuboidal	0 (0-30)	10 (0-30)	10 (0-40)
	% Low columnar	45 (20-80)	30 (10-90)	70 (50-90)
	% Columnar	50 (0-80)	60 (0-90)	10 (0-50)
	% Pseudostratified	0	0	0
	% Apical vacuole	0 (0-20)	60 (0-100)	7.5 (0-30)
	% Basal vacuole	80 (50-100)	10 (0-70)	0
Area per gland (mm2)		*0.015(0.0067)	*0.012 (0.005)	*0.0079(0.003)
Gland number /(x400hpf)		*4.4 (1.3)	*4.2 (0.8)	*4.9 (1.3)
Total gland area (mm2)/(x400hpf)		*0.059(0.0146)	*0.054(0.023)	*0.037(0.014)
Gland shape	% Tubular	0 (0-10)	0	0
	% Convoluted	100 (90-100)	100	100
Luminal secretion	% Positive	10 (0-5)	75 (25-100)	80 (10-100)
	Secretion score	10 (0-50)	75 (25-150)	80 (10-160)
Glandular invaginations (telescoping)	Percentage	0 (0-15)	0 (0-10)	0 (0-4)
Apical margin	% Smooth	77.5 (50-100)	25 (0-75)	0 (0-100)
	% Irregular	22.5 (0-50)	75 (25-100)	100 (0-100)
Stromal cellular density	Cells/17 (x1000hpf)	*29.8 (7)	*24.8 (5.9)	*37.3 (12.6)
Stromal haemorrhage	% Area	0 (0-20)	10 (0-30)	10 (10-30)
Stromal œdema	% Area	0	30 (0-50)	0 (0-10)
Decidualization	% Subepithelial	0	0	0 (0-30)
	% Uniform	0	0	0
	% Patchy	0	0	30 (0-30)
Leukocytic infiltrate		1(1-2)	1(1-3)	2(1-3)

* Mean (SD)
** Two sided unpaired *t*-test

Table (4.6.)

Histological features of the early-LP, mid-LP and late-LP of the physiological cycle endometrium. Quantitative and semi-quantitative analysis reflects, and is in agreement with the classical histological criteria.

		Women on cHRT	Physiological cycle	
Histological feature		Late bleeders (n=10) Median (range)	Late luteal (n=10) Median (range)	Significance level, P value Mann-Whitney test
Luminal epithelium	% Cuboidal	50 (20-90)	10 (0-40)	0.0007
	% Low columnar	45 (10-70)	80 (60-100)	0.0002
	% Columnar	0	5 (0-20)	0.05
	% Pseudostratified	2.5 (0-20)	0	0.05
	% Apical vacuole	5 (0-30)	5 (0-20)	0.6
	% Basal vacuole	0 (0-5)	0	0.7
Glandular epithelium	% Cuboidal	40 (30-70)	10 (0-40)	0.0004
	% Low columnar	55 (30-70)	70 (50-90)	0.05
	% Columnar	0	10 (0-50)	0.001
	% Pseudostratified	0 (0-10)	0	0.1
	% Apical vacuole	40 (5-50)	7.5 (0-30)	0.002
	% Basal vacuole	0 (0-20)	0	0.2
Area per gland (mm2)		*0.007(0.0018)	*0.0079(0.003)	**0.5
Gland number /(x400hpf)		*3.5(1.2)	*4.9 (1.3)	**0.01
Total gland area (mm2)/(x400hpf)		*0.026(0.0098)	*0.037(0.014)	**0.05
Gland shape	% Tubular	25 (0-100)	0	0.02
	% Convoluted	75 (0-100)	100	0.02
Luminal secretion	% Positive	25 (0-25)	80 (10-100)	0.001
	Secretion score	25 (0-25)	80 (10-160)	0.001
Glandular invaginations (telescoping)	Percentage	0 (0-20)	0 (0-4)	0.2
Apical margin	% Smooth	50 (50-100)	0 (0-100)	0.004
	% Irregular	37.5 (0-50)	100 (0-100)	0.004
Stromal cellular density	Cells/17 (x1000hpf)	*23 (5.3)	*37.3 (12.6)	**0.003
Stromal haemorrhage	% Area	15 (5-20)	10 (10-30)	0.8
Stromal œdema	% Area	7.5 (0-40)	0 (0-10)	0.09
Decidualization	% Subepithelial	20 (0-70)	0 (0-30)	0.2
	% Uniform	0	0	
	% Patchy	20 (0-50)	30 (0-30)	0.7
Leukocytic infiltrate		3(1-3)	2(1-3)	0.008

* Mean (SD)
** Two sided unpaired *t*-test

Table (4.7.)

Quantitative and semiquantitative comparison between the late-progestogenic phase biopsies from the LB and the late-LP endometrial biopsies. The cHRT endometrial biopsies exhibit more cuboidal, and less columnar epithelium, smaller glandular area, more tubular glands, and less secretory activity. The stroma is less dense, more decidualized, and there is more leukocytic infiltration.

Histological feature		Women on cHRT Late bleeders (n=10) Median (range)	Physiological cycle Early luteal (n=10) Median (range)	Significance level, P value Mann-Whitney test
Luminal epithelium	% Cuboidal	50 (20-90)	5 (0-40)	0.0001
	% Low columnar	45 (10-70)	80 (60-90)	0.0001
	% Columnar	0	5 (0-20)	0.02
	% Pseudostratified	2.5 (0-20)	0 (0-10)	0.07
	% Apical vacuole	5 (0-30)	0 (0-50)	0.03
	% Basal vacuole	0 (0-5)	10 (0-50)	0.0007
Glandular epithelium	% Cuboidal	40 (30-70)	0 (0-30)	0.0001
	% Low columnar	55 (30-70)	45 (20-80)	0.4
	% Columnar	0	50 (0-80)	0.0001
	% Pseudostratified	0 (0-10)	0	0.07
	% Apical vacuole	40 (5-50)	0 (0-20)	0.0001
	% Basal vacuole	0 (0-20)	80 (50-100)	0.0001
Area per gland (mm2)		*0.007(0.0018)	*0.015(0.0067)	**0.003
Gland number /(x400hpf)		*3.5(1.2)	*4.4 (1.3)	**0.12
Total gland area (mm2)/(x400hpf)		*0.026(0.0098)	*0.059(0.0146)	**0.0001
Gland shape	% Tubular	25 (0-100)	0 (0-10)	0.01
	% Convoluted	75 (0-100)	100 (90-100)	0.01
Luminal secretion	% Positive	25 (0-25)	10 (0-5)	0.4
	Secretion score	25 (0-25)	10 (0-50)	0.4
Glandular invaginations (telescoping)	Percentage	0 (0-20)	0 (0-15)	0.1
Apical margin	% Smooth	50 (50-100)	77.5 (50-100)	0.2
	% Irregular	37.5 (0-50)	22.5 (0-50)	0.002
Stromal cellular density	Cells/17 (x1000hpf)	*23 (5.3)	*29.8 (7)	**0.02
Stromal haemorrhage	% Area	15 (5-20)	0 (0-20)	0.002
Stromal œdema	% Area	7.5 (0-40)	0	0.002
Decidualization	% Subepithelial	20 (0-70)	0	0.005
	% Uniform	0	0	0.9
	% Patchy	20 (0-50)	0	0.001
Leukocytic infiltrate		3(1-3)	1(1-2)	0.005

* Mean (SD)
** Two sided unpaired *t*-test

Table (4.8.)

Quantitative and semiquantitative comparison between the late-progestogenic phase biopsies from the LB and the early-LP endometrial biopsies. Under the influence of cHRT, the luminal and the glandular epithelium has more cuboidal cells, and lower total glandular area, the glands are more tubular and less convoluted, fewer stromal cells, more stromal œdema and haemorrhage.

		Women on cHRT	Physiological cycle	
Histological feature		Late bleeders (n=10) Median (range)	Mid luteal (n=10) Median (range)	Significance level. P value Mann-Whitney test
Luminal epithelium	% Cuboidal	50 (20-90)	20 (0-30)	0.0003
	% Low columnar	45 (10-70)	70 (30-90)	0.01
	% Columnar	0	10 (0-70)	0.01
	% Pseudostratified	2.5 (0-20)	0	0.03
	% Apical vacuole	5 (0-30)	0 (0-5)	0.06
	% Basal vacuole	0 (0-5)	0 (0-10)	0.5
Glandular epithelium	% Cuboidal	40 (30-70)	10 (0-30)	0.0001
	% Low columnar	55 (30-70)	30 (10-90)	0.3
	% Columnar	0	60 (0-90)	0.0002
	% Pseudostratified	0 (0-10)	0	0.08
	% Apical vacuole	40 (5-50)	60 (0-100)	0.1
	% Basal vacuole	0 (0-20)	10 (0-70)	0.06
Area per gland (mm2)		*0.007(0.0018)	*0.012 (0.005)	**0.01
Gland number /(x400hpf)		*3.5(1.2)	*4.2 (0.8)	**0.1
Total gland area (mm2)/(x400hpf)		*0.026(0.0098)	*0.054(0.023)	**0.004
Gland shape	% Tubular	25 (0-100)	0	0.007
	% Convoluted	75 (0-100)	100	0.007
Luminal secretion	% Positive	25 (0-25)	75 (25-100)	0.002
	Secretion score	25 (0-25)	75 (25-150)	0.001
Glandular invaginations (telescoping)	Percentage	0 (0-20)	0 (0-10)	0.2
Apical margin	% Smooth	50 (50-100)	25 (0-75)	0.003
	% Irregular	37.5 (0-50)	75 (25-100)	0.003
Stromal cellular density	Cells/17 (x1000hpf)	*23 (5.3)	*24.8 (5.9)	**0.5
Stromal haemorrhage	% Area	15 (5-20)	10 (0-30)	0.007
Stromal œdema	% Area	7.5 (0-40)	30 (0-50)	0.1
Decidualization	% Subepithelial	20 (0-70)	0	0.007
	% Uniform	0	0	0.9
	% Patchy	20 (0-50)	0	0.001
Leukocytic infiltrate		3(1-3)	1(1-3)	0.008

* Mean (SD)
** Two sided unpaired t-test

Table (4.9.)

Quantitative and semiquantitative comparison between the late-progestogenic phase biopsies from the LB and the mid-LP endometrial biopsies. The cHRT biopsies exhibits more cuboidal, and less columnar epithelium, the glandular area is smaller, and there are more tubular glands, more decidualization and more leukocytic infiltrate.

Histological feature		Women on cHRT Early bleeders (n=10) Median (range)	Physiological cycle Late luteal (n=10) Median (range)	Significance level, P value Mann-Whitney test
Luminal epithelium	% Cuboidal	50 (20-90)	10 (0-40)	0.0008
	% Low columnar	45 (10-70)	80 (60-100)	0.0004
	% Columnar	0	5 (0-20)	0.05
	% Pseudostratified	2.5 (0-10)	0	0.05
	% Apical vacuole	2.5 (0-20)	5 (0-20)	0.8
	% Basal vacuole	0 (0-5)	0	0.7
Glandular epithelium	% Cuboidal	60 (20-80)	10 (0-40)	0.0004
	% Low columnar	40 (15-80)	70 (50-90)	0.01
	% Columnar	0	10 (0-50)	0.001
	% Pseudostratified	0 (0-5)	0	0.1
	% Apical vacuole	20 (10-50)	7.5 (0-30)	0.01
	% Basal vacuole	0 (0-5)	0	0.4
Area per gland (mm2)		*0.0058 (0.0015)	*0.0079(0.003)	**0.1
Gland number /(x400hpf)		*3.6 (0.8)	*4.9 (1.3)	**0.01
Total gland area (mm2)/(x400hpf)		*0.0229 (0.0086)	*0.037(0.014)	**0.01
Gland shape	% Tubular	25 (0-75)	0	0.01
	% Convoluted	75 (25-100)	100	0.01
Luminal secretion	% Positive	25 (0-100)	80 (10-100)	0.02
	Secretion score	25 (0-100)	80 (10-160)	0.01
Glandular invaginations (telescoping)	Percentage	0 (0-15)	0 (0-4)	0.2
Apical margin	% Smooth	75 (25-100)	0 (0-100)	0.003
	% Irregular	25 (0-75)	100 (0-100)	0.003
Stromal cellular density	Cells/17 (x1000hpf)	*26.5 (3.9)	*37.3 (12.6)	**0.01
Stromal haemorrhage	% Area	20 (5-30)	10 (10-30)	0.3
Stromal œdema	% Area	5 (0-20)	0 (0-10)	0.04
Decidualization	% Subepithelial	20 (0-50)	0 (0-30)	0.5
	% Uniform	0	0	
	% Patchy	20 (0-50)	30 (0-30)	0.05
Leukocytic infiltrate		3(1-3)	2(1-3)	0.08

* Mean (SD)
** Two sided unpaired *t*-test

Table (4.10.)

Quantitative and semiquantitative comparison between late-progestogenic phase biopsies from the EB (obtained before the onset of bleeding) and the late-LP endometrial biopsies. The cHRT group exhibits more cuboidal and less columnar epithelium, a smaller glandular area, poorly developed secretory activity, less dense stroma, and more subepithelial decidualization.

Chapter 5

Endometrial Leukocytes

5.1. Introduction

Leukocytes constitute a significant proportion of endometrial stromal cells, and their total and differential counts vary with the phase of the natural cycle. The precise functional significance of these changes remains unknown, but may be related to the mechanisms affecting menstruation or to preparation for implantation.

No studies, to date, have described the distribution of leukocytes in cHRT endometrium. This will be examined here with reference to the normal endometrium. If the number of leukocytes correlates with the functional state, stromal leukocytes would parallel those of the physiological cycle. Although histological assessment (Chapter 4) suggested that a different pattern may emerge.

5.1.1. Leukocyte changes in the natural cycle

Leukocytes are present within the endometrial stroma and occasionally between epithelial cells (intraepithelial lymphocytes). The leukocyte population is relatively stable throughout the cycle (10-15% of the total stromal cells), but increase premenstrually to 20-25% (85). The changes in the leukocyte population are summarised in Table (5.1., 5.2.). In the basalis, leukocytes are either scattered individually or form aggregates of B cells, T cells and macrophages. In contrast to the leukocytes in the functionalis, those in aggregates do not fluctuate with the stage of the cycle. B lymphocytes and $CD16^+$ natural killer (NK) cells are rare throughout the cycle. In the FP and the early-LP the main populations of lymphocytes are $CD3^+$ T lymphocytes (mostly $CD8^+$), $CD14^+$ macrophages, and $CD56^+$ $CD3^-$ $CD16^-$ lymphocytes. In the late-LP the number of endometrial stromal leukocytes increases, mainly due to the rise in $CD56^+$ $CD3^-$ $CD16^-$ lymphocytes, and the number of macrophages may also rise premenstrually (238).

5.1.1.1. Endometrial Granulated Lymphocytes (eGL)

Previously called 'endometrial stromal granulocytes', 'Körnchenzellen' or 'K' cells, these are the main inhabitants of the stroma. They have a round, oval, or indented hyperchromatic nucleus, are 10-12μm in diameter, and have 1-10 phloxinophilic granules which characteristically stain with phloxine tartrazine, Giemsa, toluidine blue, eosin and methyl violet. The intra-cytoplasmic granules are always round, variable in size but are mostly 1-2μ in diameter, granules vary in number within individual cells (239). Imprint preparations, because they avoid tissue traumatisation and the artefacts produced by formalin fixation, have been used in studies of these cells which lose their characteristic granules in frozen sections. eGL have an unusual antigenic phenotype, being $CD56^{+}CD38^{+}CD2^{\pm}CD3^{-}$ and $CD16^{-}$. They also express CD7 and OKT10 (240), but fail to express classical T cell or natural killer (NK) cell markers such as CD3, CD4, CD8, CD16, and CD57 (Leu-7) (241). eGL show morphological and antigenic heterogeneity in different phases of the cycle, and an infrequent occurrence is eGL cells that possess the classical NK markers as CD57 (Leu 7, HNK-1) and CD16 (Leu-11) (242).

A small population eGL cells are $CD3^{+}$, and only a minority of $CD3^{+}$ cells are $CD56^{+}$. The remaining eGL are $CD3^{-}$ and can be either $CD3^{-}CD56^{+}$ $CD2^{+}$ or $CD3^{-}$ $CD56^{+}$ $CD2^{-}$. The proportion that is $CD2^{-}$ increases in the late-LP (241). $CD2^{-}$ cells account for the increased proliferation of $CD56^{+}$ cells in the late-LP (241, 243).

Initially thought to originate from undifferentiated stromal cells (239), eGL are now believed to be largely derived from bone marrow (244), which may explain their periarteriolar localisation (244). Reports that these cells are fully differentiated, raise questions as to whether they influx from the blood (245), or differentiate in situ from bone marrow precursors. eGL exhibit proliferation although not in parallel to their number (243). The increased proliferation from the FP into the LP is not accompanied by an increase in number, and the increase eGL in the late-LP is not accompanied by increased proliferation (243). The proliferative activity reported is low, at about 1-2 mitosis/10 hpf (243). Increased proliferative activity during the late-LP, was also observed in CD3 and CD11c positive as well as negative leukocytes, this was, at least partly, attributed to proliferation in eGL(246).

5.1.1.2. Macrophages

Macrophages are bone marrow derived cells that are mostly scattered throughout the endometrium in all phases of the cycle (247). During the FP, they may comprise up to 50% of $CD45^+$ cells (242). Macrophages have been reported to represent the most prominent cell population in the mid-LP endometrium (248) and to rise before the onset of menses and that the magnitude of this rise be responsible for the pre-menstrual rise in $CD45^+$ cells (85). But this was not confirmed (247). Another study reported that macrophages increase between day 4 and day 13 after the LH surge by a factor of about 49% compared to a 62% increase in the total leukocyte count and a 92% increase in eGL (249). Although the function of these cells is not known, macrophages may have a role in phagocytosis, antigen processing, release of cytotoxic mediators such as H_2O_2, release of IL-1, TNFα, TGFβ, PDGF-A and IGF (242).

5.1.1.3. T lymphocytes

T cells ($CD3^+$, $CD5^+$, and $CD7^+$) are either scattered in the stroma and are often present in aggregates in the deeper endometrium (247). At least 75% of the T cells ($CD2^+$, $CD3^+$) are $CD8^+$, and 25% are $CD4^+$. $CD8^+$ cells increase from the FP into the LP (250).

T cells within aggregates seem to be a specialised distinct population, they proliferate and express HLA-DR, HLA-DP and HLA-DQ, and are therefore activated, but do not express IL-2 receptor (246). Their number remains relatively constant, although higher at the beginning and at the end of the cycle (247). $CD3^+$, $CD8^+$, $CD4^+$ and $CD2^+$ cells are reported to increase by 26%, 27%, 16%, and 48% respectively from day 4 to day 13 after the LH surge, but because the rate of increase is less than that of the total number of leukocytes, T cells are proportionately less during the late-LP compared to the early-LP (Table 5.2.) (249).

T lymphocytes in aggregates express ER (117), and this may mediate the œstrogen induced increase in number, similar to that reported in the rat (119). A role for progesterone cannot be excluded as peripheral blood lymphocytes express PR (120).

5.1.1.4. B lymphocytes

These are scattered in the stroma in small numbers throughout the cycle and only few are in lymphoid aggregates (249).

5.1.1.5. Polymorphonuclear leukocytes

These do not form part of the normal population but invade the endometrium a maximum of 2 days before menstruation (85, 242).

5.1.2. Modulation of Leukocytic infiltration by steroids

Sex steroids affect the function of lymphoid cells, through a yet unclear mechanism. This may be mediated through ER (**5.1.1.3.**). A role for œstrogen in regulating the number of macrophages (118), and in regulating the granulocyte-macrophage colony stimulating factor (GmCSF) have also been demonstrated in the mouse (251).

Cyclical œstrogen and progesterone treatment for women with premature ovarian failure, results in endometrium that is histologically similar to the natural cycle, and that has similar leukocytic infiltrate (252). Prolonged treatment with high doses of progestogens results in an increase in the total number of leukocytes, macrophages and eGL, and in focal tissue necrosis and the accumulation of neutrophils (253). These findings support a regulatory role for progestogen, although PR was not detected in lymphoid cells in the human endometrium (117). In a recent study, $CD45^+$ cells were reported to be ER^- and PR^-, which may indicate that the effect of steroids is indirect (254).

5.2. Aim

To quantify the total and the main subtypes of leukocytes during the late-PLP, as compared to the physiological cycle.

5.3. Material and Methods

5.3.1. Study population and regimen

Were described previously (**2.1.1., 2.1.2., 4.3.1.**).

5.3.2. Immunohistochemistry

Was described previously (**2.1.4.2., 2.1.5.2.**). The antibodies used and their specificity are detailed in Table (5.3.), and Appendix (2).

In the endometrium, anti-CD45 antibody recognises an antigen expressed exclusively on all lymphocytes. $CD56^+$ cells are predominantly granulated lymphocytes. Only a small fraction of eGL express CD3, which therefore delimits the majority of T cells. CD68 is expressed on macrophages, while CD20 is present on the majority of B cells.

5.3.3. Statistical analysis and sample size

Were considered previously (2.1.7.).

5.3.4. Image analysis

Was described previously (2.1.6.).

5.4. Results: Distribution of leukocytes

5.4.1. $CD45^+$ cells

In the physiological cycle, $CD45^+$ cells were scattered throughout the stroma with a higher density in the more superficial functionalis, and were found in aggregates near the base of the glands, with few inter-epithelial cells. Cellular density increased by about 35-40% during the mid-LP, but more than doubled (125%) during the late-LP compared to the early-LP. A similar distribution was noted during the late-PLP, but with a very pronounced increase in the total number, which was doubled compared to the late-LP (Figure 5.4., Figure 5.5., Table 5.7.). The difference between the number of $CD45^+$ cells in the late-LP and early-LP was statistically significant ($p=0.02$). The number of $CD45^+$ cells in the late-PLP was significantly higher compared to the early-LP ($p=0.0001$), the mid-LP ($p=0.0004$), and the late-LP ($p=0.0027$).

5.4.2. $CD3^+$ cells

$CD3^+$ cells were found in aggregates near the gland bases as well as throughout the stroma. Their number did not vary significantly during the mid-LP of the physiological cycle compared to the early-LP, but $CD3^+$ cellular density increased during the late-LP. There was no statistically significant difference between the number of $CD3^+$ cells in the late-LP compared to the late-PLP (Table 5.7.), the cellular distribution was also similar (Figure 5.6., 5.8.). The difference between the number of $CD3^+$ cells in the late-PLP was statistically significantly higher compared to the early-LP ($p=0.0081$), and the mid-LP ($p=0.0065$). The differences between the stages of the physiological cycle were not statistically significant (Student *t*-test).

5.4.3. $CD56^+$ cells

In the early-LP, $CD56^+$ cells were scattered throughout the stroma, but formed denser aggregated near blood vessels and glands during the late-LP. The number of cells doubled from the early to the mid-LP, with a marked (275%) increase during the late-LP. $CD56^+$ cells were the most abundant during the late-LP and the late-PLP, but in the latter their number exceeded by more than double (230%) that in the former (Figure 5.6.,Table 5.7..Figure 5.9.). The number of $CD56^+$ cells in the late-PLP was statistically significantly higher when compared to the early-LP ($p=0.0003$), the mid-LP ($p=0.0039$), and the late-LP ($p=0.0039$). The differences between the early-LP and the late-LP was also statistically significant ($p=0.0013$).

5.4.4. $CD68^+$ cells

$CD68^+$ cells were scattered evenly throughout the stroma, and their numbers did not fluctuate. There was a 40-45% increase in the number of these cells during the mid-LP and late-LP compared to the early-LP, but this was not statistically significant. There was no statistically significant difference between the number of $CD68^+$ cells during the late-LP and the late-PLP (Figure 5.6.,Table 5.7..Figure 5.10.).

5.4.5. $CD20^+$ cells

$CD20^+$ cells were only sparsely present and their numbers and distribution did not vary in the phases examined (Figure 5.6.,Table 5.7.,Figure 5.11.).

5.5 Discussion

The distribution of leukocyte subtypes during the LP of the natural cycle agrees with previous studies (249). The late-PLP exhibited a large increase in leukocytes, which is attributed to an increase in $CD56^+$ and $CD3^+$ cells. The number of $CD20^+$ cells is small and, in contrast to others remains constant during the LP and the late-PLP. Which may signal an absent response to steroids, indeed neither ER or PR have been demonstrated on $CD20^+$ cells. $CD68^+$ cells were reported to represent 30% of $CD45^+$ cells during the early-LP, and to rise by a factor of about 49% between days LH+4 and LH+13 (249). Taking into account the large standard deviations noted, this may not be significantly different from the corresponding figures of 23% and 80% in the present study. However, as the number of $CD45^+$ cells increased in this study by a factor of 126% compared to 62% reported previously (249), there were proportionately fewer macrophages (18%) in the late-LP in the current study compared to the 28% previously reported (249). The differences may be attributable to the use of frozen sections in the previous study which have their limitations (**2.1.4.1.**) (249).

eGL are characterised by possessing the CD56 antigen which has not been reported in other endometrial cells. Previous studies utilised imprint preparation and frozen sections (241), as antibodies suitable for formalin fixed tissues have only recently become available. These allowed better preservation of architecture in the present study, and this demonstrated a higher (275%) increase in the number of $CD56^+$ cells from the early-LP to the late-LP, compared to the 152% increase previously reported (241). In both studies $CD56^+$ cells, were the predominant cell types in the late-LP but they made up 40% of the total $CD45^+$ cells in this study, compared to 57.2% in the previous study (241). The increased number of leukocytes observed with cHRT underlies evidence of stromal decidualization, and more advanced stromal compared to glandular development.

In this study, $CD3^+$ cells made up a higher percentage of the total $CD45^+$ cells in both the early-LP and the late-LP (57% and 31.7% respectively), compared to 37.5% and 21.5% previously reported (241). The latter authors reported no increase in the number of $CD3^+$ cells which agrees with the non-statistically significant 11.1% increase observed here. Overall, the pattern of expression of the CD markers of the major leukocyte population was similar

in this study to other reports in literature, and the few differences may be attributable to the different antibodies used and to the use of different fixation and antigen retrieval methods.

The role of leukocytes in the endometrium is not clear, but their ability to produce enzymes, cytokines or other mediators of necrosis and repair suggests a role in tissue shedding and menstruation, or in repair. The increase in leukocytes with cHRT is rather unexpected, particularly that the group studied exhibited clinical behaviour that was similar to the physiological cycle. This suggests that the increased leukocytes may not be causally linked to bleeding.

This increase appears to be mediated either directly or indirectly by sex steroids, and could be caused by either increase in infiltration or by increased local proliferation. In support of the latter hypothesis, is the demonstration of Ki67 (Mib1) positive eGL, especially in the late-LP (243), but this has not previously been studied in women receiving cHRT.

Progestogen increases the total number of leukocytes as well as the number of $CD3^+$, $CD68^+$, and particularly the number of eGL (253). The mechanism of this effect is unknown, especially so that PR is absent from endometrial leukocytes, and although still controversial (254) ER^+ cells (mostly T cells) are localised to the lymphoid aggregates in the deeper functionalis or basalis, and only occasionally are they present scattered in the functionalis layer (117). It is possible that the effect of steroids is mediated through cytokines such as interleukin-8 (IL-8), the Monocyte chemoattractant protein-1 (MCP-1) and cyclooxygenase-2 (COX-2) which have recently been identified to increase premenstrually (255).

MCP-1 is particularly interesting, for it is a potent attractant and activator for T cells (256), and NK cells (257) both of which showed a significant increase with cHRT. Decidual cells produce COX-2 in response to IL-1β, an effect that is inhibited by progesterone (258). The inhibitory effect attributed to progesterone, and the observed increase in leukocytic infiltrate in the late-LP when levels of circulating progesterone are known to be low, led to the suggestion that progesterone has an indirect inhibitory effect and that the rise observed is secondary to release of this inhibition (255). It was proposed that PR mediates the inhibitory effect of progesterone on MCP-1 release from perivascular cells (255). This, however, would not explain the excessive

infiltrate observed under the influence of cHRT, or that observed with prolonged progestogen therapy (253), or the increased eGL in the decidua (243). Another possible explanation that does not stipulate progesterone withdrawal, could be mediated through the ability of progesterone to inhibit its own receptor (**1.12.4.4.**). Thus a higher expression of mediators could be expressed in the presence of high but 'ineffective' levels of progestogens. In cHRT, such a situation may prevail if either the dose of progestogen is higher than in the physiological state leading to receptor inhibition, or that of œstrogen is low resulting in insufficient expression during the early-PLP.

On the other hand, the lack of a rise in the number of macrophages with cHRT may be explained by the observation that granulocyte-macrophage colony stimulating factor (GmCSF), which may be responsible for macrophage migration as well as proliferation and differentiation (259), is œstrogen regulated (251), œstrogen deficiency may thus be responsible for this observation. The administration of the anti-progesterone Mifepristone, was also associated with a rise in the number of decidual macrophages, but this was attributed to the ability of mifepristone to stimulate ER and PR (260).

Cell type	Phase of the menstrual cycle		
	Proliferative	Secretory	Late luteal
Macrophages	++	++	++/+++
T-cells	+	++	+
eGL	+	+	+++
B-cells	±	± / +	?
Polymorphs	-	-	?

Table (5.1.)

Leukocyte subpopulations at the different phases of the natural cycle. (Data from Lea & Clark, 1991)(261).

Antigen	Antigen expression	% in early LP	% Increase from early to late LP	% in late LP
CD45	Common leukocyte antigen (T cell, B cell, NK/non-lineage, monocyte, macrophage, and granulocyte)	100%	62%	100%
CD3	T cells	34%	26%	27%
CD8	Maturation and T cell selection, (Sub population of T cells and NK/non-lineage cells)	23%	27%	18%
CD4	T cell helper activity	9%	16%	7%
CD56	Large granulated lymphocytes, activated T and NK cells	45%	92%	55%
CD68	Macrophages	30%	49%	28%
CD22	Sub population of B cells	2%	25%	2%
CD2	T cells, NK/non lineage cells	59%	48%	54%

Table (5.2.)

The percentage of leukocytes recognised by antibodies to each CD antigen in the early-LP (LH + 4) and the late-LP (LH + 13) of the normal menstrual cycle, and the percentage increase in the number of leukocytes between these phases. Data from Klentzeris et al., (1992)(249).

Antigen	Antibody	Comment
CD3	Dako. Rabbit polyclonal Anti-Human T cell. code No. A 452 lot 084	Is a highly specific maker for T cells (262), and no other cells are known to express the antigen except for the Purkinje cells in the cerebellum.
CD20	Monoclonal Mouse Anti-Human B Cell. clone L26. code M 0755. Lot 083	Antigen present on the majority of B cells. The antibody interacts predominantly with a 33 kDa polypeptide present in B cells and to a minor component of 30kDa polypeptide. and reacts with the majority of B cells in both peripheral blood and lymphoid tissue. with no reactivity to other haematopoietic cells.
CD45	Dako. Mouse anti-human leukocyte common antigen. M701. Lot 064. clone 2B11.	CD45 is a major cell-surface glycoprotein confined to the lymphoid and myeloid lineages. The antibody labels lymphoid cells. Macrophages and histiocytes react to a variable degree and polymorphs only weekly, many plasma cells are unreactive.
CD56	CD56/Neural Cell Adhesion Molecule-1 (NCAM),Neo markers. USA. USA	Is expressed on all eGL. and although also expressed on activated T and B cells. the majority of $CD56^{+}$ cells in the endometrium are eGL.
CD68	Mouse Anti-Human CD68. KP1. Dako Code N1577	Detects a 110kDa glycoprotein expressed on macrophages in lymphoid and non-lymphoid tissues. It also detects myeloid precursors and neutrophilic granules in bone marrow and blood. but not in other tissues. Some T-cells. believed to be of monocyte/macrophage origin. also stain positive .

Table (5.3.)

The antibodies used in this chapter and their specificity.

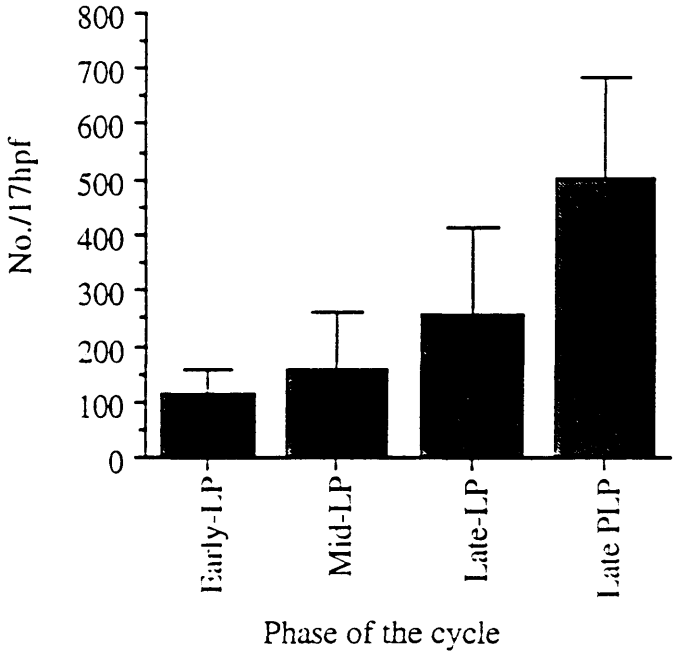
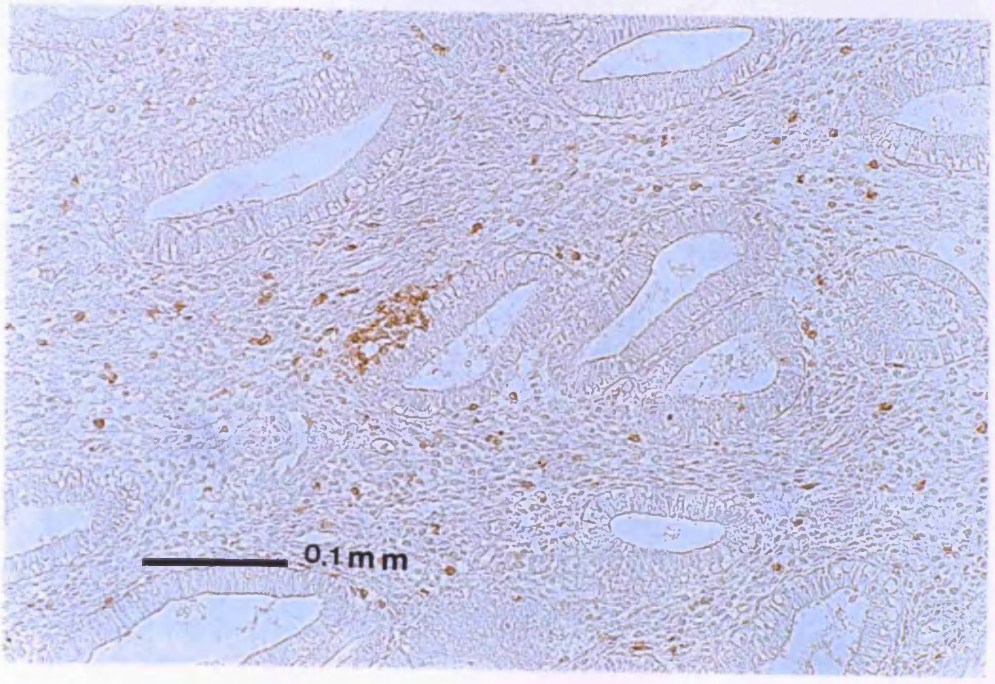
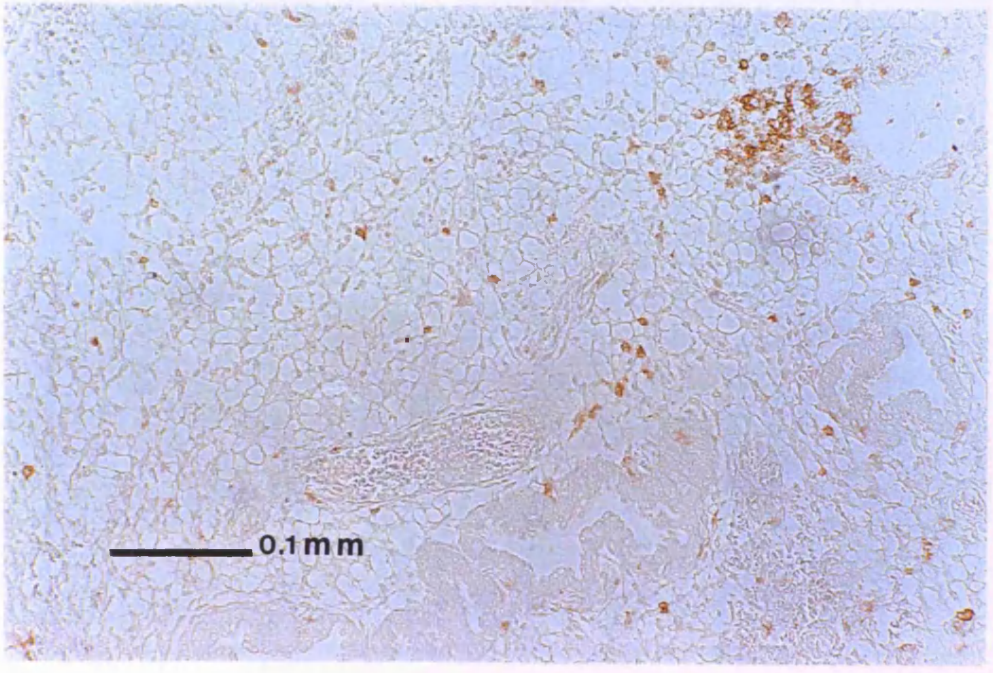


Figure (5.4.)

The number of $CD45^+$ cells in 17hpf (x400) during the different stages of the LP and the late-PLP. The total number of leukocytes increases through the stages of the LP and exhibits a dramatic increase on cHRT.



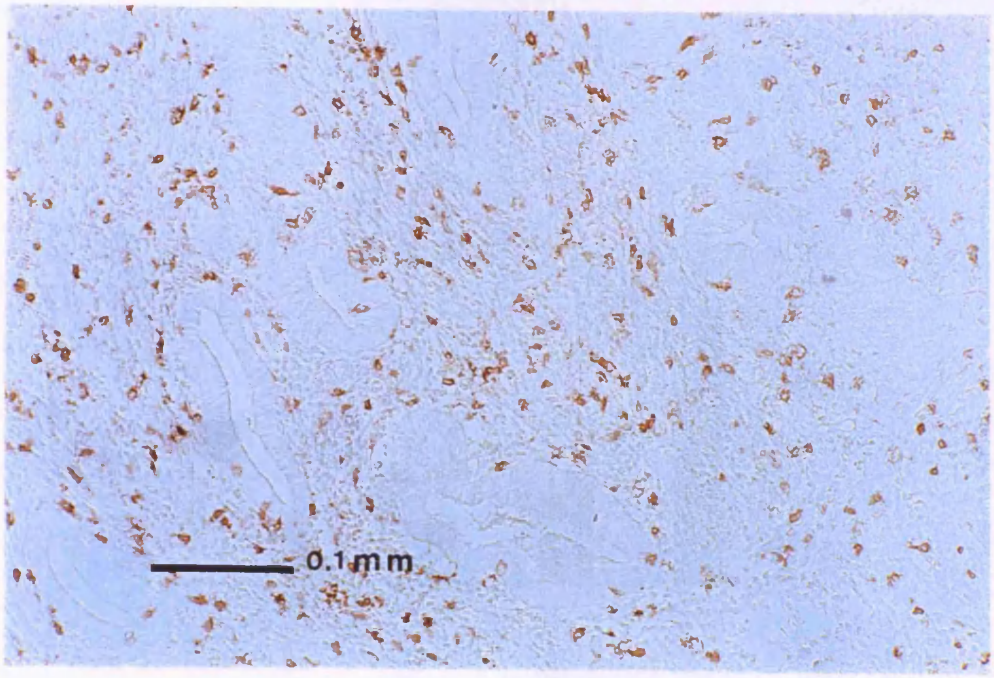
a



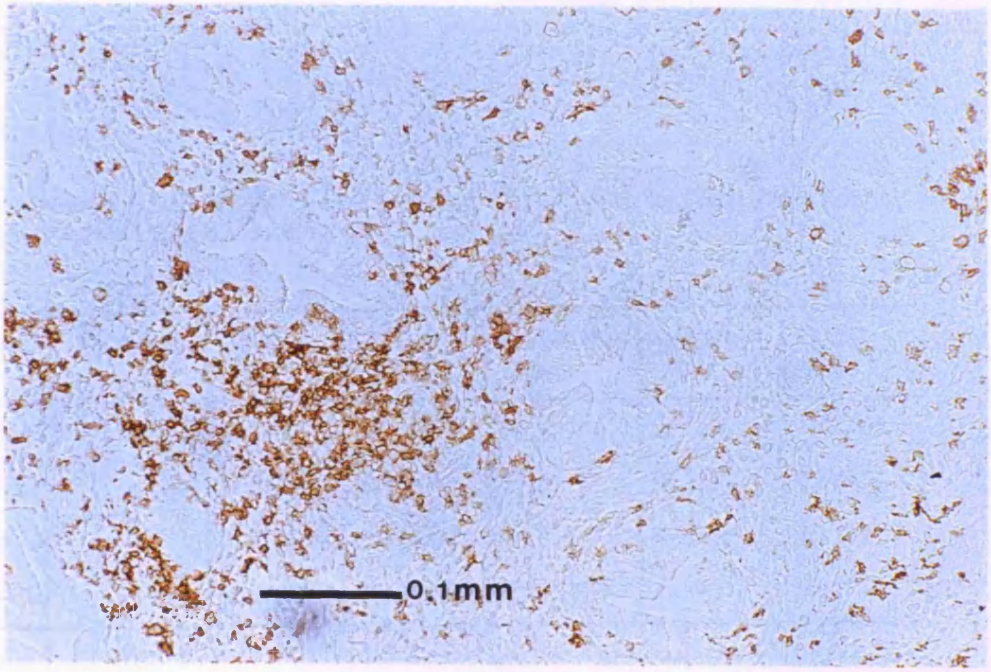
b

Figure (5.5. a,b.)

The distribution of $CD45^+$ leukocytes during the early-LP (**a**), and the mid-LP (**b**). $CD45^+$ cells appeared scattered or in small aggregates, their number increased during the mid-LP.



c



d

Figure (5.5. c,d.)

The distribution of $CD45^+$ leukocytes during the late-LP (**c**), and the late-PLP (**d**). $CD45^+$ cells appeared scattered or in small aggregates. A few intraepithelial cells are seen. A marked increase in number is noted on cHRT.

Figure (5.5.a-d.)

The distribution of $CD45^+$ cells during the different stages of the LP and the late-PLP.

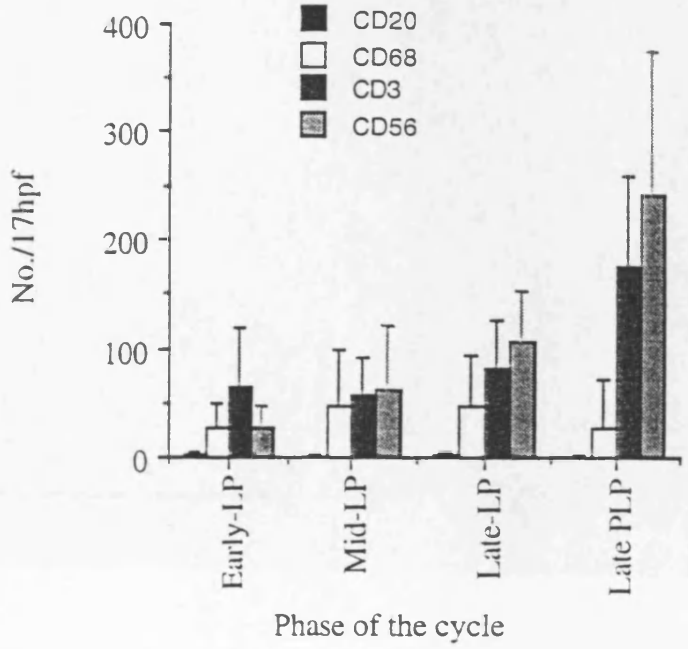


Figure (5.6.)

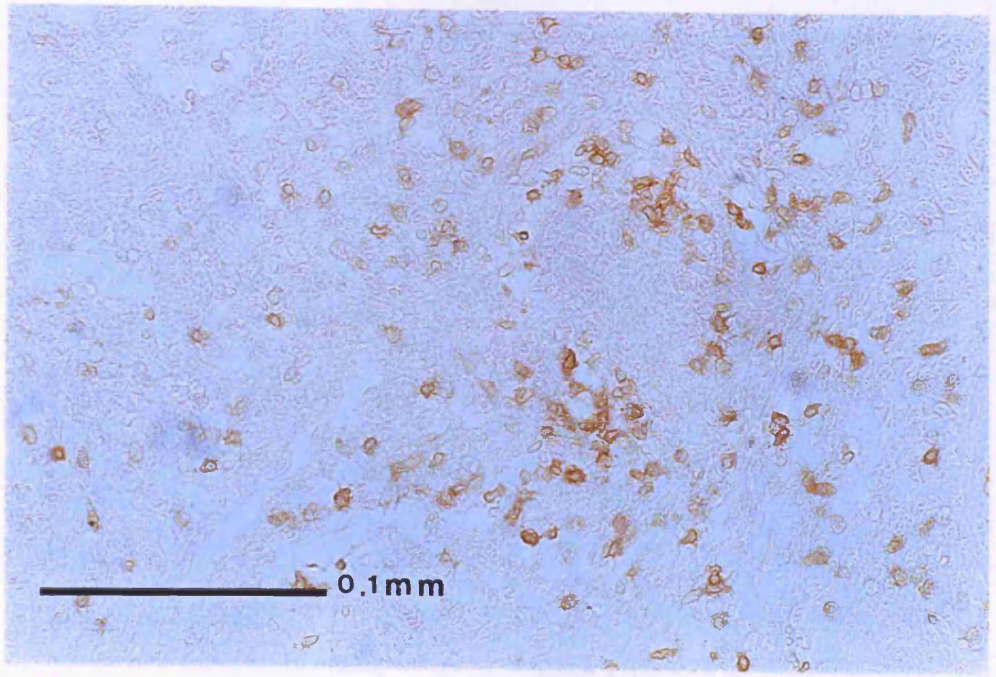
The number of $CD3^+$, $CD20^+$, $CD68^+$, and of $CD56^+$ cells in 17hpf (x400) during the different stages of the LP and the late-PLP.

Phase of the cycle	$CD45^+$ (SD)	$CD56^+$ (SD)	$CD3^+$ (SD)	$CD68^+$ (SD)	$CD20^+$ (SD)
Early -LP	114 (42)	28 (19)	64 (54)	26 (24)	1.4 (3.3)
Mid-LP	157 (104)	62 (58)	57 (35)	47 (53)	0.8 (0.9)
Late-LP	258 (155)	105 (48)	82 (44)	47 (46)	2.2 (2.5)
late-PLP	505 (183)[1]	242 (133)[2]	175 (85)[3]	28 (43)	0.5 (1.0)

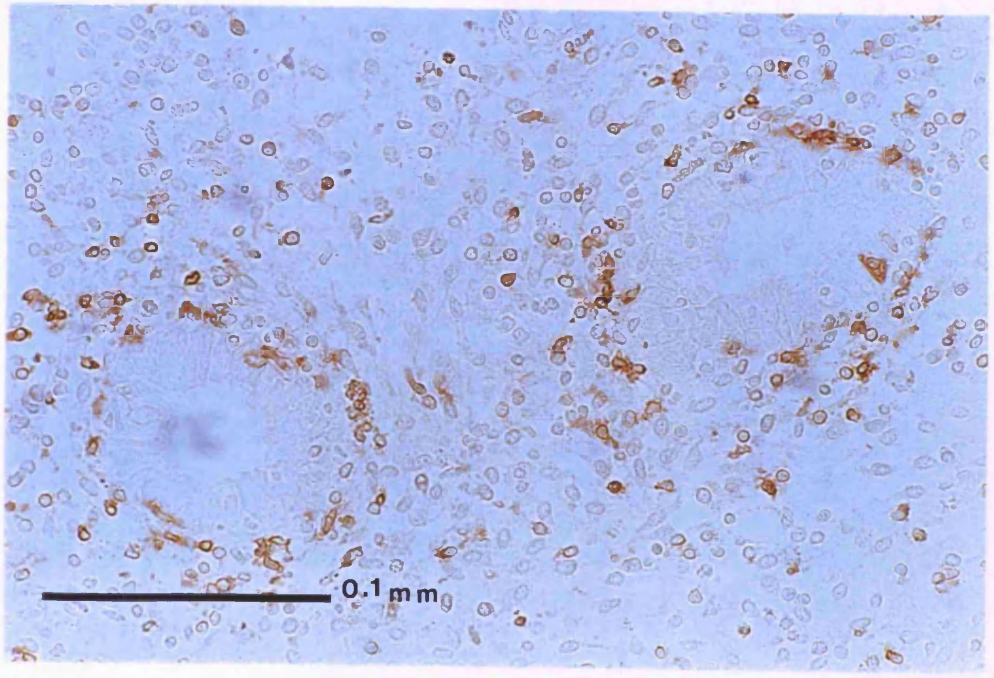
Table (5.7.)

The number and (SD) of $CD20^+$, $CD3^+$, $CD56^+$, $CD68^+$ and $CD45^+$ cells during the different stages of the LP and during the late-PLP.

[1] Statistically significantly different compared to the early-LP (0.0001), the mid-LP (p=0.0004), and the late-LP (p=0.0027).
[2] Statistically significantly different compared to the early-LP (0.0003), the mid-LP (p=0.0039), and the late-LP (p=0.0039).
[3] Statistically significantly different compared to the early-LP (0.0081), and the mid-LP (p=0.0065).



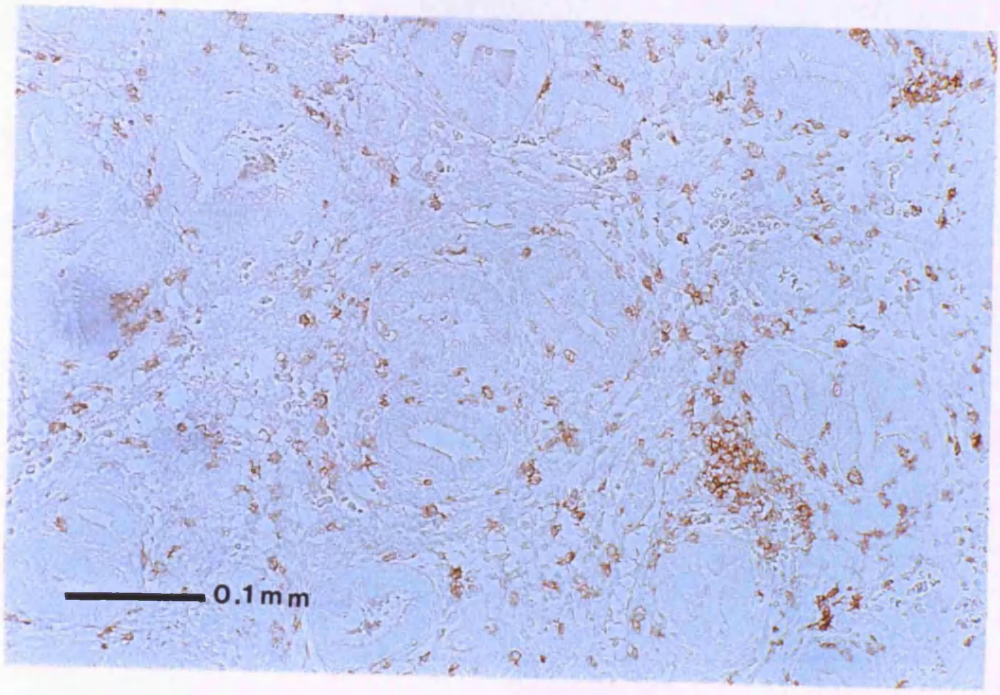
a



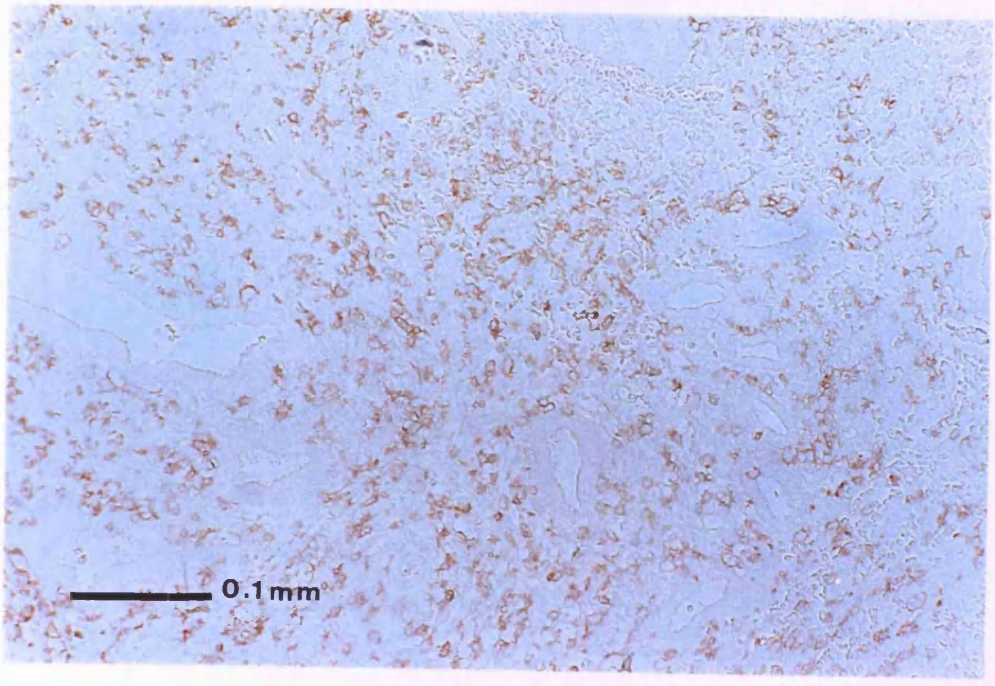
b

Figure (5.8.)

The distribution of $CD3^+$ leukocytes during the late-LP (**a**), the late-PLP (**b**). This shows similar distribution and although the number of cells was higher on cHRT it did not reach statistical significance.



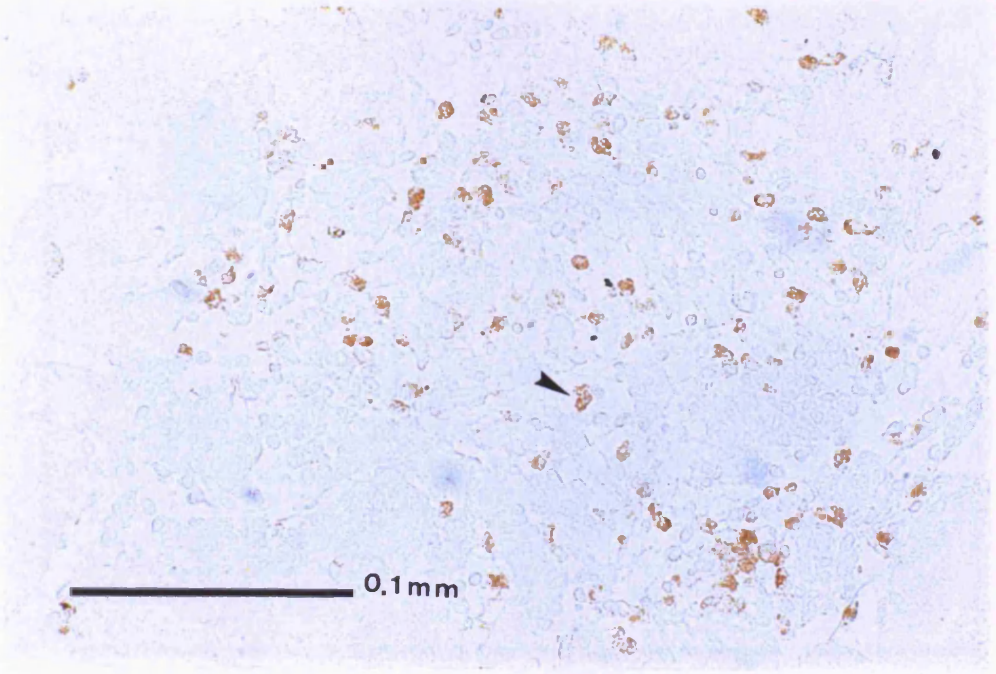
a



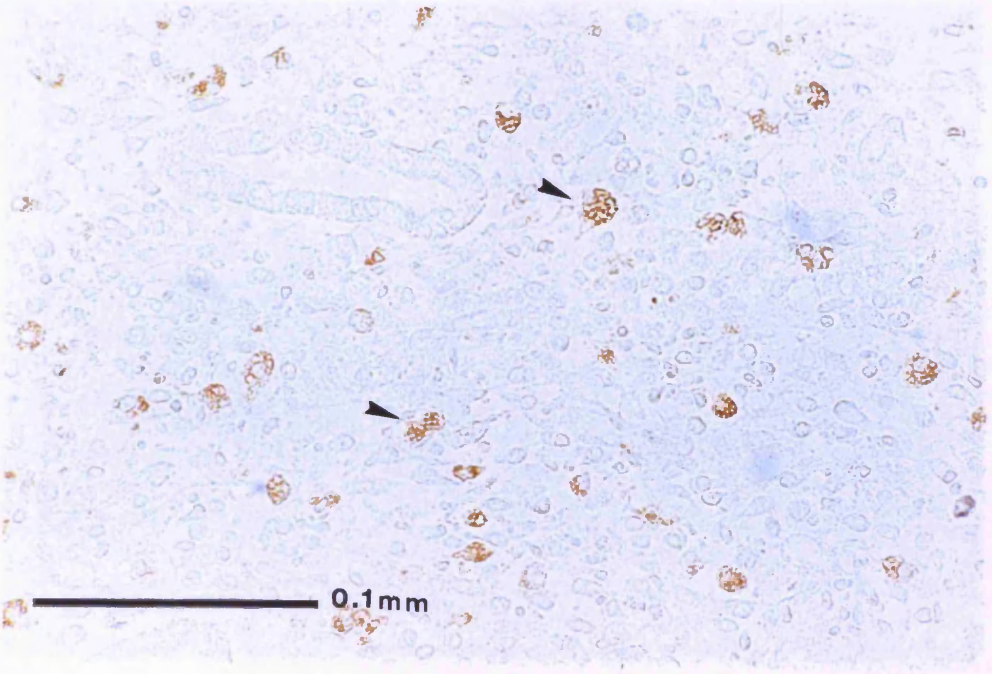
b

Figure (5.9.)

The distribution of $CD56^{+}$ leukocytes during the late-LP (**a**) and the late-PLP (**b**). The distribution of the cells was similar, but there was a dramatic and statistically significant rise under cHRT therapy.



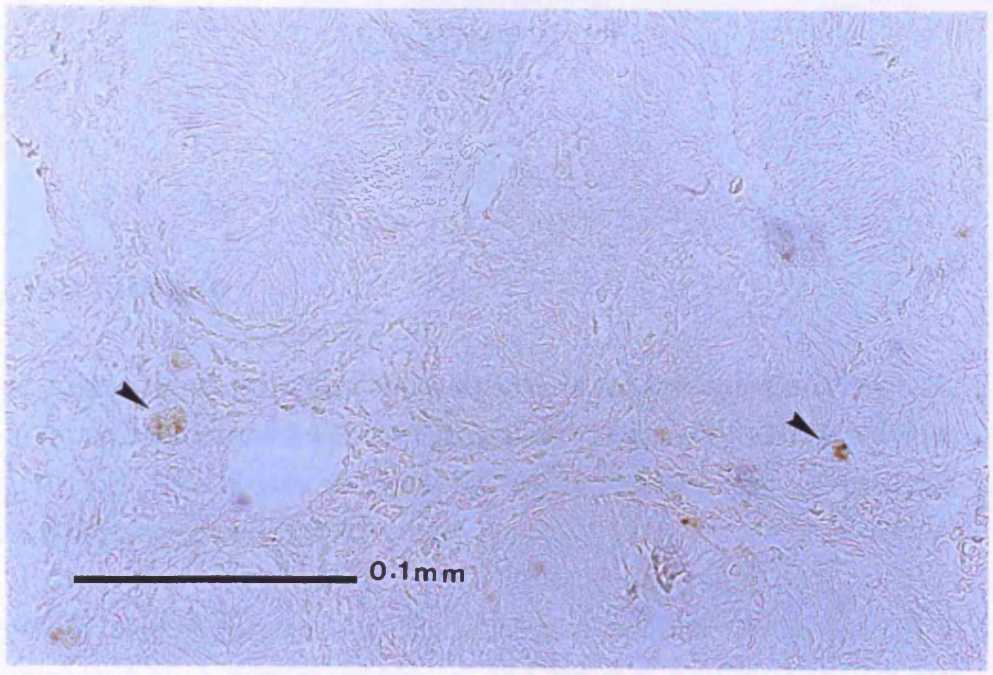
a



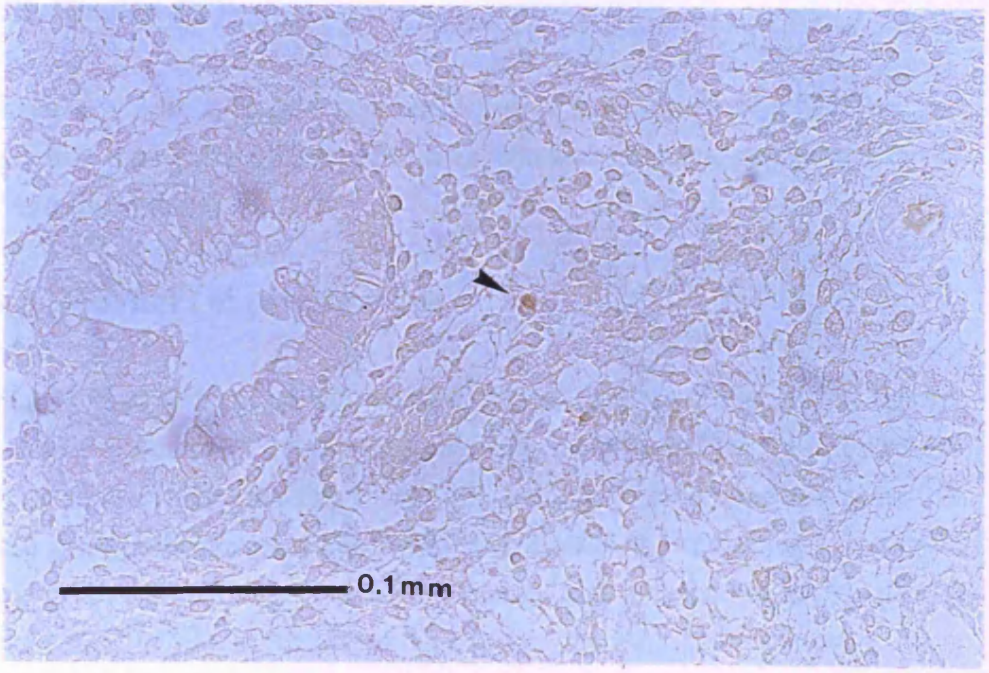
b

Figure (5.10.)

The distribution of $CD68^+$ leukocytes during the late-LP (**a**), and the late-PLP (**b**). There was no difference between these phases in the number or the distribution of $CD68^+$ cells.



a



b

Table (5.11.)

The distribution of $CD20^{+}$ leukocytes during the late-LP (**a**), and the late-PLP. These cells were sporadic and exhibited no differences in number or distribution between the two phases.

Chapter 6

Proliferative activity

6.1. Introduction

In the physiological cycle, proliferation peaks in the functionalis at day 8-10, but is reduced to near zero with the appearance of intra-glandular secretions at day 19 (263). The in-vivo proliferation rate is increased by œstrogen and reduced by progesterone (105).

The rapid decline in glandular epithelial proliferation during the LP, is accompanied by a rise in endothelial and stromal proliferation. Stromal proliferation increases markedly in the late-LP in association with increased decidualization (263).

In Chapter (5) a marked increase was noted in the number of leukocytes, and this study was conducted to determine whether this was accounted for by increased proliferation.

6.1.1. Markers of proliferation

Methods used to measure cell proliferation include counting mitotic figures: the Mitotic Index (MI), which is limited by the low frequency of mitosis in normal tissues. The Labelling Index (LI), measures DNA synthetic phase (S)-phase after incorporating radio-labelled (tritiated) thymidine or 5'-bromo-2'-deoxydridine (BrdUrd) in live cells or tissues, and is therefore not suitable for human tissues unless sustained in culture. Immunohistochemistry, using antibodies to Ki67 or to the Proliferating Cell Nuclear Antigen (PCNA/cyclin), overcome these limitations. More recently, histone ISH was introduced as a marker for the S-phase (264).

6.1.1.1. Ki67

The human Ki67 antigen is a nuclear antigen (a bimolecular complex of 345 and 395 kDa) expressed in all phases of the cell cycle except G_0 and early G_1

(265) It is a useful measure of the growth fraction. The antigen can be demonstrated in frozen (266) and formalin fixed (267) sections.

Measuring Ki67 has advantages over the PCNA which is more sensitive to fixatives and which may yield a gross overestimate of the proliferation fraction (268).

6.1.1.2. Nuclear Histone

The expression of histone mRNA in the nucleus is tightly linked to DNA synthesis. H3 mRNA is detectable in G_1 and peaks during the synthetic S-phase and rapidly disappears towards the end of G_2 (269). The short half-life of histone mRNAs makes them suitable markers for the S-phase. Histone mRNA could be detected in the nucleus and cytoplasm by ISH (269, 270). The probe used in this study hybridises to the mRNA transcripts of human histone genes H2b, H3, and H4, and has been shown to correlate with the synthetic phase as measured by BrdUrd (264).

6.1.2. The effect of steroids on endometrial proliferation

6.1.2.1. The effect of œstrogen

Œstrogen induces proliferation of endometrial epithelium in vivo, but not in vitro (271). The in vivo stimulatory effect may be mediated via growth factors as epidermal growth factor (EGF), transforming growth factor-α (TGF-α), and insulin like growth factor (IGF), while transforming growth factor β (TGF-β) has been suggested as an inhibitory mediator (147, 271). The effect on the stroma is direct or indirect (147).

6.1.2.2. The effect of progesterone

It is generally held that progesterone blocks œstrogen induced proliferation. This may be mediated through down regulation of ER or by reduction of ER binding to its nuclear acceptor, or through 17β-hydroxydehydrogenase, a progesterone-specific enzyme which converts œstrogen into œstrone and œstriol (272).

Progesterone also exerts an inhibitory effect on epithelial growth that is independent of œstrogenic stimulation and it is possible that decreased ER

content and activity are coincidental not causal to blockade of œstrogen induction of mitosis (147).

6.1.3. Proliferation in the endometrium under cHRT

Œstrogen induces epithelial proliferation, which if unopposed may lead to endometrial hyperplasia and cancer, and progestogens have a protective effect (**1.12.3.4.**). The number of dividing cells could be taken as an index of the relative potency of the œstrogenic and the progestogenic compounds in cHRT.

6.1.4. Proliferation of endometrial stromal and lymphoid cells

Tissue loss at menstruation is restored through proliferation from the basalis during the follicular phase and the early-LP. Proliferation in the stroma continues during the LP, but becomes confined to $CD45^+$ cells. This proliferation increases from the mid-FP into the LP to peak during the late-LP, and is more prominent in the scattered compared to the aggregated lymphoid cells (246). $CD3^+$ T cells and $CD11c^+$ macrophages exhibit the same pattern (246) The total number of lymphocytes and T-cells but not of B-cells or macrophages increases with œstrogen and progesterone treatment in women with premature ovarian failure (252). No work to date, reported on the proliferative activity in the endometrial leukocyte in women receiving cHRT.

6.2. Aim

To examine the pattern of proliferation, and the proliferation index (PI) of the different leukocyte populations in the endometrium under cHRT.

6.3. Material and methods

6.3.1. Study population and regimen

Were described previously (**2.1.1., 2.1.2., 4.3.1.**).

6.3.2. Immunohistochemistry and ISH

Single labelling IHC for Mib1 (Appendix 2), and ISH for nuclear Histone, were described previously (**2.1.5.2.1., 2.1.5.2.3., 2.1.5.4.**). Double labelling

(**2.1.5.2.2.**) was performed using Mib1 and each of anti-CD45, anti-CD56, and anti-CD3 (Appendix 2).

6.3.3. Statistical analysis and sample size

Were considered previously (**2.1.7.**).

6.3.4. Image analysis

Was described previously (**2.1.6.**). Cells expressing Mib1, and histone positive cells were counted in 17hpf(x400)/section. In the double labelling experiments, the same number of fields were examined to determine the number and proportion of positive cells.

6.4. Results

6.4.1. Mib1

Stromal Mib1$^+$ cells were scattered or formed clusters in the basalis. Mib1 expression was higher in the late-LP compared to the early-LP or the mid-LP ($p=0.042$ and $p=0.045$, respectively). The number of Mib1$^+$ stromal cells in the late-PLP was statistically significantly higher compared to the early-LP, mid-LP and late-LP ($p<0.0001$, $p=0.0001$, $p=0.0003$ respectively). Mib1 expression was higher in the late-LP compared to the early-LP or mid-LP ($p=0.002$ and $p=0.0068$, respectively). None of the differences in Mib1 expression in the glandular epithelium was statistically significant (Table 6.1., Figure 6.2., Figure 6.3.).

6.4.2. Histone

Histone expression in stromal cells followed the same pattern during the LP and the late-PLP as Mib1 expression (Figure 6.3., Table 6.4., Figure 6.5.). The difference between the late-PLP and the early-LP, mid-LP and late-LP was statistically significant ($p=0.0002$, $p=0.0008$, and $p=0.03$ respectively). There was a good correlation between the number of histone$^+$ and Mib1$^+$ cells in each section (Pearson's correlation coefficient, $r=0.856$, $r^2=0.732$) (Figure 6.6.).

6.4.3. Double labelling CD45 and Mib1

The number of Mib1$^+$ and of CD45$^+$ cells using double labelling (Table 6.7., Figure 6.8.) are comparable to those demonstrated using single labelling (Figure 5.4., Table 5.7.). The vast majority (88-94%) of stromal Mib1$^+$ cells expressed CD45$^+$, and are thus of haematopoietic origin (Table 6.7., Figure 6.9.). The small CD45$^-$ proportion was also morphologically similar to lymphocytes, with large rounded nuclei and sparse cytoplasm (Figure 6.10.). Between 8-37% of CD45$^+$ cells were Mib1$^+$ (Table 6.7.). The proportion of Mib1$^+$,CD45$^+$ cells is high during the mid-LP and drops during the late-LP, and is higher during the late-PLP compared to the late-LP (Figure 6.9.). The difference between the late-PLP and the early-LP and late-LP, but not the mid-LP was statistically significant ($p<0.0001$, $p=0.0001$, $p=0.39$ respectively). The difference between the early-LP and the mid-LP was also statistically significant ($p=0.037$). None of the other differences were statistically significant. The differences between the proportions of CD45$^+$, Mib1$^+$ cells in the different phases were not statistically significant.

6.4.4. Double labelling CD56 and Mib1

The proportion of CD56$^+$, Mib1$^+$ cells was relatively constant throughout the phases of the physiological cycle and similar to the late-PLP, but the proportion of Mib1$^+$, CD56$^+$ cells was 26%, 63%, 24%, and 60% during the early-LP, mid-LP, and late-LP, and the late-PLP respectively (Table 6.11., Figure 6.12., Figure 6.13.).

6.4.5. Double labelling CD3 and Mib1

Although the overall proportions are small, the proportion of Mib1$^+$, CD3$^+$ cells in the late-PLP were double that in the late-LP. The same relation was demonstrated for the proportion of CD3$^+$, Mib1$^+$ cells (Table 6.14.). The proportion of Mib1$^+$, CD3$^+$ cells was lower than the proportion of Mib1$^+$, CD56$^+$ cells in any phase of the cycle (Figure 6.12., Figure 6.15.).

6.5. Discussion

This is the first study of the proliferative activity in the leukocyte subtypes in the late-PLP. Mib1 antigen is expressed at the G_0-G_1 transition and then

throughout the cell cycle, and is maximum during the M phase. Its detection may thus over-represent the MI (273), but it may also be destroyed by fixation. Histone ISH has been shown to correlate with BrdUrd measurement of cell proliferation (199), and the good correlation between Mib1 and histone ISH in this study, argues against an over-estimate of cell proliferation.

In agreement with Tabibzadeh (1990) (246), proliferative activity in the stroma during the LP is largely confined to leukocytes. However, a small percentage of cells was $Mib1^{+}CD45^{-}$. The lineage of these remains speculative but they demonstrated morphological features similar to leukocytes, and it is possible that the inability to demonstrate CD45 antigen was due to methodological limitations.

Increased proliferation in both $CD3^{+}$ T cells, and $CD11c^{+}$ macrophages, during the LP, and an increased proliferation from the early-LP through to the late-LP, has been demonstrated (246). That study also provided evidence (though indirect), of increased proliferation of eGL. The present study provides the first direct evidence of proliferation of $CD56^{+}$ cells. But, contrary to the previous study (246), demonstrated a lower leukocyte Mitotic Index (MI) during the late-LP compared to the mid-LP. The discrepancy may be attributable to the use, in the earlier study, of the less accurate histological dating and/or of hysterectomy specimens, which may have been affected by the disease that necessitated their removal.

Leukocytes appeared scattered or, especially in the basalis, in aggregates. It is not possible to obtain an accurate calculation of the number of cells in these aggregates using image analysis. Indeed the objective of this project was to compare the functionalis (**1.14.**), where only a few aggregates were noted. There were no difference between the number or the size of these follicles in the specimens examined and these were disregarded when calculating the number of scattered cells.

Initial reports suggested that eGL do not proliferate (239). But more recently, these cells were shown using frozen sections to express Ki67 antigen(243), which suggests that these cells proliferate in situ, possibly after migrating into the endometrium as premature precursors. The maximum MI in this study preceded the peak cell density, which further supports local proliferation as the mechanism for the increase eGL population.

The increase in eGL is believed to be progesterone mediated, and the proliferative activity in these cells decrease with decidualization (243), which is again progestogen mediated. The late-PLP thus exhibits a dissociation between the two effects of progestogen as the increasing cell population was not accompanied by a reduced proliferation.

Prolonged progestogen therapy, has been shown to cause a marked increase in $CD45^+$, $CD3^+$, and $CD68^+$ cells as well as eGL (253). It is not clear how much of this increase is caused by increased leukocyte migration, or by increased local proliferation. The role of chemo-attractants was discussed previously (**5.5.**), but the finding from this study that peak proliferation precedes peak cellular density suggests that proliferation plays an important role. Little is known about the factors that regulate leukocyte proliferation. Hormonal control was suggested (246). However, peak proliferation occurs in the scattered leukocytes in the functionalis which are ER^-, rather than in the ER^+ lymphoid aggregates. Thus the effect that is assumed to be œstrogen mediated, is more likely to be achieved through mediators. DNA synthesis has been shown to decrease after several days of œstrogen administration, an effect that may be mediated by inhibitory chalone-like substances (274). Interferon-γ (IFNγ) which exerts an inhibitory effect on proliferation of the basal epithelium may have such a role (275).

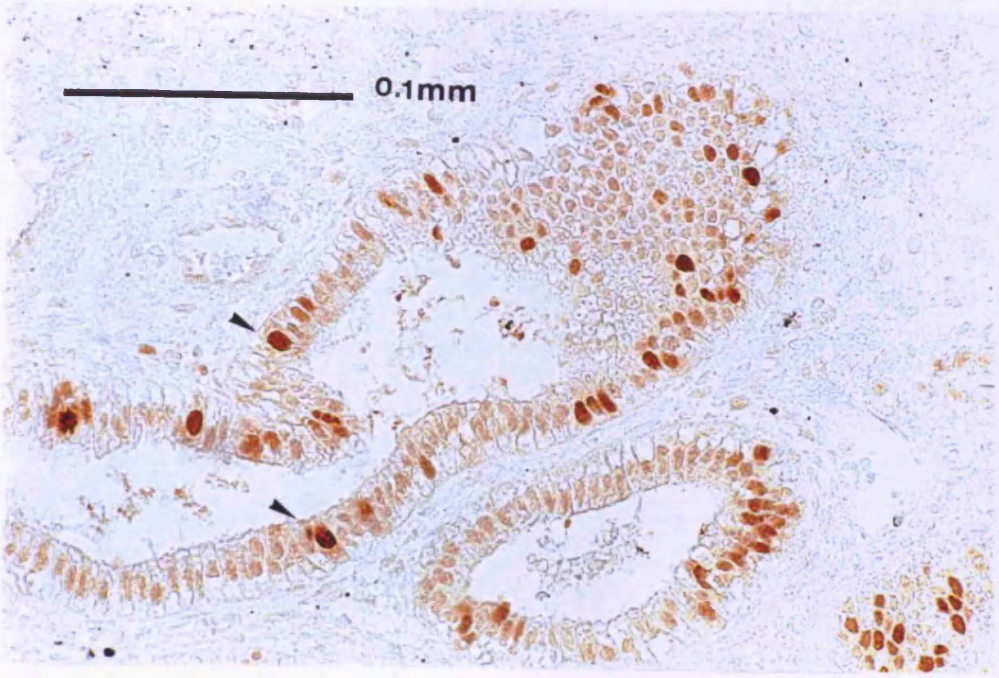
IFNγ is synthesised, under the influence of œstrogen, by T cells located in lymphoid aggregates (275, 276), and by macrophages and NK cells (277). It has been linked to the lower proliferative activity in the stroma in endometriosis (277). Increased stromal proliferation may thus be attributable to a lower level of inhibitory substances, which in turn may be a reflection of low œstrogen level.

The function of eGL is unknown, and despite similarities with peripheral blood NK cell, eGL do not exhibit NK activity (238). They contain perforin, granzyme A and TIA-1 (278, 279). These substances are capable of cytolysis, DNA fragmentation, and cytotoxicity. Activated eGL may be implicated in focal necrosis, stromal haemorrhage or normal or abnormal menstruation, and a similar role has been suggested for $CD68^+$ macrophages (253). However, the large increase in the late-PLP, and the fact that these cells exhibit proliferative activity, negates against a significant role in the regulation of bleeding.

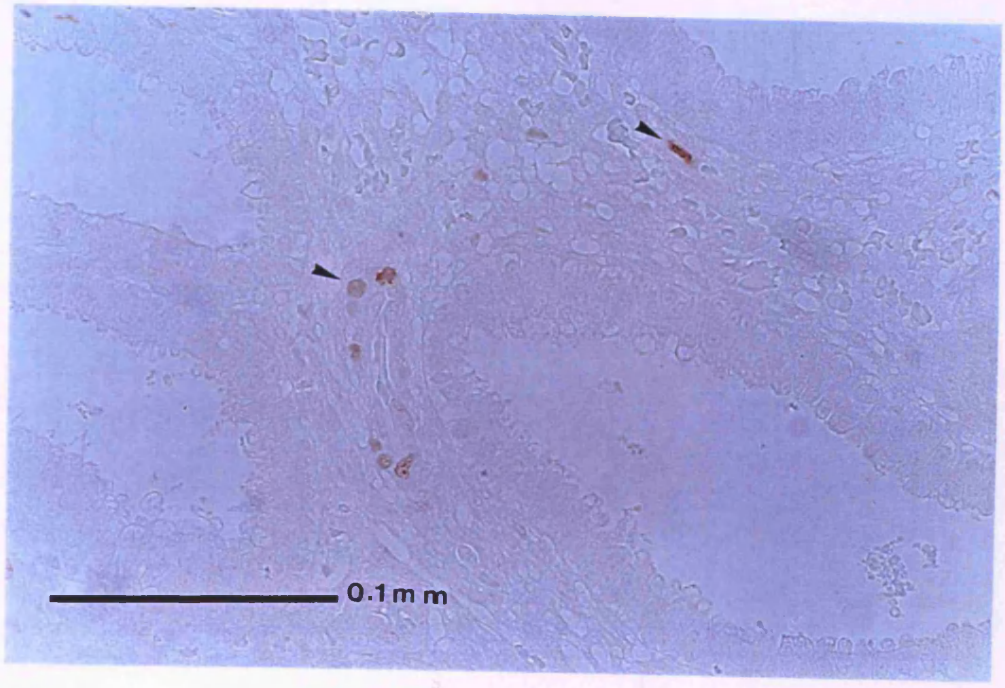
Phase of the cycle	Mib1$^+$ cells	
	Stroma Mean (SD)	Glandular epithelium Mean (SD)
Early-LP	26 (14)	12 (21)[1]
Mid-LP	26 (20)	1 (2)
Late-LP	53 (36)[2]	2 (4)
Late-PLP	302 (175)[3]	1 (2)

Table (6.1.)

Mib1$^+$ cells in the different stages of the LP and in the endometrium in the late-PLP, expressed as number and (SD)/17 random hpf (x400).
[1] Statistically significantly higher compared to all the other stages ($p<0.05$)
[2] Statistically significantly higher compared to the early-LP and the mid-LP ($p<0.05$).
[3] Statistically significantly higher compared to all stages of the LP ($p<0.05$).



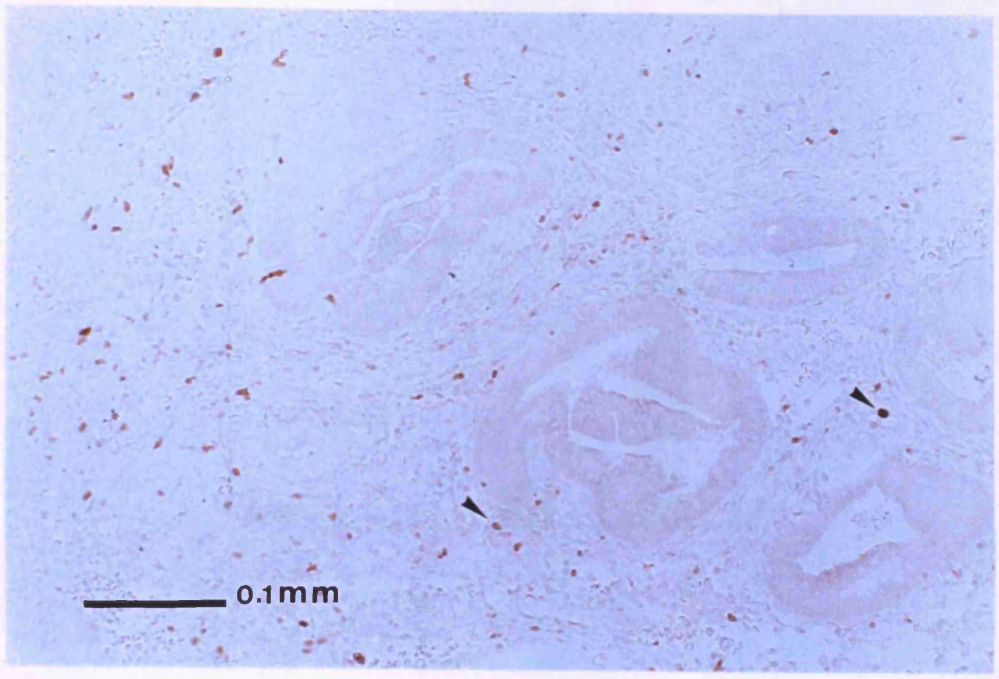
a



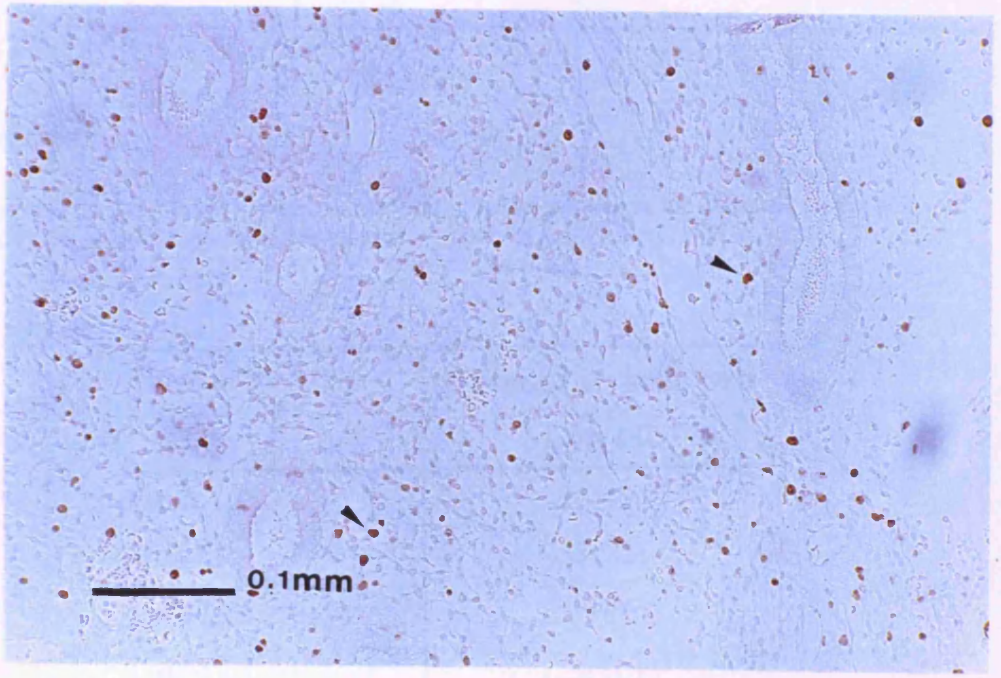
b

Figure (6.2. a,b.)

The distribution of Mib1$^+$ cells in the stroma and in the glandular epithelium in the early-LP (**a**), and the mid-LP (**b**). During the early-LP some glandular epithelial cells, but only a few stromal cells are Mib1$^+$. During the mid-LP, the glands were Mib1$^-$, whilst there was an increase in expression of Mib1 in the stroma.



c



d

Figure (6.2. c,d.)

The distribution of Mib1^{+} cells during the late-LP (**c**), and the late-PLP (**d**). There is no expression in the glandular epithelium, but a marked increase in the stroma, particularly during the late-PLP.

Figure (6.2. a-d.)

The expression of Mib1 during the different stages of the LP and the late-PLP.

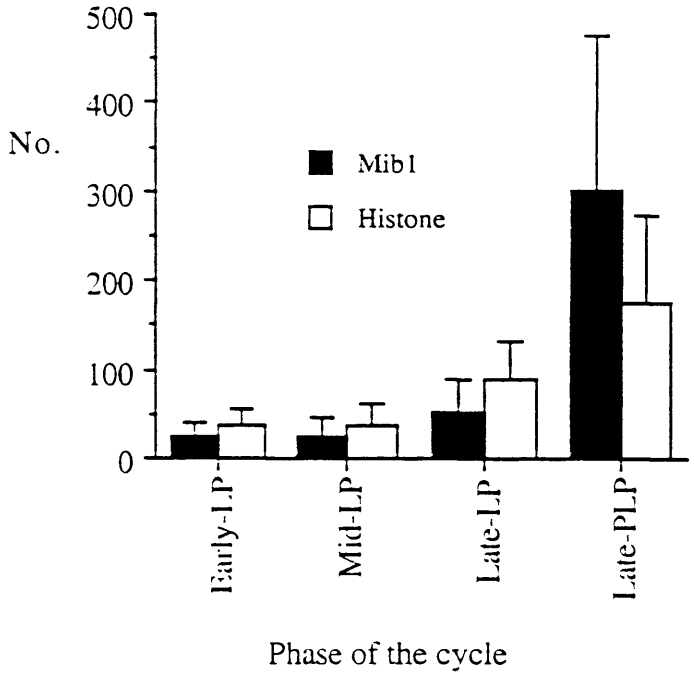


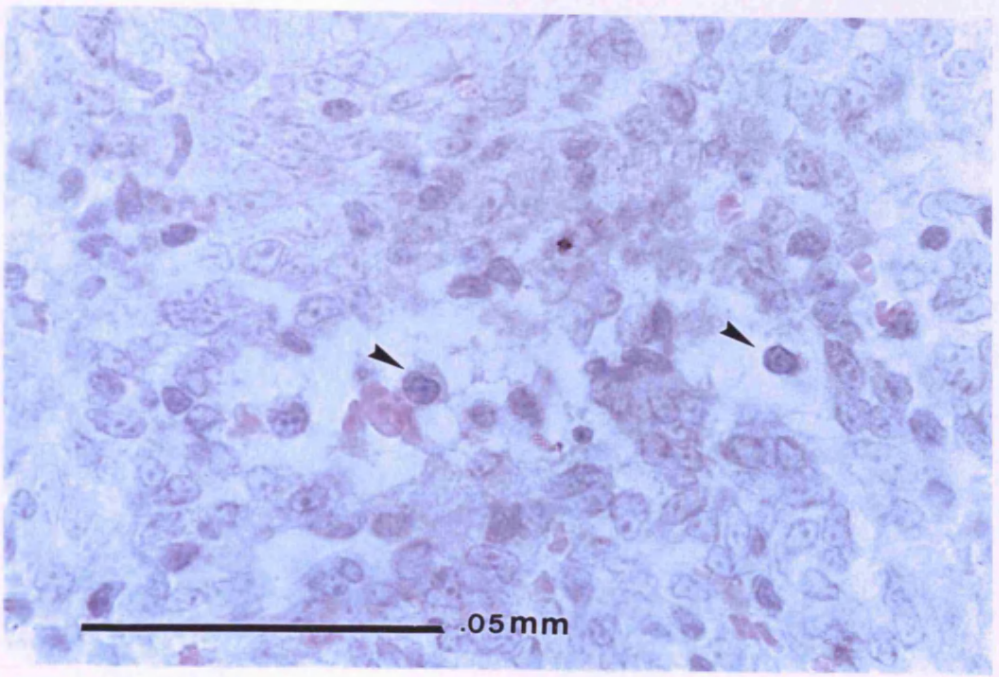
Figure (6.3.)

The distribution of Mib1$^+$ and histone$^+$ cells in the stroma in the different stages of the LP, and in the late-PLP, expressed as number of positive cells/17hpf (x400).

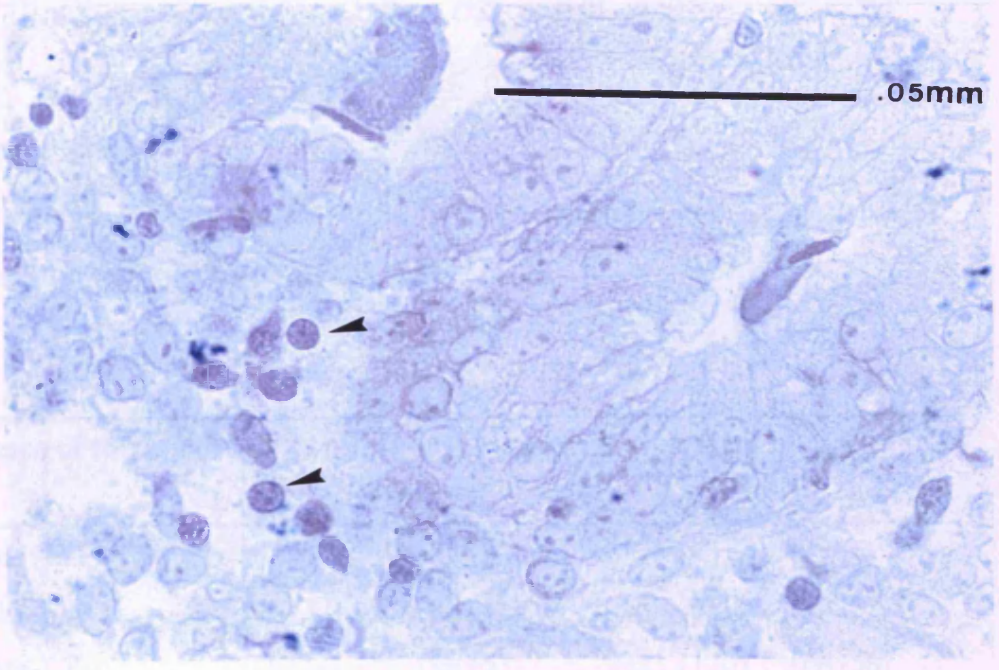
Phase of the cycle	Histone$^+$ stromal cells Mean (SD)
Early-LP	36(19)
Mid-LP	38(23)
Late-LP	88(46)
Late-PLP	175(101)[1]

Table (6.4.)

Histone positive cells during the different stages of the LP and the late-PLP, expressed as number and (SD)/17hpf (x400).
[1] Statistically significantly higher than all stages of the LP ($p<0.05$).



a



b

Figure (6.5.)

The distribution of nuclear histone positive cells in the late-LP (**a**), and the late-PLP (**b**). Epithelial cells were negative, whilst many stromal cells were positive.

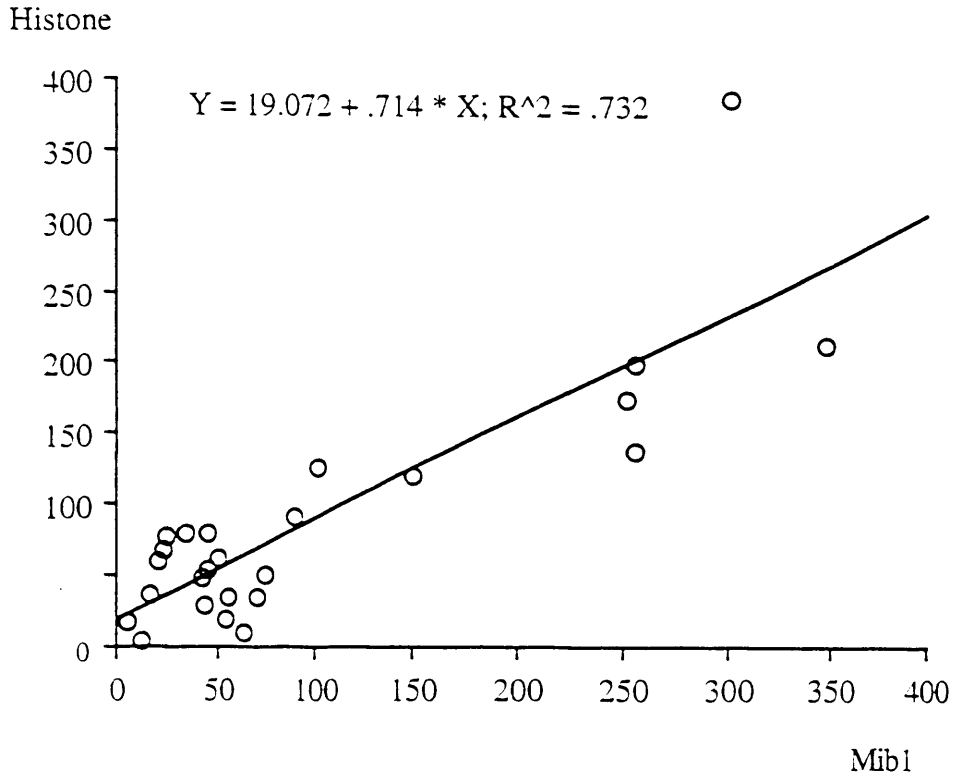


Figure (6.6.)

The correlation between the number of proliferating stromal cells measured by Mib1 and by ISH for nuclear histone.
Correlation coefficient r=0.856, r^2= 0.732.

Phase of the cycle	% Mib1$^+$, CD45$^+$ cells	% CD45$^+$, Mib1$^+$ cells	All CD45$^+$	All Mib1$^+$
Early-LP	8 (4)	94 (8)	125 (43)	15 (6)
Mid-LP	37 (37)	88 (8)	159 (91)	76 (32)
Late-LP	20 (14)	94 (5)	321 (120)	66 (58)
Late-PLP	48 (13)	92 (4)	577 (284)	310 (201)

Table (6.7.)

The total number of CD45$^+$ and of Mib1$^+$ cells, and the proportions of both that are doubly labelled. The values are expressed as means and (SD) measured in 17hpf (x400)/section.

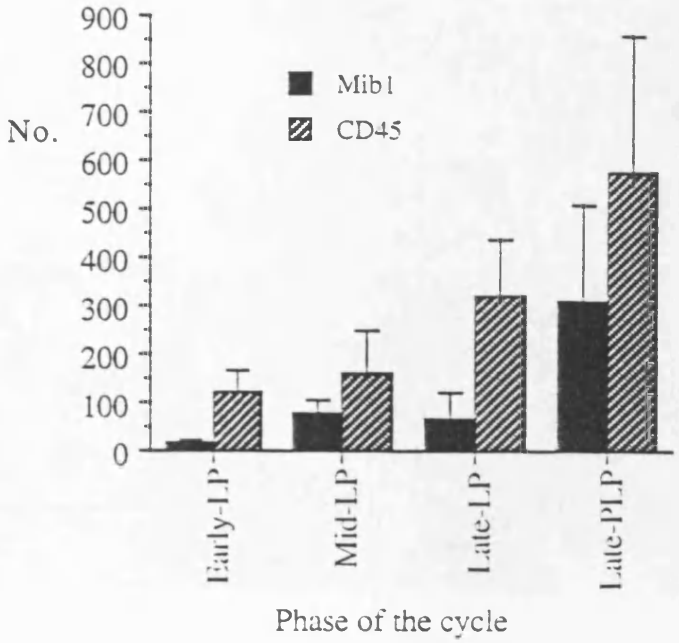


Figure (6.8.)

The number of $CD45^+$ and of $Mib1^+$ cells in the different stages of the LP and the late-PLP, in the double labelling experiment. Error bars indicate the standard deviation of the data.

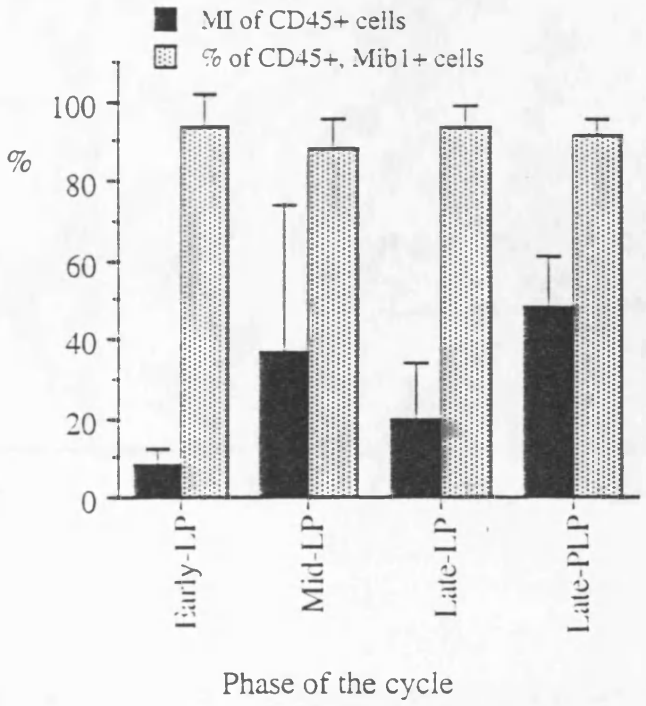
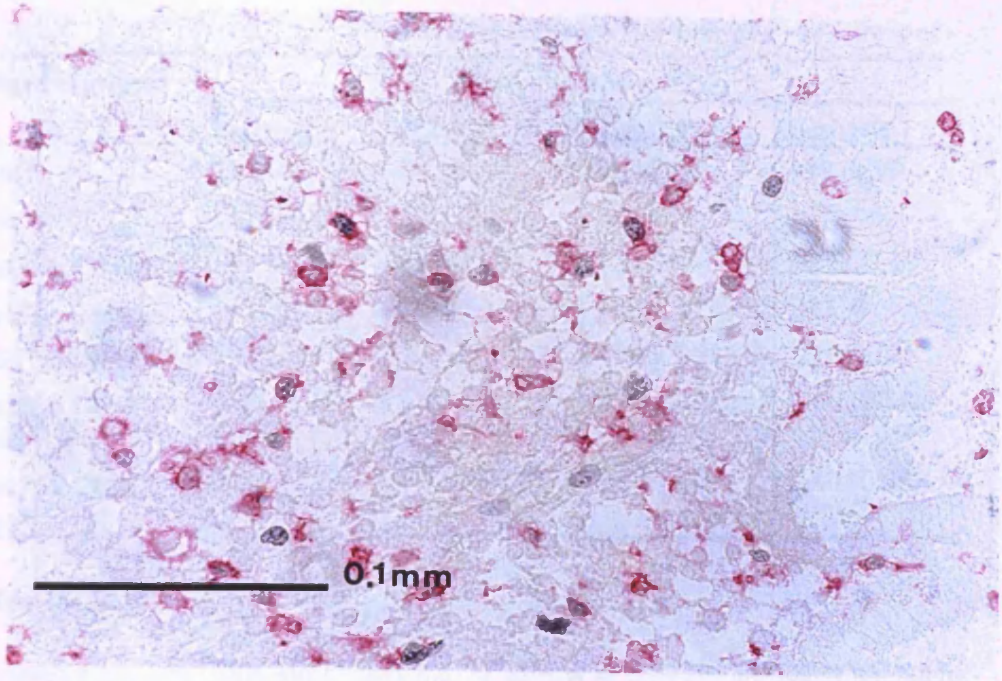
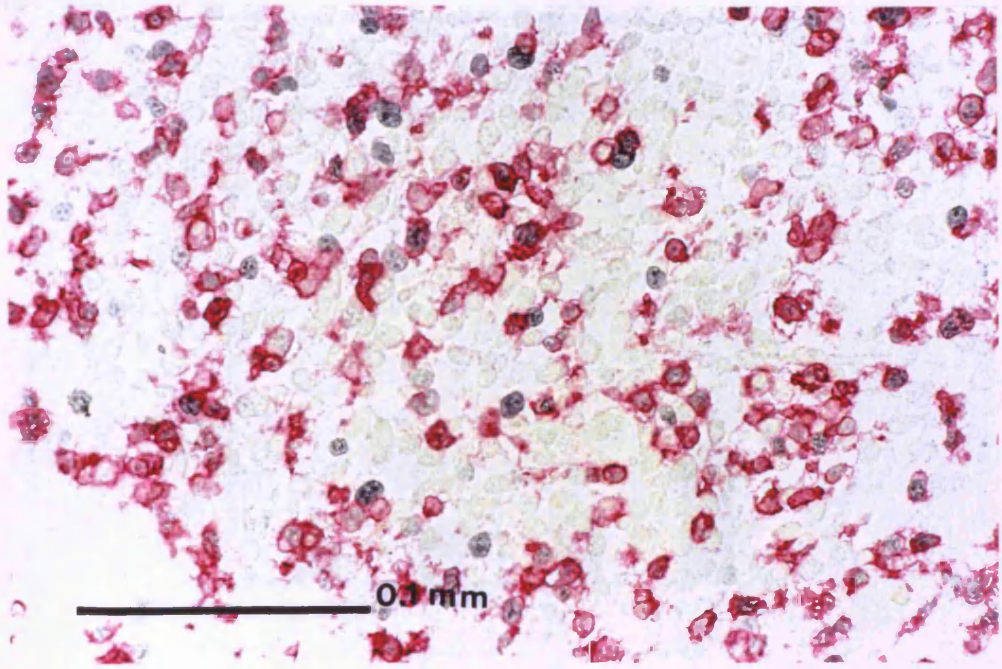


Figure (6.9.)

The proportion of $CD45^+$ cells that are $Mib1^+$, and of $Mib1^+$ cells that are $CD45^+$ in the different stages of the luteal phase and in late-PLP. Error bars indicate the standard deviation of the data. (MI: mitotic index).



a



b

Figure (6.10.)

Double labelling for CD45 (red) and Mib1 (blue-grey) during the late-LP (**a**), and the late-PLP (**b**), demonstrating co-expression of the antigens, and the higher number of proliferating $CD45^+$ cells during the late-PLP. The majority of proliferating cells were $CD45^+$.

Antigen expressed	Phase of the cycle			
	Early-LP	Mid-LP	Late-LP	Late-PLP
$CD56^+$	53 (44)	84 (98)	98 (91)	198 (125)
$Mib1^+$	26 (27)	77 (73)	44 (70)	177 (95)
$Mib1^+$ & $CD56^+$	14 (13)	48 (50.3)	18 (17)	105 (77.4)
$Mib1^+$, $CD56^-$	12 (26)	29 (46)	2 (2)	28 (29)
$CD56^+$, $Mib1^-$	34 (23)	45 (35)	79 (83)	103 (90)
$Mib1^+$ & $CD56^+$/ $CD56^+$	0.26 (0.13)	0.63 (0.23)	0.24 (0.22)	0.60 (0.34)
$Mib1^+$ & $CD56^+$/ $Mib1^+$	0.69 (0.37)	0.75 (0.38)	0.78 (0.38)	0.80 (0.08)

Table (6.11.)

The number of $CD56^+$ cells, $Mib1^+$ cells and the number of cells that are doubly labelled for both antigens per 17hpf (x400). These are expressed as the means and (SD) for each of the study groups. The proportion of the doubly labelled cells to the total number that was $CD56^+$ or $Mib1^+$ is also shown.

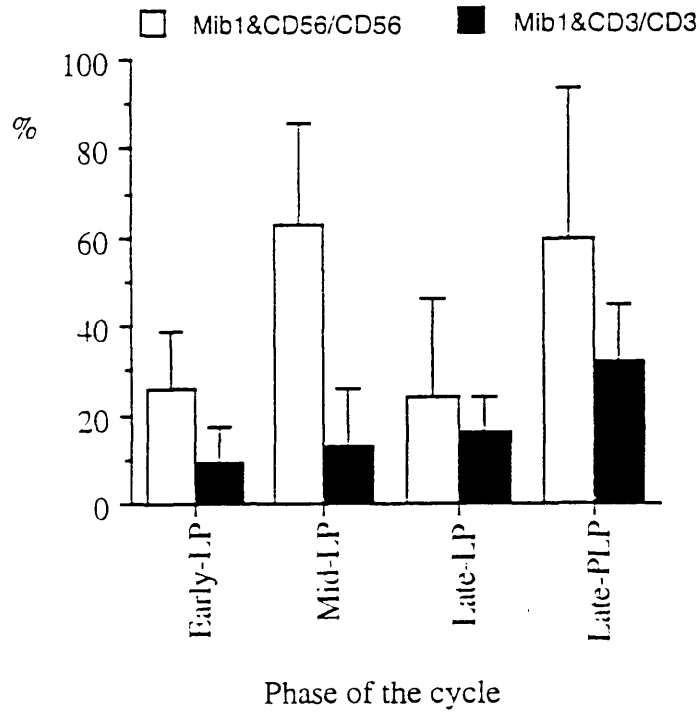
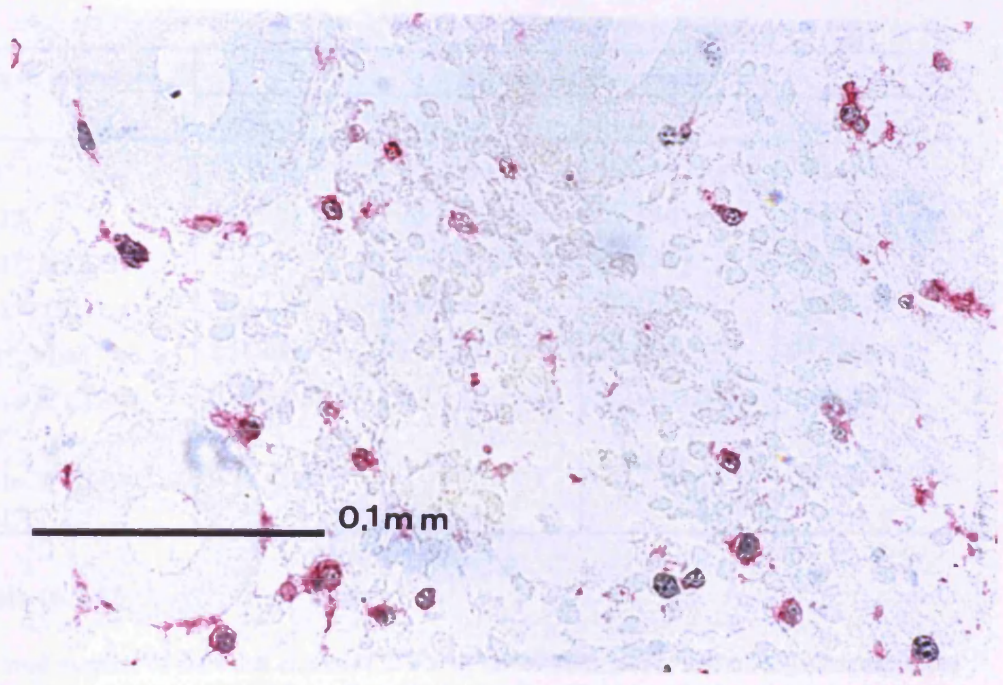
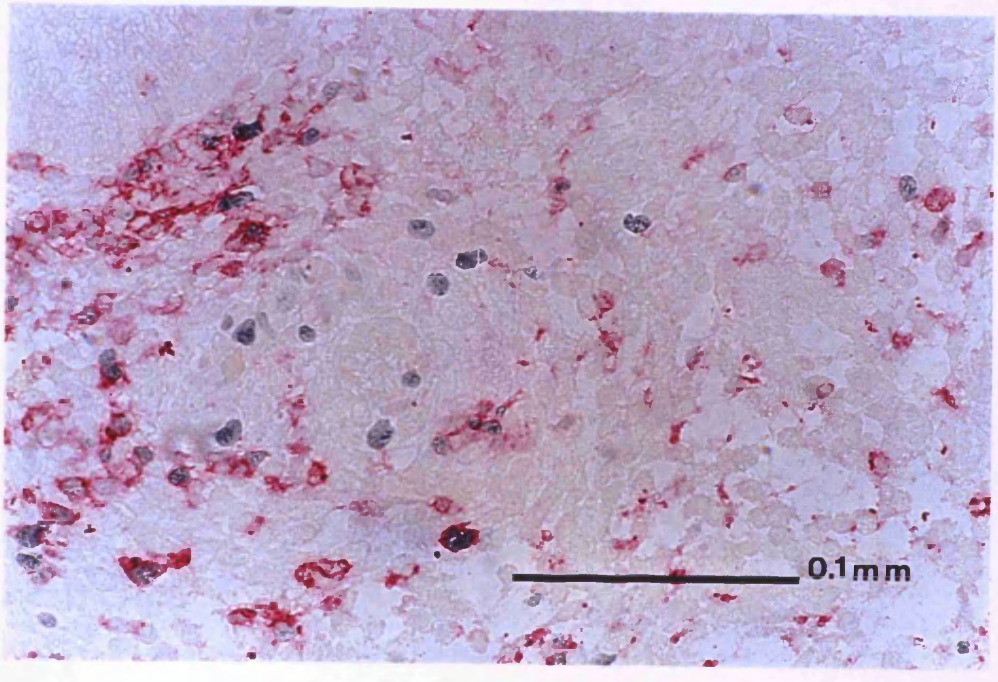


Figure (6.12.)

The proportion of $CD56^+$ and of $CD3^+$ cells are $Mib1^+$ during the different stages of the LP and during the late-PLP.



a



b

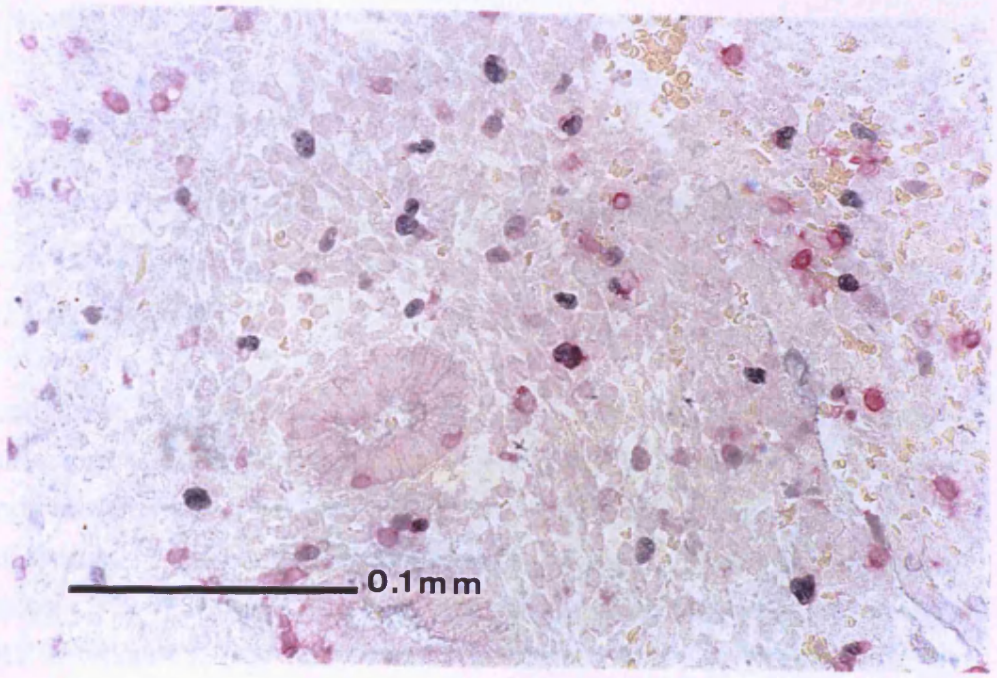
Figure (6.13.)

Double labelling for CD56 (red) and Mib1 (blue-grey) during the late-LP (**a**) and the late-PLP (**b**).

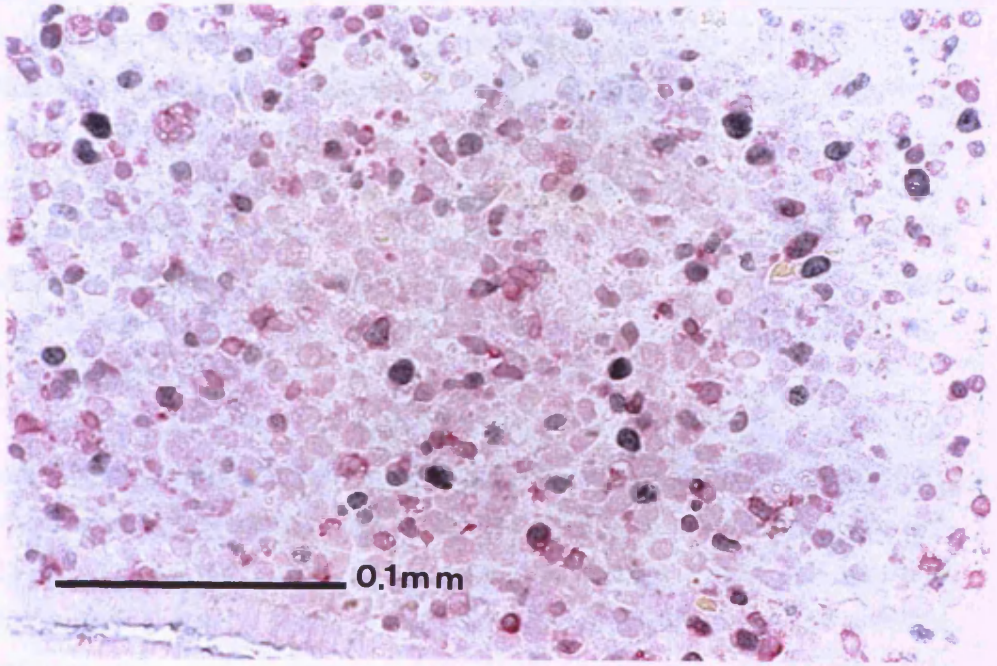
Antigen expressed	Phase of the cycle			
	Early -LP	Mid-LP	Late-LP	Late-PLP
$CD3^+$	57 (23)	42 (38)	105 (91)	148 (89)
$Mib1^+$	34 (26)	78 (78)	59 (34)	181 (119)
$Mib1^+$ & $CD3^+$	4 (3)	9 (11)	20 (31)	56 (51)
$Mib1^+$, $CD3^-$	30 (23)	69 (68)	76 (66)	114 (72)
$CD3^+$, $Mib1^-$	53 (24)	34 (28)	79 (64)	93 (39)
Mib1+ & $CD3^+$/ $CD3^+$	0.09 (0.08)	0.13 (0.13)	0.16 (0.08)	0.32 (0.13)
Mib1+ & $CD3^+$/ $Mib1^+$	0.12 (0.09)	0.07 (0.06)	0.17 (0.06)	0.3 (0.13)

Table (6.14.)

The total number of cells that express CD3 with or without Mib1, and of cells that expressed Mib1 with or without CD3, and the proportion of cells expressing both antigens in relation to those expressing the other antigen.



a



b

Figure (6.15.)

Double labelling for CD3 (red) and Mib1 (blue-grey) during the late-LP (**a**) and the late-PLP (**b**).

Chapter 7

Apoptosis and Bcl-2 expression

7.1. Introduction

According to the widely accepted model for menstruation the functionalis is totally shed, and repair occurs by re-epithelialisation from the outgrowth of glands in the basalis (263, 272, 280, 281). But as the exact amount shed is controversial, a model was constructed involving minimal tissue loss and apoptosis (98, 282). Apoptosis is affected by steroids, but its occurrence with cHRT is unknown. This study evaluates whether the high proliferation rate noted (Chapter 6) is balanced by higher apoptosis, and examines apoptosis in cHRT as a reflection of steroid action. The postulated link between apoptosis and bleeding, together with the link between apoptosis and steroid action indicate a potential as a marker of cHRT action. Women on this cHRT regimen continued to take the same dose of hormones up to the time of the biopsy, i.e. the late-PLP is in effect a sustained mid-LP, and it can be postulated that the incidence of apoptosis will be similar to that in the mid-LP. This will be examined in this chapter.

7.1.1. Apoptosis

Apoptosis eliminates cells without inducing local inflammation, thus avoiding damage to adjacent structures (283). Apoptosis may involve protein synthesis, and is controlled through regulatory genes including *c-myc, p53, apo-1/fas* and the *bcl-2* family. It plays a role in maintaining exact cell numbers, embryogenesis, and in defence against self-reactive lymphocytes, viral infected or tumour cells. Derangement of apoptosis may contribute to cancer, neuro-degenerative or autoimmune diseases, or acquired immunodeficiency (284). Its occurrence in endocrine dependent organs such as the adrenal (285), prostate (286) and endometrium (287, 288), suggests hormone dependence.

The condensation of nuclear heterochromatin and DNA cleavage into multiples of 180bp fragments which form a DNA ladder on agarose gel (289) is a characteristic but not a universal feature of apoptosis (290). Fragmentation of nuclear chromatin is affected by a Ca^{2+}/Mg^{2+} dependent endonuclease,

and is influenced by cytokines and protein kinase C which affect chromatin structure or histone phosphorylation (284). Apoptosis is associated with increased density and a decreased volume of cytoplasm, and factors that may contribute to these changes include *b*-tublin and a Ca^{2+}-dependent transglutaminase (291). Apoptosis can also be induced by other cytoplasmic proteases, as the cystein protease (ICE, interleukin-1-*b*-converting enzyme, Caspase-1) and granzyme B, which is a serine protease that shares with Caspase-1 the ability to induce cleavage after Asp residues.

7.1.1.1. Regulation of Apoptosis

Apoptosis is dependent on the balance between intrinsic and extrinsic factors. Of particular importance in relation to the endometrium is the possible role of eGL which possess in their granules both perforin (278) and granzyme A (GraA), which affects cell death (292), (Figure 7.1.).

Recent studies on the other member of the granzyme family [present in the granules of cytotoxic T lymphocytes (CTL), and natural killer (NK) cells], granzyme B (GraB) demonstrated that it induces apoptosis after entry into target cells through endocytosis, but in the absence of perforin it remains confined to cytoplasm (293). The intracytoplasmic function of GraB is proteolytic cleavage of the caspases, and although its primary target is a matter of controversy (293), it was shown to affect cleavage of caspase-10 and 7, with a weaker effect on the other caspases (294).

Another possible mediator of endometrial apoptosis is Fas antigen which is expressed throughout the cycle, particularly in the glands at the basalis (156). Mechanisms for cytotoxic T lymphocyte/NK cell induced target cell death through the Fas/Fas ligand (FasL) were demonstrated (295), these probably involve trimerisation of Fas (APO-1, CD95). Trimerisation facilitates the interaction of its cytoplasmic tail, containing the 'death domain' with the corresponding domain of the adaptor molecule Fas-associated protein with death domain (FADD), this complex interacts through the caspase FLICE (caspase-8) to activate other caspases and to induce apoptosis (293), (Figure 7.1.).

TNFα can induces apoptosis in the human endometrium (296), where both the cytokine and its receptors were demonstrated (156). TNF receptor (TNF-R1) activation allows binding of its death domain to its adaptor TRADD, which in

turn initiates caspase-10, and this in turn stimulates the level two caspase-3 (297), (Figure 7.1.).

TGFβ_1 and TGFβ2 have been implicated in the regulation of rat stromal apoptosis in vitro (298), and more recently, a related human gene *ebaf* (endometrial bleeding associated factor) which is maximally expressed before and during bleeding, has been identified (299).

Bcl-2, which has been detected in the endometrium, can prevent apoptosis (300). Recently other members of the same family were found in the endometrium, some of which inhibit (Bcl-X_L) but others stimulate (Bax, Bcl-X_S, Bad) apoptosis. Members of this family form dimeric structures, the balance of which determines the cellular response to the apoptotic stimulus (284, 301). Bcl-2 expression in target cells was shown to inhibit NK cell induced-GraB and perforin mediated-apoptosis, through inhibition of Caspase-3 (302). Although this was contradicted by others who reported inhibition only with purified substrates, and that this was bypassed by other granule components (303) these possibly include the slower agent GraA(304).

The role of *c-myc* proto-oncogene in apoptosis is complex, dependent on the interaction with other proto-oncogenes as *bcl-2* and *p53*, and growth factors it induces either proliferation or apoptosis (284). The tumour suppressor protein p53 activates apoptosis, partly through up-regulation of Bax, but this is blocked by Bcl-2 (305), and this block is partly mediated through Bcl-2 binding to p53-binding protein (306). The mechanism of *p53* induced apoptosis also involves the interaction with *p21 WAF/CIP1* gene product which inhibits cyclin-Cdk complex and thereby blocks the transition from G1 to S (284).

7.1.1.2. Apoptosis in the endometrium

Apoptosis may have a role in menstruation, (282, 288, 307), and was demonstrated in endometrial epithelium, but not in the non-decidualized stroma (308).

7.1.1.3. Apoptosis during the menstrual cycle and on HRT

The incidence of apoptosis varies with the phase of the cycle. In the epithelium, apoptosis is absent during the FP, present during the early-LP and rises gradually to a peak during the menstrual phase (296). Apoptosis is higher in the basalis of the glands, but only a few stromal cells exhibit the phenomenon at any stage (296). Apoptotic stromal cells may include $CD56^+$ cells which undergo apoptosis during the late-LP or in the decidua in cases of pregnancy failure (254). There are no studies, to date, on the incidence of apoptosis under cHRT.

Although apoptosis mainly affects epithelial cells in the basalis, and less extensively the stroma (284, 296, 309), one study reported a higher incidence in the functionalis (310).

7.1.1.4. Œstrogen and progesterone effect on apoptosis

The association between uterine epithelial apoptosis and œstrogen withdrawal was demonstrated in the mouse (311), the hamster (312), and the rabbit (287, 308). Apoptotic bodies can be detected from the 20th day of the human menstrual cycle, and by the 22nd day 80% of the curettings become positive. From the 24th day to the 28th day of the cycle apoptosis is present in 90-100% of curettings (288).

Progesterone, as well as œstrogen, affected the rate of cell death in the rabbit (287). Œstrogen decreases apoptosis and progesterone, but not œstrogen if given alone, promotes cell survival. The reduction in apoptosis induced by progesterone is more than if both hormones were co-administered (287, 308). Furthermore, the anti-progesterone RU486 increases the incidence of apoptosis, but the anti-œstrogen tamoxifen appears to be ineffective (308).

7.1.2. Bcl-2

Bcl-2 is localised in the intracytoplasmic part of mitochondrial membranes of cells characterised by apoptosis (313). It prolongs survival by protecting against apoptosis (300). Bcl-2 and other members of its family form into dimeric structures, and their net effect is dependant on the resulting balance (284). Bcl-2 acts either as a channel protein with membrane transport effect on Ca^{2+} and on proteins as the mitochondrial protease activators (cytochrome-*c* and apoptosis-inducing factor (AIF), and/or it can act as an adaptor/docking

protein that can bind to several proteins including p53-BP2, GTPases, and the protein kinase Raf-1 (306).

7.1.2.1. Bcl-2 in the endometrium

The relation between Bcl-2 and apoptosis, suggests a possible involvement in the mechanisms of bleeding (156). Bcl-2 is maximum in the uterine glandular epithelium in the late-FP, and is weaker or totally negative in the LP (309, 314). In the glandular epithelium, Bcl-2 immunoreactivity increases from the functionalis into the basalis (156). Bax protein is localised mainly in the epithelium with little staining in the stroma, and is strongly expressed during the LP in the functionalis (301, 315). Epithelial expression in the FP has been variably reported as strong (301), or modest (315). Stronger Bcl-X (both Bcl-X_{long} and Bcl-X_{short}) staining was also found in the LP especially in the functionalis (315).

7.1.3. Detection of Apoptosis and Bcl-2

In hematoxylin-eosin (H&E) stained sections, apoptotic cells are identified by marginal hyperchromatin and deep eosinophilic cytoplasm, this develops into apoptotic bodies containing nuclear fragments and a scanty cytoplasm, but identification is facilitated using TUNEL or ISEL (**2.1.5.3.**). Bcl-2 can be detected using IHC and monoclonal antibody.

7.2. Aim

To determine the incidence of apoptosis and the expression of Bcl-2 as indices of steroid action in the late-PLP, in comparison to the physiological cycle.

7.3. Material and methods

7.3.1. Study population and regimen

Were described previously (**2.1.1., 2.1.2., 4.3.1.**).

7.3.2. Immunohistochemistry and TUNEL

IHC for Bcl-2 (Appendix 2), and TUNEL, were described previously (**2.1.5.2, 2.1.5.3.**).

7.3.3. Statistical analysis and sample size

Were considered previously (**2.1.7.**).

7.3.4. Image analysis

Was described previously (**2.1.6.**).

The number of $TUNEL^+$ cells and of $Bcl\text{-}2^+$ stromal cells were counted in 17 random hpf(x400)/section. Bcl-2 staining in the glands was assessed by measuring the percentage of the glands that stained positive in 17hpf (x200). The intensity of immunoreactivity was scored subjectively as nil=0, weak=1, or strong=2. The percentage staining positive multiplied by the Intensity Score yielded the Total Staining Score.

7.4. Results

7.4.1. Apoptosis

Apoptotic cells exhibiting the characteristic hyperchromasia and margination of heterochromatin and deep eosinophilic cytoplasm, were identified in H&E sections using light microscopy. These were single subepithelial cells or small clusters within the glands. Apoptotic cells were more frequent in the late-LP and least during the FP, but the overall incidence was low (Figure 7.1.), and only very occasionally were they seen in the stroma. More often apoptotic bodies were seen near the base of the glands. The same pattern was confirmed using TUNEL. There was a statistically significant increase in the number of $TUNEL^+$ epithelial cells during the late-LP compared to the early-LP ($p=0.003$) and the mid-LP ($p=0.003$), and between the late-PLP and the early-LP ($p=0.03$) and mid-LP ($p=0.03$). There was no statistically significant difference between the late-LP and the late-PLP (Table 7.3.).

7.4.2. Bcl-2

In the stroma, the vascular endothelium and perivascular cells were negative, and immunoreactivity was localised to individually scattered round cells which were morphologically similar to leukocytes. The number of these cells remained stable during the early-LP and the mid-LP, but increased markedly during the late-LP. A comparable pattern of immunoreactivity was noted during the late-PLP. The difference between each of the late-LP or the late-PLP on one hand, and the early or the mid-LP on the other was statistically significant ($p<0.05$) (Table 7.4.).

Bcl-2 expression was also noted in the glandular epithelium, although immunoreactivity was patchy and weak, particularly during the mid-LP and the late-LP and the late-PLP (Table 7.4., Figure 7.5.). The luminal epithelium expressed Bcl-2 more consistently than the glandular epithelium in all the stages examined (Table 7.4.), but there were no statistically significant difference between the Total Staining Scores of the luminal epithelium between the different stages.

7.5. Discussion

Although TUNEL is not specific for apoptosis (198), confirmation of light microscopic features excluded the possibility of false positive readings. The pattern of distribution and the low incidence of apoptosis noted in this study is in agreement with previous reports (296), and with studies using light and electron microscopy (288), and studies on the rabbit endometrium (287, 308). A recent study (310) reported the unusually high apoptotic index (number of apoptotic cells/the total number of cells x100), during the late-LP, of 70.7%, 74.7%, and 79.7% in the surface epithelium, the glandular epithelium, and the stroma respectively, and that apoptosis is present in the functionalis but not the basalis. This in not supported by the current study or by others (156, 309). The reason for this discrepancy is unclear, but may be due to factors of tissue fixation or processing that may have increased tissue necrosis. The low incidence of apoptosis is also consistent with the known short cycle (1-3 hour) from initiation to cell elimination (197).

T cells and TNF-α may have a role in human endometrial apoptosis (156). TNF-α and IFN-γ produced by T lymphocytes (together with other cytokines), may play a role in inhibiting cellular proliferation and inducing apoptosis in a gradient diffusion model from the basal through to the more superficial layers

(156). The findings in the present study could be explained through a similar mechanism, for a relative œstrogen deficiency may induce less IFN-γ and TNF-α which may be responsible for the higher proliferative activity and the lower rate of apoptosis observed (although the latter did not reach statistical significance).

There remains some disagreement over the pattern of Bcl-2 expression in the normal endometrium (301, 314, 316, 317). In the glandular epithelium, Bcl-2 was reported present during the early-LP (314), or to disappear 2-3 days after the onset of secretory changes, with weak immunoreactivity in the late-LP (316), or to persist during the LP, although the extent and the intensity were both reduced compared to the FP (317). Strong epithelial staining was noted during the early-LP, no epithelial staining during the mid-LP or the late-LP (301). Stromal immunoreactivity was reported to increase in the predecidualized or late-LP endometrium (316, 317). This is mostly accounted for by lymphoid cells especially $CD56^+$ (301, 317). One study reported a gradual decrease in Bcl-2 expression during the early-LP to late-LP, and - in disagreement with other published work - no cyclic changes in the stroma (309).

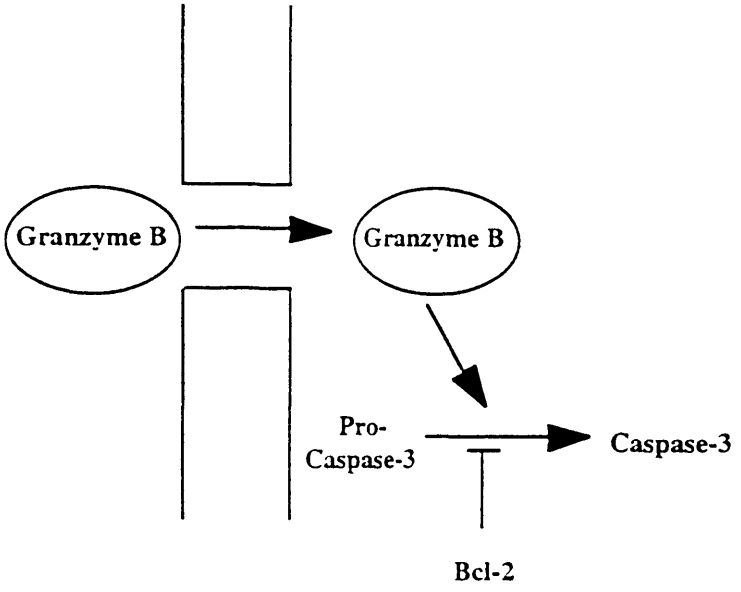
This study is in agreement with two detailed studies (156, 317) which specifically addressed the distribution of Bcl-2 in the different compartments of the endometrium. The similar pattern and intensity of Bcl-2 expression on cHRT where progestogen levels were maintained till the end of the PLP, supports the finding of a previous study (317), that HCG rescue in the late-LP had no effect on Bcl-2 expression. Apoptosis was also observed in the decidua despite high plasma progesterone and endometrial PR mRNA (318). There is thus evidence that the fall in epithelial Bcl-2 expression which is observed during the LP (although possibly initiated by progestogen), continues irrespective of a subsequent fall (as in the natural cycle), steady state (as in the HRT model), or rise (as in HCG rescue model) in progestogen, and may thus be an insensitive marker of progestogenic effect. Furthermore, the subsequent occurrence of bleeding after progestogen withdrawal and the ability of progestogens to rescue the endometrium and to prevent shedding, argues against the hypothesis that Bcl-2 has an important regulatory role with regards to bleeding. It is, however, possible that the fall in Bcl-2 has a *permissive effect* in relation to apoptosis. It is interesting that Bcl-2 expression was found not to correlate with the occurrence of apoptosis in eutopic endometrium, and

not to show cyclical fluctuations in ectopic endometrium (309), which may argue against a significant role in regulating cell death. On the other hand, clarification of the role of Bcl-2 may be achieved through the study of its interactions with other members of this family such as Bax, Bcl-X_L, Bcl-X_S, and their dimerization, but the phenomenon remains incompletely understood, and there is lack of agreement on the exact pattern of expression of this family during the cycle (301, 315).

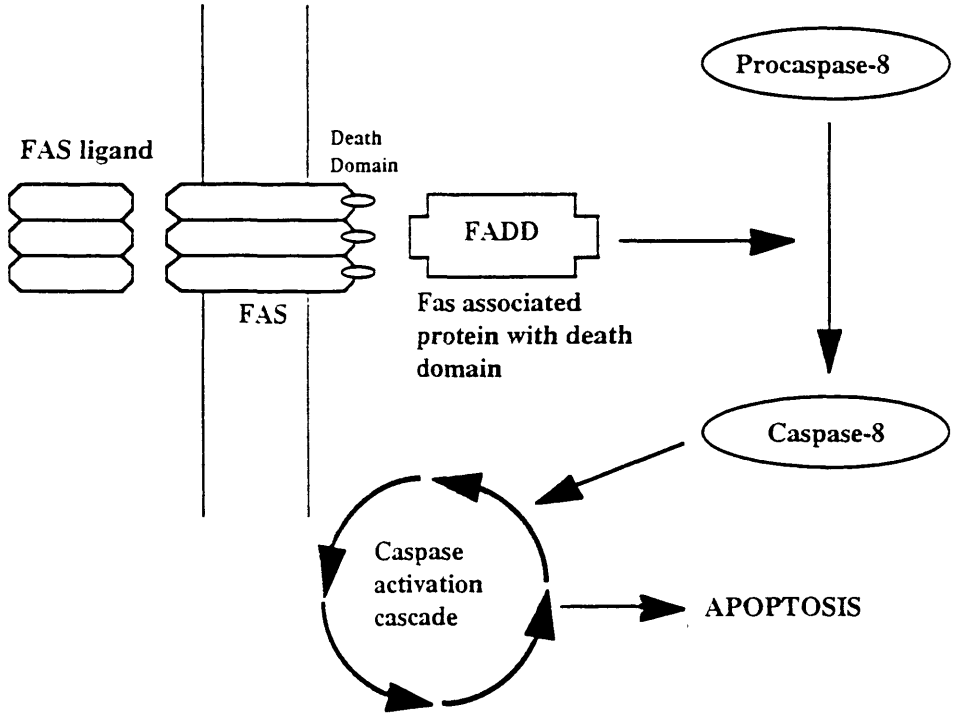
The findings in the present study of only a small number of apoptotic cells in premenstrual endometrium from both the physiological as well as cHRT cycle, is in agreement with others (309, 315), and argues against a significant role in the phenomenon of bleeding, it is possible that the role of apoptosis is in preparing for tissue repair during the FP.

Apoptosis in the endometrium may be induced through mediators as tumour necrosis factor-α (TNFα). The expression of both the cytokine and its receptors (TNFr-I and TNFrII) have been demonstrated in the endometrium (156). The level of TNFα expression in the endometrium increased gradually throughout the secretory phase to peak prior to menstruation, and although immunohistochemical and Western blot studies showed that the level of receptors did not fluctuate with the phase of the cycle, higher receptor levels were found near the base of the glands where apoptosis is most frequently found (156). Maximum Bcl-2 expression, which was reported to protect against TNFα-induced cytotoxicity (319), was also noted near the base of the glands at the same site where apoptosis is increased, this suggest that the primary role of these factors is in the regulation of proliferation and differentiation, rather than in menstruation.

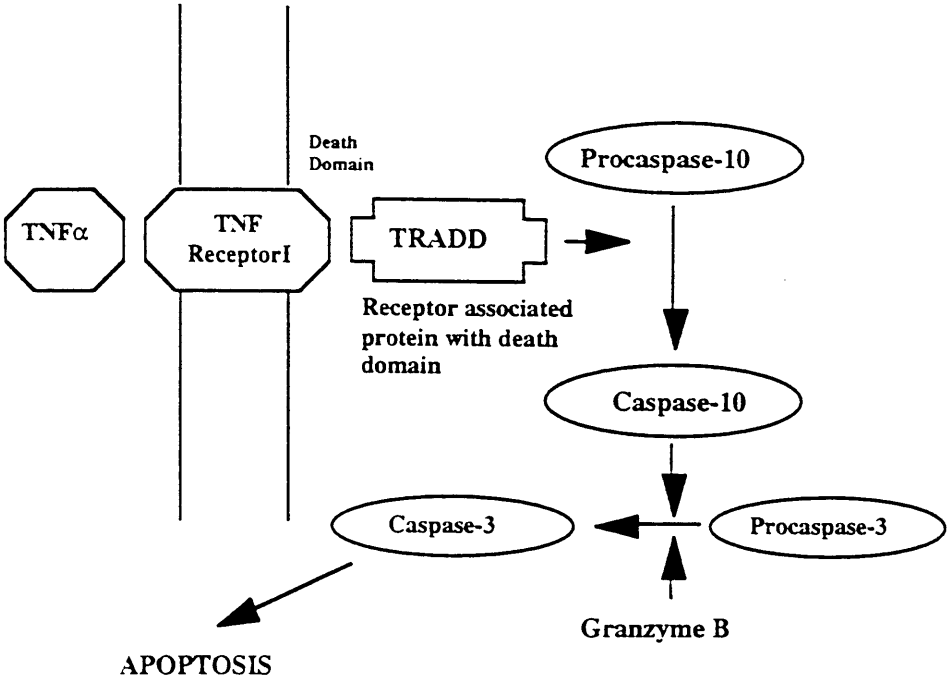
The exact factors modulating Bcl-2 expression are not clear, although its cyclicity suggests hormonal control (314). This, however, does not provide evidence as to which steroid is more influential. The data from the current study suggests that a fall in progesterone is not essential to the continued drop in Bcl-2. It is possible, however, that the continued fall is a reflection of a 'relative' progestogen deficiency secondary to low levels of receptor, or a reflection of 'programming' that was initiated by the commencement of progestogen.



A. Bcl-2 inhibits caspase-3 activation induced by granzyme B.



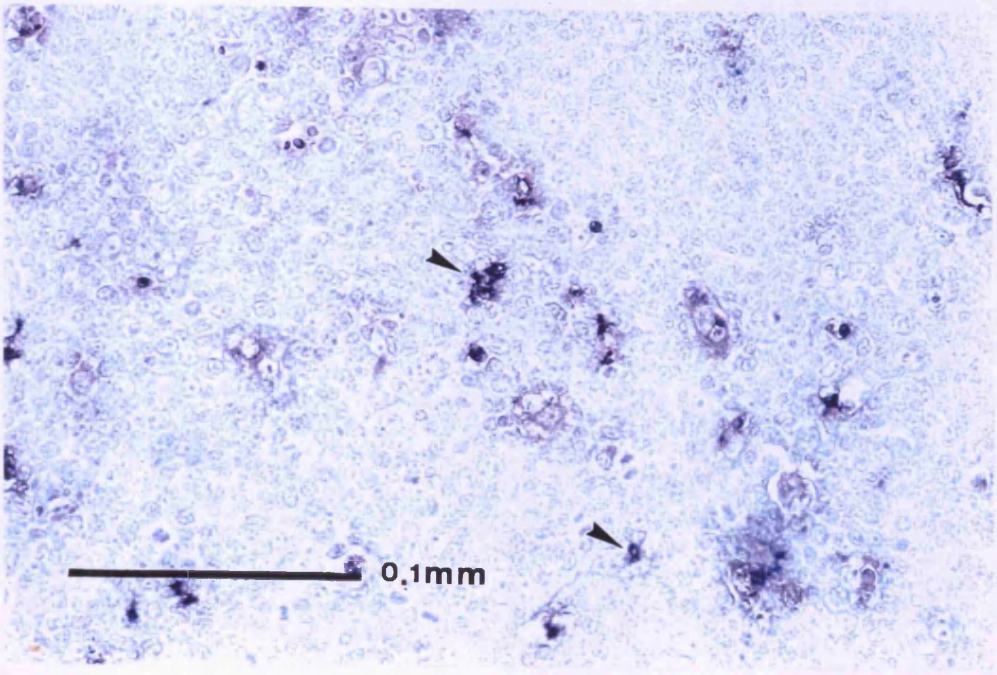
B Pathway for FAS/Fas ligand induction of apoptosis.



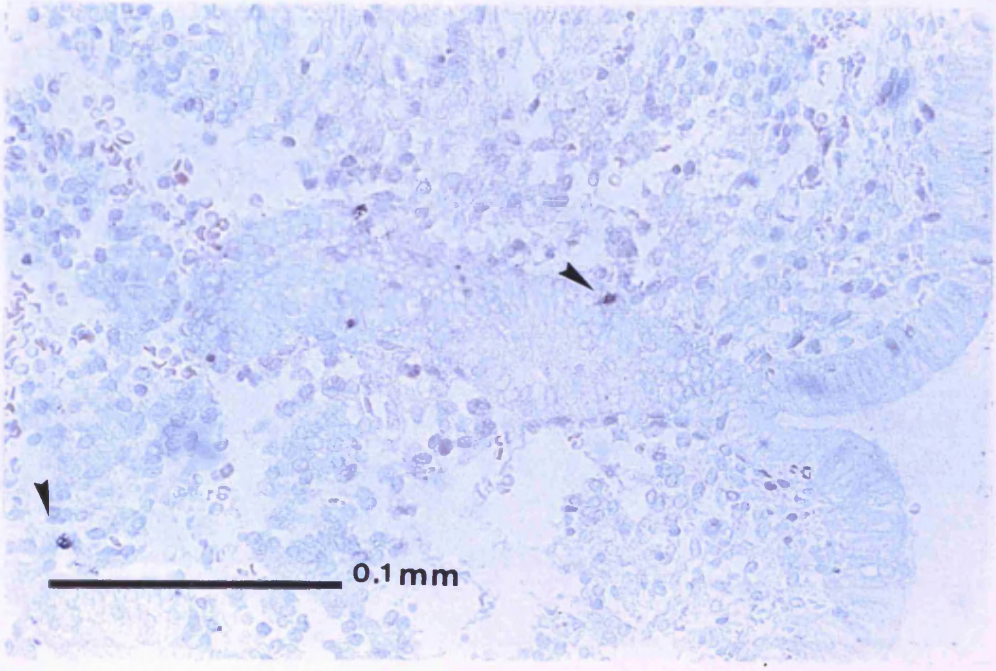
C Pathway for TNFα induced apoptosis.

Figure (7.1.)

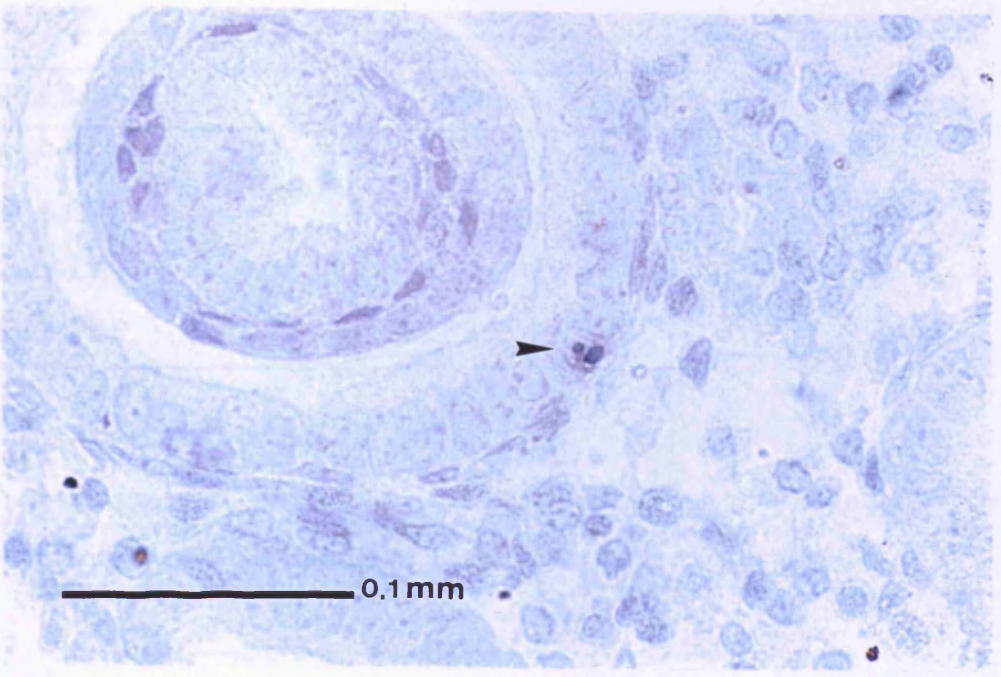
Pathways involved in apoptosis that may be relevant in the endometrium.



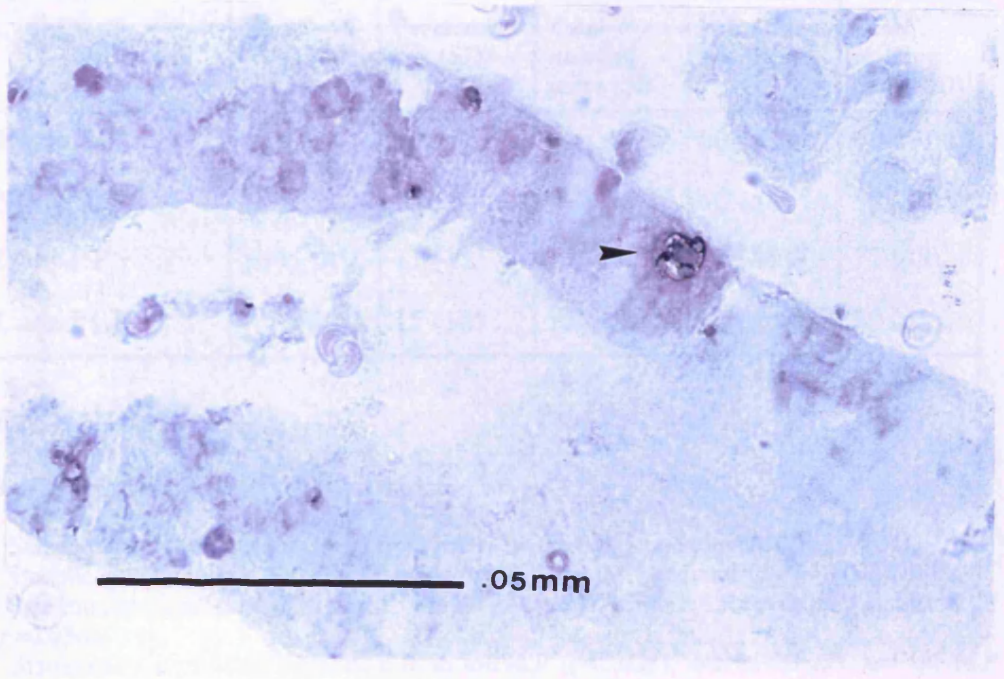
a



b



c



d

Figure (7.2. a-d.)

Apoptosis in the tonsil (**a**), and in the glandular epithelium during the late-LP (**b**), and the late-PLP (**c, d**), demonstrated by TUNEL.

Phase of the cycle	Epithelial apoptosis
Early-LP	0.5 (1.07)
Mid-LP	0.6 (1.06)
Late-LP	8.5 (6.3)[1]
Late-PLP	5.5 (6.0)[2]

Table (7.3.)

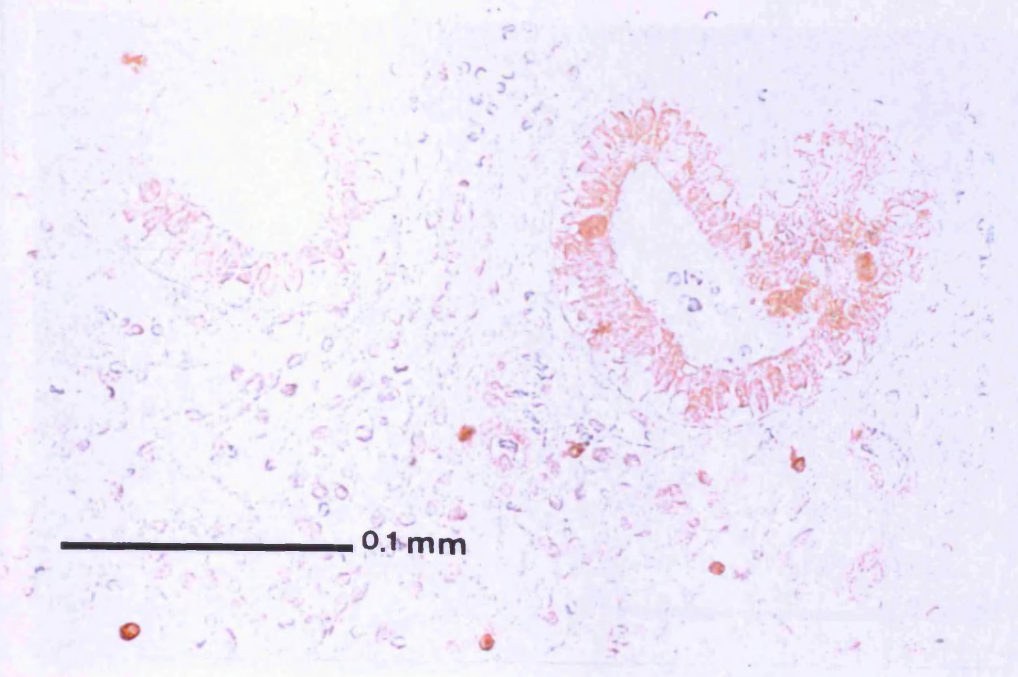
The number and (SD) of cells exhibiting apoptosis in 17hpf(x400) in the luminal and the glandular epithelium during the different stages of the LP and the late-PLP.
[1,2] Statistically significantly higher than the early-LP and the mid-LP ($p<0.05$).

Phase of the cycle	Stroma	Glands		Luminal epithelium	
	No. of +ve cells (SD)	Percentage +ve (SD)	Total staining score (SD)	Percentage +ve (SD)	Total staining score (SD)
Early-LP	31 (29)	40 (38)[3]	50 (46)[4]	60 (54)	60 (54)
Mid-LP	29 (16)	5 (8.3)	5 (8)	40 (54)	60 (89)
Late-LP	107 (79)[1]	11 (11)	11 (11)	71 (48)	71 (48)
Late-PLP	108 (62)[2]	15 (18)	15 (18)	89 (29)[5]	90 (61)

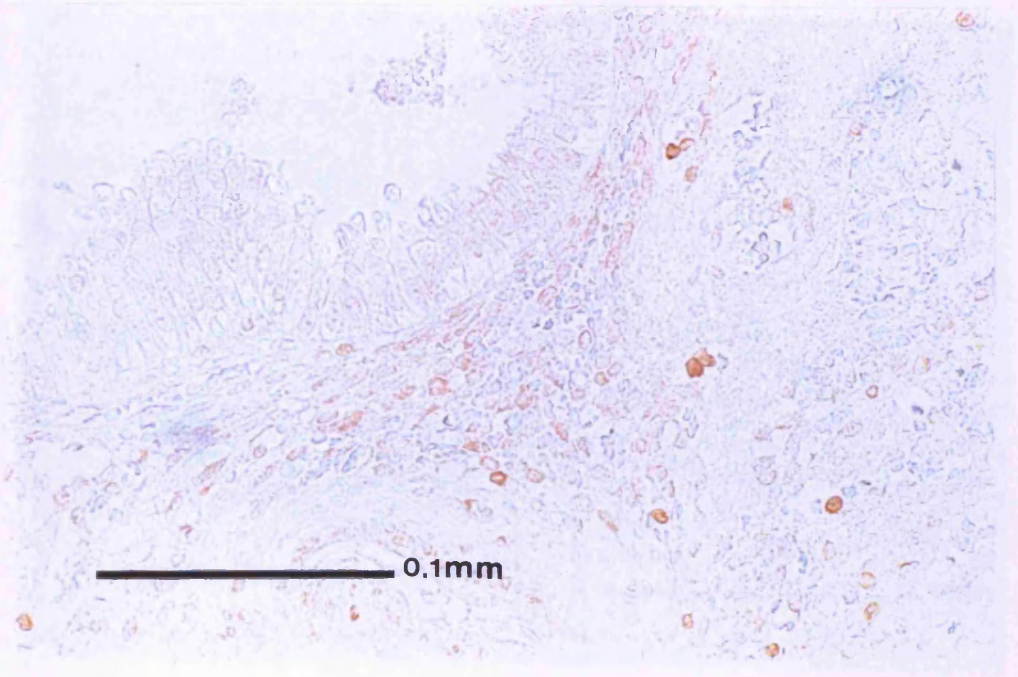
Table (7.4.)

Bcl-2 expression during the LP and the late-PLP.

[1] Statistically significant difference from early-LP ($p=0.03$), and the mid-LP ($p=0.01$).
[2] Statistically significant difference from early-LP ($p=0.001$) and mid-LP ($p=0.008$).
[3] Statistically significant difference from mid-LP ($p=0.036$), late-LP ($p=0.034$), and late-PLP ($p=0.036$).
[4] Statistically significant difference from mid-LP ($p=0.028$), late-LP (0.02), and late-PLP (0.014).
[5] Statistically significant difference from mid-LP ($p=0.009$).



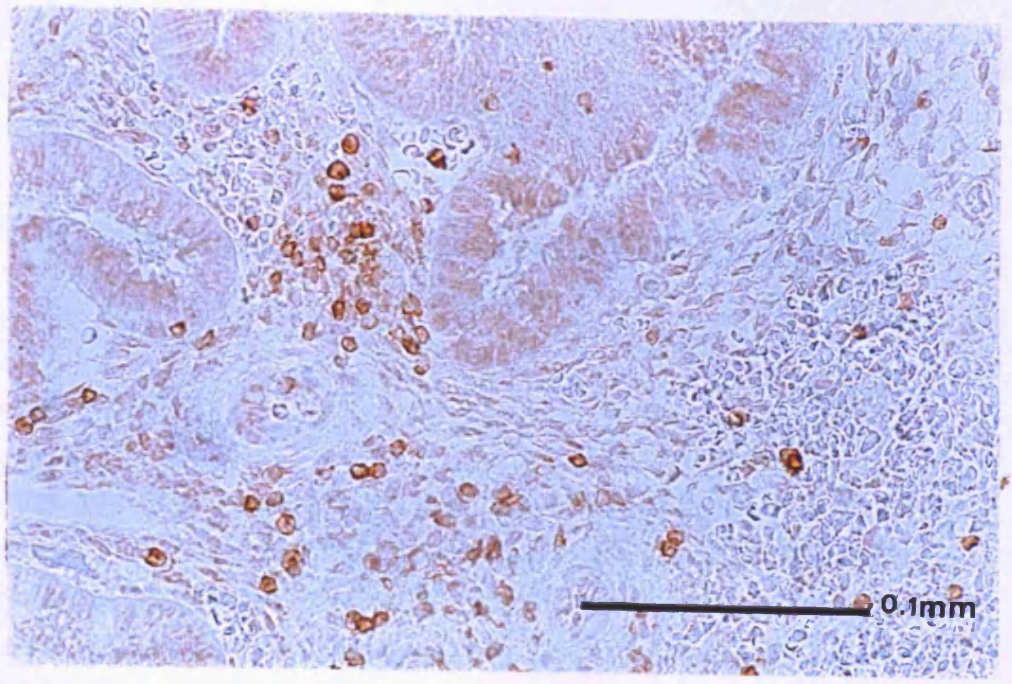
a



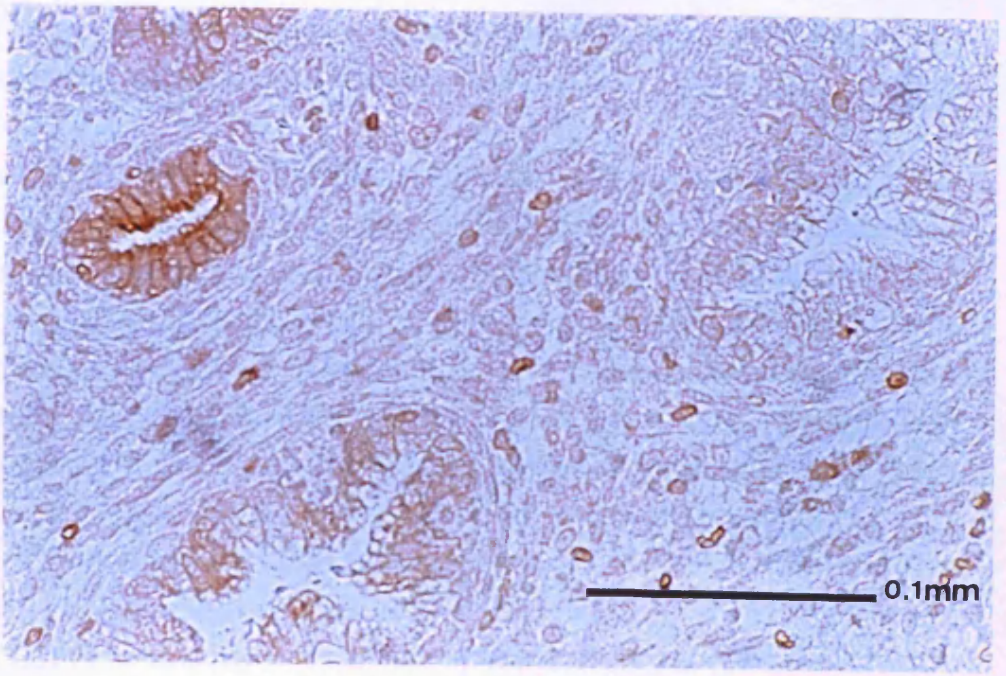
b

Figure (7.5. a,b.)

Bcl-2 expression during the early-LP, and mid-LP.



c



d

Figure (7.5. c,d.)

Bcl-2 expression during the late-LP, and late-PLP.

Figure (7.5. a-d.)

Bcl-2 expression during the LP, and late-PLP.

Chapter 8

Œstrogen receptor and Heat Shock Protein 27 expression

8.1. Introduction

The high affinity œstrogen receptor (ER) is localised to the nucleus but not the cytoplasm (320), their level is predominantly regulated by œstrogen and progesterone. In the endometrium, ER expression peaks under the influence of œstrogen during the FP, and is inhibited by progesterone in the LP (**1.12.4.2., 1.12.4.4.**). Thus the level of receptor expression may serve as a marker for steroid action (142). A new ER (ER-β), besides the classic receptor (ER-α), has been identified, and the possibility has also been raised for receptor independent mechanisms for œstrogen action (321).

Heat shock protein 27 (HSP27, synonymous with p24 and p29), is a 27kDa œstrogen regulated serine phosphoprotein. It is the only member of the small heat shock proteins (molecular weights ranging from 16-40 kDa) identified in the human (322, 323). Its exact function is unknown, but it may be involved in thermotolerance, protein degradation or as a molecular chaperone for other proteins including ER (324). HSP27 expression is associated with poor prognosis in breast cancer, but with low grade tumour in endometrial cancer (325). HSP27 is variably expressed in the endometrium during the cycle which make it a useful marker of hormonal response (322). In contrast, studies of cervical cancer showed a lack of correlation between ER and PR expression and HSP27, which may be explained by the insensitivity of cervical cancer to steroids (326), but may also indicate that HSP27 in the cervix is a proliferation/differentiation, rather than an œstrogen/progestin marker.

The previous chapters suggest a suboptimal hormonal milieu under this cHRT, with a link to a hypo-œstrogenic balance. In this chapter, the level of ER and HSP27 expression will be examined as markers of hormone effect.

8.1.1. Expression of ER in the endometrium

Immunohistochemical studies have shown that both the concentration and the distribution of ER change during the normal menstrual cycle. There is, however, some lack of agreement on the exact pattern of expression. One study (142) reported that during the mid-FP, the late-FP and the early-LP (day 7-19) about 50% of glandular and stromal cells are positive, and that during the mid-LP and the late-LP (day 21-27) ER disappears from the glandular epithelium, while faint expression remain in the stroma. Others (141, 327) reported that the peak ER expression in the glandular epithelium occurs before ovulation and declines afterwards. Stromal staining may either plateau (327) or decline (141), from the time of ovulation into the late-LP. ER expression in the stroma during the FP in women receiving cHRT, was reported to be lower compared to postmenopausal women not on cHRT (327).

Little is known about factors other than steroids, that modulate ER, but it has been reported to decline in parallel with LH induced follicular atresia in mice (328), and in response to insulin deficiency in the hamster endometrial carcinoma (329). Growth hormone (GH) and triiodothyronine (T_3) increase the transcription of ER. GH also has a regulatory role at the translational and the post-translational levels. Dexamethasone may have a predominantly inhibitory effect (330). Recently, TGFβ1 has been shown to inhibit the transcription of ER, but not its activity (331).

8.1.2. Expression of HSP27 in the endometrium

The level of HSP27 is regulated by steroid hormones and other cytokines (332). In the endometrial glandular epithelium, HSP27 appears during the late-FP and decreases after ovulation, and slightly rises premenstrually (322). This contrasts to the stronger expression in the luminal epithelium during the LP (maximum around day 21). Expression is higher in conditions of excessive œstrogen stimulation as in hyperplasia (333). The stroma remains negative till the late-LP when the predecidual cells both around the spiral arterioles and beneath the superficial epithelium become positive (322). Stroma but not glandular epithelium express HSP27 under the influence of prolonged progestin stimulation (334). Expression in the glandular epithelium is lower in women treated with cHRT compared to women in the early-FP or the late-FP, which was attributed to either the type or dose of exogenous œstrogen (334).

8.2. Aim

To compare the expression of ER and HSP27, as markers of hormone action, in the late-PLP to the physiological cycle endometrium.

8.3. Material and methods

8.3.1. Study population and regimen

Were described previously (**2.1.1., 2.1.2., 4.3.1.**).

8.3.2. Immunohistochemistry

IHC for ER and HSP27 antibodies (Appendix 2), were described previously (**2.1.5.2**).

8.3.3. Statistical analysis and sample size

Were considered previously (**2.1.7.**).

8.3.4. Image analysis

The percentage of glands expressing ER was counted in 17 random lpf (x100)/section and the percentage of luminal epithelium expressing the receptor was also calculated in the same area. The number of stromal cells expressing ER was counted using image analysis in 17 random hpf(x400), as described previously (**2.1.6.**). HSP27 staining in the glands was measured by calculating the percentage of glands that stained positive in 17lpf (x100). HSP27 staining was evaluated by determining the proportion of tissue stained (P), multiplied by the intensity of staining (i), which was assigned scores. 0=none, 1=weak, 2=distinct, and 3=strong. The total score (T/score) was calculated using the equation:

$$T/score=\sum P i\ (i+1)$$ (141, 335)

8.4. Results

8.4.1. Expression of ER

ER was uniform throughout each section in the physiological cycle, and was stronger in the luminal and glandular epithelium and in the stroma of the early-LP compared to the mid-LP or the late-LP (Figure 8.1.). There was also a gradual decline in the total number and the proportion of stromal cells that expressed ER during the later phases of the cycle (Table 8.2., Figure 8.3.). The percentage of positive stromal cells in the early-LP was significantly different from the mid-LP ($p=0.05$) and the late-LP ($p=0.05$). Also the total number of positive stromal cells was statistically significantly different between the early-LP and the mid-LP ($p=0.01$). No staining was seen in the vascular endothelium. ER expression did not exhibit any site specific preferential expression or any predilection depending on gland size or shape.

Under cHRT, the pattern of expression varied. Smaller glands were predominantly positive whilst the larger glands, which exhibited more advanced secretory features, were negative. Some glands contained positive and negative cells, although the majority were uniformly either positive or negative. Staining in the surrounding stroma followed a similar pattern, with the stroma surrounding positive glands being predominantly positive, and *vice versa*. Semi-quantitative analysis demonstrated stronger immunoreactivity in the glandular and luminal epithelium, but not the stroma (Table 8.2., Figure 8.3.). The high expression of ER in the glandular epithelium is attributable to the expression of ER in the more prevalent smaller glands. There was, however, a wide patient-to-patient variability as demonstrable by the wide standard deviation of the data. There was no difference in expression in telescopic glands. The only statistically significant differences were between the late-PLP and the early-LP in the number and the proportion of positive stromal cells ($p=0.0025$, and $p=0.01$ respectively).

8.4.2. Expression of HSP27

HSP27 expression was strong in the luminal epithelium throughout the LP, but the predominantly $HSP27^+$ epithelium was interrupted by $HSP27^-$ segments. At some gland openings, positive luminal and glandular epithelium appeared continuous. The overall expression in the glandular epithelium was very weak, and positive and negative glands were scattered in the stroma and only rarely appearing in clusters (Figure 8.4.). There was no discernible preferential expression in relation to gland size or location. Semi-quantitative assessment (Table 8.5.), demonstrated higher HSP27 in the early-LP compared to the late-LP in both the luminal and the glandular epithelium, but

the differences were not statistically significant ($p>0.05$). The stroma was predominantly negative during the early-LP and the mid-LP, but HSP27 expression was higher in the late-LP.

The pattern of expression in the luminal epithelium in the late-PLP was similar, but the HSP27$^-$ segments were larger which accounted for the weaker overall expression. These differences were statistically significant ($p=0.001$, 0.001, and 0.04 compared to the early-LP, mid-LP, and the late-LP respectively, Table 8.5.). Glandular staining followed the same pattern as in the physiological cycle, although staining was, again, very weak. Expression of HSP27 was lower in glands in the late-PLP compared to all stages of the LP, but the difference was statistically significant only in comparison to the early-LP ($p=0.05$). There was no preferential expression of HSP27 in relation to glandular size, distribution, or in the previously noted telescopic glands. The late-PLP exhibited the highest stromal HSP27 expression (Table 8.5.).

8.5. Discussion

Image analysis was used to quantify ER and PR in breast tumours (336-338), with a high agreement for ER and PR (86.7% and 91.1%, respectively), compared to quantitation using dextran charcoal-coated method (DCC) (338). Receptor expression can be measured either as the percentage of positive area (PA=positive nuclei/total nuclei), or the percentage of positive stain (PS=summation of optical density of the positive nuclear area divided by the summation of the optical density of all the nuclei studied). Measurement of PS optical density was shown not to improve the correlation between PA and DCC (338), and as the correlation between PA and the more complicated measurement PS was high ($r=0.99$ for ER; $r=0.97$ for PR), therefore PA measurement was used in this study. Thus image analysis, although semi-quantitative, combines an acceptable correlation with receptor content (correlation between the PA and DCC for ER, $r=0.71$ (338)), with the added advantage of enabling antigen localisation.

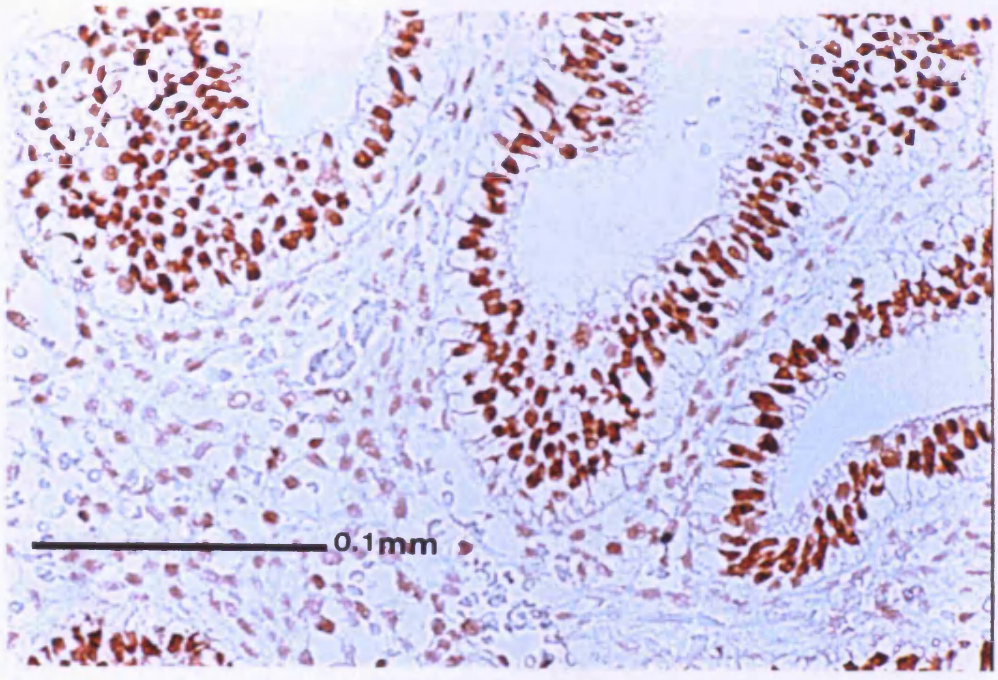
The pattern of ER expression during the physiological cycle is similar to that ascertained from the literature, but expression in the epithelium during the late-PLP was higher. This was mostly accounted for by the propensity of smaller glands lacking secretory feature. This apparent retardation of glandular development may be due to an inadequate œstrogen priming or to

poor response to progestogens. The low expression of ER in stromal cells and the response to progestogen as exhibited by HSP27 expression, suggests poor œstrogenic priming rather than inadequate progesterone. However, the latter cannot be ruled out as it has been demonstrated that œstrogens and progestogens can have a selective differential effect on each of the gland and stroma (339). Thus a dose or a preparation that is adequate for one compartment, may not be so for the other. For example, 19-nortestosterone derivatives used in oral contraceptives, have been shown to induce relatively more stromal decidualization and gland atrophy compared to progesterone derivatives (69). Also, the low HSP27 expression in the luminal epithelium and the glands during the late-PLP, may reflect poor œstrogenic response in the earlier phases, but the higher expression in the stroma may reflect adequate progestogen action (decidualization, increases stromal HSP27).

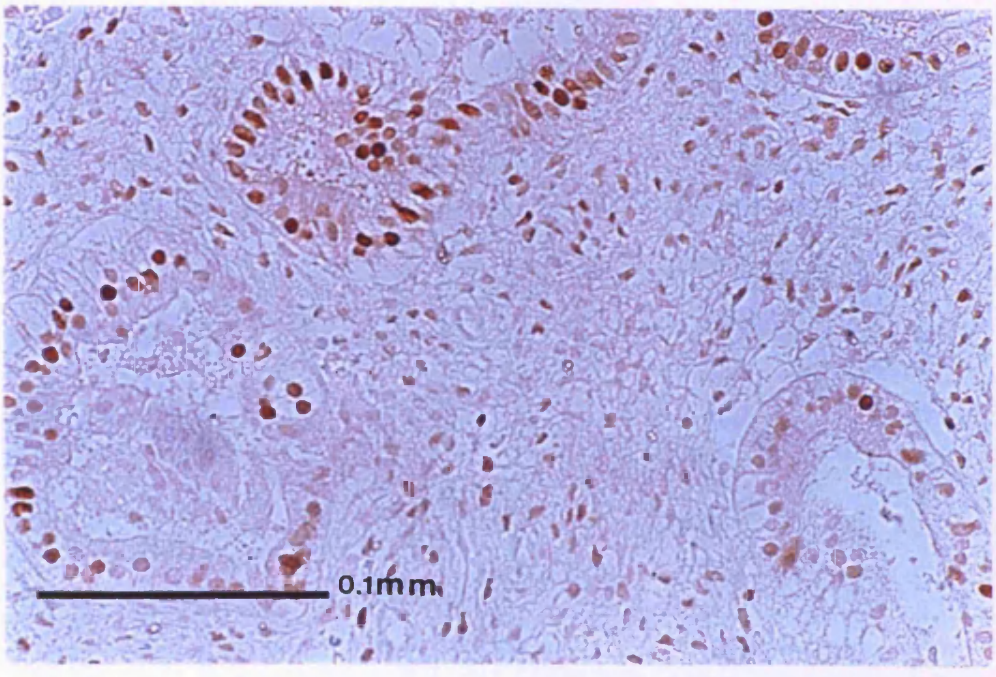
The level of HSP27 expression in the glandular epithelium during the LP was low and did not exhibit a premenstrual rise. On the other hand, expression in the luminal epithelium was constantly high and did not exhibit a mid-LP peak as reported previously (322). The discrepancy may be related to the small sample size (n=3), and non-use of statistics in the older study (322). In agreement with previous observations (141, 340) endothelial cells were ER^-.

The observation that some of the smaller diameter glands strongly expressed ER during the late-PLP whilst adjacent glands were ER^-, may be related to a threshold effect of steroids in recruiting glandular proliferation and differentiation. This in turn may be a function of both steroid levels and a stromal 'diffusion gradient' that is dependent on the proximity to blood vessels.

The different level of expression of functional markers between the two groups demonstrated dissociation between endometrial behaviour and functional markers. This is in agreement with the observation of a similar concentration of ER and PR in women with menorrhagia compared to women with normal menstrual loss (341), and may be a function of the role of second messengers as cAMP, protein kinase A, protein kinase C, AP-1, insulin-like growth factor-I (IGF-I), Epidermal growth factor (EGF), and the erbB/HER ligand, gp30 in controlling or modifying the action of steroids (342, 343).



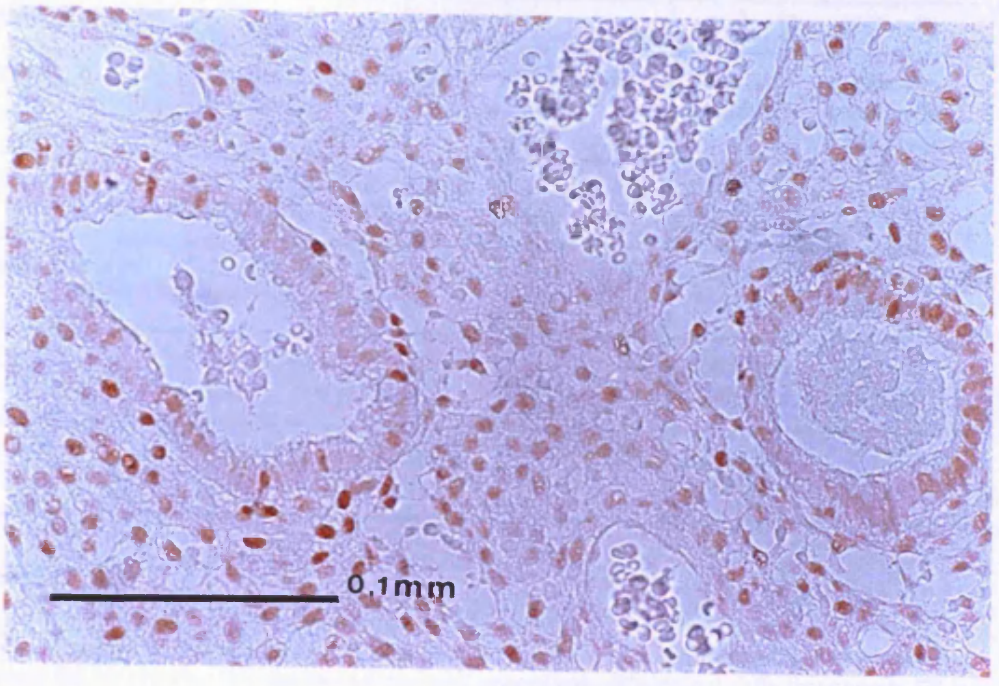
a



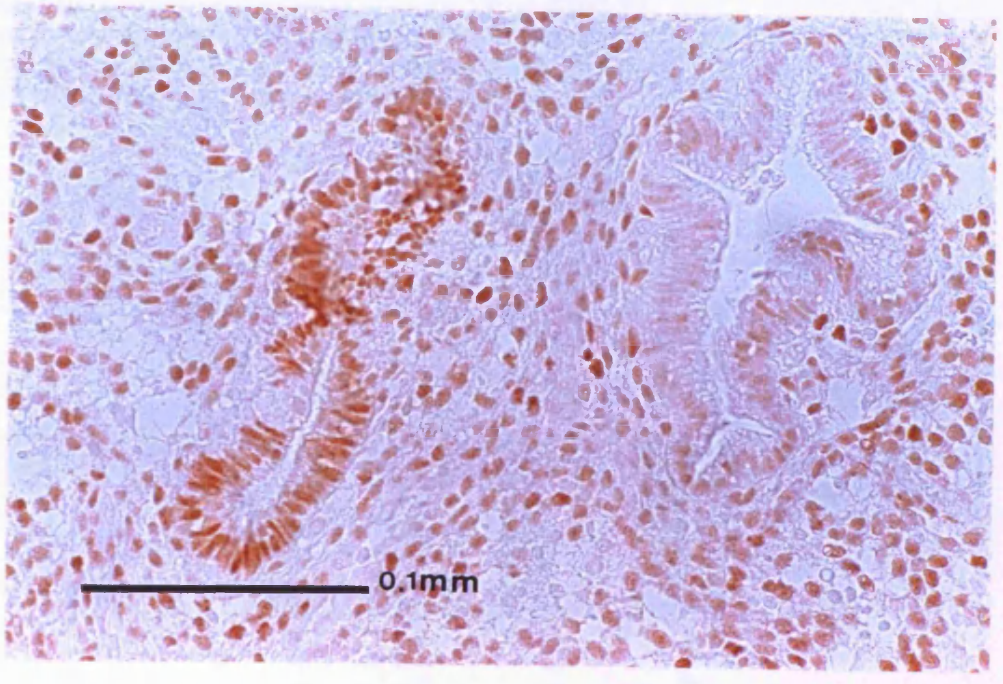
b

Figure (8.1. a,b.)

The expression of ER during the early-LP (**a**), the mid-LP (**b**).



c



d

Figure (8.1. c,d.)

The expression of ER during the late-LP (**c**), the late-PLP (**d**).

Figure (8.1. a-d.)

The expression of ER during the LP and the late-PLP.

Phase of the cycle	% Positive			+ve Stromal cells	
	Luminal epithelium	Glands	Stromal cells	Total number	Number corrected for density
Early-LP	30 (40) [0-10]	33 (40) [0-10]	47 (19) [38-56]	239 (148) [194-285]	188[1] (116) [152-224]
Mid-LP	22 (34) [0-80]	20 (27) [0-60]	27[2] (26) [0-60]	116 (113) [0-252]	109 (106) [0-238]
Late-LP	20 (34) [0-80]	20 (30) [0-80]	25[3] (29) [0-76]	160 (185) [0-480]	100 (116) [0-301]
Late-PLP	42 (45) [0-100]	40 (36) [0-80]	22[4] (25) [0-67]	86[5] (109) [0-245]	88 (112) [0-277]

Table (8.2.)

The percentage of the luminal epithelium that was ER $^{+}$, and the percentage of glands that express ER in 17lpf(x100), and the number and percentage of stromal cells that express ER in 17hpf (x400). Number, (SD), and [range].
[1] Statistically significant compared to the late-PLP (p=0.05).
[2] Statistically significant compared to the early-LP (p=0.05).
[3] Statistically significant compared to the early-LP (p=0.05).
[4] Statistically significant compared to the early-LP (p=0.01).
[5] Statistically significant compared to the early-LP (p= 0.002).

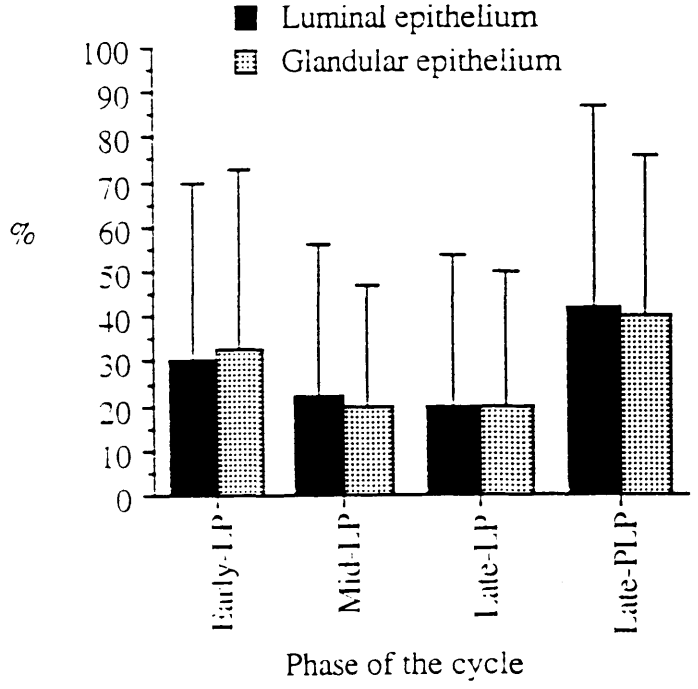
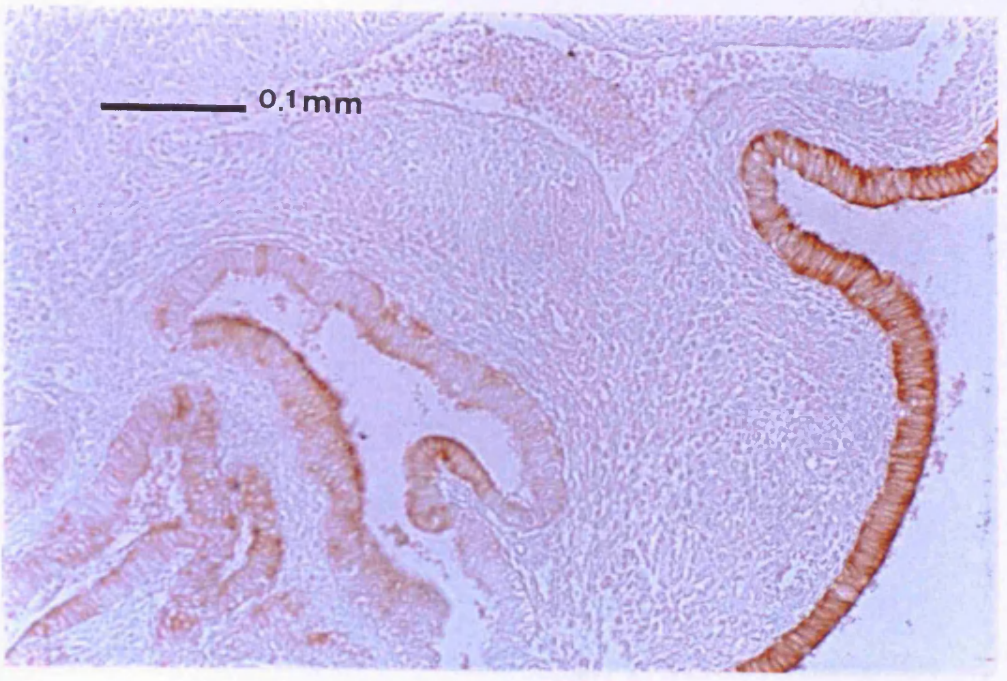
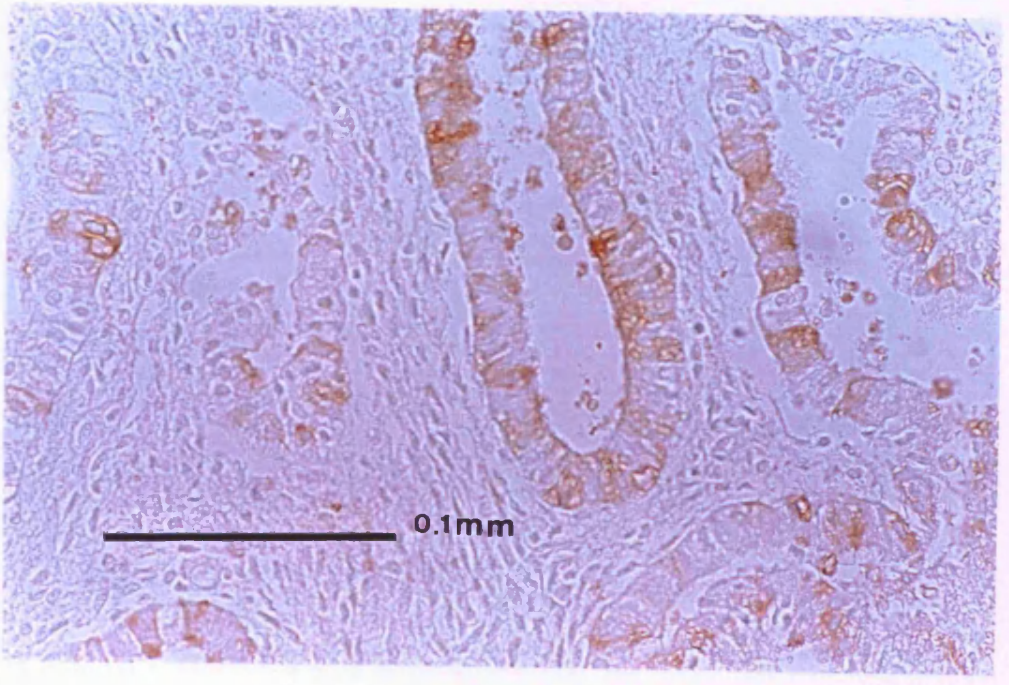


Figure (8.3.)

The expression of ER in the glandular and the luminal epithelium during the early-LP, the mid-LP, the late-LP and the late-PLP.



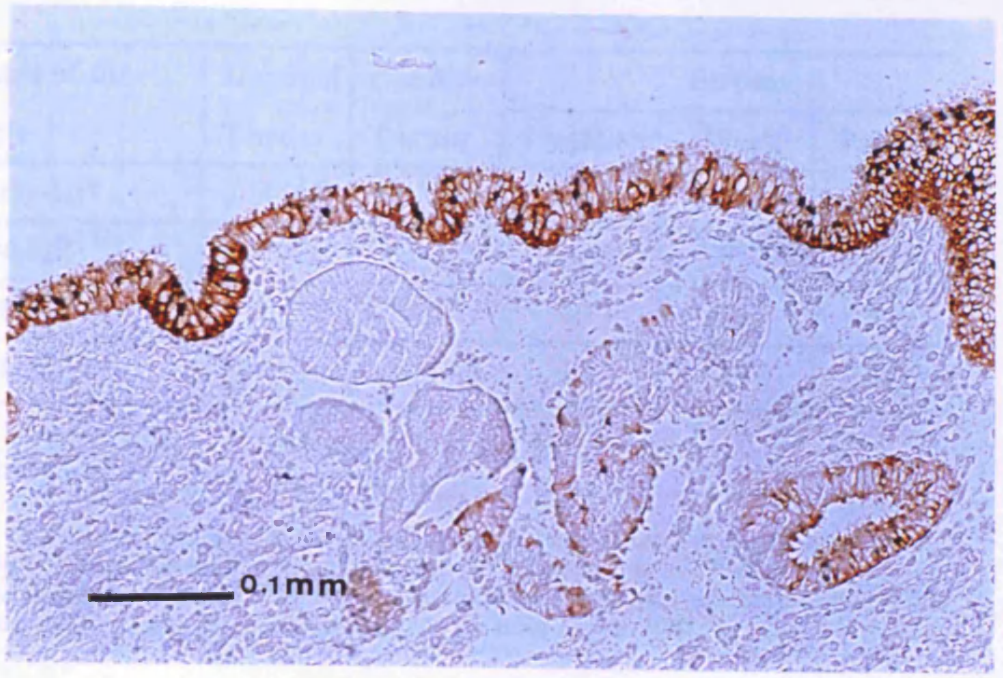
a



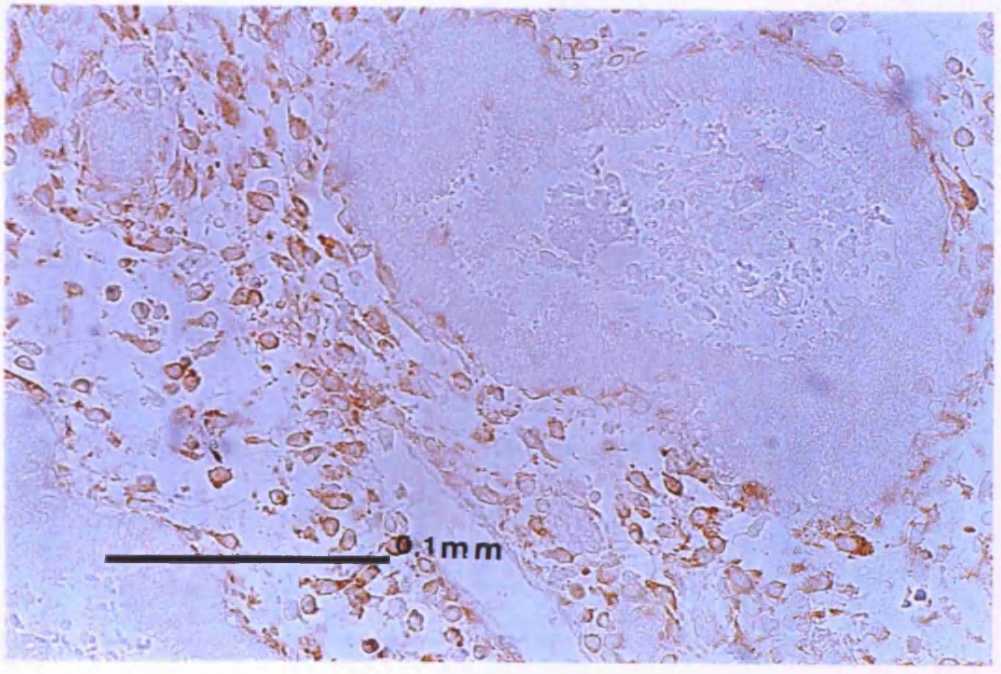
b

Figure (8.4. a,b.)

The expression of HSP27 in the early-LP (**a**), the mid-LP (**b**).



c



d

Figure (8.4. c,d.)

The expression of HSP27 in the late-LP (**c**), the late-PLP (**d**).

Figure (8.4. a-d.)

The expression of HSP27 in the LP and the late-PLP.

Phase of the cycle	Luminal T/score	Glandular T/score	Stroma		
			Negative	Weak	Positive
Early-LP	174 (47)	91 (75)	14/17	3/17	0/17
Mid-LP	170 (43)	60 (57)	14/17	3/17	0/17
Late-LP	151 (76)	58 (67)	9/17	3/17	5/17
Late-PLP	103 (38)[1]	43 (43)[2]	0/17	0/17	17/17

Table (8.5.)

The total score (T/score) of HSP27 expressed in the luminal and the glandular epithelium in 17lpf(x100), and the number and staining intensity of the stroma in 17hpf(x400).
[1] Statistically significantly lower compared to all phases of the physiological cycle ($p<0.05$).
[2] Statistically significantly lower compared to the early-LP ($p=0.05$).

Chapter 9

Expression of Progesterone Receptor & α_2-PEG

9.1. Introduction

Œstrogen, through an intragenic œstrogen-responsive element (ERE), is the main stimulator of PR gene expression. Progesterone down regulates PR through a protein-protein interaction between PR, ER and the same ERE (345). Synthesis of α_2-PEG, which is the major secretory protein in the endometrium, is progestogen dependent, and its concentration fluctuates to reach a maximum in the functionalis of the secretory endometrium. The level of PR and α_2-PEG could thus serve as markers of œstrogen/progestogen balance, which will be addressed in this chapter.

9.1.1. Expression of PR in the endometrium

In the mid-FP (day 7-8), 25% of stromal and glandular cells are PR^+. During the late-FP and the early-LP (day 9-19), the majority (75%) of glandular cells and half the stromal cells are positive. During the late-LP (day 21-27), PR disappears from the glands but remains faintly positive in the stroma (142). In a limited observation on two postmenopausal women - one of whom was on cHRT (during the œstrogenic phase) - there was strong glandular staining and moderate stromal staining for PR (327).

9.1.2. Expression of α_2-PEG in the endometrium

Pregnancy-associated endometrial α_2-globulin (α_2-PEG) also known as Placental Protein 14 (PP14), Progestogen-dependent Endometrial Protein (PEP), Endometrial Protein 15, or Progesterone-associated endometrial protein (PAEP) (344), is an α_2-globulin with a molecular weight of 56000 on gel chromatography and 28000 on polyacrylamide gel. Synthesis of α_2-PEG seems to be dependent on the development of secretory changes after ovulation. Quantitatively, it is the major secretory protein product of the decidua and the major soluble product of the endometrium during the late-LP (235). The function of α_2-PEG remains unknown, but it may have a role in

maternal immune tolerance (178). α_2-PEG suppresses T and NK cells, and inhibits IL-2 mediated T cell proliferation (344).

During in vitro incubation, a higher rate of release was found in the secretory compared to the proliferative endometrium (179). The α_2-PEG production is initiated at LH^{+6} to LH^{+7} and rises thereafter to a maximum in the functionalis zone during the late-LP (345). A rise in serum α_2-PEG was observed in postmenopausal women on cHRT during the PLP (183), but this did not correlate with the histological stage (160). The α_2-PEG rise is related to the type of progesterone (Levonorgestrel>MPA), and is dependent on œstrogen pre-priming (180), and possibly other factors (346). α_2-PEG is therefore a suitable marker of progestogenic response in the second half of the LP (235).

9.2. Aim

To study the expression of α_2-PEG and PR during the late-PLP in comparison to the LP, as an index of steroid effect.

9.3. Material and methods

9.3.1. Study population and regimen

Were described previously (**2.1.1., 2.1.2., 4.3.1.**).

9.3.2. Immunohistochemistry

IHC for PR and α_2-PEG (Appendix 2), was described previously (**2.1.5.2.**). α_2-PEG mouse monoclonal antibody (Code 2CH11) (347), was used.

9.3.3. Statistical analysis and sample size

Were considered previously (**2.1.7.**).

9.3.4. Image analysis

For PR and α_2-PEG, this was performed as described previously for ER and HSP27 respectively (**8.3.4.**). Also the total glandular area expressing α_2-PEG

was measured in 17hpf (x200) per section (equivalent area of 1.59mm^2) and expressed in mm and as a percentage.

9.4. Results

9.4.1. Expression of PR

PR expression was nuclear and was seen during all stages of the LP as well as the late-PLP. In the glands and the luminal epithelium, expression was stronger during the early-LP and gradually regressed, till it almost disappeared during the late-LP. The stroma exhibited a similar pattern but expression, although weaker, did not disappear in the late-LP. Endothelium was negative. The late-PLP exhibited little PR in the glandular and luminal epithelium, and stronger staining in the stroma. There was no expression in vascular endothelium (Figure 9.1.).

On quantitative assessment (Table 9.2.), PR was higher in the luminal epithelium during early-LP (47%) compared to the mid-LP (6.7%) and the late-LP (8.2%), and the difference between the early-LP and each of the mid-LP (p=0.0002), and the late-LP (p=0.006), but not between the mid-LP and the late-LP, was statistically significant. Similarly, in the glandular epithelium expression was maximum during the early-LP (55%) compared to the mid-LP (12%) and the late-LP (15%), and the difference between the early-LP and each of the mid-LP (p=0.001), and the late-LP (p=0.01), but not between the mid-LP and the late-LP, was statistically significant. Expression of PR in stromal cells during the normal menstrual cycle appeared uniform and was highest during the early-LP, but the differences were not statistically significant (Table 9.2.). Expressed as a function of the total number of stromal cells (Table 9.2.), the proportion that was PR$^+$ was constant through the early-LP and the mid-LP (47.7% and 48.5%, respectively), but was lower during the late-LP (34%).

Expression of PR in the glandular (18%) and the luminal (20%) epithelium during the late-PLP was lower compared to the early-LP, but higher compared to the mid-LP, or the late-LP. During the late-PLP, PR expression was higher in some of the smaller size, but not in the telescopic glands, the smallest ER$^+$ glands were PR$^-$. The difference in expression in the glands was statistically

significant compared to the early-LP, in both the luminal (p=0.02), and the glandular (p=0.006) epithelium (Figure 9.1., Table 9.2.).

The number of PR^+ stromal cells during the late-PLP was lower compared to all stages of the physiological cycle, but the difference was statistically significant only when compared to the early-LP. When expressed as a percentage of total stromal cells, PR was somewhat higher during the late-PLP but the difference was not statistically significant.

9.4.2. Expression of α_2-PEG

α_2-PEG was detected only rarely during the early-LP, and then only in a few glands. The glandular area expressing α_2-PEG increased to its peak during the mid-LP and the late-LP and most glands appeared positive (Figure 9.3., Table 9.4.). Staining in the luminal epithelium followed the same pattern but was weaker, and there was no staining in the stroma. Using image analysis, and T/score (Table 9.5.), the mid-LP peak was confirmed, and the difference between the early-LP and both the mid-LP and the late-LP was statistically significant (p=0.012 and p=0.003 respectively), the difference between the mid-LP and the late-LP was not statistically significant.

During the late-PLP, expression of α_2-PEG was noted in the majority of the glands, but a higher proportion, especially of the smaller glands, were negative. The luminal epithelium was predominantly positive but weak. The glandular area expressing α_2-PEG during the late-PLP was higher compared to the early-LP but lower compared to the mid-LP or the late-LP (Table 9.4.), and the differences were statistically significant (p=0.04, p=0.003, p=0.001, compared to the early-LP, mid-LP, and late-LP respectively). There was no difference in α_2-PEG expression in the telescopic glands, and no staining in the stroma.

Glandular secretions were positive. The highest expression was during the late-LP, compared to the mid-LP, whilst this was negative or very week during the early-LP. During the late-PLP secretions occupied a mid position between the late-LP and the mid-LP.

9.5. Discussion

The accuracy of image analysis in assessing steroid receptor expression has been addressed previously (**8.5.**). PR expression in the glandular and the luminal epithelium followed the expected pattern and the higher level was noted in the early-LP compared to the late-LP. This is mostly attributable to inhibition by progesterone. The tendency to a higher level during the late-PLP may be explained by an inability of the progestogen, in the dose prescribed, to mimic the physiological effect. An inverse relation between the expression of PR and α_2-PEG is apparent in the physiological cycle. The highest PR expression is during the early-LP which exhibit the lowest α_2-PEG, whilst low PR expression during the mid-LP and the late-LP is accompanied by the highest α_2-PEG expression. This relation seems to be retained during the late-PLP.

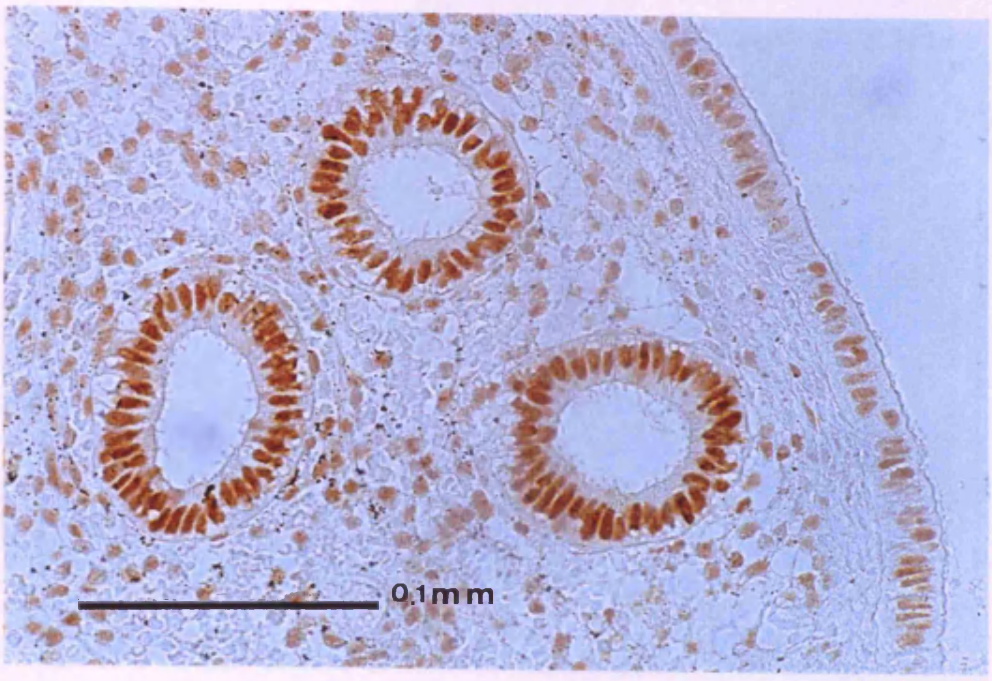
The quantitative expression of α_2-PEG demonstrated that the late-PLP is not comparable to either of the stages of the LP, being higher compared to the early-LP, but lower than the mid-LP or the late-LP. This demonstrates a suboptimal progestogenic response, which may be secondary to a suboptimal œstrogenic induction of progesterone receptor, or a function of the type of progestogen used or both. But the higher expression of PR during the late-PLP, suggests that the suboptimal progestogenic response may be related to the type or dose of progestogen used (or to both). Lower expression of ER in the stroma of the late-PLP (**8.4.1.**), together with evidence of suboptimal progestogenic response suggest that suboptimal production of the ER, rather than excessive progestogen mediated suppression is the more likely explanation. This is supported by the persistence of PR expression in a proportion of the glands during the late-PLP. In turn, it is also possible that a suboptimal PR expression (which may parallel low ER levels) during the early stages of the PLP may have contributed to the poor progestogenic response. The suboptimal PR expression may be due to an overall low level or receptor or to an alteration of the PR_A/PR_B balance in the endometrium.

It was argued that the late expression of α_2-PEG (luteal day 5), suggests that it is not directly dependent on either œstrogen or progesterone, but by 'endocrine regulators for decidualization' (236). Factors known to affect serum levels of α_2-PEG include the antiœstrogen tamoxifen, the antiprogestin RU486, clomiphene citrate, HCG and the GnRH analogue buserelin (344). Unidentified factors also influencing serum α_2-PEG levels (348). A glucocorticoid/progesterone regulatory elements (PREs) have been found in the α_2-PEG gene, and these elements bind purified PR in vitro (349). It is thus

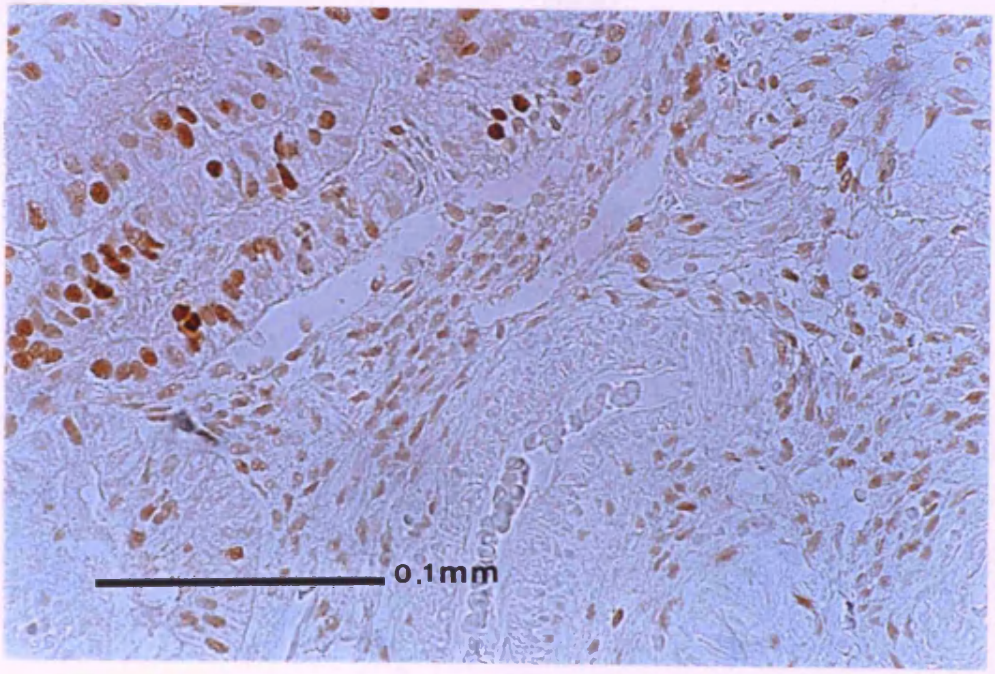
possible that the other factors that affect α_2-PEG secretion may act by modifying the response of this PRE, thus α_2-PEG secretion would reflect the balance of these factors. In cHRT specimens, α_2-PEG production was reduced despite evidence of stromal decidualization, which emphasises the dissociation between stromal and gland development, and between gland development and menstrual function.

This study demonstrates a suboptimal progestogenic response in cHRT treated endometrium, despite somewhat higher PR expression. It is possible that cHRT treatment produces:

1- Adequate PR but that the progestogen is inadequate to suppress PR or to produce α_2-PEG.
2- PR levels during the early-PLP are less than during the physiological cycle and that this may have contributed to the inadequate α_2-PEG synthesis.
3- Combination of 1 and 2
4- The total PR levels are adequate but the balance between PR_A/PR_B is not.



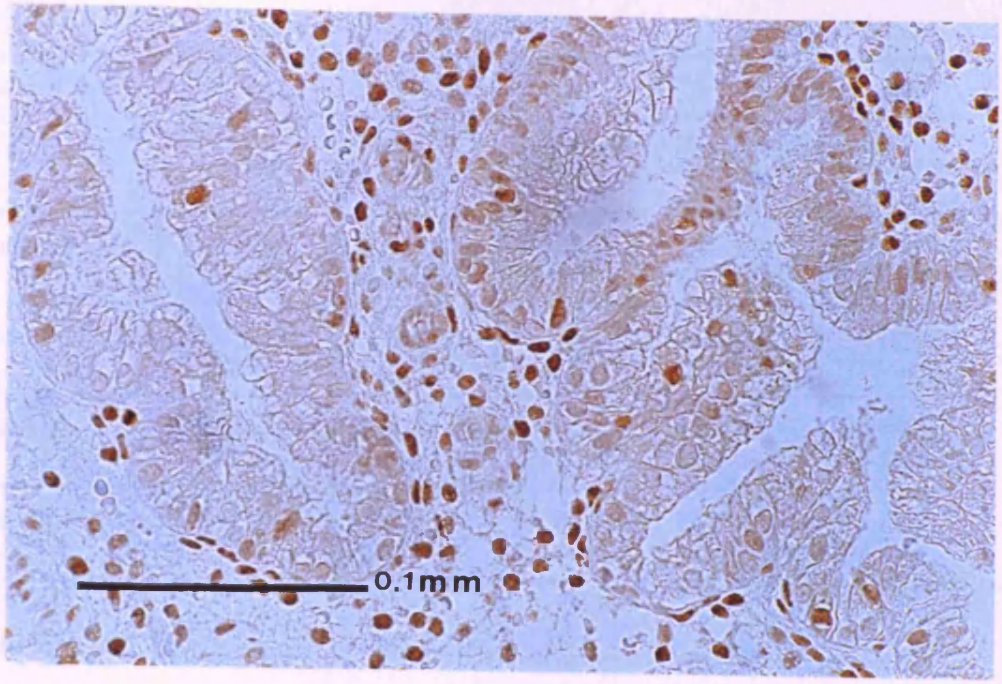
a



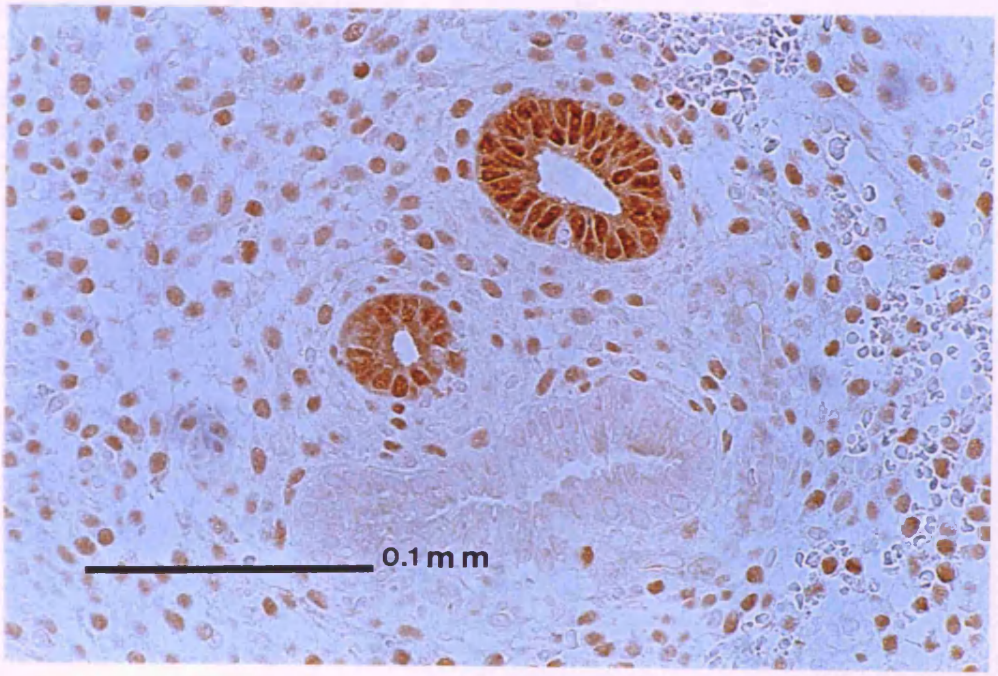
b

Figure (9.1. a,b.)

The expression of PR in the early-LP (**a**), and the mid-LP (**b**).



c



d

Figure (9.1. c,d.)

The expression of PR in the late-LP (**c**), and the late-PLP (**b**).

Figure (9.1. a-d.)

The expression of PR in the LP and the late-PLP.

Phase of the cycle	% Positive			+ve Stromal cells	
	Luminal epithelium	Glands	Stromal cells	Total number	Number corrected for density
Early-LP	47[1] (48) [20-100]	55[2] (48) [20-100]	47.7% (16) [35-75]	242 (81) [176-383]	190 (64) [139-302]
Mid-LP	6.7 (9.8) [0-20]	12 (19) [0-55]	48.5% (18) [27-75]	205 (87) [135-365]	194 (82) [127-343]
Late-LP	8.2 (13) [0-40]	15 (21) [0-60]	34% (9.7) [22-52.5]	217 (62) [108-329]	136[4](38) [68-207]
Late-PLP	20 (25) [0-70]	18 (29) [0-75]	42.5% (16) [22.5-80]	167[3](65) [91-313]	171 (66) [93-320]

Table (9.2.)

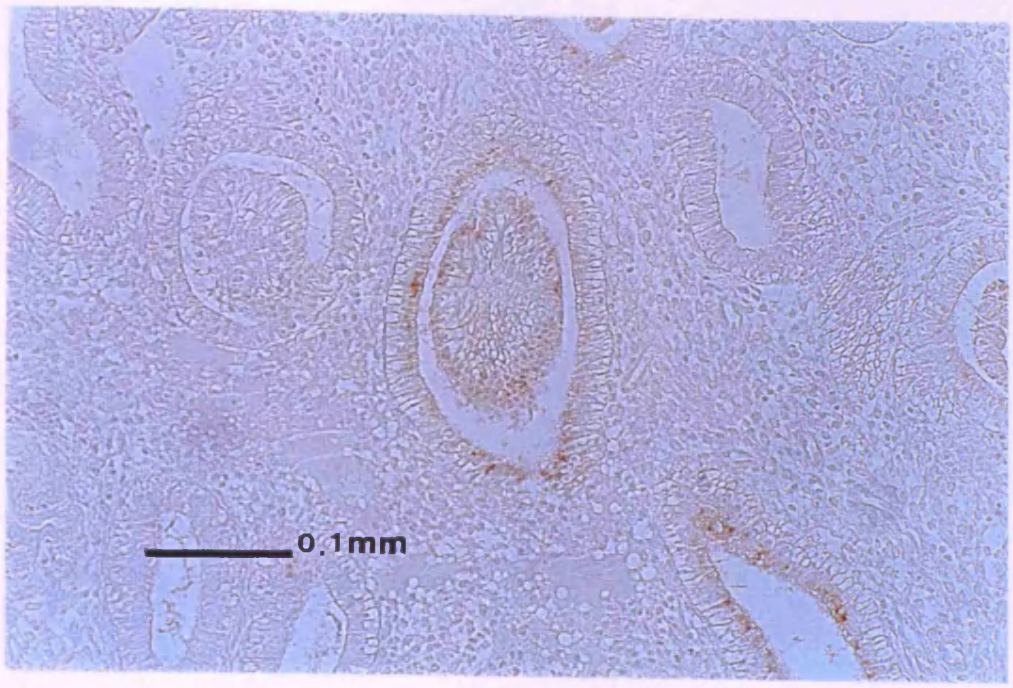
The percentage of the luminal epithelium that was PR $^{+}$, and the percentage of glands that express PR in 17lpf(x100), and the number and percentage of stromal cells that express PR in 17hpf (x400). Number, (SD), and [range].

[1] Statistically significantly higher than the mid-LP, late-LP, and the late-PLP ($p<0.05$).

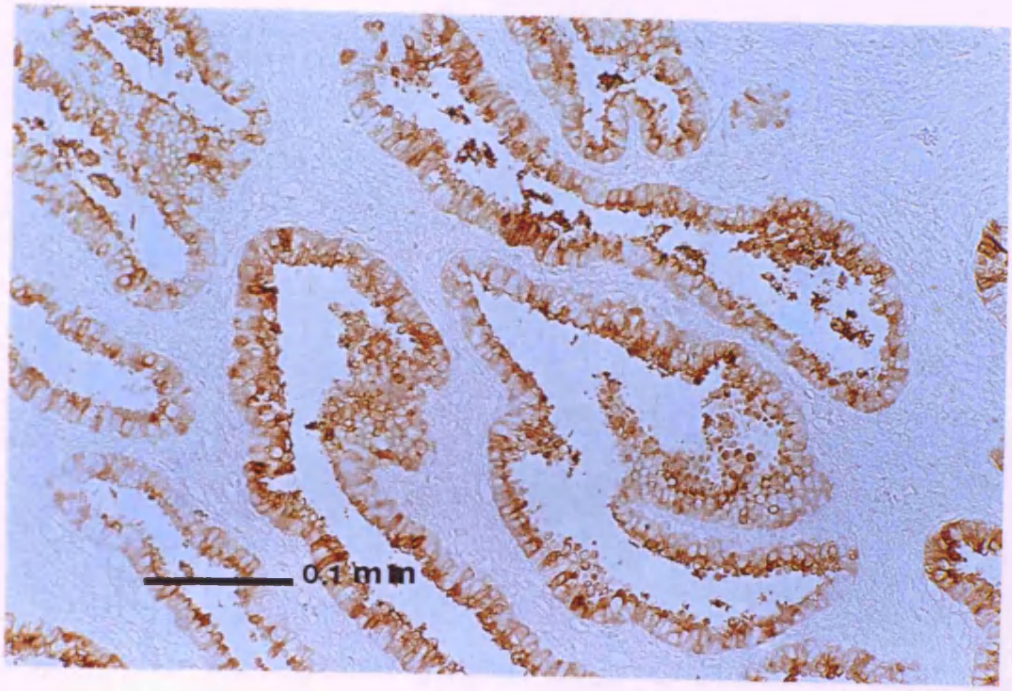
[2] Statistically significantly higher than the mid-LP and late-LP and the late-PLP ($p<0.05$).

[3] Statistically significantly lower than the early-LP ($p=0.023$).

[4] Statistically significantly lower than the early-LP ($p=0.024$).



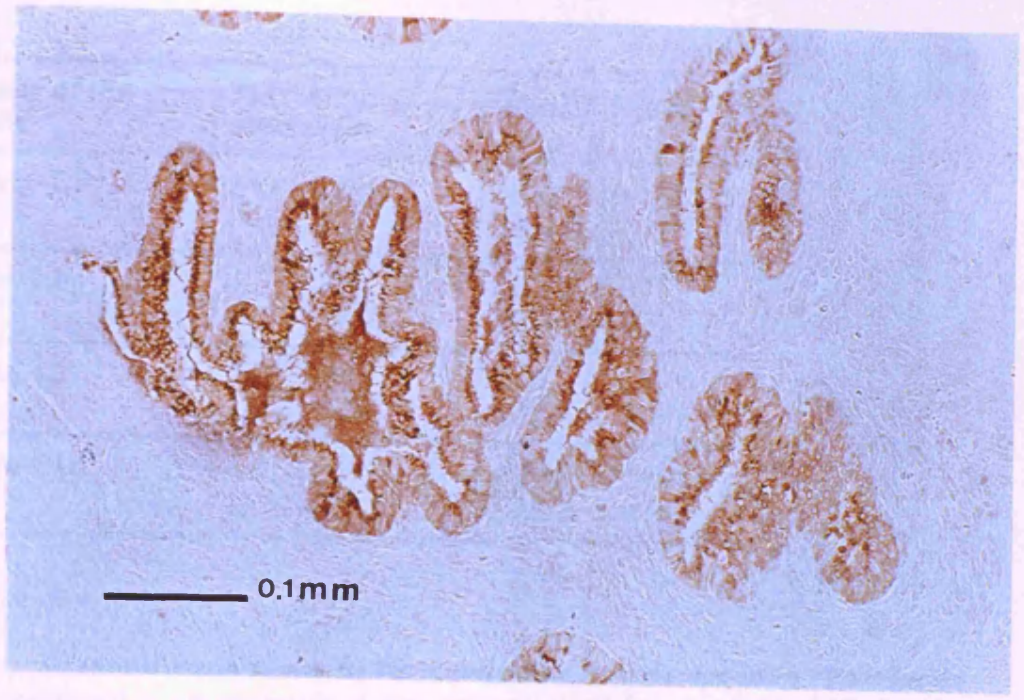
a



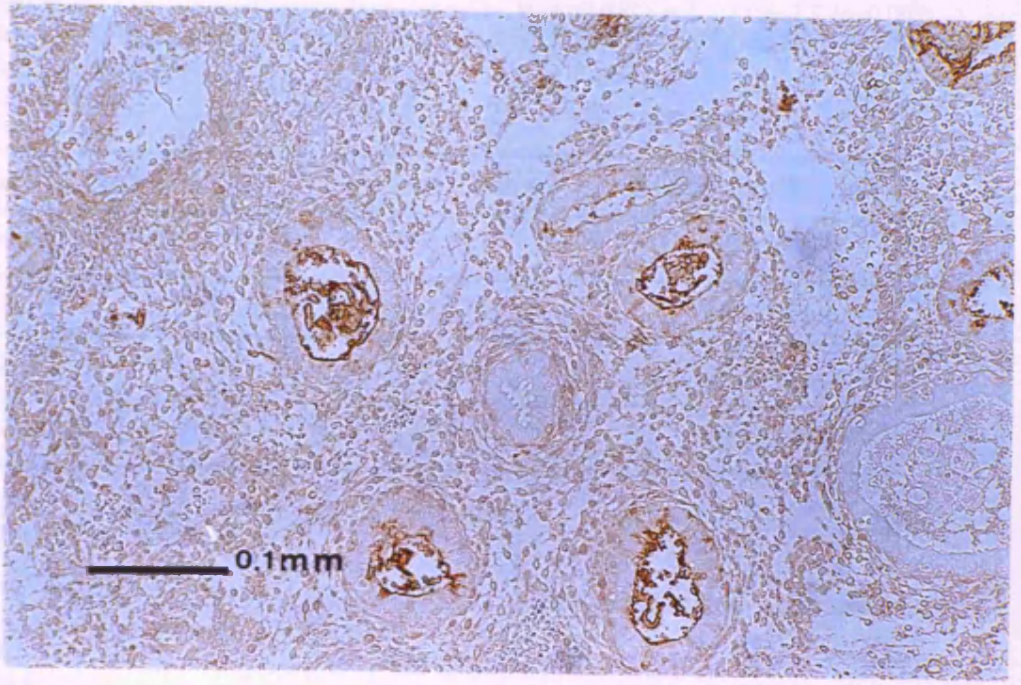
b

Figure (9.3. a,b.)

The expression of α_2-PEG in the early-LP (**a**), the mid-LP (**b**).



c



d

Figure (9.3. c,d.)

The expression of α_2-PEG in the late-LP (**c**), the late-PLP (**d**).

Figure (9.3. a-d.)

The expression of α_2-PEG in the LP, and the late-PLP.

Phase of the cycle	Percentage of total area	$mm^2/1.59mm^2$
Early-LP	0.06[1](0.82) [0.0-1.89]	0.001[1] (0.013) [0.0-0.03]
Mid-LP	8.57 (4.6) [0.077-14.7]	0.136 (0.073) [0.001-0.23]
Late-LP	7.68 (3.34) [2.02-12.8]	0.122 (0.053) [0.032-0.204]
Late-PLP	3.02[2](1.2) [0.52-4.8]	0.048[2](0.019) [0.008-0.076]

Table (9.4.)

The total glandular area expressing α_2-PEG during the different stages of the LP and the late-PLP, expressed as the percentage of area stained per section, and in mm^2/17hpf (x200), equivalent area of $1.59mm^2$. Values shown are the mean, (SD), and [range].

[1] Statistically significant difference from the mid-LP (p=0.012) and the late-LP (p=0.003).
[2] Statistically significant difference from the early-LP (p=0.001), the mid-LP (p=0.04), and the late-LP (p=0.003).

Phase of the cycle	Luminal T/score	Glandular T/score
Early-LP	14 (13)	33 (53)[1]
Mid-LP	49 (63)	140 (60)
Late-LP	68 (48)[2]	120 (61)
Late-PLP	22 (11)	60 (40)[3]

Table (9.5.)

The Total score (T/score) for α_2-PEG expression in the luminal and the glandular epithelium in 17hpf (x100). All stroma was negative.

[1] Statistically significantly lower compared to the mid-LP (p=0.008), and the late-LP (p=0.004).
[2] Statistically significantly higher compared to the early-LP (p=0.0.1), and the late-PLP (p=0.03).
[3] Statistically significantly lower compared to the mid-LP (p=0.006), and the late-LP (p=0.003).

Chapter 10

Expression of von Willebrand Factor (vWF) and alpha Smooth Muscle Actin (αSMA)

10.1. Introduction

Blood vessels undergo sequential changes during the menstrual cycle which are believed to be pivotal to menstruation (**1.12.5.**), and it is possible that vasculature plays a similar role in bleeding on cHRT. The development of the endometrial vasculature is, either directly or indirectly, under steroid control. But the development of vessels under the influence of cHRT has not been previously studied. The previous chapters have demonstrated differences between glandular and stromal features of the late-LP and the late-PLP. Here the endothelial marker, vWF, and the demonstration of αSMA in vascular smooth muscle cell (VSMC) (350) will be used to study endometrial vasculature.

Some endometrial stromal cells differentiate to exhibit light microscopic and ultrastructural features of smooth muscle cells (351). These cells are similar to myofibroblasts (352, 353), which suggests a role in regeneration and maintaining tissue integrity that is similar to that of myofibroblasts elsewhere in the body (352). Myofibroblasts which may be induced in response to tissue stress (353), share with smooth muscle cells, myoepithelial cells, and pericytes α-SMA, which is detected by the anti-αSMA-1 antibody (354), and has been shown to be under hormonal control (353).

10.1.1. Expression of Endothelial markers

Various markers, each with its own staining profile, have been used to identify endothelial cells. vWF gives good staining in larger blood vessels but may be absent in capillaries which stain positive for CD34, CD31, or CD36 (92, 355). CD34 stained more vessels per unit area than vWF in the physiological cycle but not in progestogen treated endometrium (92).

10.1.2. Expression of vWF

vWF is a large multimeric glycoprotein which is a component of normal haemostasis. It is localised in Weibel-Palade bodies in the vascular endothelial cell cytoplasm. Endometrial vWF activity during the physiological cycle has been variably shown to be constant (92, 355) or to vary (356, 357). Staining in the menstrual and the late-LP may be absent (356), or low (357). Reactivity, increases from the early-FP and decrease from the early-LP (357). Vascular density does not vary with the phases of the cycle, but is reduced with prolonged high dose progestogen (92). Although correction for glandular size demonstrated an increased vascular stromal density in the LP (358).

vWF expression is affected by various factors including œstrogen, which increases expression, and progesterone (92, 357). The effect of œstrogen is not mediated through ER and may be direct (359). Progestogens increase the prevalence of dilated endometrial venules (92), and low dose (20mg/day) levonorgestrel-releasing vaginal ring, was shown to reduce arteriolar concentration, with no significant change in capillaries or venules (360). But changes in endometrial vasculature with progestogens are not always consistent with the degree of the suppression of non-vascular compartments (90, 360).

10.1.3. Expression of αSMA

During the FP, stromal cells are diffusely positive for αSMA, this becomes focal and scattered in the LP (352). αSMA^+ cells form scattered complete or incomplete cuffs around endometrial glands mostly in the basalis, and around dilated or cystic glands, but also occasionally in the more superficial layers and around some non-dilated glands (353). Glandular involvement is seen in atrophic, proliferative and hyperplastic endometria, but is less prominent in secretory endometrium where glandular cuffing is mostly in the basal inactive layer, or around single non-secretory glands, which suggests modulation by steroids (353). Endometrial blood vessels are also surrounded by αSMA^+ cells (353). Around arterioles, they can be up to 3 layers thick, while smaller pre-capillaries or developing arterioles are surrounded by one layer. Staining intensity does not vary with the phase of the cycle in younger women, but expression is higher in the mid-FP in older women, and no modulation occurs during the LP (350).

10.2. Aim

To compare the expression and distribution of αSMA and vWF, as markers of stromal differentiation, during the late-PLP of regularly cycling women with the expression during the LP.

10.3. Material and methods

10.3.1. Study population and regimen

Were described previously (**2.1.1., 2.1.2., 4.3.1.**).

10.3.2. Immunohistochemistry

IHC for vWF and αSMA antibodies (Appendix 2), was described previously (**2.1.5.2**).

10.3.3. Statistical analysis and sample size

Were considered previously (**2.1.7.**).

10.3.4. Assessment

The distribution of αSMA^+ cells in relation to the glands, the blood vessels, the subepithelial stroma, and the scattered stromal cells was described on a scale of none=0, minimal=1, moderate=2, and intense=3, and the number of vessels surrounded by 1 or >1 VSMC layer were counted (350). The number of blood vessels stained for vWF was counted in 17 random hpf(x400)/section, and the intensity of staining was calculated on the same scale as above.

10.4. Results

αSMA^+ cells were noted around arterioles, venules and pre-capillaries throughout the stages of the LP and in the late-PLP. During the early-LP, αSMA^+ cells were noted around blood vessels, arterioles and a few dilated venules or capillaries, and only occasionally around glands and these did not form complete cuffs (Figure 10.1.). Only a few scattered stromal cells were positive. During the mid-LP, αSMA^+ cells surrounding blood vessels were more prominent sometimes forming a double layer (Table 10.2., Table 10.3.),

few glands appeared encapsulated with αSMA$^+$ cells, individually scattered positive stromal cells were more prominent. The features were similar during the late-LP, with only few glands surrounded with an αSMA$^+$ cuff. During the late-PLP, expression was more prominent around glands and many were surrounded by a complete cuff of αSMA$^+$ cells, but there was no difference in the distribution of αSMA$^+$ cells surrounding blood vessels.

On semiquantitative assessment, the late-PLP expressed more periglandular staining compared to all stages of the LP, and more stromal staining compared to the mid-LP (Table 10.2.). There were no statistically significant differences between the different phases of the physiological cycle.

There were no statistically significant differences in the number of blood vessels stained with vWF, between any of the stages of the LP or the late-PLP (Table 10.4., Figure 10.5.). Although vascular density was slightly higher during the late-PLP this difference was offset by the smaller glandular diameter. Also, there was no statistically significant difference between the intensity of staining in the different stages of the LP and the late-PLP. In the late-PLP, there were more dilated capillary spaces particularly beneath the luminal epithelium.

10.5. Discussion

Heterogeneity of endothelial cells is widely accepted (355, 357). But it is not clear whether differential staining with different endothelial markers represents true subpopulations or is due to expression below the threshold of detection of the respective antibodies (357). This, however, would not affect the interpretation of this comparative study.

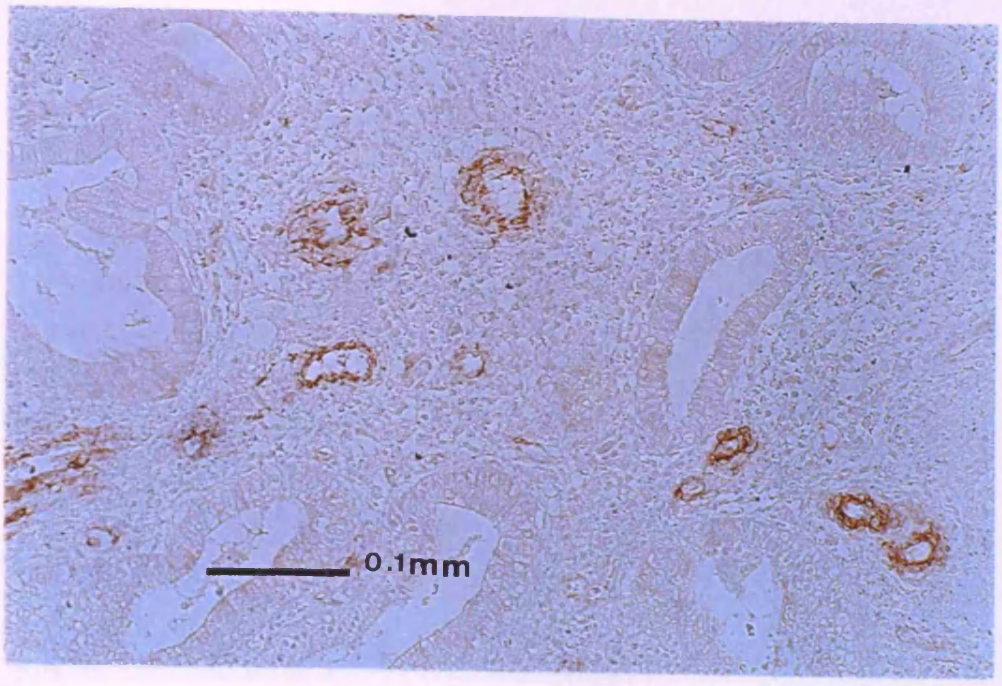
The higher expression of αSMA around the glands in the late-PLP may be a reflection of the higher prevalence of retarded non-secretory glands (353). The function of peri-glandular myofibroblasts is not known, but as they are not constant, they cannot be related to growth or differentiation (353). Stromal myofibroblasts may have a role in tissue remodelling together with tenascin which is often co-expressed around αSMA positive cells (353, 361). TGFβ induces αSMA expression in smooth muscle cells and pericytes (361), and it is possible that the higher expression of αSMA around some glands and in isolated stromal cells may reflect higher TGFβ expression in the stroma.

The lack of variation in vWF expression during the LP is consistent with other studies (92, 355). Expression of vWF in this study was strong compared to the very low level reported previously (356), this may be a reflection of the sensitivity of the assay used. Little is known about the factor(s) that regulate vWF expression but œstradiol stimulates vWF synthesis in the human endometrial endothelial cells in culture (362). The effect of steroids in vivo is believed to be indirect (92), and the similarity in expression of vWF between the different phases examined, whilst contrasts to other markers used in this thesis, may be related to this different mode of œstrogen action.

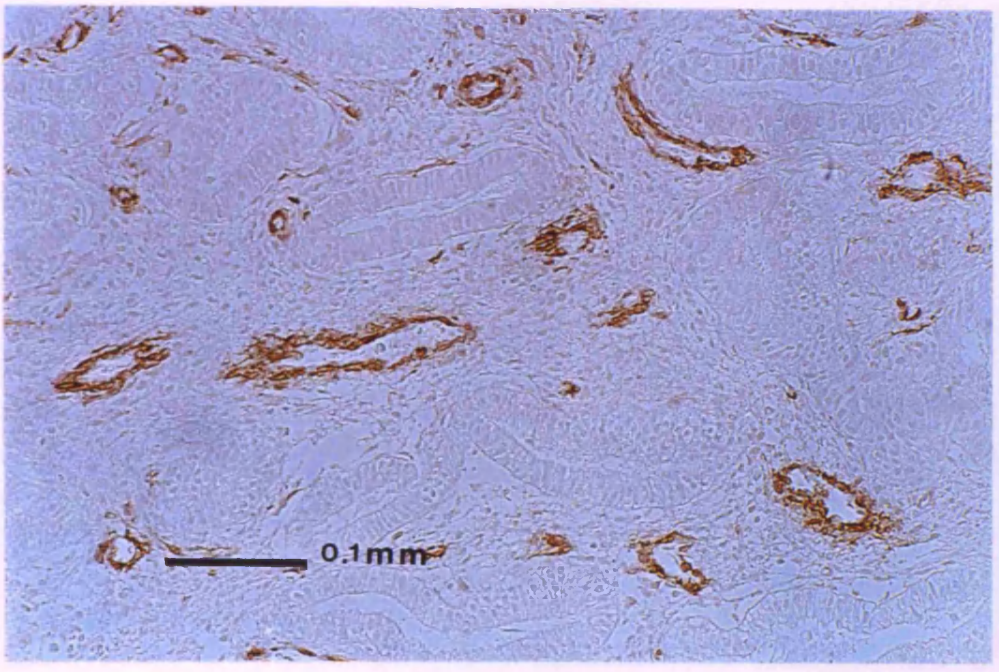
The density of vWF^+ vessels did not vary during the LP, which is in agreement with the findings of others (90, 92, 358). However, taking the area fraction of stroma into account (358) vW^+ vascular density increases in the LP compared to the FP. Ultrastructural studies (363, 364), and studies on progestogen implant contraceptives (Norplant) (90), have suggested that progestogen is a net angiogenic promoter in the endometrium. Furthermore it has also been demonstrated that patients with elevated endogenous œstradiol had lower microvascular density compared to other Norplant users (90).

Many factors are known to affect angiogenesis in the endometrium, including acidic and basic fibroblast growth factors, but the principal regulator is likely to be the vascular endothelial growth factor (VEGF), whose mRNA and protein expression increase under the influence of œstrogen, progestogen, or both (365).

It is possible that the similarities seen in the vasculature are related to the similar bleeding pattern. This, however, awaits further investigation in order to establish the link between the observed structural and functional similarities. This is particularly important as microvascular density did not correlate with the occurrence of breakthrough bleeding in Norplant users (90). However, as the two regimens are dissimilar, extrapolations cannot be made.



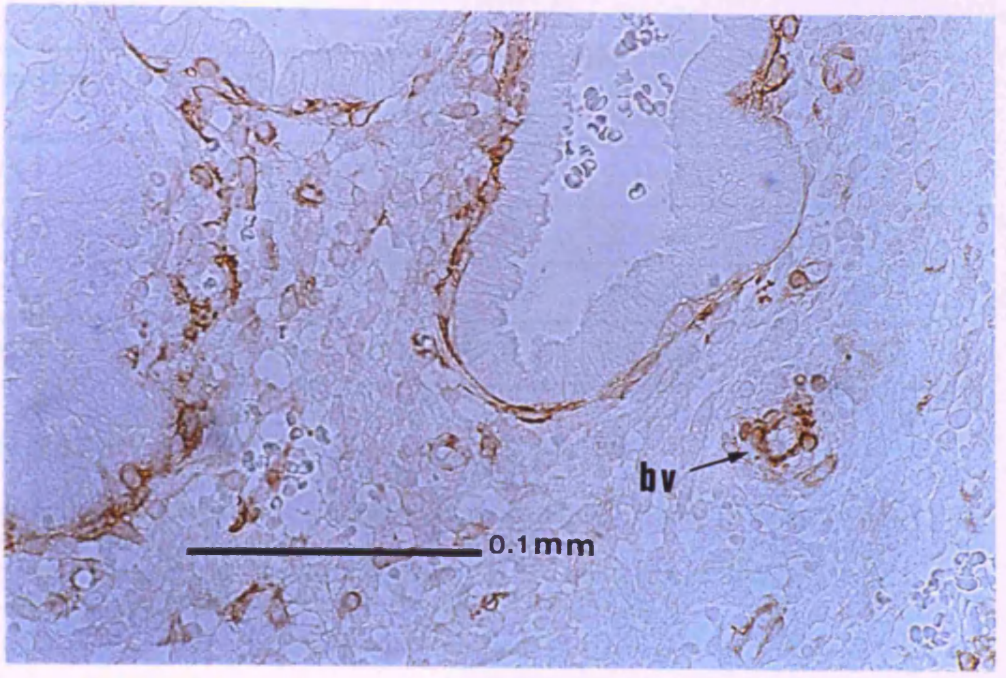
a



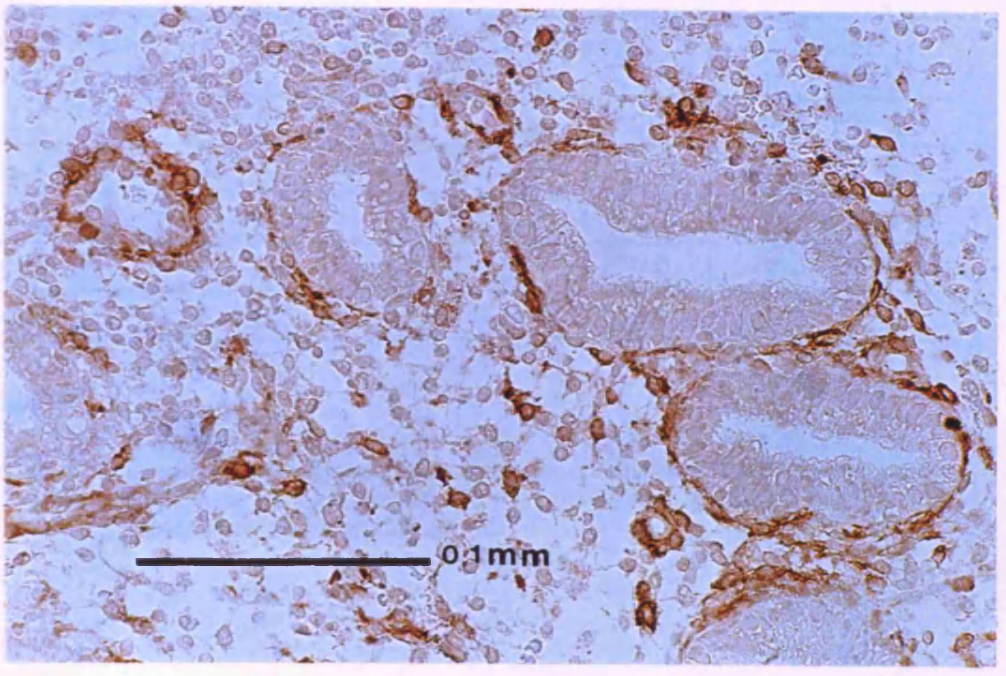
b

Figure (10.1. a,b.)

The expression of αSMA positive cells during the early-LP (**a**), and the mid-LP (**b**).



c



d

Figure (10.1. c,d.)
The expression of αSMA positive cells during the late-LP (**c**), and the late-PLP (**d**).

Figure (10.1. a-d.)

The expression of αSMA positive cells during the LP and the late-PLP.

Phase of the cycle	Peri-glandular	Sub-epithelial	Peri-vascular	Stromal
Early-LP	0.43 (0-1)	0	2.3 (1-3)	1.6 (1-2)
Mid-LP	0.5 (0-1)	0.6 (0-2)	2.3 (1-3)	1.2 (0-2)
Late-LP	0.7 (0-2)	0.8 (0-3)	2.4 (1-3)	1.4 (0-3)
Late-PLP	1.6 (0-3)[1]	0.8 (0-3)	2.3 (1-3)	2.1 (0-3)[2]

Table (10.2.)

Semiquantitative assessment of αSMA immunostaining in the LP and the late-PLP, expressed as mean and (range).
1 Statistically significant compared to the early-LP, mid-LP, and late-LP ($p<0.05$).
2 Statistically significant compared to the mid-LP ($p<0.05$).

Phase of cycle	Single layer		>1 layer		Total	
	Mean	SD	Mean	SD	Mean	SD
Early-LP	57	13.5	15	11.6	72	22.1
Mid-LP	58	7.5	24	12.8	82	17.6
Late-LP	55	16.7	21	8.6	77	19.2
Late-PLP	50	13.2	20	9.8	70	25.6

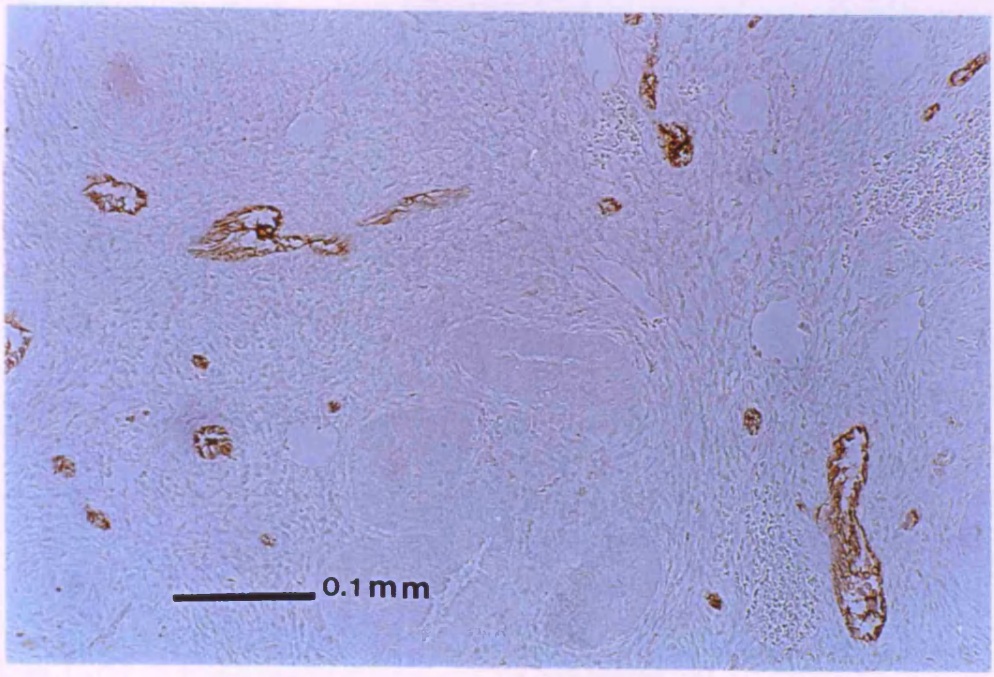
Table (10.3.)

The number of endometrial blood vessels surrounded by either one or more than one layer of αSMA during the different stages of the LP and the late-PLP. Calculated in 17hpf (x200). None of the differences were statistically significant.

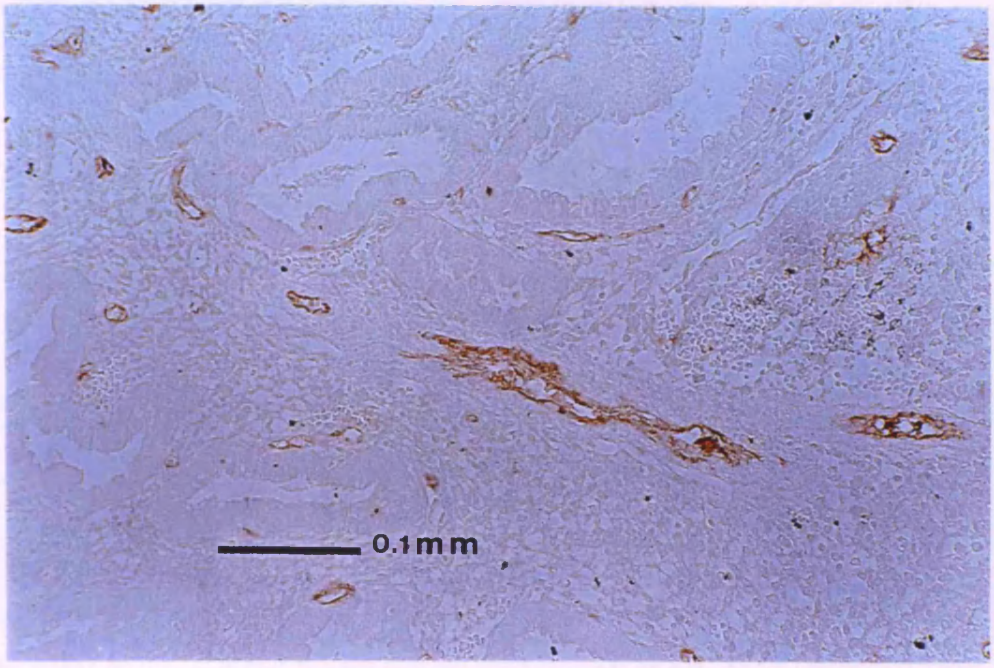
Phase of the cycle	Number		Intensity	
	Mean	SD	Mean	Range
Early-LP	66	10.3	1.8	(1-3)
Mid-LP	58	29.5	1.6	(1-2)
Late-LP	57	21.2	2	(1-3)
Late-PLP	70	20.7	2	(1-3)

Table (10.4.)

The number of vWF positive blood vessels per 17hpf (x200), and the mean staining intensity. There were no statistically significant differences.



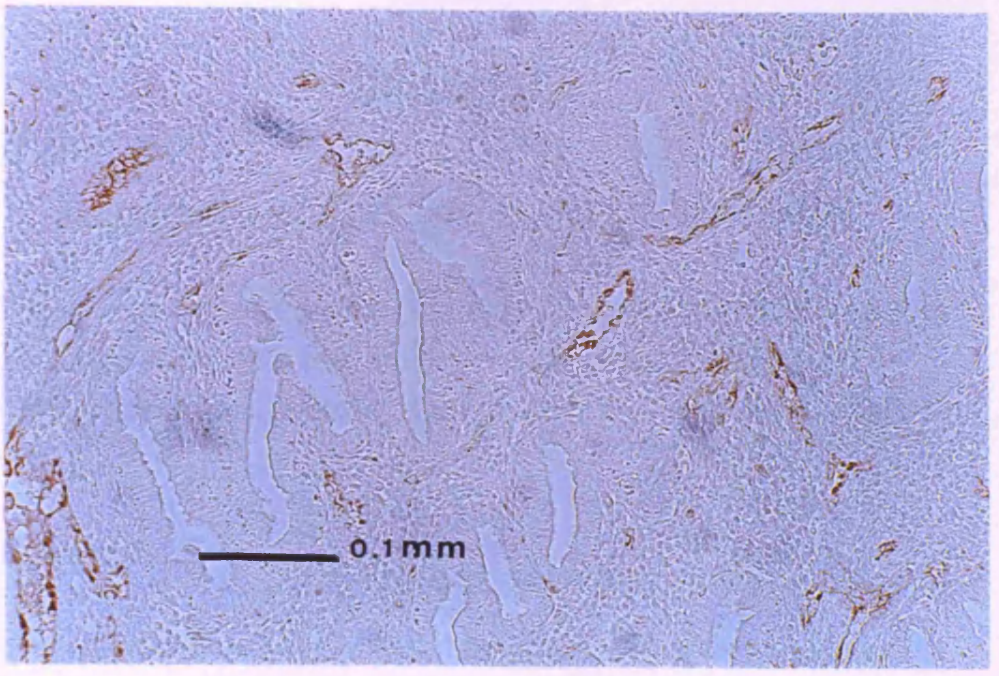
a



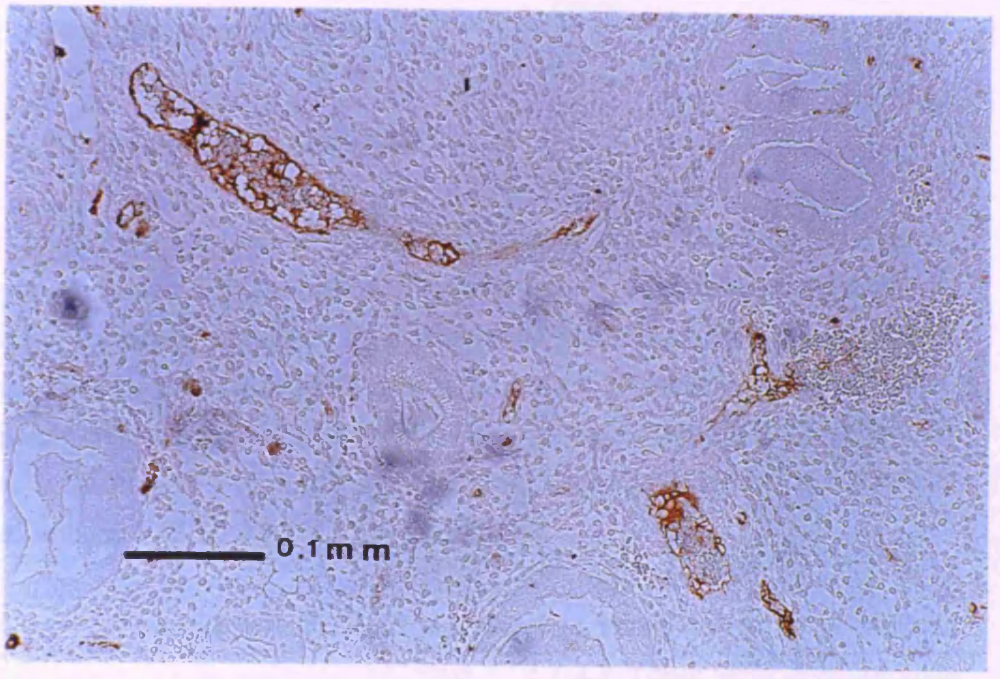
b

Figure (10.5. a,b.)

The expression of vWF during the early-LP (**a**), and the mid-LP (**b**).



c



d

Figure (10.5. c,d.)

The expression of vWF during the late-LP (**c**), and the late-PLP (**d**).

Figure (10.5. a-d.)

The expression of vWF during the LP, and the late-PLP.

Chapter 11

Generation of Anti-Endometrial Antibodies

1- A mouse monoclonal antibody, LDS60, detects a ~200kDa membrane antigen on the surface of human endometrial glandular epithelial cells.
2- A mouse monoclonal antibody , LDS10, detects an endothelial cell related antigen.

11.1. Introduction

Besides the known markers of endometrial function tested previously, the objective of this project was to identify new cell-type specific and/or phase specific antigenic markers, that may be useful for monitoring endometrial response to hormones and to cHRT.

11.2. Material and methods

Cell membrane was prepared as described previously (**2.2.1.1.**) from pooled benign endometrial tissue removed from hysterectomy specimens, where the operation was performed for non-endometrial pathology. The concentration of membrane protein (12mg/ml) was calculated (Appendix 4). Membrane-adjuvant mixture was used to immunise 6-10 weeks old, (MF1 x Balb/C)F1 mice in groups of 2 (**2.2.1.**). After culling, splenocytes were hybridised to myeloma cells (**2.2.1.2.**) and cloned (**2.2.1.3.**).

Immunohistochemistry (**2.1.5.2.1.**) was used to verify the immune response and to screen for the antibodies of interest (**2.2.2.**). These were developed into monoclonals (**2.2.1.3.**) and ascites (**2.2.1.5.**). The epitope was identified using Western blotting (**2.2.3.2.**) and IHC on endometrium and other body tissue sections.

11.3. Procedure

IHC was performed at the following stages:

1- To verify an immune response using whole serum obtained through a tail bleed. Serial dilutions were used with preimmunisation serum from same animal as negative control and anti-CD45 antibody (Appendix 2), as positive control.

2- After fusion, culture supernatant from growing cells was tested (usually between 40-60 wells per fusion). Immune serum, and culture medium were used as positive and negative controls respectively. Antibody producing wells (A) were identified (usually between 3-12). Cells from A were expanded into wells (B) (**2.2.1.3.**).

3- Supernatant from wells (B), that grew single clones (usually between 12-24/well A) were tested. Supernatant from A and culture medium were used as positive and negative controls respectively. Wells (B) that exhibited the best staining (C) were identified (usually 2-3).

4- Supernatant from (C) was further tested prior to storage (**2.2.1.4.**) or developing into ascites (**2.2.1.5.**).

11.4. Criteria

Screening aimed to identify antibodies that were:

1- Cell type specific, i.e. stain either the epithelium, the endothelium, or the stroma, and:
2- Cycle phase specific, i.e. bind selectively, or more strongly, or that have a different pattern of staining to either the proliferative or the secretory endometrium.

11.5. Results

Sixteen mice were immunised as above, of these 2 did not develop an adequate immune response, and 3 fusions were unsuccessful. Eleven fusions were performed, cells from 2 of these died after the initial screening. From the remaining 9 successfully grown fusions, 64 wells were selected and monoclonal antibodies were developed from 19, and two (LDS60, LDS10) were developed into ascites (**2.2.1.5.**).

11.5.1. LDS60

Immunohistochemical localisation in the endometrium (Figure 11.1.) demonstrated that LDS60 identifies an epitope that is specific to epithelial cells. During the FP, staining is intracytoplasmic in the form of small vesicles near to the base of the glands, and minimal staining appeared at the luminal border, but only a few glands are positive. During the early-LP immunolocalisation is also intracytoplasmic, either near the base, or in a position about 2/3 distance from the base of the cell. During the mid-LP, micro-vesicles appeared to accumulate nearer to the apices. During the late-LP there is no intracytoplasmic staining, immunostaining localises to the luminal border of the epithelium, and some staining appeared in small 'vesicular structures' within the gland lumen. The luminal epithelium was positive throughout the LP, and staining was confined to the apical border of the cells. The late-PLP exhibited strong LSD60 staining in the luminal epithelium and the apical border of the epithelial cells in some of the glands. There was minimal expression in the gland lumen.

IHC also demonstrated that this epitope is expressed in other tissues in the body (Table 11.2., Figure 11.3.), but more specifically to some mucous secreting cells.

Antibody isotyping was performed using the monoclonal isotyping kit (cat. no. MMTRC1, Serotec, Oxford, UK) according to the manufacturer's protocol. LSD60 was identified as an IgM antibody. On Western blotting (**2.2.3.2., 2.2.3.3.**) the epitope was identified as a ~200kDa antigen (Figure 11.4.).

11.5.2. LDS10

LDS10 identified an epitope that was specifically and strongly expressed on all endothelial cells in both the endometrium and the myometrium. Expression appeared to be phase specific, it was -ve in 3/4 of the proliferative samples examined but +ve in 10/12 of the secretory samples (Figure 11.5.). Expression did not vary between the early-LP, mid-LP, or late-LP. There was no expression in the stroma or the epithelium. The late-PLP was $LDS10^{+}$ and exhibited the same pattern as the late-LP (Figure 11.5.). The endothelium was positive in the breast, tonsil, and testis, and so were the seminiferous tubules, but other body tissues examined were negative (Table 11.6., Figure 11.7.).

11.6. Discussion

LSD60 antibody identifies a ~200kDa antigen that is expressed in the endometrium where it exhibits modulation during the cycle. It is also expressed in cHRT endometrium. This modulation suggests that it may be useful in monitoring the response to steroids, although further characterisation will be necessary in order to understand its function

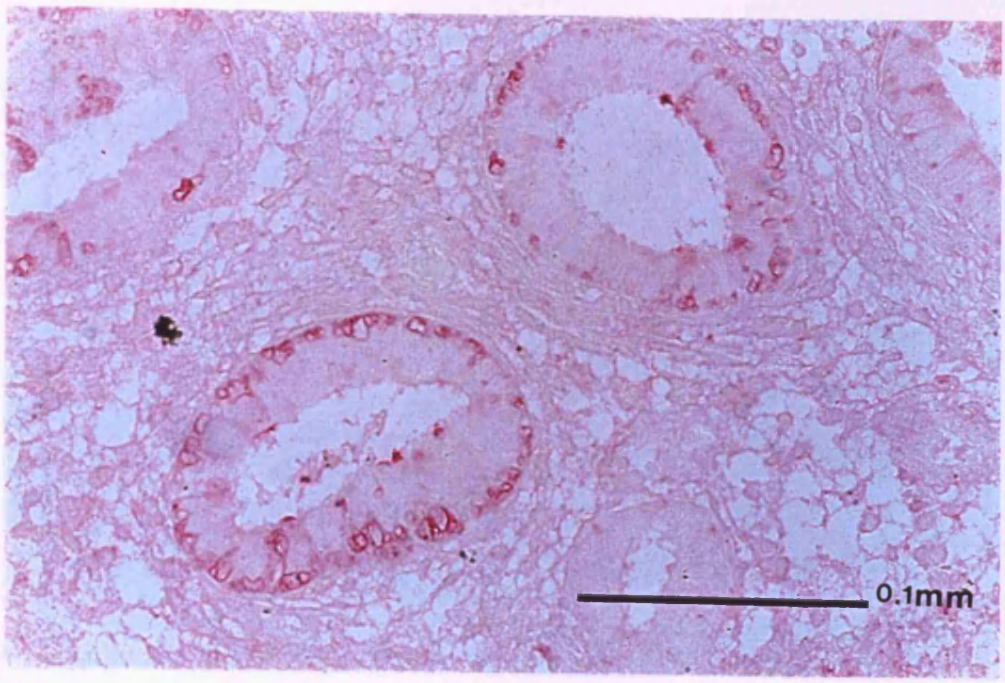
The antigen is not endometrium specific. It also identifies the Neck mucous glands in the stomach, tongue mucous glands, the colonic goblet cells, and type II pneumocytes in the lung, which will limit its use systemically. Also its size and localisation do not support a putative role as a serum marker.

Both the molecular weight and the distribution, together with it being an IgM antibody, suggest that the antibody is raised against a glycosylated molecule. Also the epitope identified using the LDS60 antibody shows distributional similarities to that identified by the antibody D9B1 (366). It may therefore be related to MUC-1 or to some other surface-associated mucin.

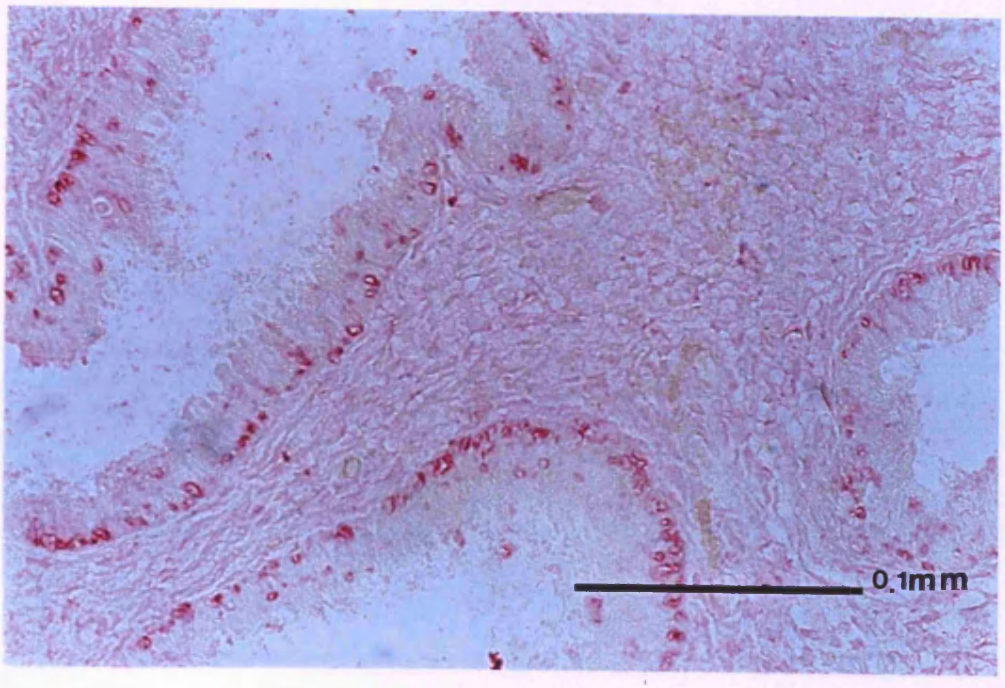
The membrane location of this antibody raises interest in its use for local tissue targeting which could be employed for photo-sensitisation during Laser Ablation of the endometrium. A procedure designed to destroy the endometrial lining and consequently to resolve the problem of heavy or irregular bleeding which is a common problem in cHRT (**1.9.2.**).

LDS10 identifies the endothelium of the endometrium during the secretory phase and less so during the proliferative phase, this suggested hormonal modulation. It is, however, unusual in that it was expressed in the endothelium in the myometrium, which raised interest in its expression in other body sites. Most body tissues were negative, but the expression in the breast and the testis also raised interest in possible hormonal modulation, but this is unlikely to be the case in view of the expression in the tonsil. The pattern thus suggests an antigen that may be individual, rather than tissue specific. This finding will require further clarification, but although interesting, it reduces the utility of LDS10 in relation to the current study. However, further characterization may be hampered at the IHC level by the need to use different tissues obtained from individuals with LDS10^{+} endometrium. An alternative approach may be

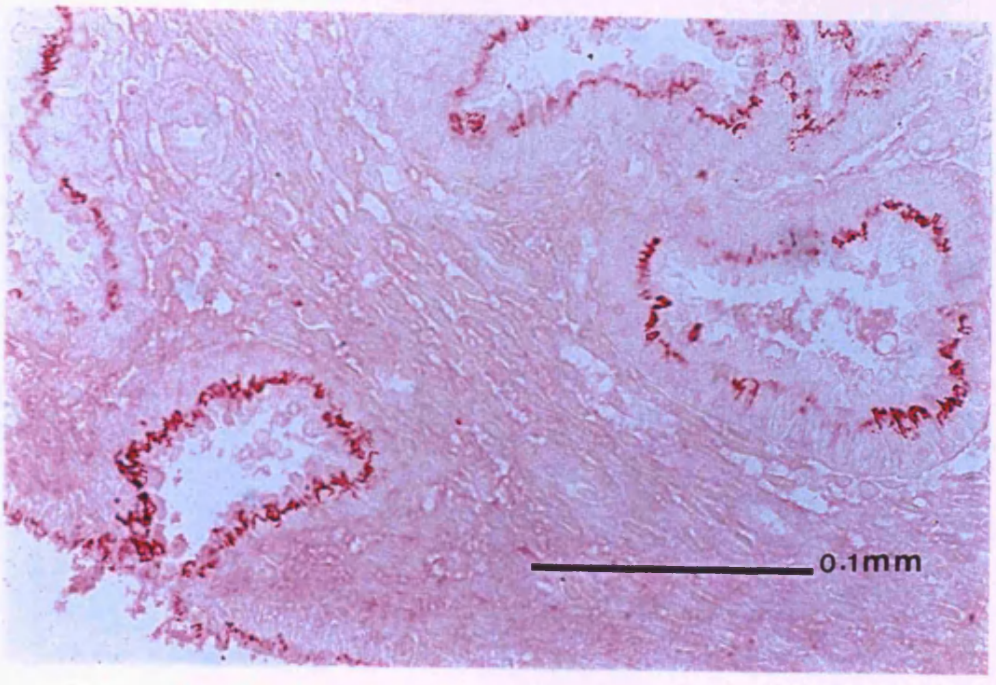
through antigen characterisation, but as the expression did not vary in relation to the use of cHRT, this was not pursued any further in this thesis.



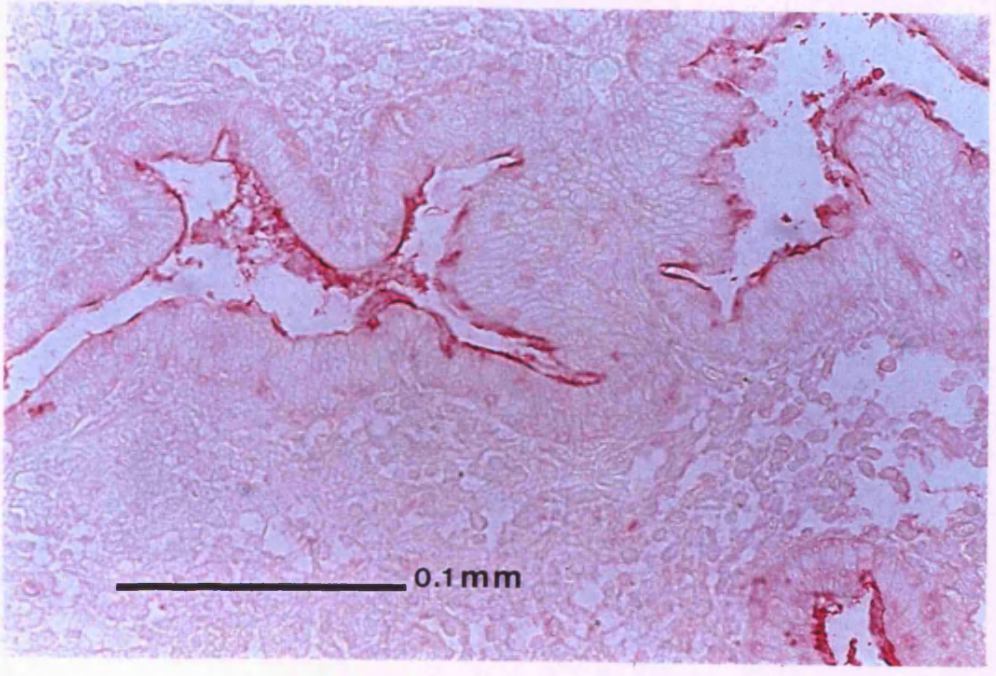
a- Expression of LDS60 during the follicular phase.



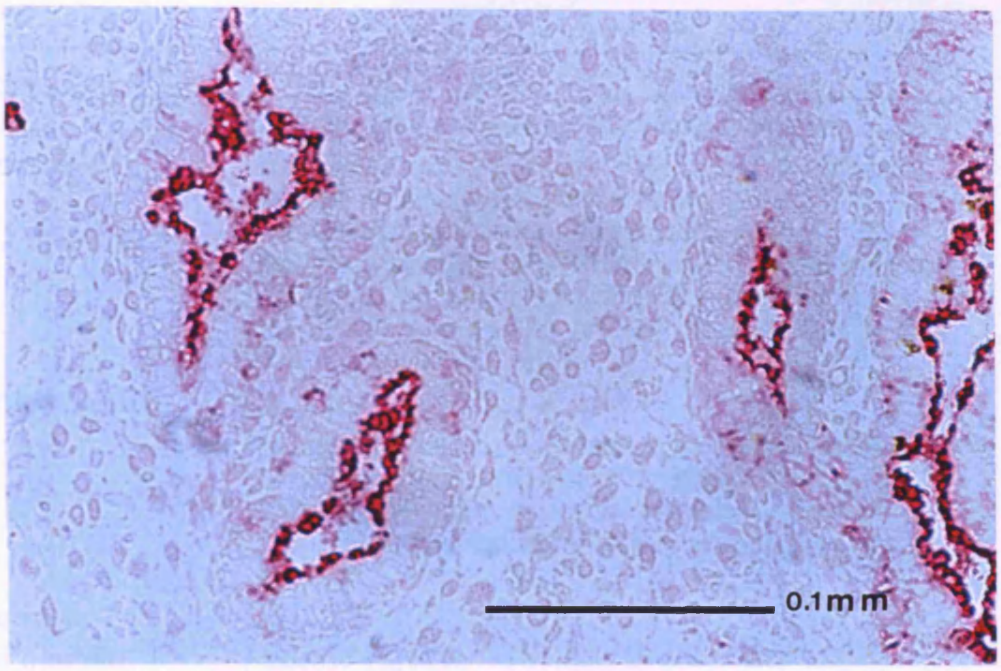
b- Expression of LDS60 during the early-LP.



c- Expression of LDS60 during the mid-LP.



d- Expression of LDS60 during the late-LP.



e- Expression of LDS60 during the late-PLP.

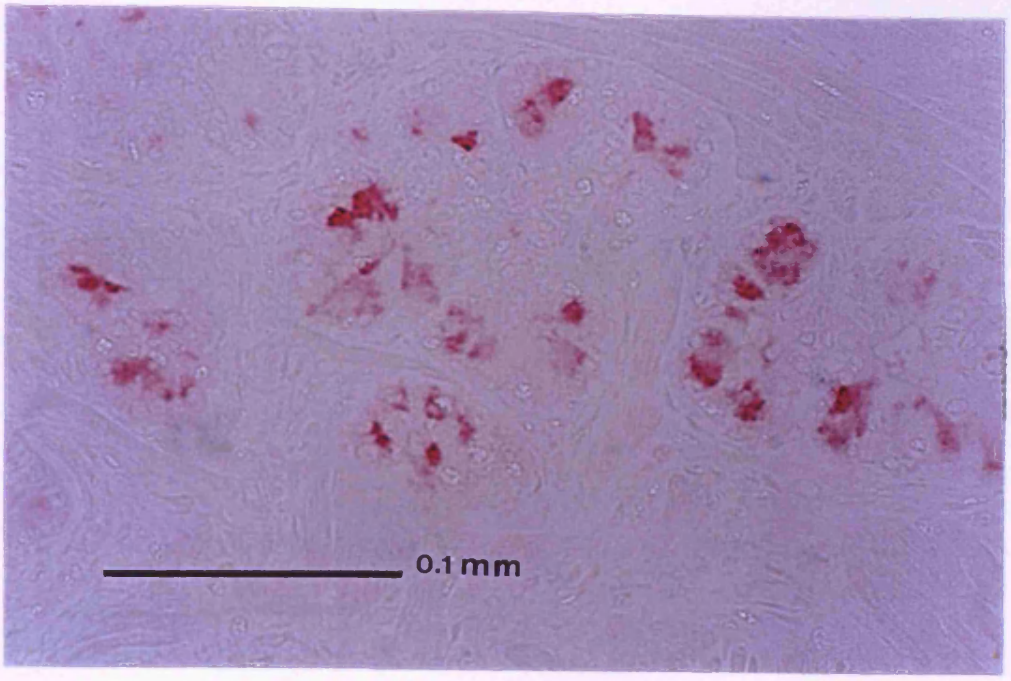
Figure (11.1. a-e.)

The expression of LSD60 during the FP, the LP, and late-PLP.

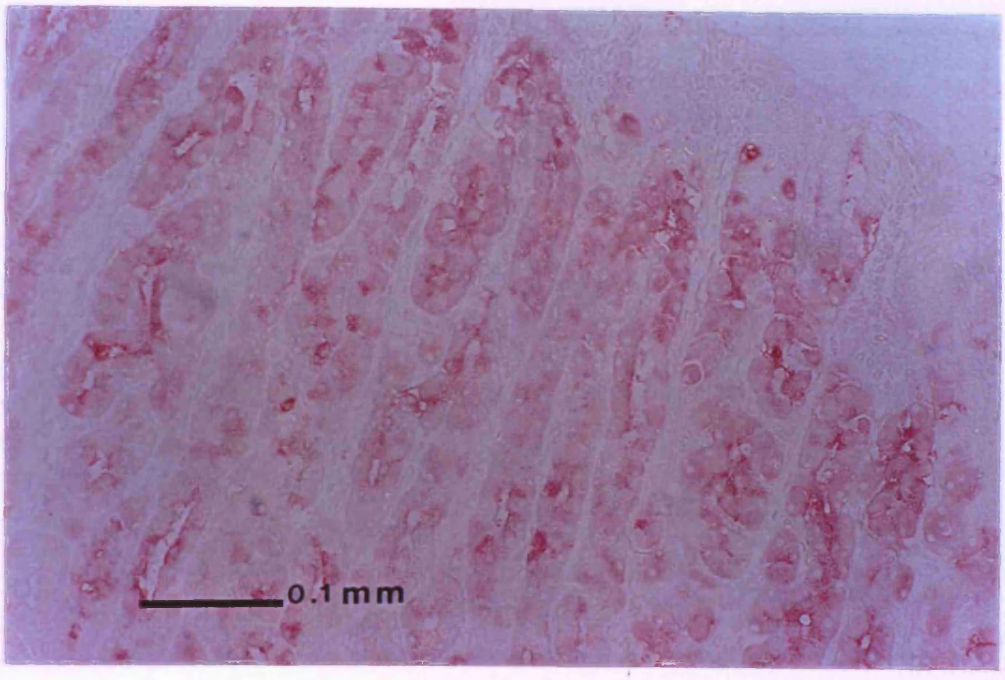
Tissue	Pattern of expression
Tongue	mucous glands below the surface epithelium.
Stomach	mucous-secreting cells at the neck and the isthmic portion of the gastric glands. Staining was intracellular in location. These cells secrete a less viscous type of mucous which may function to protect the glands from autodigestion. These are known as the Neck Mucous Cells, and are characterised by their less intense staining by PAS.
Small intestine	negative
Colon	goblet cells which predominate the base of colonic glands, and which represent the mucous secreting glands in the colon, staining was intracellular, but also positive, were some individually scattered cells in the mucosa.
Pancreas	negative
Gall bladder	mucosa negative
Liver	few distinct cells scattered between liver lobules, not Kupffer cells
Thyroid	few cells between the follicles were positive (?parafollicular cells)
Adrenal gland	negative
Anterior-pituitary	Chromophilic cells stained positive
Lung	stained lung pneumocytes, which secrete the surface-active material: surfactant, which functions to reduce surface tension within alveoli
Breast	negative
Ovary	negative
Testis	negative
Kidney	some renal tubules were positive, staining was more at the luminal border, but was present all-around the cell
Tonsil	negative
Spleen	few scattered leukocytes were positive
Cardiac muscle	negative
Striated muscle	negative
Brain	negative
Medulla oblongata	few distinct round bodies stained positive
Cerebellum	few distinct round bodies stained positive

Table (11.2.)

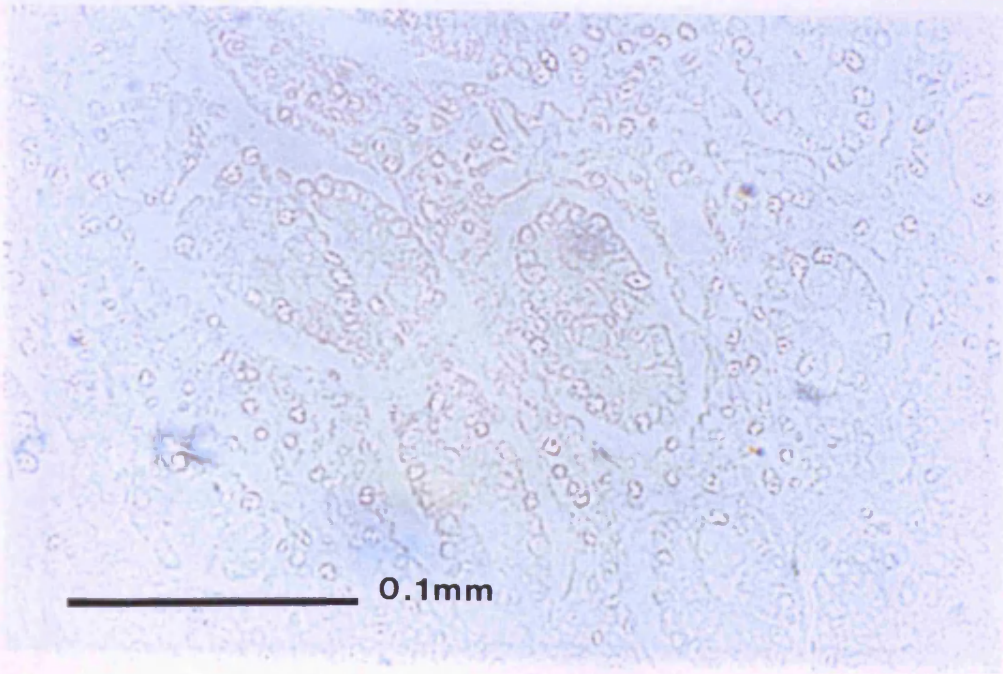
IHC localisation of LDS60 antibody in different body tissues.



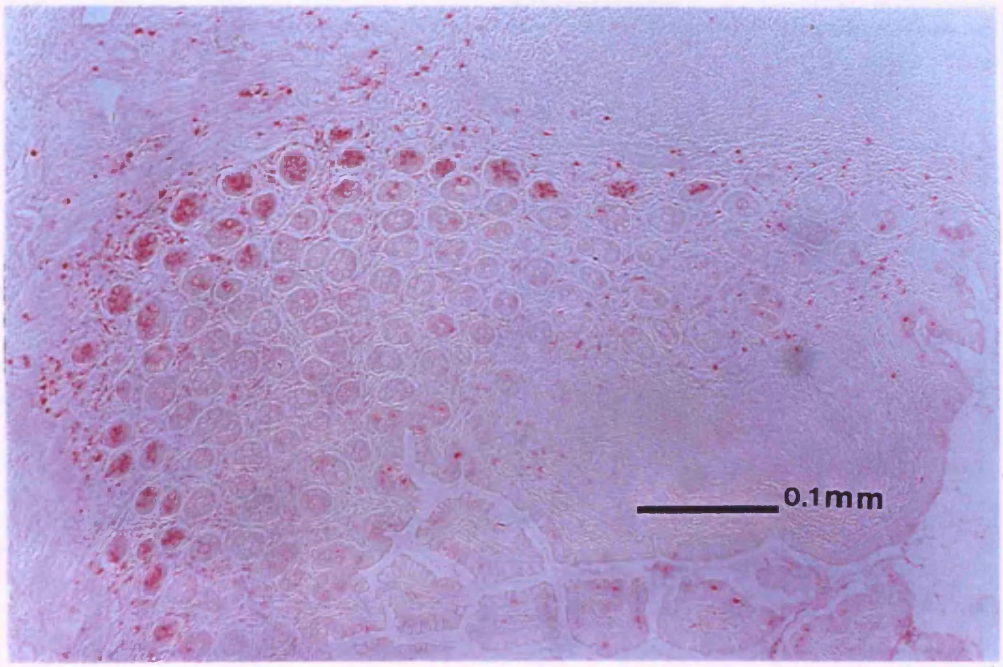
a- Expression of LDS60 in the tongue.



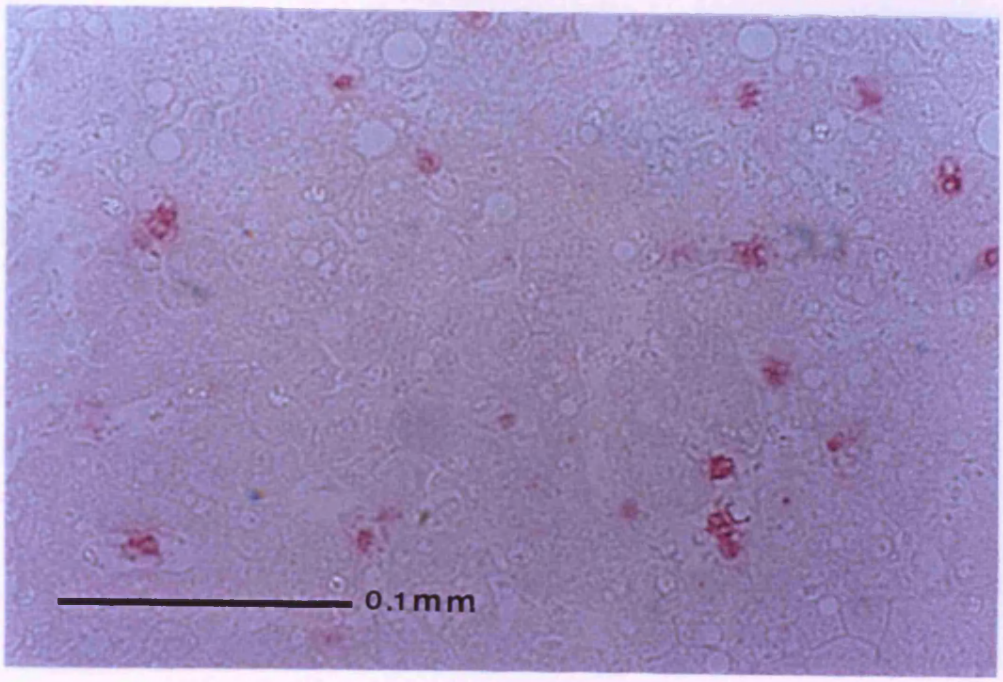
b- Expression of LDS60 in the stomach.



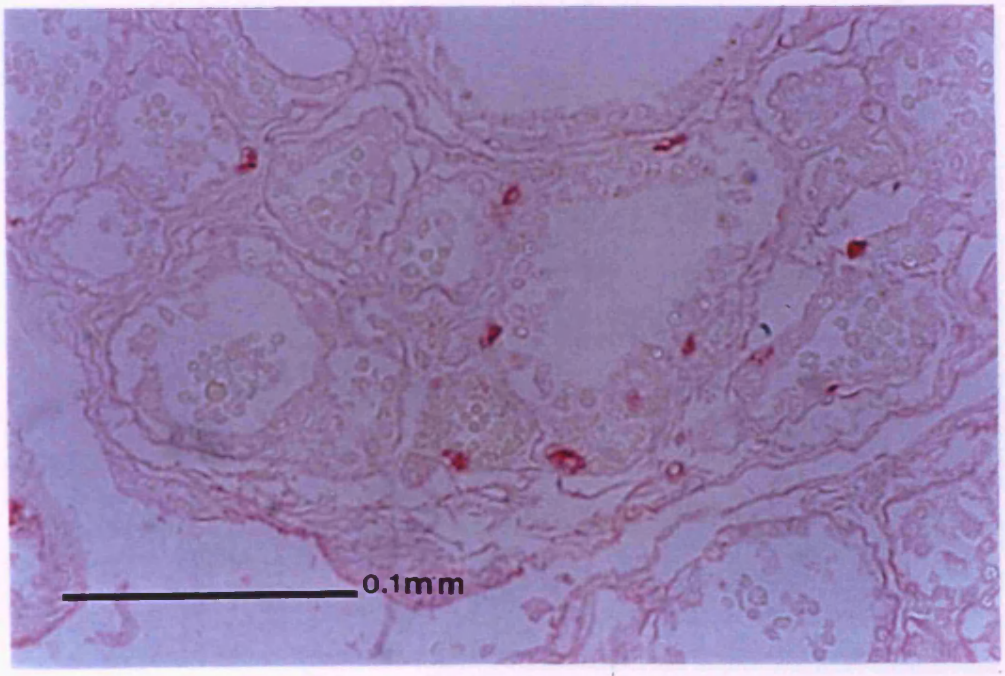
c- Expression of LDS60 in the small intestinal mucosa.



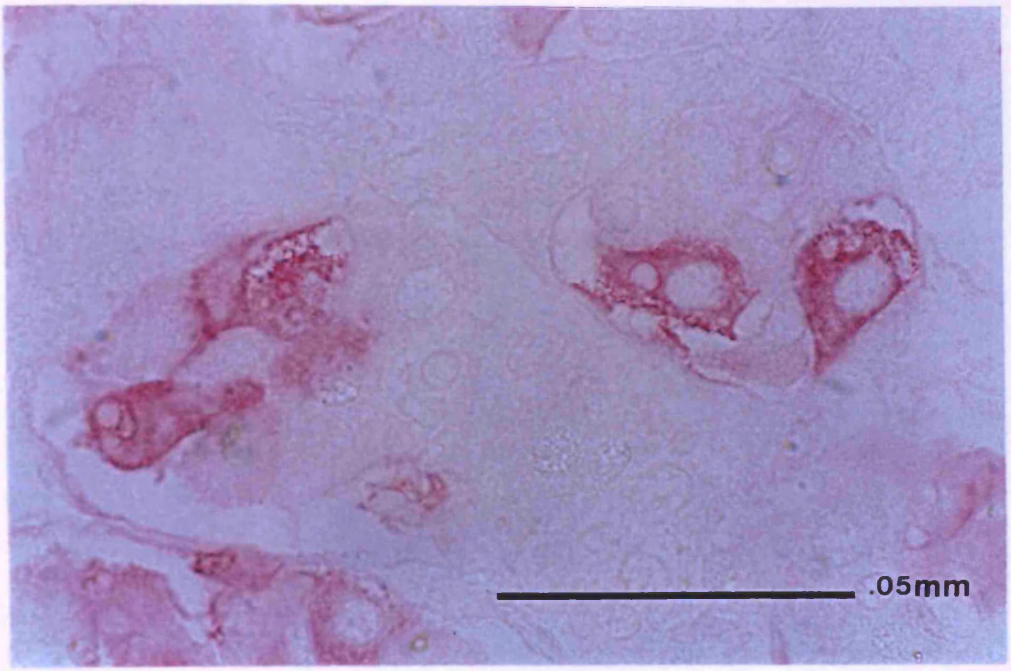
d- Expression of LDS60 in the colonic mucosa.



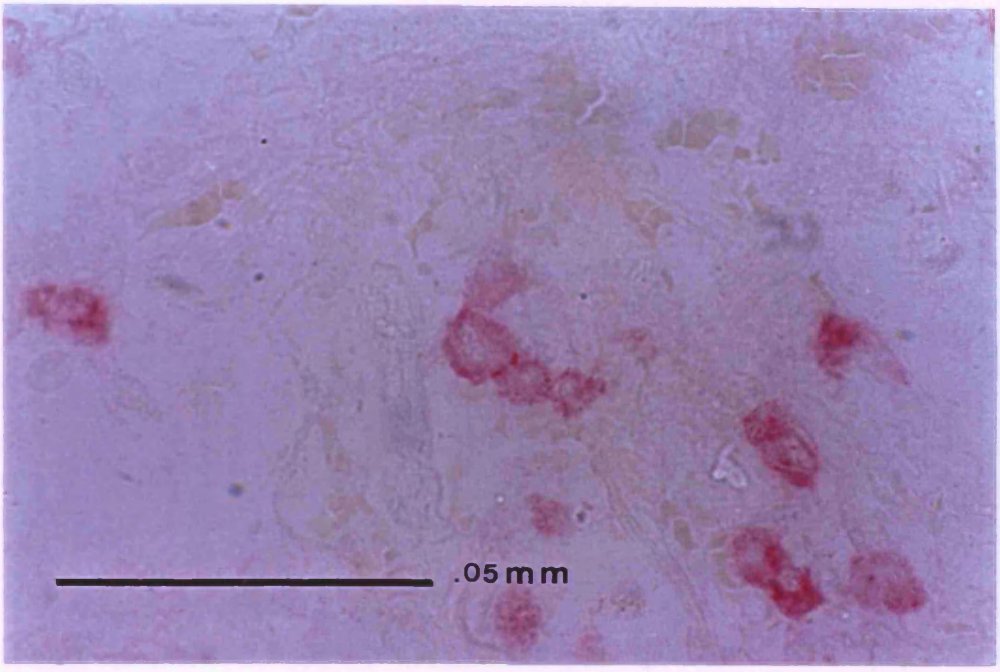
e. Expression of LDS60 in the liver.



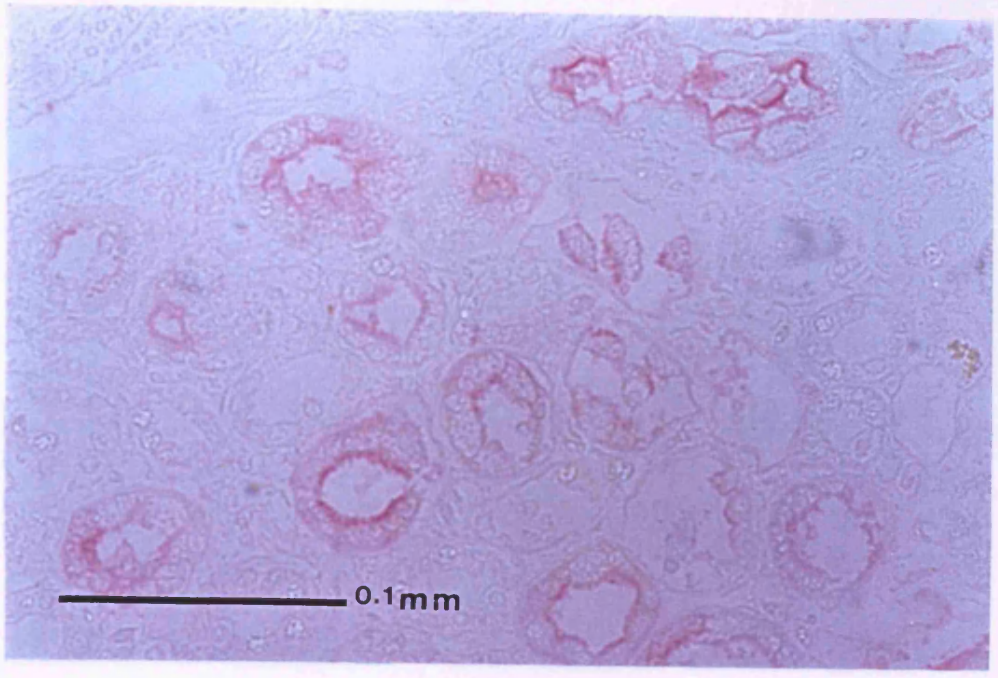
f- Expression of LDS60 in the thyroid gland.



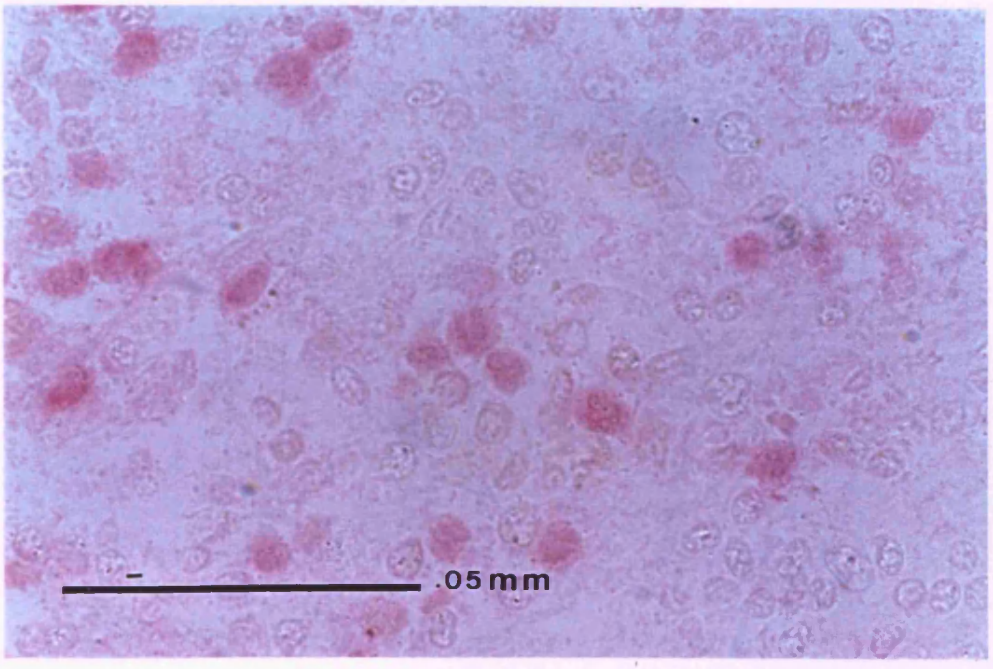
g- Expression of LDS60 in the anterior pituitary.



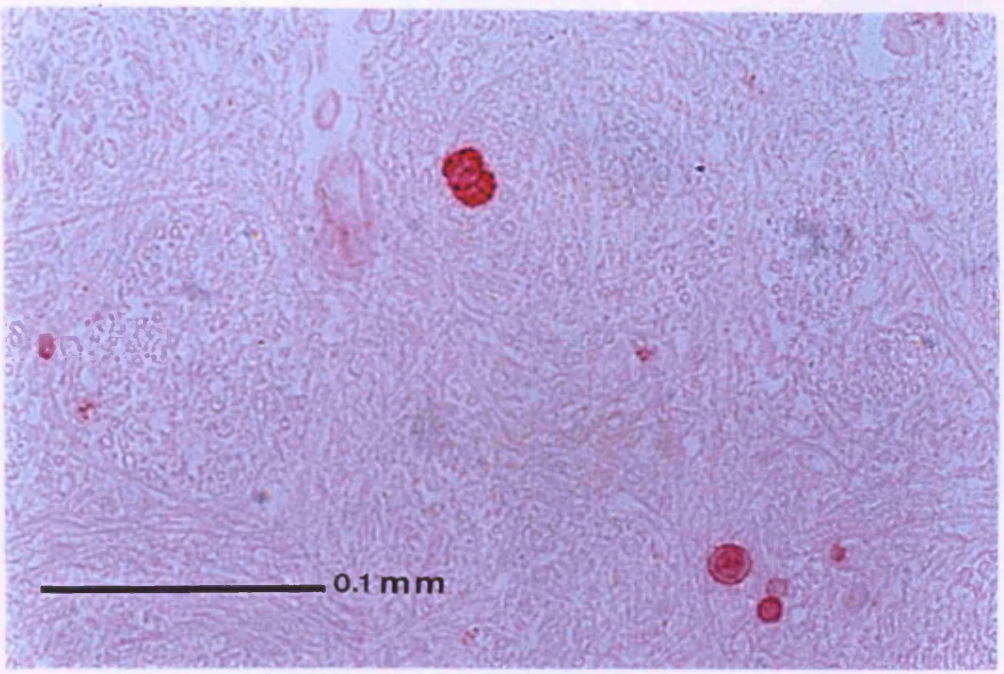
h- Expression of LDS60 in the lung.



i - Expression of LDS60 in the kidney.



j- Expression of LDS60 in the spleen.



k- Expression of LDS60 in the medulla oblongata.

Figure (11.3. a-k.)

IHC expression of LSD60 in different body tissues.

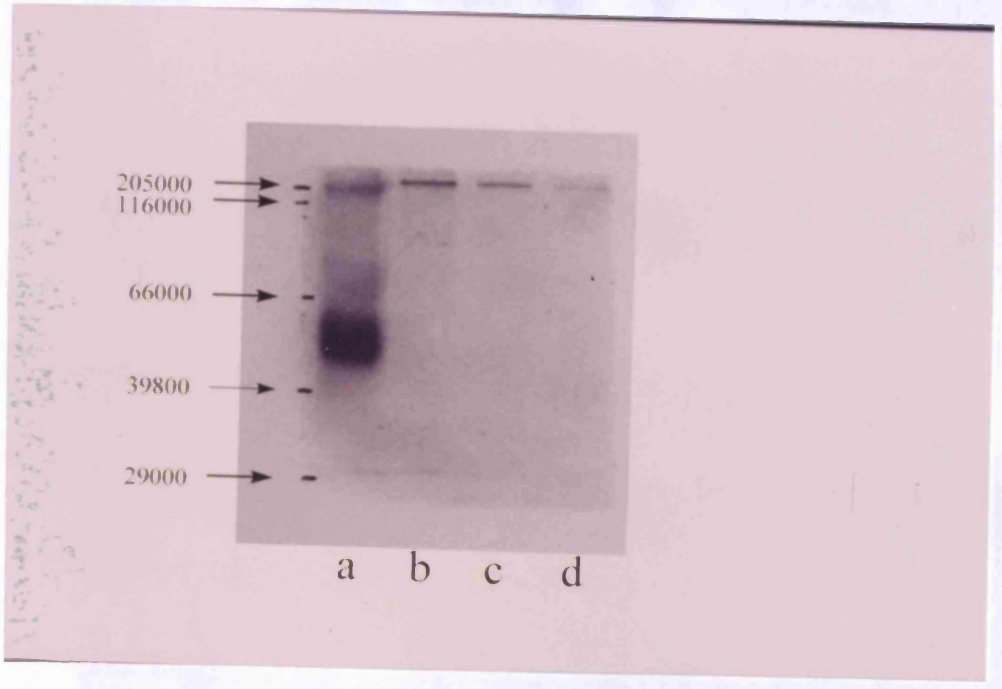
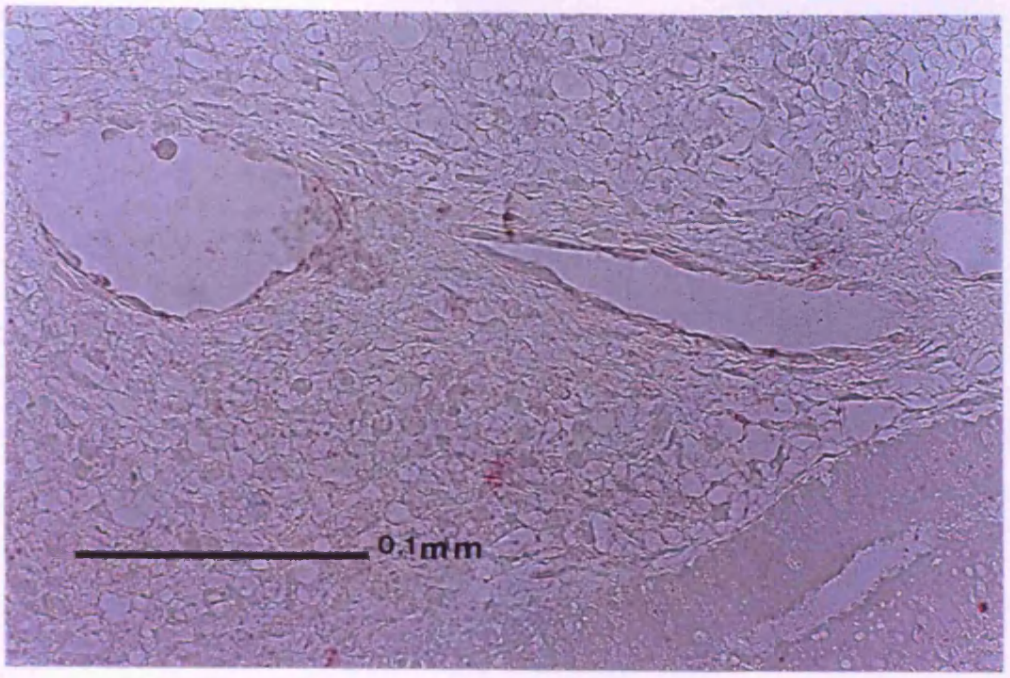
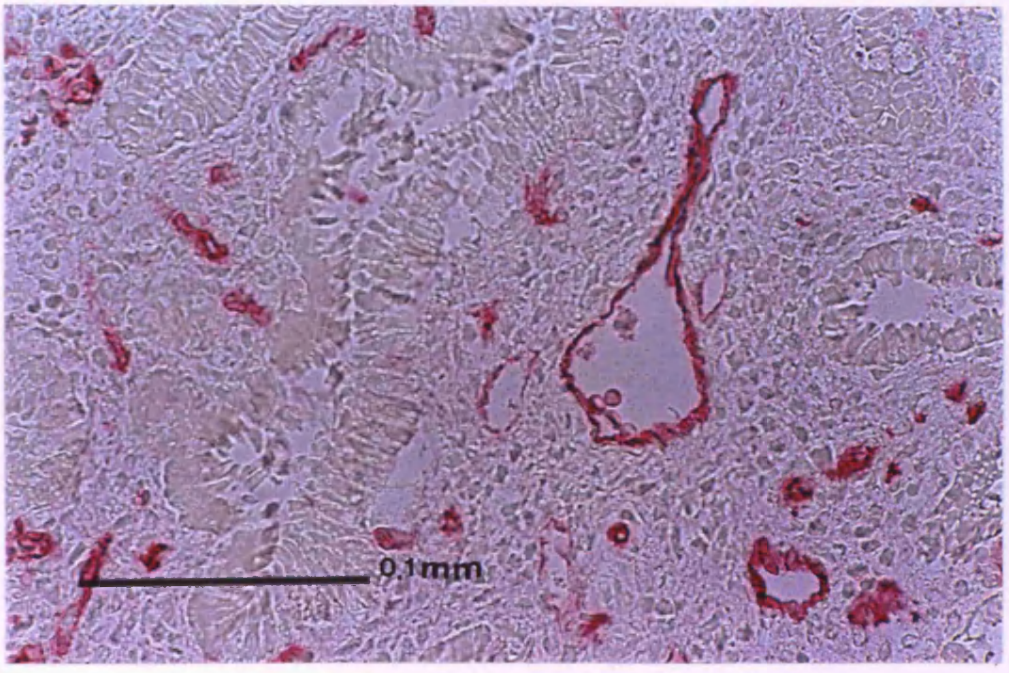


Figure (11.4.)

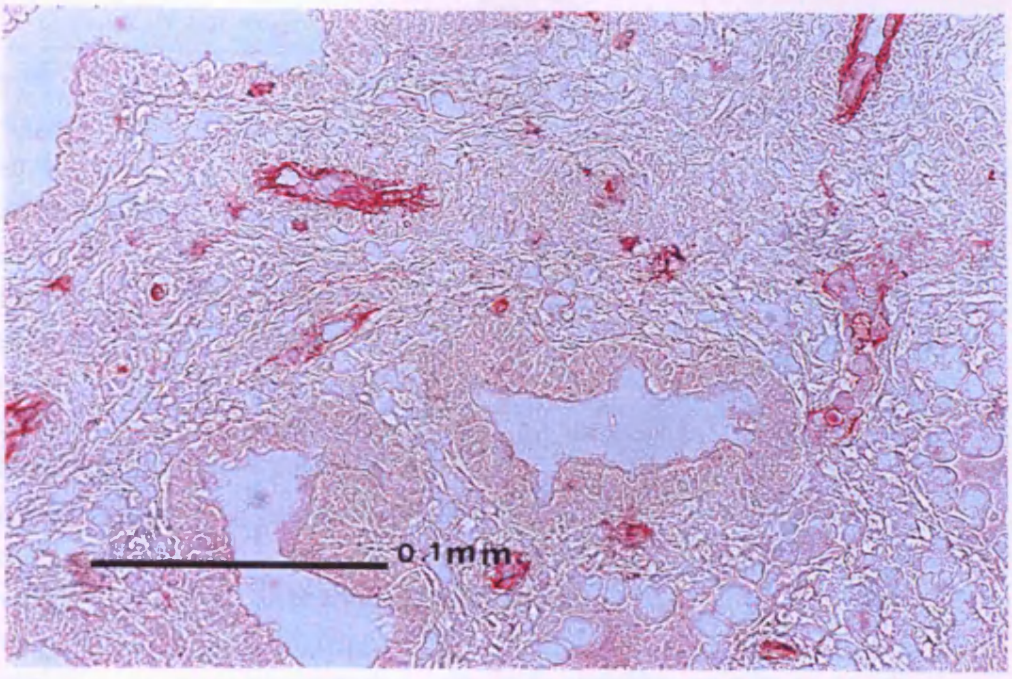
Western blot of LDS60 demonstrating a band of MW≅200 000.
a- Homogenised endometrial tissue 1:5.
b- Membrane preparation 1:2.
c- Membrane preparation 1:4.
d- Membrane preparation 1:8.



a- Expression of LDS10 in the proliferative endometrium.



b- Expression of LDS10 in the secretory endometrium.



c- Expression of LDS10 in the late-PLP of cHRT therapy.

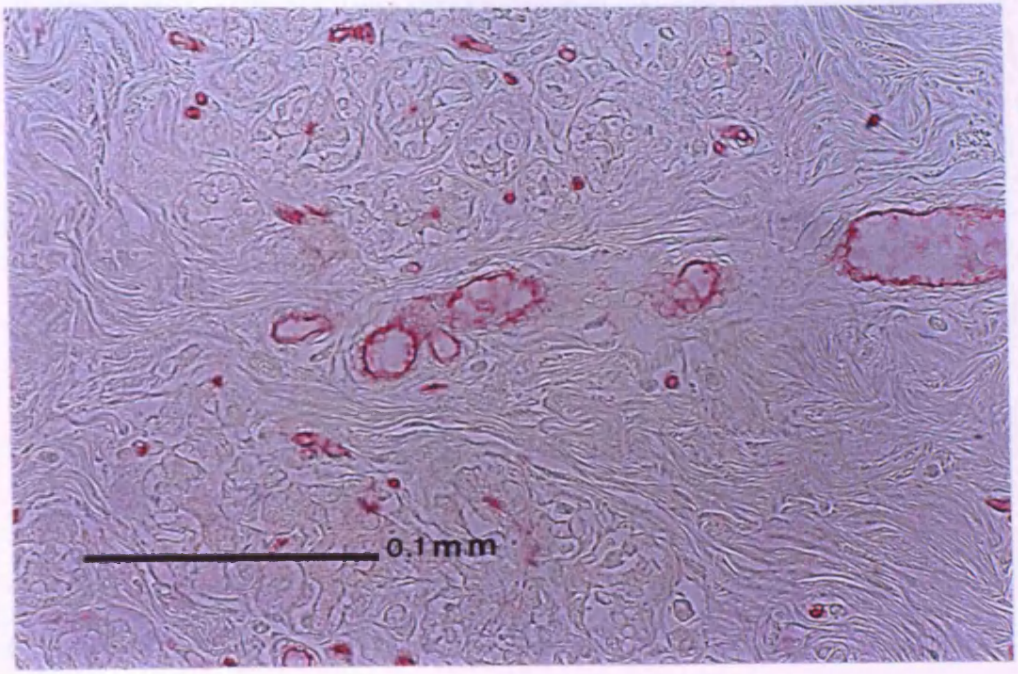
Figure (11.5.)

The expression of LSD10 during the FP, the LP, and the late-PLP.

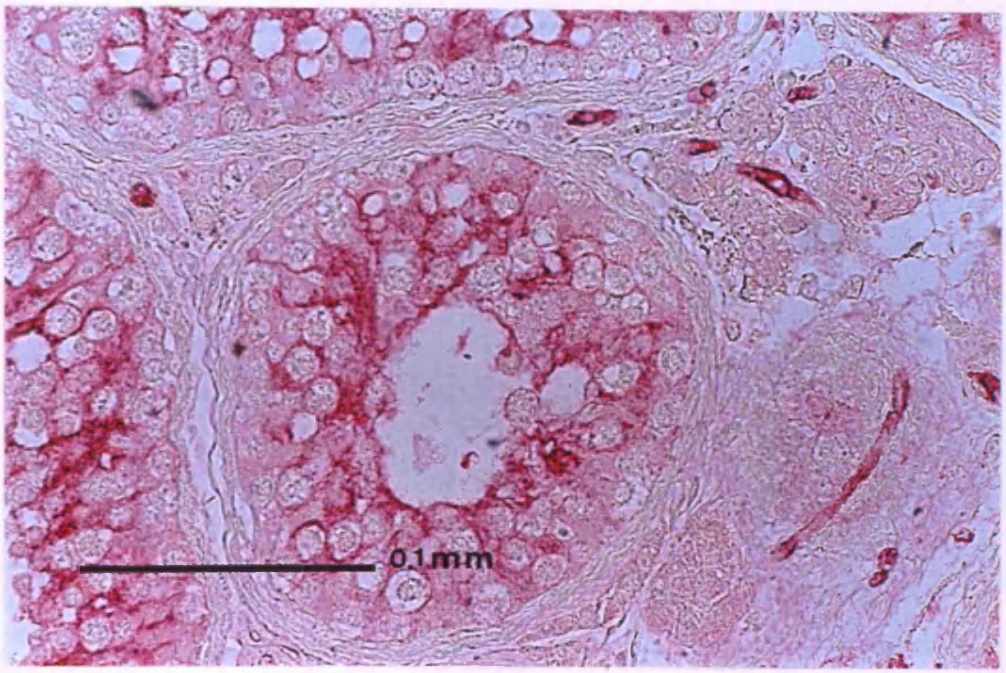
Tissue	Pattern of expression
Tongue	negative
Stomach	negative
Small intestine	negative
Colon	negative
Pancreas	negative
Gall bladder	negative
Liver	negative
Thyroid	negative
Adrenal gland	negative
Anterior-pituitary	negative
Lung	negative
Breast	endothelial cells positive
Ovary	negative
Testis	endothelial cells and seminiferous tubules positive
Kidney	negative
Tonsil	positive endothelial cells
Spleen	few scattered leukocytes were positive
Cardiac muscle	negative
Striated muscle	negative
Brain	negative
Medulla oblongata	negative
Cerebellum	negative

Table (11.6.)

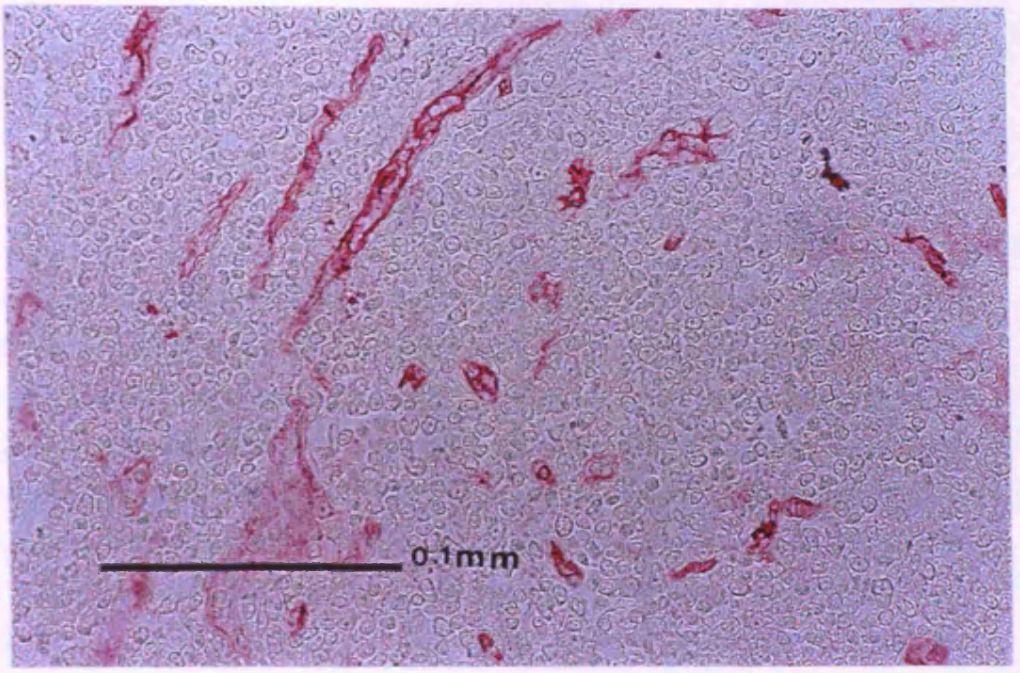
Immunohistochemical localisation of LDS10 antibody in different body tissues.



a- Expression of LDS10 in the breast.



b- Expression of LDS10 in the testis.



c- Expression of LDS10 in the tonsil.

Figure (11.7.)

The expression of LSD10 in the breast, testis, and the tonsil.

Chapter 12

Discussion

This study utilised histological sections and immunohistochemical localisation of different antigens, and their expression e.g. the stronger expression of ER in smaller glands. In general histological features were more uniform in the normal cycle compared to the area to area and the gland to stromal dysynchrony observed under the influence of cHRT. The distribution of leukocytes showed diffuse infiltrate and although there were a few aggregates and a few intraepithelial lymphocytes, the pattern was similar in cHRT when compared to the physiological cycle. In this study assessment was limited to the functionalis, and although deeper layers including the endometrial/myometrial junctional zone have become accessible following the introduction of the Leicester Endometrial Needle Sampler (LENS) that was developed during the work on this thesis (Al-Azzawi et al., 1997), the functionalis is the more responsive layer of the endometrium and is therefore more likely to reflect the hormonal effect which was the subject of this thesis. Examination of tissue sections allowed identification of $Mib1^+$ cells as being predominantly leukocytes and demonstrated that proliferating leukocytes followed the same distribution as the other leukocytes. Apoptosis and Bcl-2 expression exhibited the same pattern of expression as in the physiological cycle. Expression of ER differentiated between 2 types of glands: smaller ones were apparently retarded and out of phase for the cycle, these were also PR^- and α_2PEG^- and were also surrounded by a thicker αSMA^+ layer . As discussed earlier this may be related to a 'threshold' effect but may be explained by the relative proximity to endometrial blood vessels. It is possible that glands further from blood vessels may not develop to the same degree. The comparative findings between the late-PLP and the luteal phase endometrium is represented diagramatically in Figure (12.1. a-e).

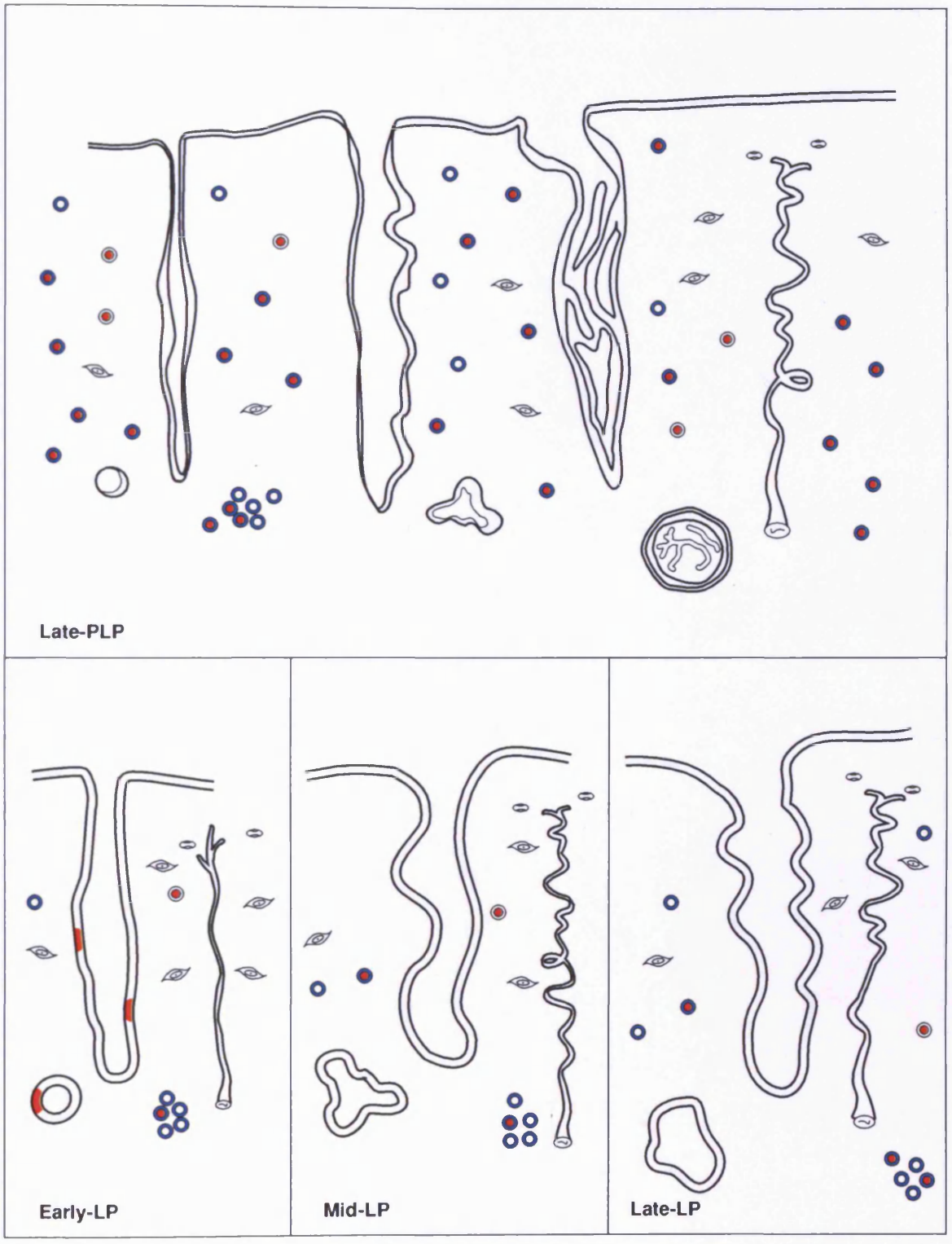


Figure (12.1. a): Expression of MiB1 (red) and CD45 (blue).

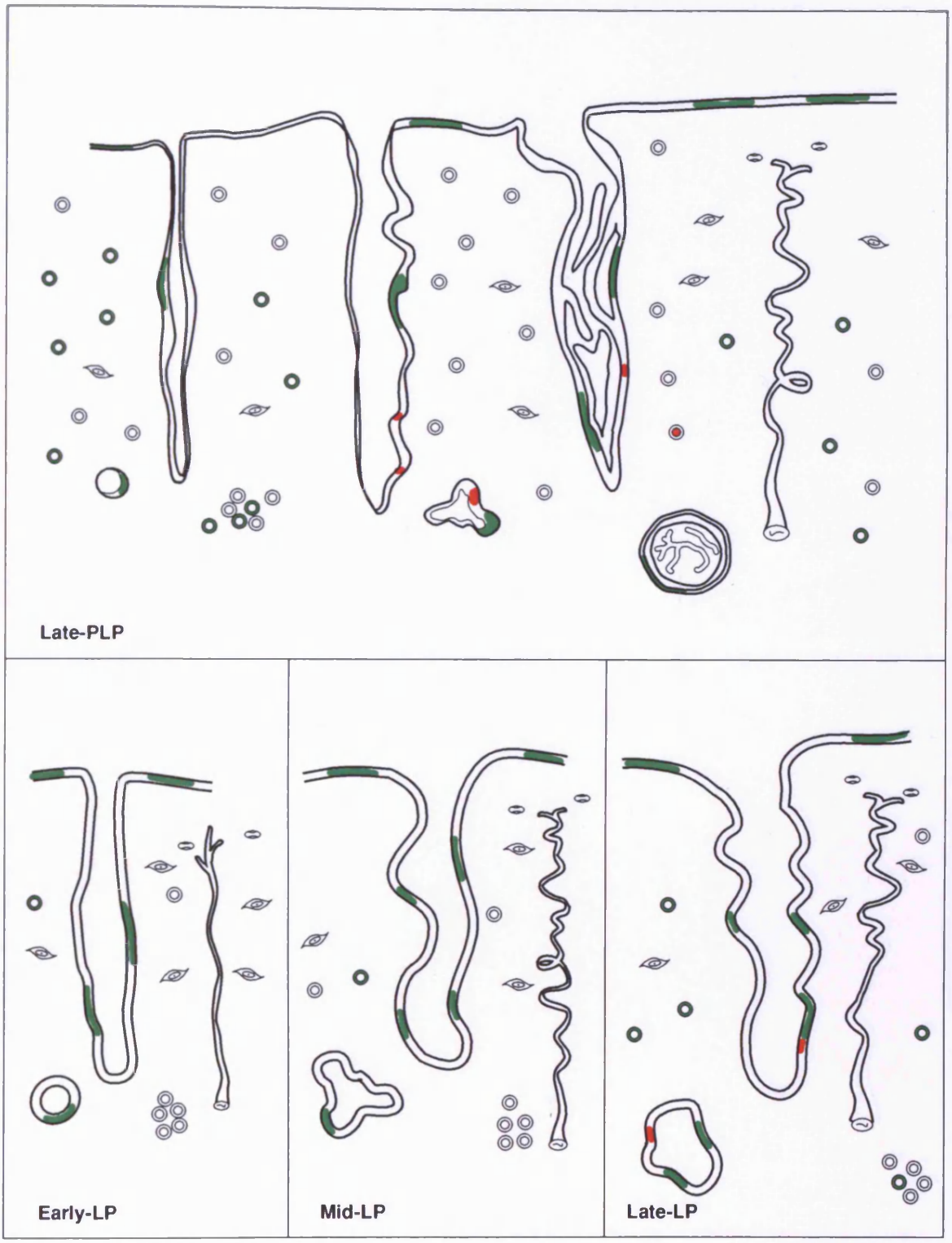


Figure (12.1. b): Expression of ISEL (red) and Bcl_2 (green).

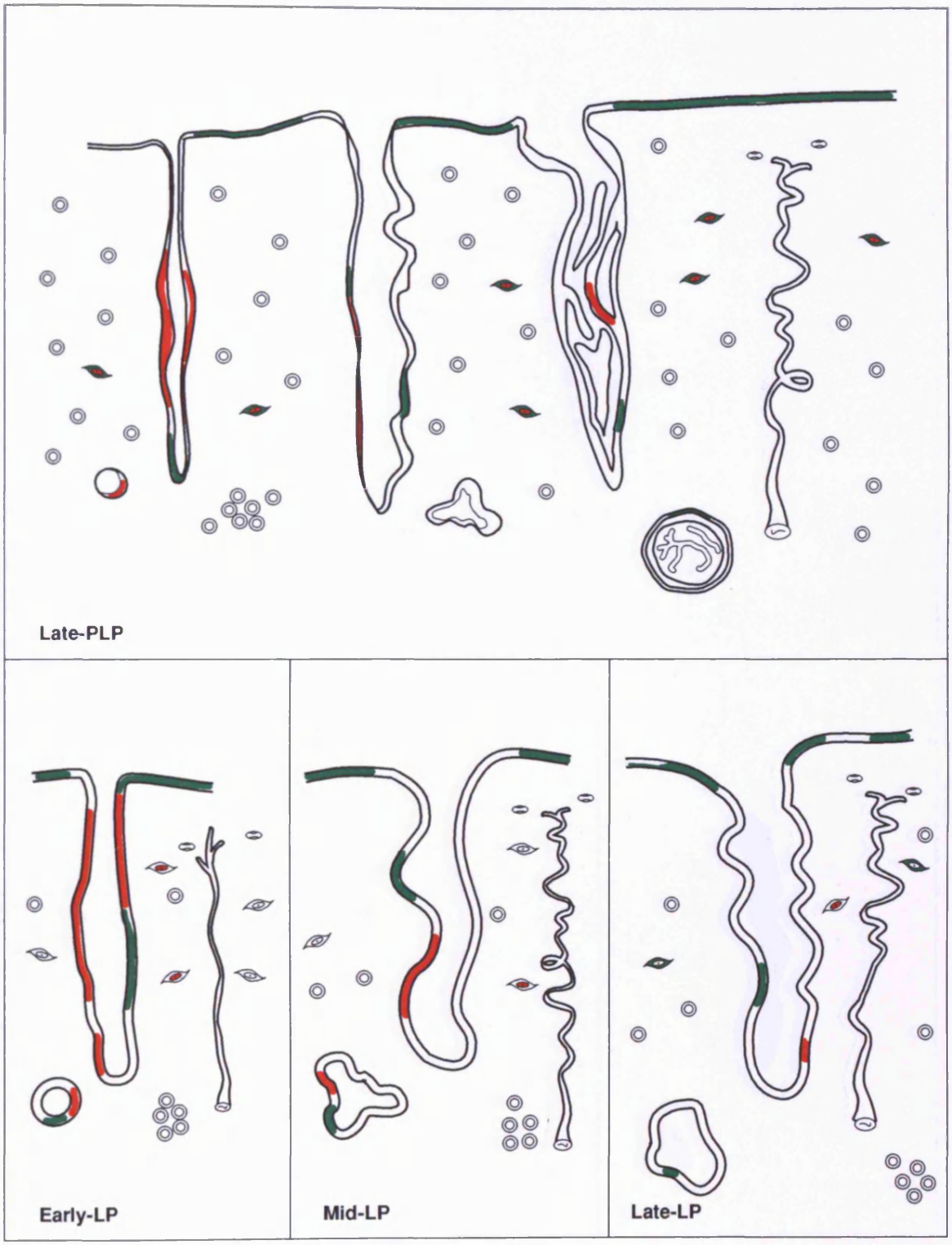


Figure (12.1. c): Expression of HSP27 (green) and ER (red).

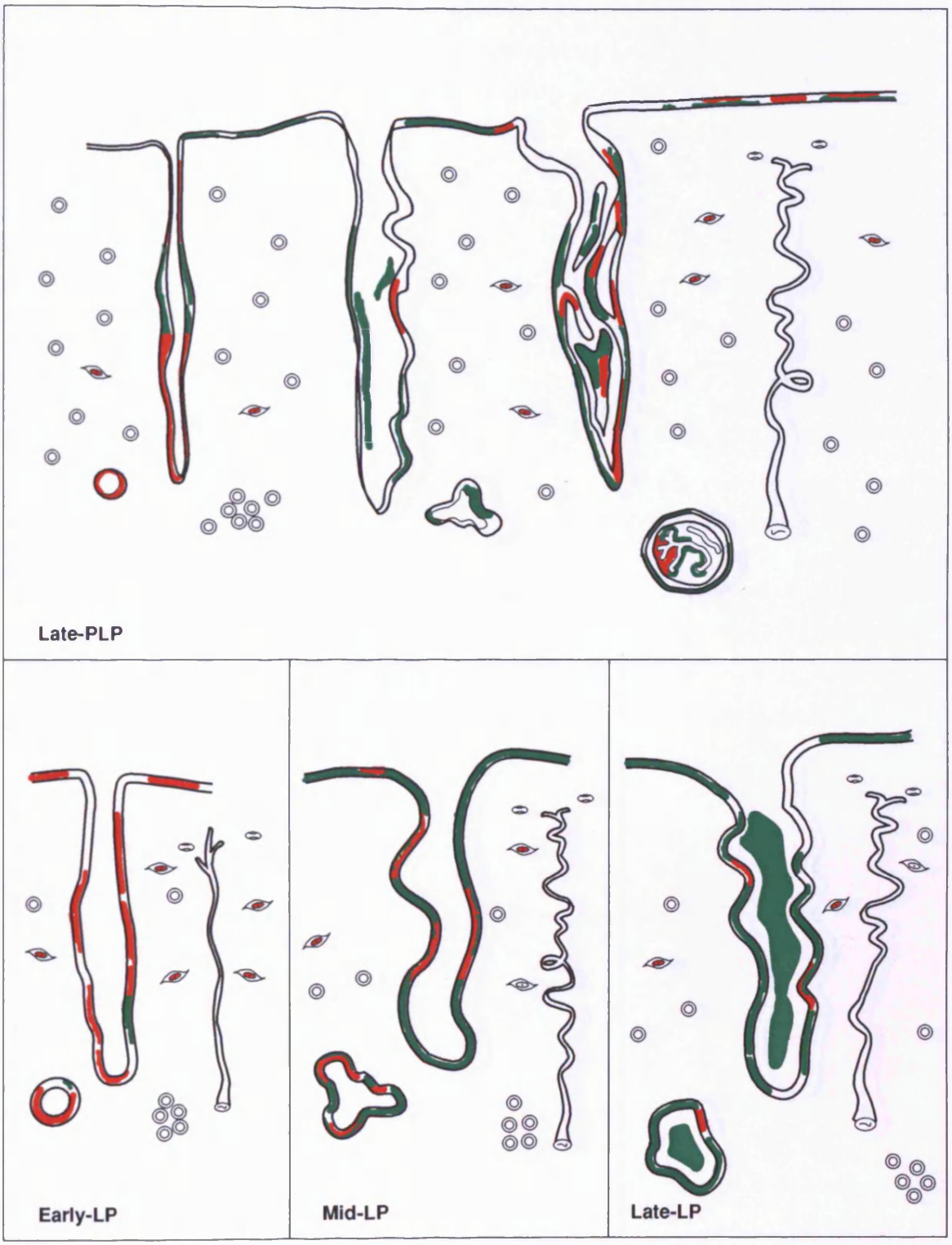


Figure (12.1. d): Expression of PR (red) and α_2PEG (green).

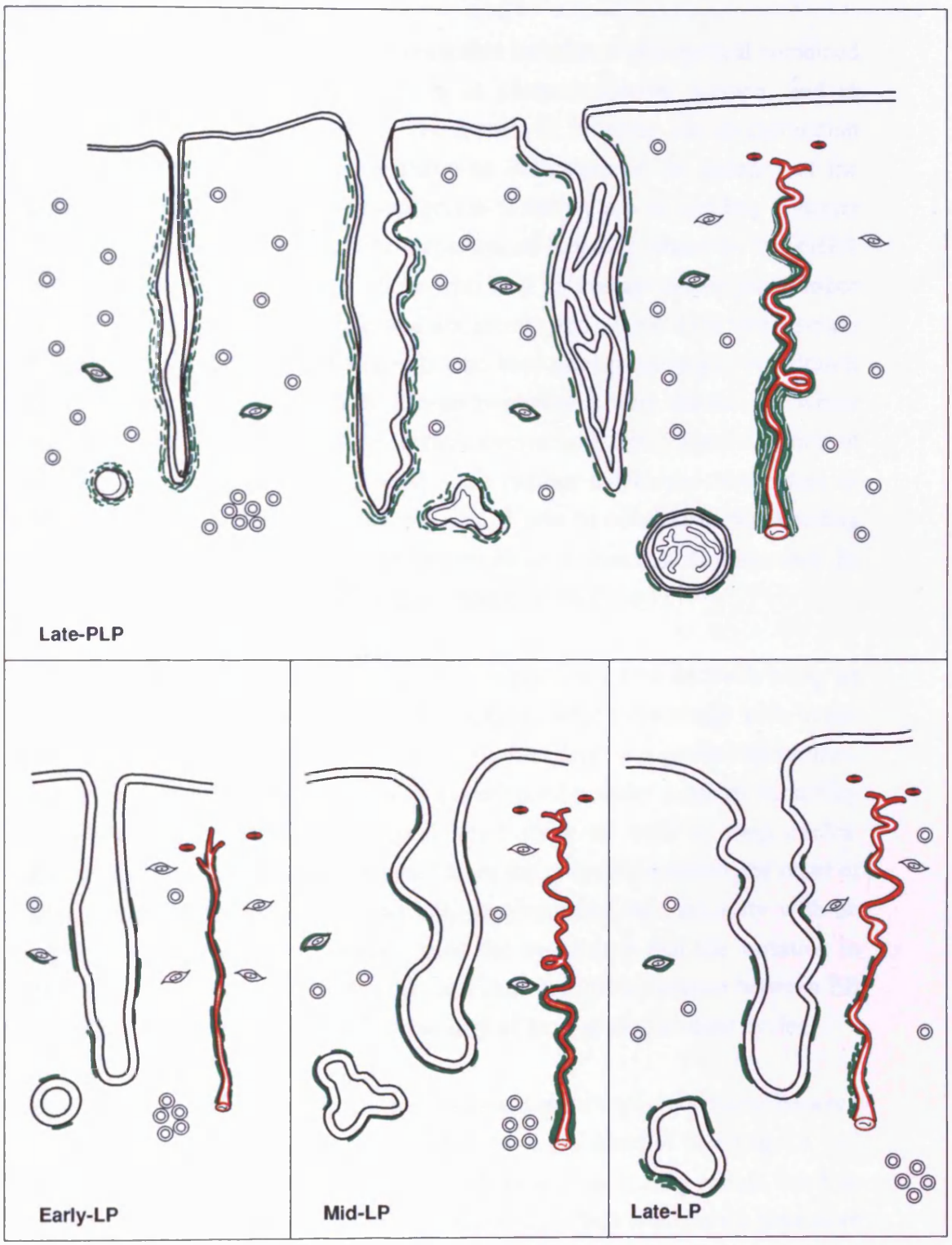


Figure (12.1. e): Expression of vWF and αSMA.

Figure (12.1. a-e):
Antigen Expression during the different stages of LP and during the late-PLP.

12.1. Aspects of endometrial structure and molecular markers

This investigation was designed to examine the effects of a cyclical combined HRT regimen on the endometrium in postmenopausal women, and to determine its biological potency. The pattern of bleeding and its correlation with endometrial changes were taken as indicators of the potency of the compounds used. The initial investigation of the pattern of bleeding (Chapter 3) demonstrated that women who experienced bleeding whilst on this cHRT regimen belong to two groups: those who bled on average before progestogen withdrawal (progestogen intake was not interrupted), these were termed early bleeders (EB), and the second group who bled after progestogen withdrawal were termed late bleeders (LB). These two groups were distinct for whilst early bleeders experiences more erratic cycles and a prolonged duration of bleeding, late bleeders experienced more regular cycles and fewer days of bleeding. The regularity of bleeding in LB was in contrast to the bleeding pattern of the group as a whole and to previous studies that demonstrated the unpredictable pattern of bleeding in women on cHRT (160).

Further analysis of the bleeding pattern suggested a link between being an early-bleeder or a non-bleeder and smoking, which correlates with hypo-œstrogenism. However, although the 'early bleeders' as a group experienced, on average, shorter cycles, they also experienced a wider range of variability in cycle length. They thus experienced short as well as long cycles. Comparing the endometrium obtained from the long cycles before the onset of bleeding in both groups: EB and LB, demonstrated the similarity with no significant differences. This confirmed the hypothesis that the variation in bleeding behaviour is cycle specific, and thus that the difference between EB and LB resides in the statistical probability of having long or short cycles.

It would have been of interest if the endometrium could be compared between the two groups at a similar stage in relation to the onset of bleeding, i.e. 1-2 days before the onset of bleeding in short as well as in long cycles. Such an investigation would be useful in demonstrating which features are predictive of, or are necessarily associated with, the onset of bleeding. Such an investigation, however, is hampered by the unpredictability of bleeding and the inability to prospectively predict the length of any particular cycle.

The association between menstruation and cyclic fluctuation in steroid levels has long been recognised. Exogenously administered steroids are also recognised to affect the characteristics of withdrawal bleed, and this has been extensively researched in the field of oral contraceptive pills (367). These investigations were stimulated by the adverse effect irregular bleeding has on compliance, which is similar to the case in HRT (368). It is nevertheless recognised that OCP, in general, are associated with a predictable bleeding pattern, and that this is dose related, the best cycle control being linked to preparations with higher doses of œstrogen. Thus preparations containing less than 0.05 mg Ethinyl-œstradiol are associated with a higher incidence of inter-menstrual bleeding/spotting, compared to higher dose preparations, and shorter and more predictable bleeding compared to the natural cycle. Studies of OCP, have utilised the 90 day bleeding interval method which was adopted by the WHO and which is most suited for comparative studies (163, 223). Its drawback is that its focus is on episodic bleeding rather than on cyclicity (222), and is thus less useful in addressing the factors that affect cycle rhythm. Another factor that was taken into account in analysing the menstrual diaries in this thesis is that in order for comparisons to the natural cycle to be accurate, these need to take into account (and compensate for) the factors that cause variability in the length of the follicular phase, which account for the major part of the variation in the length of the cycle. In order to avert this problem, the assumption was made in this study that regular intake of medication should result in regular bleeding, and although no attempt was made to predetermine the range of cycle length or of variability to be regarded as 'normal', the hypothesis allowed comparison within each woman and within the group as a whole.

Following the identification of the group of late bleeders, who had predictable bleeding and whose onset of bleeding coincided with progestogen withdrawal, the hypothesis was developed that such an optimal bleeding pattern is predictive of optimal histological changes, that are similar to those in the physiological cycle. This was viewed in the context of the clinical assumptions that cHRT reverses the adverse effects of œstrogen deficiency and that regular bleeding is an index that no further interference is necessary, i.e. a satisfactory response.

Histological and feature analysis examination of endometrial biopsies taken before the onset of bleeding from the late bleeders refuted the original hypothesis of a similarity between the physiological cycle and cHRT treated

endometrium (Chapter 4), and demonstrated that despite the similar and optimal bleeding pattern the endometrium exhibited significant histological differences. The significance of this findings obtains from the demonstration that the bleeding pattern is not an adequate index of the underlying structural changes. The implications of this are far reaching, for it raised important questions regarding the influence of cHRT on other body tissues, and emphasises that the assumption of a 'physiological' response that underpins cHRT as a physiological 'replacement' for ovarian steroids cannot be sustained without further verification. Neither could such an assumption be made based on a particular clinical parameter e.g. bleeding. This emphasises the need for thorough investigation of all aspects of hormone action including both functional and structural correlates before the conclusion could be made of a 'physiological' response.

Another aspect highlighted in this study is that the difference in histological features would pose a challenge to pathologists attempting to define abnormal histological states e.g. hyperplasia. For although hyperplasia is defined with reference to the architectural changes and the degree of crowding of the glands in comparison to the physiological cycle, the demonstration of significant differences between the endometrium under cHRT, and the physiological state emphasises the need to redefine the point of reference. This could be decided utilising image analysis, and the reference values from this study, but it remains to be determined whether the same values would apply to other cHRT regimens. This is particularly important as most endometrial assessments done in clinical practice are aimed at preventing endometrial hyperplasia which is regarded as a precursor of cancer. This is particularly relevant taking into account that although cHRT in the current combinations utilising 12-14 days of progestogen are considered safe (369), the evidence is not overwhelmingly conclusive. This was emphasised by Rees (1996) who argued that the small overall relative risk (RR) for endometrial cancer of 0.8 (CI 0.6-1.2) shown in meta-analysis tends to conceal the difference between the cohort studies that demonstrated a RR or 0.4 (CI 0.2-0.6) and the case control studies that demonstrated a RR of 1.8 (CI 1.1-3.1), and that although cohort studies are considered methodologically superior, published studies only included a small number (n = 10) of patients with cancer (33).

There is little in the literature to suggest the correct action in women who experience unscheduled bleeding whilst using cHRT, apart from the suggestion that increasing the number of progesterone treatment days may be

appropriate (79). This was based on the observation of non-secretory endometrium in women who bled early. This observation was later disputed in another study that demonstrated non-secretory endometrium even in those who experienced long cycles (80). The findings in this thesis contradict previous work and demonstrate evidence of progestogen action in all adequate specimens that were optimally timed, and it is argued that the presence of proliferative or atrophic endometrium in previous studies may be due to either incorrect timing or to biopsies obtained from the uterine isthmus which is known to be less responsive to hormones (370). Relevant to this, it is important to note that the previously proposed hypothesis of a relation between early onset of bleeding and inadequate progestogen administration has not been tested despite more that 10 years elapsed since its publication.

As mentioned previously, studies of the endometrium, because of its sensitivity to hormones and also because of its accessibility, provide a model for the study of the relative potency of the hormones used in cHRT. Previous work (79) suggested that early bleeding in women receiving cHRT is an index of inadequate progestogen, but the findings in this study of secretory changes in early bleeders as well as in late bleeders, and the statistically significant difference in the smoking habits between the two groups, and between the late bleeders and those who did not bleed on cHRT suggests (but does not prove) that a relative hypo-œstrogenic state may be responsible for early onset of bleeding (371) (**3.5.**).

The role, if any, of the relative hyper-androgenism in postmenopausal women (372), remains unknown, this is particularly relevant with the use of norethisterone with its known androgenic profile, which is partly mediated through interaction with androgen receptor (373). Low œstrogen levels may further enhance the androgenic effects of norethisterone through reducing the level of 5α-reductase (374), which converts norethisterone to 5α-norethisterone. 5α-norethisterone has been shown to exhibit less androgenic (373) and less progestogenic (375) potency than norethisterone, and although the same mechanism has the potential for enhancing the progestogenic effect, this may be hampered by inadequate PR expression.

One possible explanation for the discrepant histology and antigen expression is the effect of ageing on the endometrium. On one hand, it is possible that this be controlled for in a different experimental design involving women with premature menopause, especially if that was surgically induced. This,

however, would not only be difficult in view of the small number who would fulfil the criteria, but may perhaps be unnecessary. For evidence exist that the endometrium of older women remains responsive to steroids in the normal way.

It is well established that women's fertility declines with age (376), and some clinical reports from IVF and ovum donation programmes have attributed this to the existence of endometrial ageing (377-380). Alternatively the poor fecundity may be related to a declining ovarian activity with poor oocyte quality and the production of oocytes with a higher rate of chromosomal abnormalities (381-384). Animal studies have demonstrated marked age related endometrial changes, mainly an increase in collagen and a reduction in stromal cells (385, 386), and a reduction of ER (387).

The evidence in humans is less conclusive and may be suggestive that such an effect does not exist. Comparison between endometrial histology and the distribution of CD34, PCNA, PR and ER, and PAS positive reaction and Masson Trichrome stain in 2 groups of normally cycling women - the first <30 year old and the second >40 year old, demonstrated no differences (388). Lectin binding was also similar in the peri-implantation endometrium of younger (<30 year) and older (>40 year) women (389). So although a decline in uterine receptivity with age cannot be ruled out, available evidence suggests that the histological differences noted in cHRT specimens cannot be explained based on the effect of age.

The observed response of the endometrium to exogenous steroids suggests that they are the main determinants of endometrial features and behaviour, and that changing the dose and/or the type of the administered hormones may result in the development of different features within the endometrium. Although other factors e.g. androgens or gonadotrophins, may affect the development of the endometrium, the influence of these, if any, remains unclear.

Under the influence of cHRT, the epithelium, both glandular and luminal, featured predominantly cuboidal and low columnar and fewer columnar cells. There was also a higher incidence of apical secretory vacuoles which appeared underdeveloped and were not released into the lumen in accordance with the phase of the cycle. Although the difference in glandular size did not reach statistical significance, the total glandular area was smaller with cHRT

therapy. The glands were more tubular and less convoluted, and contained less secretions. Glandular and stromal dysynchrony was also noted. These features are similar to those noted with OCP use, and which have been attributed to premature interruption of the proliferative phase by the progestogen, resulting in an early arrest of glandular growth and differentiation (69). A reduced glandular area was also reported in IVF cycles following clomiphene citrate treatment but not with high œstrogen regimens (390). These histological similarities are unexpected particularly that the length of the œstrogenic phase in his cHRT was 16 days compared to the 5 days progestogen free interval on OCP, and also in view of the underlying assumption of an adequate œstrogen and absence of anti-œstrogens.

The apparent similarity between the endometrium under cHRT and that of combination OCP occurs even though the two regimens of steroid administration are different. On this cHRT regimen, women received a daily dose of œstrogen with progestogen added for 12 out of every 28 days. Users of OCP receive a daily dose of œstrogen and progestogen for 21 days with 7 tablet free days, during which the ovaries may commence into a new cycle of follicular development and œstrogen production. As mentioned above, it has been hypothesised that the effect of OCP is due to premature introduction of progestogen, a factor that is not operational in this cHRT regimen, an alternative explanation may be in a slower rate of development during the œstrogenic phase of cHRT treatment resulting in a less well developed endometrium at the end of this phase, which suggests inadequate effective œstrogen. Alternatively, the progestogen in cHRT preparations may cause regression of endometrial glands similar to that noted with prolonged high dose progestogen treatment. This is however an unlikely explanation, as norethisterone (like other 19-nortestosterone derivatives) has a predilection to induce stromal differentiation, and a dose or duration of therapy that is sufficient to cause glandular atrophy would induce a higher degree of decidualization than that observed in this regimen and that was similar to the degree of decidualization observed in the physiological cycle (the underlying assumption being that the dose of œstrogen is adequate).

The high αSMA expression around the glands and in the stroma under cHRT indicates a persistent follicular phase feature, and/or tissue remodelling or stress, which could be a characteristic of the progestogen used. The functional significance of this observation remains unknown, but it signifies that stromal development under cHRT differs from that in natural cycle. αSMA^+ cells are

associated with stroma from tissues characterised by contracture, but not in normally healing granulation tissue (391). This may denote stromal sclerosis, which may in turn constrain gland growth resulting in the appearance of glandular invaginations. Reduced remodelling may also result from the absence of a phase of gradual progestogen withdrawal. It is thus possible that the effect of the progestogen may persist into the early œstrogenic phase, which creates a relative MMP deficient environment. Glandular invaginations on cHRT often reach a high degree of tortuousity with secondary or tertiary invaginations, and although many authors have referred to these as fixation artefact (69, 392), this is not universally accepted, and it has been suggested that they represent evidence of differential polypoidal growth originating near the base of a gland and that they are secondary to circulatory imbalance (237)

The expression of functional markers as α_2-PEG, HSP27, ER, and PR (Chapter 8 & 9), all demonstrated that the endometrial epithelium exhibits different characteristics under the influence of cHRT. Glandular epithelium on cHRT during the late-PLP expressed lower levels of α_2-PEG compared to the mid-LP, and the late-LP, but higher levels compared to the early-LP. It expressed the same level of PR compared to the mid-LP or the late-LP but lower levels compared to the early-LP. cHRT treated endometrium expressed lower levels of HSP27 compared to all phases of the physiological cycle, there was no statistically significant difference in the level of ER expression, although smaller glands were strongly positive.

Persistence of some small proliferative type glands, which were ER^+ but PR^- represents failure of recruitment into the more advanced proliferative phase, and suggests inadequate œstrogenic stimulation. This is consistent with the finding of a lower α_2-PEG level compared to the late-LP as low œstrogen effect could result in inadequate PR expression, and consequently in weaker progestogenic influence. However, analysing the results should also take into account that norethisterone has some properties (e.g. its androgenic effect) that are different from those of progesterone, and it could be that these antagonise the progestogenic potency resulting in inadequate α_2-PEG expression and the incomplete suppression of PR.

Stromal cellular density is significantly reduced on cHRT compared to the early-LP and the late-LP of the physiological cycle, but is similar to that at the peak of stromal œdema during the mid-LP. Even after making allowance for

the degree of stroma œdema noted, and considering that a larger proportion of stromal cells in cHRT endometrium are in fact leukocytes, a true reduction of the number of stromal fibroblasts is observed with cHRT, this again suggests a reduced œstrogenic influence. Other features seen in the stroma that are consistent with a progestogenic effect are that the level of PR and of ER are similar to those of the late-LP but lower than that of found during the early-LP of the physiological cycle. The presence of stromal œdema and evidence of decidualization and the higher expression of HSP27 is consistent with a preferential effect of norethisterone on the stroma.

Another interesting observation in cHRT treated endometrium is the presence of glands at various stages of development within the same field e.g. the persistence of small diameter ER^+, α_2-PEG^- glands. This indicates the inability of these glands to reach the stage where they can respond appropriately to administered progestogen. This may be due to a threshold like effect, with at one extreme, none of the glands shows evidence of steroid action and the endometrium remains atrophic, and which was represented by the group of non-bleeders. At the other end of the scale, is the endometrium where all glands respond adequately to œstrogen and progesterone resulting in well developed secretory changes as occur in the physiological endometrium, and between these two ends of the scale, individual glands respond either fully or only partially. Within this model HRT treated endometrium would show variable response depending on the availability of steroids, with glandular units reaching different developmental stages.

12.2. The mechanism of bleeding

The normal menstrual cycle is regulated by ovarian steroids and is defined as a mean cycle length of 28 days (±7 days), and a duration of bleeding of 4 days (±2-3 days). Exogenous steroids used in OCPs induce a similar pattern of bleeding, with a tendency to reduced loss, whilst cHRT has been associated with more irregular bleeding (160). Hormonal changes that precipitate bleeding in these situations are different. Bleeding in the natural cycle and with OCP results from the simultaneous withdrawal of œstrogen and progestogen, but in cHRT bleeding occurs in response to withdrawal of progestogen (despite continued œstrogen) from an œstrogen primed endometrium. It is also known that the rapid, but not the gradual, withdrawal

of œstrogen can result in bleeding but this is often heavier and less well controlled (69, 158).

There remain many unsolved questions regarding the control of menstruation. Stromal cells differentiate under continued œstrogen and progestogen to form the predecidual cell, and the decidual cell of pregnancy. Decidualization is initiated in the endometrium under the luminal epithelium which has a rich vascular capillary plexus and also around spiral arterioles. These cells are therefore in a unique position to influence the process of bleeding.

The exact trigger for menstruation remains a subject of considerable research, and there is strong evidence to implicate the decidua. An analogy was drawn between decidualization and inflammation (393). This model requires inhibition of promoters of tissue breakdown during the phase of increased leukocyte infiltration, and also requires the presence of mechanisms that can mediate stromal disintegration at the time of menstruation. Recent immunohistochemical studies and studies utilising in-vitro cultured stromal cells, demonstrated that human endometrial stromal cells are capable of this regulatory role (394). Factors implicated in endometrial haemostasis include tissue factor (TF), that acts via thrombin generation, and plasminogen activator inhibitor-1 (PAI-1) which inhibits fibrinolysis. Both of these factors are enhanced by MPA after œstrogen priming. MPA also reduces the activity of tissue-type plasminogen activator (tPA), the primary fibrinolytic agent (395). Expression of the extracellular matrix (ECM) degrading proteases urokinase (uPA) and metalloproteinases (MMPs) is also inhibited in stromal cells under the influence of progestogens (395).

Efficient ECM degradation is thought to reflect the interplay of the plasminogen activators (PAs) and MMPs following their release into the stroma. In-vitro studies have demonstrated the release of these factors in response to steroid withdrawal (395). PAs generate plasmin, which affects the breakdown of matrices and the activation of MMPs, which in turn degrade the scaffolding structure of the ECM (394). MMP-1, -2, -3, -9, -10, and -11 are produced by endometrial stromal cells, MMP-7 is produced by the epithelium, and MMP-9 is localised to neutrophils (396). The regulation of MMP is depicted in Figure (12.2.).

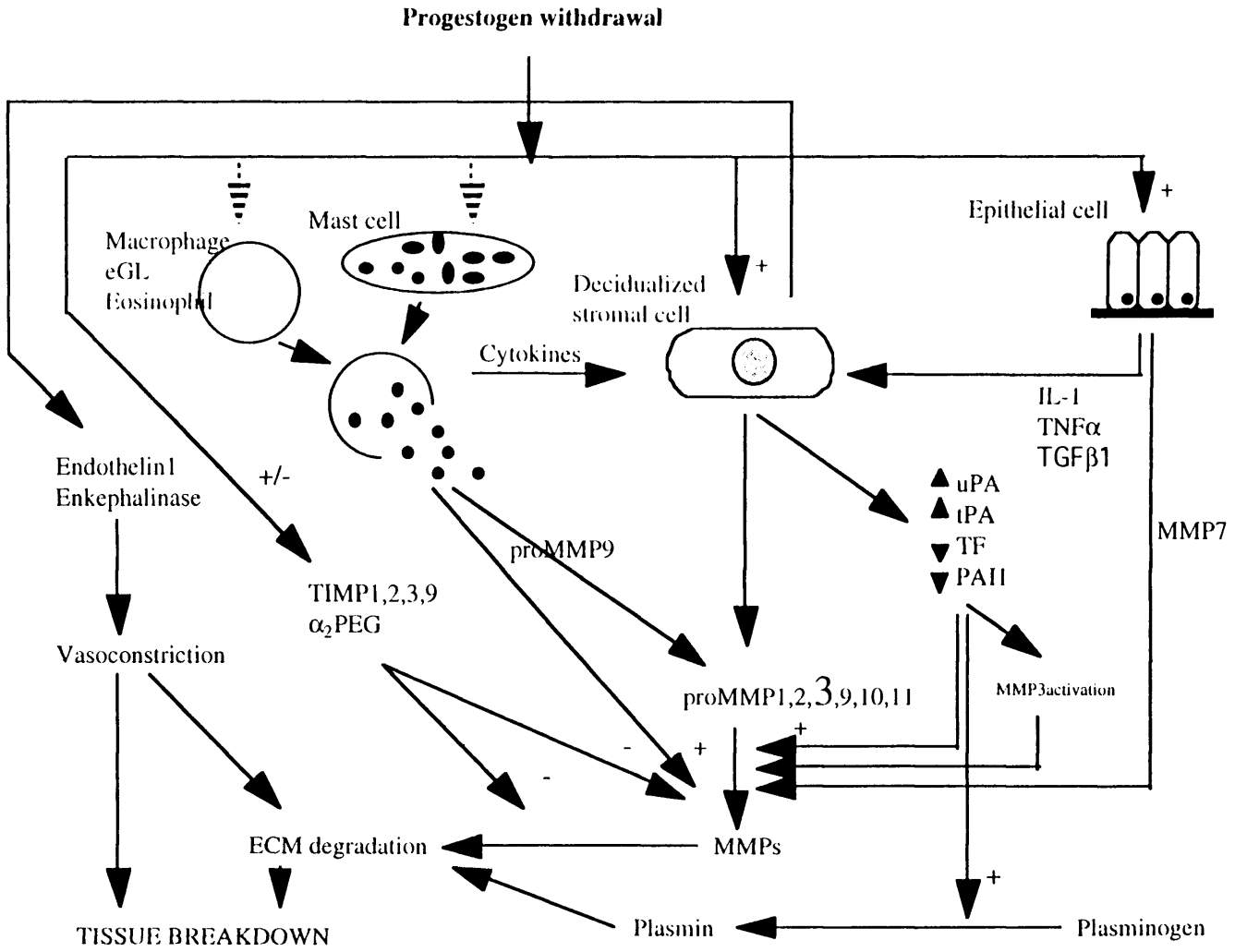


Figure (12.2.)

Pathways for ECM degradation and tissue breakdown (396-399).

The distribution of MMPs during the different phases of the menstrual cycle suggests modulation by steroids and particularly inhibition by progesterone (400). This is consistent with the structure of the MMP genes which contain promoters that have sequence resemblance to the steroid hormone-response elements (401, 402). Factors that interact with these receptors and with the AP-1 site, were shown to affect MMP expression (403, 404). It is possible, but not yet demonstrated that the effect of progesterone is mediated through these hormone-response elements, although mediators as TGF-β were demonstrated to affect MMP expression (399). Another mechanism for progesterone effect may be through post-translational modification or through regulation of MMP inhibitors such as the TIMPs and PAI-1 (399). Œstrogen has little effect on MMP expression although it induces PR and stimulates the transcription of AP-1 (399).

Among the MMP family, MMP3 utilises a wide range of ECM components and can also activate other members, which may indicate a central role. Experiments utilising in-vitro endometrial stromal cells demonstrated that combined œstrogen and MPA inhibit expression of MMP3, and that withdrawal of these hormones enhance its expression both at the protein and the mRNA level (405). These observations, and others, support the role of decidualized stromal cells in both inhibiting extracellular matrix degradation when under the influence of œstrogen and progesterone during the LP, and in enhancing degradation upon withdrawal of these hormones (395).

Decidualization was observed in this study during the late-PLP and may thus have a similar role in relation to bleeding on cHRT. In cultured endometrial stromal cells, the level of MMP3 and its mRNA were low when cultures were maintained under combined œstrogen and MPA, and these remained inhibited with continued œstrogen after MPA withdrawal, but became elevated after the withdrawal of both hormones or after the addition of RU486 (405). The onset of bleeding in the LB occurred within 2.4±1.3 days from the end of the cycle, i.e. <2days of progestogen withdrawal, despite continued œstrogen. The stabilising effect of œstrogen is thought to be through enhanced PR expression, and may thus be dose related. It is thus possible that the observed effect of œstrogen on maintaining the inhibition of MMP3 expression in cultured stromal cells is dose related. This effect of œstrogen, however, was not maintained and MMP3 levels rose, albeit by a small degree, after 8-12days of MPA withdrawal in the cultured stromal cells model. Analogous to this, it is possible that bleeding on cHRT in response to progestogen withdrawal is

also a reflection of the inability of the œstrogen used to sustain the endometrium. This may be enhanced by the slight reduction of the ER level observed on cHRT. On the other hand, bleeding commenced in EB despite continuation of both hormones, and it remains to be determined whether the same or different mechanisms are involved. In cultured endometrial stromal cells, the level of MMP3 started to rise after œstrogen and MPA withdrawal, but paralleled that induced by RU486 only after 8-12days (405). This contrasts with the relative rapid onset of bleeding after progestogen withdrawal that was observed in this study. The difference may be related to binding properties of MPA and norethisterone to PR. LB continue to express PR during the late-PLP, which argues against an 'effective' progestogen withdrawal as a trigger for bleeding, but indicates that the stroma remains hormone dependent, and thus sensitive to hormone withdrawal during that phase. In the natural cycle, bleeding occurs after about 7 days of gradual progesterone withdrawal, and the long time lag may argue against the role of MMPs in menstruation (396). Bleeding on cHRT is thus more akin to the stromal cell culture model, although this model suggests that bleeding on cHRT occurs before the peak MMP production.

Activation of MMPs may occur through the release of granules from mast cells. These, although only a minor population in the endometrium, were shown to degranulate before the onset of bleeding (406). Leukocytes, particularly neutrophils, monocyte-macrophages, and eosinophils express MMP9 (399), and possibly others. They also contain proteases capable of activating proMMPs. This suggests a role in menstruation (407). But the role, if any, of the increased leukocytic infiltrate noted in the late-PLP remains to be determined.

Perivascular endometrial vascular smooth muscle cells express ER and PR, although this expression has been shown to be highly variable between specimens and not to vary during the phases of the menstrual cycle (408). This led to the suggestion that vascular smooth muscle cells may belong to different population subsets with regards to ER and PR expression and the level of these receptors in response to steroids (408). The significance of this remains to be explored, particularly in relation to the recent report that progesterone and MPA up-regulate both PR_A and PR_B in the endometrial stroma (409). Steroid hormone receptor expression in these cells and their origin from stromal fibroblasts may also indicate that they are capable of producing MMPs. This together with their perivascular location makes them

ideally suited to influence perivascular ECM degradation and consequently bleeding.

Progesterone withdrawal also results in reduced stromal tissue factor (TF) and of plasminogen activator inhibitor (PAI-1), which result in a reduction in decidual haemostatic potential and enhanced fibrinolytic and proteolytic capacity (410). Progesterone withdrawal also releases the potent vasoconstrictors glandular-derived $PGF_{2\alpha}$, and decidual-derived endothelin. The overall effect is an increased vascular injury and inadequate haemostasis. It is not known whether the same or different mechanism(s) operate to control bleeding on cHRT, but increased expression of vasoactive agents resulting in bleeding can occur if œstrogen stimulation is inadequate and/or if progestin results in down regulation of stromal PR (410). It is not clear which of these mechanisms is operational in cHRT induced bleeding, for PR levels in the stroma continue to be higher than that in the late-LP if expressed as a proportion although not so if expressed as absolute number of positive cells. Progestogen withdrawal is not the mechanism of bleeding in early bleeders, but it remains to be clarified what effect œstrogen levels will have on this interaction.

Underlying the mechanism(s) of bleeding is the influence that steroids exert via their receptors. Recently, un-liganded human PR has been shown to stimulate the activity of the composite transcription factor activating protein 1 (AP-1), transactivation of which has been linked to cell proliferation (411). This stimulatory effect on cell proliferation is inhibited in the presence of ligand (411). However, AP-1 is also an essential promoter for MMPs (with the possible exception of MMP2) (399). This would seem to contradict the observation that the withdrawal of MPA alone (which would increase unliganded PR), but not the withdrawal of œstrogen and MPA (which would reduce unliganded PR), results in increased MMP3. It also contradicts the observation that RU486, which has an inhibitory effect on AP-1 (411), results in an even higher increase in MMP3 (405). The reason for this discrepancy may be related to the particular PR isoform (PR_A or PR_B) induced in stromal cell cultures and in the endometrium under different conditions. Emerging evidence suggests that at least in the Macaque expression of the isoforms in the endometrium is not affected by progesterone and that PR_A is 5 times higher than PR_B (412). On the other hand œstradiol enhanced PR_B but not PR_A in endometrial cancer cell lines (413). The differential expression of the two types of PR in the endometrium both in the stroma and in the epithelium

await further exploration. This is particularly important with the demonstration that bound PR_A and PR_B can exhibit different antagonistic effects on ER-mediated transcription (414).

The molecular mechanisms of MMP release after œstrogen and MPA withdrawal from endometrial stromal cells grown in culture remain to be clarified, but withdrawal of both hormones will result in a gradual reduction of unliganded-PR, a more rapid reduction will follow from the addition of RU486, whereas withdrawal of MPA alone will result in an increase in unliganded-PR. The level of this unliganded-PR will be dependent on the degree of œstrogenic stimulation. If unliganded-PR is essential for MMP inhibition, it could be possible that withdrawal of progestogen in a low œstrogen environment will result in levels of PR that cannot sustain MMP inhibition. It may also be possible that relatively lower levels of œstrogen and high progestogen will result in even fewer unliganded-PR which can result in bleeding despite continued progestogen as observed in the EB.

Although stromal leukocytes were difficult to quantify using hematoxylin and eosin staining (Chapter 4), quantitative and qualitative study based on their CD antigens was possible using immunohistochemistry and image analysis (Chapter 5). cHRT use was associated with a large increase in the total leukocytic infiltrate. This was accounted for by an increase of $CD56^+$ eGL and $CD3^+$ T cells. The pattern of distribution of leukocytes followed that noted during the physiological cycle, but there were also few aggregates of leukocytes surrounding some glands, which may represent tissue reaction to injury. The significance of the general increase in leukocyte infiltrate is not clear, but it indicates that the initiation of bleeding is not dependant on the achievement of a critical concentration of leukocytes. Furthermore, despite the higher concentration there was no apparent difference in the clinical manifestation of bleeding, which argues against a significant role. It is not clear what effect, if any, such a high infiltrate would have on implantation if embryo transfer were to be attempted, but implantation in the physiological cycle occurs 7 days post-ovulation at a stage when the local infiltrate is lower compared to the late-PLP in cHRT, and in view of the ability of eGL to lyse trophoblast, at least in the mouse (241), implantation may be hindered. It is interesting that no or only low NK cell activity was demonstrated in isolated non-pregnant endometrial leukocytes despite a high $CD56^+$ cell content (241), which suggestes that these cells may require the presence of either trophoblast or a pregnancy-like hormonal milieu for their activation (241). It is thus

possible that the leukocyte infiltrate during cHRT treatment has no specific function, but is rather a by-product of this therapy, and that its significance only stems from it being a manifestation of the hormonal balance as discussed previously (**5.5.**).

Shrinkage of the stroma, possibly secondary to release of proteases, together with the rise in vasoactive molecules, results in vascular constriction and stasis. This culminates in tissue ischaemia, breakdown and bleeding. In this study, there were no statistically significant differences in the number of endometrial vessels, or in the expression of vWF, and although this does not prove that the vasculature is identical in both states, yet this similarity is observed despite differences in most other histological features and may signify that the different components of the endometrium may be regulated by different mechanisms (e.g. the effect of hypoxia as a regulator of vascular development, or the non-receptor mediated direct effect of œstrogen). The similarity may also be a reflection of the role of vasculature in the control of bleeding, and that the similarity is a reflection of the fact that late bleeders were selected because of an optimal bleeding pattern and thus optimal vascular development. The similarities observed in this study emphasise the role of the vasculature in relation to endometrial bleeding. It is difficult, though, to extrapolate assumptions regarding the effect of cHRT on vasculature elsewhere in the body, for there is no known link between optimal 'menstruation' and for example 'cardiovascular protection'.

The role of apoptosis in the endometrium remains unclear, for if the whole functionalis is shed as is suggested in many studies (263, 272, 280, 281), apoptosis would appear to be biologically wasteful. Apoptosis was observed in this study both in the physiological cycle endometrium (in agreement with others), and under the influence of cHRT. It is interesting that the rate of apoptosis during the late-PLP was comparable to that observes during the late-LP endometrium, and higher compared to the mid-LP. This observed increase occurs despite continued administration of œstrogen (throughout the whole therapy period) and progestogen (up to the time of biopsy), which suggests epithelial instability, and that the administered dose in unable to sustain epithelial integrity. It is not possible to determine from this study whether this instability is a premenstrual phenomenon, or whether its occurrence is a constant feature in cHRT treated endometrium. Study of this phenomenon during other phases of the cHRT treated endometrium may clarify whether the occurrence of apoptosis is linked to the onset of bleeding. This, however,

seems unlikely as apoptosis is primarily an epithelial phenomenon, and its occurrence at the base of the glands, suggests that its role is primarily to affect regression of the glandular epithelium in areas that will not shed during menstruation. Very few sporadic apoptotic cells were noted scattered in the stroma, these appeared as individual cells with no discernible pattern. They may represent effete leukocytes as was suggested previously (317). The recently demonstrated mechanisms for T-cells regulation of apoptosis (through mediation of TNF-α and IFN-γ) (**7.1.1., 7.1.1.1.**) (275), and for granulated lymphocyte regulated apoptosis (through granzyme A (GraA) and perforin and/or Fas/Fas-ligand mediation) (**7.1.1.1.**), are of interest in relation to the regulation of the phenomenon in the endometrium. The role of leukocytes in the regulation of apoptosis in cHRT treated endometrium awaits further investigation. This could be of major interest particularly in relation to the use of continuous combined preparations where the endometrium is subjected to a daily dose of œstrogen and progestogen and remains in a histologically 'inactive' state.

The significance of apoptosis in relation to the mechanism of menstruation and to withdrawal bleeding on cHRT is not clear. But it is interesting that despite persistent œstrogen and progestogen treatment up to the time of the biopsy, the endometrial epithelium exhibited an incidence of cell death that was similar to that observed in the late-LP which is characterised by hormone withdrawal. This degree of instability may indicate that persistent stimulation by steroids does not protect from apoptosis. Despite a higher T cell and eGL infiltrate, the incidence of apoptosis is not higher than that noted in the late-LP. This may cast some doubts on the role, if any of T cells and of eGL in endometrial epithelial apoptosis. On the other hand, this may be a reflection of the higher percentage of these cells that are in active proliferation, and which may therefore not be activated.

During the late-LP and the late-PLP, the pattern of expression of Bcl-2 paralleled that of HSP27 except in the luminal epithelium where expression of Bcl-2 was higher in the physiological cycle. These similarities may be related to a similar role in protecting against apoptosis (415). But the functional significance of their down-regulation prior to menstruation is not clear. HSP27 inhibits Fas/APO-1 induced apoptosis, whereas Bcl-2 has only a weak effect on this pathway, contrasted to a stronger effect against p53-mediated apoptosis which is not affected by HSP27 (415). In order to ascribe a

physiological pro-apoptotic function to the observed reduction in HSP27 and Bcl-2 expression, a common pathway will need to be found.

This study represents a model for menstruation, where two patterns of bleeding (and a group of non-bleeders) have emerged in response to the same treatment regimen, it also suggested a possible mechanism for this difference in clinical behaviour. The model could be utilised to demonstrate the effect of variation of the dosage of œstrogen and/or progestogen on the bleeding pattern and on the expression of vasoactive substances and of proteases, which could greatly enhance the understanding of the mechanisms regulating the onset and duration of bleeding.

Whilst glandular epithelial proliferation was inhibited during the late-LP and late-PLP, there was a large proliferative activity in the stroma. This phenomenon is interesting for is demonstrates that different control mechanisms operate to affect this change. Stromal proliferation is largely, if not totally, confined to the leukocyte population, although the nature of the $CD45^-$ cells that were actively proliferating remains to be confirmed. It is interesting that while stromal cell proliferation declined during the late-LP, cHRT endometrium continued to exhibit a proliferative activity similar to that noted at the peak of proliferation in the mid-LP. Thus progestogen is not a down regulator of leukocyte proliferation, but may be stimulatory, possibly through cytokines or other mediators. That continued progestogen administration releases an inhibitory signal secondary to its action on down regulating its own receptor, is unlikely to be the mechanism of this effect as stromal PR level is proportionately increased in the late-PLP compared to the late-LP . On the other hand the higher rate of proliferation may be secondary to lower levels of IFNγ consequent to low œstrogen level (**6.5.**). Prolonged progestogen therapy has been demonstrated to increase leukocyte number in the endometrial stroma (253), and it was concluded that increased leukocyte infiltration is a function of the progestogen used that may be separate from decidualization, although the degree of infiltrate did not correlate with the dosage of progestogen and the only significant correlation with the duration of therapy was observed with $CD3^+$ cells (253). The work presented in this thesis supports a dissociation between the extent of decidualization and of leukocyte infiltration. It remains to be determined whether a similar degree of infiltrate could be induced with the use of progesterone or other progestogens. It would be interesting to examine the effect of continuation with progestogen in this regimen on the proliferation index in order to determine the point at

which a steady state is achieved. Administration of a high dose progestogens for a duration ranging from 8 days to 6 months demonstrated a 3 fold increase in $CD45^+$ cells (253), whilst the rise observed in this work was about 4.5 fold, this together with the high proliferation index at the end of 12days of therapy, suggests that further rise in leukocyte infiltrate is likely. One possible mechanism which would disallow this occurrence is the increased tissue necrosis observed with continued administration (253), this ultimately results in the development of the thin and atrophic endometrium with suppressed glandular development and dense-pseudo-decidualization (69). A similar regimen of continuous administration is used in continuous combined HRT formulations and has been associated with a high incidence of breakthrough bleeding, particularly during the first three months of therapy (416), and it is possible that necrosis is the mechanism of bleeding with these regimens. A high incidence of breakthrough bleeding also occurs with continuous progestogen only contraception (whether orally or parenterally), but this has been attributed to either prominent superficial venules or to bleeding from endometrial-myometrial plexus (417).

The relative importance of cell infiltration vs. local proliferation in causing the observed increase in the leukocyte population is not clear. But double labelling for the proliferation marker Mib1 (Chapter 7), has demonstrated that local proliferation of $CD45^+$ and of both the $CD56^+$ and the $CD3^+$ cells does occur throughout the luteal phase of the physiological cycle, and that the rate of proliferation is higher during the late-PLP. It is possible that increased migration of peripheral lymphocytes also contributes to the increase in leukocyte number, and many mediators have been suggested that may influence this effect (**5.5.**). It is also significant that increased leukocyte population has been noted with the use of synthetic steroids (69, 253), which may have a particular ability to attract leukocytes or to induce their local proliferation. It would, therefore, be of interest to examine the effect of different progestogens in order to determine whether they differ in their ability to attract or to increase proliferation of leukocytes.

In contrast to the earlier report (117), a recent study demonstrated that endometrial leukocytes do not express ER or PR (254). The stimulus for leukocyte proliferation, therefore, remains unknown. But putative agents include IL-2 and IL-15 (254), whilst IL-8, MCP-1 and prostaglandins may function as chemoatractants (255). It was considered previously whether these mediators may be increased under conditions of effective progestogen

deficiency. Against this is the finding of high levels of PR in the stroma during the late-PLP, and the recent report that progestogens induce PR expression in the stroma (409). However, it is still unknown which type of PR is expressed during that phase.

12.3. Mechanism of steroid action

Œstrogenic hormones exert their effect through interaction with their receptors which are members of a large superfamily of nuclear receptors that function as ligand-activated transcription factors. A different mechanism for steroid action where receptors have not been localised within target genes involves the activation of AP1 sites, this involves activation of *c-jun* and *c-fos* genes (418). It has been suggested that steps downstream of DNA binding may be responsible for distinguishing between agonist- and antagonist-activated receptors (419). More recently, it has been demonstrated that ligands induce a differential stimulatory or inhibitory effect depending on the type of receptor (ERα or ERβ) when the effect is dependent on AP1 site (420). For example, at the AP1 site 17β-œstradiol stimulates transcription with ERα, but inhibits transcription with ERβ. The effects of anti-œstrogens also vary depending on the receptor type (420). Whether the effect on ERα or ERβ or on the AP1 site of œstrone differs from the effect of 17β-œstradiol is unknown. But œstrone level is higher in women receiving cHRT compared to the physiological cycle (421) and a differential effect may account for the observed differences. The differential effect referred to above, demonstrates that different compounds cannot be compared in terms of a single index of 'potency' and emphasises the complex nature of any comparison. The need thus arises to identify the parameters upon which comparisons can be made. Some of the effects of œstrogen may be mediated directly on cell membrane (422), or through the mediation of stimulatory or inhibitory second messengers (138). Norethisterone has progestogenic, androgenic and week œstrogenic potency, the latter may be induced through the binding of its reduced metabolites to the ER (423), but it remains to be explored whether these interfere with what is considered to be the agonistic or the antagonistic properties of œstrogen. The magnitude of rise in plasma œstradiol level following the oral administration of 2mg œstradiol valerate to postmenopausal women was reported to be only minimal (424) or to be significant (425). There is however agreement that the greater rise is in the level of the less active œstrone and a preservation of the postmenopausal œstradiol/œstrone ratio. The significance of the higher rise in

œstrone and other metabolites is difficult to assess based on 'relative œstrogenic potency' which emphasises the significance of in vivo studies to assess the overall effect. Thus the net œstrogenic effect of cHRT is dependent not only on the level of œstradiol, but on the balance between agonists and antagonists. In the postmenopausal woman this involves a balance between administered œstrogen and progesterone and their metabolites on one hand and the woman's endogenous hormones on the other hand.

Androgen receptors (AR) have been demonstrated in the endometrial stroma but not the epithelium (426), and it is possible that these mediate the androgenic properties of norethisterone. The level of AR has been shown to be lower during the late-FP and to be absent during the late-LP of the natural cycle (426). AR level is under the influence of dihydrotestosterone (DHT). In the natural cycle, it is possible that the competition between progesterone and testosterone for 5α-reductase results in diminished DHT and inhibition of AR level. Whether norethisterone exerts a similar effect is unknown, but norethisterone and possibly its metabolites have an androgen like potency. The effect of 5α-reductase on norethisterone is to reduce its progestogenic potency which may contribute to the weaker progestogenic effect of cHRT.

The menopause itself is a hyper-androgenic state with a higher level of androstenedione, testosterone, and dehydroepiandrosterone, and may thus be associated with a relative abundance of AR. It is possible that this remains so during the late-PLP. However, in the absence of data on the effect of AR stimulation on endometrial structure and function it remains unknown to what degree the androgenic potency of norethisterone may have contributed to the differences observed in this study. A diagrammatic representation of the possible androgenic influences is depicted in Figure (12.3.).

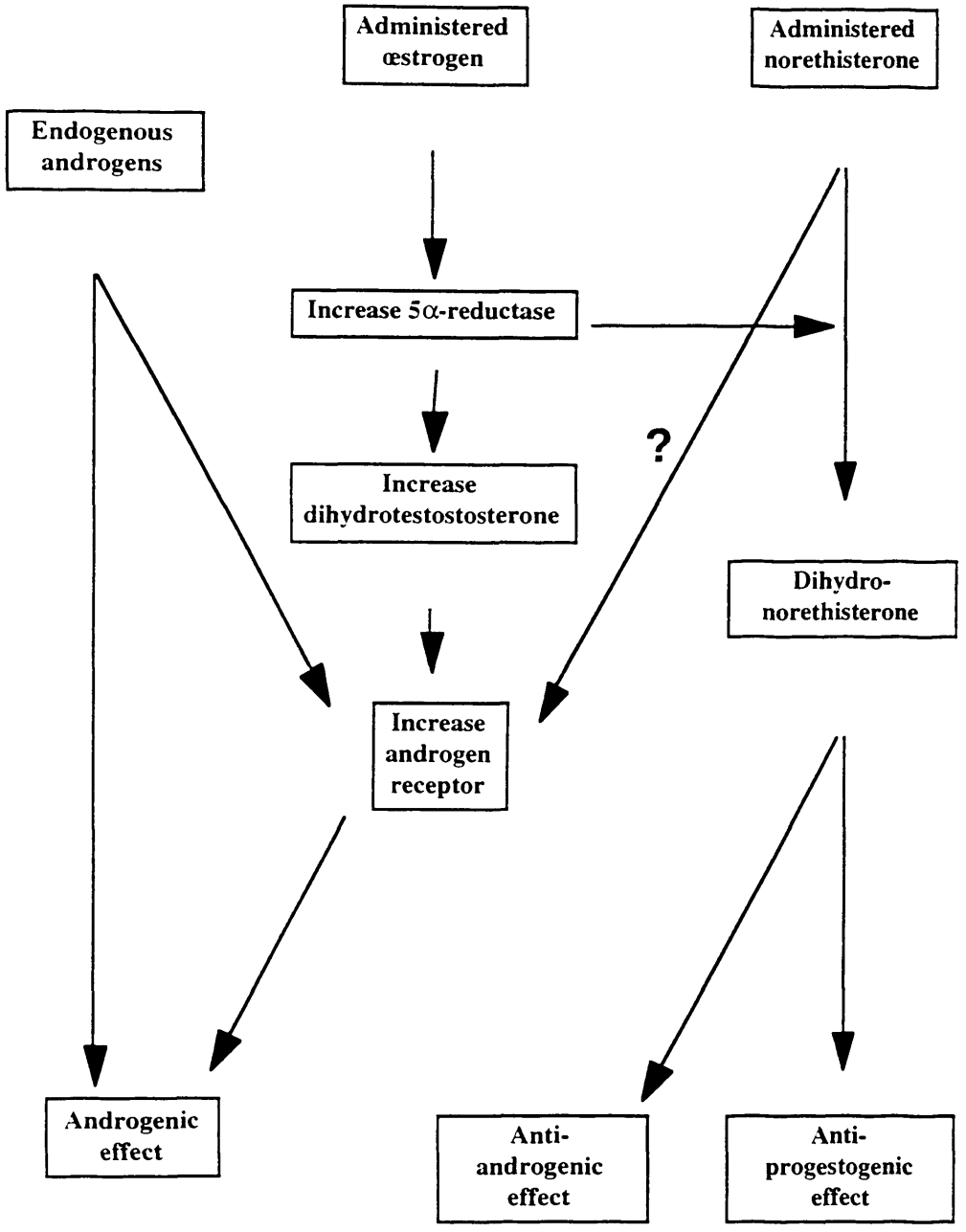


Figure (12.3)

The pathways that may be involved in the androgenic effect of cHRT on the endometrium.

Similar to œstrogen, progesterone exerts its effect on the endometrium through interaction with PR, although direct membrane effects have also been reported (427). The effect of progesterone is dependant on the factors as œstrogen, progesterone, growth factors as insulin like growth factor, and EGF, as well as on the interaction with PR which involves other proteins as hsp70 and hsp90. Norethisterone in large doses together with its metabolites (5α-dihydronorethisterone and 3β, 5α-tetrahydronorethisterone) have been shown to exhibit receptor mediated anti-progestational and anti-implantational effects similar to those of RU-486 (375).

The demonstration of different nuclear receptor subtypes that respond differently to ligands, and that are differentially regulated by steroids and stimulated by steroid antagonists adds a layer of complexity to the analysis of the pharmacology of cHRT. It may also be the key to explaining the complex discrepancies noted in this study. This complexity may be resolved if specific transcriptionally modulated genes were identified that are linked to desired therapeutic properties.

Despite the histological differences and the different antigen expression in the endometrium. This regimen was associated with a favourable bleeding pattern, and it is possible, that the differences observed are not associated with harmful effects, this is supported by the low incidence of side effects. Nevertheless, investigating tissue response may improve the chance of obtaining a more favourable risk/benefit balance.

Both the luminal and the glandular epithelial cells during the late-PLP are predominantly cuboidal and low columnar compared to all stages of the LP. The poor development of these cells and the reduced total glandular area, indicate hypo-œstrogenism. Stromal cellularity is also reduced reflecting inadequate development during the proliferative phase. Persistent apical vacuoles during the late-PLP is comparable to the mid-LP of the physiological cycle, and the less convoluted glands, and the diminished glandular secretions indicate retarded secretory development. Overall this may indicate a balance between inadequate proliferation and incomplete secretory transformation. This is supported by the lower α_2PEG expression, but not by the higher leukocytic infiltration ($CD45^+$, $CD56^+$ and $CD3^+$ cells) compared to all stages of the LP. This demonstrates that whilst some features are retarded, others are more advanced, and indicates a differential effects of the hormones given in cHRT, a concept which has hitherto only been used to describe the

recently introduced selective receptor modulators. The differential effect also indicates that endometrial features are not linked, thus one cannot deduce indicators about a particular feature from the presence or absence of another.

The dissociation between features is also demonstrated from the finding that 48% of $CD45^+$ cells express Mib1 compared to 8%, 37%, and 20% during the early-LP, mid-LP and the late-LP of the physiological cycle. $Mib1^+$ $CD56^+$ cells were proportionately similar during the late-PLP (60%) compared to the mid-LP (63%) but higher than that during the late-LP (24%), and the same was observed for $Mib1^+$ $CD3^+$ cells. This may be a function of continued strong progestogenic stimulation, however, this is not supported by the lower the α_2PEG level. It is possible that leukocyte infiltrate is less sensitive than glandular secretion to the factors that operate during the FP and which may be responsible for the poor glandular development, on the other hand the differential effect may be related to the type of progestogen. The higher HSP27 expression in the endometrial stroma during the late-PLP compared to the late-LP, supports the contention that the administered progestogen in this regimen has a selective stromal effect.

The expression of ER in the late-PLP was comparable to that observed during the mid-LP and late-LP but lower than the early-LP. This may be a reflection of either adequate down regulation or lower initial levels, but the demonstration of the persistent receptor expression in the small glands demonstrates that adequate down regulation cannot be the only factor. Progesterone receptor expression in the glands and in the luminal epithelium was lower compared to the early-LP but similar to that observed in the mid-LP and late-LP. This may be a reflection of adequate inhibition by progesterone, but taken together with the evidence derived from ER expression is unlikely to be the only factor. It is significant that PR in the stroma does not exhibit the same degree of inhibition noted in the glands. If the higher leukocytic infiltrate indicates a strong progestogenic influence a lower PR level would be expected. The reason for the discrepant effect is unclear but it is possible that norethisterone has a specific leukocyte attractant potency. Stromal proliferation in this study was largely if not totally confined to lymphocytes, which indicates receptor occupancy, and may argue against an inadequate progestogenic effect on the stroma. The apparent discrepancy between the stromal effect and the inadequate secretory effect on the epithelium remains to be further investigated. It may be mediated either through different binding characteristics of norethisterone or its metabolites to PR, or to its ability to

induce different mediators. In support of this is the finding that different progestogens have different effects on intracellular organelles (**1.11.5.**).

The different effects of progestogens compared to progesterone cannot be explained solely by their relative binding affinity (RBA) to PR, as both norethisterone and MPA exhibited stronger glycogen induction compared to progesterone, although compared to progesterone, norethisterone had a weaker and MPA a stronger RBA (428). More recently the two metabolites 5α-dihydro-norethisterone and 3β-5α-tetrahydro-norethisterone have been shown to possess anti-progestational properties in the rabbit endometrium (429), and it is possible that similar effects may occur in humans. Serum concentration of norethisterone was reported to vary by a factor of 9 after oral administration (430), and the serum half life ranged from 6.1-12.3hours.(430). The apparent lack of correlation between this wide variability and clinical behaviour remains to be explored, but it is significant that no correlation were drawn between this and the efficacy of OCPs.

12.4. Implications for the use of HRT

The combination of an œstrogen (e.g. premarin, œstradiol valerate, 17β-œstradiol, etc.) and a progestin (e.g. norethisterone, medroxyprogesterone acetate, etc.) in cHRT, are administered with the aim of compensating the natural loss of these hormones after the menopause. The results from this thesis, demonstrate that what was suggested to be 'physiological' effects are not so, in at least as far as the endometrium in affected by these hormones. The effects on other body tissues may indeed be different from the effects that occur in the natural cycle. A related observation is that the pituitary hormones, FSH and LH, remain elevated in women receiving cHRT, and are not suppressed to the levels observed in premenopausal women. This raises the important question of what constitutes a "replacement" therapy. This thesis demonstrated that this HRT preparation, cannot be defined as such based on similarities between its effect on the endometrium and that of the natural cycle. A need therefore arises for the identification of the characteristic(s) that compounds should have in order to be described as "replacement" hormone therapies. This is particularly relevant, with the introduction of non-œstrogen/progestogen compounds like Tibolone (Organon, Cambridge, UK), or the newly introduced ER modulators. For while these compounds share some of the properties of œstrogen, they differ in significant aspects. On the

other hand, the expression may be altered to encompass hormonal and non-hormonal preparation each with its own properties.

Furthermore, it has been demonstrated in this thesis that women respond differently to the same preparation e.g. EB vs. LB which indicates the need for the development of means for monitoring individual response. This thesis challenges the value of the bleeding pattern, endometrial architecture, or the other parameters assessed, as the majority exhibited significant variations compared to the physiological cycle, and it remains unknown whether attempting to optimise one or the other of these parameters would result in a more optimal HRT. It is also apparent that optimising response e.g. the bleeding pattern cannot be achieved through a uniform dose prescription, and that an optimal dose for one effect may have different effects on other cells even within the same tissue. The need for monitoring will not only have cost implications, but importantly requires the identification of appropriate markers for the required response. As steroids have a wide effect on most body tissues, the use of 'selective' receptor stimulators may be successful in inducing the desired effect, without adversely affecting other hormone sensitive organs. The need for monitoring the endometrium will be facilitated if protein(s) could be isolated in the uterine fluid or in the cervicovaginal secretions that could be measured through less invasive methods than obtaining a biopsy, and that would allow repeat sampling at intervals during the same cycle.

The significance of this is emphasised by the observation that despite loss of ovarian activity, untreated postmenopausal women retain lower case specific mortality (**1.2.**) compared to men. The higher cancer mortality in women during the early and mid reproductive years compared to men, and the reversal of this pattern after the menopause (**1.4.2.**) demonstrates a biological disadvantage for women that may be related to œstrogen. This together with the observed non-physiological effects on the endometrium in cHRT users demonstrate a need for close monitoring in order to establish a favourable risk/benefit ratio.

The results indicate that this cHRT regimen cannot be interpreted based on an equilibrium between œstrogenic and progestogenic potency, but that other properties of this compound need further exploration. These indeed may play a significant role. Œstradiol valerate, and/or norethisterone are constituents of at least 10 other HRT preparations available in the UK. The findings therefore

have implications beyond the single product subject of this study, and highlight the need for similar work on other combinations.

This study identified smoking as one factor that affects the pattern of bleeding on cHRT. It is possible that other factors are also influential. A positive correlation was demonstrated between œstrogen absorption and body fat and with increased age (431), although no such a correlation was found in this study. The discrepancy may be due to the narrow inclusion criteria adopted in this work, and if such a correlation was confirmed this may translate into an even larger range of variability.

cHRT exerted a physiological effect on some features as the bleeding pattern, but did not on others e.g. α_2PEG or on leukocyte infiltrate. It remains to be determined whether HRT exerts the same effect on the breast epithelium, bone, and other body tissues. cHRT protects against endometrial cancer (**1.10.1.**), the mechanism for this may be mediated through the ability of progestogen to inhibit proliferation and induce differentiation (although the secretory effect was blunted), or through increasing apoptosis. But if a similar effect of increased apoptosis were to occur in the breast, one would expect a reduction rather than an increased incidence of breast cancer (**1.7.2.**).

cHRT has been shown to induce some beneficial as well as some detrimental effects on known cardiovascular risk markers (60), this is in agreement with the results observed in this study on the endometrium. But it remains to be determined which of these risk markers specifically correlate with the degree of cardiovascular protection attributed to cHRT.

Because of its inaccessibility bone tissue remains the least studied in relation to HRT, and despite the increase in bone density observed in postmenopausal women using HRT, there remain a percentage who do not respond to steroids (**1.8.**). The exact relation between increased bone density and the risk of fractures remain a matter of controversy, and if the observations noted on cHRT endometrium were to apply to the effects on bone, no deduction should be drawn from the study of histology on function, which will make a correlation between function and the gross features of bone density more problematic.

Good and Moyer (1968) introduced a two dimensional model for the balanced effect of œstrogen and progesterone on the endometrium (72). The work in

this thesis demonstrates that this model is inadequate in the study of cHRT and that other influences need to be taken into account. This is depicted diagrammatically in Figure (12.4.). In order to achieve optimal endometrial development the relative androgenicity needs to be optimised, furthermore the influence of other factors for example the gonadotrophins may need to be considered.

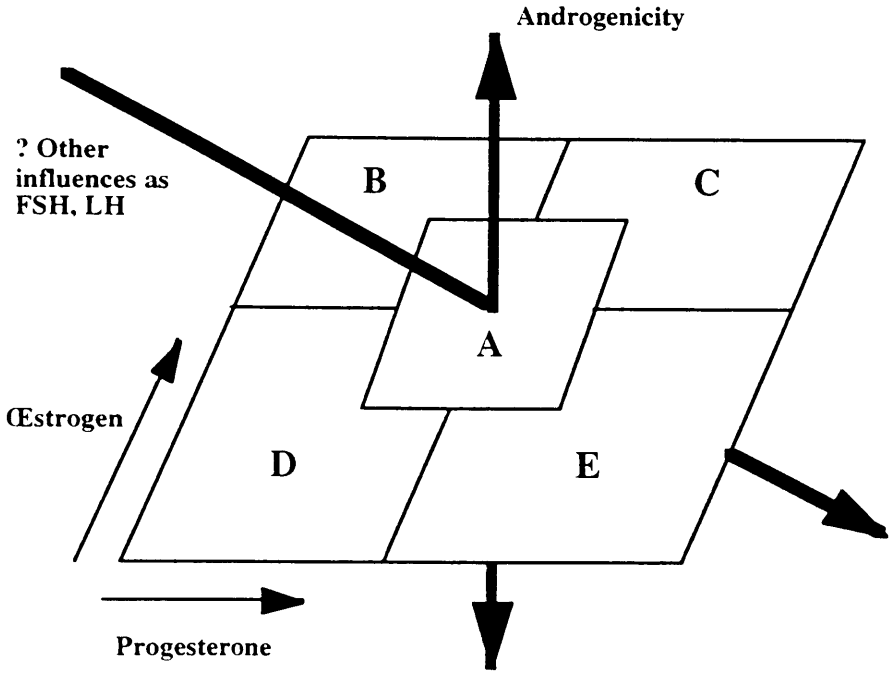


Figure (12.4.)

A model for endometrial development, modified from the two dimensional model of Good and Moyer (1968). This model depicts that other hormonal influences are important for the development of endometrium that is morphologically similar to that of the physiological cycle.

A: Secretory endometrium like that of the normal cycle
B: Glands proliferated and dilated; epithelium pseudostratified, stroma underdeveloped
C: Progesterone predominant: glands involuting; stroma predecidual
D: Glands and stroma underdeveloped
E: Glands underdeveloped; stroma predecidual

12.5. Future research

This study highlighted some of the deficiencies in the current understanding of the effects of exogenous steroids on the endometrium. This has wider implications, as similar deficiencies exist in relation to the other systemic effects of the steroids used in HRT. It is proposed that future research should be directed at exploring some of the following areas :

1- The study of the effect on the endometrium, of varying the dose of exogenously administered œstrogen, in order to ascertain the possibility of producing more 'physiological' structural changes as well as to examine the effect of this on functional markers. A comparison could be made between the follicular phase and the œstrogenic phase of HRT. One suggestion will aim at introducing phasic preparations where the administered œstrogen dose will vary to parallel the natural cycle.

2- It is unlikely that varying the œstrogen alone will mimic the physiological LP. But this may be facilitated by studying the effect of other progestogens on the endometrium using the same approach described in this work. It is proposed that this be analysed with reference to their androgenic properties.

3- Evaluation of the effect of androgen receptor stimulation on the endometrium, in order to ascertain the extent by which the structural and functional parameters tested are influenced by the androgenic properties of administered steroids.

4- Evaluation of the role of PR_A and PR_B, and of ERα and ERβ in the endometrium, and their relative induction by different steroids.

5- The study of the effect of progestogen and œstrogen metabolites on the endometrium, taking into account the effect of systemic as well as local tissue metabolism. Also the extent by which endogenous steroids interact with administered hormones.

6- The search for functional markers that reflect endometrial development as well as markers for systemic desired/adverse effects. These would be most useful in monitoring the response to HRT. The identification of such markers can help to define more accurately the end objective of HRT administration,

and may thus be useful in defining the goal for future therapeutic developments.

7- Similarly, there is a need for such functional markers in relation to the skeletal and the cardiovascular system. This is particularly important as some of the selective œstrogen receptor modulators, may have weak or no endometrial effect.

8- Exploring the HRT model to examine the mechanism(s) involved in bleeding, and also how bleeding could be induced/delayed or modified in its duration and/or amount by steroids. This would enhance our understanding of the physiological process of menstruation and its derangement.

9- The study of the factor(s) that lead to the development of the telescopic glands, as this may help the understanding of the process of development of endometrial polyps or hyperplasia.

10- The need for monitoring of HRT highlights the need to develop less invasive methods for endometrial assessment, possibly the use of uterine fluid or uterine secretions in the cervicovaginal area in conjunction with 'dipstick' technology to identify particular marker(s).

11- The development of antibodies (as LDS60) for targeted therapy of the endometrium possible in conjunction with the use of photo-sensitisers which could be used during Laser ablation of the endometrium. This approach may help to resolve the problem of abnormal bleeding.

Appendix 1

Contents

1- Buffers

1.1. Citrate Buffer (10mM Citrate Buffer pH 6.0)

Stock solution :

A- 0.1M citric acid ($C_6H_8O_7.H_2O$)	21.01gm /1000ml
B- 0.1M sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	29.41gm /1000ml

Working solution: Add 9ml of A to 41ml of B, and 450ml of dH_2O.

1.2. Gluteraldehyde (4%) in Sorensons Phosphate Buffer

25% gluteraldehyde solution	16ml
Sorensons phosphate buffer (pH 7.4)	84ml

Method:

1- Transfer the gluteraldehyde solution into the fume cupboard.
2- Using a measuring cylinder place 84 ml of Sorensons buffer into a conical flask.
3- Add 16 ml of the 25 % gluteraldehyde solution in the fume cupboard. 50mM
4- Transfer to the storage bottle using a funnel.
5- Store at 4°C. Keeps for 6 months.

1.3. PBS Phosphate Buffered Saline, 0.1M, pH 7.6

Sodium chloride (NaCl)	80gm
Disodium hydrogenorthophosphate (Na_2HPO_4)	13.7gm
Potassium dihydrogenorthophosphate (KH_2PO_4)	2gm

Distilled water (dH_2O)to	500ml
Adjust pH to	7.6
dH_2O to	1000ml

Stock remains stable for 6 months at RT in Duran bottle.
Make up to 10 litre with distilled water before use.
For 10x PBS for molecular biology make up to 1litre, treat with DEPC and autoclave for 15 minutes at 15lb $sq.in.^{-1}$ (in pressure cooker).

1.4. SDS Gel-Loading Buffer (For Electrophoresis)

Tris.HCl (pH 6.8)	50mM
Dithiothreitol	100mM
SDS	2%
Bromophenol blue	0.1%
Glycerol	10%

1.5. %. 1M Sodium Borate Buffer, pH 8.5

Sodium borate	38.11gm
dH_2O	100ml
Heat to aid dissolution, then pH to	8.5

DEPC treat and autoclave.

1.6. Sorensons Phosphate Buffer, pH 7.4

Solution A	
Di-sodium hydrogen orthophosphate	9.465gm
dH_2O	1litre
Solution B	
Potassium di-hydrogen orthophosphate	9.07gm
dH_2O	1litre

Method:
1- Add 800 ml of solution A to 200ml solution B.
2- Adjust to pH 7.4.
3-Store at 4°C. Keeps for 1month.

1.7.TB Buffer

Sodium chloride	300mM
Sodium citrate	30mM

1.8. TBS , Trisma Buffered Saline, pH 7.6

Tris	71gm
NaCl	85gm
dH_2O to	500ml
Adjust to	pH 7.6
MgCl	4gm
dH_2O to	1litre

Stock stable for 6 months at RT in Duran bottle. Make up to 10l with dH_2O prior to use.

1.9. TBS-BSA

Bovine Serum Albumin (BSA, Fraction V, Sigma)	1gm
TBS	100ml

1.10. TdT Buffer

Sodium cacodylate	22.4gm
Cobalt chloride	0.238gm
dH_2O	1litre
pH with concentrated HCl	7.0

Keeps for 1 month at 4°C.

1.11. TdT Buffer in Trisma Base, pH 7.2

Trisma base	30mM
Sodium cacodylate	140mM
Cobalt chloride	1mM

1.12. 10x TE buffer

Tris HCl pH 8.0, 1.211g in 100mls	100mM
EDTA, 0.292g in 100mls	10mM

Dissolve the Tris in DEPC water in treated glassware, add the EDTA. Treat pH probe.
Adjust pH to 8.0.

1.13. Transfer Buffer (For Electrophoresis)

Glycine	39mM
Tris base	48mM
SDS	0.037%

Methanol	20%

1.14. 1M Tris HCl, pH 9.5

Trisma base/dH_2O	15.76gm/100ml

Autoclave

1.15. 1M Tris, pH 7.65

Trisma base/dH_2O	12.114gm/100ml

Dilute 1:20 for 0.05M Tris for Proteinase K dilutions.
Make up in DEPC water and autoclave.

1.16. Tris-glycine Electrophoresis Buffer

Trisma base	25 mM
Glycine (pH 8.3)	250mM
SDS	0.1%

2- Enzymes

2.1. Pepsin (0.4%)

Porcine pepsin 1:2500 (Sigma)/dH_2O	0.4gm/100ml
5M Hydrochloric acid	200µl

2.2. Proteinase K (10µg/ml)

1mg/ml solution of proteinase K in dH_2O	10µl
0.05M TrisHCL pH 7.6	990µl

2.3. Trypsin (0.1%)

Trypsin	0.3gm
Warm (37°C) dH_2O	300ml
CaCl	0.36gm
Adjust pH to	7.8

3- Fixatives

3.1. Buffered Neutral Formalin (1%)

40% formaldehyde	100ml
$NaH_2PO_4.2H_2O$	4gm
Na_2HPO4	6.5gm
NaCl	8gm
Tap water	900ml

3.2. Paraformaldehyde Solution (0.4%)

10x Stock	
Paraformaldehyde (Sigma, Dorset, UK)	4gm
10x PBS	100ml

Heat to 70°C and add few drops NaOH. Store at 4°C. Store for 2 weeks but keep check for crystals. Paraformaldehyde solution in DEPC is made up 100ml of 4% in 10x PBS, diluted with DEPC water and stored at 4°C for up to 2 weeks.

4- Solutions

4.1. BCIP (5-Bromo-4-chloro-3-indolyl Phosphate) stock

50mg BCIP in 1ml DMF (Dimethylformamide).

4.2. Blocking solution

TBS	100ml
Bovine serum albumin	3gm
Triton-X-100	100µl

Filter before use. Prepare fresh or store at 4°C for 24hours.

4.3. DEPC (diethyl pyrocarbonate) water

For RNAse free DEPC treated solutions or DEPC 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. Autoclave for 15 minutes at 15lb sq.in.$^{-1}$ (in pressure cooker) or boil for 30 minutes. All steps performed in the fume hood.

Note: Tris based solutions cannot be DEPC treated in this way and must be made up in treated glassware using autoclaved DEPC water and then autoclaved.

To treat glassware etc. soak for at least 1 hour in 3% Hydrogen Peroxide and dry thoroughly in the fan oven at 37°C.

4.4. Dextran Sulphate (50%)

Dextran sulphate	25gm
DEPC water	to 50 ml

Dissolve at 65°C. Do not autoclave.

4.5. EDTA* 0.5M

EDTA/dH_2O	18.61gm/100ml
pH (6M NaOH)	8

DEPC treat and autoclave.

4.6. 1M Levamisole

Levamisole/dH_2O	1.204gm/5ml

4.7. 1M MgCl*

Magnesium chloride/dH_2O	20.331g/100ml

4.8. 10mM Manganese chloride*

Manganese chloride/dH_2O	0.198gm/100ml

4.9. 5M NaCl*

Sodium chloride/dH_2O	29.22gm/100ml

4.10. NBT stock

NBT (Nitroblue tetrazolium)	75mg
DMF 70% (Dimethylformamide)	1ml

4.11. 10x PE

Tris (hydroxymethyl methylamine)	6.058gm
EDTA	1.862gm
DEPC water	70ml
pH (concentrated HCl) to	7.5
Add (All from Sigma)	
Tetra Sodium Pyrophosphate	1gm
Polyvinyl Pyrrolidone	2gm
Ficoll	2gm

To aid dissolution heat to 65°C. Once dissolved hold at this temperature for 15 minutes, then make up to 100ml in a volumetric flask. Autoclave for 15 minutes at 15lb/sq.in.

4.12. Pre-hybridisation solution

DEPC water	132.5µl
5M NaCl	60µl
10x PE	50µl
50% Dextran sulphate	100µl
10mg/ml sonicated & denatured salmon sperm DNA	7.5µl
30% Formamide	150µl

4.13. Sephadex Slurry*

Sephadex slurry (G50)	5g
1x TE Buffer	100ml

4.14. Sodium cacodylate

1.2M solution, pH adjusted to 7.2 with hydrochloric acid.

4.15. 20x Standard Saline Citrate (SSC)*

Sodium chloride, 3M	175.32gm
Sodium citrate, 0.3M	98.03gm
dH_2O to	1litre
pH to	7.0

*Solutions used for molecular biology were all DEPC treated and autoclaved.

5- Stains

5.1. Mayer's Hematoxylin

Hematoxylin	1gm
Aluminium potassium sulphate	50gm
Sodium iodate	0.2gm
Citric acid	1gm
dH_2O	1litre

Appendix 2

Antibodies used in the thesis

Primary antibody Antigen	Supplier	Antibody type/Clone	Ig isotype	Pre-treatment, time	Dilution, time, temp
CD3	Dako	Rabbit PcAb anti-hum T cell		Pep, 40 min, 37C	1:50, Ovn, 4C
CD20	Dako	McAb mouse anti-hum B Cell, L26	IgG2a/ kappa	Pep, 30 min, RT	1:50, Ovn, 4C
CD45 (LCA)	Dako	McAb mouse anti-hum, 2B11&PD7/26	IgG1/ kappa	Pep, 20 min, 37C	1:150, 2h..37C
CD56/Neural Cell Adhesion Molecule-1	Neo-markers	McAb mouse anti-hum NCAM, 123C3D5	IgG1/ kappa	Mv, CB, 30 min	1:50, 2h..37C
CD68	Dako	McAb mouse anti-hum, KP1	IgG1/ kappa	Pep, 30 min, 37C	neat, Ovn, RT
Bcl-2	Dako	McAb mouse anti-hum, 124.(3)	IgG1/ kappa	Mv, 40 min	1:25, Ovn,4C
Ki67 (Mib1)	Oncogene Science	McAb mouse anti-Ki67 fragment, Mib1	IgG1	Mv, 40 min	1:50, Ovn, RT
Estrogen Receptor (ER)	Neo-markers	McAb mouse anti-hum, AER314	IgG1/ lambda	Mv, 30 min	1:50, Ovn, 4C
Heat Shock Protein 27 (hsp27)	Neo-markers	McAb mouse anti-HSP27, G3.1	IgG1	Pep, 20 min, 37C	1:100, 2h, 37C
Progesterone recpetor (PR)	Neo-markers	McAb mouse anti-hum, hPRa 3	IgG1	Mv, 30 min	1:50, Ovn, 4C
α2PEG	Dr SC Bell[1]	McAb mouse anti-hum, Code 2CH11		none	1:2000, 2h, 37C
Smooth Muscle Actin	Dako	McAb mouse anti-hum αSMA, 1A4 (1)	IgG2a/ kappa	Mv, 30 min	1:50, Ovn, 4C
Von Willebrand Factor	Serotec	McAb mouse anti-hum, 21-43	IgG1	Mv, 30 min	1:50, Ovn, 4C

Secondary Antibody	Supplier	Type/ clone	Dilution, time, temp
Alkaline phosphatase-conjugated rabbit anti-mouse immunoglubulins	Dako	PcAb	1:150, 30min, RT
Anti-Goat/Sheep IgG1, peroxidase	Sigma	McAb/ GT-34	1:3000, 1h,.37C
Biotinylated rabbit anti-mouse immunoglobulins	Dako	PcAb	1:150, 30min, RT
Biotinylated F(ab)2 fragmented sheep anti-mouse immunoglobulins	Sigma	PcAb	1:2000 1h, 37C
Biotinylated F(ab)2 fragmented swine anti-rabbit immunoglobulins	Dako	PcAb	1:150, 30 min, RT

C=°C, CB=citrate buffer, h=hour, hum=human, McAb=monoclonal antibody, min=minute, Mv=microwave, Ovn=overnight, PcAb=polyclonal antibody, Pep=pepsin, RT=room temperature.

[1] Kindlly rovided by Dr. SC Bell, University of Leicester.

Appendix 3

Reagents used in this thesis

Reagent	Supplier	Lot/ Catalogue no.
ECL detection reagent (RPN2106)	Amerhsam ,UK	RPN2106
Fast Red TR/Naphthol AS-MX Alkaline phosphatase substrate tablet set	Sigma, UK	F-4523
Levamisole solution	Vector Laboratories, USA	SP-5000
Normal Rabbit Serum	Dako, Bucks, UK	Lot 054
Swine Serum	Dako, Bucks, UK	Lot 063
Vectastain ABC-AP Kit	Vector Laboratories, USA	AK-5000
Vectastain ABC Kit (peroxidase)	Vector Laboratories, USA	PK-4000
Vectastain ABC elite Kit (peroxidase)	Vector Laboratories, USA	PK-6100
Vector alkaline phosphatase substrate kit (BCIP/NBT)	Vector Laboratories, USA	SK-5400
Vector DAB substrate kit (peroxidase)	Vector Laboratories, USA	SK-4100
Vector SG substrate Kit (peroxidase)	Vector Laboratories, USA	SK-4700

Appendix 4

Bradford protein assay

1) Laboratory reagents:
Human serum albumin Fr. V (Sigma,) for protein standard 1mg/ml in water.

2) Bradford reagent: 100mg Coomassie brilliant blue G250 (ICN, Thame, UK) in 50ml ethanol, add 100ml 85% orthophosphoric acid (50ml orthophosphoric acid S.G. 1.75 + 50ml distilled water).
Dilute to 1 litre with water and filter through Whatman no.1 filter paper.

3) Assay: 0.2 ml standard/ sample mixed with 5ml reagent and stand for 10 minutes.
4) Read absorbency at 595nm.
5) Standard curve:

Human serum albumin (µg)	Human serum albumin (ml)	Water (ml)
2	0.010	0.990
5	0.025	0.975
10	0.050	0.950
15	0.075	0.925
20	0.100	0.900
25	0.125	0.875
30	0.150	0.850
35	0.175	0.825
40	0.200	0.800
50	0.250	0.750
60	0.300	0.700

References

1. WHO Scientific Group on Research on the Menopause in the 1990s. Research on the menopause in the 1990s: Report of the WHO scientific group. WHO technical report series. Report number 866. WHO Geneva, 1996.

2. WHO Scientific Group. Research on the menopause. Report of the WHO Scientific Group. WHO Technical Report Series. Report number 670. WHO Geneva, 1981.

3. Khaw KT. Epidemiology of the menopause. British Medical Bulletin 1992;48(2):249-261.

4. WHO. World Health Statistics Annual 1992. World Health Organization, Geneva, 1993.

5. OPCS. 1992 Mortality Statistics, General, England and Wales. Office of Population Censuses and Surveys. Series DHI no. 27, London: HMSO, 1994.

6. Nelson JF. Puberty, gonadal steroids and fertility: Potential reproductive markers of aging. Exp Gerontol 1988;23:359-367.

7. Snowdon DA, Kane RL, Beeson WL, et al. Is early natural menopause a biological marker of health and aging? Am J Publ Health 1989;79(6):709-714.

8. Snowdon DA. Early natural menopause and the duration of postmenopausal life. J Am Ger Soc 1990;38:402-408.

9. WHO. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. Report of a WHO Study Group. WHO technical report series. Report number 843. Geneva: World Health Organization, 1994.

10. Theintz G, Buchs B, Rizzoli R, et al. Longitudinal monitoring of bone mass accumulation in healthy adolescents; evidence for a marked reduction after 16 years of age of the levels of lumbar spine and fermoral neck in female subjects. Journal of Clinical Endocrinology and Metabolism 1992;75(4):1060-1065.

11. Kelly PJ, Twomey L, Sambrook PN, Eisman JA. Sex differences in peak adult bone mineral density. Journal of Bone and Mineral Research 1990;5(11):1169-1175.

12. Riggs BL, Melton LJI. Involutional osteoporosis. New England Journal of Medicine 1986;314:1676-1686.

13. Spector TD. The relationship between sex steroids and bone mineral content in women soon after the menopause. Clinical Endocrinology 1991;34:37-41.

14. Wark JD. Osteoporosis: Pathogenesis, diagnosis, prevention and management. Bailliere's Clinical Endocrinology and Metabolism 1993;7:157-181.

15. Grodstein F, Stampfer M. The epidemiology of coronary heart disease and estrogen replacement in postmenopausal women. Progress in Cardiovascular Diseases 1995;38(3):199-210.

16. Stampfer MJ, Colditz GA, Willett WC. Menopause and heart disease: a review. Annals of the New York Academy of Sciences1990;592:193-203.

17. Taylor RW. The mechanism of action of œstrogen in the human female. Journal of Obstetrics and Gynæcology of the British Commonwealth 1974;81:856-866.

18. Notelovitz M. Gynecologic problems of menopausal women: part 1. Changes in genital tissue. Geriatrics 1978;33:24-30.

19. Leiblum S, Bachmann G, Kemmann E, Colburn D, Swartzman L. Vaginal atrophy with post-menopausal women: The importance of sexual activity and hormones. JAMA 1983;249(16):2195-2198.

20. Langlois PL. The size of the normal uterus. Journal of Reproductive Medicine 1970;4:220-228.

21. Jolleys JV. Reported prevalence of urinary incontinence in women in a general practice. British Medical Journal 1988;296:1300-1302.

22. Avis NE, Kaufert PA, Lock M, McKinlay SM, Vass K. The evolution of menopausal symptoms. Bailliere's Clinical Endocrinology and Metabolism 1993;7:17-32.

23. Holte A. Influence of natural menopause on health complaints: a prospective study of healthy Norwegian women. Maturitas 1992;14(2):127-141.

24. Kaufert PA, Gilbert P, Tate R. The Manitoba project: A reexamination of the link between menopause and depression. Maturitas 1992;4(2):143-156.

25. Martin MC, Block JE, Sanchez SD, Arnaud CD, Beyene Y. Menopause without symptoms: the endocrinology of menopause among rural Mayan Indians. American Journal of Obstetrics and Gynecology 1993;168(6 pt 1):1839-1845.

26. Hunter MS. The South East England longitudinal study of the climacteric and postmenopause. Maturitas 1992;14:117-126.

27. Burger HG. The menopausal transition. Bailliere's Clinical Obstetrics and Gynæcology 1996;10(3):347-359.

28. Sitruk-Ware R, Utian WH. Risks and benefits of hormone replacement therapy. In: Sitruk-Ware R. and Utian WH. ed. The menopause and hormonal replacement therapy. New York, Basel: Marcel Dekker, 1991: 283-288.

29. Daly E, Roche M, Barlow D, Gray A, McPherson K, Vessey M. HRT: An analysis of benefits, risks and costs. British Medical Bulletin 1992;48(2):368-400.

30. Bush TL, Miller-Bass K. Œstrogen therapy and cardiovascular disease: do the benefits outweigh the risks? Bailliere's Clinical Obstetrics and Gynæcology 1991;5(4):889-913.

31. BNF, Joint Formulary Committee. British National Formulary.London: British Medical Association, Royal Pharmaceutical Society of Great Britain, 1995:299-303.

32. DTB. Hormone replacement therapy. Drug and Therapeutics Bulletin 1996;34(11):81-84.

33. Rees MCP. The uterus and the menopause. Bailliere's Clinical Obstetrics and Gynæcology 1996;10(3):419-432.

34. Harris RB, Laws A, Reddy VM, King A, Haskell WL. Are women using postmenopausal estrogens? A community survey. American Journal of Public Health 1990;80(10):1266-1268.

35. Gusberg SB. Estrogen and endometrial cancer: An epilogue á la Recherche du Temps Perdu. Gynecologic Oncology 1994;52:3-9.

36. Bush TL, Miller VT. Effect of pharmacologic agents used during menopause. Impact on lipids and lipoproteins. In: Mishell D, ed. Menopause: Physiology and pharmacology. Chicago, IL: Year Book Medical, 1986: 187-208.

37. Miller VT, Muesing RA, La Rosa JC, et al. Effects of conjugated equine estrogen with and without three different progestogens on lipoproteins, high density lipoprotein subfractions, and apolipoprotein A-I. Obstetrics and Gynecology 1991;77(2):235-240.

38. Walsh BW, Schiff I, Rosner B, et al. Effects of postmenopausal estrogen replacement on the concentration and metabolism of plasma lipoproteins. New England Journal of Medicine 1991;325(17):1196-1204.

39. Bourne T, Hillard TC, Whitehead MI, Crook D, Campbell S. Œstrogens, arterial status, and postmenopausal women. Lancet 1990;335:1470-1471.

40. Gilligan DM, Quyyumi AA, Cannon RO. Effects of physiological levels of estrogen on coronary vasomotor function in postmenopausal women. Circulation 1994;89:2545-2551.

41. Mugge A, Riedel M, Barton M, et al. Endothelium independent relaxation of human coronary arteries by 17β-œstradiol in vitro. Cardiovascular Research 1993;27(11):1939-1942.

42. Steinleitner A, Stanczyk FZ, Levin JH, et al. Decreased in vitro production of 6-keto-prostaglandin $F_{1\alpha}$ by uterine arteries from postmenopausal women. American Journal of Obstetrics and Gynecology 1989;161:1677-1681.

43. Barrett-Connor E, Laakso M. Ischemic heart disease risk in postmenopausal women. Effects of estrogen use on glucose and insulin levels. Arteriosclerosis 1990;10:531-534.

44. Van der Mooren MJ, Wouters MG, Blom HJ, et al. Hormone replacement therapy may reduce high serum homocysteine in postmenopausal women. European Journal of Clinical Investigation 1994;24(11):733-736.

45. Sarrel PM. Blood flow. In: Lobo R, ed. Treatment of the postmenopausal woman. New York, NY: Raven, 1994: 251-262.

46. Lindsay R. The menopause: Sex steroids and osteoporosis. Clinical Obstetrics and Gynecology 1987;30:847-859.

47. Kleerekoper M, Sullivan JM. Osteoporosis as a model of the long-term clinical consequences of the menopause. Progress in Cardiovascular Diseases 1995;38(3):181-188.

48. Lindsay R. Why do œstrogens prevent bone loss? Bailliere's Clinical Obstetrics and Gynæcology 1991;5(4):837-852.

49. Christiansen C, Riis B, Nilas L, Rodbro P, Deftos L. Uncoupling of bone formation and resorption by combined œstrogen and progestogen therapy in postmenopausal osteoporosis. Lancet 1985;2:800-801.

50. Kanis JA. The menopause and the skeleton: Key issues. Bailliere's Clinical Obstetrics and Gynæcology 1996;10(3):469-481.

51. Coope J, Thomson J. Effects of 'natural œstrogens' on menopausal symptoms and blood clotting. British Medical Journal 1975;4:139-143.

52. Marsh MS, Whitehead MI. Management of the menopause. British Medical Bulletin 1992;48(2):426-457.

53. Reynolds JEF. Martindale. The extrapharmacopoeia. (Thirtieth Edition ed.) London: The Pharmaceutical Press, 1993:1191-1193.

54. Hunt K, Vessey M. Use of hormone replacement therapy and breast cancer risk. In: Sitruk-Ware R, and Utian WH. ed. The menopause and hormonal replacement therapy. New York, Basel, Hong Kong: Marcel Dekker, 1991: 143-160.

55. Daly E, Vessey MP, Hawkins MM, et al. Risk of venous thromboembolism in users of hormone replacement therapy. Lancet 1996;348:977-80.

56. Jick H, Derby LE, Wald Myers M, et al. Risk of hospital admission for idiopathic venous thromboembolism among users of postmenopausal œstrogens. Lancet 1996;348:981-3.

57. Grodstein F, Stampfer MJ, Goldhaber SZ, et al. Prospective study of exogenous hormones and risk of pulmonary embolism in women. Lancet 1996;348:983-987.

58. Sitruk-Ware R. Hormonal replacement therapy: What to prescribe, how, and for how long. In: Sitruk-Ware R, and Utian WH. ed. The menopause and hormonal replacement therapy. New York, Basel: Marcel Dekker, 1991: 259-282.

59. Stevenson JC, Hillard TC, Lees B, et al. Postmenopausal bone loss: does HRT always work ? International Journal of Fertility and Menopausal Studies 1993;38(Suppl. 2):88-91.

60. Habiba M, Akkad A, Al-Azzawi F. Effect of a new cyclical sequential postmenopausal HRT on lipoprotein, apoprotein and thrombophilia profile. European Journal of Obstetrics and Gynecology and Reproductive Biology 1995;62(1):89-95.

61. Marsh MS, Whitehead MI. The practicalities of hormone replacement therapy. Bailliere's Clinical Endocrinology and Metabolism 1993;7(1):183-202.

62. Guirgis RR. Œstradiol implants : What dose, How often ? Lancet 1987;2(8563):856.

63. Studd JWW, Savvas M, Watson NR, Dooley M. Monitoring œstradiol implantation. Lancet 1987;2:1404-1405.

64. Thomson J, Oswald I. Effect of œstrogen on the sleep, mood, and anxiety of menopausal women. British Medical Journal 1977;2(6098):1317-1319.

65. Nachtigall LE. Enhancing patient compliance with hormone replacement therapy at menopause. Obstetrics and Gynecology 1990;75:77S-80S.

66. Rivlin ME. Handbook of drug therapy in reproductive endocrinology and infertility. Boston, Toronto: Little, Brown and Company, 1990:15-43.

67. Nachtigall LE. Compliance with hormone replacement therapy: where we stand today. In: Berg GaH M., ed. The modern management of the menopause. A perspective for rhe 21st century. The proceedings of the VII international congress on the menopause. New York, London: Parthenon, 1994: 439-443.

68. Hahn RG, Nachtigall RD, Davies TC. Compliance difficulties with progestin supplemented replacement therapy. Journal of Family Practice 1984;18:411-414.

69. Dallenbach-Hellweg G. *Histopathology of the Endometrium* (Fourth edition). Berlin Heidelberg, New York: Springer-Verlag, 1987:1-25.

70. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Fertility and Sterility 1950;1:3-25.

71. Johannisson E. The influence of contraceptive steroids on the histochemistry and cytochemistry of the normal endometrium. In: Diczfalusy E, Fraser IS, Webb FTG, ed. Endometrial Bleeding and Steroidal Contraception. Proceedings of a Symposium on Steroid Contraception and Mechanisms of Endometrial Bleeding organized by the WHO in Geneva on 12-14 September, 1979. Bath, England: Pitman Press Ltd, 1980: 174-190.

72. Good RG, Moyer DL. Estrogen-progesterone relationships in the development of secretory endometrium. Fertility and Sterility 1968;19(1):37-49.

73. Dallenbach-Hellweg G. The influence of contraceptive steroids on the histological appearance of the endometrium. In: Diczfalusy E, Fraser IS, Webb FTG, ed. Endometrial Bleeding and Steroidal Contraception. Proceedings of a Symposium on Steroid Contraception and Mechanisms of Endometrial Bleeding organized by the WHO in Geneva on 12-14 September, 1979. Bath, England: Pitman Press Ltd, 1980: 153-173.

74. Smith DC, Prentice R, Thompson DJ, Herrmann WL. Association of exogenous estrogen and endometrial carcinoma. New England Journal of Medicine 1975;293:1164-1167.

75. Ziel HK, Finkle WD. Increased risk of endometrial carcinoma among users of conjugated estrogens. New England Journal of Medicine 1975;293:1167-1170.

76. Barrett-Connor E. Hormone replacement and cancer. British Medical Bulletin 1992;48:345-355.

77. Creasy GW, Kafrissen ME, Upmalis D. Review of the endometrial effects of estrogens and progestins. Obstetrical and Gynecological Survey 1992;47(9):654-678.

78. Whitehead MI, Townsend PT, Pryse-Davies J, et al. Effects of estrogen and progestins on the morphology and biochemistry of the postmenopausal endometrium. New England Journal of Medicine 1981;305:1599-1605.

79. Padwick ML, Pryse-Davies J, Whitehead MI. A simple method for determining the optimal dosage of progestin in postmenopausal women receiving estrogens. New England Journal of Medicine 1986;315:930-934.

80. Sturdee DW, Barlow DH, Ulrich LG, et al. Is timing of withdrawal bleeding a guide to endometrial safety during sequential œstrogen-progestagen replacement therapy? Lancet 1994;343(8928):979-982.

81. Tindall VR. Jeffcoate's Principles of Gynæcology.London: Butterworths, 1987:512-531.

82. Robertson WB. The Endometrium.London, Boston: Butterworths, 1981:7-44.

83. Rock J, Hertig AT. Information regarding the time of human ovulation derived from a study of 3 unfertilized and 11 fertilized ova. American Journal of Obstetrics and Gynecology 1944;47:343.

84. Zuckerman S. The menstrual cycle. Lancet 1949;i:1031-1034

85. Kamat BR, Isaacson PG. The immunocytochemical distribution of leukocytic sub-populations in human endometrium. American Journal of Pathology 1987;127:66-73.

86. Sheppard BL, Bonnar J. The development of vessels of the endometrium during the menstrual cycle. In: Diczfalusy E, Fraser IS, Webb FTG, ed. Endometrial Bleeding and Steroidal Contraception. Proceedings of a Symposium on Steroid Contraception and Mechanisms of Endometrial Bleeding organized by the WHO in Geneva on 12-14 September, 1979. Bath, England: Pitman Press Ltd, 1980: 65-85.

87. Markee JE. Menstruation in intraocular endometrial transplants in the rhesus monkey. Contrib. Embryol. Carnegie Inst. Wash. 1940;28:219-308.

88. Hourihan HM, Sheppard BL, Brosens IA. Endometrial hemostasis. In: D'Arcangues C, Fraser IS, Newton JR, Odlind, V, ed. Contraception and Mechanisms of Endometrial Bleeding. Scientific basis of fertility regulation. Proceedings of a symposium organized by the special programme of research, development and research training in Human reproduction of the WHO in Geneva on 28 November-2 December 1988. Cambridge: Cambridge University Press, 1990: 95-116.

89. Shaw ST, Macaulay LK, Hohman WR. Vessel density in endometrium of women with and without intrauterine contraceptive devices: a morphometric evaluation. American Journal of Obstetrics and Gynecology 1979;135:202-206.

90. Rogers PAW, Au CL, Affandi B. Endometrial microvascular density during the normal menstrual cycle and following exposure to long-term levonorgestrel. Human Reproduction 1993;8(9):1396-1404.

91. Kelly FD, Tawia SA, Rogers PAW. Immunohistochemical characterization of human endometrial microvascular basement membrane components during the normal menstrual cycle. Human Reproduction 1995;10(2):268-276.

92. Song JY, Markham R, Russell P, et al. The effect of high-dose medium- and long- term progestogen exposure on endometrial vessels. Human Reproduction 1995;10(4):797-800.

93. Ancla M, Belaish J, De Brux J. Action of chorionic gonadotrophin on cellular structures in the human endometrium in the secretory phase. Journal of Reproduction and Fertility 1969;19:291-297.

94. Maqueo M. Vascular and perivascular changes in the endometrium of women using steroidal contraceptives. In: Diczfalusy E, Fraser IS, Webb FTG, ed. Endometrial Bleeding and Steroidal Contraception. Proceedings of a Symposium on Steroid Contraception and Mechanisms of Endometrial Bleeding organized by the WHO in Geneva on 12-14 September, 1979. Bath, England: Pitman Press Ltd, 1980: 138-149.

95. Johannisson E, Landgren B-M, Diczfalusy E. Endometrial morphology and peripheral steroid levels in women with and without intermenstrual bleeding during contraception with the 300µg norethisterone (NST) minipill. Contraception 1982;25:13-30.

96. Johannisson E, Landgren B-M, Rohr HP, Diczfalusy E. Endometrial morphology and peripheral hormone levels in women with regular menstrual cycles. Fertility and Sterility 1987;38:564-571.

97. Dockery P, Li TC, Rogers AW, Cooke ID, Lenton EA. The ultrastructure of the glandular epithelium in the timed endometrial biopsy. Human Reproduction 1988;3(7):826-834.

98. Dockery P, Rogers AW. The effects of steroids on the fine structure of the endometrium. Bailliere's Clinical Obstetrics and Gynæcology 1989;3(2):227-248.

99. Spornitz UM. The functional morphology of the human endometrium and decidua. Advances in Anatomy, Embryology and Cell Biology 1992;124(1):1-99.

100. Dockery P, Tidey RR, Li TC, Cooke ID. A morphometric study of the uterine glandular epithelium in women with premature ovarian failure undergoing hormone replacement therapy. Human Reproduction 1991;6(10):1354-1364.

101. Dockery P, Pritchard K, Taylor A, et al. The fine structure of the human endometrial glandular epithelium in cases of unexplained infertility: a morphometric study. Human Reproduction 1993;8(5):667-673.

102. Kohorn EI, Rice SI, Hemperly S, Gordon M. The relation of the structure of progestational steroids to nucleolar differentiation in human endometrium. Journal of Clinical Endocrinology and Metaboilsm 1972;34:257-264.

103. Gompel C, Silvererg SG. Pathology in gynecology and obstetrics.Philadelphia: Lippincott, 1985:149-277.

104. McBride JM. The normal postmenopausal endometrium. Journal of Obstetrics and Gynæcology of the British Empire. 1954;61:691.

105. Ferenczy A, Bergeron C. Histology of the human endometrium: from birth to senescence. Annals of the New York Academy of Sciences 1991;622(6):6-27.

106. Palter SF, Olive DL. Reproductive physiology. In: Berek JS, Adashi EY, Hillard PA, ed. Novak's gynecology. Baltimore: Williams and Wilkins, 1996: 149-174.

107. Sherman ME, Silverberg SG. Advances in endometrial pathology. Gynecologic pathology 1995;15(3):517-543.

108. Egger H, Kindermann G. Effect of estrogens at high dosage on the human endometrium. Arch Gynakol 1974;216:399.

109. Dickey RP, Stone SC. Progestational potency of oral contraceptives. Obstetrics and Gynecology 1976;47:106.

110. Kim-Bjorklund T, Landgren BM, Johannisson E. Morphometric studies of the endometrium, the fallopian tube, and the corpus luteum during contraception with 300µg norethisterone (NET) minipill. Contraception 1991;43:459-474.

111. Ludwig H. The morphologic response of the human endmetrium to long-term treatment with progestational agents. American Journal of Obstetrics and Gynecology 1982;142:796-808.

112. Johannisson E. Endometrial morphology during the normal cycle and under the influence of contraceptive steroids. In: D'Arcangues C, Fraser IS, Newton JR, and Odlind V, ed. Contraception and mechanisms of endometrial bleeding. Scientific basis of fertility regulation. Proceedings of a symposium organized by the special programme of research, development and research training in Human reproduction of the WHO in Geneva on 28 November-2 December 1988. Cambridge: Cambridge University Press, 1990: 53-80.

113. Luukkainen T, Haukkamaa M. Concentrations of levonorgestrel in endometrium and myometrium after oral and intrauterine administration. In: D'Arcangues C, Fraser IS, Newton JR, and Odlind V, ed. Contraception and mechanisms of endometrial bleeding. Scientific basis of fertility regulation. Proceedings of a symposium organized by the special programme of research, development and research training in Human reproduction of the WHO in Geneva on 28 November-2 December 1988. Cambridge: Cambridge University Press, 1990: 181-186.

114. Li T-C, Dockery P, Cooke ID. Effect of exogenous progesterone administration on the morphology of normally developing endometrium in the pre-implantation period. Human Reproduction 1991;6:641-644.

115. Kaufman C. Echte menstruelle Blutung bei kastrierten Frauen nach Zufuhr von Follikel-and Corpus luteum-Hormon. Klin Wochenschr 1933;12:217.

116. Ferin J, Schilkker E. Artificial cycles in oophorectomized women. International Journal of Fertility 1960;5:19.

117. Tabibzadeh S, Saytaswaroop PG. Sex steroid receptors in lymphoid cells of human endometrium. American Journal of Clinical Pathology 1989;91:656-663.

118. De M, Wood GW. Influence of œstrogen and progesterone on macrophage distribution in the mouse uterus. Journal of Endocrinology 1990;126:417-424.

119. Zheng Y, Zhao-Zong Z, Lyttle R, Teuscher C. Immunohistochemical characterization of the estrogen-stimulated leukocyte influx in the immature rat uterus. Journal of Leukocyte Biology 1988;44:27-32.

120. Szekeres-Bartho J, Csernus V, Hadnagy I, Pasca AS. Immunosuppressive effect of serum progesterone during pregnancy depends on the progesterone binding capacity of the lymphocytes. Journal of Reproductive Immunology 1983;5:81-88.

121. Hatcher RA, Trussell J, Stewart F, et al. Contraceptive Technology. (Sixteenth ed.) New York: Irvington, 1994:223-284.

122. Rudel HW, Martinez-Manautou J, Maqueo M. Comparison of the antiestrogenic activity of several progestogens in women. Journal of New Drugs 1966;6:126-127.

123. Goldzieher JW, Becerra C, Gual C, et al. New oral contraceptive. Sequential estrogen and progestin. American Journal of Obstetrics and Gynecology 1964;90:404-411.

124. Ober WB. Synthetic progestagen-estrogen preparations and endometrial morphology. Journal of Clinical Pathology 1966;19:138-147.

125. Morris JM. Mechanisms involved in progesterone contraception and œstrogen interception. American Journal of Obstetrics and Gynecology 1973;117:167-176.

126. De Ziegler D, Fanchin R, Massonneau M, et al. Hormonal control of endometrial receptivity. The egg donation model and controlled ovarian hyperstimulation. Annals of the New York Academy of Sciences 1994;734:209-220.

127. De Ziegler D, Frydman R. Different implantation rates after transfers of cryopreserved embryos originating from donated oocytes or from regular in vitro fertilization. Fertility and Sterility 1990;54:682-688.

128. Check JH. The use of the donor oocyte program to evaluate embryo implantation. Annals of the New York Academy of Sciences 1994;734:198-208.

129. Check JH, Nowroozi K, Chase J, Nazari A, Braithwaite C. Comparison of pregnancy rates following in vitro fertilization-embryo transfer between the donors and the recipients in a donor oocyte program. Journal of Assisted Reproduction and Genetics 1992;9:248-250.

130. Novat D, Bergh PA, Williams MA, et al. Poor oocyte quality rather than implantation failure as a cause of age-related decline in female fertility. Lancet 1991;337:1375-1377.

131. Novat D, Anderson K, Droesch K, Scott R, Kreiner D, Rosenwaks Z. Hormonal manipulation of endometrial maturation. Journal of Clinical Endocrinology and Metabolism 1989;68:801-807.

132. Li TC, Warren MA, Hill CJ, Saravelos H. Morphology of the human endometrium in the peri-implantation period. Annals of the New York Academy of Sciences 1994;734:169-184.

133. Antinori S, Versaci C, Hossein Gholami G, et al. Oocyte donation in menopausal women. Human Reproduction 1993;8(9):1487-1490.

134. Whitehead MI, King RJB, McQueen J, Campbell S. Endometrial histology and biochemistry in climacteric women during estrogen and estrogen/progestogen therapy. Journal of the Royal Society of Medicine 1979;72:322-327.

135. Thom MH, White PJ, Williams RM, Paterson MEL, Wade-Evans T, Studd JWW. Prevention and treatment of endometrial disease in climacteric women receiving œstrogen therapy. Lancet 1979;2:455-457.

136. Grady D, Gebretsadik T, Kerlikowske K, et al. Hormone replacement therapy and endometrial cancer risk: a meta-analysis. Obstetrics and Gynecology 1995;85(2):304-313.

137. Auchus RJ, Fuqua SAW. The œstrogen receptor. Bailliere's clinical endocrinology and metabolism 1994;8(2):433-450.

138. Bern HA. Is estrogen a cellular signal for female genital tract epithelium? In: Lavia LA, ed. Cellular Signals Controlling Uterine Function. New York: Plenum Press, 1991: 5-9.

139. Lavia LA. Qualitative and quantitative morphology of induction in endometrial epithelium. In: Lavia LA, ed. Cellular Signals Controlling Uterine Function. New York: Plenum Press, 1991: 31-47.

140. Stancel GM, Chiappetta C, Gardner RM, et al. Interactions of estrogens, protooncogenes and growth factors. In: Lavia LA, ed. Cellular Signals Controlling Uterine Function. New York: Plenum Press, 1990: 49-61.

141. Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL, McCarty KSJ. Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. Journal of Clinical Endocrinology and Matabolism 1988;67:334-340.

142. Garcia E, Bouchard P, De Brux J, et al. Use of immunocytochemistry of progesterone and estrogen receptors for endometrial dating. Journal of Clinical Endocrinology and Matabolism 1988;67(1):80-87.

143. Tachi C, Tachi S, Linder HR. Modification by progesterone of œstradiol-induced cell proliferation, RNA synthesis and œstradiol distribution in the rat uterus. Journal of Reproduction and Fertility 1972;31:59.

144. Hsueh AJW, Peck EJJ, Clarke JH. Progesterone antagonism of the œstrogen receptor and œstrogen-induced uterine growth. Nature 1975;254:337.

145. Okulicz WC, Evans RW, Leavitt WW. Progesterone regulation of the occupied form of nuclear estrogen receptor. Science 1981;213:1503.

146. Zahradnik HP. The mechanism of effect of gestagens. In: Dallenbach-Hellweg G, ed. Functional morphologic changes in female sex organs induced by exogenous hormones. Berlin, Heidelberg, New York: Springer-Verlag, 1980: 74-79.

147. Bigsby RM. Reciprocal tissue interactions in morphogenesis and hormonal responsiveness of the female reproductive tract. In: Lavia LA, ed. Cellular Signals Controlling Uterine Function. New York: Plenum Press, 1991: 11-29.

148. Kastner P, Krust A, Turcotte B, et al. Two distinct estrogen-regulated promoters generate transcripts encoding the 2 functionally different human progesterone-receptor form-A and form-B. EMBO Journal 1990;9(5):1603-1614.

149. Tora L, Gronemeyer H, Turcotte B, Gaub MP, Chambon P. The N-terminal region of the chicken progesterone receptor specifies target gene activation. Nature 1988;333(6169):185-188.

150. Song JY, Fraser IS. Effects of progestogens on human endometrium. Obstetrical and Gynecological Survey 1995;50(5).

151. Bouchard P, Schaison G, Marraoui J, et al. Evaluation of endometrial maturation with the immunocytolocalization of estradiol and progesterone receptors. In: D'Arcangues C, Fraser IS, Newton JR, and Odlind V, ed. Contraception and mechanisms of endometrial bleeding. Scientific basis of fertility regulation. Proceedings of a symposium organized by the special programme of research, development and research training in Human reproduction of the WHO in Geneva on 28 November-2 December 1988. Cambridge: Cambridge University Press, 1990: 163-179.

152. Finn CA, Pope M. Vascular and cellular changes in the decidualized endometrium of the ovariectomized mouse following cessation of hormone treatment. A possible model for menstruation. Journal of Endocrinology 1984;100(3):295-300.

153. Cornillie FJ, Lauweryns JM, Brosens IA. Normal human endmetrium. An ultrastructural survey. Gynecologic and Obstetric Investigation 1985;20:113-129.

154. Henzl MR, Smith RE, Boost G, Taylor ET. Lysosomal concept of menstrual bleeding in humans. Journal of Clinical Endocrinology and Metabolism 1972;34:860-875.

155. Cameron IT, Davenport AP, van Papendorp C, et al. Endothelin-like immunoreactivity in human endometrium. Journal of Reproduction and Fertility 1992;95(2):623-628.

156. Tabibzadeh S, Zupi E, Babaknia A, et al. Site and menstrual cycle dependent expression of proteins of the tumour necrosis factor (TNF) receptor family, and Bcl-2 oncoprotein and phase-specific production of TNFα in human endometrium. Human Reproduction 1995;10(2):277-286.

157. Charnock-Jones DS, Sharkey AM, Rajput-Williams J, et al. Identification and localization of alternately spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. Biology of Reproduction 1993;48(5):1120-1128.

158. Smith SK. The physiology of menstruation. In: D'Arcangues C, Fraser IS, Newton JR, and Odlind V, ed. Contraception and mechanisms of endometrial bleeding. Scientific basis of fertility regulation. Proceedings of a symposium organized by the special programme of research, development and research training in Human reproduction of the WHO in Geneva on 28 November-2 December 1988. Cambridge: Cambridge University Press, 1990: 33-51.

159. Al-Azzawi F, Hawley J, Parsons A, Hart DM. Investigation of the bleeding patterns of postmenopausal women treated with Estrapak-50. Maturitas 1994;18:115-125.

160. Habiba M, Al-Azzawi F. Analysis of menstrual calendars and serum α2-PEG in women on hormone replacement therapy for 12 months. European Journal of Obstetrics and Gynecology and Reproductive Biology 1994;57:37-42.

161. Stock RJ, Kanbour A. Prehysterectomy curettage. Obstetrics and Gynecology 1975;45:537-541.

162. Grimes DA. Diagnostic dilatation and curettage: a reappraisal. American Journal of Obstetrics and Gynecology 1982;142:1-6.

163. Rodrigues GC, Yaqub N, King ME. A comparison of the pipelle device and the vabra aspirator as measured by endometrial denudation in hysterectomy specimens: The pipelle device samples significantly less of the endometrial surface than the vabra aspirator. American Journal of Obstetrics and Gynecology 1993;168:55-59.

164. Noyes RW. Uniformity of secretory endometrium. Obstetrics and Gynecology 1956;7:221-227.

165. Taylor PJ, Graham G. Is diagnostic curettage harmful in women with unexplained infertility ? British Journal of Obstetrics and Gynæcology 1982;89:296.

166. Novak E. A suction curet apparatus for endometrial biopsy. JAMA 1935;104:1497.

167. Randall LM. Endometrial biopsy. Proceedings Mayo Clinic 1935;10:143-144.

168. Twiggs LS, DiSaia PJ, Morrow CP, et al. Gravlee jet irrigator: Efficacy in diagnosis of endometrial neoplasia. JAMA 1976;235(25):2748-2750.

169. DiSaia PJ, Creasman WT. Clinical Gynecologic Oncology.St Louis, Washington, D.C.: C.V.Mosby Company, 1989:161-197.

170. Pradhan S, Chenoy R, O'Brien PMS. Dilatation and curettage in patients with cervical polyps: a retrospective analysis. British Journal of Obstetrics and Gynæcology 1995;102:415-417.

171. Sturdee DW, Wade-Evans T, Paterson MEL, et al. Relations between bleeding pattern, endometrial histology, and œstrogen treatment in menopausal women. British Medical J ournal 1978;i:1575-1577.

172. Habiba M, Akkad A, Al-Azzawi F. The role of pipelle endometrial biopsy in patients with postmenopausal bleeding. British Journal of Obstetrics and Gynæcology 1995;102:262.

173. De Cecco L, Gerbaldo D, Cristoforoni P, et al. Morphological aspects of human endometrium during hormone replacement therapy. Annals of the New York Academy of Sciences 1994;734:263-270.

174. Fay TN, Grudzinskas JG. Human endometrial peptides: a review of their potential role in implantation and placentation. Human Reproduction 1991;6(9):1311-26.

175. Johannisson E, Parker RA, Landgren BM, Diczfalusy E. Morphomeric analysis of the human endometrium in relation to peripheral hormone levels. Fertility and Sterility 1982;38:564-571.

176. Strinden ST, Shapiro SS. Progesterone-altered secretory proteins from cultured human endometrium. Endocrinology 1983;112:862-870.

177. Santoro N, MacLaughlin DT, Bauer HH, et al. In vitro protein production by the human endometrium. Biology of Reproduction 1989;40:1047-1055.

178. Bell SC, Drife JO. Secretory proteins of the endometrium - potential markers for endometrial dysfunction. Bailliere's Clinical Obstetrics and Gynæcology. 1989;3(2):271-291.

179. Julkunen M, Koistinen R, Sjoberg J, et al. Secretory endometrium synthesizes placental protein 14. Endocrinology 1986;118(5):1782-1786.

180. Li TC, Dalton C, Bolton AE, et al. An analysis of the variation of plasma concentrations of placental protein 14 in artificial cycles. Fertility and Sterility 1992;57(4):776-82.

181. Bell SC, Patel SR, Kirwan PH, Drife JO. Protein synthesis and secretion by the human endometrium during the menstrual cycle and the effect of progesterone in vitro. Journal of Reproduction and Fertility 1986;77:221-231.

182. Julkunen M, Apter D, Seppala M, et al. Serum levels of placental protein 14 reflect ovulation in nonconceptional menstrual cycles. Fertility and Sterility 1986;45:47-50.

183. Seppala M, Ronnberg L, Karonen SL, Kauppila A. Micronized oral progesterone increases the circulating level of endometrial secretory PP14/β–lactoglobulin homologue. Human Reproduction 1987;2:453-455.

184. Byrjalsen I, Thormann L, Meinecke B, et al. Sequential estrogen and progestogen therapy: Assessment of progestational effects on the postmenopausal endometrium. Obstetrics and Gynecology 1992;79:523-528.

185. McRae MA, Galle PC, Joshi SG. The role of measurement of progestagen-associated endometrial protein in predicting adequate endometrial differentiation. Human Reproduction 1991;6(6):761-5.

186. Julkunen M, Koistinen R, Aalto-Setala K, et al. Primary structure of human insulin-like growth factor-binding protein/placental protein 12 and tissue specific expression of its mRNA. FEBS Letters 1988;236(2):295-301.

187. Bell SC, Patel S, Hales W, et al. Immunochemical detection and characterization of pregnancy-associated endometrial α1-and α2-globulins secreted by the human endometrium and decidua. Journal of Reproduction and Fertility 1985;74:261-270.

188. Yeoh S-I, Baxter RC. Metabolic regulation of growth hormone indepenent insulin-like growth factor binding protein in human plasma. Acta Endocrinologica 1988;119:465-473.

189. Li TC, Ling E, Dalton C, et al. The techniques and complications of outpatient uterine washing in the assessment of endometrial function. Human Reproduction 1993;8:343-346.

190. Hirsch PJ, Fergusson ILC, King RJB. Protein composition of human endometrium and its secretion at different stages of the menstrual cycle. Annals of the New York Academy of Sciences1977;286:233.

191. Smolen A. Image analytic techniques for quantification of immunohistochemical staining in the nervous system. In: Conn P, ed. Qunatitative and qualitative microscopy. San Diego: Academic Press, 1990: 208-229.

192. Park-Sarge O-K, Mayo K E. Molecular biology of endocrine receptors in the ovary. In: Findlay JK, ed. Molecular biology of the female reproductive system. San Diego, New York: Academic Press, 1994: 153-205.

193. Jackson P, Blythe D. Immunolabelling techniques for light microscopy. In: Beesley JE, ed. Immunocytochemistry. A practical approach. (Rickwood D, Hames BD, ed. The Practical Approach Series; vol 122). Oxford: IRL Press, 1993: 15-42.

194. Bancroft JD, Stevens A. Theory and practice of histological techniques. 2nd edition. Edinburgh, London: Churchill Livingstone, 1982.

195. Stevens A. Microorganisms. In: Bancroft JD, Stevens A, ed. Theory and practice of histological techniques. Edinburgh, London, Melbourne: Churchill Livingstone, 1982: 278-296.

196. Shi S-R, Key ME, Kalra KL. Antigen Retrieval in formalin-fixed, paraffin-embedded tissue: An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. Histochemistry and Cytochemistry 1991;39:741-748.

197. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. Journal of Cell Biology 1992;119(3):493-501.

198. Ansari B, Coates PJ, Greenstein BD, Hall PA. In situ end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. Journal of Pathology 1993;170:1-8.

199. Alison M, Chaudry Z, Baker J, et al. Liver Regeneration: A comparison of *in situ* Hybridization for Histone mRNA with Bromodeoxyuridine Labelling for the detection of S-phase Cells. Histochemistry and Cytochemistry 1994;42:1603-1608.

200. Malak TM. Structural biology of the human fetal membranes [PhD]. University of Leicester, 1995.

201. Lowe D. Planning for medical research: A practical guide to research methods. Holding Hands Series. Middlesbrough: Astraglobe limited, 1993.

202. Machin D, Campbell MJ. Statistical tables for the design of clinical trials.Oxford, London, Edingburgh: Blackwell Scientific Publications, 1987:79-88.

203. Galfre G, Milstein C. Preparation of monoclonal antibodies: Strategies and Procedures. In: Langone JJ, Van Vunakis H, ed. Immunochemical techniques. Part B. (Colowick SP, Kaplan NO, ed. Methods in Enzymology; vol 73). New York, London: Academic Press, 1981: 3-46.

204. Srkalovic G, Wittliff JL, Schally AV. Detection and partial characterization of receptors for [D-Trp^6]-Luteinizing Hormone-releasing hormone and epidermal growth factor in human endometrial carcinoma. Cancer Research 1990;50(6):1841-1846.

205. Gallagher G, Al-Azzawi F, Walsh LP, Wilson G. 14C1, an antigen associated with human ovarian cancer, defined using a human IgG monoclonal antibody. Clinical and Experimental Immunology 1991;83:92-95.

206. Chiazze L, Brayer FT, Macisco JJ, et al. The length and variability of the human menstrual cycle. JAMA 1968;203(6):377-380.

207. Munster K, Schmidt L, Helm P. Length and variation in the menstrual cycle - a cross sectional study from a Danish county. British Journal of Obstetrics and Gynæcology 1992;99:422-429.

208. Harlow SD, Matanoski GM. The association between weight, physical activity, and stress and variation in the length of the menstrual cycle. American Journal of Epidemiology 1991;133:38-49.

209. Goldzieher JW, Henkins AE, Hamblen EC. Characteristics of normal menstrual cycle. American Journal of Obstetrics and Gynecology 1947;54:668-675.

210. Lenton EA, Landgren B-M, Sexton L. Normal variation in the length of the luteal phase of the menstrual cycle: identification of the short luteal phase. British Journal of Obstetrics and Gynæcology 1984;91:685-689.

211. Beitins IZ, McArthur JW, Turnbull BA, et al. Exercise induces two types of human luteal dysfunction: Confirmation by urinary free progesterone. Journal of Clinical Endocrinology and Metabolism 1991;72:1350-1358.

212. Treloar AE, Boynton RE, Behn BG, Brown BW. Variation of the human menstrual cycle through reproductive life. International Journal of Fertility 1967;12:77-126.

213. Gray RH. Patterns of bleeding associated with the use of steroidal contraceptives. In: Diczfalusy E, Fraser IS, Webb FTG, ed. Endometrial Bleeding and Steroidal Contraception. Proceedings of a Symposium on Steroid Contraception and Mechanisms of Endometrial Bleeding organized by the WHO in Geneva on 12-14 September, 1979. Bath, England: Pitman Press Ltd, 1980: 14-49.

214. Schwarz BE. Reference period analysis of vaginal bleeding with triphasic oral contraceptive agents containing norethindrone or levonorgestrel: A comparison study. International Journal of Fertility 1992;37(3):176-182.

215. Sporrong T, Rybo G, Mattsson LA. An objective and subjective assessment of uterine blood loss in postmenopausal women on hormone replacement therapy. British Journal of Obstetrics and Gynæcology 1992;99:399-401.

216. Harlow SD, Campbell BC. Host factors that influence the duration of menstrual bleeding. Epidemiology. 1994;5:352-355.

217. Guillebaud J, Bonnar J. Longer though lighter menstrual and intermenstrual bleeding with copper as compared to inert intrauterine devices. British Journal of Obstetrics and Gynæcology 1978;85:707-712.

218. Hallberg L, Hogdahl AM, Nilsson L, Rybo G. Menstrual blood loss - a population study. Acta Obstetricia et Gynecologica Scandinavica 1966;45:320-351.

219. Ji G, Li-yuan M, Su Z, et al. Menstrual blood loss in healthy Chinese women. Contraception. 1981;23:591-601.

220. Collett ME, Wertenberger GE, Fiske VM. The effect of age upon the pattern of the menstrual cycle. Fertility and Sterility 1954;45:437-448.

221. Kirchengast S. Intercorrelation between menstrual cycle patterns and body dimensions in Austrian women. Journal of Biosocial Science 1994;26:207-216.

222. Harlow SD, Zeger SL. An application of longitudinal methods to the analysis of menstrual diary data. Journal of Clinical Epidemiology 1991;44:1015-1025.

223. Belsey EM, Farley TMM. The analysis of menstrual bleeding patterns: A review. Appl Stochastic Models Data Anal 1987;3:125-150.

224. Lindquist O, Bengtsson C. Menopausal age in relation to smoking. Acta Medica Scandinavica 1979;205:73-77.

225. Andersen FS, Transbol I, Christiansen C. Is cigarette smoking a promotor of the menopause ? Acta Medica Scandinavica 1982;212:137-139.

226. Doll R, Gray R, Hafner B, Peto R. Mortality in relation to smoking: 22 years' observations on female British doctors. British Medical Journal 1980;280:967-971.

227. Weir HK, Sloan M, Kreiger N. The relationship between cigarette smoking and the risk of endometrial neoplasms. International Journal of Epidemiology 1994;23:261-266.

228. Jensen J, Christiansen C. Effects of smoking on serum lipoproteins and bone mineral content during postmenopausal hormone replacement therapy. American Journal of Obstetrics and Gynecology 1988;159:820-825.

229. Baron JA, Vecchia CL, Levi F. The antiestrogenic effect of cigarette smoking in women. American Journal of Obstetrics and Gynecology 1990;162:502-514.

230. Michnovicz JJ, Hershcopf RJ, Naganuma H, et al. Increased 2-hydroxylation of estradiol as a possible mechanism for the anti-estrogenic effect of cigarette smoking. New England Journal of Medicine 1986;315:1305-1309.

231. Jensen J, Christiansen C, Rodbro P. Cigarette smoking, serum estrogens, and bone loss during hormone replecement therapy early after menopause. New England Journal of Medicine 1985;313x:973-975.

232. Khaw K-T, Tazuke S, Barrett-Connor E. Cigarette smoking and levels of adrenal androgens in postmenopausal women. New England Journal of Medicine 1988;318:1705-1709.

233. Vollman RF. The menstrual cycle. In: Major problems in Obstetrics and Gynaecology. Philadelphia: W. B. Saunders, 1977.

234. Buckley CH, Fox H. Biopsy pathology of the endometrium. New York: Raven Press, 1989:11-30.

235. Bell SC. Assessment of endometrial differentiation and function. British Medical Bulletin 1990;46(3):720-32.

236. Manners CV. Endometrial assessment in a group of infertile women on stimulated cycles for IVF: immunohistochemical findings. Human Reproduction 1990;5(2):128-132.

237. Hampson F, Gerlis LM. Some form variations in endometrial tubules. Journal of Obstetrics and Gynæcology of the British Empire 1954;61:744-749.

238. Bulmer JN, Longfellow M, Ritson A. Leukocytes and resident blood cells in endometrium. Annals of the New York Academy of Sciences 1991;622:57-68.

239. Hamperl H, Hellweg G. Granular endometrial stroma cells. Obstetrics and Gynecology 1958;11(4):379-387.

240. Ritson A, Bulmer JN. Endometrial granulocytes in human decidua react with a natural-killer (NK) cell marker, NKH1. Immunology 1987;62:329-331.

241. Bulmer JN, Morrison L, Longfellow M, Ritson A, Pace D. Granulated lymphocytes in human endometrium: histochemical and immuno-histochemical studies. Human Reproduction 1991;6(6):791-798.

242. Clark DA, Daya S. Macrophages and other migratory cells in endometrium: relevance to endometrial bleeding. In: D'Arcangues C, Fraser IS, Newton JR, and Odlind V, ed. Contraception and mechanisms of endometrial bleeding. Scientific basis of fertility regulation. Proceedings of a symposium organized by the special programme of research, development and research training in Human reproduction of the WHO in Geneva on 28 November-2 December 1988. Cambridge: Cambridge University Press, 1990: 363-382.

243. Pace D, Morrison L, Bulmer NJ. Proliferative activity in endometrial stromal granulocytes throughout menstrual cycle and early human pregnancy. Journal of Clinical Pathology 1989;42:35-39.

244. Bulmer JN, Hollings D, Ritson A. Immunocytochemical evidence that endometrial stromal granulocytes are granulated lymphocytes. Journal of Pathology 1987;153:281-288.

245. Marzusch K, Ruck P, Geiselhart A, et al. Distribution of cell adhesion molecules on CD56++, CD3-, CD16- large granular lymphocytes and endothelial cells in first-trimester human decidua. Human Reproduction 1993;8(8):1203-1208.

246. Tabibzadeh S. Proliferative activity of lymphoid cells in human endometrium throughout the menstrual cycle. Journal of Clinical Endocrinology and Metabolism 1990;70(2):437-443.

247. Starkey PM, Clover LM, Rees MPC. Variation during the menstrual cycle of immune cell populations in human endometrium. European Journal of Obstetrics and Gynecology and Reproductive Biology 1991;39(3):203-207.

248. Haller H, Radillo O, Rukavina D, et al. An immunohistochemical study of leucocytes in human endometrium, first and third trimester basal decidua. Journal of Reproductive Immunology 1993;23:41-49.

249. Klentzeris LD, Bulmer JN, Warren A, et al. Endometrial lymphoid tissue in the timed endometrial biopsy: morphometric and immunohistochemical aspects. American Journal of Obstetrics and Gynecology 1992;167(3):667-674.

250. Bulmer JN, Ritson A. The decidua in early pregnancy. In: Beard RW, Sharp F. ed. Early Pregnancy Loss Mechanism and Treatment. London: Royal College of Obstetricians and Gynæcologists, 1988:171-180.

251. Robertson SA, Mayrhofer G, Seamark RF. Ovarian steroid hormones regulate granulocyte-macrophage colony-stimulating factor synthesis by uterine epithelial cells in the mouse. Biology of Reproduction 1996;54:183-196.

252. Booker SS, Jayanetti C, Karalak S, Hsiu J-G, Archer D. The effect of progesterone on the accumulation of leukocytes in the human endometrium. American Journal of Obstetrics and Gynecology 1994;171:139-142.

253. Song jY, Russell P, Markham R, Manconi F, Fraser IS. Effect of high dose progestogens on white cells and necrosis in human endometrium. Human Reproduction 1996;11(8):1713-1718.

254. King A, Gardner L, Loke YW. Evaluation of œstrogen and progesterone receptor expression in uterine mucosal lymphocytes. Human Reproduction 1996;11(5):1079-1082.

255. Jones RL, Kelly RW, Critchley HOD. Chemokine and cyclooxygenase-2 expression in human endometrium coincides with leukocyte accumulation. Human Reproduction 1997;12(6):1300-1306.

256. Roth SJ, Woldemar Carr M, Springer TA. C-C chemokines, but not the C-X-C chemokines interleukin-8 and interferon-gamma inducible protein-10, stimulate transendothelial chemotaxis of T lymphocytes. European Journal of Immunology 1995;25:3482-3488.

257. Allavena P, Bianchi G, Zhou D, et al. Induction of natural-killer-cell migration by monocyte chemotactic protein-1, protein-2, and protein-3. European Journal of Immunology 1994;24(12):3233-3236.

258. Ishihara O, Matsuoka K, Kinoshita K, et al. Interleukin-1β-stimulated PGE_2 production from early first trimester human decidual cells is inhibited by dexamethasone and progesterone. Prostaglandins 1995;49(1):15-26.

259. Wang JM, Colella S, Allavena P, Mantovani A. Chemotactic activity of human recombinant granulocyte-macrophage colony-stimulating factor. Immunology 1987;60(3):439-444.

260. Critchley HOD, Kelly RW, Lea RG, et al. Sex steroid regulation of leukocyte traffic in human decidua. Human Reproduction 1996;11(10):2257-2262.

261. Lea RG, Clark DA. Macrophages and migratory cells in endometrium relevant to implantation. Bailliere's Clinical Obstetrics and Gynæcology 1991;5(1):25-59.

262. Campana D, Thompson JS, Amlot P, et al. The cytoplasmic expression of CD3 antigens in normal and malignant cells of the T lymphoid lineage. Journal of Immunology 1987;138:648-55.

263. Ferenczy A, Bertrand G, Gelfand MM. Proliferation kinetics of human endometrium during the normal menstrual cycle. American Journal of Obstetrics and Gynecology 1979;133:859-867.

264. Alison M, Chaudry Z, Baker J, et al. Liver regeneration: A comparison on *in situ* Hybridization for Histone mRNA with bromodeoxyuridine labeling for the detection of S-phase cells. Histochemistry and Cytochemistry 1994;42:1603-1608.

265. Yu C-W, Woods AL, Levison DA. The assessment of cellular proliferation by immunohistochemistry: a review of currently available methods and their applications. Histochemical Journal 1992;24:121-131.

266. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. International Journal of Cancer 1983;31:13-20.

267. Gerdes J, Becker MHG, Key G. Immunohistological detection of tumour growth fraction (Ki-67 antigen) in formalin fixed and routinely processed tissue. Journal of Pathology 1992;168:85.

268. Schwarting R. Little missed markers and Ki-67. Laboratory Investigation 1993;68:597.

269. Chou MY, Chang ALC, McBride J, Donoff B, Gallagher GT, Wong DTW. A rapid method to determine proliferation patterns of normal and malignant tissues by H3 mRNA in situ hybridization. American Journal of Pathology 1990;136:729.

270. Bosch FX, Udvarhelyi N, Venter E, et al. Expression of the histone H3 gene in benign, semi-malignant and malignant lesions of the head and neck: a reliable proliferation marker. European Journal of Cancer 1993;29A(10):1454-1461.

271. Leake R, Carr L, Rinaldi F. Autocrine and paracrine effects in the endometrium. Annals of the New York Academy of Sciences 1991;622:145-148.

272. Ferenczy A. Studies on the cytodynamics of human endometrial regeneration. I.Scanning electron microscopy. American Journal of Obstetrics and Gynecology 1976;124(1):64-74.

273. Scott RJ, Hall PA, Haldane JS, et al. A comparison of immunohistochemical markers of cell proliferation with experimentally determined growth fraction. Journal of Pathology 1991;165(2):173-8.

274. Gorski J, Stormshak F, Harris J, Wertz N. Hormonal regulation of growth: Stimulatory and inhibitory influences of estrogen on DNA synthesis. Journal ofToxicology and Environmental Health 1977;3(271-9).

275. Tabibzadeh S. Regulatory roles of IFN-γ in human endometrium. Annals of the New York Academy of Sciences 1994;734:1-6.

276. Stewart CJR, Farquharson MA, Foulis AK. The distribution and possible function of gamma interferon-immunoreactive cells in normal endometrium and myometrium. Virchows Archiv A Pathological Anatomy and Histopathology 1992;420:419-424.

277. Klein NA, Pergola GM, Tekmal RR, et al. Cytokine regulation of cellular proliferation in endometriosis. Annals of the New York Academy of Sciences 1994;734:322-332.

278. King A, Wooding P, Gardner L, Loke YW. Expression of perforin, granzyme A and TIA-1 by human uterine CD56+ NK cells implies they are activated and capable of effector functions. Human Reproduction 1993;8:2061-2067.

279. Hameed A, Fox WM, Kurman RJ, et al. Perforin expression in endometrium during the menstrual cycle. International Journal of Gynecological Pathology 1995;14:143-150.

280. Ferenczy A. Studies on the cytodynamics of human endometrial regeneration. II.Transmission electron microscopy and histochemistry. American Journal of Obstetrics and Gynecology 1976;124(6):582-595.

281. Wynn RM. Histology and ultrastructure of the human endometrium. In: Wynn RM, ed. Biology of the uterus. New York, London: Plenum Press, 1977: 341-376.

282. Wilborn WH, Flowers CE. Cellular mechanisms for endometrial conservation during menstrual bleeding. Seminars in Reproductive Endocrinology 1984;2(4):307-341.

283. Kerr JF, Wyllie AH, Currie AH. Apoptosis, a basic biological phenomenon - with wider implications in tissue kinetics. British Journal of Cancer 1972;26:239-245.

284. Vinatier D, Dufour P, Subtil D. Apoptosis: A programmed cell death involved in ovarian and uterine physiology. European Journal of Obstetrics and Gynecology and Reproductive Biology 1996;67:85-102.

285. Wyllie AH, Kerr JF, Macaskill IAM, Currie AR. Adrenocortical cell deletion: the role of ACTH. Journal of Pathology 1973;111:85-94.

286. Kyprianou N, Isaacs JT. Activation of programmed cell death in the rat ventral prostate after castration. Endocrinology 1988;122:552-562.

287. Nawaz S, Lynch MP, Galand P, Gerschenson LE. Hormonal regulation of cell death in rabbit uterine epithelium. American Journal of Pathology 1987;127:51-59.

288. Hopwood D, Levison DA. Atrophy and apoptosis in the cyclical human endometrium. Journal of Pathology 1976;119:159-166.

289. Arends MJ, Wyllie AH. Apoptosis: mechanisms and roles in pathology. International Review of Experimental Pathology 1991;32:223-254.

290. Cohen Gm, Sun X-M, Snowden RT, et al. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochemical Journal 1992;286:331-334.

291. Piacentini M, Fesus L, Farrace MG, et al. The expression of tissue translutaminase in two human cancer cell lines is related with programmed cell death apoptosis. European Journal of Cell Biology 1991;(54):246-254.

292. Shiver JW, Su L, Henkart PA. Cytotoxicity with target DNA breakdown by rat basophilic leukaemia cells expressing both cytolysin and granzyme A. Cell 1992;71:315-322.

293. Moretta A. Molecular mechanisms in cell-mediated cytotoxicity. Cell 1997;90:13-18.

294. Talanian RV, Yang XH, Turbov J, et al. Granule-mediated Killing: Pathways for Granzyme B-initiated Apoptosis. Journal of Experimental Medicine 1997;186(8):1323-1331.

295. Baker SJ, Reddy EP. Transducers of life and Death: TNF receptor superfamily and associated proteins. Oncogene 1996;12:1-9.

296. Tabibzadeh S, Kong QF, Satyaswaroop PG, et al. Distinct regional and menstrual cycle dependent distribution of apoptosis in human endometrium. Potential regulatory role of T cells and TNF-α. Endocrine Journal 1994;2:87-95.

297. Peter ME, Heufelder AE, Hengartner MO. Advances in apoptosis research. Proceedings of the National Academy of Sciences USA 1997;94:12736-12737.

298. Moulton B. Transforming growth factor-β stimulates endometrial stromal apoptosis in vitro. Endocrinology 1994;134(3):1055-1060.

299. Kothapalli R, Buyuksal I, Wu S-Q, et al. Detection of *ebaf*, a novel human gene of the transforming growth factor-β superfamily. Association of gene expression with endometrial bleeding. Journal of Clinical Investigation 1997;99(10):2342-2350.

300. Hockenbery DM, Nunez G, Milliman C, et al. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 1990;348:334-336.

301. McLaren J, Prentice A, Charnock-Jones DS, et al. Immunolocalizatoin of the apoptosis regulating proteins Bcl-2 and Bax in human endometrium and isolated peritoneal fluid macrophages in endometriosis. Human Reproduction 1997;12(1):146-152.

302. Renvoizé C, Roger R, Moulian N, Bertoglio J, Bréard J. Bcl-2 expression in target cells leads to functional inhibition of caspase-3 protease family in human NK and lymphokine-activated killer cell granule-mediated apoptosis. Journal of Immunology 1997;159:126-134.

303. Sutton VR, Vaux DL, Trapani JA. Bcl-2 prevents apoptosis induced by perforin and granzyme B, but not that mediated by whole cytotoxic lymphocytes. Journal of Immunology 1997;158:5783-5790.

304. Shi L, Kam C-M, Powers CJ, et al. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. Journal of Experimental Medicine 1992;176:1521-1526.

305. Spencer SJ, Cataldo NA, Jaffe RB. Apoptosis in the human female reproductive tract. Obstetrical and Gynecological Survey 1996;51(5):314-323.

306. Reed JC. Double identity for proteins of the Bcl-2 family. Nature 1997;387:773-776.

307. Bartelmez GW. The phases of the menstrual cycle and their interpretation in terms of the pregnancy cycle. American Journal of Obstetrics and Gynecology 1957;74:931-955.

308. Rotello RJ, Hocker MB, Gerschenson LE. Biochemical evidence for programmed cell death in rabbit uterine epithelium. American Journal of Pathology 1989;134(3):491-495.

309. Watanabe H, Kanzaki H, Narukawa S, et al. Bcl-2 and Fas expression in eutopic and ectopic human endometrium during the menstrual cycle in relation to endometrial cell apoptosis. American Journal of Obstetrics and Gynecology 1997;176(2):360-368.

310. Kokawa K, Shikone T, Nakano R. Apoptosis in the human uterine endometrium during the menstrual cycle. Journal of Clinical Endocrinology and Matabolism 1996;81(11):4144-4147.

311. Finn CA, Publicover M. Hormonal control of cell death in the luminal epithelium of the mouse uterus. Journal of Endocrinology 1981;91:335-340.

312. Sandow BA, West NB, Norman R, Brenner RM. Hormonal control of apoptosis in Hamster uterine luminal epithelium. American Journal of Anatomy 1979;156:15-36.

313. Hockenbery DM, Zutter M, Hickey W, et al. BCL-2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proceedings of the National Academy of Sciences USA 1991;88:6961-6965.

314. Otsuki Y, Misaki O, Sugimoto O, et al. Cyclic *bcl-2* gene expression in human uterine endometrium during menstrual cycle. Lancet 1994;344:28-29.

315. Tao X-J, Tilly KI, Maravei DV, et al. Differential expression of members of the *bcl-2* gene family in proliferative and secretory human endometrium: glandular epithelial cell apoptosis is associated with increased expression of *bax*. Journal of Clinical Endocrinology and Metabolism 1997;82(8):2738-2746.

316. Gompel A, Sabourin J-C, Martin A, et al. Bcl-2 expression in the endometrium during the menstrual cycle. Second conference on The Endometrium, Sept 1993. Bologna, Italy 1993; 10.

317. Koh EAT, Illingworth PJ, Duncan WC, Critchley HOD. Immunolocalization of bcl-2 protein in human endometrium in the menstrual cycle and simulated early pregnancy. Molecular Human Reproduction 1995;1:1557-1562.

318. Gu Y, Jow G, Moulton B, et al. Apoptosis in decidual tissue regression and reorganization. Endocrinology 1994;135(3):1272-1279.

319. Hennet T, Bertoni G, Richter C, Peterhans E. Expression of bcl-2 protein enhances the survival of mouse fibroblastoid cells in tumor necrosis factor-mediated cytotoxicity. Cancer Research 1993;53:1456-1460.

320. Press MF, Nousek-Goebl NA, Greene GL. Immunoelectron microscopic localization of estrogen receptor with monoclonal estrophilin antibodies. Journal of Histochemistry and Cytochemistry 1985;33(9):915-924.

321. Gustafsson J-A. Estrogen receptor β - Getting in on the action? Nature Medicine 1997;3(5):493-494.

322. Ciocca DR, Asch RH, Adams DJ, McGuire WL. Evidence for modulation of a 24K protein in human endomtrium during the menstrual cycle. Journal of Clinical Endocrinology and Matabolism 1983;57:496-499.

323. McGuire SE, Fuqua SA, Naylor SL, et al. Chromosomal assignments of human 27kDa heat shock protein gene family. Somatic Cell and Molecular Genetics 1989;15:167-171.

324. Ciocca DR, Oesterreich S, Chamness GC, et al. Biological and clinical implications of heat shock protein 27000 (Hsp27): a review. Journal of the National Cancer Institute 1993;85:1558-1570.

325. McGuire WL, Dressler LG, Sledge GW, et al. An estrogen-regulated protein in normal and malignant endometrium. Journal of Steroid Biochemistry 1986;24(1):155-159.

326. Ciocca DR, Puy LA, Fasoli LC. Study of estrogen receptor, progesterone receptor, and the estrogen-regulated Mr 24,000 protein in patients with carcinomas of the endometrium and cervix. Cancer Research 1989;49:4298-4304.

327. Amso NN, Crow J, Shaw RW. Comparative immunohistochemical study of œstrogen and progesterone receptors in the fallopian tube and uterus at different stages of the menstrual cycle and the menopause. Human Reproduction 1994;9(6):1027-1037.

328. Richards J. Estradiol receptor content in rat granulosa cells during follicular development: Modification by estradiol and gonadotropins. Endocrinology 1975;97(5):1174-1184.

329. Chaudhuri PK, Chaudhuri B, Patel N. Modulation of estrogen receptor by insulin and its biologic significance. Archives of Surgery 1986;121:1322-1325.

330. Freyschuss B, Sahlin L, Masironi B, Eriksson H. The hormonal regulation of the œstrogen receptor in rat liver: an interplay involving growth hormone, thyroid hormones and glucocorticoids. Journal of Endocrinology 1994;142:285-298.

331. Stoica A, Saceda M, Fakhro A, et al. The role of transforming growth factor-β in the regulation of estrogen receptor expression in the MCF-7 breast cancer cell line. Endocrinology 1997;138:1498-1505.

332. Baniahmad A, Ming-Jer T. Mechanisms of transcriptional activation by steroid hormone receptors. Journal of Cell Biochemistry 1993;51:151-156.

333. Ciocca DR, Puy LA, Edwards DP, et al. The presence of an estrogen-regulated protein detected by monoclonal antibody in abnormal human endometrium. Journal of Clinical Endocrinology and Matabolism 1985;60:137-143.

334. Padwick ML, Whitehead M, King RJ. Hormonal regulation of HSP27 expression in human endometrial epithelial and stromal cells. Molecular and Cellular Endocrinology 1994;102(1-2):9-14.

335. Budwit-Novotny DA, McCarty SKS, Cox EB, et al. Immuno-histochemical analyses of estrogen receptor in endometrial adenocarcinoma using a monoclonal antibody. Cancer Research 1986;46(10):5419.

336. Bacus S, Flowers JL, Press MF, et al. The evaluation of estrogen receptor in primary breast carcinoma by computer-assisted image analysis. American Journal of Clinical Pathology 1988;90:233-239.

337. Aziz DC. Quantitation of estrogen and progesterone receptors by immunocytochemical and image analysis. American Journal of Clinical Pathology 1992;98:105-111.

338. Esteban JM, Kandalaft PL, Mehta P, et al. Improvement of the quantification of estrogen and progesterone receptors in paraffin-embedded tumors by image analysis. American Journal of Clinical Pathology 1993;99:32-38.

339. King RJB, Townsend PT, Whitehead MI, et al. Biochemical analyses of separated epithelium and stroma from endometria of premenopausal and postmenopausal women receiving estrogen and progestins. Journal of Steroid Biochemistry 1981;14:979-987.

340. Perrot-Applanat M, Groyer-Picard MT, Garcia E, et al. Immuno-cytochemical demonstration of estrogen and progesterone receptors in muscle cells of uterine arteries in rabbits and humans. Endocrinology 1988;123:1511-1519.

341. Critchley HOD, Abberton KM, Taylor NH, et al. Endometrial sex steroid receptor expresion in women with menorrhagia. British Journal of Obstetrics and Gynæcology 1994;101:428-434.

342. Katzenellenbogen BS. Estrogen receptors: bioactivities and interactions with cell signaling pathways. Biology of Reproduction 1996;54:287-293.

343. Saceda M, Grunt TW, Colomer R, et al. Regulation of estrogen receptor concentration and activity by an erbB/HER ligand in breast carcinoma cell lines. Endocrinology 1996;137:4322-4330.

344. Seppala M, Koistinen R, Rutanen E-M. Uterine endocrinology and paracrinology: insulin-like growth factor binding protein-1 and placental protein-14 revisited. Human Reproduction 1994;9(5):917-925.

345. Waites GT, Wood PL, Walker RA, Bell SC. Immunohistological localization of human endometrial secretory protein, 'pregnancy-associated endometrial α_2-globulin' (α_2-PEG), during the menstrual cycle. Journal of Reproduction and Fertility 1988;82(2):665-72.

346. Seppala M, Angervo M, Koistinen R, et al. Human endometrial protein secretion relative to implantation. Bailliere's Clinical Obstetrics and Gynæcology 1991;5(1):61-72.

347. Waites GT, James RFL, Bell SC. Immunohistological localisation of the human endometrial secretory protein "pregnancy associated endometrial α_1 globulin" (α_1-PEG) an insulin-like growth factor binding protein, during the menstrual cycle. Journal of Clinical Endocrinology and Metabolism 1988;67:1100-1104.

348. Check JH, Nowroozi K, Check ML, Vetter B. Correlation of serum progestagen-associated endometrial protein levels with endometrial biopsies, serum steroid levels and therapy for luteal phase defects. Human Reproduction 1991;6:511-514.

349. Vaisse C, Atger M, Potier B, Milgrom E. Human placental protein 14 gene: sequence and characterization of a short duplication. DNA and Cell Biology 1990;9:401-413.

350. Abberton KM, Taylor NH, Healy NH, Rogers PAW. Vascular smooth muscle α-actin distribution around endometrial arterioles during the menstrual cycle: increased expression during the perimenopause and lack of correlation with menorrhagia. Human Reproduction 1996;11(1):204-211.

351. Bird CC, Willis RA. The production of smooth muscle by the endometrial stroma of the adult human uterus. Journal of Pathology and Bacteriology 1965;90:75-81.

352. Franquemont DW, Frierson HF, Mills SE. An immunohistochemical study of normal endometrial stroma and endometrial stromal neoplasms. American Journal of Surgical Pathology 1991;15(9):861-870.

353. Czernobilsky B, Remadi S, Gabbiani G. Alpha-smooth muscle actin and other stromal markers in endometrial mucosa. Virchows Archiv A Pathological Anatomy and Histopathology 1993;422(4):313-7.

354. Skalli O, Ropraz P, Trzeciak A, et al. A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. Journal of Cell Biology 1986;103:2787-96.

355. Rees MCP, Heryet AR, Bicknell R. Immunohistochemical properties of the endothelial cells in the human uterus during the menstrual cycle. Human Reproduction 1993;8(8):1173-1178.

356. Zhu P, Gu Z. Observation of the activity of Factor VIII in the endometrium of women with regular menstrual cycles. Human Reproduction 1988;3:273-275.

357. Au CL, Rogers PAW. Immunohistochemical staining of von Willebrand factor in human endometrium during normal menstrual cycle. Human Reproduction 1993;8(1):17-23.

358. Morgan KG, Wilkinson N, Buckley H. Angiogenesis in normal, hyperplasic, and neoplastic endometrium. Journal of Pathology 1996;179:317-320.

359. Harrison RL, Mckee PA. Estrogen stimulates von Willebrand factor production by cultured endothelial cells. Blood 1984;63:657-665.

360. Hourihan HM, Sheppard BL, Belsey EM, Brosens IA. Endometrial vascular features prior to and following exposure to levonorgestrel. Contraception 1991;43:375-385.

361. Vollmer G, Siegal GP, Chiquet-Ehrismann R, et al. Tenascin expression in the human endometrium and in endometrial adenocarcinomas. Laboratory Investigation 1990;62:725-730.

362. Johannisson E. Effects of œstradiol and progesterone on the synthesis of DNA and the anti-haemophilic factor VIII antigen in human endometrial endothelial cells in vitro: a pilot study. Human Reproduction 1986;1:207-212.

363. Kaiserman-Abramof IR, Padykula HA. Ultrastructural epithelial zonation of the primate endometrium (Rhesus Monkey). American Journal of Anatomy 1989;184:13-30.

364. Roberts DK, Parmley TH, Walker MJ, Horbelt DV. Ultrastructure of the microvasculature in the human endometrium throughout the normal menstrual cycle. American Journal of Obstetrics and Gynecology 1992;166:1393-1406.

365. Shifren JL, Tseng JF, Zaloudek CJ, et al. Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the

menstrual cycle and in the pathogenesis of endometriosis. Journal of Clinical Endocrinology and Metabolism 1996;81(8):3112-3118.

366. Aplin JD, Seif MW, Graham RA, et al. The endometrial cell surface and implantation. Annals of the New York Academy of Sciences 1994;734:103-121.

367. Odlind V, Fraser IS. Contraception and menstrual bleeding disturbances: a clinical overview. In: D'Arcangues C, Fraser IS, Newton JR, and Odlind V, ed. Contraception and mechanisms of endometrial bleeding. Scientific basis of fertility regulation. Proceedings of a symposium organized by the special programme of research, development and research training in Human reproduction of the WHO in Geneva on 28 November-2 December 1988. Cambridge: Cambridge University Press, 1990: 5-32.

368. Al-Azzawi F, Habiba M. Regular bleeding on hormone replacement therapy: a myth? British Journal of Obstetrics and Gynæcology 1994;101:661-662.

369. Woodruff JD, Pickar JH. Incidence of endometrial hyperplasia in postmenopausal women taking conjugated estrogens (Premarin) with medroxyprogesterone acetate or conjugated estrogens alone. American Journal of Obstetrics and Gynecology 1994;170(5):1213-1223.

370. Habiba MA, Bell SC, Al-Azzawi F. Endometrial responses to hormone replacement therapy: Histological features compared to late luteal phase endometrium. Human Reproduction 1998:In Press.

371. Habiba MA, Bell SC, Abrams K, Al-Azzawi F. Endometrial responses to hormone replacement therapy: The bleeding pattern. Human Reproduction 1996;11:503-508.

372. Speroff L, Glass RH, Kase NG. Clinical Gynecologic Endocrinology and Infertility. Baltimore, Hong Kong, London, Sydney: Williams and Wilkins, 1989:91-120.

373. Lemus AE, Enriquez J, Garcia GA, et al. 5α-Reduction of norethisterone enhances its binding affinity for androgen receptors but diminishes its androgenic potency. Journal of Steroid Biochemistry and Molecular Biology 1997;60(1-2):121-129.

374. Suzuki K, Takezawa Y, Suzuki T, et al. Synergistic effects of estrogen with androgen on the prostate. Prostate 1994;25:169-176.

375. Castro I, Cerbon MA, Pasapera AM, et al. Molecular mechanisms of the antihormonal and anti-implantation effects of norethisterone and its A-ring reduced metabolites. Molecular Reproduction and Development 1995;40(2):157-163.

376. Tietze C. Reproductive span and rate of reproduction among Hutteritc women. Fertility and Sterility 1957;8:89-93.

377. Levran D, Ben-Shlomo I, Dor J, et al. Ageing of endometrium and oocytes: Observations on conception and ablation rates in an egg donation model. Fertility and Sterility 1991;56(6):1091-1094.

378. Check JH, Askari HA, Fisher C, Vanaman L. The use of a shared donor oocyte program to evaluate the effect of uterine senescence. Fertility and Sterility 1994;61(2):252-256.

379. Cano F, Simon C, Remohi J, Pellicer A. Effect of ageing on the female reproductive system: Evidence for a role of uterine senescence in the decline in female fecundity. Fertility and Sterility 1995;64:584-589.

380. Pellicer A, Simon C, Romohi J. Effects of ageing on the female reproductive system. Human Reproduction 1995;10:77-82.

381. Navot D, Bergh PA, Williams MA, et al. Poor oocyte quality rather than implantation failure as a cause of age related decline in female fertility. Lancet 1991;337:1375-1377.

382. Sauer MV, Paulson RJ, Lobo RA. Reversing the natural decline in human fertility. J AMA 1992;268:1275-1279.

383. Wramsby H, Freolga K, Liedholm P. Chromosome analysis of human oocytes recovered from preovulatory follicles in stimulated cycles. New England Journal of Medicine 1987;316:121-124.

384. Richardson SJ, Nelson JF. Follicular depletion during the menopausal transition. Annals of the New York Academy of Sciences 1990;592:13-18.

385. Wilcox AJ. Female fecundity as a function of age. New England Journal of Medicine 1982;307:373-378.

386. Craig SS, Jollie WP. Age changes in density of endometrial stromal cells of the rat. Experimental Gerontology 1985;20:93-96.

387. Han Z, Hokkonen CC, Roth GS. Effect of ageing in populations of estogen receptor-containing cells in the rat uterus. Experimental Cell Research 1989;180:234-239.

388. Noci I, Borri P, Chietti O, et al. I Ageing of the human endometrium: A basic morphological and immunohisto chemical study. European Journal of Obstetrics and Gynecology and Reproductive Biology 1995;63(2):181-185.

389. Noci I, Gheri G, Gheri Bryk SG, et al. II Ageing of human endometrium peri-implantation phase endometrium does not evidence any age dependent variation in lectin binding. European Journal of Obstetrics and Gynecology and Reproductive Biology 1996;64(1):11-27.

390. Rogers PA, Hosie MJ, Ortis A, et al. Uterine glandular area during the menstrual cycle and the effect of different in-vitro fertilization related hormonal treatments. Human Reproduction 1996;11:376-379.

391. Skalli O, Schurch W, Seemayer T, et al. Myofibroblasts from diverse pathologic settings are heterogeneous in their content of actin isoforms and intermediate filament proteins. Laboratory Investigation 1989;60(2):275-285.

392. Demopoulos RI. Normal endometrium. In: Blaustein A, ed. Pathology of the female genital tract. 2nd edition. New York, Heidelberg, Berlin: Springer-Verlag, 1982: 235-278.

393. Bell SC. Decidualization and relevance to menstruation. In: D'Arcangues C, Fraser IS, Newton JR, and Odlind V, ed. Contraception and mechanisms of endometrial bleeding. Scientific basis of fertility regulation. Proceedings of a symposium organized by the special programme of research, development and research training in Human reproduction of the WHO in Geneva on 28 November-2 December 1988. Cambridge: Cambridge University Press, 1991: 187-212.

394. Schatz F, Krikun G, Hausknecht V, et al. Physiological and clinical implications of decidualization-associated protease activity. Annals of the New York Academy of Sciences 1997;828:175-179.

395. Lockwood CJ, Kirkun G, Hausknecht V, et al. Decidual cell regulation of hemostasis during implantation and menstruation. Annals of the New York Academy of Sciences 1997;828:188-193.

396. Salamonsen LA, Woolley DE. Matrix metalloproteinases in normal menstruation. Human Reproduction 1996;11(Supplement 2):124-133.

397. Marshburn PB, Arici AM, Casey ML. Expression of transforming growth factor-β1 messenger ribonucleic acid and the modulation of deoxyribonucleic acid synthesis by transforming growth factor-β1 in human endometrial cells. American Journal of Obstetrics and Gynecology 1994;170:1152-1158.

398. Salamonsen LA, Butt AR, Hammond FR, et al. Production of endometrial matrix metalloproteinases, but not their tissue inhibitors, is modulated by progesterone withdrawal in an in Vitro model for menstruation. Journal of Clinical Endocrinology and Metabolism 1997;82(5):1409-1415.

399. Hulboy DL, Rudolph LA, Matrisian LM. Matrix metalloproteinases as mediators of reproductive function. Molecular Human Reproduction 1997;3(1):27-45.

400. Rogers WH, Matrisian LM, Giudice LC, et al. Pattern of matrix metalloproteinases expression in cycling endometrium imply differential functions and regulation by steroid hormones. Journal of Clinical Investigation 1994;94:946-953.

401. Frisch S, Ruley H. Transcription from the stromelysin promoter is induced by interleukin-1 and repressed by dexamethasone. Journal of Biological Chemistry 1987;262:16300-16304.

402. Gaire M, Magbanua Z, McDonnell S, et al. Structure and expression of the human gene for the matrix metalloproteinase matrilysin. Journal of Biological Chemistry 1994;269:2032-2040.

403. Jonat C, Rahmsdorf H, Park K, et al. Anti-tumor promotion and anti-inflammation: Down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell 1990;62:1189-1204.

404. Nicholson R, Mader S, Nagpal S, et al. Negative regulation of the rat stromelysin gene promoter by retinoic acid is mediated by an AP-1 binding site. EMBO Journal 1990;9:4443-4454.

405. Schatz F, Papp C, Aigner S, et al. Biological mechanisms underlying the clinical effects of RU 486: modulation of cultured endometrial stromal cell stromelysin-1 and prolactin expression. Journal of Clinical Endocrinology and Metabolism 1997;82(1):188-93.

406. Jeziorska M, Nagase H, Salamonsen LA, Woolley DE. Immunolocalization of the matrix metalloproteinases gelatinase B and stromelysin 1 in human endometrium throughout the menstrual cycle. Journal of Reproduction and Fertility 1996;107:43-51.

407. Campbell S, Cameron IT. The origins and physiology of menstruation. In: Cameron IT, Fraser IS, Smith SK, ed. Clinical disorders of the endometrium and menstrual cycle. Oxford, New York, Tokyo: Oxford University Press, 1998: 13-30.

408. Rogers PAW, Lederman F, Kooy J, Taylor NH, Healy DL. Endometrial vascular smooth muscle œstrogen and progesterone receptor distribution in women with and without menorrhagia. Human Reproduction 1996;11(9):2003-2008.

409. Tseng L, Zhu HH. Regulation of progesterone receptor messenger ribonucleic acid by progestin in human endometrial stromal cells. Biology of Reproduction 1997;57(6):1360-1366.

410. Lockwood CJ, Krikun G, Papp C, et al. The role of progestationally regulated stromal cell tissue factor and type-1 plasminogen activator inhibitor (PAI-1) in endometrial hemostasis and menstruation. Annals of the New York Academy of Sciences 1994;734:57-79.

411. Bamberger A-M, Bamberger CM, Gellersen B, Schulte HM. Modulation of AP-1 activity by the human progesterone receptor in endometrial adenocarcinoma cells. Proceedings of the National Academy of Sciences USA 1996;93:6169-6174.

412. Bethea CL, Widmann AA. Differential Expression of progestin receptor isoforms in the hypothalamus, pituitary, and endometrium of Rhesus Macaques. Endocrinology 1998;139(2):677-687.

413. Leslie KK, Kumar NS, Richer J, et al. Differential expression of the A and B isoforms of progesterone receptor in human endometrial cancer cells. Annals of the New York Academy of Sciences 1997;828:17-26.

414. Giangrande PH, Pollio G, McDonnell DP. Mapping and characterization of the functional domains responsible for the differential activity of the A and B isoforms of the human progesterone receptor. Journal of Biological Chemistry 1997;272(52):32889-32900.

415. Guenal I, Sidoti-de Fraisse C, Gaumer S, Mignotte B. Bcl-2 and HSP27 act at different levels to suppress programmed cell death. Oncogene 1997;15:347-360.

416. Al-Azzawi F, Habiba MA, Akkad AA, Phipps B. Endometrial histology and the bleeding pattern of postmenopausal women on continuous combined HRT compared to Tibolone. 8th International Conference on the Menopause, 3-7 November 1996. Abstract P235. Maturitas 1997;27 (Suppl):190.

417. Hickey M, Fraser I. Menstrual disturance and contraceptive steroids: mechanisms and management. In: Cameron IT, Fraser IS, Smith SK, ed. Clinical disorders of the endometrium and menstrual cycle. Oxford, New York, Tokyo: Oxford University Press, 1998: 399-414.

418. Alkhalaf M, Murphy LC. Regulation of c-*jun* and *jun*-B by progestins in T-47D human breast cancer cells. Molecular Endocrinology 1992;6:1625-1633.

419. McDonnell DP, Lieberman BA, Norris J. Development of Tissue-Selective estrogen receptor modulators. In: Baird DT, Schutz G, Krattenmacher R, ed. Organ-selective actions of steroid hormones. Ernst Schering Research Foundation Workshop 16. Berlin: Springer, 1995: 1-28.

420. Paech K, Webb P, Kulper GGJM, et al. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science 1997;277(5331):1508-1510.

421. Van Erpecum KJ, Van Berge Henegouwen GP, Verschoor L, Stoelwinder B, Willekens FL. Different hepatobiliary effects of oral and transdermal estradiol in postmenopausal women. Gastroenterology 1991;100(2):482-488.

422. Tuohimaa P, Blauer M, Pasanen S, et al. Mechanisms of action of sex steroid hormones: Basic concepts and clinical correlations. Maturitas 1996;23 Suppl:S3-S12.

423. Oropeza MV, Campos MG, Lemus AE, et al. Estrogenic actions of norethisterone and its A-ring reduced metabolites. Induction of in vitro uterine sensitivity to serotonin. Archives of Medical Research 1994;25(3):307-10.

424. Anderson ABM, Sklovsky E, Sayers L, Steele PA, Turnbull AC. Comparison of serum œstrogen concentrations in post-menopausal women taking œstrone sulphate and œstradiol. British Medical Journal 1978;i:140-142.

425. Aedo A-R, Landgren B-M, Diczfalusy E. Pharmacokinetics and biotransformation of orally administered œstrone sulphate and œstradiol valerate in post-menopasual women. Maturitas 1990;12:333-343.

426. Mertens HJ, Heineman MJ, Koudstaal J, et al. Androgen receptor content in human endometrium. European Journal of Obstetrics and Gynecology and Reproductive Biology 1996;70(1):11-3.

427. Schumacher M. Rapid membrane effects of steroid hormones: an emerging concept in neuroendocrinology. Trends in Neuroscience 1990;13:359-361.

428. Shapiro SS, Dyer RD, Colas AE. Synthetic progestins: In vitro potency on human endometrium and specific binding to cytosol receptors. American Journal of Obstetrics and Gynecology 1978;132:549-554.

429. Pasapera AM, Cerbon MA, Castro I, et al. Norethisterone metabolites modulate the uteroglobin and progesterone receptor gene expression in prepubertal rabbits. Biology of Reproduction 1995;52(2):426-32.

430. Back DJ, Breckenridge AM, Crawford FE, et al. Kinetics of norethindrone in women. i. Radioimmunoassay and concentrations during multiple dosing. Clinical Pharmacology and Therapeutics 1978;24(4):439-447.

431. Hartmann BW, Kirchengast S, Albrecht A, et al. Absorption of orally supplied natural estrogens correlated wit hage and somatometric parameters. Gynecological Endocrinology 1994;8:101-107.

Endometrial Responses to Hormone Replacement Therapy

Abstract

Combinations of an œstrogen and a progestogen are being increasingly used to correct the hypo-œstrogenic postmenopausal state. Underpinning this use is the assumed causal link between œstrogen deficiency and increased morbidity and mortality, and that 'replacement' using exogenous steroids will reverse the adverse effects of hypo-œstrogenism. In this thesis, the endometrial effects of cyclical combined HRT were used as an index of its systemic action. A subgroup of women was identified that exhibited a bleeding pattern reminiscent of the physiological cycle, but study of known structural and functional markers in the endometrium of this subgroup, demonstrated significant differences compared to the endometrium obtained from a corresponding phase of the physiological cycle. These differences were demonstrated both in the glands and the stroma, and were unexpected considering the similarity in clinical behaviour.

This dissociation between endometrial bleeding and known structural and functional markers, demonstrates the unreliability of bleeding as an indicator of a physiological effect on the endometrium, and by corollary, elsewhere in the body. Furthermore, features in the endometrium indicated a non-physiological effect. The differences in individual response indicate individual monitoring, and the lack of correlation between different markers indicates monitoring each effect. As this thesis refutes the hypothesis of a physiological effect of HRT, the need emerges for the identification of correlates of function in each body tissue.

With the view to develop new markers of function, a monoclonal antibody was developed that may be potentially useful in gauging the effect of HRT on the endometrium. But it was not possible to interpret the overall endometrial effects based on a two dimensional relation between œstrogen and progesterone, which may signify the importance of the androgenic properties of the administered progestogen as well as the effect(s) of the endogenous postmenopausal hormonal milieu.

SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN

Human Reproduction vol.11 no.3 pp.503–508, 1996

Endometrial responses to hormone replacement therapy: the bleeding pattern

M.A.Habiba[1,2], S.C.Bell[1,2], K.Abrams[3] and F.Al-Azzawi[1,2,4]

[1]Menopause Research Unit, [2]Department of Obstetrics and Gynaecology, [3]Department of Epidemiology and Public Health, University of Leicester, School of Medicine, Leicester LE2 7LX, UK

[4]To whom correspondence should be addressed at: Menopause Research Unit, Department of Obstetrics and Gynaecology, Robert Kilpatrick Building, Leicester Royal Infirmary, PO Box 65, Leicester LE2 7LX, UK

Little information is available concerning the response of the endometrium to exogenous sex steroid therapy, particularly in the post-menopausal state. In this study we examined the variability of the bleeding pattern in 103 post-menopausal women receiving cyclical sequential combined hormone replacement therapy (HRT) over 6 months. All patients kept menstrual diary cards to record the onset, duration and subjective assessment of the severity of bleeding. We defined a cycle as starting from the commencement of treatment till the day of onset of bleeding. Two groups were identified amongst 99 women who experienced bleeding: those with a mean cycle length of 29 or more days (late bleeders, n = 50) and those with shorter mean cycle length (early bleeders, n = 49). The former were characterized by less variability in cycle length and bleeding that was of shorter duration. Four women experienced no bleeding. There were no significant differences between the two groups in age, year since the menopause, weight, height, body mass index (BMI), parity, or in the previous use of HRT. The only significant difference was in their smoking habits. This suggests a possible link of a hypo-oestrogenic state to poor cycle control.

Key words: cycle variability/hormone replacement/menopause/menstrual bleeding pattern

Introduction

An average woman should expect to live one-third of her life after the menopause (Ferguson *et al.*, 1989). Most women will benefit from oestrogen replacement therapy, which confers cardiovascular protection, prevention of osteoporosis, and the relief from many of the distressing symptoms associated with the menopause (Daly *et al.*, 1992)

The addition of progestogen to oestrogen replacement regimens for the protection against endometrial hyperplasia and carcinoma has introduced hormone 'withdrawal' bleeding, which, especially if heavy or irregular, ranks among the most important causes for discontinuation of therapy. Up to 50% of those who stop treatment do so because of bleeding problems (Nachtigall, 1990). Despite this, our understanding of the mechanisms underlying bleeding in hormone replacement therapy (HRT) cycles remains limited.

The paucity of detailed studies of the bleeding pattern on HRT (Al-Azzawi *et al.*, 1994; Habiba and Al-Azzawi, 1994) is surprising, especially when this is compared to studies on normal menstrual patterns (Zuckerman, 1949; Treloar *et al.*, 1967; Chiazze *et al.*, 1968; Munster *et al.*, 1992) and bleeding associated with the use of oral contraceptive pills (Belsey, 1988; Belsey *et al.*, 1988).

Studies of the bleeding pattern on HRT regimens are important not only because unpredictable bleeding will adversely affect the acceptance of HRT (Al-Azzawi and Habiba, 1994), but also because they may provide insights into the mechanism(s) responsible for the control of bleeding *per se*, whether HRT-induced or in natural cycles

In the present study we have investigated the bleeding pattern in a group of women receiving HRT over 6 months of observation. As both the variation from cycle to cycle within an individual as well as the differences between women contribute significantly to our understanding of HRT cycles, both will be examined in this analysis.

We shall use an analytical model (Harlow and Zeger, 1991) that enables us to investigate the cyclical nature of HRT-induced bleeding, rather than the 90 day bleeding interval method. The latter method was adopted by the World Health Organization (WHO) in studies of menstrual bleeding patterns (Rodriguez *et al.*, 1976; Belsey and Farley, 1987; Belsey and Carlson, 1991). However, as its focus was on the effect of different modalities of contraceptives, some of which do not induce cyclical bleeding, its methodology and terminology regarded the menstrual cycle as consisting of a conglomerate of separate events which resulted in the separation of the concept of episodic bleeding from that of cyclicity (Harlow and Zeger, 1991). The 90 day bleeding interval method focuses on the collective numbers of cycles or 'bleeding episodes' experienced by the whole group rather than the experience of the individual woman. Although helpful in comparing different drug regimens, this approach is less helpful when we try to gain insight into the behaviour of the individual woman and into the mechanism(s) that influence cycle length and blood loss.

By defining the HRT cycle as the interval between the commencement of medication and the day of onset of bleeding, this paper attempts to answer two questions: firstly to determine intra-patient variability or cyclicity and secondly to determine what is the average cycle length and how cycles vary about this average in the whole group of patients. These questions

Table I. Reason for not being enrolled into the study following screening

	No.
High oestradiol (< 30 pg)	9
Changed her mind re participating	6
Age below or above specified	3
Lost to follow up	3
Administrative	1
11 months post menopausal	1
Entry biopsy not performed	1
Contraindication to HRT	5
Elevated liver enzymes	2
Developed thrombophlebitis	1
Bowel cancer	1

HRT = hormone replacement therapy.

are distinct (Burch *et al.*, 1967) and in addressing them we aimed to gain insight into different aspects of the cycle control on HRT.

Materials and methods

This study was an open design, one centre study with ambulatory patients. All women were recruited from among those referred to The Menopause Research Unit at Leicester Royal Infirmary, UK by their General Practitioner. All participants signed an informed consent approved by the local ethics committee. Participants adhered to a rigid schedule, the first consultation being a pre-study assessment for inclusion, 2–3 weeks prior to commencement of the 168 days of active medication. During the treatment period women attended clinic every 56 days. Our objective was to evaluate 100 completed patients. A total of 164 post-menopausal women were screened, between December, 1992 and September, 1994, according to the study protocol. Thirty-three patients subsequent to screening were found not to be eligible to continue with the study (Table I). The inclusion criteria were healthy non-hysterectomized post-menopausal women, aged between 44 and 62 years, who were at least 12 months since their last normal menstrual period, with serum oestradiol of < 100 pmol/l and serum follicle stimulating hormone (FSH) > 40 IU/l. None had received oral or topical oestrogen, progestogen or androgen within the previous 3 months. Women who had ever received oestradiol implants were excluded. Also excluded were women with contraindications to HRT. All women underwent routine investigations for parameters of liver, renal and thyroid function and those patients who had evidence of abnormalities were excluded. All women had a normal mammogram and a normal cervical smear before commencing therapy. The normality of the endometrium was confirmed by an endometrial pipelle biopsy (Laboratoire CCD, Paris, France) prior to enrolment into the study. Patients were to be excluded if this biopsy could not be obtained because of administrative or technical reasons or if the baseline biopsy showed evidence of endometrial hyperplasia or malignancy. A pelvic examination was performed at the beginning of the study; patients with abnormalities on vaginal examination were excluded. Additional oestrogenic, progestogenic or androgenic preparations were not allowed during the study. Drugs known to influence the metabolic clearance of steroids by the liver were contraindicated.

All women were prescribed a regimen of 2 mg of oestradiol (Solray Pharma, GmbH) valerate daily with the addition of 1 mg of norethisterone (Solray Pharma, GmbH) from day 17 through to day 28 of each cycle.

The final visit was planned to be at the end of the last 2 days of tablet intake (day 27 or 28 of cycle 6) when a general physical examination, blood safety tests and an endometrial biopsy were performed.

Bleeding episodes: records and description

Patients filled in Diary Cards during the study period to document intake of study medication and to record the days of bleeding and its severity. The card design was such as to minimize any chances of inaccuracy. Bleeding was graded as: no bleeding (0), spotting (1), normal (2), heavy (3). The total bleeding score was represented by the summation of the daily scores. This total bleeding score was divided by the duration of bleeding to calculate the average bleeding score.

Diaries were collected by the investigators at each visit and checked for accuracy and clarity. Adherence to the regimen was verified by checking the patients' diary cards and by collecting the returned medication packs and counting any remaining tablets. The documentation on the diaries was verified against the returned blister packs. All adverse events and concomitant medications were recorded.

For the description of bleeding episodes, we adopted the following criteria: bleeding was defined as any vaginal blood loss requiring the use of such protection as pads or tampons. Spotting was defined as any vaginal blood loss not requiring sanitary protection. A period was defined as at least 2 consecutive days during which blood loss (bleeding or spotting) was entered on the calendar record and that was bounded by more than one bleeding/spotting-free day. Breakthrough bleeding was defined as any bleeding/spotting episode that occurred between consecutive periods and was separated from the period days by > 1 bleeding/spotting-free day. For the purpose of this analysis, we took the day of commencement of the oestrogenic phase of the cycle as the fixed point of reference. We thus defined the cycle as starting on the day of commencement of oestrogen to the day of onset of bleeding.

Bleeding episodes: data analysis

One objective was to describe the variability in cycle length in individual women. We assessed this relationship by estimating the probability of transition from a given cycle length to various alternative cycle lengths and by examining the degree of correlation between length of the current cycle and length of subsequent cycles.

Cycles were divided into five categories according to length, < 26, 26, 27, 28, 29, 30, 31, and > 31 days. To determine the variability in cycle length in an individual woman, we estimated the probability of the woman having a similar or an alternative cycle length in the following cycle. This transition probability was estimated as follows. The probability of transition from category i to category j was calculated as being the ratio of the total number of times the kth woman started in category i and moved to j, to the total number of times the kth woman started in category i. These ratios are then arranged over the total number of women who ever began in category i.

To determine the variability in the difference between cycle lengths, we similarly calculated the probability for a woman having a variation in cycle length of 0, 1 and 2 or more days between two consecutive cycles of having a similar or a different variation in cycle lengths in her subsequent cycles.

Statistical computations were performed by repeat measurements analysis of variance, and Student t-test for group differences using the SuperANOVA software (Abacus Concepts, Inc., Berkeley, CA, USA) for the Apple Macintosh computer. Results are shown as mean ± SD.

Results

Patient description

Of the 131 patients who commenced therapy there were 28 withdrawals (21%). The primary reasons for withdrawal of

Table II. The primary reason for withdrawal after enrolment into the study (% of the total number enrolled)

Primary reason for withdrawal	Total no.	%
Period related	6	4.5
Heavy bleeding	5	
Not wanting periods	1	
Side effects	11	8.3
Fluid retention	3	
Poor symptom control	3	
Skin rash whilst on therapy	1	
Breast tenderness	1	
Depression	1	
Dysmenorrhoea	1	
Headaches	1	
Administrative	8	6.1
Declined end biopsy	3	
Poor compliance	3	
Lost to follow-up	1	
Changed address/unable to attend	1	
Other	3	2.2
Breast cancer	1	
Superficial thrombophlebitis	1	
Widespread cancer	1	
Total	28	21.3

these patients is listed in Table II. Only five (3.8%) stopped because they experienced heavy periods; a sixth patient stopped treatment as she decided that she did not wish to have periods. Only three stopped treatment for poor symptom control; those who completed the study reported adequate symptom relief.

There were 103 patients who completed the 6 months on therapy. Their mean age was 53.4 years (range 44.2–61.5 years). The average parity was 2.3 (range 0–5), weight 67.9 kg (range 48–96 kg), height 160 cm (range 144–177 cm) and body mass index (BMI: kg/m^2) was 26.3 (SD = 4.3). They were all at least 1 year past the menopause, with a median of 40 months (range 12–240 months). Twenty-five women in this group (24.2%) were smokers; for these the average number of cigarettes smoked was 15 cigarettes per day. None of the representative samples of endometrium that were taken at the end of the study showed proliferative, hyperplastic or neoplastic changes. The final biopsy in all patients but 12 showed secretory endometrium. Of these, six were insufficient or scanty, one sample was taken during the proliferative phase and another was taken after the patient had stopped therapy— these proved to be proliferative and atrophic endometrium respectively. Four other samples (three in the early bleeder group and one in the late bleeder group) showed inactive endometrium. A detailed study of these biopsies is currently underway.

Four patients did not experience any bleeding whilst on therapy. Their mean age (52.8 years), weight (60.7 kg), height (160.2 cm), BMI (23.5) and the number of previous HRT regimens they used (mean 1.25, range 0–2) were not statistically different from the rest of the group.

The bleeding pattern

Figure 1 illustrates the frequency distribution of the cycle length in all individuals. We excluded the last cycle for all patients to obviate the probability of over-representing short

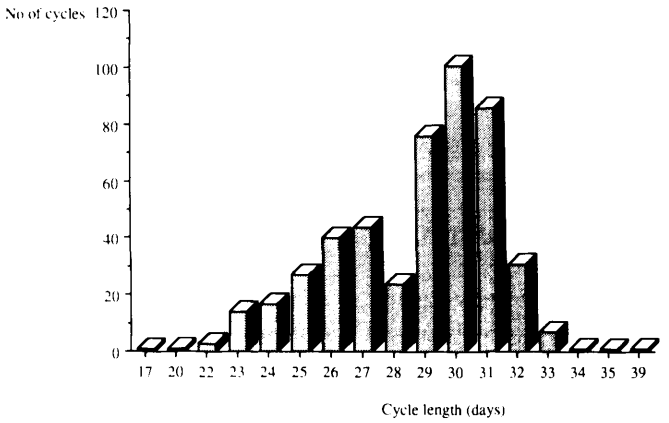


Figure 1. The distribution of cycle length of all women excluding the last cycle. Cycle length was calculated from commencing HRT to the onset of bleeding.

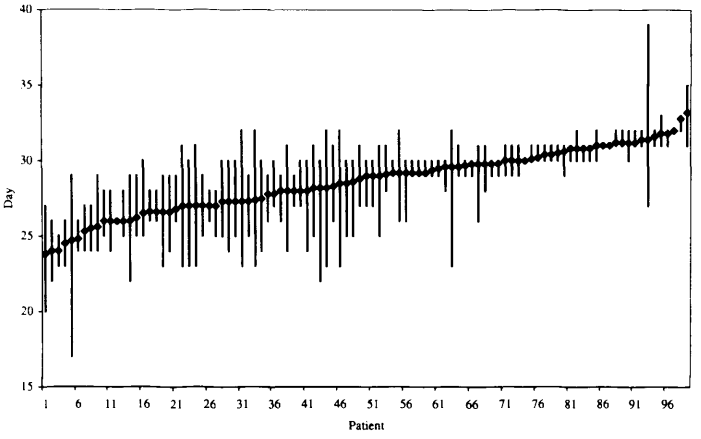


Figure 2. The mean and the range of cycle length of individual women. The means are plotted in the order of increasing magnitude.

cycles. There were 475 completed cycles, data were missing in six cycles and in 32 instances there was no bleeding, 20 of which were from four women. The mean cycle length in these cycles was 28.7 ± 2.7 days and the median 29 days (range 17–39). Out of these 475 cycles, 171 (36%) commenced before day 29, and 304 (64%) commenced on or after day 29.

Figure 2 shows the relation between the mean cycle length for each patient and the individual range of cycle lengths. A mean onset of bleeding of 29 or later appeared to be associated with less cycle to cycle variability within an individual. This is shown dramatically in Figure 3 when the mean cycle lengths of all women were plotted versus the intra-individual standard deviations. The mean cycle length for those who bled before day 29 was 27.0 ± 2.3 days, and for those who bled on or after day 29 was 30.4 ± 1.3 days. The significant difference between the cycle lengths in the two groups was consistent when all the cycles were considered ($P < 0.0001$, F value 161.7, repeat measurements ANOVA). Of the women who had a mean cycle length of <29 days ($n = 49$), only six had a standard deviation (SD) of <1. The mean cycle length in these six patients was 24, 24.8, 26, 26.6, 26.6, and 27 days. Thirty patients in this group had a SD of <2 days. Of the women who had a mean cycle length of 29 days or more ($n = 50$),

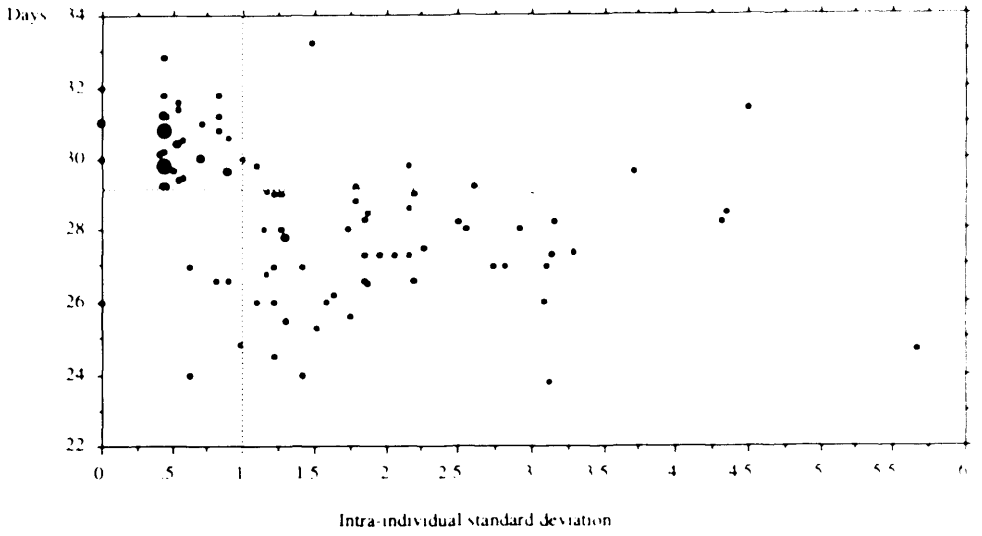


Figure 3. Mean cycle length (days) of individual women with the intra-individual standard deviation. The larger circles indicate overlapping observations.

Table III. Demographic and cycle characteristics of patients who had short cycles, i.e. < 29 days (early bleeders), long cycles, i.e. equal or > 29 days (late bleeders), and the non-bleeders who experienced no bleeding on this regimen

	Study population			
	Late bleeders (n = 50) Mean (SD)	Early bleeders (n = 49) Mean (SD)	Non bleeders (n = 4) Mean (SD)	P
Age	54.3 (44.2–60*)	52.7 (46.4–60*)	52.8 (51–56.3*)	
Weight (kg)	67.9 (10.5)	68.5 (13.6)	60.7 (10.2)	0.81
Height (cm)	159.2 (6.8)	161.4 (6.32)	160.2 (7.6)	0.2
BMI	26.7 (3.8)	26.2 (4.8)	23.5 (2.2)	0.55
Systolic BP	135.9 (17.1)	132.9 (17.3)	105 (17.3)	0.39
Diastolic BP	79.7 (8.8)	80.2 (9.4)	69 (8.1)	0.8
Pulse	72.5 (9.0)	71.4 (7.6)	64.5 (5.22)	0.51
Previous HRT**	0.9 (0–3*)	0.9 (0–3*)	1.2 (0–2*)	
Months since LMP	57.8 (16–160*)	52.3 (8–240)	45.7 (18–60*)	
No. of cigarettes per day	0.74 (3.2)	6 (9.2)	13 (12.5)	0.0002
Duration of bleeding (days)	5.03 (1.57)	6.53 (3.0)	N/A	
Total bleeding score ***	7.89 (3.11)	11.05 (4.6)	N/A	
Average bleeding score ****	1.6 (0.94)	1.58 (0.33)	N/A	

BMI body mass index; BP blood pressure; HRT hormone replacement therapy; LMP last monthly period
*Range
**Number of preparations used in the past
***The sum of the bleeding score per bleeding episode
****The bleeding score per episode divided by the duration of bleeding
P value refers to the difference between early and late bleeders
N/A non-applicable

39 had a SD of < 1 day, 45 had a SD of < 2 days and 5 had a SD of more than 2 days. The mean cycle length of these five patients was 29, 29.2, 29.6, 29.8, and 31.4 days. Twenty-nine out of this group had a range of variability between the shortest and the longest cycle of 0 or 1 days, 10 had a range of 2 days, and four had a range of 3 days. Seven out of the 49 women who bled early had a range of variability of 2 or fewer days, and 11 had a range of 3 days.

There was no statistically significant difference between the group of women who had a mean cycle length of < 29 days (n = 49), termed 'early bleeders', and those who bled on or after day 29 (n = 50), termed 'late bleeders', in respect to the duration of the menopause, weight, height, BMI, systolic or diastolic blood pressures or in the number of HRT preparations they used in the past (Table III). Early bleeders exhibited significantly longer duration of bleeding (P < 0.0001, F value, 19.702) and had higher total bleeding scores than late bleeders (P < 0.0001, F value 22.3). These differences were consistent throughout all treatment cycles (Table III). However, there was no significant difference in the average bleeding score.

There was a significant difference between the SD of the cycle lengths (a measure of cycle variability) of early and late bleeders (mean ± SD of early bleeders 1.996 ± 1.061 and late bleeders 0.865 ± 0.861, P < 0.0001).

We found that early bleeders smoked significantly more cigarettes than late bleeders (P < 0.0002). Non-bleeders (n = 4) smoked an even higher mean number of cigarettes per day. The difference between non-bleeders and late bleeders was statistically significant (P < 0.003).

Women with the least cycle to cycle variability between two consecutive cycles and those with most variability continued to exhibit similar characteristics in any subsequent third cycle (Table IV). Table V demonstrates the probability for a woman with a particular cycle length of having the same or a different cycle length in the following cycle. It demonstrates a tendency for the short and the long cycles to be followed by similar cycles, e.g. cycles shorter than 26 days had a 0.65 probability of being followed by cycles shorter than 28 days and 30–31 day cycles had a 0.79 probability of being followed by cycles longer than 27 days. The higher variability in patients with shorter cycles is exemplified by the 0.3 versus the 0.44 probability of a 26–27 day cycle and a 30–31 day cycle respectively, to be followed by a cycle of similar length.

Discussion

We have demonstrated that women under the same HRT regimen respond differently in terms of bleeding patterns: the first is the group of 'late bleeders' who bled at or later than day 29 and whose cycles tend to be less variable as demonstrated by the narrow SD and by the higher probability of their having the same cycle length in subsequent cycles, with less cycle to cycle variability. This group also bled for a shorter duration and had a lower total bleeding score per cycle. The second group of 'early bleeders' had cycles shorter than 29 days. These women exhibited a wide range of variability of the day of onset of bleeding, a longer duration of bleeding and larger amount of blood loss. The two responses point to differences in the mechanisms of cycle control. Whilst the first group is more reminiscent of menstrual bleeding, there is no obvious analogy for early bleeders, who bleed early and for a longer duration.

In the normal menstrual cycle, a wide variability in length has been documented. This variability persists even after anovulatory cycles and early miscarriages are taken into account (Goldzieher *et al.*, 1947). This can be attributed to the wide variability in the length of the pre-ovulatory phase that contrasts with the duration of the luteal phase which has been shown to have a much smaller variability (Lenton *et al.*, 1984). Also, the wide range of biological variables that affect natural cycles are not operational in HRT induced bleeding. Because of these differences, we cannot extrapolate a range of acceptable variability from natural cycles that can be applied to HRT induced bleeding. Indeed it could be argued that HRT induced cycles should be under tighter control, and should be subject to a narrower range of variability both between individuals and within cycles of the same individual, and in this context be more akin to the control of the luteal phase.

With the exception of a few studies on the duration of menstrual bleeding on oral contraceptives, there has been little research into the variations in the duration of menstrual flow in natural cycles (Harlow and Campbell, 1994). Geographical (Hallberg *et al.*, 1966; Ji *et al.*, 1981) and age-related (Collett *et al.*, 1954) variability have been documented. The duration of bleeding is also influenced by body weight and by other factors that influence the length of the cycle (Harlow and Matanoski, 1991; Kirchengast, 1994). No similar work has been directed to the duration of HRT-induced bleeding. In this study we have demonstrated that a shorter duration of bleeding characterizes longer cycles which may be a reflection of the hormonal balance. Contrary to the studies on natural cycles, we did not find significant differences in factors of weight, height, BMI, age or parity between the early and the late bleeders.

The statistically significant difference in the smoking habits, between the group of late bleeders and the early bleeders, and between the late bleeders and those who did not bleed on HRT (albeit a small group) is striking. Women who smoke have been shown to have an earlier menopause (Lindquist and Bengtsson, 1979; Andersen *et al.*, 1982). The lower incidence of breast cancer, and of endometrial cancer (Doll *et al.*, 1980; Weir *et al.*, 1994), and the higher risk of osteoporosis in smokers (Jensen and Christiansen, 1988), have been attributed to anti-oestrogenic properties of cigarette smoking and may be a reflection of a lower level of circulating oestrogen (Baron *et al.*, 1990). The 'oestradiol 2-hydroxylation pathway', which irreversibly metabolizes oestradiol to inert catechol oestrogens, has been demonstrated to be more active in smokers as compared to non-smokers and has been proposed as a possible

Table IV. The estimated probability of the following cycle to be 0, 1, or 2 or more days shorter or longer, if the variability between the previous two cycles was 0, 1, or 2 or more days

Variability in cycle length* (days)	Number of transitions	Probability of variability in subsequent cycle		
		0	1	≥2
0	62	0.42	0.38	0.2
1	59	0.41	0.28	0.31
≤2	51	0.24	0.31	0.45

* Between any 2 consecutive cycles.

Table V. The probability for a woman with a cycle length t, of having her subsequent cycle of the same or different length

Cycle length t (days)	Total number of women (k)	No bleeding	Cycle length t + 1 (days)				
			≤25	26–27	28–29	30–31	≥32
No bleeding	11	0.187	0.062	0.312	0.25	0.125	0.062
≤25	33	0.07	0.23	0.42	0.13	0.12	0.03
26–27	44	0.02	0.27	0.3	0.16	0.14	0.11
28–29	52	0.02	0.16	0.16	0.19	0.23	0.25
30–31	65	0.04	0.03	0.14	0.25	0.44	0.1
≥32	19	0.06	0.15	0.03	0.02	0.43	0.3

SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN

underlying factor for the hypo-oestrogenic state (Michnovicz *et al.*, 1986). Altered oestrogen metabolism has been demonstrated in post-menopausal smokers receiving HRT (Jensen *et al.*, 1985). The correlation between cycle characteristics and the smoking habits may reflect the significance of the oestrogenic priming of the endometrium in relation to the day of onset of bleeding in HRT regimens. Alternatively, relative hyperandrogenism has been proposed as the underlying mechanism for the anti-oestrogenic properties of smoking (Khaw *et al.*, 1988). The influence, if any, of this on the bleeding pattern is unknown.

The lower than expected drop-out rate attributable to bleeding in this study was fortunate as it reduced the effect of selection bias. This allowed us a unique opportunity to investigate the endometrial response in a model that combined menstrual-like control with early bleeding. This response is patient-specific and may be a reflection of a critical hormonal balance.

A key observation is that patients using the same HRT regimen had a different response to treatment. This study of the bleeding pattern suggested a different end organ response, and/or possibly different effective hormone levels.

Acknowledgement

We would like to thank Mrs B. Phipps, Menopause Nurse Specialist and Staff at the Menopause Research Unit for help and support and Solvay Health Care for financial support.

References

Al-Azzawi, F. and Habiba, M. (1994) Regular bleeding on hormone replacement therapy: a myth? *Br. J. Obstet. Gynaecol.*, **101**, 661–662.

Al-Azzawi, F., Hawley, J., Parsons, A. and Hart, D. M. (1994) Investigation of the bleeding patterns of post-menopausal women treated with Estrapak 50. *Maturitas*, **18**, 115–125.

Andersen, E.S., Transbol, I. and Christiansen, C. (1982) Is cigarette smoking a promotor of the menopause? *Acta Med. Scand.*, **212**, 137–139.

Baron, J.A., Vecchia, C.L., and Levi, F. (1990) The antiestrogenic effect of cigarette smoking in women. *Am. J. Obstet. Gynecol.*, **162**, 502–514.

Belsey, E.M. (1988) Vaginal bleeding among women using one natural and eight hormonal methods of contraception. *Contraception*, **38**, 188–207.

Belsey, E.M. and Carlson, N. (1991) The description of menstrual bleeding patterns: towards fewer measures. *Stat. Med.*, **10**, 267–284.

Belsey, E.M. and Farley, T.M.M. (1987) The analysis of menstrual bleeding patterns: a review. *Appl. Stochastic Models Data Anal.*, **3**, 125–150.

Belsey, E.M., Peregoudov, S. and Task Force on Long-Acting Systemic Agents for Fertility Regulation (1988) Determinants of menstrual bleeding patterns among women using natural and hormonal methods of contraception. I. Regional variation. *Contraception*, **38**, 227–242.

Burch, T.K., Macrsco, J.J. and Parker, M.P. (1967) Some methodologic problems in the analysis of menstrual data. *Int. J. Fertil.*, **12**, 67–76.

Chiazze, L., Brayer, F.T., Macrsco, J.J., Parker, M.P. and Duffy, B.J. (1968) The length and variability of the human menstrual cycle. *JAMA*, **203**, 89–92.

Collett, M.E., Wertenberger, G.E. and Fiske, V.M. (1954) The effect of age upon the pattern of the menstrual cycle. *Fertil. Steril.*, **45**, 437–448.

Daly, E., Roche, M., Barlow, D., Gray, A., McPherson, K. and Vessey, M. (1992) HRT: an analysis of benefits, risks and costs. *Br. Med. Bull.*, **48**, 368–400.

Doll, R., Gray, R., Hafner, B. and Peto, R. (1980) Mortality in relation to smoking: 22 years' observations on female British doctors. *Br. Med. J.*, **280**, 967–971.

Ferguson, K.J., Hoegh, C. and Johnson, S. (1989) Estrogen replacement therapy: a survey of women's knowledge and attitudes. *Arch. Int. Med.*, **149**, 133–136.

Goldzieher, J.W., Henkins, A.F. and Hamblen, E.C. (1947) Characteristics of normal menstrual cycle. *Am. J. Obstet. Gynecol.*, **54**, 668–675.

Habiba, M. and Al-Azzawi, F. (1994) Analysis of menstrual calendars and serum 6-PFG in women on hormone replacement therapy for 12 months. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **57**, 37–42.

Hallberg, L., Hogdahl, A.M., Nilsson, L. and Rybo, G. (1966) Menstrual blood loss—a population study. *Acta Obstet. Gynecol. Scand.*, **45**, 320–351.

Harlow, S.D. and Campbell, B.C. (1994) Host factors that influence the duration of menstrual bleeding. *Epidemiology*, **5**, 382–388.

Harlow, S.D. and Matanoski, G.M. (1991) The association between weight, physical activity, and stress and variation in the length of the menstrual cycle. *Am. J. Epidemiol.*, **133**, 38–49.

Harlow, S.D. and Zeger, S.L. (1991) An application of longitudinal methods to the analysis of menstrual diary data. *J. Clin. Epidemiol.*, **44**, 1015–1025.

Jensen, J. and Christiansen, C. (1988) Effects of smoking on serum lipoproteins and bone mineral content during post-menopausal hormone replacement therapy. *Am. J. Obstet. Gynecol.*, **159**, 820–825.

Jensen, J., Christiansen, C. and Rodbro, P. (1985) Cigarette smoking, serum estrogens, and bone loss during hormone replacement therapy early after menopause. *N. Engl. J. Med.*, **313**, 973–975.

Ji, G., Liyuan, M., Su, Z., Huimin, F. and Lihua, H. (1981) Menstrual blood loss in healthy Chinese women. *Contraception*, **23**, 591–601.

Khaw, K.T., Tazuke, S. and Barrett-Connor, E. (1988) Cigarette smoking and levels of adrenal androgens in post-menopausal women. *N. Engl. J. Med.*, **318**, 1705–1709.

Kirchengast, S. (1994) Intercorrelation between menstrual cycle patterns and body dimensions in Austrian women. *J. Biosoc. Sci.*, **26**, 207–216.

Lenton, E.A., Landgren, B.M. and Sexton, L. (1984) Normal variation in the length of the luteal phase of the menstrual cycle: identification of the short luteal phase. *Br. J. Obstet. Gynaecol.*, **91**, 685–689.

Lindquist, O. and Bengtsson, C. (1979) Menopausal age in relation to smoking. *Acta Med. Scand.*, **205**, 73–77.

Michnovicz, J.J., Hershcopf, R.J., Naganuma, H., Bradlow, H.L. and Fishman, J. (1986) Increased 2-hydroxylation of estradiol as a possible mechanism for the anti-estrogenic effect of cigarette smoking. *N. Engl. J. Med.*, **315**, 1305–1309.

Munster, K., Schmidt, L. and Helm, P. (1992) Length and variation in the menstrual cycle, a cross-sectional study from a Danish county. *Br. J. Obstet. Gynecol.*, **99**, 422–429.

Nachtigall, I.E. (1990) Enhancing patient compliance with hormone replacement therapy at menopause. *Obstet. Gynecol.*, **75**, 77S–80S.

Rodriguez, G., Faundes, L.atham, A. and Atkinson, L.E. (1976) An approach to the analysis of menstrual patterns in the critical evaluation of contraceptives. *Stud. Fam. Plan.*, **7**, 42–51.

Treloar, A.E., Boynton, R.E., Behn, B.G. and Brown, B.W. (1967) Variation of the human menstrual cycle through reproductive life. *Int. J. Fertil.*, **12**, 77–126.

Weir, H.K., Sloan, M. and Kreiger, N. (1994) The relationship between cigarette smoking and the risk of endometrial neoplasms. *Int. J. Epidemiol.*, **23**, 261–266.

Zuckerman, S. (1939) The menstrual cycle. *Lancet I*, 1031–1038.

Received on October 10, 1995; accepted on December 12, 1995

Human Reproduction vol.13 no.6 pp.1674–1682, 1998

Endometrial responses to hormone replacement therapy: histological features compared with those of late luteal phase endometrium

M.A.Habiba, S.C.Bell and F.Al-Azzawi[1]

Menopause Research Unit, Department of Obstetrics and Gynaecology, Leicester University School of Medicine, Leicester, UK

[1]To whom correspondence should be addressed at: Department of Obstetrics and Gynaecology, Clinical Sciences Building, Leicester Royal Infirmary, PO Box 65, Leicester LE2 7LX, UK

We evaluated the histological features of the endometrium in relation to the bleeding pattern in a group of women receiving oral cyclical combined hormone replacement therapy (HRT), and compared the histological features with those of luteinizing hormone (LH)-dated endometrial biopsies obtained from healthy women at the time of sterilization. A total of 103 women completed 6 months of HRT therapy. All received a regimen of 2 mg oestradiol valerate daily, with 1 mg norethisterone added for the last 12 days of every 28-day cycle. Endometrial biopsies were scheduled for the end of the study (days 27–29 of the last cycle of therapy). Using the classical histological criteria, secretory endometrial changes were demonstrated in the majority (n = 89) of cases. The remaining were insufficient or inactive (n = 12), proliferative (n = 1) or atrophic (n = 1). Forty-nine women had a mean cycle length of less than 29 days (early bleeders), 50 women experienced cycles of more than 29 days (late bleeders) and four did not experience any bleeding. When the individual histological structures were examined, using image analysis, there were no statistically significant differences in the histological features when the long cycles in early bleeders were compared with those in late bleeders. LH-dated endometrium showed a high degree of homogeneity that was consistent with cycle day as described by the classic criteria, but HRT-treated endometrium exhibited a wide range of variability. HRT-treated endometrium from the subset of women who bled on or after day 29, and whose biopsies were obtained before the onset of bleeding, differed significantly from the endometrium taken at the corresponding phase of the physiological cycle. We conclude that the use of classical histological criteria, which are used in relation to the physiological cycle, in the study of HRT-treated endometria is inappropriate.

Key words: endometrium/histology/hormone replacement therapy/image analysis/menopause

Introduction

During the menstrual cycle, the endometrium undergoes a series of well-defined histological changes programmed by the steroid hormones of the ovarian cycle. These histological changes are presumed to reflect sequential functional states of the endometrium, i.e. proliferation, a state 'receptive' to implantation during the mid luteal phase, and the changes programming the endometrium to a menstrual 'bleed' after regression of the corpus luteum and hormone withdrawal during the late luteal phase.

It is still not clear what histological features specifically reflect these states and this has impeded the assessment of the effects of exogenous steroid regimens on endometrial function. The focus of research in in-vitro fertilization (IVF) programmes has been to produce an optimally receptive endometrium by employing changes in the luteal phase which have been suggested to be associated with the receptive state, e.g. glandular and blood vessel maturation (Seif *et al.*, 1992). Although no morphological features have been unequivocally associated with implantation (Rizk *et al.*, 1992: Rogers *et al.*, 1996), there is evidence that functional protein expression may be more predictive of outcome (Rizk *et al.*, 1992; Lessey *et al.*, 1995).

In contrast, the focus in hormone replacement therapy (HRT) research programmes has been directed towards preventing the histological appearance of features of hyperplasia, which has been traditionally perceived as the precursor of cancer. Endometrial hyperplasia has been linked with the use of unopposed oestrogen therapy (Whitehead *et al.*, 1979) and, although originally a focus of controversy, it has become widely accepted that unopposed oestrogen replacement therapy is associated with a 3–25-fold increase in the risk of endometrial cancer (Ziel and Finkle, 1975; Smith *et al.*, 1975; Barrett-Connor, 1992). To guard against these developments, the current practice is to add a minimum of 10 days (Whitehead *et al.*, 1981), but preferably 12–14 days, of progestogen to every 28-day treatment cycle (Woodruff and Pickar, 1994). This, however, introduces a monthly 'hormone withdrawal' bleed which, especially if it becomes heavy, irregular or unpredictable, ranks among the most important causes for the discontinuation of therapy (Hahn *et al.*, 1984; Nachtigall, 1990) and adversely affects the uptake of HRT (Al-Azzawi and Habiba, 1994).

Little information is available concerning the response of the endometrium to exogenous sex steroid therapy, particularly in the post-menopausal state. However, in order to produce more effective exogenous steroidal treatment modalities, attempts have been made to correlate features of endometrial histology with the bleeding associated with HRT. In the normal menstrual cycle, histological changes occur in the endometrium during the late luteal phase, which may be associated with the onset of menstruation. Paradoxically, endometrial histological

findings spanning the whole spectrum of atrophic, inactive, proliferative, early and late secretory, and hyperplastic endometrium have been reported in biopsies from the late progestogenic phase whilst on HRT from women exhibiting apparently 'normal' bleeding patterns (Padwick *et al.*, 1986; Sturdee *et al.*, 1994). It may be that histological changes in the late luteal phase of normal menstrual cycle are not causally linked with bleeding, or that the mechanism(s) of bleeding associated with exogenous steroids, in the dose ranges employed in HRT, is different from that during the menstrual cycle.

In a previous study, we analysed the bleeding pattern in 103 women who received a 6-month treatment with a cyclical sequential combined HRT. We classified these women as 'early' or 'late' bleeders. The former were characterized by experiencing an increased frequency of 'short' cycles, i.e. cycle lengths of less than 29 days, an increased cycle length variability, a longer duration of bleeding, and a larger amount of blood loss compared with those of the latter group (Habiba *et al.*, 1996). Here, we report on the endometrial biopsies taken from these women during the late-progestogenic, i.e. 'pseudoluteal' phase, and we compare these with biopsies obtained during the luteal phase of the normal LH-dated cycle. Our aim was to determine whether features could be identified that may be more predictive of subsequent endometrial behaviour. If these were to be identified, they would be useful for monitoring women's responses to HRT, and might also have implications for the utility of the criteria traditionally used in endometrial assessment when applied to HRT-treated endometrium.

Materials and methods

Women on HRT were prescribed a regimen of 2 mg of oestradiol valerate daily with the addition of 1 mg of norethisterone from day 17 through to day 28 of each cycle (medication supplied by Solvay Pharma Hannover GmbH). Participants adhered to a rigid schedule with a pre-study assessment 2–3 weeks prior to commencement of the 168 days of active medication and with visits to clinic every 56 days. The inclusion and exclusion criteria and patient characteristics have been reported previously (Habiba *et al.*, 1996). An endometrial pipelle biopsy (Laboratoire CCD, Paris, France) was obtained prior to enrolment into the study. Patients were excluded if this biopsy could not be obtained because of administrative or technical reasons, or if the baseline biopsy showed evidence of endometrial hyperplasia or malignancy.

Patients filled in diary cards during the study period to document their intake of study medication and to record the days of bleeding and its severity. Day 1 of the cycle was taken as the beginning of the oestrogenic phase. Compliance was verified by checking the patients' diary cards and by collecting the returned empty medication packs. The documentation on the diaries was verified against the returned blister packs.

A total of 164 post-menopausal women was screened between December 1992 and September 1994. Subsequent to screening, 33 of them were found not to be eligible to continue and were withdrawn. Of 131 patients enrolled, 103 patients completed 6 months of treatment. The inclusion and exclusion criteria and the reasons for drop-out have been discussed previously (Habiba *et al.*, 1996). Another endometrial biopsy was planned for the final clinic visit that was scheduled for days 27 to 29 of the sixth cycle. The demographic characteristics of the study population and the bleeding pattern are detailed elsewhere (Habiba *et al.*, 1996). Patients were classified according to their cycle length characteristics into three groups: (i) early bleeders (n = 49) who had a mean cycle length of less than 29 days (range 23.8–28.8, mean = 27.0, SD = 2.3); (ii) late bleeders (n = 50) who had a mean cycle length of 29 or more days (range 29.0–33.2, mean = 30.4, SD = 1.3); and (iii) non-bleeders (n = 4) who experienced no bleeding whilst on therapy.

The LH-dated natural cycle biopsies (n = 30) were obtained by dilatation and curettage from healthy women with regular cycles at the time of scheduled tubal sterilization. They were all given a urine ovulation detection kit (Clearplan, Unipath, Bedford, UK) and instructed to its use. Sterilization and dilatation and curettage were performed in the early (day 2–6 after the LH peak), the mid (day 7–11 after the LH peak), or the late (day 12–14 after the LH peak) luteal phases, with the day of LH peak taken as day 0.

None of the women was using hormonal contraception or an intrauterine device, or had any pregnancies in the previous 6 months. Their mean age was 34.7 years (range 26–39 years), and mean parity was 3.2 (range 2–6). All women gave written consent to the procedure that was approved by the local ethics committee.

All endometrial samples were fixed in formalin and routinely processed and stained with haematoxylin and eosin. Endometrial sections were assessed according to the histological classification following standard criteria (Noyes *et al.*, 1950; Demopoulos, 1982; Buckley and Fox, 1989) and by using image analysis.

Quantitative and semi-quantitative feature analysis

Biopsies were examined quantitatively or semi-quantitatively using image analysis in order to assess each histological feature seen in tissue sections. Five groups were compared: (i) late bleeders: the first 10 biopsies from the late bleeders' group from whom the biopsies were obtained between days 27–29 and before the onset of bleeding in the sixth treatment cycle; (ii) early bleeders: the 10 biopsies from the patients in the early bleeders' group from whom the biopsies were obtained between days 27–29 and from whom the biopsies were obtained before the onset of bleeding in the sixth treatment cycle; (iii) early luteal phase biopsies (n = 10); (iv) mid luteal phase biopsies (n = 10); and (v) late luteal phase biopsies (n = 10).

The image analysis system comprised an Axioplan (Carl-Zeiss) microscope connected to a video camera (Sony DXC-151P) and an Apple Macintosh computer with NIH image™ (version 1.51) software. Assessment was performed as follows:

(i) Epithelial height was measured, and the percentage of the luminal and of the glandular epithelium lined by each type of epithelium (cuboidal, low columnar, columnar, and pseudostratified columnar) and the percentage showing apical or basal vacuoles were estimated in 17 random high-power fields (×400). The mean for each section was calculated.

(ii) The glandular diameter of 17 randomly selected glands was measured employing high-power microscopy (×200) and the measurements converted to metric scale using a measurement grid.

(iii) The proportion of tubular and convoluted glands was calculated in a random selection of 20 glands per section.

(iv) The glandular density was counted in 17 random high-power fields (×400) per section.

(v) The proportion of glands (out of 20 glands) containing luminal secretions was counted, and the amount of secretion was graded using a semi-quantitative scale as: minimal = 1; moderate = 2; and abundant = 3. The amount of secretion was multiplied by the proportion of glands that were positive to obtain a 'secretion score'.

(vi) The percentage of glands containing glandular invaginations (telescoping) was counted in the whole section.

Table I. Histological features of the endometrium before and after 6 months of hormone replacement therapy

Endometrial histology	Pre-enrolment	Final biopsy	Timing of final biopsy (days)			
			< 27	27–29	30–32	Other
Insufficient	20	8	1	6[a]	1	0
Atrophic	54	1	0	0	0	1[b]
Inactive (non-secretory)	23	4	0	4[c]	0	0
Secretory	0	89	9	75	2	3[d]
Proliferative	6	1	1[e]	0	0	0
Adenocarcinoma	0	0	0	0	0	0
Simple (cystic) hyperplasia	0	0	0	0	0	0
Total	103	103	11	85	3	4

[a]Two of these also exhibited endocervical epithelium.
[b]Woman stopped treatment prior to biopsy.
[c]Non-bleeders.
[d]Within 2 days of day 28 but cannot be exactly identified.
[e]Taken on day 13 of the cycle.

(vii) The glandular apical margins were described as either smooth or irregular and the percentage exhibiting each feature was estimated in 20 glands using high-power (×400) microscopy.
(viii) The stromal cellular density was calculated by counting the number of nuclei in 17 random high-power fields (×1000) per section under oil immersion.
(ix) The presence or absence of decidualization, and whether this feature was present in the upper 1/3 of the stroma, was diffuse, or whether it was only present in a perivascular location was described.
(x) The number of leukocytes per section was assessed semi-quantitatively on a grading of: minimal/few = 1; average = 2; and heavy infiltrate = 3.

Statistical analysis

As the standard deviation of the test parameters was unknown, the number of fields randomly selected from each section (n = 17) was calculated in order to satisfy a one-sample t-test, with a power $1-b$ = 0.90, and a two-sided α = 0.01 to detect the population mean with a standardized difference d_t = 1 (Machin and Campbell, 1987). The number of sections to be examined was calculated in order to satisfy a two-sided t-test with a power $1-b$ = 0.90 and a two-sided α = 0.05 to detect a 20% difference between the means. A pilot calculation gave the estimated d_t = 1. The results were analysed using the two-sided unpaired t-test, and the Mann–Whitney test.

Results

Standard histological assessment

The histological classification of the pre-enrolment biopsies is shown in Table I. The end biopsy material was insufficient for assessment in only eight cases (two of these included endocervical epithelium), and no cases exhibited hyperplastic or neoplastic changes. The only proliferative sample was the biopsy obtained on day 13 of the cycle. In 89 (86.4%) of patients who completed the study, a secretory histology was recorded. Those patients who had biopsies taken on days 27–29 included the only four patients who did not experience bleeding during the study, and it was only from these patients that endometrial biopsies classified as inactive were obtained at the end of the study (Table I). Thus 100% of the patients from whom biopsies were taken on days 27–29 (n = 85), and who exhibited bleeding during the study period (n = 81), and in whom sufficient endometrial tissue was obtained (n = 75) had secretory endometrium on histology.

The relation between the timing of the biopsy and the onset of bleeding in the index cycle in the early (n = 49), the late (n = 50), and the non-bleeder (n = 4) groups is shown in Table II. Out of the 85 biopsies which were taken on days 27–29, 75 exhibited secretory endometrium and, of these, 33 were early bleeders and 42 were late bleeders. The pre-study biopsies of these 75 women exhibited atrophic endometrium in 41 cases, inactive endometrium in 15 cases and proliferative endometrium in three cases and was insufficient in 16 cases. Thus, in all these patients, the secretory endometrial histology was induced in response to HRT. The pre-study endometrium in the four patients who did not experience withdrawal bleeding exhibited atrophic endometrium in one case and inactive endometrium in three cases.

Qualitative histological assessment

Although the majority of biopsy specimens were classified as 'secretory endometrium' using the standard histological criteria, the endometrial sections from HRT-treated women, which were classified as such, were characterized by wide variability as regards the presence of 'secretory' features throughout the sections. This was in contrast to the more uniform features exhibited by sections of secretory endometrial specimens obtained from the early, mid, and late luteal phase of the physiological cycle (Figure 1).

The glands featured variable development: some were well developed, i.e. tubular or convoluted, but others were of a narrow and variable calibre. The epithelial lining exhibited a variety of secretory changes, which ranged from glands with abundant supra-nuclear vacuolation to exhausted secretory changes, although these characteristics were poorly developed in the majority of cases. Most glands were lined with inactive or cuboidal columnar epithelium. Few glands exhibited subnuclear vacuolation. Apoptotic bodies were sometimes seen. Glandular telescoping was noted in a large proportion (40%) of sections.

Table II. The histological classification of the endometrium from women on hormone replacement therapy in relation to the bleeding pattern

Bleeding pattern classification	Number	Timing of biopsy in relation to day of bleeding in biopsy cycle	Number	Histological assessment	Number
All biopsies (n = 103)					
Early bleeders	49[a]	After onset	32	Insufficient	6
				Secretory	26
		Before onset	15	Insufficient	1
				Secretory	14
Late bleeders	50	After onset	4	Insufficient	0
				Secretory	4
		Before onset	43	Insufficient	1
				Secretory	42
		Not known	3	Secretory	3
Non-bleeders	4	Not applicable	4	Inactive	4
Days 27–29 biopsies (n = 85)					
Early bleeders	39	After onset	28	Insufficient	5
				Secretory	23
		Before onset	11	Insufficient	1
				Secretory	10
Late bleeders	42	After onset	3	Insufficient	0
				Secretory	3
		Before onset	39	Insufficient	0
				Secretory	39
Non-bleeders	4	Not applicable	4	Inactive	4

[a]Includes one biopsy performed on day 13 of cycle (proliferative endometrial histology) and one patient who terminated treatment prior to biopsy (atrophic endometrial histology).

In some cases glands appeared to be surrounded by an intense leukocytic infiltrate (Figure 2).

The stroma featured small round or spindle cells and, although sometimes the stroma exhibited well-developed decidualization, this was often variable and sometimes absent. Stromal oedema or breakdown was apparent in some of the specimens. Vasculature was of narrow calibre sometimes with thin muscular walls, but on occasions this was well developed. Dilated capillaries were often noted. Area-to-area dysynchrony as well as gland-to-stroma dysynchrony was seen to some extent in all sections examined. The presence of leukocytes in some sections was extensive but in others was difficult to distinguish.

Quantitative and semi-quantitative histological assessment

The results of the quantitative and semi-quantitative analysis are shown in Tables III and IV. Comparing the late pseudoluteal phase endometrium of HRT-treated cycles between the early and the late bleeders showed no significant differences (Table III).

Since late bleeders from whom biopsies were obtained before the onset of bleeding in the biopsy cycle and whose biopsies exhibited 'secretory endometrium' represent the equivalent of the late luteal phase endometrium of the natural cycle, comparison was made between these two groups. The features seen in the endometrial epithelium, the glands and the stroma exhibited some significant differences. The luminal epithelium contained more cuboidal and pseudostratified cells and less low columnar and columnar cells. Glandular epithelial cells were also predominantly shorter, but contained more apical vacuoles. The glands were less numerous but of similar diameter, and the total glandular area was smaller in HRT-treated endometrium. The glands were also more tubular, less convoluted, and contained less luminal secretion, and the apical margins of the glandular cells were smoother than those found in the natural cycle. The stroma was less cellular than in the natural cycle, but there was no statistically significant difference in the degree of stromal oedema, haemorrhage or decidualization. Leukocyte infiltrate was significantly higher in HRT-treated endometrium.

Comparison between the late bleeders in the pseudoluteal phase of HRT and the early, mid and late luteal phases of the normal cycle, demonstrated that the HRT-treated endometrium shared some features with the early luteal phase (e.g. the characteristics of the apical margins of the glandular epithelial cells, the incidence of pseudostratification in the luminal epithelium and of low columnar cells in the glandular epithelium, and the gland number). Some features were shared with the mid luteal phase (e.g. the incidence of apical vacuoles in the glandular and luminal epithelium) and some features were similar to the late luteal phase (e.g. gland diameters). On the other hand, the high incidence of cuboidal cells, and the low incidence of low columnar and columnar cells were unique to HRT-treated endometrium. The stroma under the influence of HRT contained some of the features of the mid luteal phase, but mostly exhibited features of the late luteal phases of the natural cycles.

Discussion

All adequate biopsies obtained between days 27–29, from women experiencing regular cycles, were secretory as assessed by the standard histological criteria and, in all cases where diagnosable biopsies were obtained, the endometrium exhibited

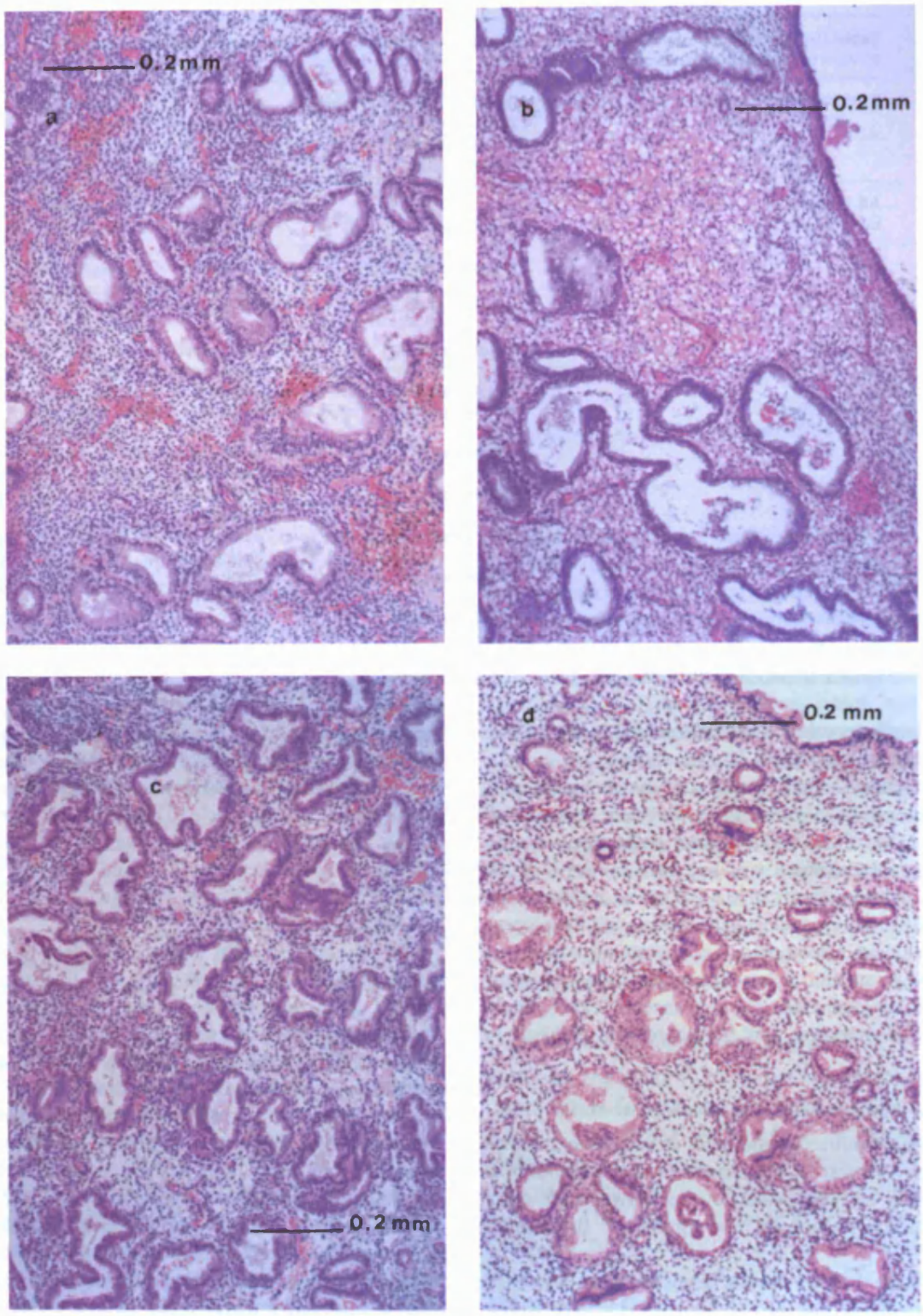


Figure 1. Haematoxylin and eosin stained sections of (**a**) early, (**b**) mid, (**c**) late secretory endometrium, and (**d**) late pseudoluteal phase of hormone replacement therapy (HRT)-treated endometrium, demonstrating the uniformity and 'in-phase' development of physiological cycle endometrium compared with the variable development on HRT sections (see text). Original magnification ×100.

a histological response to HRT, except in those four patients who did not bleed. These four patients' endometrial biopsies were inactive. Interestingly, these patients were heavy smokers and a lack of a histological response might be related to the hypo-oestrogenic state in heavy smokers (Habiba *et al.*, 1996). Our data, thus, do not support the previously published studies that indicated that a significant proportion of biopsies taken in the pseudoluteal phase of the HRT cycle from patients having regular cycles exhibit proliferative or atrophic histology, e.g. 13% in the study by Sturdee *et al.* (1994) and 16% in the study by Padwick *et al.* (1986). In addition, in these studies, 27% and 13% of patients, respectively, could not be assessed

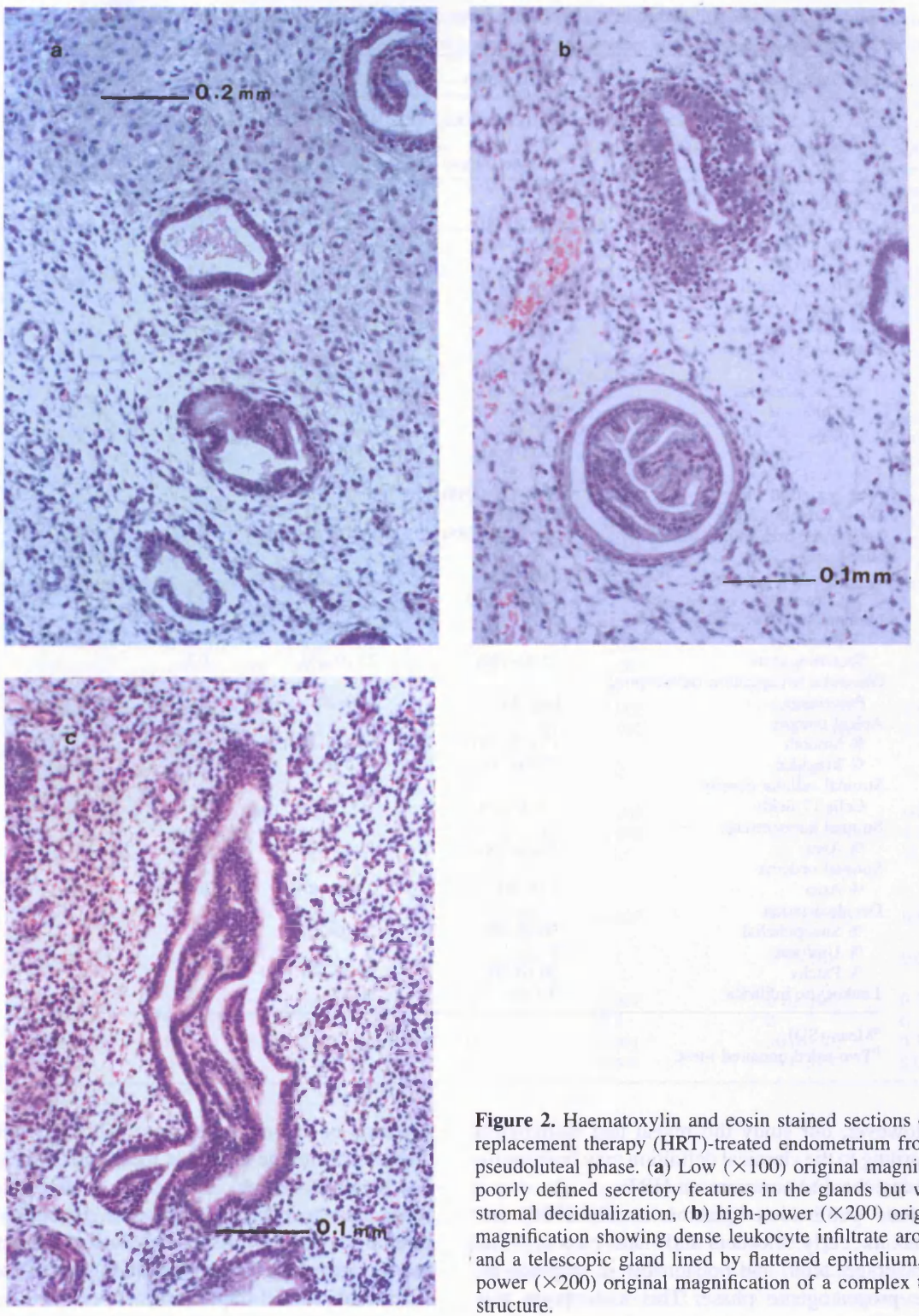


Figure 2. Haematoxylin and eosin stained sections of hormone replacement therapy (HRT)-treated endometrium from the late pseudoluteal phase. (**a**) Low (×100) original magnification showing poorly defined secretory features in the glands but well-developed stromal decidualization, (**b**) high-power (×200) original magnification showing dense leukocyte infiltrate around one gland and a telescopic gland lined by flattened epithelium, and (**c**) high-power (×200) original magnification of a complex telescopic gland structure.

because of scant or absent biopsy material, which was considered to indicate the presence of atrophic or inactive endometrium. The apparent lack of an endometrial response to HRT in women with regular cycles, cited in the previously published work, would be difficult to explain if the biopsies were adequate and optimally timed. The presence of endocervical epithelium in two of our patients whose biopsies were insufficient for adequate histological assessment suggests that the lack of response was because the biopsies were obtained from the less responsive isthmus.

Endometrial biopsies obtained on days 11–13 of the pseudoluteal phase (cycle days 27–29) were classified according to standard histological assessment as secretory, irrespective of whether the biopsies were obtained from 'early' or 'late' bleeders. This supports a lack of correlation between classically defined histological features and the time of bleeding in

Table III. Comparison between the histological features of the early and the late bleeders using image analysis

Histological feature	Study group		
	Early bleeders (n = 10) median (range)	Late bleeders (n = 10) median (range)	Significance level P value Mann Whitney test
Luminal epithelium			
% Cuboidal	50 (20–90)	50 (20–90)	0.7
% Low columnar	45 (10–70)	45 (10–70)	>0.99
% Columnar	0	0	
% Pseudostratified	2.5 (0–10)	2.5 (0–20)	0.6
% Apical vacuole	2.5 (0–20)	5 (0–30)	0.3
% Basal vacuole	0 (0–5)	0 (0–5)	>0.99
Glandular epithelium			
% Cuboidal	60 (20–80)	40 (30–70)	0.2
% Low columnar	40 (15–80)	55 (30–70)	0.3
% Columnar	0	0	
% Pseudostratified	0 (0–5)	0 (0–10)	0.5
% Apical vacuole	20 (10–50)	40 (5–50)	0.2
% Basal vacuole	0 (0–5)	0 (0–20)	0.7
Area per gland (mm^2)	[a]0.006 (0.0018)	[a]0.007 (0.0018)	[b]0.15
Gland number (/field)	[a]3.6 (0.8)	[a]3.5 (1.2)	[b]0.7
Total gland area (mm^2) (/field)	[a]0.022 (0.0085)	[a]0.026 (0.0098)	[b]0.46
Gland shape			
% Tubular	25 (0–75)	25 (0–100)	0.7
% Convoluted	75 (25–100)	75 (0–100)	0.7
Luminal secretion			
% Positive	25 (0–100)	25 (0–25)	0.3
Secretion score	25 (0–100)	25 (0–25)	0.3
Glandular invagination (telescoping)			
Percentage	0 (0–15)	0 (0–20)	0.8
Apical margin			
% Smooth	75 (25–100)	50 (50–100)	0.16
% Irregular	25 (0–75)	37.5 (0–50)	0.17
Stromal cellular density			
Cells/17 fields	[a]26.5 (3.9)	[a]23 (5.3)	[b]0.12
Stromal haemorrhage			
% Area	20 (5–30)	15 (5–20)	0.1
Stromal oedema			
% Area	5 (0–20)	7.5 (0–40)	0.9
Decidualization			
% Subepithelial	20 (0–50)	20 (0–70)	0.6
% Uniform	0	0	
% Patchy	20 (0–50)	20 (0–50)	0.8
Leukocytic infiltrate	3 (1–3)	3 (1–3)	0.9

[a]Mean (SD).
[b]Two-sided unpaired t-test.

HRT cycles. However, our study did reveal that histological assessment according to the classical definition may be inappropriate for assessing the endometrium on HRT.

In our previous publication (Habiba *et al.*, 1996), we classified patients as 'early' bleeders and 'late' bleeders. The latter bled, on average, after, and presumably in response to, the end of the progestogenic phase. This assumption was supported by the lesser cycle variability exhibited by these patients. When we compared the endometrium from the whole group of 'early' and 'late' bleeders (results not shown), a few features were found to exhibit differences in frequency. These differences, however, were those associated with the occurrence of bleeding (e.g. stromal haemorrhage). These differences were not found when all women who had bled by the time of the biopsy were excluded. This suggests that the difference between the 'early bleeders' and the 'late bleeders' is in the statistical probability of short or long cycles in each group, rather than a difference between 'early' and 'late' bleeders *per se* or in variation of end-organ response. This difference may thus be due to extrauterine factors in these patients, e.g. steroid uptake and metabolism, which may also exhibit temporal variation. Since cycle variability is reflected clinically in unpredictable bleeding, which is associated with discontinuance of HRT and in low compliance, identifying these factors will have significant clinical implications.

In contrast to the physiological cycle endometrium, HRT-treated endometria from the pseudoluteal phase exhibited a high degree of heterogeneity, which may represent a poor control of endometrial proliferation and differentiation with the intake of exogenous steroid hormones. The total number of leukocytes is increased in the HRT-treated endometrium, but whether this represents an increase in the characteristic CD56-enriched population of the late luteal phase endometrium, or represents a different subpopulation, awaits further investigation.

The histological features of HRT-treated endometrium

Table IV. Comparison between the histological features of the endometrium from early and late bleeders and from the physiological cycle using image analysis

Histological feature	Study group						
	Late bleeders (n = 10) median (range)	Early luteal (n = 10) median (range)	Difference between early-luteal and late bleeders (P-value)	Mid luteal (n = 10) median (range)	Difference between mid-luteal and late bleeders (P-value)	Late luteal (n = 10) median (range)	Difference between late-luteal and late bleeders (P-value)
Luminal epithelium							
% Cuboidal	50 (20–90)	5 (0–40)	0.0001	20 (0–30)	0.0003	10 (0–40)	0.0007
% Low columnar	45 (10–70)	80 (60–90)	0.0001	70 (30–90	0.01	80 (60–100)	0.0002
% Columnar	0	5 (0–20)	0.02	10 (0–70)	0.01	5 (0–20)	0.05
% Pseudostratified	2.5 (0–20)	0 (0–10)	0.07	0	0.03	0	0.05
% Apical vacuole	5 (0–30)	0 (0–50)	0.03	0 (0–5)	0.06	5 (0–20)	0.6
% Basal vacuole	0 (0–5)	10 (0–50)	0.0007	0 (0–10)	0.5	0	0.7
Glandular epithelium							
% Cuboidal	40 (30–70)	0 (0–30)	0.0001	10 (0–30)	0.0001	10 (0–40)	0.0004
% Low columnar	55 (30–70)	45 (20–80)	0.4	30 (10–90)	0.3	70 (50–90)	0.05
% Columnar	0	50 (0–80)	0.0001	60 (0–90)	0.0002	10 (0–50)	0.001
% Pseudostratified	0 (0–10)	0	0.07	0	0.08	0	0.1
% Apical vacuole	40 (5–50)	0 (0–20)	0.0001	60 (0–100)	0.1	7.5 (0–30)	0.002
% Basal vacuole	0 (0–20)	80 (50–100)	0.0001	10 (0–70)	0.06	0	0.2
Area per gland (mm^2)	[a]0.007 (0.0018)	[a]0.015 (0.0067)	[b]0.003	[a]0.012 (0.005)	[b]0.01	[a]0.0079 (0.003)	[b]0.5
Gland number (/field)	[a]3.5 (1.2)	[a]4.4 (1.3)	[b]0.12	[a]4.2 (0.8)	[b]0.1	[a]4.9 (1.3)	[b]0.01
Total gland area (mm^2) (/field)	[a]0.026 (0.0098)	[a]0.059 (0.0146)	[b]0.0001	[a]0.054 (0.023)	[b]0.004	[a]0.037 (0.014)	[b]0.05
Gland shape							
% Tubular	25 (0–100)	0 (0–10)	0.01	0	0.007	0	0.02
% Convoluted	75 (0–100)	100 (90–100)	0.01	100	0.007	100	0.02
Luminal secretion							
% Positive	25 (0–25)	10 (0–5)	0.4	75 (25–100)	0.002	80 (10–100)	0.001
Secretion score	25 (0–25)	10 (0–50)	0.4	75 (25–150)	0.001	80 (10–160)	0.001
Glandular invagination							
Percentage	0 (0–20)	0 (0–15)	0.1	0 (0–10)	0.2	0 (0–4)	0.2
Apical margin							
% Smooth	50 (50–100)	77.5 (50–100)	0.2	25 (0–75)	0.003	0 (0–100)	0.004
% Irregular	37.5 (0–50)	22.5 (0–50)	0.002	75 (25–100)	0.003	100 (0–100)	0.004
Stromal cellular density: cells/17 fields	[a]23 (5.3)	[a]29.8 (7)	[b]0.02	[a]24.8 (5.9)	[b]0.5	[a]37.3 (12.6)	[b]0.003
Stromal haemorrhage							
% Area	15 (5–20)	0 (0–20)	0.002	10 (0–30)	0.007	10 (10–30)	0.8
Stromal oedema							
% Area	7.5 (0–40)	0	0.002	30 (0–50)	0.1	0 (0–10)	0.09
Decidualization							
% Subepithelial	20 (0–70)	0	0.005	0	0.007	0 (0–30)	0.2
% Uniform	0	0	0.9	0	0.9	0	0.9
% Patchy	20 (0–50)	0	0.001	0	0.001	30 (0–30)	0.7
Leukocytic infiltrate	3 (1–3)	1 (1–2)	0.005	1 (1–3)	0.008	2 (1–3)	0.05

[a]Mean (SD).
[b]Two-sided unpaired t-test.

differed significantly from those noted in any stage of the luteal phase of the menstrual cycle. The glandular component exhibited a mixture of features, some of which were similar to those seen in the early, the mid or in the late luteal phase, as well as unique features that are not normally noted in the luteal phase, whilst the stroma exhibited mostly advanced features. This supports the qualitative impression of glandular to stromal dysynchrony in HRT-treated endometrium. This suggests that there may be a disproportion between the doses and/or the effect of the oestrogen and the progestogen in this regimen (Good and Moyer, 1968). Comparing the histology of the group who experienced regular withdrawal bleeding whilst on HRT to the endometrium of the late luteal phase of the physiological cycle, demonstrated that the main similarities between the two groups was in the histological features of the stroma. This suggests that one or more of the stromal features may be the important determinant of bleeding. It is interesting that the endometrial samples obtained after the onset of bleeding on HRT exhibited secretory features. This may be explained by patchy endometrial maturation/shedding, which may, in turn, be related to the fact that hormone administration in this regimen was continued to complete the 12 days of combined oestrogen and progestogen, irrespective of the onset of bleeding, and that the vast majority of these biopsies were obtained within 1–2 days after the onset of bleeding.

The reduced glandular area reported in the present study is similar to that reported in IVF cycles (Rogers *et al.*, 1996). Reduced glandular area has been reported with clomiphene citrate treatment, but was not found in high oestrogen regimens, which supports a central role for oestrogen in glandular development (Rogers *et al.*, 1996). This supports our hypothesis that the regimen we employed is relatively hypo-oestrogenic.

Some of the observed histological discrepancies may be related to the use of the synthetic progestogen norethisterone. Norethisterone is widely used in HRT preparations but has mild androgenic effects, which may be mediated through the androgen receptors that are present in the endometrial epithelium as well as in the stroma (Mertens *et al.*, 1996). It is also possible that some of the histological discrepancies that we observed in HRT endometrium may be due to the fact that, with the use of HRT, the endometrium is exposed to 12 days of continuous progestogen rather than the fluctuations that occur in the physiological cycle.

This study has demonstrated the dissociation between the classical histological features in the endometrium and the occurrence of bleeding. This dissociation between morphology and function has far-reaching implications, as it raises important questions concerning the utility of traditional histological classification in the assessment of the different states of the endometrium under HRT. This conclusion is supported by the previously described discrepancy between endometrial histological features and immunohistochemical markers (Bell, 1990; Rizk *et al.*, 1992).

Our study also indicates that future research should be focused on parameters known to be related to function, and should aim to discover new parameters. Only if such relationships are known and measured, can we adequately monitor current preparations, and design new HRT preparations for use in clinical practice.

References

Al-Azzawi, F. and Habiba, M. (1994) Regular bleeding on hormone replacement therapy: a myth? *Br. J. Obstet. Gynaecol.*, **101**, 661–662.

Barrett-Connor, E. (1992) Hormone replacement therapy and cancer. *Br. Med. Bull.*, **48**, 345–355.

Bell, S.C. (1990) Assessment of endometrial differentiation and function. *Br. Med. Bull.*, **43**, 720–732.

Buckley, C.H. and Fox, H. (1982) *Biopsy Pathology of the Endometrium*. Raven Press, New York, pp. 11–30.

Demopoulos, R.I. (1982) Normal endometrium. In Blaustein, A. (ed.), *Pathology of the Female Genital Tract*. Second Edition. Springer-Verlag, New York, pp. 235–278.

Good, R.G. and Moyer, D.L. (1968) Estrogen-progesterone relationships in the development of secretory endometrium. *Fertil. Steril.*, **19**, 37–49.

Habiba, M.A., Bell, S.C., Abrams, K. and Al-Azzawi, F. (1996) Endometrial responses to hormone replacement therapy: the bleeding pattern. *Hum. Reprod.*, **11**, 503–508.

Hahn, R.G., Nachtigall, R.D. and Davies, T.C. (1984) Compliance difficulties with progestin supplemented replacement therapy. *J. Fam. Pract.*, **18**, 411–414.

Lessey, B.A., Castelbaum, A.J., Sawin, S.W. and Sun, J. (1995) Integrins as markers of uterine receptivity in women with primary unexplained infertility. *Fertil. Steril.*, **63**, 535–542.

Machin, D. and Campbell, M.J. (1987) *Statistical Tables for the Design of Clinical Trials*. Blackwell Scientific Publications, Oxford, pp. 79–88.

Mertens, H.J., Heineman, M.J., Koudstaal, J. *et al.* (1996) Androgen receptor content in human endometrium. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **70**, 11–13.

Nachtigall, L.E. (1990) Enhancing patient compliance with hormone replacement therapy at menopause. *Obstet. Gynecol.* **75** (suppl.), 77–80.

Noyes, R.W., Hertig, A.T. and Rock, J. (1950) Dating the endometrial biopsy. *Fertil. Steril.*, **1**, 3–25.

Padwick, M.L., Pryse-Davies, J. and Whitehead, M.I. (1986) A simple method for determining the optimal dosage of progestin in postmenopausal women receiving oestrogens. *N. Engl. J. Med.*, **315**, 930–934.

Rizk, B., Manners, C.V., Davies, M.C. *et al.* (1992) Immunohistochemical expression of endometrial proteins and pregnancy outcome in frozen embryo replacement cycles. *Hum. Reprod.*, **7**, 413–417.

Rogers, P.A., Hoste, M.J., Ortis, N. *et al.* (1996) Uterine glandular area during the menstrual cycle and the effect of different in vitro fertilization related hormonal treatments. *Hum. Reprod.*, **11**, 376–379.

Seif, M.W., Pearson, J.M., Ibrahim, Z.H.Z. *et al.* (1992) Endometrium in in vitro fertilization cycles: morphological and functional differentiation in the implantation phase. *Hum. Reprod.*, **7**, 6–11.

Smith, D.C., Prentice, R., Thompson, D. and Herrmann, W.L. (1975) Association of exogenous estrogen and endometrial carcinoma. *N. Engl. J. Med.*, **293**, 1164–1167.

Sturdee, D.W., Barlow, D.H., Ulrich, L.D. *et al.* (1994) Is timing of withdrawal bleeding a guide to endometrial safety during sequential oestrogen progestagen replacement therapy? *Lancet*, **343**, 979–982.

Whitehead, M.I., King, R.J.B., McQueen, J. and Campbell, S. (1979) Endometrial histology and biochemistry in climacteric women during oestrogen and oestrogen-progestogen therapy. *J. Roy. Soc. Med.*, **72**, 322.

Whitehead, M.I., Townsend, P.T., Pryse Davies, J. *et al.* (1981) Effects of estrogens and progestins on the morphology and biochemistry of the post menopausal endometrium. *N. Engl. J. Med.*, **305**, 1599–1605.

Woodruff, J.D. and Pickar, J.H. (1994) Incidence of endometrial hyperplasia in postmenopausal women taking conjugated estrogens (Premarin) with medroxyprogesterone acetate or conjugated estrogens alone. *Am. J. Obstet. Gynecol.*, **170**, 1213–1223.

Ziel, H.K. and Finkle, W.D. (1975) Increased risk of endometrial carcinoma among users of conjugated estrogens. *N. Engl. J. Med.*, **293**, 1167–1170.

Received on December 23, 1997; accepted March 18, 1998