THE MEDICAL TREATMENT OF ENDOMETRIOSIS WITH DANAZOL AND GESTRINONE

A STUDY OF THEIR CLINICAL, ENDOCRINE AND IN VITRO EFFECTS

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ABSTRACT

This thesis describes an investigation of the clinical efficacy and mechanisms of action of danazol and the newer steroidal agent, gestrinone in the medical management of endometriosis.

A prospective randomized double-blind study was performed on 51 patients treated with either danazol or gestrinone. The efficacy and tolerance of the two drugs were shown to be similar.

The endocrine effects of danazol and gestrinone were found to be analogous. Treatment with both drugs resulted in early follicular phase levels of luteinizing hormone, follicle stimulating hormone and oestradiol, a fall in sex hormone binding globulin and an increase in percent free testosterone and the concentration of free testosterone. The latter is significantly related to the improvement in endometriosis seen during treatment. Although vaginal bleeding and oestradiol levels during therapy are also significantly related, neither of these parameters correlate with effective elimination of endometriosis.

The effect of gestrinone, danazol and the two major metabolites of danazol, ethisterone and 2-hydroxymethyl ethisterone were tested on endometrial tissue cells, used as a model for endometriotic tissue, cultured in vitro. In view of the increase in free testosterone observed during treatment with the drugs, the effect of testosterone was

also examined. Danazol and testosterone at one and ten times the normal circulating concentrations caused a significant suppression of endometrial cell growth in vitro, but gestrinone, ethisterone and 2-hydroxymethyl ethisterone caused no effect.

This thesis is dedicated to my parents,

Joyce and Cyril Rose.

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ABBREVIATIONS

A4	Androstenedione
AFS	American Fertility Society
AR	Androgen receptor
C	Complement
CHW	Chelsea Hospital for Women
c.v.	Coefficient of variation
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandosterone sulphate
DHT	Dihydrotestosterone
E	Ethisterone
E1	Oestrone
E2	Oestradiol
ER	Oestrogen receptor
e.s.r	Erythrocyte sedimentation rate
FSH	Follicle stimulating hormone
GR	Glucocorticoid receptor
^{3}H	Tritiated
Ig	Immunoglobulin
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
MR	Mineralocorticoid receptor
2OHME	2 Hydroxymethyl ethisterone
P	Progesterone
PBS	Phosphate buffered saline
PGE	Prostaglandin E
PGF	Prostaglandin F
PR	Progesterone receptor

QC	Quality control
RIA	Radioimmuoassay
SD	Standard deviation
s.e.m	Standard of the mean
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SHBG	Sex hormone binding globulin
SHBG BC	Sex hormone binding globulin binding capacity
SLE	Systemic lupus erythamatosis
T	Testosterone
TXB_2	Thromboxane B_2
TRH	Thyrotrophin releasing hormone
WHO	World Health Organisation

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SECTION 1

INTRODUCTION

CHAPTER 1

THE AETIOLOGY, EPIDEMIOLOGY AND HISTIOGENESIS OF ENDOMETRIOSIS

Endometriosis is a medical enigma. The puzzle of its aetiology has led to speculations without positive proof and unfortunately any "unproven theory" may perpetuate a fallible concept. Attempts to understand the condition, the search for treatments and their mode of action, further provokes research and this thesis attempts to elicit some of the facts from the fantasies in the application of medical treatment for endometriosis.

HISTORY AND HISTOGENESIS

Endometriosis is defined as active endometrial tissue in an ectopic site. The term endometriosis was first coined by Sampson in 1921 (Sampson, 1921), but the condition was described, but unnamed, for decades, and maybe even centuries before this. In the Papyrus Ebers, 1600 B.C. (Ridley, 1968), the complaint of dysmenorrhoea is described as an "incantation" of "crushed onion, subt (sic) and sawdust of pine" was prepared and " applied to the hypogastric area". Perhaps this was the first known attempt to treat endometriosis.

The first accurate description of endometriosis is credited to Von Rokitansky in 1860 (Von Rokitansky, 1860), when he described an adenomyoma, but citings by Cruveilhier

(1835) report similar findings. By 1870, Wadeyer suggested that adnexal cysts might arise from germinal epithelium and in 1894, Breus first used the term "chocolate cyst". Endometrial tissue in the ovary was first described by Russell (1899). Theories of aetiology continued to abound at this time, until 1919 when Meyer suggested the coelomic metaplasia theory. This remained unchallenged, as Meyer was so eminent in his day, until Sampson's theory of 1921. Sampson's paper has become one of the corner stones in the history of endometriosis and his further publication in 1927, set out the theory of retrograde menstruation and subsequent "seeding". Meyer refused to accept Sampson's theory and thus two hypotheses were perpetuated.

Since that time, a number of theories and variations have been postulated (Table 1.1). Sampson's theory has remained the most popular concept and many observations have supported this. The presence of blood in the peritoneal cavity at the time of menstruation has been demonstrated in women undergoing peritoneal dialysis (Blumenkrantz et al, 1981), at the time of laparoscopy and by ultrasound. Endometrial cells have been identified in the aspirates of this blood stained peritoneal fluid (Ridley, 1968). The presence of menstrual blood in the peritoneal cavity would seem to occur in all menstruating women (Halme et al, 1984b) and the endometrial cells are viable (Keetel and Stein, 1951, Ridley and Edwards, 1958). The fact that only 25% of women develop the disease (and this is only an estimate) means that retrograde menstruation is only a prerequisite to

TABLE 1.1: THEORIES OF THE AETIOLOGY OF ENDOMETRIOSIS

1. Transplantation theories

   Implantation (spontaneous)
   Transplantation (surgical)
   Metastases
   Direct Extension

2. Metaplasia theories

   Coelomic metaplasia
   Ovarian metaplasia
   Embryonic rests

3. Induction theories

the disease, not the aetiology. Women without uteri do not develop pelvic endometriosis.

The occurence of endometriosis in scars appears to be secondary to the unintentional transfer of endometrial cells from the uterus during a surgical procedure. Alternative theories of metastases explain the other distant sites of endometriosis. This may be by venous or lymphatic spread and proof of this mode of transport was provided by Javert (1949).

The theory propounded by Meyer that endometriosis originated in the coelomic membrane via a process of metaplasia following a metaplastic induction factor, was supported by Novak (1931). He believed that the induction agent was hormonal and the existence of endometriosis in males (Oliker and Harris, 1971) and in females without a uterus (El-Mahgoub and Yaseen, 1980) lends clinical support to the hypothesis.

Metaplasia may occur in almost any tissue and McVeigh (1955) suggested that endometriosis resulted from metaplastic change in the shed corona radiata following ovulation. The metaplasia theory has also been suggested as an explanation for the more distant sites in that embryonic rest cells may undergo change to endometriosis with the appropriate stimuli as the cells are totipotential.

Finally, the induction theory of Lavender (1941) proposes that chemical substances released by the endometrium stimulate undifferentiated mesenchyme to undergo metaplastic transformation. Experimental evidence to support

this theory was offered by Merrill (1966) who claimed to induce endometriosis in rabbits and monkeys using cell free endometrial extracts.

It would seem that Sampson's theory of retrograde menstruation being a prerequisite to pelvic endometriosis is accepted. Perhaps, lymphatic spread and metaplastic change both lead to endometriosis being found in distant sites, but the induction of the metaplasia by hormonal or other chemicals in the pelvic peritoneum is less likely to be an explanation. The presence of endometrial cells in the peritoneal cavity does not explain the aetiology of the disease and further studies will be required to understand this process fully.

PREVALENCE

The exact prevalence of endometriosis is unknown. There is a general belief that the prevalence is rising but as no longitudinal population studies have been performed, it may only represent an increased awareness of the disease and greater ease with which the diagnosis can be made. In a prospective study from the Mayo Clinic (Williams and Pratt, 1977), the authors attempted to document the prevalence of endometriosis in 1000 patients undergoing a laparotomy for an unrelated cause. They found endometriosis in 50% of cases. A series published by Strathy et al, (1982), found endometriosis in 2% of patients undergoing sterilisation and ten times that figure in infertile patients. These two studies demonstrate the wide range of reported prevalence of

the disease and Houston (1984) in an overview of 78 publications was unable to reach a consensus of opinion. The variation is almost certainly a reflection of the choice of study population and as yet there are no studies considering the rate at which newly diagnosed disease appears in defined populations initially free from the disease.

Ninety percent of women diagnosed as having endometriosis are between the ages of 20 and 50, but the diagnosis of endometriosis in teenagers is increasing with the demand for investigation. The youngest recorded case is a 10 year old girl and the oldest, a 76 year old woman (Houston, 1984).

It is generally believed that endometriosis is a disease primarily of white, caucasian women and it is uncommon in black women (Kistner, 1975). However, the problem of such observations is the paucity of studies examining different racial groups under similar circumstances. The highest prevalence of the disease is reported in orientals, especially Japanese women (Hassan, 1976, Miyazawa, 1976).

Clinicians are in agreement that socioeconomic factors influence the risk of developing endometriosis. It seems to be a disease more commonly seen in civilized communities, and highest in the financially successful. The typical woman is described as ambitious and career orientated and one who delays marriage and more especially child bearing until later in her reproductive years. The reasons for the socio-economic association remain unclear.

Possible genetic factors in the prevalence of endometriosis have been considered. Simpson et al (1980) and Malinak et al (1980) performed systematic pedigree analysis of relatives of patients with endometriosis and found a 6.8% occurrence of endometriosis in first degree relatives as opposed to 1% of controls. They postulated a polygenic/multifactorial pattern of inheritance although they could not exclude other genetic mechanisms. In 1984, a further study by Simpson failed to reveal an association between HLA status and endometriosis. Whether some other genetic factor confers an increased susceptibility to the disease remains speculative.

PATHOGENESIS

Having established the presence of endometrial tissue in the peritoneal cavity, an explanation as to the factors which lead to the establishment of ectopic sites of growth has long been sought. These various theories will be discussed in relationship to peritoneal fluid and its constituents, immunological factors, ovarian function and the hypothalamo-pituitary axis.

ENDOMETRIOSIS AND PERITONEAL FLUID

The relationship between the peritoneal environment and endometriosis is poorly understood, but the association with infertility has led several investigators to consider peritoneal fluid changes in an attempt to understand the

disease further. The findings of the various groups do not always concur and hence the enigma continues.

Peritoneal Fluid Volume

The peritoneal fluid volume is maintained at low, but measurable levels, to act presumably, as an intra-abdominal lubricant. Most studies estimate aspirated fluid volume from the pouch of Douglas and in men, postmenopausal women, women on oral contraceptives (Maathius et al, 1978, Koninckx et al, 1980a, Donnez et al, 1982), the mean volume (1.0-5.5ml) remains constant. In ovulatory cycles, however, the volume of fluid increases during the follicular phase, rapidly rises at the time of ovulation and remains elevated during the luteal phase until 3 to 4 days before menstruation when the levels fall (Maathius et al, 1978, Koninckx et al, 1980a, Donnez et al, 1982), (Table 1.2). The absence of the uterus and the occlusion of the fallopian tubes failed to influence the volume (Koninckx et al, 1980a)

The variation in volume throughout the menstrual cycle has been explained either as alterations in vascular permeability under the influence of oestradiol or as a primary result of functional ovarian activity, (Maathius et al, 1978). The first theory is probably incorrect as protein levels in serum and peritoneal fluid remain unaltered in their ratio during the cycle. The role of the change of fluid volume is unexplained but it may be important for tubal motility and ovum pick-up.

TABLE 1.2: VOLUME OF PERITONEAL FLUID IN NORMAL OVULATORY WOMEN

DAYS OF MENSTRUAL CYCLE	PERITONEAL FLUID VOLUME (ML)
1-6	4.2 ± 0.7 (25)
7-11	5.7 ± 0.7 (18)
12-13	9.6 ± 1.3 (25)
14-16	22.5 ± 4.8 (20)
17-19	17.2 ± 2.5 (17)
20-22	16.5 ± 1.0 (29)
23-25	12.9 ± 3.2 (15)
26-28	7.0 ± 2.0 (9)

(all values are mean ± SEM
numbers in parantheses indicate number of samples)

Ref. Donnez et al, 1982

The undisputed fact that changes do occur, led to estimates of fluid volumes in patients with endometriosis. Koninckx et al (1980a) were the first to comment and suggested that in the early follicular phase, the volume was significantly higher. The number of patients in the normal and the endometriosis groups was 5 and 4 respectively and the statistics on these numbers must be questioned. Throughout the rest of the cycle, there was no significant difference. Drake et al (1980) however, suggested a significantly higher volume in endometriotics, but the sampling was allocated to 9-12 day intervals in the cycle and the data meaned. The changes in the cycle normally are considerable over such time intervals (Table 1.2) and this may explain their findings. Subsequently, Rock et al (1982) confirmed the findings of Koninckx and co-workers (1980a) and showed no significant difference from controls. When the severity of the disease was classified into mild, moderate and severe, all investigators have failed to demonstrate any significant difference (Koninckx et al, 1980a, Rock et al, 1982, Sgarlata et al, 1983, Dawood et al, 1984). It would seem, therefore, that there is no real evidence that the presence of endometriosis effects the peritoneal volume.

Steroid Environment

During the normal menstrual cycle, the levels of oestradiol and progesterone in plasma and peritoneal fluid are very similar in the early and mid follicular phases. As the follicle rapidly increases in size during the late

follicular phase, the intrafollicular levels of oestradiol rapidly rise, as do the levels in peritoneal fluid, (McNatty et al, 1975). The rise in plasma steroids, although considerable, is less marked than the change in the intraovarian or peritoneal environment, thereby suggesting that there is a direct ovarian transudate (Maathius et al, 1978, Koninckx et al, 1980a). In the postovulatory period, the levels of oestradiol are 8-10 times the peripheral levels and progesterone is up to 50 times higher than that found in plasma (Maathius et al, 1978, Donnez et al, 1982, Zorn et al, 1982).

In endometriosis, the peritoneal steroid hormone levels when compared to controls have shown no significant difference (Donnez et al, 1983, Dhont et al, 1984). The levels of oestradiol and progesterone have been suggested to be decreased in moderate and severe disease (Donnez et al, 1983), but this may merely reflect poor follicular growth. They suggest that this may explain the poor fertile potential in these patients as opposed to those with mild disease, but moderate and severe disease is probably multifactorial when affecting fertility potential. Koninckx and Brosens (1982) suggest that the luteinized unruptured follicle (LUF) syndrome is an aetiological factor in infertility in endometriosis patients, reflected by decreased levels of progesterone in the peritoneal fluid. Although similar results were found by Lesorgan et al (1984), Dhont and co-workers (1984) have failed to substantiate the association of LUF and lower peritoneal

progesterone. Thus the conclusions about variations in peritoneal steroids and endometriosis remain debated, and no certainty can be drawn from the data currently available.

Prostaglandins

These arachidonic acid derivatives have an as yet ill-defined role in reproduction. In other mammals their role has been demonstrated; in the mechanism of ovulation in rabbits (Kobayashi et al, 1981), and luteolysis in sheep (Henderson and McNatty, 1975) and monkeys (Kirton et al, 1970). Prostaglandins are produced by endometrium (Downie, 1974), developing follicles (Ylikorkala et al, 1984) and peritoneal macrophages.

Endometriotic deposits have also been demonstrated to produce prostaglandins when cultured in vitro (Ylikokala et al, 1983). Schenken and Asch (1980) induced endometriosis in the rabbit and showed that peritoneal levels of prostaglandin F (PGF) were markedly elevated and that this was associated with decreased ovulation and fertility. This led many investigators to look at peritoneal prostaglandin levels in humans with endometriosis. In humans, endometrium produces mainly prostaglandin E (PGE) in the follicular phase and $PGF_{2\alpha}$ in the luteal phase, and thus the time in the cycle when sampling occurs is important in interpreting the results. The circulating PGE and $PGF_{2\alpha}$ are rapidly converted to their metabolites, 6-keto prostaglandin $F_{2\alpha}$ and 13,14, dihydro-15-keto prostaglandin $F_{2\alpha}$ and E_2 and thromboxane B_2 (TXB_2). Drake et al (1981) measured TXB_2 and

6-keto prostaglandin $F_{1\alpha}$ (a breakdown product of prostacyclin) in patients with and without endometriomas and they found higher levels in patients with endometriosis. The study can be criticised as the time in the cycle was not consistent in the two groups. Subsequently, Sgarlata et al (1983) repeated the study with cycle date control and showed no significant difference in the two groups. Badawy et al (1982) measured 13,14-dihydro-15 keto $FG_{2\alpha}$ and $PGE_{2\alpha}$, and Rock et al (1982), in addition, measured TXB_2 and again showed no significant difference in endometriotics and controls.

In the study published by Dawood et al (1984), the claim for a role of prostaglandins in endometriosis is again investigated. However, although elevated levels of 6-keto $PGF_{1\alpha}$ were found, the other metabolites, TXB_2, $PGF_{2\alpha}$ and PGE_2 were unchanged. The authors offer varying cycle day as an explanation for the elevated 6-keto $PGF_{1\alpha}$ and thus failed to associate prostaglandins and endometriosis. The only recent publication supporting the role of prostaglandins is by Ylikorkala et al (1984) with significant levels of 6-keto $PGF_{1\alpha}$ and TXB_2, who also suggest that the levels are also higher when the disease is more advanced. The diversity of results reported casts doubt on the theory that prostaglandins have a role in endometriosis and this hypothesis is at present in abeyance.

Macrophages

Macrophages constitute the majority (85%) of cells

found in the peritoneal fluid, and these cells are capable of secreting products responsible for an inflammatory response but, perhaps more importantly, they play a vital role in antigen recognition and the development of the appropriate immune response.

Haney et al (1981) found an increase in the total number of macrophages in patients with endometriosis as opposed to controls, but unfortunately no relationship to peritoneal volume is included and thus the significance in terms of concentration of cells could not be judged. Halme et al (1982) subsequently demonstrated that there is a cyclical fluctuation in the number of macrophages, the time of menstruation being associated with an increase in macrophage concentration, provided the fallopian tubes were patent. This suggests that retrograde menstruation influences the macrophage population. Muscato et al (1982), found that the macrophages from endometriotic patients showed a significantly greater ability to phagocytose normal sperm than controls and they suggested that this could be the cause of infertility in patients with endometriosis. These macrophages have also been found in the fallopian tube and this has led to the suggestion that the ovum as well as the sperm could be influenced by their presence (Haney et al, 1983). Halme et al (1981) showed in their study, an increased concentration of macrophages between day 13 and 21 of the cycle in patients with endometriosis.

In an attempt to identify the role of the macrophages, Halme et al (1984a) looked at enzyme activity

of these macrophages and showed greater activity in macrophages from patients with endometriosis as opposed to controls. The phagocytic activity per se was not, however, different. The macrophage population has recently been studied using flow cytometry and two distinct cell populations have been found. Endometriosis was associated with a significantly increased number of peritoneal macrophages and a higher proportion of large macrophages (Halme et al, 1987). These workers also showed increased cell surface antigen activity in these large macrophages suggesting an increased expression of both lysosomal and associated enzymes. The hypothesis proposed is that circulating monocytes are recruited to become peritoneal macrophages and in the normal situation phagocytosis occurs to remove menstrual debris, the cell membrane function being low. In endometriosis, the macrophage population undergoes increased maturation and activation and the cell membrane secretes proteases, acid hydrolases and prostaglandins. It may be that cells produce putative growth factors which stimulate endometrial cell growth which leads to implantation in ectopic sites in the peritoneum.

The role of macrophages in the aetiology of endometriosis or in the manifestation of infertility seems plausible and further immunological studies will be required to confirm or refute this.

IMMUNOLOGICAL MECHANISMS

Humoral Factors

The primary humoral defence mechanism of the body comprises the antigen-antibody reaction in combination with complement (C). Complement is a group of at least 20 plasma proteins, 9 of which are of major importance (C1 to C9), and they circulate as inactive molecules. Complement activation gives rise to a series of active fragments which are generated during the proteolytic cascade. This may be induced in two ways, either by antigen-antibody complexes (IgG1, IgG2, IgG3 and IgM), DNA and C-reactive protein, or via the "alternative pathway" by IgA. Activation of the complement system leads to cytolytic destruction, increased vascular permeability, contraction of smooth muscle, release of lysosomes, granulocyte aggregation and chemotaxis.

Weed and Arquembaurg (1980) first reported the presence of C3 in the mid cycle uterine endometrium in endometriosis, with an absence in control samples. This finding has not been confirmed in subsequent work by Bartosik et al (1984), but interestingly, they did show a negative correlation between C3 and C4 and the stage of the disease. In stage 1, 79% of patients had C3 or C4, 50% with stage 2, and 40% with stage 3 disease, although the reason for this is uncertain.

Immunoglobulins are found in human endometrium, IgG being prominent and this is highest in the secretory phase of the cycle. IgA is also found, again predominantly in the

secretory phase (Tourville et al, 1970). It seems, however, that IgA is probably of greater concentration in the secretory phase than the proliferative phase, and the levels of IgG and IgM are of much less significance (Rebello et al, 1975, Kelly and Fox, 1979).

In patients with endometriosis, the reports are somewhat contradictory. Mathur et al (1982) found large quantities of endometrial IgG in endometriosis as compared to controls, although no mention is made about the time of the cycle. Bartosik et al (1984) found IgG in 74% of all patients with endometriosis as against 73% of normal women, and they also report an inverse relationship with the stage of the disease; 70% in stage 2 and 60% in stage 3. IgA, however, was significantly lower in patients with endometriosis (29%) as opposed to controls (60%). Again there was an inverse relationship with increasing severity of the disease.

It is difficult to speculate as to the specific role these compounds play in endometrial function, but increased IgG and IgA are required by the mouse to facilitate implantation (Bernard et al, 1981). As the immunoglobulin levels decrease with increasing severity of the disease, failure of implantation may result and thus contribute to the infertile state.

Mathur et al (1982) also demonstrated the presence of high levels of autoantibodies in 11 of 13 patients with endometriosis. They found elevated levels of anti-whole ovary, anti-granulosa cell and anti-theca cell antibodies

and 7 of 13 had anti-endometrial cell antibodies. They also demonstrated a 100% binding of sera from endometriosis patients to normal endometrium, 9 out of 13 having IgG and IgA antibodies; 3 had IgG and IgM antibodies, one having IgM alone.

Recently, Grimes et al (1985) demonstrated that patients with endometriosis exhibited twice the normal risk of developing systemic lupus erythematosis (SLE) as compared to controls and an increased spontaneous abortion rate is seen in patients with SLE (Lubbe et al, 1984). Endometriosis is reported as being associated with a higher spontaneous abortion rate (Wheeler et al, 1983, Groll, 1984) suggesting that the disease may have a systemic effect. Further recently published work by Gleicher et al (1987) confirms the relationship between endometriosis and abnormal B lymphocyte function classically associated with autoimmune disease. This was most commonly seen in those patients who were positive for lupus anticoagulant.

This work strongly suggests an autoimmune basis to endometriosis; the question which remains unanswered is however, which came first, the disease or the autoantibodies?

Cellular Immunity

Important work by Dmowski et al (1981) studied cellular immune mechanisms in endometriosis. In rhesus monkeys, a group of animals were found with spontaneous

endometriosis. Intradermal injections of preparations of a) endometrial antigens, b) peritoneal antigens, and c) antigens raised from ectopic endometrium were given to control animals and those with endometriosis. The delayed hypersensitivity reaction was measured and animals with endometriosis showed significantly reduced inflammmatory response. In vitro studies using lymphocytes from the two groups showed a similar reduced cellular immune response. Steele et al (1984) further studied the cellular immunity by measuring cytolytic function of lymphoid cells. Lymphocytes from control patients were significantly more efficient in their cytolytic effect on endometrial cells than those from endometriosis patients. This effect was only evident in patients with moderate or severe disease.

It is difficult to establish the significance of these findings as the cellular immune response may be moderated through prostaglandins, although again it is difficult to know whether this is the primary or the secondary response. It may be attractive to suggest that endometriosis develops in susceptible individuals because of deficient cellular immune mechanisms but further work is necessary to substantiate this.

ENDOMETRIOSIS AND OVARIAN FUNCTION

There have been a number of associations of ovarian dysfunction with endometriosis which have led to hypotheses about the development of endometriosis. Little substantiated evidence exists to confirm these theories.

Anovulation

The prevalence of anovulation coexisting with endometriosis varies from 10%-27% of infertile women, (Soules et al, 1976, Dmowski et al, 1976, Badawy et al, 1981). However, all of the studies suffer from: a) having no control group and b) laparoscopies were probably performed in patients with anovulation after attempts to induce ovulation, thereby biasing the study group. The prevalence of anovulation in the normal population may be as high as 10% and thus no firm association can be postulated.

Luteal Phase Defects

There have been several studies looking at this association, again without any conclusion. There was no proven association in the retrospective study by Cheesman et al (1983) of 66 patients and in the prospective study by Pittaway et al (1983) on 143 ovulatory infertile patients, 9% of endometriosis patients and 5% of non-endometriosis patients had evidence of luteal phase defects, the difference not being significant. The association remains questionable.

Luteinized Unruptured Follicle Syndrome

It was Jewelewicz (1975) who first coined the term, luteinized unruptured follicle (LUF) syndrome in which the oocyte remains trapped inside a ripened follicle which fails to rupture. The follicle is luteinized and thereafter produces progesterone, and therefore LUF cannot be diagnosed

endocrinologically. It is defined either at laparoscopy after ovulation by the absence of an ovulatory stigma on the corpus luteum, or by serial ultrasound scans which detect failure of follicular collapse after the LH surge and the persistance of a follicular cyst. However, there is considerable doubt as to the reliability of these techniques to diagnose LUF as a problem. Vanrell et al (1982) inspected the ovaries of 15 patients undergoing sterilisation in the luteal phase of the cycle, and failed to identify the ovulatory stigma in 47% of cases. Furthermore, in a study of 325 infertile patients in the luteal phase, 90% had a corpus luteum, but only 18% had an identifiable stigma. Two patients with a corpus luteum and no stigma became pregnant in the laparoscopy cycle (Portuondo et al, 1981). Therefore, considerable doubt exists as to the significance of the LUF syndrome.

The use of ultrasound in evaluating the prevalence of the LUF syndrome reveals an overall lower rate. In the study of Kerin et al (1983), they found a prevalence of 14% in 66 patients (5% of 183 cycles) when daily ultrasound was used to monitor follicular growth. There was only one cycle of recurrence in 35 additional cycles. They conclude that LUF is a sporadic, infrequent phenomenon. Daly et al (1985) found similar results in 33 patients with a rate of 9%, and a 6% rate of repeat LUF in a subsequent cycle. Gibbons et al (1984) studied 153 patients through 263 cycles with ultrasound and found a 9% incidence of LUF. Only the paper by Luikkonen et al (1982) disagrees with the above reports,

and they reported an 84% incidence of LUF in unexplained infertility, and 48% had repetitive findings. In the light of other studies showing a prevalence of 9-14%, one must question the validity of this report.

The first study examining LUF and endometriosis was published by Brosens et al (1978). He evaluated 34 endometriosis patients and 28 control subjects (tubal and male factors) and in spite of minimal endocrine differences, found that 21% of endometriosis patients had ovarian stigma versus 94% in the controls. It was concluded that endometriosis associated with infertility may result from LUF. The same authors reported in 1980 (Koninckx et al, 1980b) on 81 patients with infertility (37 with endometriosis) and suggested that LUF may cause endometriosis. The peritoneal fluid was assayed for oestradiol, progesterone, total protein and SHBG, and they found that there was an increase in peritoneal concentrations of progesterone in normal cycles as opposed to those with LUF. They suggested that the higher level of progesterone was protective against peritoneal implantation of endometrial cells. Two follow up studies (Donnez et al, 1983 and Dhont et al, 1984), have failed to confirm these findings, both studies being compared with matched controls. It was interesting to note that Donnez et al (1983) found a higher incidence of LUF in patients with more severe disease, and it may be that adhesion formation around the ovary could have a role in preventing follicular rupture. Studies in monkeys by Schenken et al (1984) support this

finding. In 21 monkeys with surgically induced endometriosis, lower term pregnancy rates were found with moderate and severe disease and LUF was found in 46% of moderate disease and 50% of severe disease. The animals with mild disease and the control animals had no evidence of LUF.

Evidence for LUF has been found in normal, fertile women and it is therefore doubtful whether it has any role in infertility. The increased observed prevalence in humans and other primates with moderate and severe endometriosis may reflect ovarian adhesion formation rather than an aetiology in endometriosis. Furthermore, better controlled studies are necessary to establish whether LUF syndrome is a significant aetiological factor.

ENDOMETRIOSIS AND THE HYPOTHALAMO-PITUITARY AXIS

In 1978, Hirschowitz et al suggested that there was a syndrome called the "galactorrhoea-endometriosis syndrome" which existed with normoprolactinaemia, but demonstrable galactorrhoea in 8 of 9 patients with endometriosis. Five had galactorrhoea prior to danazol therapy and three either on therapy or post therapy. No further reports of this association, however, have been forthcoming.

Interestingly, Muse et al (1982) suggested that hyperprolactinaemia through stress, may play a role in endometriosis. Fourteen infertile patients with endometriosis and 12 control women having sterilisation had baseline prolactin levels measured, and a thyrotrophin

releasing hormone (TRH) stimulation test. There was a significant difference in peak values after TRH, higher in the endometriotic patients. There was no difference in basal levels. Again, no follow up studies have confirmed these findings.

These limited reports are in themselves interesting, but failure to substantiate the data on prolactin casts doubt on any relationship with endometriosis.

Gonadotrophin secretion has also been investigated for signs of disruption. Urinary luteinizing hormone (LH), conjugated oestriol and pregnanediol were measured in 53 infertile women, 29 of whom had endometriosis. Twenty six of the endometriosis patients were found to have two distinct LH surges, two to three days apart. As there was no elevation in the pregnanediol until after the second surge, these patients were assumed to have a short luteal phase, (Cheesman, 1982). The control group in this study could not be described as normal however, as they all had infertility problems. So, although the authors suggested a poor ovarian response to the first LH surge leading to a disruption in positive feedback, there are no subsequent publications to confirm this.

The LH receptors in follicles and corpora lutea were studied by Ronnberg et al (1984) in patients with endometriosis and controls. They found that the LH receptor level was lower in the early and late follicular phases in endometriotic patients, suggesting that there may be an abnormality at the end organ, perhaps explaining the double

LH peak suggested by Cheesman et al (1982) or even as an explanation for LUF syndrome.

ENDOMETRIOSIS AND THE UTERUS

Whilst it seems that retrograde menstruation is a pre-requisite to the establishment of pelvic endometriosis, the uterine environment seems to be affected in the disease also.

Adenomyosis

This condition is characterised by endometrial tissue being found either in localised areas or diffusely spread in the myometrium. It is these histological changes which have led to the association between endometriosis and adenomyosis, both being ectopic sites of endometrium. The aetiology and pathogenesis of adenomyosis remains obscure, although pregnancy and curettage have been implicated. As adenomyosis is a diagnosis made by a pathologist, the aetiology is difficult to study, but some 10-20% of patients with adenomyosis are found to have endometriosis at the time of surgery, (Dewhurst, 1979).

STEROID RECEPTORS

Shortly after the advent of the establishment of cytosol oestogen receptor assays in human endometrium (Spona et al, 1979), investigators began to look at a comparison of receptor concentration in endometriotic tissue and endometrium. Tamaya et al (1979) reported on oestrogen

receptors (ER) and progesterone receptors (PR) in 7 cases of ovarian endometriosis. The levels of ER and PR were substantially lower than in corresponding endometrium, the level of androgen receptors (AR) remaining constant. In 1981, Janne et al reported that endometriosis was characterised by low or absent ER content but high PR levels. In the same year, Berqvist et al (1981) found lower concentrations of ER and PR in endometriosis as compared with normal endometrium. However, in 12 of 20 cases no ER, and in 7 out of 9 cases no PR could be detected, thus casting doubt on the conclusions from their data. The data by Tamaya (1979) are difficult to interpret as no mention is made of cycle day. The subsequent results seem to be rather contradictory, and two further publications fail to clarify the situation. Kauppila et al (1985) suggested similar results to Berqvist et al (1981) with lower levels of ER and PR, whereas Saracoglu et al (1985) claim no significant difference between ER and PR in endometriotic tissue and endometrium. Such discrepancies in results suggest that biochemical steroid receptor assays are unreliable as they require large tissue samples and their sensitivity may be insufficient to detect receptors in samples with low receptor-containing cells. In the light of this finding, Berqvist et al (1984) used a histochemical technique, thereby identifying steroid receptor binding on individual cells. They discovered distinct and indifferent binding in endometriotic tissue and endometrium obtained simultaneously from the same patients. Thus, it would seem that

endometriosis cells show little difference at the ER and PR level. Androgen receptor concentration is mentioned solely in the paper by Tamaya et al (1979) and showed no differences.

CHAPTER 2

MEDICAL TREATMENT OF ENDOMETRIOSIS

ANDROGEN THERAPY

Until the 1940s, the only treatment available for endometriosis was surgery, either conservative or radical. The first medical treatment to be used was androgens, either methyl testosterone or testosterone proprionate (Hirst, 1947, Creadick, 1950, Preston and Campbell, 1953). It was used during the 1940s and 1950s on the premise that it was anti-oestrogenic and at that time, endometriosis was thought to be due to excess oestrogens (Preston and Campbell, 1953). All the above authors reported symptomatic improvement in approximately 80% of patients and pregnancy rates of 11-20%. Although no serious virilising side effects were observed, this is an obvious disadvantage of androgen therapy. Surprisingly, as late as 1978, Hammond et al performed a prospective study using methyl testosterone, citing that its advantage was that "it did not suppress ovulation and therefore conception could occur during treatment". Apart from the risk of virilisation to a female fetus, they achieved only a 11% pregnancy rate and observed a rapid recurrence of symptoms on cessation of therapy.

OESTROGENS, PROGESTOGENS AND COMBINED THERAPY

Following the impression gained by clinicians that pregnancy improved endometriosis because of the production

of a decidual reaction, treatments aimed at inducing a state of "pseudopregnancy" were investigated. It was proposed that in the areas of decidualisation in the endometriotic lesions, subsequent necrosis and fibrosis would occur (Kistner, 1958). As the decidual reaction was related to high levels of oestradiol (E2) and progesterone (P), these have been tried both separately and together as methods of treating the disease medically.

Initially, incrementally higher doses of stilboestrol were administered to patients, often without proven endometriosis (Karnaky, 1948, Haskins and Woolf, 1955). Although subjective improvement occurred, side effects were common, as were the recognised complications of prolonged unopposed oestrogen therapy, i.e. break through bleeding, endometrial hyperplasia, metropathia. Further Haskens and Woolf (1955) admitted that they were unable to detect any clinical change in endometriotic lesions.

After this, various combinations of oestrogen and progesterone were tried. Symptomatic relief in 36% (Noble and Letchworth, 1980) to 89% (Kourides and Kistner, 1968) of patients has been described with this regimen. Both Kistner (1958) and Andrews et al (1959) observed decidual transformation of the endometrium and endometriotic tissue in all their cases, but the latter group found pseudodecidual cast expulsion was a problem if treatment was stopped prior to 10 weeks. Pregnancy rates following treatment range from 29% to 43% (Kourides and Kistner, 1968, Andrews and Larson, 1974, Noble and Letchworth, 1980). (A

more detailed discussion on the comparability and value of pregnancy rates quoted in the literature is found in Chapter 3). Side effects were a serious problem in up to 87% of patients (Noble and Letchworth, 1980), especially weight gain, breast tenderness, nausea and vomiting, break-through bleeding and, more seriously, thrombosis. Further, at follow up operation, 19 of 21 patients treated by Andrews and Larson (1974) still had active endometriosis, and compared to a group of patients who were treated by operation in the first instance, the subsequent need for surgical treatment was not reduced.

In view of the concern over side effects, mostly attributed to the oestrogen component of the combined therapy, different natural and synthetic progestins have been used, both cyclically and continuously. Most authors have described rapid and effective relief of symptoms in up to 89% of patients and pregnancy rates of 40-90% (Ullery et al, 1963, Johnson, 1976, Moghissis and Boyce, 1976). Although side effects occurred less frequently, break-through bleeding has been the major drawback. Gunning and Moyer (1967) tried using intramuscular Depo-provera, a long acting progestational agent, to overcome this, and despite observing a decidual reaction with necrobiosis in the endometriotic tissue of their patients, they all suffered from some degree of continuous vaginal bleeding or spotting. Also, cyclical menstruation did not return for 4-7 months after cessation of therapy, which may be considered unacceptable by patients with infertility.

An interesting study by Scott and Wharton (1962) on rhesus monkeys with experimentally induced endometriosis showed that treatment with progesterone caused an increase in the areas of endometriosis, which remained unaffected microscopically, with spotting and abnormal bleeding occurring during therapy. They then investigated the effect of a synthetic progestin (Norethindrone) which induced amenorrhoea and although decidual changes with necrosis and patchy fibrosis occurred, abundant stroma and glands remained and the endometriotic lesions did not change in size. They recommend the use of progestogens only prior to surgery to aid visualisation of endometriotic implants and to help dissection through tissue planes, and their findings question the value of progestogens in the treatment of endometriosis. Indeed, there is a lack of prospective, controlled or comparative trials of "pseudopregnancy" treatment and also of long term recurrence rates following this method of therapy.

LUTEINIZING HORMONE RELEASING HORMONE (LHRH) ANALOGUES

As surgical castration and the menopause result in the regression of endometriosis, a more recent alternative approach to treatment has been the temporary induction of a "pseudomenopausal" state. LHRH analogue treatment causes desensitisation of the pituitary and a reversible suppression of gonadotrophin secretion. These analogues have been synthesized and administered for the treatment of endometriosis by the intranasal and subcutaneous injection

routes. They cause anovulation, with menopausal levels of E2, and following an initial surge in luteinizing hormone (LH) and follicle stimulating hormone (FSH), these levels fall to low baseline values (Meldrum et al, 1982, Lemay et al, 1984, Zorn et al, 1986).

Symptomatic improvement has been seen in 72-90% of patients and clinical improvement in 88% (Lemay et al, 1984, Matta and Shaw, 1987). At repeat laparoscopy, an 83-100% improvement in endometriosis has been observed (Shaw et al, 1983, Lemay et al, 1984, Matta and Shaw, 1987), and Lemay et al (1984) found atrophy or weak proliferation in all their endometrial biopsies performed on the last day of 6-8 months of treatment. Studies of long term pregnancy and recurrence rates are still in progress, but Matta and Shaw (1987) suggest a 20% pregnancy rate after 6 months and Jelley (1987) a 30% recurrence rate at 2-8 months.

Side effects relating to the hypo-oestrogenic state are common (i.e. hot flushes, sweats, atrophic vaginitis) and although many clinicians are optimistic about this new method of treatment, there is concern over the risks of long term oestrogen deficiency e.g. osteoporosis.

DANAZOL AND GESTRINONE

Currently the most widely used drug in the treatment of endometriosis is danazol. An interest has developed in the use of a new steroidal drug, gestrinone, which was originally investigated as a potential contraceptive agent.

The mechanism of action and metabolism of danazol are now discussed. The clinical effects of these drugs are described in Section 2.

MODE OF ACTION AND METABOLISM OF DANAZOL AND GESTRINONE

It has been repeatedly stated in the literature that "danazol has strong antigonadotrophic properties and mild androgenic effects, with no other hormonal properties". However, this is derived from Greenblatt et al's (1971) original paper on danazol, which was based on experiments in rodents and that did not include the direct measurement of serum gonadotrophin concentrations. Current knowledge indicates that the pharmacology of both danazol and gestrinone is far more complex.

The proposed mechanisms of action described for these drugs include:

1) An antigonadotrophic effect on the hypothalamo-pituitary axis.

2) A reduction in sex hormone binding globulin (SHBG) binding capacity resulting in an increase in the biologically active, free fraction of testosterone (T).

3) A direct effect via steroid receptors.

4) An antiprogestogenic effect.

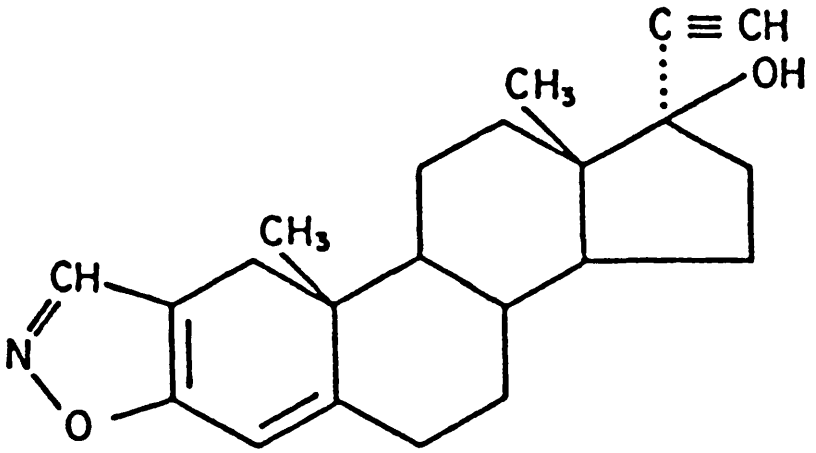
5) An effect on steroidogenesis in the ovary and also possibly in the adrenal gland.

6) An immunosuppressive effect.

The data available for each of these proposed mechanisms will be discussed in detail. In order to consider also the possible contribution of the metabolites of these drugs, the metabolism of danazol and gestrinone will first be described.

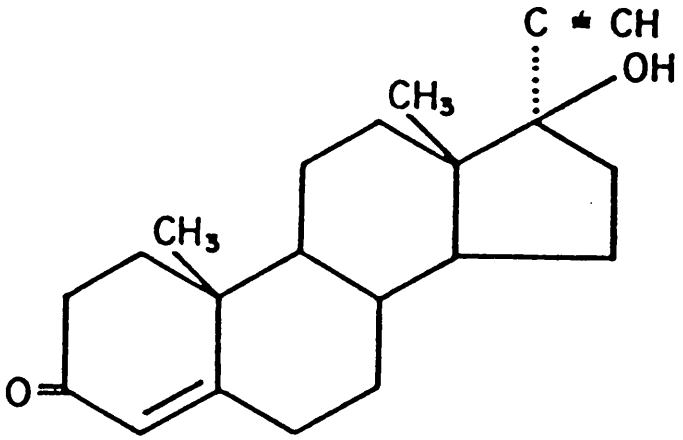
METABOLISM OF DANAZOL

Danazol (Figure 1.1) is well absorbed orally, achieving a steady state plasma concentration of 1000-2000 nmol/l at doses of 600-800mg daily (Lloyd Jones et al, 1977). The circulating half-life of danazol is reported as 15 hours in humans (Petersen et al, 1978), although the tissue half-life of some of its derivatives is longer, probably days (Goldman and Kenneck, 1970). It is rapidly metabolised and approximately 60 end products have been found in monkeys' urine (Davison et al, 1976). Six of these have been identified of which 2-hydroxymethyl ethisterone (2OHME), ethisterone (E) and Δ^1-2-2OHME are the major metabolites. Their structures and chemical formulae are illustrated in Figure 1.2. Trace amounts of 2-ketoethisterone, 6β-hydroxy-2-OHME and Δ6β-hydroxy-2OHME have also been demonstrated, and these 6 named metabolites represent approximately 11% of the excreted drug (Potts, 1977). Potts tested these metabolites for antigonadotrophic properties at doses at which danazol is effective and found they were inactive, and concluded that danazol per se was the active steroid. However, Desualles and Krahenbuhl (1964)

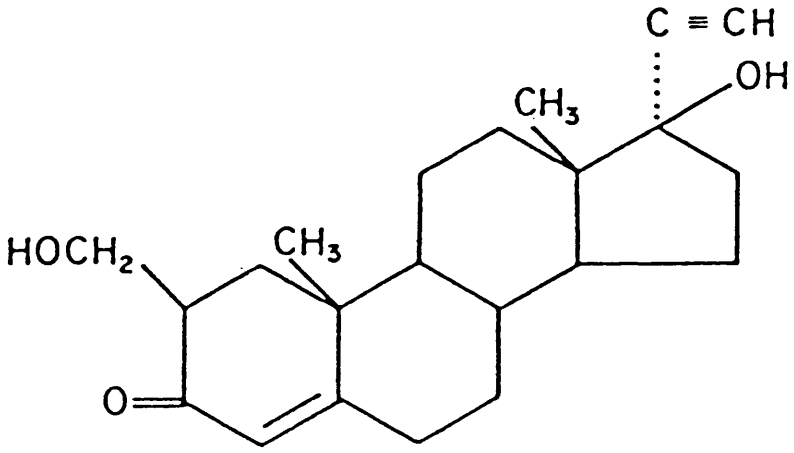


DANAZOL

FIGURE 1.1: The Chemical structure of Danazol
(17-α-pregn-4-en-2yno-(2,3-d) isoxazol-17-ol)



A) Ethisterone



B) 2-Hydroxymethyl ethisterone

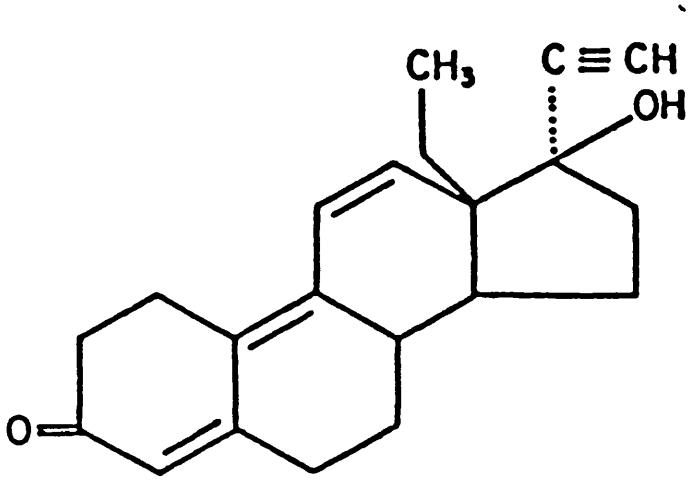
FIGURE 1.2: The chemical structure of Ethisterone and 2-Hydroxymethyl ethisterone

observed progestational and androgenic effects from 2OHME and that E minimally suppressed LH and FSH.

In the monkey little unchanged danazol is excreted in the urine or faeces at pharmacological doses, but metabolites have been found in both, whereas in the rat, excretion only occurs in the faeces (Davison et al, 1976). In both these animals, concentrations of danazol greater than those in plasma were found only in the liver, kidneys and adrenal.

METABOLISM OF GESTRINONE

Gestrinone (Figure 1.3) is also well absorbed orally, but has an elimination half-life of 26.8hrs and hence, can be administered much less frequently than danazol. Its most important metabolic pathway is hydroxylation which creates four metabolites whose biological activity is similar to, but less than gestrinone. The majority of gestrinone is excreted in the urine. Similar excretion has been demonstrated in guinea pigs, rats and rabbits (Salmon et al, 1984).



GESTRINONE

FIGURE 1.3: The chemical structure of Gestrinone (17α)-13-ethyl-17α ethinyl-17 hydroxy-gona-4,9,11-trien-3-one

The antigonadotrophic effect (1) and that on SHBG binding capacity and percent (%) free T (2) are discussed in the introduction of Section 3 (Review of the endocrine effects of danazol and gestrinone).

3) A DIRECT EFFECT VIA RECEPTORS

Danazol has been shown to bind with a relatively high affinity to androgen receptors (AR) in rat prostate (Barbieri et al, 1979, Chamness et al, 1980, Moguilewsky and Philibert, 1984), and also in human endometrium and myometrium (Punnonen and Lukola, 1982, Jenkin et al, 1983, Tamaya et al, 1984). Chamness et al (1980) further demonstrated that danazol translocated the AR into the target cell nuclei and that nuclear receptor levels remain elevated for several hours. These findings are consistent with the androgenic effects of stimulation of prostate, seminal vesicle and levator ani weights observed in castrated male rats in vivo (Potts, 1977).

Danazol has also been shown to bind with a moderate affinity to progesterone receptors (PR) in rat and rabbit uteri (Barbieri et al, 1979, Chamness et al, 1980), which is 3.3% that of P (Kokko, 1983), and binds to PR in human endometrium (Punnonen and Lukola, 1982, Jenkin et al, 1983, Tamaya et al, 1984). Chamness was unable to demonstrate translocation of the PR, but Tamaya et al (1984) found that the danazol-PR complex translocated into the nucleus at a

very slow rate but was unable to stimulate RNA synthesis. Moguilewsky and Philibert (1984) were unable to demonstrate an interaction of danazol with PR in in vivo experiments.

Danazol was not shown to bind to oestrogen receptors (ER) in rat uteri (Barbieri et al, 1979), nor in human uteri (Wood et al, 1975, Punnonen and Lukola, 1982). However at high doses, low affinity to ER was demonstrated by Jenkin et al (1983) and Tamaya et al (1984). The latter suggest that as circulating concentrations of danazol are 11000-23000 times greater than oestradiol, danazol may interfere with normal ER dynamics and even be antioestrogenic.

Danazol has also been shown to have significant glucocorticoid receptor (GR) binding activity in vitro and in vivo (Barbieri et al, 1979).

Gestrinone has been shown to bind to AR, PR, ER, GR and mineralocorticoid (MR) receptors in tissues of the rat, mouse and rabbit in vitro, with a more marked relative binding affinity to AR and PR. These findings have been confirmed in in vivo experiments in which nuclear translocation was seen with ER and PR binding (Raynaud et al, 1975, Moguilewsky and Philibert, 1984).

4) AN ANTIPROGESTOGENIC EFFECT

There is much debate as to whether danazol exerts progestational or antiprogestational activity, but some of the confusion may exist because of the progestational effect

of the metabolite, ethisterone (Desualles and Krahenbuhl, 1964).

Potts (1977) observed a dose related anti-progestational effect produced by danazol in the Clauberg assay test. However, Henig et al (1988) noticed that in castrated female rats treated with danazol, serum and endometriotic explant cytosolic ER and PR concentrations were significantly higher than in other castrated groups. They suggested that the increase seen in ER concentrations may reflect progestational like properties of danazol or its metabolites, or alternatively may have been secondary to danazol AR binding which may have affected ER and PR replenishment and resulted in a net increase in receptor concentrations. Prior to this Wentz et al (1976) had also described a progestational effect on the endometrium of ovariectomized patients pretreated with oestrogens, but Schweppe et al (1981) strongly disputed the interpretation of their findings.

Tamaya et al (1983) observed decreased ER and PR levels in the endometrium of patients treated with danazol for 10 days, but increased levels if therapy continued for longer than one month. However Musich et al (1981) found the converse in rat uteri, where short term treatment increased both ER concentration and binding and long term medication caused a marked decrease in ER binding capacity. They infer from these findings that danazol has weak oestrogenic and strong anti-oestogenic effects on uterine tissue, but they did not perform their experiments on castrated animals.

In conclusion, danazol is thought to have mixed agonist/antagonist effects on the PR system, probably due to the differing effects of the drug and its metabolites, especially E.

In the Clauberg test, gestrinone has antioestrogenic and antiprogestogenic effects. In vivo, in rats, it was found to be slightly progestomimetic at very low doses (Azadian-Boulanger et al, 1984) and further experiments demonstrated oestrogenic, antiprogestogenic and androgenic activities of this drug (Sakiz and Azadian-Boulanger, 1970).

Reel et al (1979) also demonstrated mixed progestational and antiprogestational effects from gestrinone and suggested that it bound to PR in the uterus where it behaved as a mixed agonist/antagonist. However, Cornillie et al (1985) examining endometriotic lesions in patients treated with gestrinone observed enhanced lysosomal activity in epithelial cells which mimicked the premenstrual lysosomal degradation seen in endometrium and concluded that gestrinone was competitively binding with PR causing an antiprogestogenic effect. In contrast, Kaupilla et al (1985) found that gestrinone treatment for one month caused a reduction in ER and PR concentration and an induction of 17β hydroxysteroid dehydrogenase in endometrium, a typical progestin effect, although no corresponding effect was seen in endometriotic tissue from the same patient.

In summary, there appears to be evidence of

progestogenic and antiprogestogenic activity by gestrinone.

5) EFFECT ON STEROIDOGENESIS

There is evidence for inhibition by danazol by many enzyme systems, in vitro and in vivo.

Danazol inhibited E2 and P production in rat luteal cells (Menon et al, 1980, Henderson and Tsang, 1980) and from cultured granulosa cells (Olsson et al, 1986). Rabe et al (1983) also showed that human placental P synthesis could be inhibited in vitro by danazol. Although Asch et al (1980) observed lowered P levels in monkeys having menstrual cycles, treated with danazol, Lieberman et al (1977) concluded danazol had no direct inhibitory action on P production by the corpus luteum in vivo, as danazol administered after ovulation failed to lower P levels.

Barbieri et al (1977) found that danazol inhibited multiple enzyme systems in rat organs in vitro; 17α -hydroxylase, 17,20-lyase, 3β-hydroxysteroid dehydrogenase in the ovary; the afore mentioned and 17β-hydroxysteroid dehydrogenase in the testis and 3β-hydroxysteroid dehydrogenase, 21-hydroxylase and 11β-hydroxylase in the adrenal. They also describe a rapid fall in serum T in adult rats following a subcutaneous injection of danazol which they attribute to an in vivo inhibition of T production. This reduction in T synthesis has also been demonstrated in vitro in the mouse testis (Asch et al, 1980), and in vivo in men (Sherins et al,1971) and in women with a complete form of testicular feminisation

(Reyniak and Gurpide, 1982). Stillman et al (1980) also produced evidence to suggest that danazol inhibits the enzymes, 3B-hydroxysteroid dehydrogenase and 11B-hydroxylase in the adrenal in vivo, by assessing the concentrations of various steroids of adrenal origin before and after acute ACTH stimulation. Inhibition of steroid sulphatase activity has also been demonstrated in vitro by Carlstrom et al (1984).

Recently, Olsson et al (1988) have shown that danazol rapidly concentrates in follicular fluid in a dose-dependent manner (73% serum concentration after 2 days). Hence, local drug concentrations are attained capable of inhibiting ovarian follicular steroid production. This supports the possibility that the inhibition of steroidogenesis seen in in vitro experiments (at therapeutic danazol doses) may occur in vivo.

Gestrinone has been tested in vitro on 5 enzyme systems involved in steroid synthesis and also on prostaglandin synthase, in order to detect a possible inhibitory action. Gestrinone was found to be only a weak inhibitor in all the systems examined and it was concluded that the mechanism of action of gestrinone is not due to a direct alteration of steroid biosynthesis (Belghmi et al, 1984). These investigators did simultaneously confirm the inhibition of enzymes of steroid biosynthesis by danazol, previously described.

6) IMMUNOSUPPRESSIVE EFFECT

Hill et al (1987) demonstrated an inhibition of lymphocyte proliferation in cultures activated by T-cell mitogens and allogenic mixed lymphocyte culture by danazol. The inhibition observed was comparable to that obtained by dexamethasone and cortisol. If the aetiology of endometriosis proves to be immunological, this may explain the efficacy of danazol in its treatment, as well as its use in other autoimmune diseases such as systemic lupus erythematosus (Agnello et al, 1983), idiopathic thrombocytopaenic purpura (Ahn et al, 1983) and angioneurotic oedema (Gelfand et al, 1976).

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AIM OF THE STUDY

The mechanisms of action of danazol and gestrinone in the treatment of endometriosis are still incompletely understood. The aim of this thesis is to further elucidate the means by which these drugs effect their action.

Firstly, a study of the endocrine changes induced by the drugs will be performed, with particular reference to the androgenic effects. The resultant endocrinology will then be correlated with the clinical changes in patients treated with the drugs in a randomized double-blind trial.

Secondly, the direct effect of the two drugs will be examined in vitro, using cultured endometrial cells as a model for endometriotic tissue.

Finally, the clinical results, the endocrine effects

and the tissue culture observations will be correlated in an attempt to clarify further the understanding of the mode of action of danazol and gestrinone.

SECTION 2

THE CLINICAL EFFECTS OF DANAZOL AND GESTRINONE. A RANDOMISED DOUBLE-BLIND STUDY

CHAPTER 3

REVIEW OF THE CLINICAL EFFECTS OF DANAZOL AND GESTRINONE IN THE TREATMENT OF ENDOMETRIOSIS

It is clear that the mechanisms of action of danazol and the newer steroidal agent, gestrinone, are incompletely understood.

The aims of this thesis were to investigate the effects of these drugs at a clinical, endocrinological and in vitro level, so as to understand better and consequently optimise their use in the treatment of endometriosis. In order to interpret the endocrinological changes observed during therapy, it was essential to correlate the results with the clinical situation. Patients were therefore assessed by their symptomatic, clinical and laparoscopic response to treatment.

A prospective randomised double-blind controlled study was therefore conducted on 51 patients with endometriosis, who were treated with either danazol or gestrinone. The clinical data obtained also contributed to a multicentre randomized double-blind trial conducted by Roussel Laboratories Ltd,(Uxbridge, United Kingdom) who required clinical information on the efficacy of gestrinone. This was performed in collaboration with Winthrop Laboratories (Guildford, United kingdom) who supplied the danazol. It was appreciated that the clinical data obtained from the 51 patients studied at the Chelsea Hospital for

Women would be unlikely to provide large enough numbers to answer questions about the relative efficacy of the two drugs.

DANAZOL

Danazol, an isoxazole-derivative of 17α -ethinyl testosterone (17- α -pregn-4-en-2yno-(2,3-d) isoxazol-17-ol) was first synthesized at the Sterling Winthrop Research Institute in 1963 by Manson et al. It was originally used in 1969 in clinical trials, initially in animals, and then in male and female volunteers, followed by therapeutic trials in patients with a variety of gynaecological and endocrinological disorders (Greenblatt et al, 1971). The preliminary data suggested that danazol may be "of value in the management of endometriosis, breast disorders and precocious puberty". Danazol has been available in the United Kingdom since 1974.

DANAZOL IN THE TREATMENT OF ENDOMETRIOSIS

There are many reports on the clinical effect of danazol in the treatment of endometriosis (Table 2.1). These all claim a symptomatic, clinical and laparoscopic observed response, at varying doses and durations of treatment. A symptomatic response of 80-100%, clinical response of 66-100% and laparoscopic response of 61-95% is described. A number of observations and conclusions regarding danazol therapy are made by these authors:

1) Endometriosis should be confirmed by direct

TABLE 2.1: SYMPTOMATIC, CLINICAL AND LAPAROSCOPIC ASSESSMENT OF DANAZOL THERAPY. RESULTS OF PREVIOUS SERIES

REFERENCE	DANAZOL DOSE	NO OF PATIENTS	RESPONSE (%) SYMPTOMATIC	CLINICAL	LAPAROSCOPIC	COMMENTS
GREENBLATT ET AL (1971)	800mg	15	100	66		
FRIEDLANDER (1973)	800mg	39	93		87	
DMOWSKI AND COHEN (1975)	800mg	39	100	100	85	
LAUERSON ET AL (1975)	800mg	32	88	88		
CHALMERS AND SHERVINGTON (1977)	600mg	20	80			
INGERSLEV (1977)	400mg	6	100	100		4 cases of intestinal endometriosis
YOUNG AND BLACKMORE (1977)	200 - 800mg	370	96	87	95	multicentre study (10 centres)
DMOWSKI AND COHEN (1978)	800mg	80	100	85		
BARBIERI ET AL (1982)	800mg	100	89		94	
PULEO AND HAMMOND (1983)	800mg	39	97			
BUTTRAM ET AL (1985)	400mg 800mg	107 96		61		no difference betwen dose groups

visualisation at laparoscopy or laparotomy prior to treatment (Barbieri et al, 1982).

2) Treatment should continue for a period not less than 6 months. However, recently, Brosens et al (1987) have provided new evidence from biopsies of endometriosis lesions showing a maximum response at 2 months, suggesting that shorter term therapy may be as effective.

3) Symptomatic improvement is seen from 2-8 weeks after commencement of the drug (Lauerson et al, 1975, Chalmers and Shervington, 1977, 1979).

4) Ovarian endometriomas respond poorly to danazol (and other medical) treatment, and are better treated surgically when greater than 1cm in diameter (Barbieri et al, 1982, Buttram et al, 1985).

5) Bleeding episodes occur variably during treatment, but are commonest during the early months. Amenorrhea develops in about 66% of patients after one month to more than 95% after three months of therapy, (Greenblatt et al, 1971, Young and Blackmore, 1977, Dmowski and Cohen, 1978).

6) Patients should be advised not to conceive during danazol therapy because of the risks of teratogenicity.

PREGNANCY RATES FOLLOWING DANAZOL THERAPY

The beneficial effect of medical treatment on patients with infertility associated with endometriosis, especially mild endometriosis, has not been proven in any properly controlled randomised trials. There are however, many reports in the literature quoting pregnancy rates for

such patients following treatment with danazol (Table 2.2). The published pregnancy rates vary between 28% and 53%.

It is extremely difficult however, to compare or assess these results for a number of reasons:

1) Some authors have failed to distinguish the severity of the endometriosis in relation to the conception rates, whilst others have categorised patients and analysed their data appropriately.

2) The assessments of endometriosis were performed using a number of different scoring systems, e.g. Acosta et al (1973), American Fertility Society (1979, 1985).

3) Selection of patients with endometriosis as the sole cause of infertility is rare and many papers include patients with additional factors contributing to their infertility.

4) The duration of post treatment follow up was variable.

5) The dose of danazol medication differed.

6) Some of the trials were performed prospectively, others were retrospective, and few had patients randomly allocated to treatment or variable dose groups. Only Siebel et al (1982) used a control group of untreated patients with which to compare the effect of danazol.

There did not appear to be any significant differences in reported pregnancy rates at doses between 100-800mg per day (Moore et al, 1981, Dmowski et al, 1982, Low et al, 1984) but success following treatment with danazol was directly related to the severity of the disease. The overall opinion was that danazol should only be used to

TABLE 2.2: FERTILITY RATES FOLLOWING DANAZOL THERAPY
RESULTS OF PREVIOUS STUDIES

REFERENCE	DOSE OF DANAZOL	NO TREATED	NO CONCEIVING	PREGNANCY RATE (%)	CORRECTED PREGNANCY RATE (%)*	MISCARRIAGE (1ST TRIMESTER LOSS) %
FRIEDLANDER (1973)	800mg	22	9	41		
DMOWSKI + COHEN (1978)	800mg	84	39	46	72	
GREENBLATT + TZINGOUNIS (1979)	800mg	30	10	33	50	20
MOORE ET AL (1981)	100-600mg	28	8	28		
BARBIERI ET AL (1982)	800mg	56	26	45		13
SEIBEL ET AL (1982)	800mg dec to 400mg	20	6	30	36	14
PULEO + HAMMOND (1983)	800mg	29	9	33	56	0
GUZICK + ROCK (1983)	800mg	91	30	33		10
BUTLER ET AL (1984)	400-800mg	75	21	28	29	
BUTTRAM ET AL (1985)	400 + 800mg	157	83	53		7
RANGE				28-53	36-72	0-29

*Corrected pregnancy rate = pregnancy rate in patients with infertility solely due to endometriosis

treat infertility patients with mild or moderate disease, and that those with severe disease required surgical intervention (Barbieri and Ryan, 1981, Schmidt, 1985).

Danazol has also been used either prior to surgery, or post operatively in the treatment of infertility patients with endometriosis (Daniell and Christianson, 1981, Wheeler and Malinak, 1981, and Auderbert, 1979), or in the three-step therapy protocol advocated by Mettler and Semm (1979). These authors suggest that the combination of medical and surgical treatment produces better pregnancy rates (45%), but this is contested by Buttram et al (1982).

It has even been suggested that danazol is effective in the treatment of unexplained infertility, (Van Dijk, 1979), but no further studies have been published to support this theory.

RECURRENCE RATE FOLLOWING DANAZOL THERAPY

One of the most disappointing aspects of the treatment of endometriosis, is the frequency with which the disease recurs.

The recurrence rate is variable, depending on whether the recurrence has been judged by symptomatic, clinical or laparoscopically observed return of the disease. A review of the literature on the recurrence of endometriosis following danazol therapy at various doses is shown in Table 2.3. Although an overall 30-40% recurrence rate is apparent with a follow up period of up to 60 months, Moore et al (1981) claimed a 51% recurrence of symptoms at

TABLE 2.3: RECURRENCE RATE AFTER DANAZOL TREATMENT. RESULTS OF PREVIOUS STUDIES

REFERENCE	NO OF PATIENTS	DOSE OF DANAZOL	RECURRENCE (%) SYMPTOM	CLINICAL	LAPARO-SCOPIC	TIME OF ASSESSMENT (MONTHS)	REC *
DMOWSKI + COHEN (1978)	99	800mg	39	33		37	15
GREENBLATT + TZINGOUNIS (1979)	49	800mg	33			78	1-35
DANIELL + CHRISTIANSON (1981)	19	800mg 1/12, then 600mg			23	6	
MOORE ET AL (1981)	38	100-600mg	51			12	
BIBEROGLU + BEHRMAN (1981)	32	100-600mg	36			19	
BARBIERI ET AL (1982)	100	800mg	33	33		60	
PULEO + HAMMOND (1983)	39	800mg	38			6.9	
RANGE			33-51	33	23		

* = Average time of recurrence

12 months post treatment. However, the latter results included patients treated on low doses of danazol, and Biberoglu and Behrman (1981) who investigated similar low doses concluded that symptomatic recurrence was dose-dependent.

Dmowski and Cohen (1978) and Barbieri et al (1982) both reported a 15-20% recurrence of endometriosis in the first year following danazol treatment, with a subsequent 5% (3-8%) recurrence rate per year in patients treated with 800mg daily. However, a second look laparoscopy performed immediately on completion of 6 months danazol therapy by Dmowski and Cohen (1975) revealed 15% of patients had residual endometriosis. Further, Buttram et al (1985) performing either laparoscopy or laparotomy on 127 patients on completion of danazol therapy found 38-45% residual peritoneal disease and 56-58% residual ovarian disease (the variation depending on danazol dose, either 400mg or 800mg). In conclusion, it may be that the high number of so called "recurrences" reported in the first year post treatment, includes a significant number of patients in whom the endometriosis has not been completely eliminated. This is further supported by the observation by Puleo and Hammond (1983) that more advanced disease is associated with an increased likelihood and rapidity of recurrence.

SIDE EFFECTS OF DANAZOL

Side effects on danazol have been reported in up to 85% of patients (Barbieri et al, 1982). However, the

majority of the side effects are minor, and in a large prospective study, Buttram et al (1985) claimed that side effects lead to a cessation of therapy in only 7% of patients.

The most commonly occuring side effect is weight gain and oedema (Friedlander, 1973, Lauersen et al, 1975, Chalmers and Shervington, 1977, Barbieri et al, 1982,), which may be between two and 10kgs, with a mean of 3kgs (Dmowski and Cohen, 1978, Noble and Letchworth, 1977). Weight gain is reported in up to 55% of patients treated with danazol (Barbieri and Ryan, 1985). Friedlander (1973) and Lauersen et al (1975) actually used concurrent diuretic treatment in some patients and successfully reduced this. Weight loss has been described rarely during danazol therapy (Lauersen et al, 1975, Young and Blackmore, 1977).

The next most frequently described side effects are acne, seborrhoea and greasy hair (13-27%), (Dmowski and Cohen, 1975, Young and Blackmore, 1977). These effects are attributable to the androgenic activity of danazol, which also causes hirsutism (5-10%), and more rarely clitoromegaly and deepening of the voice (3-10%), (Spooner, 1977, Barbieri et al, 1982, Buttram et al, 1985). This voice change was first observed in 3 out of 62 patients by Greenblatt et al (1971) in his original paper on the clinical use of danazol. Initially these changes, described as a lowering of pitch, were thought to be reversible on cessation of danazol, but in 1983, Wardle and Whitehead published a report of a woman who developed loss of pitch control and voice weakness,

which remained for 16 months after discontinuation of treatment. Discrepancy still exists in the literature with regard to the effect of danazol on the voice: Nordenskjold and Fex (1984) were unable to demonstrate any significant vocal changes in 23 treated patients, but Mercaitis et al (1985) showed persistent effects one year after ceasing medication.

Because of the androgenic side effects of danazol therapy, all patients taking the drug should use a barrier form of contraception, as potentially there could be virilisation of a female fetus. Transient androgenisation has been described following danazol exposure in utero by Castro-Magana et al (1981) and several case reports exist associating in utero exposure to danazol with female pseudohermaphroditism, (Duck and Katayama, 1981, Peress et al, 1982, Shaw and Farquhar, 1984).

Other side effects, such as decrease in breast size, hot flushes and sweats, decrease in libido and vaginitis, (Spooner, 1977), may be assumed to result from reduced circulating oestrogen levels occurring during danazol therapy.

A variety of other side effects may also occur during danazol ingestion, including: muscle cramps, skin rashes, nausea, vomiting, change in bowel habit, headaches, lethargy, mood swings, irritability, tremors and dizziness, (Young and Blackmore, 1977, Barbieri and Ryan, 1981, Barbieri et al, 1982, and Schmidt, 1985). Rare side effects described are alopecia (Duff and Mayer, 1980), and liver

toxicity (Pearson and Zimmerman, 1980).

A further consideration is the increased low density lipo-proteins (cholesterol) and decreased high density lipoproteins which have been reported with danazol usage (Allen and Fraser, 1981, Luciano et al, 1983, Schweppe and Assman, 1984). These findings have important implications as they may be associated with atheromatous arterial disease, and this, therefore, contraindicates the long term use of danazol.

In summary, it is difficult to quote exact prevalences for the side effects that occur in danazol treated patients. Generally, however, these adverse effects are reversible on ceasing danazol (Ansbacher, 1975, Young and Blackmore, 1977, Chalmers and Shervington, 1977, and Greenblatt and Tzingounis, 1979), and rarely severe enough to cause discontinuation of medication.

DOSE OF DANAZOL

Initially, when danazol was available for general use in the United States of America, the Food and Drugs Administration recommended a dose of 800mg daily. Consequently, the early papers describing the use of danazol relate to the 800mg dose, (Greenblatt et al,1971, Friedlander, 1973, Lauersen et al, 1975, Dmowski and Cohen, 1978, Greenblatt and Tzingounis, 1979). Although in their original paper, Greenblatt et al (1971) commented "side effects were few", the subsequent authors mentioned above, described many side effects, and Barbieri et al (1982) found

significant side effects in 85% of patients treated at the 800mg dose of danazol.

In view of the high prevalence of side effects, and the cost of the drug, a number of studies have been performed to examine the effect of lower dosages. Doses between 50-800mgs have been investigated. Although some symptomatic relief was obtained at a dose of 100mg daily, this was not considered adequate for the treatment of endometriosis, (Biberoglu and Behrman, 1981, Moore et al, 1981, Dmowski et al, 1982). Chalmers (1982) and Low et al (1984) concluded from their studies that provided endometriosis was not severe, 200mg danazol daily was adequate therapy, with a concurrent reduction in the development of side effects. However the opposite view was held by Dmowski et al (1982), who using their own laparoscopic scoring system to asses endometriotic regression, found a 40% improvement in endometriosis at doses of 100mg and 200mg, a 74% improvement at doses of 400mg and 600mg compared with an 85% improvement at a dose of 800mg. They concluded that danazol was less effective at doses below 800mg, and recommended this as a starting dose.

The majority of studies on danazol dosage versus efficacy suggest 400mg as the starting dose (Biberoglu and Behrman, 1981, Barbieri and Ryan, 1981), although some specify that this relates only to mild and moderate disease and exclude cases with endometriomas (Moore et al, 1980, 1981) and others suggest altering the dose later as clinically indicated (Ward, 1977, 1979). Controversy exists

as to whether this lower dose actually does reduce the occurrence of side effects. Young and Blackmore (1977), Chalmers and Shervington (1979) and Chalmers (1982), all found a reduced incidence of side effects at lower doses of danazol, but Biberoglu and Behrman (1981) and Buttram et al (1985) found no difference between the 400mg and 800mg dose. Biberoglu and Behrman (1981) further suggested that symptomatic recurrence was dose dependent.

The development of amenorrhoea increases with higher doses of danazol (Biberoglu and Behrman, 1981), but the importance of amenorrhoea for the regression of endometriosis also remains unresolved. Low et al (1984) and Doberl et al (1984) failed to find a correlation between persistant or frequent breakthrough bleeding and the eventual healing of endometriotic implants during danazol treatment. Breakthrough bleeding occuring in danazol treated patients has been demonstrated by Guillebaud et al (1977) to coincide with falls in the level of oestradiol.

In conclusion, the literature does not seem to provide a definitive answer for the optimal dose of danazol for the treatment of endometriosis. Taking into consideration the previous experience described in the literature with regard to efficacy and side effects, and also the cost of the drug, a dose of 400mg of danazol daily was chosen for this study.

GESTRINONE

Gestrinone (R-2323, Roussel-UCLAF, Uxbridge) was first filed for patent in France in 1965. It is a synthetic trienic 19-norsteroid with a chemical formula (17α) -13-ethyl- 17α-ethinyl- 17hydroxy-gona- 4,9,11-trien- 3-one.

It was first studied as a potential contraceptive agent because of its antiprogestogenic activity (Sakiz and Azadian-Boulanger, 1970, Mora et al, 1974, Sakiz et al, 1974). Although offering a failure rate of (2-5%), further development was abandoned after Phase II trials for financial reasons (Deltour et al, 1984). However from the clinical and endocrinological observations made during these early trials, gestrinone was considered a potential agent for the treatment of endometriosis, and further investigations were undertaken.

As this steroid is a newer therapeutic agent for the management of endometriosis, there are far fewer reports of its use in the literature.

GESTRINONE IN THE TREATMENT OF ENDOMETRIOSIS

Symptomatic relief has been reported in 85-91% of patients with endometriosis treated with gestrinone after 2-3 months (Coutinho, 1982, 1985, Azadian-Boulanger et al,1984, Tamaya et al, 1985). These authors also suggested that amenorrhoea occured in the "majority" of patients after 2 months of therapy. However Thomas and Cooke (1987a), who observed a significant improvement in endometriosis, judged by the American Fertility Society scoring system at

laparoscopy, in gestrinone treated patients compared to untreated controls, found only 44% of the gestrinone group became amenorrhoeic after 6 months.

PREGNANCY RATES AFTER GESTRINONE TREATMENT

Pregnancy rates for patients treated in uncontrolled trials are also found in the literature for gestrinone. These vary from 56-66% (Coutinho, 1982, 1984, Azadian-Boulanger et al, 1984, Mettler and Semm, 1984). However, in 1987, Thomas and Cooke (1987b) published the first prospective double blind randomised placebo controlled trial with gestrinone 2.5mg twice weekly for six months. All the patients in this trial had asymptomatic endometriosis and infertility, and other possible causes for failure to conceive had been excluded. They found no significant difference in the cumulative conception rates at 12 months, which were 25% (5/20) in those patients treated with gestrinone and 24% (4/17) in those given placebo, and furthermore the elimination of endometriosis was not related to the likelihood of pregnancy.

RECURRENCE RATES FOLLOWING GESTRINONE TREATMENT

Information regarding recurrence rates following gestrinone treatment is limited, but varies from 15-31% at 12-24 months (Azadian-Boulanger et al, 1982, Mettler and Semm, 1984, Coutinho, 1984).

SIDE EFFECTS OF GESTRINONE

Weight gain, on average 2-2.5kgs is described by all authors using 2.5 or 5mg gestrinone twice weekly for 6 months. Seborrhoea and acne are the major side effects, followed by voice hoarseness. Other side effects include hirsutism, reduction in breast size and libido, increased vaginal discharge, appetite, headaches, nausea, leg pain and oedema (Coutinho, 1982, 1984, Azadian-Boulanger et al, 1984, Mettler and Semm, 1984). These side effects all reversed on ceasing therapy.

There is an obvious qualitative similarity between the side effects of gestrinone and those discussed for danazol.

DOSE OF GESTRINONE

Gestrinone has been used at doses of 5mg twice weekly, 2.5mg twice and three times weekly in the treatment of endometriosis. The conclusions of the investigators using these different doses, was that the 2.5mg twice weekly dose gave a satisfactory clinical response with the minimum of side effects. They observed that the side effects of gestrinone were dose dependent, although vaginal bleeding was also more common at the lower dose, (Azadian-Boulanger et al, 1984, Mettler and Semm, 1984, Coutinho, 1984).

In view of the evidence that 2.5mg gestrinone twice weekly is adequate for the treatment of endometriosis, and that the associated side effects are less at this dose than at higher doses, it was selected for comparison with

danazol 200mg twice daily.

CHAPTER 4

STUDY DESIGN AND METHODS

This study was conducted at the Chelsea Hospital for Women, from January 1984 until February 1987, when 51 patients had been recruited, treated and followed up for one year. Involvement in the study required each patient to have a pre- and post-treatment laparoscopy, six months danazol or gestrinone therapy (randomly allocated) and monthly assessments during this and 12 months post-treatment follow up, to assess recurrence and conception rates.

ETHICAL CONSIDERATIONS AND CONSENT

As gestrinone and danazol have both been shown to exert a significant clinical improvement on the disease process, it was considered unethical to use a placebo control group in this study.

Laparoscopy is a procedure not without significant morbidity and mortality, the mortality risk from one laparoscopy being 8 per 100,000 (Chamberlain and Brown, 1978). However the only true method of assessing improvement in endometriosis is by direct visualisation. Each potential patient for admission into the trial was seen on the day following laparoscopy, and again within one month in the endometriosis clinic, and on each occasion their condition, the possible treatments available and the trial were fully explained. Inclusion in the trial was voluntary, and if

agreed, written consent to take part and also to use a barrier form of contraception during the therapy period, was obtained. It was also made clear to patients that they were free to withdraw from the trial at any time.

The protocol, consent and patient education forms were submitted to and approved by the Queen Charlottes'and Hammersmith Special Health Authority Ethical Committee prior to commencement of the study.

CLINICAL INVESTIGATORS

This clinical study was initiated at the Chelsea Hospital for Women by Mr Kevin Forbes, F.R.A.C.O.G. who conducted the first nine months of the project. He was responsible for the enrollment and he conducted the requisite laparoscopies and collection of data and blood samples during that time of the first 20 patients entered into the trial. All of the subsequent part of the study was conducted by myself. This included the continued management and/or follow-up of all patients recruited by Mr Forbes. Thus, I was involved at some time with all patients who participated in the trial.

PATIENT SELECTION

Patients enrolled into the study were under the care of consultant gynaecologists, primarily Mr D. K. Edmonds, at the Chelsea Hospital for Women, having been referred by their general practitioners. Patients with endometriosis were eligible for the trial whether suffering from symptoms

or infertility. Infertility was defined as failure to conceive with the same partner for more than one year. Inclusion criteria were:

1) aged between 18-45 years
2) endometriosis confirmed by laparoscopy or laparotomy
3) presentation with a first or recurrent episode of endometriosis
4) suitable for hormone treatment

Exclusion criteria were:

1) endometriotic condition warranted surgical excision
2) a known history of hepatic or renal impairment
3) current hormone therapy
4) hormone therapy less than two months previously
5) inability to comply with the required hospital assessment visits or the repeat laparoscopy
6) serious systemic disease
7) requirement for other long term medical therapy
8) anticoagulation therapy
9) unacceptability of a barrier form of contraception
10) previous failure to respond to danazol therapy
11) danazol treatment for endometriosis within the last six months.

LAPAROSCOPY

All laparoscopies were either performed or checked by myself or Mr. Kevin Forbes, under general anaesthetic. If endometriosis was found during a routine diagnostic laparoscopy performed in the Chelsea Hospital for Women by

another member of staff, one of us was called to the operating theatre to assess the degree of endometriosis personally, in order to avoid inter-operator variability.

In the operating theatre, a Verres needle was introduced through a small vertical sub-umbilical incision. A 2 litre pneumoperitoneum was induced using carbon dioxide. The laparoscope was then inserted through the same incision. The Verres needle was reintroduced just above the pubic hair line, in the midline, to use as a manipulator of the pelvic organs and to ensure proper visualisation and assessment, especially of the posterior aspects of the ovaries. Anteversion of the uterus during the examination was obtained by an assistant with a vaginally inserted uterine sound. The pelvis was systematically examined to ensure all areas of endometriosis and adhesion formation were observed and recorded. Endometriosis was diagnosed when the classically described bluish-black or reddish-brown lesions were seen on the pelvic peritoneum or ovaries (Mettler and Semm, 1979). The size of each lesion was assessed approximately by subjective visual assessment, together with the degree of associated adhesion formation. The endometriosis and adhesions were recorded using the American Fertility Society (A.F.S.) scoring system (The American Fertility Society, 1979), which was considered the most acceptable scoring system available at that time (Figure 2.1). However, there has been criticism of this and other previously used scoring systems, e.g. Acosta et al (1973), and recently the A.F.S. scoring system has been revised (The

U K 8 3 3 2 3 0 1 Patient No. 1

PATIENT'S NAME : _____ DATE Day Month Year

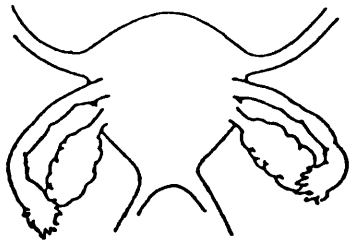
LAPAROSCOPY / LAPAROTOMY FINDINGS:

Severity of adhesions (to be assessed before division of adhesions) (38)
(0 = None, 1 = Slight, 2 = Moderate, 3 = Severe)

AMERICAN FERTILITY SOCIETY CLASSIFICATION OF ENDOMETRIOSIS

Stage I (Mild) 1–5
Stage II (Moderate) 6–15
Stage III (Severe) 16–30
Stage IV (Extensive) 31–54

Total Score: (39-40)



PERITONEUM	ENDOMETRIOSIS	<1 cm	1–3 cm	>3 cm
		1	2	3
	ADHESIONS	filmy	dense w/ partial cul-de-sac obliteration	dense w/ complete cul-de-sac obliteration
		1	2	3
OVARY	ENDOMETRIOSIS	<1 cm	1–3 cm	>3 cm or ruptured endometrioma
	R	2	4	6
	L	2	4	6
	ADHESIONS	filmy	dense w/ partial ovarian enclosure	dense w/ complete ovarian enclosure
	R	2	4	6
	L	2	4	6
TUBE	ENDOMETRIOSIS	<1 cm	>1 cm	tubal occlusion
	R	2	4	6
	L	2	4	6
	ADHESIONS	filmy	dense w/ tubal distortion	dense w/ tubal enclosure
	R	2	4	6
	L	2	4	6

ADDITIONAL OBSERVATIONS : (✓ box)

UTERO-SACRAL LIGAMENTS	ENDOMETRIOSIS	<1 cm	1–3 cm	>3 cm
	R			
	L			
	ADHESIONS	slight	Moderate	Severe
	R			
	L			

(41-42)

(43-44)

Additional pathology: _____ (45-48)

_____ (49-52)

Investigator's Signature:

FIGURE 2.1: The American Fertility Society score card for recording laparoscopy findings

American Fertility Society, 1985), in an effort to standardise further results published regarding the treatment of endometriosis (Buttram, 1985).

No biopsies were taken from endometriotic lesions at the time of laparoscopy for histological confirmation of the disease. This was because there is good evidence to support a strong association between the visual and histological diagnosis of endometriosis, (Lauersen et al, 1975, Vasquez et al, 1984), and usual clinical practice depends on observed endometriosis. Further, the biopsy itself may have variably altered the amount of endometriosis present or altered adhesion formation.

Any patient being investigated for infertility had methylene blue dye injected through the uterus to assess tubal patency. Endometrial biopsies were not performed unless clinically indicated.

DATA COLLECTION

Patients were assessed in the endometriosis clinic before the start of treatment, at monthly intervals during the six month trial period, monthly for the first three months following the post treatment laparoscopy and thereafter at 3 monthly intervals for a further 9 months. During treatment, patients were asked to complete a diary card to record daily vaginal bleeding patterns and drug ingestion.

Once patients had undergone their diagnostic laparoscopy and consented to take part in the trial, they

were consecutively allocated a study number to which either gestrinone 2.5mg twice weekly or danazol 200mg twice daily was assigned, using random numbers. For both drugs, medication commenced on the first day of the next menstrual cycle and continued for 24 weeks. The investigator, the patient and the dispensing hospital pharmacist were unaware of which drug any individual received. The drugs were stored and issued by our own pharmacy in identical unit dose blister packs. Each pack contained the twice weekly and the twice daily non-identifiable capsules, consisting of one of the drugs and a placebo.

The various assessments and data collected at each visit are described below.

Pretreatment Assessment

The following information was collected from patients:

1) name, age, obstetric and drug history

2) gynaecological history including: date of last menstrual period, menstrual cycle, duration of infertility (if present), duration of endometriosis, any previous treatment for endometriosis, severity of the symptoms: dysmenorrhoea, dyspareunia and pelvic pain

3) pre-existence of any of the possible side effects of the drugs, specifically acne or seborrhoea, hirsutism, hoarseness, ankle oedema, hot flushes or sweats, loss of libido, leg cramps, nausea or vomitting, anorexia or hunger, dizziness or giddiness, tiredness, faintness or skin rashes

4) clinical assessment including weight, pulse, blood

pressure, general physical, abdominal and vaginal examination and evaluation of hirsutism, using the scoring system of Ferriman and Gallwey, (1961).

Blood was also taken for:

a) full blood count, including platelets and erythrocyte sedimentation rate (ESR)

b) urea, electrolytes and creatinine

c) liver function tests (including, serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase, total protein, albumin, cholesterol, and bilirubin)

d) endocrine hormone profile i.e.

luteinizing hormone and follicle stimulating hormone, prolactin, oestradiol and progesterone, sex hormone binding globulin, testosterone, androstenedione, 5α dihydrotestosterone, dehydroepiandrostenedione sulphate, % free testosterone. These were performed in order to study the endocrinological changes, particularly with reference to androgens. They will be discussed in greater detail in Section 3.

Assessments during Treatment

The patients were reviewed every 4 weeks during the treatment period. At each visit, the following were recorded:

1) patient's assessment of treatment, including any new symptoms

2) vaginal bleeding pattern

3) severity of the symptoms previously described

4) any new medical disease or treatment

5) regularity of taking medication

6) details of side effects from the check list already described

7) examination as for pretreatment visit.

The patients' own diary sheet was checked on each occasion to confirm the bleeding history given and compliance to therapy. Patients were also asked to return the empty drug packs to ensure these correlated with the information supplied.

Blood samples were taken at all these visits for endocrinological analyses.

A repeat laparoscopy was performed 3 weeks after completion of the drug treatment. This was performed in the same manner as the first laparoscopy and the findings again documented using the A.F.S. scoring system.

Follow Up Assessments

Patients were assessed monthly for the first three months after completion of therapy, and 3 monthly thereafter for a total of 12 months. At each visit information was collected about:

1) date of the last menstrual period, cycle length and duration of bleeding

2) assessment of symptom severity

3) conception (if desired) and, if appropriate, a

pregnancy test performed

4) clinical examination as previously

Blood was taken at the first follow up visit only and all parameters examined at the pretreatment visit repeated.

Withdrawals

Data were also collected from any patient withdrawn from the trial, with an explanation for the reason for cessation of therapy and the likelihood of an association.

STATISTICAL METHODS

The admission data for the gestrinone and danazol groups were compared using Student's t Test for unpaired data to determine whether the two groups were significantly different in any important aspect before treatment started.

Laparoscopy scores were analysed for both AFS scores and "pure" endometriosis scores (i.e. scores for endometriotic spots but not adhesions) in patients who underwent pre- and post-treatment laparoscopies. The Wilcoxon Rank Sum Test for paired non-parametric data was carried out to test for (1) any difference between the groups pre- and post-treatment (2) a treatment effect of each drug and (3) differences between drug-related effects. These comparisons were also performed on the weight data using a repeated measures analysis of variance.

The effect of each drug on symptoms and side effects (except for weight) was assessed by comparing the

pre-treatment score and that at the completion of the drugs, i.e. month 6, using the Wilcoxon Rank Sum Test. (If month 6 was not available, the value for month 5 was used). For each comparison the categories of none, mild, moderate and severe were given numerical values of 0, 1, 2 and 3, respectively. The effect of gestrinone and danazol on each symptom and side effect was compared by first calculating the difference between the pretreatment and the month 5/6 scores for each patient. These " values" were then compared using the Mann Whitney Test for unpaired non-parametric data. The validity of these approaches was confirmed by the statistician, Ms Angela Lee, B.Sc, M.Sc.

CHAPTER 5

RESULTS

Fifty one patients were recruited into the trial: 26 to gestrinone and 25 to danazol treatment. The randomisation code for the drugs was broken only when the last patient had completed 12 months follow up after treatment.

PATIENTS WITHDRAWN FROM THE TRIAL

Five patients (19.2%) were withdrawn from the gestrinone group and 3 (12.0%) from the danazol group. The reasons for stopping treatment and withdrawal from the trial were as follows:

GESTRINONE

Patient No. 7 (after 36 days): an itchy rash

Patient No. 14 (after 58 days): the patient sought a second opinion and was treated surgically

Patient No. 17 (after 117 days): excessive tiredness, depression and irritability

Patient No. 35 (after 88 days): severe migraine headaches

Patient No. 44: became pregnant in the cycle prior to commencing treatment.

DANAZOL

Patient No. 19 (after 55 days): patient noticed a change in her voice persisting for two weeks

Patient No. 33 (after 135 days): patient was an opera singer and noticed a voice change

Patient No. 38 (after 41 days): leg cramps, dry mouth, tiredness and depression.

In addition two patients who completed therapy and the second-look laparoscopy were omitted from the analysis of endometriosis scores. These were patient number 30 (treated with gestrinone) who continued on medication for a further 3 months (i.e. a total of 9 months treatment) as was clinically indicated and patient number 45 (treated with danazol) who took a one month break from the drug after the first 4 weeks due to a misunderstanding of the instructions.

ADMISSION DATA

Median age was 30 years for both groups and the ranges were 21-38 years for gestrinone and 23-44 years for danazol. The duration of endometriosis was estimated according to the following criteria:

a) In asymptomatic patients, it was taken from the time of diagnostic laparoscopy

b) From the time of onset of symptoms strongly suggestive of endometriosis in symptomatic women

Infertility alone was not used to indicate the duration of the history.

The history of endometriosis ranged from 0-10 years in the gestrinone group and 0-6 years in the danazol group; these were not significantly different. In 66% of the

gestrinone and 75% of the danazol treated patients, the women were unaware of their endometriosis until it was diagnosed at their first laparoscopy. A history of infertility (failure to conceive after one year) was given by 85% of the gestrinone group patients, ranging from 1-10 years, and by 76%, ranging from 1.8-8.3 years in the danazol group. However, in both these groups several of the patients had other factors associated with subfertility such as sperm or mucus abnormalities. Two patients in the gestrinone group had received previous medical treatment for endometriosis with danazol and 3 patients in the danazol group had had drug therapy in the past, 2 with danazol and one with gynovlar. In all cases this treatment had been completed more than 6 months prior to entry into the trial.

The 2 groups were comparable for all the parameters discussed above. They were also similar for parity, weight, length of menstrual cycle and number of days of menstrual loss per month. Both groups had a median of 5 days bleeding and a 28 day cycle.

SYMPTOMS

The distribution of symptoms pre-treatment and at each month during treatment are shown in Tables 2.4-2.7 and in the form of histograms in Figures 2.2-2.5. The 2 treatment groups were comparable for each of the symptoms. The subjective assessment of each symptom was recorded as none, mild, moderate or severe.

Pre-treatment, 88% of the patients treated with

TABLE 2.4: SYMPTOMS BEFORE AND DURING TREATMENT WITH GESTRINONE AND DANAZOL: DYSMENORRHOEA

TREATMENT GROUP	VISIT						
	Pre-treatment	1	2	3	4	5	6
GESTRINONE							
None	3(12%)	7(29%)	11(58%)	9(60%)	6(46%)	7(54%)	6(60%)
Mild	5(19%)	9(38%)	4(21%)	4(27%)	3(23%)	4(31%)	2(20%)
Moderate	6(23%)	4(17%)	3(16%)	1(7%)	4(31%)	2(15%)	1(10%)
Severe	12(46%)	4(17%)	1(5%)	1(7%)	0	0	1(10%)
N/A	0	1	5	8	9	6	8
Unknown	0	0	0	0	0	0	6
NUMBER OF PATIENTS	26	25	24	23	22	19	24
DANAZOL							
None	2(8%)	9(39%)	11(61%)	11(79%)	12(92%)	11(85%)	4(80%)
Mild	6(24%)	6(26%)	1(6%)	1(7%)	0	0	0
Moderate	5(20%)	4(17%)	2(11%)	1(7%)	0	1(8%)	1(20%)
Severe	12(48%)	4(17%)	4(22%)	1(7%)	1(8%)	1(8%)	0
N/A	0	1	5	8	8	9	7
Unknown	0	0	0	0	0	0	10
NUMBER OF PATIENTS	25	24	23	22	21	22	22

N/A = not applicable

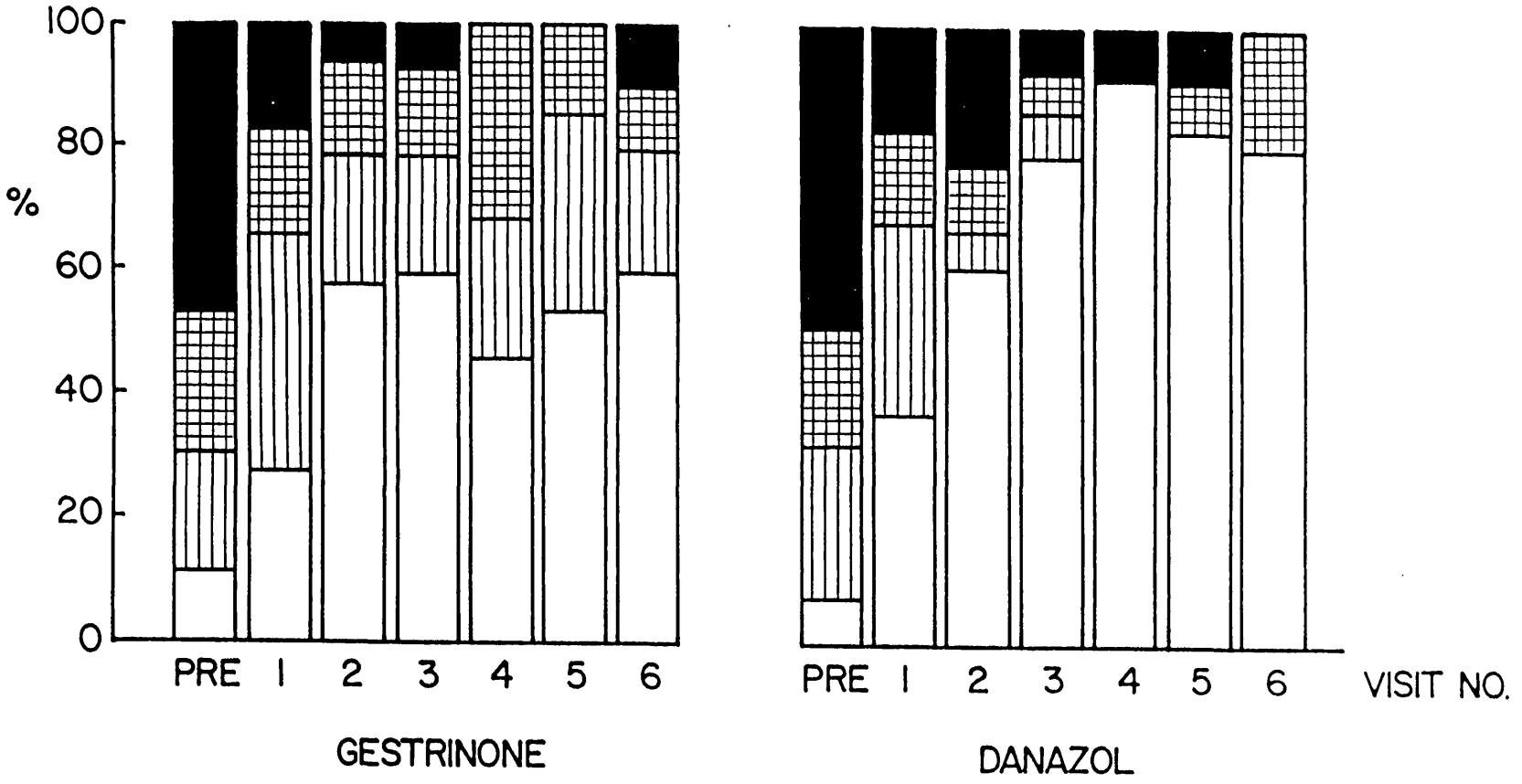


FIGURE 2.2: The effect of Gestrinone and Danazol treatment on the percentage of patients complaining of dysmenorrhoea
(□none, ▥ mild, ⊞moderate, ■severe)

TABLE 2.5: SYMPTOMS BEFORE AND DURING TREATMENT WITH GESTRINONE AND DANAZOL: DEEP DYSPAREUNIA

TREATMENT GROUP	VISIT						
	Pre-treatment	1	2	3	4	5	6
GESTRINONE							
None	14(54%)	15(63%)	17(71%)	20(91%)	17(85%)	15(83%)	15(79%)
Mild	5(19%)	4(17%)	3(13%)	1(5%)	3(15%)	2(11%)	2(11%)
Moderate	2(8%)	2(8%)	2(8%)	1(5%)	0	0	2(11%)
Severe	5(19%)	3(13%)	2(8%)	0	0	1(6%)	0
N/A	0	1	0	1	2	1	0
Unknown	0	0	0	0	0	0	5
NUMBER OF PATIENTS	26	25	24	23	22	19	24
DANAZOL							
None	11(46%)	15(68%)	18(82%)	18(86%)	16(80%)	18(82%)	10(83%)
Mild	4(17%)	3(14%)	2(9%)	2(10%)	2(10%)	3(14%)	2(17%)
Moderate	4(17%)	4(18%)	1(5%)	1(5%)	1(5%)	0	0
Severe	5(21%)	0	1	0	1(5%)	1(5%)	0
N/A	1	2	1	1	1	0	0
Unknown	0	0	0	0	0	0	10
NUMBER OF PATIENTS	25	24	23	22	21	22	22

N/A = not applicable

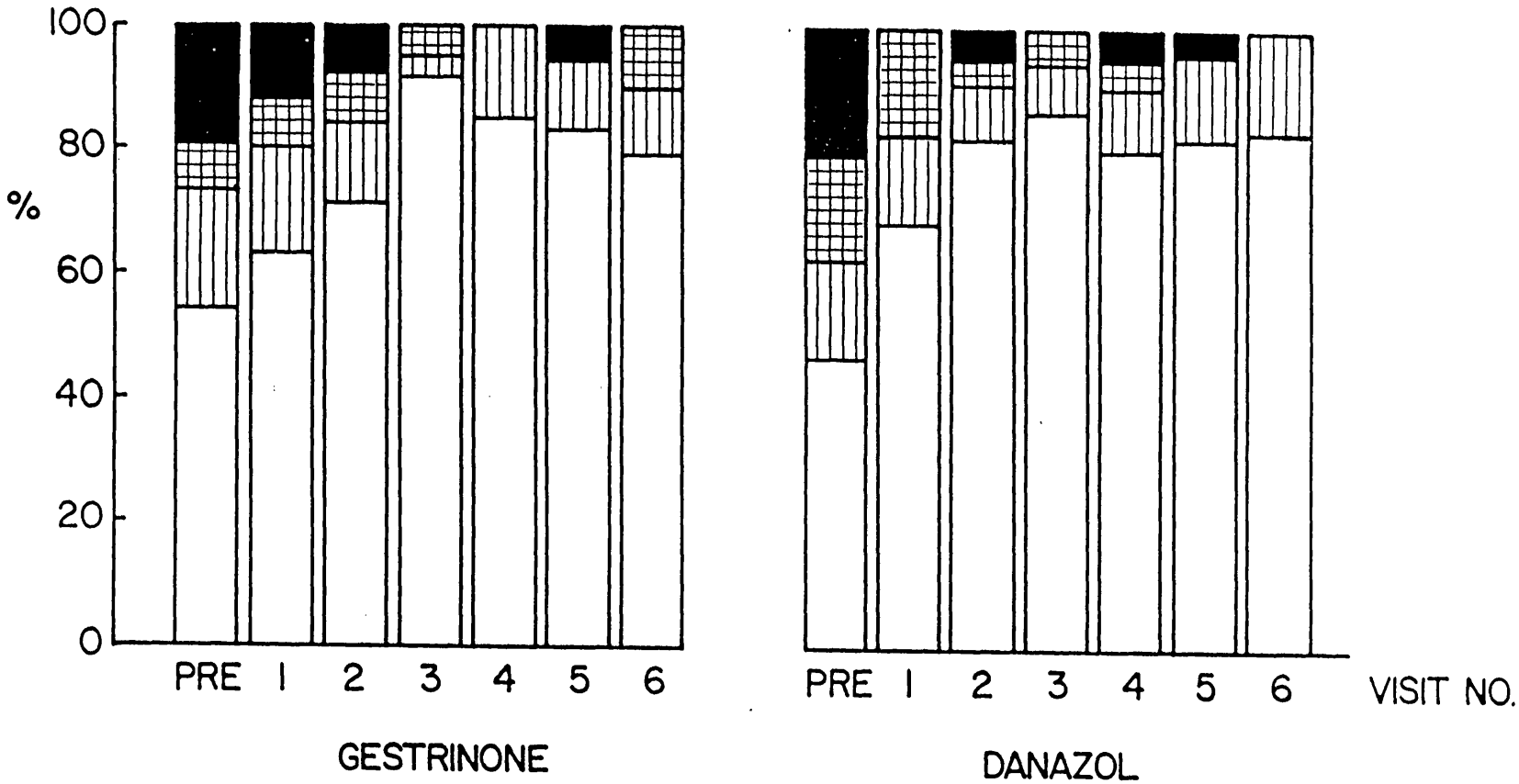


FIGURE 2.3: The effect of Gestrinone and Danazol treatment on the percentage of patients complaining of deep dyspareunia
(□none, ▥ mild, ▦ moderate, ■ severe)

TABLE 2.6: SYMPTOMS BEFORE AND DURING TREATMENT WITH GESTRINONE AND DANAZOL: SUPERFICIAL DYSPAREUNIA

TREATMENT GROUP	VISIT						
	Pre-treatment	1	2	3	4	5	6
GESTRINONE							
None	21(81%)	23(96%)	23(96%)	21(95%)	20(100%)	17(100%)	19(100%)
Mild	2(8%)	1(4%)	1(4%)	0	0	0	0
Moderate	3(12%)	0	0	1(5%)	0	0	0
Severe	0	0	0	0	0	0	0
N/A	0	1	0	1	2	1	0
Unknown	0	0	0	0	0	1	5
NUMBER OF PATIENTS	26	35	24	23	22	19	24
DANAZOL							
None	21(91%)	20(91%)	20(91%)	19(90%)	19(95%)	20(91%)	12(100%)
Mild	1(4%)	1(5%)	2(9%)	1(5%)	0	1(5%)	0
Moderate	1(4%)	1(5%)	0	0	0	0	0
Severe	0	0	0	1(5%)	1(5%)	1(5%)	0
N/A	1	2	1	1	1	0	0
Unknown	1	0	0	0	0	0	10
NUMBER OF PATIENTS	25	24	23	22	21	22	22

N/A = not applicable

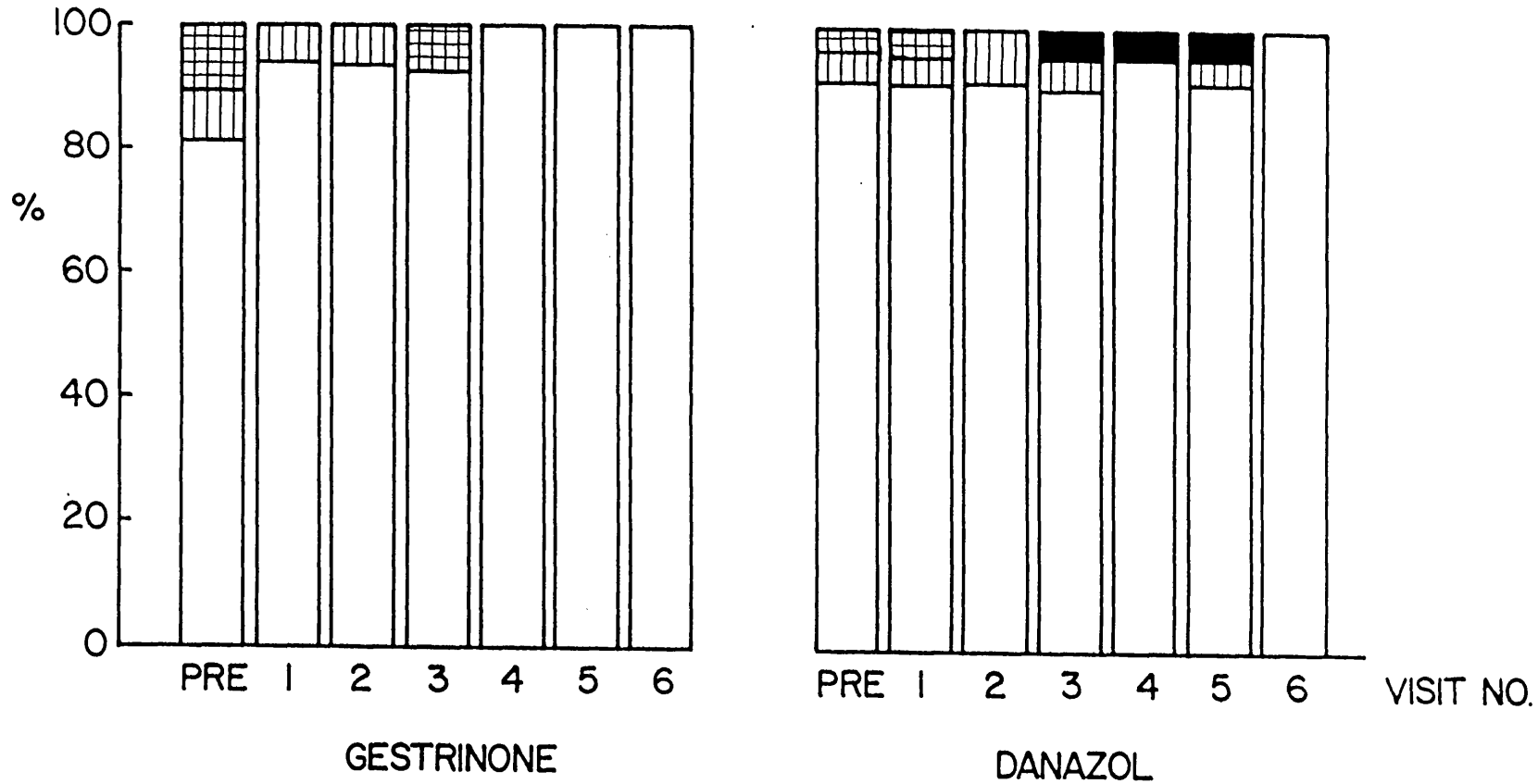


FIGURE 2.4: The effect of Gestrinone and Danazol treatment on the percentage of patients complaining of superficial dyspareunia
(□ none, ▥ mild, ⊞ moderate, ■ severe)

TABLE 2.7: SYMPTOMS BEFORE AND DURING TREATMENT WITH GESTRINONE AND DANAZOL: LOWER ABDOMINAL PAIN (not related to periods)

TREATMENT GROUP	VISIT						
	Pre-treatment	1	2	3	4	5	6
GESTRINONE							
None	11(42%)	16(64%)	17(71%)	18(78%)	14(64%)	16(89%)	17(89%)
Mild	8(31%)	5(20%)	2(8%)	3(13%)	5(23%)	1(6%)	0
Moderate	5(19%)	2(8%)	3(13%)	1(4%)	3(14%)	1(6%)	2(11%)
Severe	2(8%)	2(8%)	2(8%)	1(4%)	0	0	0
N/A	0	0	0	0	0	0	0
Unknown	0	0	0	0	0	1	5
NUMBER OF PATIENTS	26	25	24	23	22	19	24
DANAZOL							
None	11(44%)	13(54%)	14(61%)	16(73%)	10(53%)	15(68%)	10(83%)
Mild	8(32%)	6(25%)	5(22%)	5(23%)	7(37%)	6(27%)	2(17%)
Moderate	3(12%)	3(13%)	4(17%)	0	0	0	0
Severe	3(12%)	2(8%)	0	1(5%)	2(11%)	1(5%)	0
N/A	0	0	0	0	2	0	0
Unknown	0	0	0	0	0	0	10
NUMBER OF PATIENTS	25	24	23	22	21	22	22

N/A = not applicable

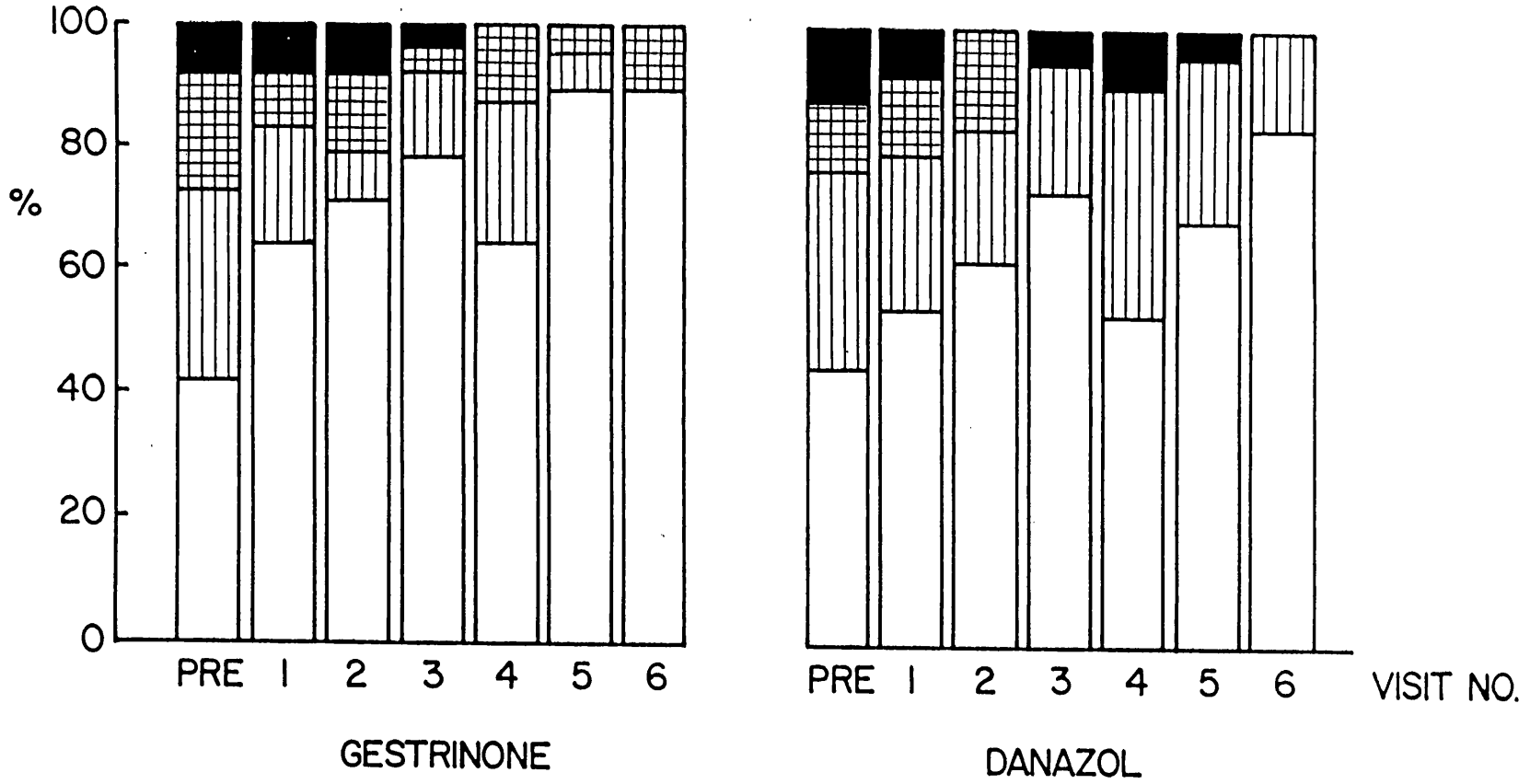


FIGURE 2.5: The effect of Gestrinone and Danazol treatment on the percentage of patients complaining of lower abdominal pain
(□none, ▥ mild, ⊞ moderate, ■ severe)

gestrinone and 92% with danazol suffered from dysmenorrhoea, which was classified as severe by 46% of the former and 48% of the latter.

Deep dyspareunia was a more frequent complaint than superficial dyspareunia: 46% in the gestrinone group and 54% in the danazol group reported degrees of deep dyspareunia compared with 20% and 8% in the gestrinone and danazol groups respectively describing superficial dyspareunia.

Lower abdominal pain which was not related to menstruation was reported in 58% of the gestrinone group and 56% in the danazol group.

CLINICAL EXAMINATION

Pulse, blood pressure and general systematic examination revealed no serious abnormality in any patient included in the study, either pre- or during treatment, except for patient 33 in whom a breast lump was discovered at the time that she was withdrawn from the study because of a change in her voice. (Subsequent mammography confirmed this to be benign). Rectal examination, although included in the original protocol was abandoned at an early stage as it did not provide a valuable contribution to the data and was not found acceptable by many of the patients. Vaginal examination was performed at most visits but was not found to be specific as a measure of endometriosis and was therefore not analysed.

SIDE EFFECTS

A surprisingly large number of patients complained of many of the previously reported side effects of the drugs prior to commencing treatment. Change in weight and seven of the side effects for which data were collected were examined in detail, with particular interest in the androgenic complaints for comparison with the androgenic endocrine changes. The side effects of acne, seborrhoea, hirsutism, voice change, headaches, leg cramps and hot flushes were selected and were categorised into none, mild, moderate and severe. At the pre-treatment assessment, these symptoms occurred in patients as shown in Table 2.8.

Table 2.8: The incidence of pre-existing conditions prior to treatment with gestrinone and danazol. The groups were not statistically significantly different.

	GESTRINONE	DANAZOL
acne	43%	20%
seborrhoea	15%	40%
hirsutism	8%	24%
voice change	4%	0%
headaches	69%	48%
leg cramps	23%	16%
hot flushes	19%	16%

Although objective data were obtained on hirsutism using the scoring system of Ferriman and Gallwey (1961),

because of shaving and other hair removing techniques used by the patients, it was inappropriate to analyse this information.

COMPLIANCE

Compliance was checked on the patients' diary card and also by examining the empty drug packets. The majority of patients who completed the treatment were reliable in their drug taking and omitted only the occasional forgotten tablet. Any patient who had more than a 10 day gap in therapy was excluded from the analysis, and this occurred only with patient 45.

LAPAROSCOPY ASSESSMENTS

The assessment of endometriosis at laparoscopy was described using the American Fertility Society (AFS) scoring method and the information was also recorded diagrammatically (Figure 2.1). The data were analysed for the total AFS scores and also for the pure endometriosis scores i.e. endometriotic deposits without visible associated scarring, as adhesions were not expected to be changed by medical therapy. The individual scores for each patient are shown in Tables 2.9-2.11 (gestrinone) and 2.12-2.14 (danazol). In the gestrinone group, pre-treatment AFS scores ranged from 1-18 (median 4.5) and the pure endometriosis scores from 1-12 (median 3). In the danazol group pre-treatment AFS scores ranged from 1-26 (median 2) and the endometriosis scores from 1-15 (median 2). The 2

TABLE 2.9: INDIVIDUAL TOTAL AFS SCORES
PRE AND POST-TREATMENT WITH GESTRINONE

PATIENT NO	PRE-TREATMENT TOTAL	POST-TREATMENT TOTAL
2	7	4
3	6	2
8	12	12
10	1	0
11	2	2
12	3	2
18	17	1
23	6	5
25	1	2
28	2	3
29	1	5
31	18	20
36	5	2
39	2	0
40	6	2
41	4	7
43	5	0
48	3	5
49	2	1
321	12	23

N = 20

TABLE 2.10: INDIVIDUAL ENDOMETRIOSIS SCORES
PRE AND POST-TREATMENT WITH GESTRINONE

PATIENT NO	PRE-TREATMENT ENDOMETRIOSIS	POST-TREATMENT ENDOMETRIOSIS
2	3	2
3	6	2
8	4	4
10	1	0
11	1	1
12	3	0
18	4	1
23	2	5
25	1	2
28	2	2
29	1	1
31	9	10
36	5	1
39	2	0
40	5	1
41	2	1
43	5	0
48	3	5
49	2	1
321	12	12

N = 20

TABLE 2.11: INDIVIDUAL ADHESIONS SCORES
PRE AND POST-TREATMENT WITH GESTRINONE

PATIENT NO	PRE-TREATMENT ADHESIONS	POST-TREATMENT ADHESIONS
2	4	2
3	0	0
8	8	8
10	0	0
11	1	1
12	0	2
18	13	0
23	4	0
25	0	0
28	0	1
29	0	4
31	9	10
36	0	1
39	0	0
40	1	1
41	2	6
43	0	0
48	0	0
49	0	0
321	0	11

N = 20

TABLE 2.12: INDIVIDUAL TOTAL AFS SCORES PRE AND POST-TREATMENT WITH DANAZOL

PATIENT NO	PRE-TREATMENT TOTAL	POST-TREATMENT TOTAL
1	2	1
4	11	6
5	2	0
6	7	1
9	1	0
13	1	0
15	1	0
16	25	17
20	2	2
21	2	2
22	2	18
24	15	6
26	26	0
27	5	5
32	3	1
34	2	2
37	2	1
42	3	11
46	2	0
47	2	2
50	3	4

N = 21

TABLE 2.13: INDIVIDUAL ENDOMETRIOSIS SCORES PRE AND POST-TREATMENT WITH DANAZOL

PATIENT NO	PRE-TREATMENT ENDOMETRIOSIS	POST-TREATMENT ENDOMETRIOSIS
1	1	0
4	3	4
5	2	0
6	3	1
9	1	0
13	1	0
15	1	0
16	9	1
20	1	0
21	2	2
22	2	2
24	15	6
26	8	0
27	4	4
32	1	0
34	2	2
37	1	0
42	2	1
46	2	0
47	2	2
50	3	3

N = 21

TABLE 2.14: INDIVIDUAL ADHESION SCORES PRE AND POST-TREATMENT WITH DANAZOL

PATIENT NO	PRE-TREATMENT ADHESIONS	POST-TREATMENT ADHESIONS
1	1	1
4	8	2
5	0	0
6	4	0
9	0	0
13	0	0
15	0	0
16	16	16
20	1	2
21	0	0
22	0	16
24	0	0
26	18	0
27	1	1
32	2	1
34	0	0
37	1	1
42	1	10
46	0	0
47	0	0
50	0	1

N = 21

groups were not significantly different (AFS scores: p=0.35, endometriosis scores: p=0.19). According to the AFS criteria, the patients were categorised into mild (1-5), moderate (6-15) and severe (16-30) disease. The patients fell into these groups as shown in Table 2.15.

Table 2.15: The distribution of endometriosis scores into none, mild, moderate and severe, in gestrinone and danazol treated patients. The two groups were not different, statistically.

	GESTRINONE	DANAZOL
None	0 (0%)	0 (0%)
Mild	12 (60%)	16 (76.2%)
Moderate	6 (30%)	3 (14.3%)
severe	2 (10%)	2 (9.2%)

Post-treatment AFS scores for the gestrinone group ranged from 0-23 (median 2) and for endometriosis scores from 0-12 (median 1). This showed a significant improvement in the endometriosis score, ($0.02<p<0.05$), but not for the AFS score, ($p>0.1$). In the danazol group, the post-treatment laparoscopy scores ranged form 0-18 (median 2) and the endometriosis scores from 0-6 (median 1) and again significant improvement occurred in the endometriosis score, ($p<0.01$), but not for the AFS score, ($0.05<p<0.1$). There was no difference between the drugs in the improvement seen in

either the AFS score, (p=0.28), or in the endometriosis scores, (p=0.17).

When examined more closely, the endometriosis scores in 55% of the gestrinone patients had improved (35%) or were cured (20%) compared to 68% (19% improved and 48% cured) in the danazol group (Table 2.16). However 20% of the gestrinone and 5% of the danazol treated patients deteriorated during the 6 month therapy period. As expected the majority of the adhesion scores either remained unchanged (gestrinone 50%, danazol 62%) or became worse (gestrinone 35%, danazol 19%). The increase in adhesions present most likely resulted from scar formation at old sites of endometriosis.

The post-treatment scores were distributed into groups of severity as shown in Table 2.17.

Table 2.17: The distribution of endometriosis scores into none, mild, moderate and severe, after treatment with gestrinone or danazol. The groups were not statistically different.

	GESTRINONE	DANAZOL
None	3 (15%)	6 (28.6%)
Mild	13 (65%)	10 (47.6%)
Moderate	2 (10%)	3 (14.3%)
Severe	2 (10%)	2 (9.5%)

TABLE 2.16: THE CHANGE IN ENDOMETRIOSIS, AFS AND ADHESION SCORES FOLLOWING SIX MONTHS' TREATMENT WITH GESTRINONE AND DANAZOL

SCORE	CHANGE WITH TREATMENT	GESTRINONE		DANAZOL	
		NO	%	NO	%
PURE ENDOMETRIOSIS	CURED	4	20	10	48
	IMPROVED	7	35	4	19
	SAME	5	25	6	29
	WORSE	4	20	1	5
AFS	CURED	3	15	6	29
	IMPROVED	8	40	7	33
	SAME	2	10	5	24
	WORSE	7	35	3	14
ADHESIONS	CURED	0	0	2	10
	IMPROVED	3	15	2	10
	SAME	10	50	13	62
	WORSE	7	35	4	19

VAGINAL BLEEDING DURING TREATMENT

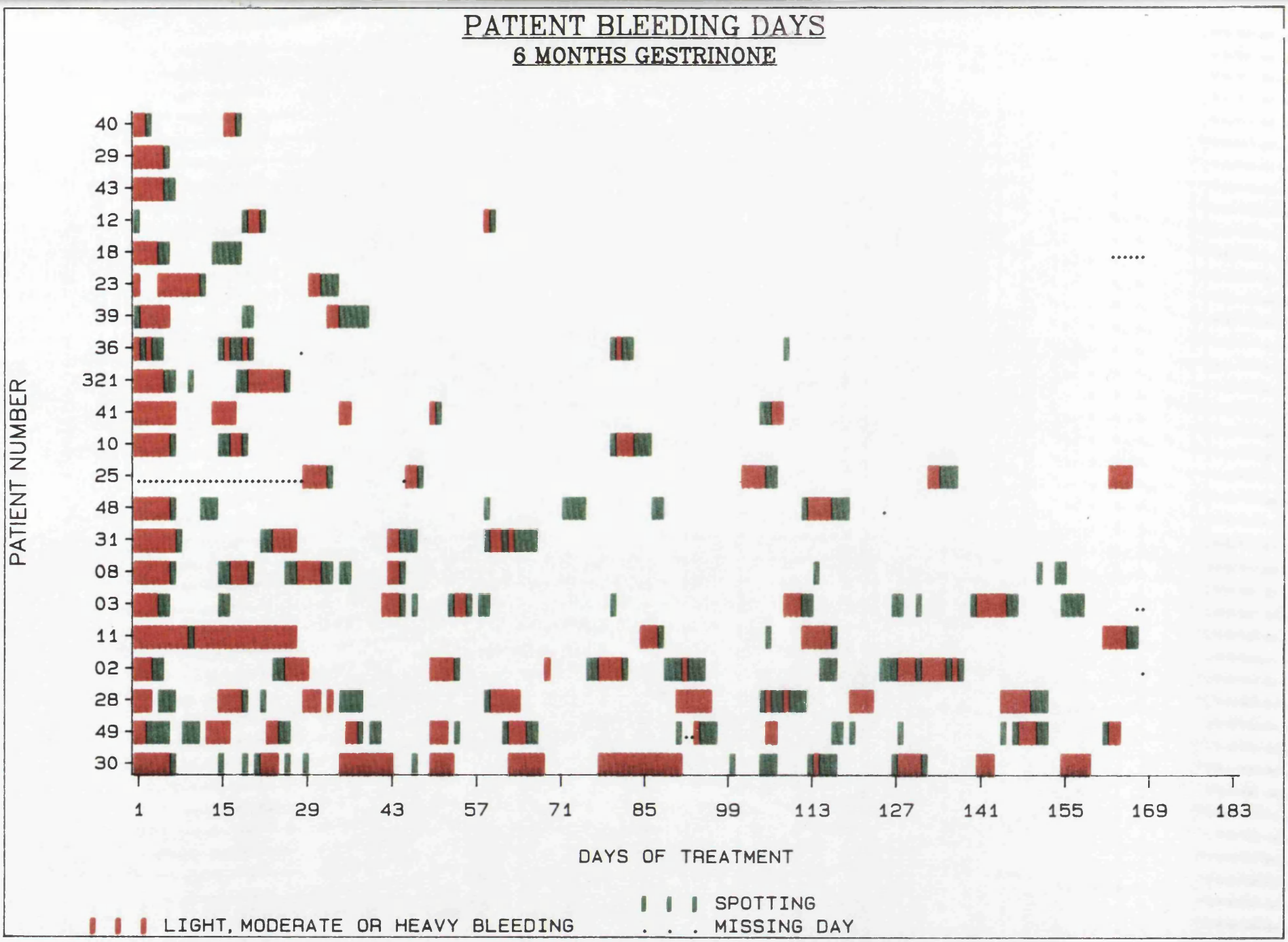
A record of vaginal bleeding and spotting was kept in the diary cards by patients during treatment. The bleeding patterns of the individual patients are shown in Figures 2.6 (gestrinone) and 2.7 (danazol). Excluding the first 28 days of treatment (during which all patients had a menstrual bleed), the gestrinone group had an a mean of 8.8 bleeding days (total 177) and 7.2 (total 145) days of spotting, whereas the danazol group had a mean of 4.3 (total 91) bleeding and 7.5 (total 157) spotting days. Although the incidence of bleeding appeared to be higher in the gestrinone treated patients, there was no statistically significant difference between the groups. Only 5 (25%) patients treated with gestrinone and 6 (28%) of those treated with danazol were amenorrhoeic after the first month throughout the following 5 months.

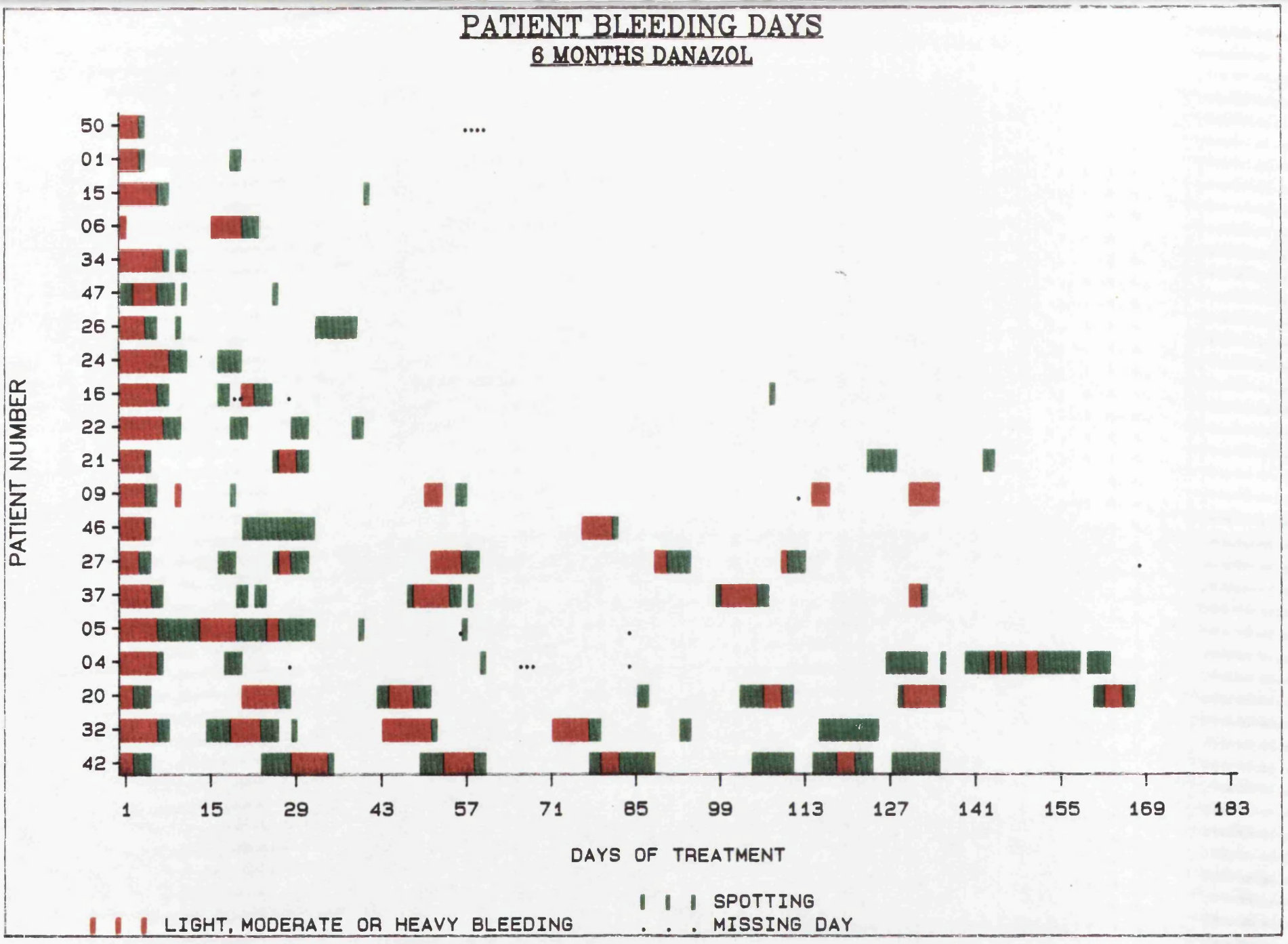
CHANGE IN SYMPTOMS WITH TREATMENT

Symptoms were assessed subjectively as severe, moderate, mild or none. For analysis these were scored as 3, 2, 1 and 0, respectively.

Dysmenorrhoea (Table 2.4, Figure 2.2)

Before treatment, 88% in the gestrinone group and 92% in the danazol group complained of dysmenorrhoea. For the majority of patients, this was severe: 46% in the gestrinone group and 48% in the danazol group. After 6 months medication, 40% in the gestrinone group and 20% in


PATIENT BLEEDING DAYS
6 MONTHS GESTRINONE
PATIENT NUMBER
40
29
43
12
18
23
39
36
321
41
10
25
48
31
08
03
11
02
28
49
30
1
15
29
43
57
71
85
99
113
127
141
155
169
183
DAYS OF TREATMENT
LIGHT, MODERATE OR HEAVY BLEEDING
SPOTTING
MISSING DAY


PATIENT BLEEDING DAYS
6 MONTHS DANAZOL
PATIENT NUMBER
50
01
15
06
34
47
26
24
16
22
21
09
46
27
37
05
04
20
32
42
1
15
29
43
57
71
85
99
113
127
141
155
169
183
DAYS OF TREATMENT
LIGHT, MODERATE OR HEAVY BLEEDING
SPOTTING
MISSING DAY

the danazol group continued to experience dysmenorrhoea. Although only small numbers of patients were available for analysis because the majority of patients on both danazol and gestrinone were amenorrhoeic, both drugs caused a significant improvement in dysmenorrhoea, (gestrinone: p=0.038, danazol: p=0.006). However, there was no significant difference in the effect produced by the two drugs (p=0.14).

Dyspareunia (Tables 2.5, 2.6, Figures 2.3, 2.4)

Deep dyspareunia improved significantly during treatment in patients treated with both drugs, its occurrence decreasing from 46% (19% severe) to 21% (none severe) in the gestrinone group (p=0.046) and from 54% (21% severe) to 17% (none severe) in the danazol group (p=0.0014). The difference in effect between the two drugs was not significant (p=0.48).

The majority of patients (81% of the gestrinone group and 91% of the danazol) did not suffer from superficial dyspareunia at the commencement of the trial. After 6 months of medical therapy, no patient in either group complained of this symptom, but this change was not significant.

Lower abdominal pain (Table 2.7, Figure 2.5)

A highly significant decrease in the incidence of lower abdominal pain occurred at the end of treatment, from 58% to 11% in the gestrinone group (p=0.005) and from 56% to 17% in the danazol group (p=0.013). There was no significant difference between the two drugs.

SIDE EFFECTS

Weight

The mean weight of patients in the gestrinone group was 59.8 ± 9.2 (standard deviation <SD>); (range 45.5-81.0) and 60.0 ± 9.7 (range 40.5-79.5) in the danazol group. At month 5/6 the mean weights had increased to 63.5 ± 9.5 for the former and 61.7 ± 10.8 for the latter. There was no difference between the treatments but a significant time effect for each drug (p=0.0001).

Acne (Table 2.18, Figure 2.8)

Forty three per cent of the gestrinone group and 20% of the danazol group reported some degree of acne before treatment. Moderate and severe acne was more frequently described during treatment with both gestrinone and danazol, but this effect was not statistically significant for either drug (gestrinone: p=0.1, danazol: p=0.58). The analysis showed no significant difference in the change in acne between the treatment groups.

Seborrhoea (Table 2.19, Figure 2.9)

As with acne, there appeared to be an increased incidence of moderate and severe seborrhoea occurring during treatment, especially in the gestrinone group (Figure 2.9). However, this increase was not significant for gestrinone (p=0.19) or danazol (p=0.76), and there was no difference between the drug effects (p=0.38).

Hirsutism (Table 2.20, Figure 2.10)

Subjective assessment revealed a marked increase in

TABLE 2.18: SUBJECTIVE SIDE EFFECTS BEFORE AND DURING TREATMENT WITH GESTRINONE AND DANAZOL: ACNE

TREATMENT GROUP	VISIT						
	Pre-treatment	1	2	3	4	5	6
GESTRINONE							
None	15(88%)	16(64%)	14(58%)	10(43%)	7(32%)	12(63%)	8(57%)
Mild	9(35%)	9(36%)	5(21%)	9(39%)	11(50%)	2(11%)	2(21%)
Moderate	2(8%)	0	4(17%)	4(17%)	3(14%)	5(26%)	2(14%)
Severe	0	0	1(4%)	0	1(5%)	0	1(7%)
Unknown	0	0	0	0	0	0	5
NUMBER OF PATIENTS	26	25	24	23	22	19	19
DANAZOL							
None	20(80%)	19(79%)	15(65%)	14(64%)	17(81%)	12(55%)	8(80%)
Mild	5(20%)	3(13%)	6(26%)	6(27%)	2(10%)	8(36%)	2(20%)
Moderate	0	1(4%)	1(4%)	2(9%)	1(5%)	2(9%)	0
Severe	0	1(4%)	1(4%)	0	1(5%)	0	0
Unknown	0	0	0	0	0	0	10
NUMBER OF PATIENTS	25	24	23	22	21	22	20

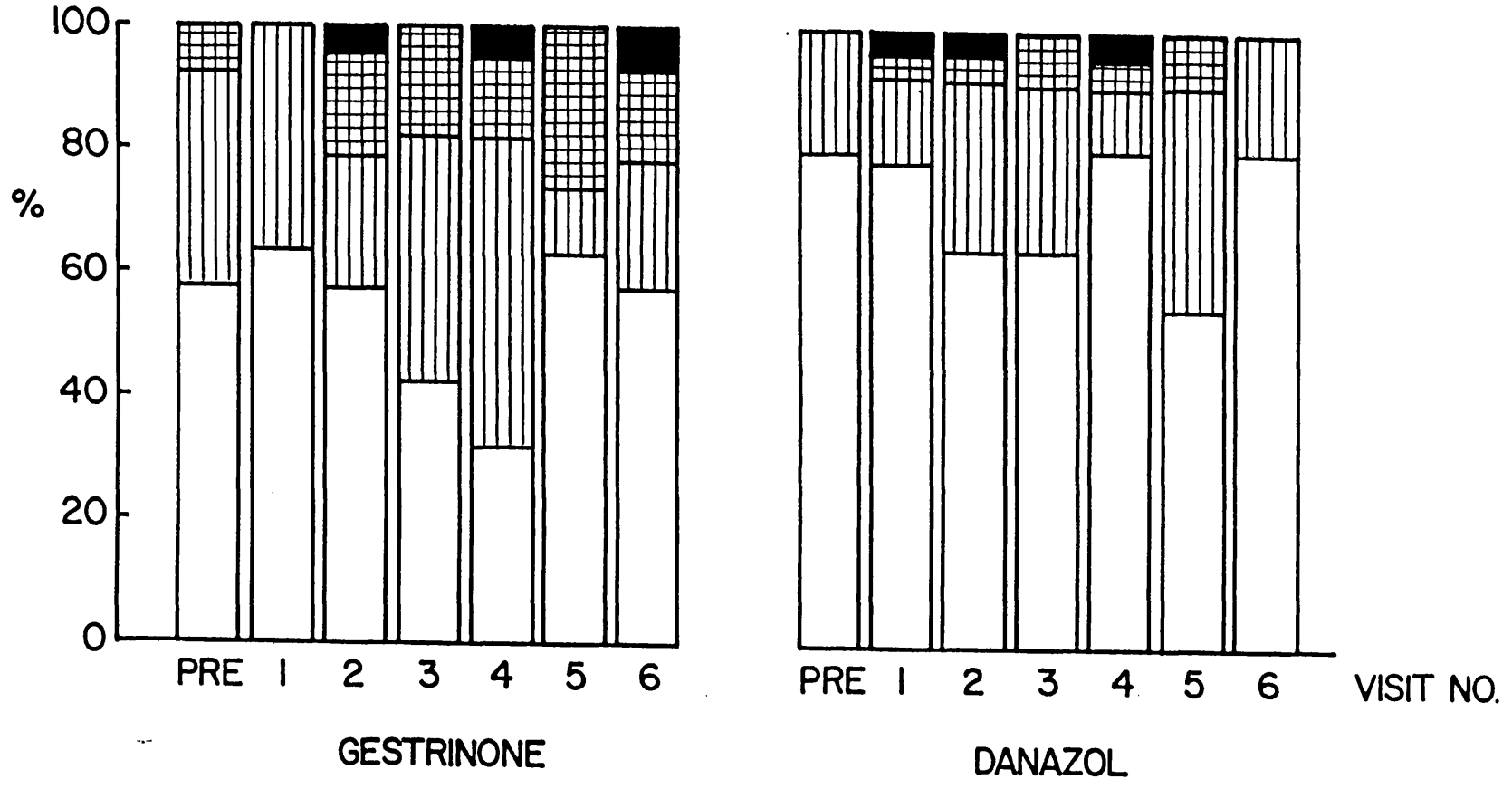


FIGURE 2.8: The effect of Gestrinone and Danazol treatment on the incidence of acne
(□none, ▥mild, ⊞moderate, ■severe)

TABLE 2.19: SUBJECTIVE SIDE EFFECTS BEFORE AND DURING TREATMENT WITH GESTRINONE AND DANAZOL: SEBORRHOEA

TREATMENT GROUP	VISIT						
	Pre-treatment	1	2	3	4	5	6
GESTRINONE							
None	22(85%)	17(68%)	13(54%)	13(57%)	13(59%)	13(68%)	8(57%)
Mild	2(8%)	7(28%)	7(29%)	8(35%)	6(27%)	3(16%)	3(21%)
Moderate	2(8%)	1(4%)	4(17%)	2(9%)	2(9%)	2(11%)	2(14%)
Severe	0	0	0	0	1(5%)	1(5%)	1(7%)
Unknown	0	0	0	0	0	0	5
NUMBER OF PATIENTS	26	25	24	23	22	19	19
DANAZOL							
None	15(60%)	19(79%)	16(70%)	14(64%)	15(71%)	14(64%)	7(70%)
Mild	10(40%)	3(13%)	4(17%)	7(32%)	4(19%)	6(27%)	2(20%)
Moderate	0	2(8%)	3(13%)	1(5%)	1(5%)	2(9%)	1(10%)
Severe	0	0	0	0	1(5%)	0	0
Unknown	0	0	0	0	0	0	10
NUMBER OF PATIENTS	25	24	23	22	21	22	20

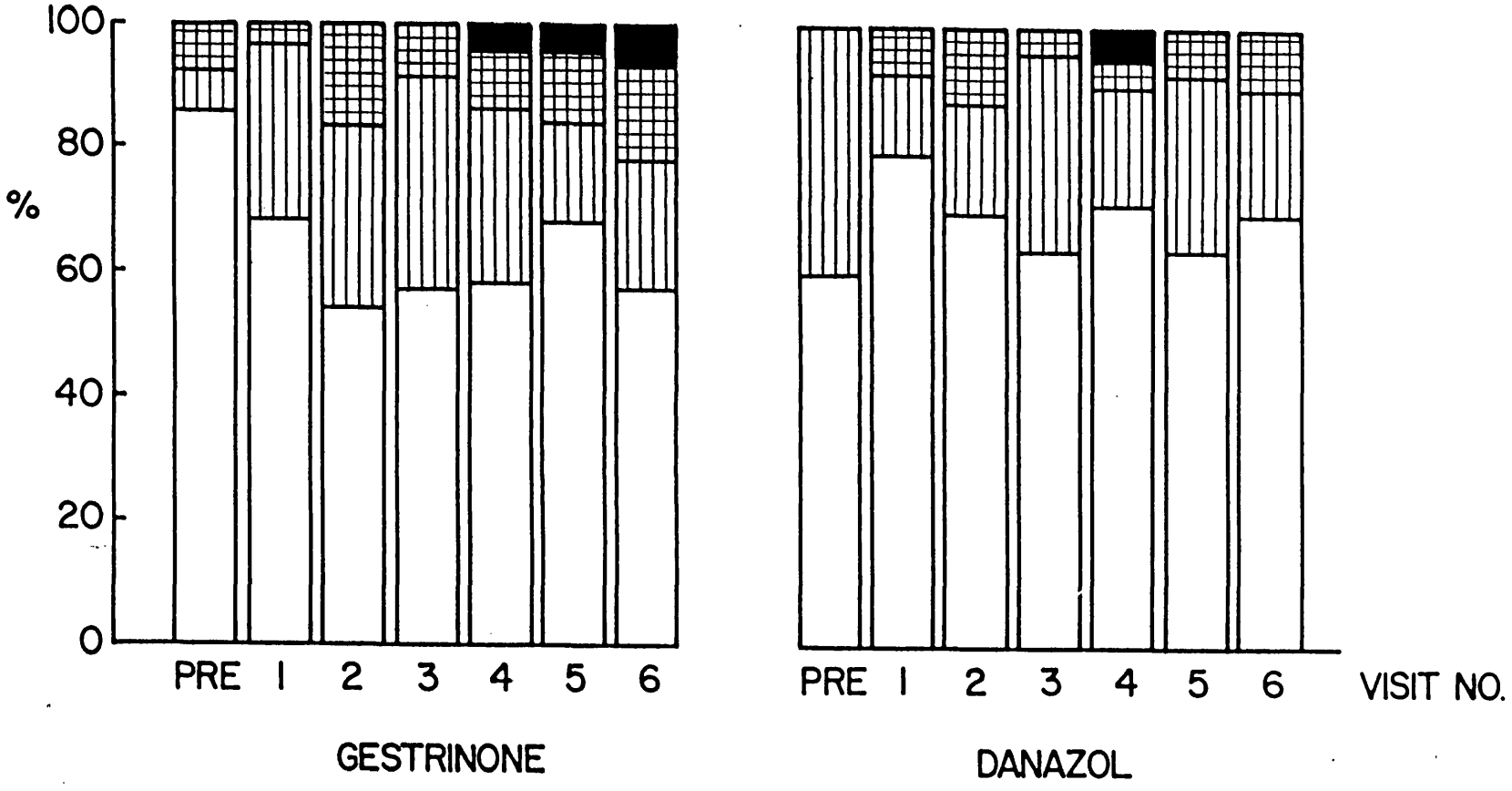


FIGURE 2.9: The effect of Gestrinone and Danazol treatment on the incidence of seborrhoea
(□ none, ▥ mild, ⊞ moderate, ■ severe)

TABLE 2.20: SUBJECTIVE SIDE EFFECTS BEFORE AND DURING TREATMENT WITH GESTRINONE AND DANAZOL: HIRSUTISM

TREATMENT GROUP	VISIT						
	Pre-treatment	1	2	3	4	5	6
GESTRINONE							
None	24(92%)	25(100%)	17(71%)	11(48%)	13(59%)	9(47%)	5(36%)
Mild	1(4%)	0	7(29%)	9(39%)	4(18%)	7(37%)	7(50%)
Moderate	0	0	0	2(9%)	5(23%)	2(11%)	2(14%)
Severe	1(4%)	0	0	1(4%)	0	1(5%)	0
Unknown	0	0	0	0	0	0	5
NUMBER OF PATIENTS	26	25	24	23	22	19	19
DANAZOL							
None	19(76%)	21(88%)	20(87%)	17(77%)	20(95%)	19(86%)	8(80%)
Mild	4(16%)	3(13%)	2(9%)	3(14%)	1(5%)	2(9%)	1(10%)
Moderate	2(8%)	0	1(4%)	2(9%)	0	1(5%)	1(10%)
Severe	0	0	0	0	0	0	0
Unknown	0	0	0	0	0	0	10
NUMBER OF PATIENTS	25	24	23	22	21	22	20

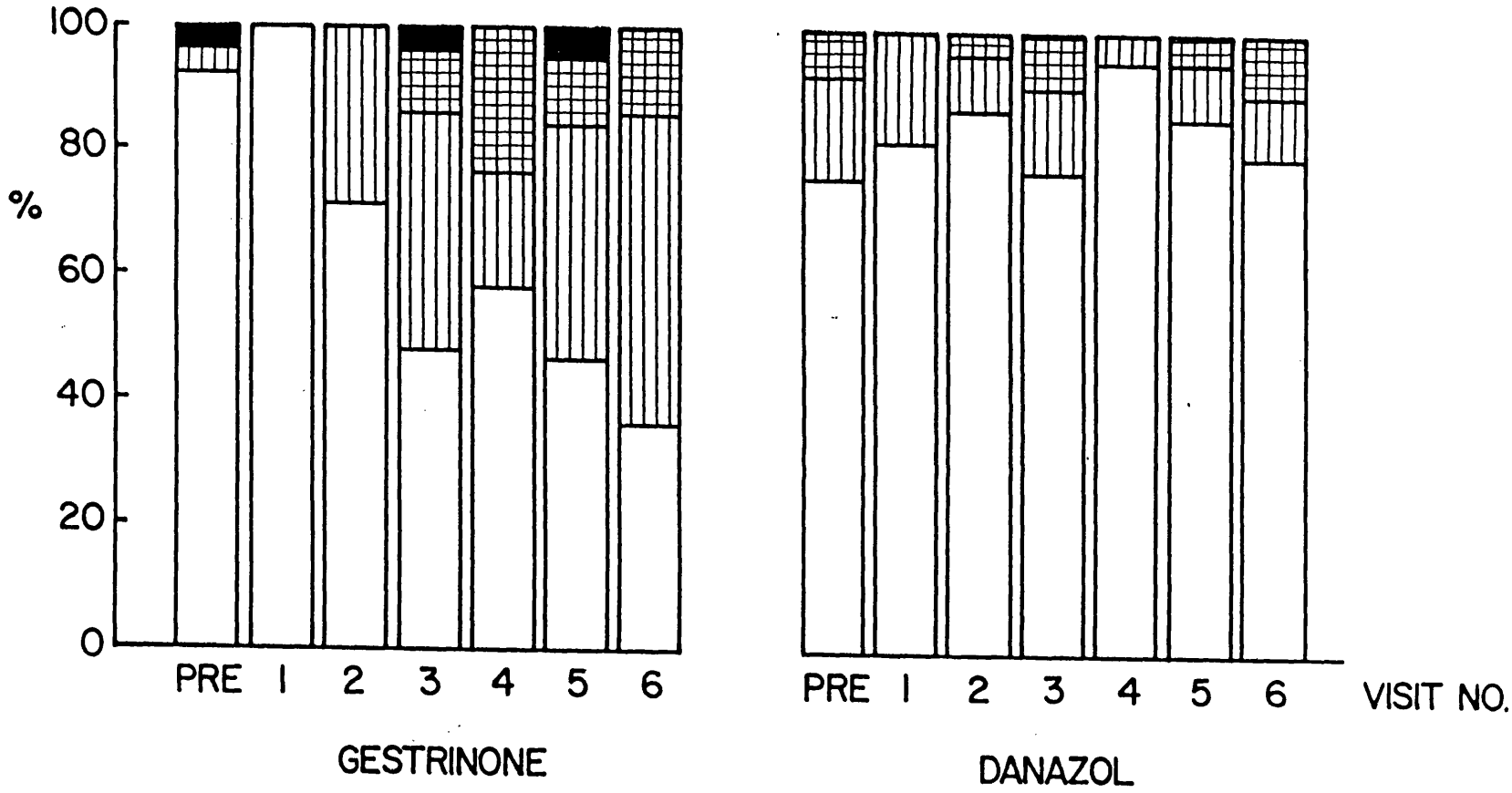


FIGURE 2.10: The effect of Gestrinone and Danazol treatment on the incidence of hirsutism
(□none, ▥mild, ⊞moderate, ■severe)

the incidence of hirsutism during treatment with gestrinone (p=0.003). There was no significant change in hirsutism scores during treatment with danazol (p=0.1) with no patient showing a worsening of hirsutism at month 5/6 in comparison with pretreatment, and this difference between the two treatment effects was highly significant (p=0.0002).

Voice Change (Table 2.21, Figure 2.11)

In the danazol group, 2 patients complained of a voice change during treatment and were consequently withdrawn from the trial. Voice changes occurring during gestrinone therapy were only temporarily reported. The very small number of events did not merit statistical analysis.

Headaches (Table 2.22, Figure 2.12)

There was no significant change in the incidence of headaches during treatment with gestrinone (p=0.12) or danazol (p=0.28), and no difference between the drug effects (p=0.33).

Leg Cramps (Table 2.23, Figure 2.13)

Although little change was observed in the incidence of leg cramps with gestrinone treatment, there appeared to be a noticeable increase in severe leg cramps in the danazol group. However, neither of the changes in leg cramp scores were statistically significant (gestrinone: p=0.58, danazol: p=0.11), and there was no significant difference between the drug effects (p=0.5).

TABLE 2.21: SUBJECTIVE SIDE EFFECTS BEFORE AND DURING TREATMENT WITH GESTRINONE AND DANAZOL: VOICE CHANGE

TREATMENT GROUP	VISIT						
	Pre-treatment	1	2	3	4	5	6
GESTRINONE							
None	25(96%)	24(96%)	24(100%)	22(96%)	20(91%)	19(100%)	14(100%)
Mild	1(4%)	1(4%)	0	1(4%)	0	0	0
Moderate	0	0	0	0	1(5%)	0	0
Severe	0	0	0	0	1(5%)	0	0
Unknown	0	0	0	0	0	0	5
NUMBER OF PATIENTS	26	25	24	23	22	19	19
DANAZOL							
None	25(100%)	24(100%)	21(91%)	22(100%)	21(100%)	21(95%)	10(100%)
Mild	0	0	2(9%)	0	0	1(5%)	0
Moderate	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0
Unknown	0	0	0	0	0	0	10
NUMBER OF PATIENTS	25	24	23	22	21	22	20

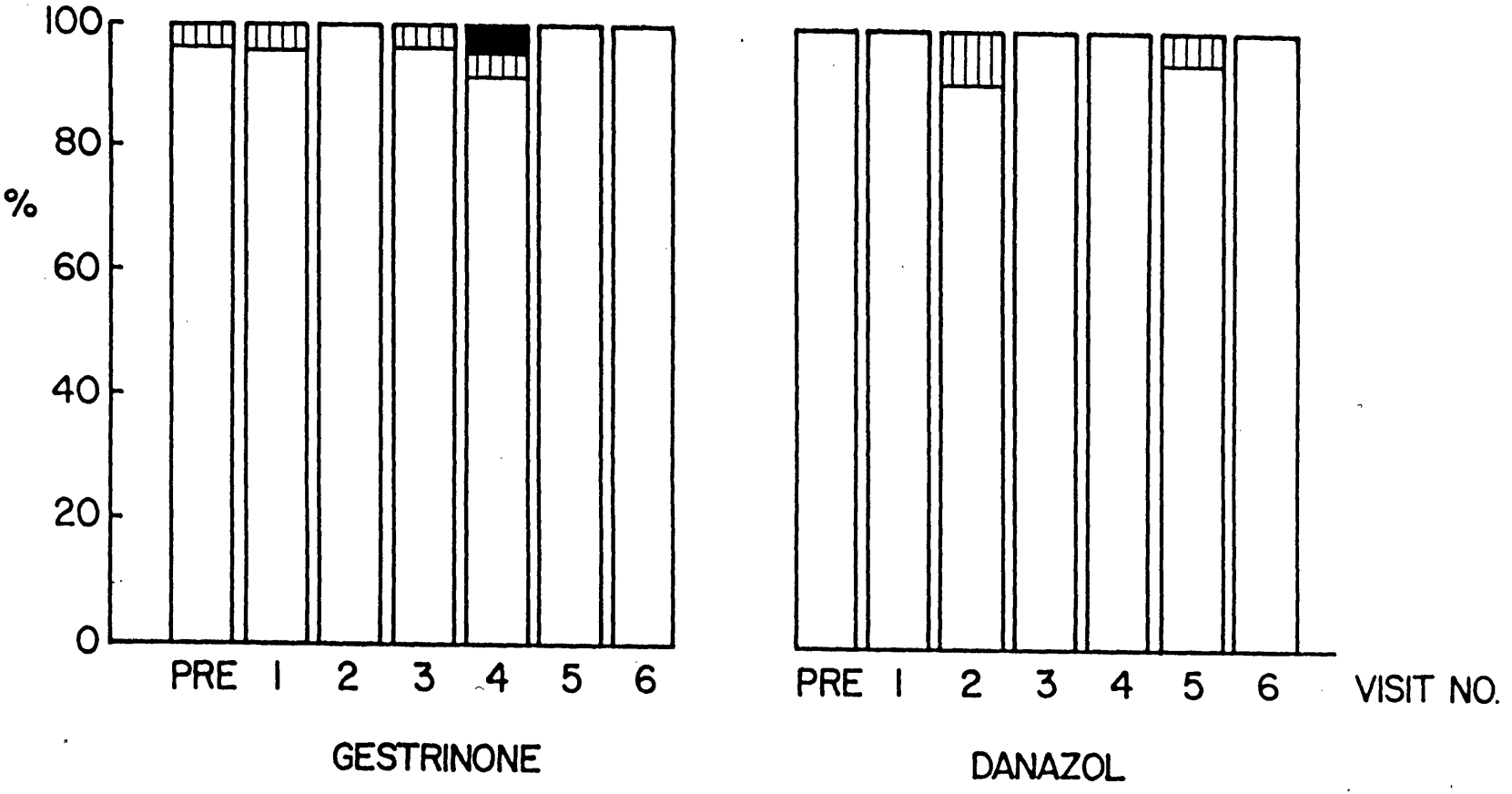


FIGURE 2.11: The effect of Gestrinone and Danazol treatment on the incidence of voice change
(□none, ▥mild, ⊞moderate, ■severe)

TABLE 2.22: SUBJECTIVE SIDE EFFECTS BEFORE AND DURING TREATMENT WITH GESTRINONE AND DANAZOL: HEADACHES

TREATMENT GROUP	VISIT						
	Pre-treatment	1	2	3	4	5	6
GESTRINONE							
None	8(31%)	14(56%)	17(71%)	20(87%)	14(64%)	15(79%)	11(79%)
Mild	10(38%)	4(16%)	4(17%)	2(9%)	5(23%)	1(5%)	2(14%)
Moderate	4(15%)	6(24%)	2(8%)	1(4%)	1(5%)	1(5%)	0
Severe	4(15%)	1(4%)	1(4%)	0	2(9%)	2(11%)	1(7%)
Unknown	0	0	0	0	0	0	5
NUMBER OF PATIENTS	26	25	24	23	22	19	19
DANAZOL							
None	13(52%)	14(58%)	15(65%)	16(73%)	16(76%)	15(68%)	6(60%)
Mild	6(24%)	6(25%)	5(22%)	4(18%)	4(19%)	4(18%)	1(10%)
Moderate	2(8%)	2(8%)	2(9%)	2(9%)	1(5%)	3(14%)	1(10%)
Severe	4(16%)	2(8%)	1(4%)	0	0	0	2(20%)
Unknown	0	0	0	0	0	0	10
NUMBER OF PATIENTS	25	24	23	22	21	22	20

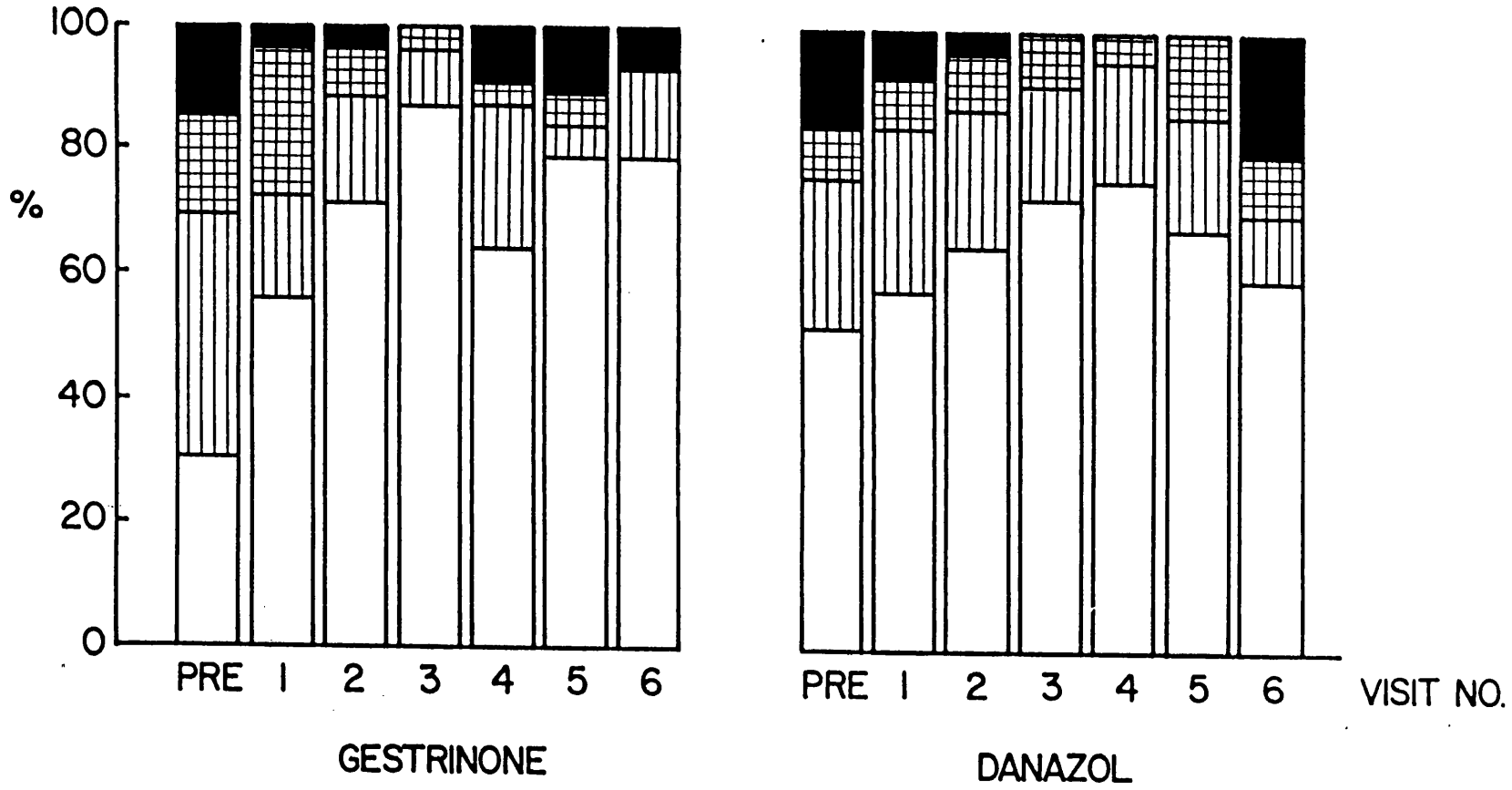


FIGURE 2.12: The effect of Gestrinone and Danazol treatment on the incidence of headaches
(□none, ▥ mild, ▦ moderate, ■ severe)

TABLE 2.23: SUBJECTIVE SIDE EFFECTS BEFORE AND DURING TREATMENT WITH GESTRINONE AND DANAZOL: LEG CRAMPS

TREATMENT GROUP	VISIT						
	Pre-treatment	1	2	3	4	5	6
GESTRINONE							
None	20(77%)	20(80%)	20(83%)	16(70%)	17(77%)	15(79%)	12(86%)
Mild	3(12%)	2(8%)	3(13%)	5(22%)	3(14%)	2(11%)	0
Moderate	2(8%)	3(12%)	1(4%)	2(9%)	2(9%)	2(11%)	1(7%)
Severe	1(4%)	0	0	0	0	0	1(7%)
Unknown	0	0	0	0	0	0	5
NUMBER OF PATIENTS	26	25	24	23	22	19	19
DANAZOL							
None	21(84%)	14(58%)	16(70%)	16(73%)	17(81%)	15(68%)	7(70%)
Mild	4(16%)	6(25%)	2(9%)	4(18%)	2(10%)	2(9%)	1(10%)
Moderate	0	1(4%)	1(4%)	1(5%)	0	3(14%)	0
Severe	0	3(13%)	4(17%)	1(5%)	2(10%)	2(9%)	2(20%)
Unknown	0	0	0	0	0	0	10
NUMBER OF PATIENTS	25	24	23	22	21	22	20

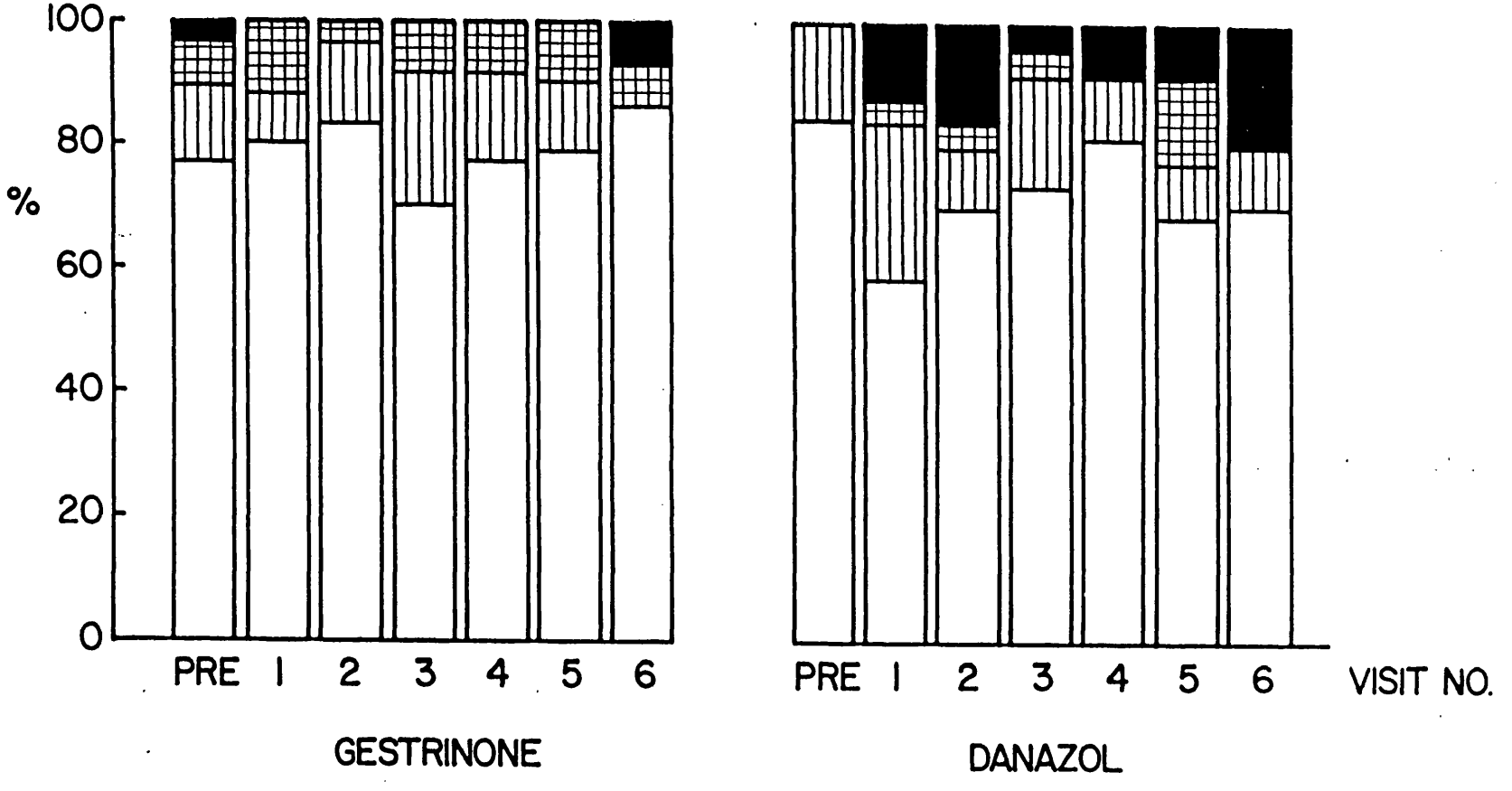


FIGURE 2.13: The effect of Gestrinone and Danazol treatment on the incidence of leg cramps
(□none, ▥mild, ▦moderate, ■severe)

Hot Flushes (Table 2.24, Figure 2.14)

There appeared to be little change in either group in the incidence of hot flushes during treatment, (gestrinone: p=0.66, danazol: p=0.86), and no significant difference between the treatment groups, (p=1.0).

SUMMARY

Two comparable groups of patients with endometriosis were treated for 24 weeks with gestrinone or danazol. The efficacy, determined by laparoscopic assessment and symptomatic relief, and the tolerance in terms of side effects and withdrawal from treatment, were similar for both drugs except for the effect of gestrinone in promoting hirsutism. However, minor differences between the drugs were unlikely to be detected with the small numbers in our study.

In the next sections of this thesis, investigations of the similarities and differences in the mechanisms of actions of these drugs in producing these effects are reported.

TABLE 2.24: SUBJECTIVE SIDE EFFECTS BEFORE AND DURING TREATMENT WITH GESTRINONE AND DANAZOL: HOT FLUSHES

TREATMENT GROUP	VISIT						
	Pre-treatment	1	2	3	4	5	6
GESTRINONE							
None	21(81%)	17(68%)	20(83%)	19(83%)	20(91%)	16(84%)	14(100%)
Mild	4(15%)	7(28%)	4(17%)	3(13%)	1(5%)	2(11%)	0
Moderate	1(4%)	1(4%)	0	0	0	0	0
Severe	0	0	0	1(4%)	1(5%)	1(5%)	0
Unknown	0	0	0	0	0	0	5
NUMBER OF PATIENTS	26	25	24	23	22	19	19
DANAZOL							
None	21(84%)	16(67%)	18(78%)	17(77%)	18(86%)	18(82%)	8(80%)
Mild	2(8%)	5(21%)	4(17%)	5(23%)	3(14%)	1(5%)	1(10%)
Moderate	2(8%)	3(13%)	1(4%)	0	0	3(14%)	0
Severe	0	0	0	0	0	0	1(10%)
Unknown	0	0	0	0	0	0	10
NUMBER OF PATIENTS	25	24	23	22	21	22	20

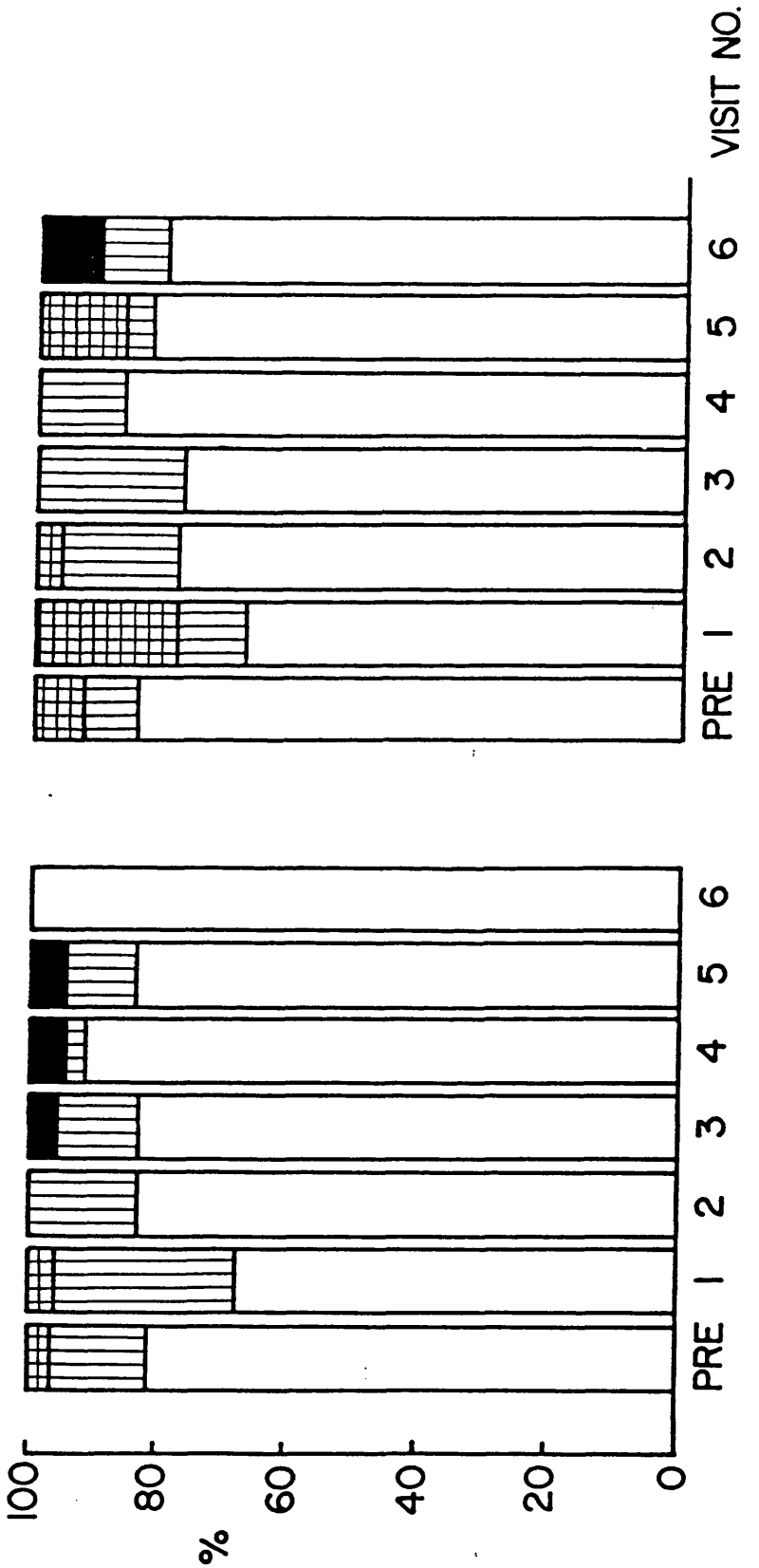


FIGURE 2.14: The effect on Gestrinone and Danazol treatment on the incidence of hot flushes
(□none, ▨mild, ▦moderate, ■severe)

CHAPTER 6

DISCUSSION

This treatise compares the efficacy and tolerance of the two therapeutic compounds, gestrinone and danazol, in 51 women with proven endometriosis using a randomised double-blind technique.

The two groups of patients on gestrinone and danazol therapy were comparable prior to treatment in all respects examined and it may be assumed that any differences that subsequently occurred are likely to have been as a result of the drug.

The diagnosis of endometriosis was made by laparoscopy. Ideally, all of these laparoscopies should have been performed by one person to avoid inter-operator error. However, due to the length time over which patients were recruited, the first 20 observations were made by another surgeon, i.e. Mr. Forbes. As the randomisation code for either gestrinone or danazol was performed in subgroups of 10, any inter-observer difference in the scoring techniques should have affected both treatment groups equally. The AFS scoring system was devised to prevent inter-observer bias and its use in this study should negate this problem. A major criticism of the AFS system is demonstrated here as theoretically a weighted numbering system should have ensured that the severity of the endometriosis could have been compared between individual patients. However, in the

system points are given for endometriosis and adhesions, and medical treatment would not be expected to change the adhesions already present. Therefore, to assess the effect of the drug therapies, the actual endometriosis scores are of greater importance. No significant improvement occurred in the total AFS scores during treatment, but a significant improvement in endometriosis scores was seen as a result of treatment with both gestrinone and danazol.

The timing of both the pre and post treatment laparoscopy also warrants comment. Drug treatment was started within two months of the diagnostic laparoscopy which, in view of the natural history of the disease, was necessary for the two groups to remain comparable. Thomas and Cooke (1987a) found that endometriosis deteriorated in 8 (47%) of 17 untreated patients during a 6 month period compared to none of 18 patients treated with gestrinone 2.5mg weekly, but were unable to predict in which patients this would occur. In this study, the post-treatment laparoscopy was performed 3 weeks after stopping medication i.e. prior to the first menstrual bleed. Although this would have been expected to reveal the maximum effect of the drugs, criticism may be made as at this time only approximately 50% of patients in each group had oestradiol levels compatible with ovarian activity. Since oestradiol stimulation may have re-activated otherwise masked quiescent areas of endometriosis, the early timing of the laparoscopy may have resulted in an underestimation of residual disease. It also prevents any conclusion being drawn as regard the

long term benefit of the drug therapy.

In this study, 55% and 67% of patients treated with gestrinone and danazol respectively, improved or were cured at laparoscopic assessment, which appears initially to be an inferior response than the 85-95% reported in the literature (see Table 2.1). However, in the majority of these studies, 800mg danazol has been used. Thus, the poorer response may have resulted from the lower 400mg dose of danazol chosen for this study. Although Buttram et al (1985) did not show any significant difference in the response observed in laparoscopically assessed patients treated with 400mg or 800mg danazol, Dmowski et al (1982) demonstrated a dose related response. They observed a 42% improvement with 100mg, 39% at 200mg daily and 74% at 400mg and 600mg daily, which was less than the 85% improvement they had previously described using 800mg daily (Dmowski and Cohen, 1975). It must be noted that they also had small numbers of patients, with only 5-8 patients in each treatment group. Biberoglu and Berhman (1981) similarly found a dose related improvement in laparoscopy scores, with the disappearance of endometriotic implants occurring in 14%, 63%, 57% and 87% respectively in groups receiving 100mg, 200mg, 400mg and 600mg of danazol and hence the 400mg dose may have been inadequate for some of the patients.

Although the results of this study indicate that the efficacy of the drugs is similar, a percentage of patients appears to remain resistant to treatment: 45% of gestrinone patients and 34% of danazol. Unfortunately, there does not

seem to be any method of predicting patients who will fail to respond to medical therapy at the time of diagnosis, other than patients with large endometriomas. It is claimed that danazol can reduce the size of endometriomas, but rarely causes complete regression of these lesions, which invariably require surgical excision (Dmowski and Cohen, 1978, Biberoglu and Berhman, 1981, Barbieri et al, 1982). Consequently any patients with large endometriomas were excluded from the trial. If morphological or receptor patterns in endometriotic biopsies taken at the time of laparoscopy would identify poor responders to drug therapy better patient selection for medical treatment would be possible.

The adhesion scores remained unchanged or increased after treatment in both groups. Scar tissue would not be expected to improve with medical therapy, and the increase seen in some patients may result from the development of adhesions during the healing of endometriotic lesions. This scar formation may be significant for future fertility function if the ovaries or fallopian tubes are involved.

Improvement rather than cure in the disease occurred in 35% of gestrinone and 19% of danazol treated patients suggesting that these patients may have been inadequately treated. This is a cause of concern, especially as the recognised recurrence rate of 30-50% may actually be due to incomplete treatment rather than the development of new implants. Biberoglu and Berhman (1981) have further suggested that recurrence rates may be dose related and if

cure rates are also dose linked, this provides additional evidence that recurrence is the result of inadequate treatment. The large percentage of patients who failed to respond completely in this study may further indicate that the doses chosen (gestrinone 2.5mg twice weekly and danazol 400mg daily) were insufficient for optimal treatment of the endometriosis. Alternatively, they may represent a group of patients with endometriosis which is inherently insensitive to hormonal therapy. Furthermore, the patients who are allegedly cured are only so judged on visual assessment of the pelvic peritoneum. As Murphy et al (1986) have demonstrated, endometriotic deposits may only be visible microscopically and thus the assessment of eradication of the disease may equally be compared to the problem of eradication of malignant cells following chemotherapy. In both circumstances, the dose/cure response is almost impossible to calculate.

Pregnancy rates subsequent to treatment were not considered in this study. This was because patients with infertility due to other factors were not excluded from the trial and thus their endometriosis may or may not have been significant. This was a design fault of the trial, as it prevented analysis of the outcome of treatment on subsequent conception rates. However, Thomas and Cooke (1987b) who published the first randomised double blind placebo controlled trial on the impact of medical treatment on conception rates in patients suffering from asymptomatic

endometriosis, failed to demonstrate any improvement in conception rates from 6 months of gestrinone therapy. This suggests that there is no value in using pregnancy rates as an endpoint for the efficacy of drug treatment, at least for mild disease.

Vaginal bleeding occurred in 75% of gestrinone patients and 72% of the danazol group after the first 28 days. This compares with an incidence of 55% reported by Thomas and Cooke (1987a) and approximately 50% by Mettler and Semm (1984) and Tamaya et al (1985). At a higher dose of 5mg twice weekly, Coutinho (1982) reported amenorrhoea in 100% of patients after the first 2 months, but commented on an associated higher incidence of side effects. It has often been stated that absolute amenorrhoea is a requirement for the effective elimination of endometriosis. However, a linear regression analysis of number of bleeding days during treatment (excluding the first 28 days during which all patients experienced some blood loss) and the improvement of endometriosis scores failed to demonstrate any relationship. Although irregular and frequent bleeding was found to be inconvenient and a cause for concern for many of the patients, it may be concluded that vaginal bleeding does not prevent the benefit obtained from medical treatment and that the mechanism of action of the drugs is not dependent on the development of amenorrhoea.

Although significant symptomatic improvement

occurred in dysmenorrhoea, deep dyspareunia and lower abdominal pain, complete relief of pain did not occur in all patients in either the gestrinone or danazol group. Approximately 80% (range 60-89%) were asymptomatic at the completion of therapy. These figures are less than the 80-100% described in the literature (Table 2.1), although some of these papers confuse improvement and cure. This provides further evidence that treatment for 6 months at the doses used in this trial may be inadequate for complete resolution of endometriotic lesions in all patients, and suggests that a greater variation of both the dose and the duration of therapy tailored to individual requirements may improve the medical management of endometriosis.

Examination of the frequency of reported side effects during treatment revealed a surprisingly high occurrence of many of the expected complaints prior to the onset of therapy. This demonstrates the importance of defining the incidence of any expected side effect prior to the commencement of any drug trial.

The mean weight gain during treatment was similar to that already described (Noble and Letchworth, 1977, Dmowski and Cohen, 1978, Coutinho, 1982, 1984, Azadian-Boulanger et al, 1984, Barbieri and Ryan, 1985). In an attempt to prevent this increase in weight from occurring, patients were advised at the start of their medication to reduce their calorific intake, but despite their efforts many gained weight, almost certainly due to the anabolic properties of

both of the drugs.

With regard to the androgenic side effects, the only significant observation was the increase in hirsutism seen in the gestrinone group. Only Mettler and Semm (1984) and Coutinho (1984) have previously described hirsutism during gestrinone treatment, and neither of these have quantified its occurrence. The hirsutism may result from androgen receptor stimulation, either by the drug itself or by the endocrinological changes produced by it, but a similar trend might have been expected in acne and seborrhoea, assuming they are equally reflective of hyperandrogenism. This variation may reflect the lack of precision when using a subjectively reported response, or these two aspects of hyperandrogenism may be less sensitive to exogenous stimuli than hair growth. However, as none of the recognised side effects of danazol (Noble and Letchworth, 1977, Young and Blackmore, 1977, Barbieri et al, 1982) occurred in significant numbers of patients, it is most likely that the insignificant development of side effects, other than hirsutism in the gestrinone group, was simply a reflection of the small numbers of patients in each treatment group of the trial. A further possibility may be that the side effects could be dose dependent, although Barbieri et al, 1982, failed to demonstate any difference in adverse effects between 400 and 800mg of danazol daily. These findings will be further discussed together with the concurrent endocrinological changes in Section 3.

Voice change occurred in an equal number of patients

in each group, but in view of the possible long term effects reported (Wardle and Whitehead, 1983, Mercaitis et al, 1985), this was considered an indication to stop medication if persistent.

Withdrawal from the trial because of side effects was only necessary in 3 patients (12%) from each group. Although the tolerance of individual patients may vary, it would appear that the acceptability of the two drugs is similar.

In summary, it would appear that gestrinone and danazol are very similar in terms of efficacy and tolerance in the treatment of endometriosis. The only significant difference observed was the greater incidence of hirsutism in the gestrinone group. However, the larger multicentre study may provide more data regarding the efficacy and side effects produced by these drugs (results as yet unpublished). They both appear to be effective in the treatment of endometriosis in 60-80% of cases, depending on the end point used. However a percentage of unidentifiable patients remain resistant to drug therapy or are incompletely treated. This may result from inadequate doses used for this study or alternatively to an inherent insensitivity of some endometriotic lesions. Greater flexibility in the dose and duration of treatment, selected according to an individual patient's requirement is difficult to accommodate in a clinical trial but may improve the quality of care.

In view of the similarity of the 2 drugs, it seems unlikely that patients who have failed to respond to one or developed unacceptable side effects would benefit from the other, but this could only be established in a trial in which patients crossed-over between treatments. It would be interesting to observe the results of treatment with drugs whose mode of action is totally different, such as the LHRH analogues, in these resistant cases.

In the next parts of this thesis, the mechanisms by which gestrinone and danazol may produce their effects will be examined.

SECTION 3

THE ENDOCRINE EFFECTS OF DANAZOL AND GESTRINONE.

CHAPTER 7

REVIEW OF THE ENDOCRINE EFFECTS OF DANAZOL AND GESTRINONE

Since the advent of medical treatment for endometriosis, much research time has been dedicated to studying the changes in the endocrine milieu during treatment. This quest to explain the action of the drugs was based on the belief that pregnancy and the menopause were curative, and that endometriotic lesions were oestrogen dependent. Thus, it is pertinent to consider the reported endocrine effects of danazol and gestrinone in this context.

DANAZOL, GESTRINONE AND HYPOTHALAMO-PITUITARY FUNCTION

Danazol (Greenblatt et al, 1971) and gestrinone (Coutinho, 1982), have both been referred to as antigonadotrophins. This is however, a misnomer since an antigonadotrophin is a substance which antagonizes the action of gonadotrophins. It is not this property which is usually described by the term "antigonadotrophin", but the alleged reduction in their secretion. The majority of the literature supports the finding that baseline levels of LH and FSH remain unchanged during danazol administration (Greenblatt et al, 1971, Wood et al, 1975, Lieberman et al, 1977, Guillebaud et al, 1977, Ronnberg et al, 1979, Van Dijk et al, 1979, Hirschowitz et al, 1979, Floyd, 1980, Stillman et al, 1980, Luciano et al, 1981, Reyniak and Gurpide, 1982, and others). Evidence does exist, however, suggesting that

in frequently sampled women, LH remains normal but a decrease in FSH has been noted (Braun et al, 1983). Bevan et al (1984) describe a significant rise in LH in 7 out of 8 patients by the end of the first week of treatment, but variable subsequent changes, with no evidence of suppression. In women receiving gestrinone, again gonadotrophin levels are unchanged (Robyn et al, 1984, Kaupilla et al, 1985, Tamaya et al, 1985), although Mettler and Semm (1984) showed depressed LH and FSH levels on continuous therapy and Thomas and Cooke (1987a) suggest decreased levels but without data to justify the claim.

From these data, it would be incorrect to suggest that danazol and gestrinone had no effect on gonadotrophins. Most premenopausal women on danazol or gestrinone have decreased levels of oestradiol (E2) but there is no compensatory increase in LH or FSH. This would suggest that there is a drug induced block on gonadotrophin response.

Interestingly, Dmowski et al (1983) found a reduction in the frequency of LH pulses in premenopausal women during danazol (800mgs daily) therapy, with an increase in mean pulse amplitude and increment, and a co-existing reduction in the frequency of oestradiol pulses and increment. They found that mean LH concentrations were significantly suppressed in only 4 of their 8 patients and concluded that the persistence of LH pulses may explain inconsistent results and invalidate mean values. They were unable to demonstrate unequivocal FSH pulses. Braun et al (1983) also observed a reduction in both mean LH levels and LH pulse

frequency, but neither of these reached statistical significance.

In an attempt to identify any change in pituitary sensitivity to gonadotrophin releasing hormone, several investigators have studied the pituitary response to stimulation with a bolus dose of LHRH. No difference in the amplitude and frequency of the response was seen in normal women (Fraser et al, 1982, Braun et al, 1983), nor in female rats (Skane et al, 1978, Luciano et al, 1981, Rannevik and Thorell, 1984).

Shane et al (1978) found dose-dependent decreased levels of LH in castrated female rats and concluded that the mechanism of this action was primarily by inhibition of hypothalamic secretion. Having observed an absence of LH suppression in androgen receptor deficient pseudohermaphrodite or flutamide treated male rats, Krey et al (1981) further implied that the inhibition of LH release was due to danazol-androgen receptor interactions within the hypothalamo-pituitary axis. Similar results with LHRH stimulation were found in gestrinone treated patients (Mettler and Semm, 1984) although Robyn et al (1984) noted an exaggerated response. The animal data for gestrinone are less comparable to the human than those for danazol. Proulx et al (1984) found gestrinone significantly inhibited the LH response to a bolus of LHRH in ovariectomized rats . and further showed with in vitro experiments on cultured anterior pituitary cells that gestrinone stimulated LH release in oestrogen primed cultures as effectively as

progestins and inhibited LH release in unprimed cultures. This group concluded that gestrinone may exert some activity at the anterior pituitary level in the female rat.

Overall, the literature suggests that in humans, danazol and gestrinone mediate their effect on gonadotrophins at the level of the hypothalamus. However, it is accepted that progestins and androgens both influence gonadotrophin secretion at the same level and thus the effect may be indirect (see androgen section).

Although basal levels of LH and FSH remain unchanged, Greenblatt et al (1971) in his original paper on danazol, noticed the absence of a midcycle surge. This has subsequently been confirmed by many other authors, (Young and Blackmore, 1977, Mettler and Semm, 1979, Luciano et al, 1981). Luciano et al (1981) suggested that this absence of the LH and FSH surge may be due to a lack of ovarian follicular maturation and a consequent lack of the preovulatory rise in oestradiol rather than to a direct inhibitory effect of danazol. The data on gestrinone are similar (Robyn et al, 1984, Kaupilla et al, 1985, Tamaya et al, 1985).

Finally, there is conflicting evidence in the literature on the effect of danazol on prolactin. Most authors found no change in prolactin levels during therapy (Franchimont and Cramillion, 1977, Ronnberg et al, 1979, Hirschowitz et al, 1979, Floyd, 1980). However, Rannevik and Thorell (1984) and Braun et al (1983) found a significant lowering of prolactin levels, and Van Dijk et al (1979), who

observed unexpectedly high levels of prolactin in a group of patients with unexplained infertility, found that these "normalised" during danazol therapy. The decrease in prolactin described by some authors may be secondary to the low levels of oestradiol occurring during danazol administration. Plasma prolactin levels remain unchanged during gestrinone administration (Robyn et al, 1984). There is no demonstrable effect of danazol on adrenocorticotrophic hormone, thyroid stimulating hormone or growth hormone (Franchimont and Cramillion, 1977).

THE EFFECT OF DANAZOL ON OESTROGEN AND PROGESTERONE

Most investigators have only examined the effect of danazol on oestradiol (E2), and only rarely included oestrone (E1). The consensus of opinion is that E2 and E1 levels remain in the low follicular range during danazol treatment (Andrews and Wentz, 1975, Wood et al, 1975, Lieberman et al, 1977, Braun et al, 1983, Meldrum et al, 1983, Rannevik and Thorell, 1984). Young and Blackmore (1977) agreed with this, except that they observed a rise in E2 levels during the first month of therapy. Hirschowitz et al (1979) described significantly increased mean levels of E2 throughout treatment but the validity of their study design and assay methods were admittedly unacceptable.

Bevan et al (1984) described a fall in E2 levels during the first month of treatment, and Ronnberg et al (1979) agreed. Both papers may be criticised for comparing

ovulatory cycles, and on close inspection of their data, oestradiol levels "fall" on treatment to normal early follicular phase levels, as previously described data suggests. Floyd (1980) found E2 and E1 levels decreased to 60% of pretreatment follicular phase levels, but that this was not dose dependent.

Oestradiol levels have been shown to decrease in patients on gestrinone (Deltour et al, 1984, Robyn et al, 1984, Tamaya et al, 1985, Thomas and Cooke, 1987a) although Kaupilla et al (1985) observed unchanged mean E2 concentrations.

The lack of ovarian activity may reflect inappropriate gonadotrophin stimulation as outlined above or a direct effect of danazol on steroidogenesis. It has been noted that at lower doses of danazol, E2 production may be less inhibited (Rannevik and Thorell, 1984) suggesting a dose-related response. As described in Chapter 2, danazol has been shown to inhibit directly enzyme activity related to the steroid pathway in the ovary. Gestrinone however, has not been shown to have the same inhibitory effect on steroidogenesis (Belghmi, 1984) and the inhibition of ovarian activity may be via its receptor blocking capacity.

THE EFFECT OF DANAZOL ON SEX HORMONE BINDING GLOBULIN AND OTHER PLASMA PROTEINS

The major high-affinity transport protein for sex steroids is sex hormone binding globulin (SHBG), with the following order of high affinity binding: Dihydrotestosterone (DHT) > testosterone (T) > oestradiol (E2) > oestrone > androstenedione (A4) (Dunn et al, 1981). It has a half-life of 15 $\pm$ 5.7 (SD) days (Gershagen et al, 1984). Changes in SHBG, result in alterations in the free biologically available fraction of the sex steroids, with a consequent change in clearance rates (Anderson, 1974).

In the normal human female approximately 60% of circulating T is bound to SHBG and danazol also binds to SHBG with a high affinity (Nilsson et al, 1983). In computer derived estimates, it has been found that danazol would be expected to displace 83% of the concentration of T bound to SHBG (Pugeat et al, 1981). Laurell and Rannevik (1979) found that 14 of 25 plasma proteins they investigated were significantly affected by danazol. More importantly, Haning et al (1982) showed that the two principal metabolites of danazol, ethisterone (E) and 2-hydroxymethylethisterone (2OHME) also displaced T from SHBG. They claimed serum levels of danazol, with an oral dose of 800mgs, are 1300 times greater than that of T in the normal female, whilst Nilsson et al (1982), claim a 1000 times greater level with a 600mgs dose. Therefore, during treatment with normal therapeutic doses of danazol, the SHBG binding capacity is greatly exceeded. However, 2OHME and E have twice and 10

times higher affinity for SHBG than danazol respectively. Since the relative concentrations of E and 2OHME are greater than danazol, they may be more potent than danazol as a competitive displacer of T (Haning et al, 1982). Nilsson et al (1983) also found a reduction from 60.2% of T bound to SHBG to 17.9% in danazol treated patients, but a rise in albumin-bound T from 38.6% to 79.4%. Hence, albumin buffers T, diminishing the potential rise in % free T. Free T rose from 1.28% to 2.64%. Most authors agree that albumin levels are not affected by danazol (Laurell and Rannevik, 1982, Damber et al, 1984), but the bioavailability of the albumin bound fraction of T has been controversial. Manni et al in 1985, showed that 55% of albumin bound T entered tissues and that as the bioavailable T exceeded the free T, the albumin fraction should be considered biologically important.

All investigators have found SHBG levels decreased by danazol (Laurell and Rannevik, 1979, Meldrum et al, 1983, Damber et al, 1984). Bevan et al (1984) observed a significant fall in SHBG in the first week of treatment, which continued for 4 weeks, and this was confirmed later by Forbes et al (1986), who also described a dose relationship during the first 2 months of therapy. Gershagen et al (1984) observed this fall within 24 hours of starting medication, at doses from 200-800mgs, in pre and postmenopausal women.

It is unlikely that the low follicular phase E2 levels found in patients on danazol therapy are the causes of the low SHBG levels seen, since amenorrhoeic patients with equivalent low E2 levels did not have similarly reduced

SHBG levels (Laurell and Rannevik, 1979). A direct inhibitory effect on hepatic biosynthesis has been proposed as a more likely explanation. Hepatocytes have been shown to contain steroid receptors and therefore a direct androgenic effect by danazol may alternatively mediate this effect (Laurell and Rannevik, 1979, Gershagen et al, 1984).

It would therefore appear that danazol has 2 effects on SHBG:

1) an effective reduction in the binding capacity of SHBG, by competitive binding by danazol itself and its 2 major metabolites, E and 2OHME

2) a reduction of SHBG biosynthesis in the liver.

A dramatic decrease in SHBG has also been observed in patients treated with gestrinone (Pugeat et al, 1984, Kaupilla et al, 1985, Thomas and Cooke, 1987a). The decrease in SHBG was observed within the first month of therapy and directly mimicked the pattern seen with danazol. Again, this is thought to result from reduced hepatic synthesis, but unlike danazol, gestrinone has not been demonstrated to displace T from SHBG (Dowsett et al, 1986). In the study of Dowsett et al (1986), comparing the endocrine effects of danazol and gestrinone, % free T after 7 days therapy was significantly higher in the danazol group, but SHBG levels at this time were identical. By one month, levels of % free T were not significantly different and the authors suggest that displacement of T from SHBG within the first week was largely due to E, as in vitro experiments confirmed. After 4 weeks treatment the circulating level of SHBG had fallen to

such a low level that the % free T was close to the theoretical maximum with both drugs and the additional direct effect of the drugs and/or their metabolites on displacement of T was less important.

EFFECT OF DANAZOL ON TESTOSTERONE AND % FREE TESTOSTERONE

A fall in T has been described in women by several authors (Haning et al, 1982, Carlstrom et al, 1983, 1984, Forbes et al, 1986), and a rise in % free T has unanimously been agreed (Haning et al, 1982, Nilsson, 1982, 1983, Forbes et al, 1986). There was conflicting evidence on the effect of danazol on testosterone, because the earlier investigators did not appreciate the cross reaction that occurs between danazol and T in immunoassays, and the similar cross-reactivity with E and 2OHME (Dowsett et al, 1986, Forbes et al, 1986). Bevan et al (1984) found the cross reaction rate between danazol and T to be 0.12% in their assay, which invalidated T measurements unless separation methods were performed.

Ronnberg et al (1979) and Floyd (1980) both reported a significant rise in total T concentration during danazol treatment. Luciano et al (1981) agreed with this finding and further described a concommitant 80% increase in urinary 17-ketosteroid excretion. All these authors appear to have been aware of the the possibility of cross reaction to danazol occurring in their assays, but as Luciano et al (1981) had used a very specific antisera with a cross

reactivity of less than 0.001% with danazol for the measurement of T, they considered interference may have been by the metabolites.

Damber et al (1984) found levels of total T were unchanged, but that % free T increased by 80%. Nilsson et al (1983) were in accordance with these findings, using chromatographic celite microcolumns to separate danazol and avoid cross reactivity.

Forbes et al (1986) found no dose relationship in the changes observed in the concentration of total T, free T or DHT. When all dose levels were pooled they described a significant fall in total T in the first week, followed by a further significant fall at 2 months, but there was no change thereafter. This fall may be explained by an increase in the metabolic clearance rate of T resulting from the decrease in SHBG bound T (Forbes et al, 1986). However, the concentration of free T rose during the first week of therapy and then fell, although it remained at levels higher than pretreatment. DHT levels fell during the first week, but these did not change again during the subsequent 23 weeks of medication. In this study, percentage (%) of free T in pooled samples rose during the first week, but then remained unchanged, but when examined in separate groups, they did see a dose relationship for the rate of change in % free T.

Although, the evidence shows that % free T is elevated by danazol, the literature is more confused with regard to total T. However, the earlier reported 'rises' in

T were almost certainly due to cross reaction from danazol and its metabolites, and the later studies which describe a fall are probably more representative.

The total T concentration falls progressively during the first 3 months of gestrinone therapy and % free T markedly increases by 60% by 2-3 weeks (Pugeat et al, 1984, Robyn et al, 1984, Kaupilla et al, 1985, Tamaya et al, 1985). The theoretical prediction of Pugeat et al (1984) that less than 10% of plasma T bound to SHBG might be expected to be displaced by gestrinone has been confirmed by Dowsett et al (1986).

THE EFFECT OF DANAZOL ON ANDROSTENEDIONE (A4), DEHYDROEPIANDROSTERONE (DHEA) AND DEHYDROEPIANDROSTERONE SULPHATE (DHEAS)

Floyd (1980) observed no change in A4 levels with varied doses and durations of danazol therapy. A significant rise in A4 was described by Stillman et al (1980) and Luciano et al (1981), but again, cross reaction in their assays from danazol and its metabolites cannot be excluded. In men, Sherins et al (1971) found dose related falls in A4, and this has also subsequently been seen in women (Carlstrom et al, 1984, Forbes et al, 1986). Forbes et al (1986) found no significant fall in the first week of treatment, as opposed to the changes seen in other androgens after this time, but the decrease was apparent after 2 months. However, the A4 results were related to menstruation, and those patients with continued normal bleeding patterns during

therapy, usually on low doses of danazol, had significantly higher levels. As 60% of the circulating concentration of A4 in women is derived from the ovary, it was suggested that the relationship of A4 to menstruation involves decreased ovarian activity during treatment.

DHEAS rises during danazol administration (Floyd, 1980, Luciano et al, 1981, Carlstrom et al, 1983, 1984), and DHEA falls in women (Carlstrom et al, 1983, 1984) and men (Sherins et al, 1971). DHEAS binds only with albumin significantly, and as previously discussed, albumin remains unchanged during danazol therapy. Hence, the changes observed in DHEAS cannot be attributed to changes in plasma protein (including SHBG) levels (Carlstrom et al, 1983). Carlstrom et al (1984) also describe a fall in the DHEA/DHEAS ratio. They conclude that these effects are caused by an inhibition of hepatic sulphatase activity, as they also saw an increase in alanine aminotransaminase levels, and the divergent patterns of DHEA and DHEAS makes an effect on adrenal biosynthesis less likely.

Only Stillman et al (1980) found elevated levels of DHEA and they conversely suggest that this is consistent with the inhibition of adrenal enzymes of cortisol synthesis by danazol.

Robyn et al (1984) have shown that A4 decreases in gestrinone treated patients but DHEAS remains unchanged.

POST TREATMENT CHANGES

Despite the numerous and varied endocrinological

changes observed during danazol and gestrinone treatment, it is important to note that all the authors discussed above, agree that each of the parameters investigated return to pretreatment levels within 1 month of ceasing therapy.

CHAPTER 8

MATERIALS AND METHODS

PATIENTS

Blood samples for hormone assays were taken from the patients entered into the clinical trial described in Chapter 4 and were assayed without breaking the code. Samples were withdrawn pre-treatment, at monthly intervals during the 24 weeks of drug therapy and 3 weeks post-treatment. Where possible, the pre-treatment sample was taken during the third week of the cycle, and four weekly thereafter, but this was not possible in all cases, due to difficulties in patient attendance. The endometriosis clinic was held between 2.00 and 5.00 p.m. on the same day of the week throughout the trial and samples were taken during this period. The samples were all centrifuged and the serum was separated at the end of the clinic and was stored at -20C until analysis. All assays were performed by laboratory technicians in the Biochemical Endocrinology Laboratory at the CHW. However, the SHBG binding capacity (SHBG BC) and total T assays were performed by myself on a few occasions, in order to obtain a proper understanding of the methods and because of their relevance in investigating the mechanism of action of gestrinone and danazol. These, the thin layer chromatography and the modified percent free T method which were developed in our laboratory are described in detail in this section.

GONADOTROPHIN ASSAYS

Serum LH and FSH assays were performed using the double antibody Chelsea Kit radioimmunoassay (RIA), (Ferguson et al, 1982). These were the routine assays used by the hospital. The reference materials were the established World Health Organisation (WHO) International Reference Preparations for LH (68/40) and FSH (78/549). The coefficients of variation (c.v.) for LH were between batch <10% over a working range (2-50IU/L) and a mean within batch of 6% over the same range. For FSH the between batch c.v. was <10% over a working range (1-20IU/L), with a mean within batch c.v. of 4% over the same range.

OESTRADIOL (E2) AND PROGESTERONE (P) ASSAYS

Serum E2 and P were also measured by the routine laboratory RIAs, using reagents provided by the WHO Matched Reagent Scheme (Sufi et al, 1982). For oestradiol, the within- and between-assay coefficients of variation were 4.7% and 14.0% respectively, at a serum concentration of 250pmol/L. For P the within assay c.v. was 4.1% and the between assay c.v. was 12.5%.

SEX HORMONE BINDING GLOBULIN BINDING CAPACITY (SHBG BC)

SHBG BC was measured using the dual-column method of Iqbal and Johnson (1977), with the modifications published by Dowsett et al (1985). This technique involves the removal of serum albumin-bound steroid from samples by dye-linked

Sepharose in the upper section of a "two-tier" column and removal of free steroid from the lower tier by a gel of Sephadex LH-20, so that only specifically SHBG bound steroid is obtained in the eluate. By adding a known saturating concentration of ^{3}H-DHT to the sample, the SHBG BC can be calculated after radioactive counting of the eluate from the column.

20ngs of 5α DHT (Sigma) and 125nCi (4.6MBq) of (1,2,4,5,6,7,-^{3}H) 5αDHT (Amersham International, 138Ci/mmol (5 X 10^{15} Bq/mol)) were added in 400ul of tris buffer (0.05mol/l, pH7.4) containing $CaCl_2$ (0.01mol/l) to 100ul of plasma or to 100ul of a 5% solution of human serum albumin (HSA, Sigma A-1887) in tris buffer and allowed to equilibrate for 45min. Of this mixture, 100ul was applied in duplicate to columns of Sephadex LH-20 (bottom-layer) and Cibacron Blue F3G-A-Sepharose 6B (top-layer, affinity gel for albumin), which were formed in disposable 5ml plastic pipette tips (LIP services). The columns were washed with 2.8ml of tris buffer and the radioactivity in the eluates (containing the SHBG-bound 5αDHT) was estimated by liquid scintillation counting. The SHBG BC (nmol/l) was calculated as (cpm (sample eluate) - cpm (HSA eluate)) X 689/total cpm added. Samples with binding capacities of less than 10nmol/l were re-assayed using 5ng (rather than 20ng) of 5αDHT which gave an assay sensitivity of 0.7 nmol/l. For serum quality control pools having mean SHBG binding capacities of 33 and 71 nmol/l, the within-assay c.v. were found to be 2.8% and 2.7% (n=11) and between-assay c.v. were 3.1% and 2.9% (n=25)

respectively.

Some assays were repeated after treatment of samples with charcoal (Norit SX plus, 0.2% w/v at 37C for 30 min) to remove potentially interfering drug metabolites.

The effect of the drugs and the metabolites of danazol on SHBG BC

In order to determine the extent to which gestrinone (Roussel Laboratories, Uxbridge, Middlesex, UK), danazol, 2OHME (Sterling Winthrop, Guildford Surrey, UK) or ethisterone (Sigma, Poole, Dorset, UK) interfered with the measurement of BC, a pool of normal female serum was assayed with and without the addition of:

gestrinone	3×10^{-7} mol/l
danazol	3×10^{-6} mol/l
ethisterone	3×10^{-7} mol/l and 10^{-6} mol/l
2OHME	10^{-5} mol/l,

all in triplicate. These concentrations are approximately three times the upper limit of their respective therapeutic ranges. The effect of the metabolites of gestrinone were not investigated, as on structural considerations, there are no known metabolites of this steroid that would be expected to have a higher affinity for SHBG than gestrinone itself (information from Roussel Laboratories).

ANDROGEN ASSAYS

It has been recognised that danazol and its major metabolites, which are present in significant amounts in

serum, may interfere with steroid RIAs (Creange and Potts, 1974). Further, as these are lipophilic, they will be retained after solvent extraction procedures (Dowsett, 1985). Hence a thin-layer chromatographic system (benzene/ethyl acetate/methanol 80/20/2 on silica gel, Merck, Darmstadt, West Gemany) was developed to separate T from danazol, E and 2OHME, as well as from A4 and DHT (Forbes et al, 1986). Two markers, 11-deoxycorticosterone and a purple dye (1,4 diaminoanthraquinone), were used to identify the position of the separated steroids. Initially, this purification step was used in conjunction with an antiserum raised against T-3-CMO-BSA (WHO, Chelsea Hospital for Women, London) which gave cross reactions: danazol 0.02%, E 0.07% and 2OHME 0.01%. However, on considering possible cross reactions from other unidentified metabolites, the antiserum was changed to one raised to a T-7-CMO-BSA conjugate (Miles Laboratories, Slough), which has a cross reaction with danazol of only 0.001% (Dowsett, 1985). The other androgen antisera used were raised to A-7-carboethythio-ether ovalbumin (HP/S/665/A, Guildhay, Guildford) and 5α -DHT-3-CMO-BSA (Chelsea Hospital for Women). The results were corrected according to the recovery of ^{3}H-T, ^{3}H-A4 and ^{3}H-DHT, about 1000cts/min of each having been added before extraction of 1ml of serum. The within (n=10) assay and between (n=17) assay c.v.s were for T 5.6% and 11.7%, for A4 8.9% and 14.7% and for DHT 10.1 and 12.1%, respectively.

DEHYDROEPIANDROSTERONE SULPHATE

DHEAS was assayed without extraction on samples diluted 100-fold using an antiserum that cross-reacted 100% with DHEA. This enabled the use of (1,2,6,7,-^{3}H) dehydro epiandrosterone as tracer ligand. The antiserum was kindly donated by Dr B.T. Rudd (Birmingham and Midland Hospital for Women, Birmingham, England). Significant cross reactions were 17% for epiandosterone sulphate and 3% for androsterone sulphate. The circulating levels of these steroids were much lower than DHEAS, and this reduced their potential contribution to less than 1%. The within- and between assay c.v.s were 5.0% and 11.4%, respectively, at a sample concentration of 620 ng/ml. The sensitivity limit was 20 ng/ml (Harris et al, 1982).

% FREE TESTOSTERONE

A number of laboratories have described formulae relating SHBG to % free T, that for the Chelsea laboratory based on real data in patients being:

log(% free T) = 0.44log8.52 + logSHBG concentration. (Dowsett et al, 1985)

However, free steroid concentrations in serum are small as the majority of the circulating hormones are bound to SHBG and albumin. Also, free levels may vary between individuals and be affected by danazol, causing displacement of T from SHBG. More importantly, this calculation does not take account of displacement by danazol or gestrinone. Hence, it was considered preferable to measure the free fraction of T

directly.

Percent free T was measured by the centrifugal ultrafiltration method of Hammond et al (1980), with the modifications recommended by Dowsett et al (1984). In this method, % free steroid can be estimated in undiluted serum, by incubation of the ^{3}H-steroid, (in this case T), with ^{14}C glucose, which is subjected to centrifugal ultrafiltration through a dialysis membrane. The % of free T can be estimated by comparing the ratio of ^{3}H-T to ^{14}C glucose in the ultrafiltrate with the corresponding ratio in the serum retained by the dialysis membrane. This technique is reproducible and approximates to the situation in vivo.

The (1,2,6,7,-^{3}H)T (107Ci/mmol) and D-(U-^{14}C)glucose (249mCi/mmol) were obtained from Amersham International. The steroid isotopes were freshly purified on columns of Lipidex 5000 (chloroform/hexane/methanol, 5/95/0.5) prior to each assay. Filtration cells were made from 30mm long glass tubing, 9mm in diameter, covered at one end with Visking dialysis membrane, which was stretched across and held in place with a rubber band. This membrane had been first washed twice with 95% ethanol, once with 3% $NaHCO_3$ 0.1% EDTA and twice with distilled water. The cells were placed into Packard polyethylene 6ml counting vials containing 1 X 1cm diameter glass fibre discs (Whatman). The use of glass fibre rather than filter paper discs, and polyethylene rather than polypropylene vials, were the modifications made by Dowsett et al (1984) to the original method of Hammond et al (1980). The changes were made on the basis that these materials

reduced absorption of (^{3}H)-T from the protein-free solution and eliminated the discrepancy between observed and expected results for % free T in infiltrates.

A 450ul aliquot of serum was incubated with 100nCi of (^{3}H)T and 5nCi (^{14}C)glucose for 30mins at 37C. From each sample, two 200ul portions were then transferred to the filtration cells, which were placed immediately into the polyethylene counting vials in a prewarmed (37°C) centrifuge. Duplicate plasma samples and two quality control (QC) samples for each assay were then centrifuged at 3000g for one hour. The filtration cells were then removed from the counting vials and 30ul of the retained fluid placed in a second counting vial containing the glass fibre disc. Four counting vials, each with a glass fibre disc had 5nCi ^{14}C glucose added to act as a crossover control during each dual channel count. 350ul of water and 4ml of scintillation cocktail were added to the dialysate, the aliquot of retained fluid and the crossovers. The vials were vigorously mixed to ensure the breakdown of the glass fibre discs, thereby minimising quenching of any radioactive material retained by the disc.

The vials were counted for ^{3}H and ^{14}C (10mins per vial), and after correction for crossover of ^{14}C into the ^{3}H channel and background counts, the % free T was calculated as:

$$\frac{^{3}\text{Hcpm(filtered)}}{^{14}\text{Ccpm(filtered)}} \div \frac{^{3}\text{Hcpm(retained)}}{^{14}\text{Ccpm(retained)}} \times 100$$

The within-assay c.v. (n=10) was 8.1% at a % free T of 1.12.

The effect of the drugs and the metabolites of danazol on % free T in vitro

The effect of gestrinone, danazol, ethisterone and 2OHME on % free T was estimated in duplicate at a range of concentrations, either side of the therapeutic ranges, in a pool of normal female serum before and after it had been heated to 60C for 30min to reduce SHBG binding (Westphal, 1971). The relationship between SHBG BC and % free T was determined by similar heat-treatment of a pool of female serum with an initial SHBG BC of 126nmol/l and mixing portions of the heated and unheated pools to give samples with varying levels of SHBG BC but which were otherwise essentially identical. Percent free T was measured in duplicate portions of these samples.

CONCENTRATION OF FREE T

This was calculated from the following equation:

$$\text{conc free T} = \frac{\text{\% free T X conc total T}}{100}$$

STATISTICAL METHODS

Shapiro-Wilk's Normality Test was applied to all the endocrine results to examine the distribution of the data. As the data from the measurement of LH, prolactin and E2 exhibited moderate and T mild departure from normality, a logarithmic transformation was performed on the data on these four hormones. The Shapiro-Wilk's normality Test was then re-applied to the geometric data and normal distribution of the transformed data was verified prior to further analysis. All other data was compared using untransformed data.

Statistical evaluation of the data was carried out using the SAS statistical package running on the Amdahl computer at the University of London Computer Centre. The GLM (General Linear Model) procedure was used to perform a repeated measures analysis of variance on serum levels. A between group factor (drug treatment) and within groups factor (sampling time) were included in the model to assess the effect of gestrinone with danazol on blood levels over time. Student's t test was used for individual time comparisons.

In the figures, error bars are not shown since these are not inferential in the presentations made.

CHAPTER 9

RESULTS

Serum samples for LH, FSH, prolactin, oestradiol and SHBG were analysed pre-treatment, at each month during treatment and one month post-treatment. Androgen assays were peformed pre- and post-treatment, but only at months 1, 2, and 6 during treatment. This was because preliminary analysis of samples failed to show any further change in androgen levels after month 2, as had previously been reported from our laboratory (Forbes et al, 1986).

The mean values for each hormone (with normally distributed results) during drug treatment are shown in Table 3.1 (gestrinone) and Table 3.2 (danazol) and for those requiring logarithmic transformation, the geometric means are shown in Table 3.3 and 3.4 for gestrinone and danazol respectively. These are all graphically illustrated in Figures 3.1-3.10 and 3.14). The individual results from each patient are shown in the Appendix, Tables A.1-A.22.

Although an attempt was made to ensure that blood samples were taken in the third week of the pretreatment cycle and four weekly thereafter, this was not always possible. Samples from patients that withdrew from the trial were only included in the analysis up until the time at which they ceased taking the medication. The patients withdrawn from the trial are described in the clinical results section, chapter 5. Also omitted from the analysis

TABLE 3.1: SUMMARY OF THE EFFECTS OF GESTRINONE ON MEAN ±SEM SERUM CONCENTRATION OF HORMONES (NORMAL DISTRIBUTION)

	TIME ON TREATMENT							
HORMONE	PRE	M1	M2	M3	M4	M5	M6	M7
FSH								
MEAN	4.9	4.7	5.3	4.8	4.8	5.8	4.5	7.2
±SEM	0.80	0.46	0.41	0.59	0.55	0.77	0.54	1.2
SHBG								
MEAN	53	9.0	5.8	5.2	5.7	5.2	4.6	30
±SEM	5.1	1.3	0.69	0.65	0.65	0.61	0.92	3.4
A4								
MEAN	3.3	2.7	2.4	-	-	-	2.7	2.8
±SEM	0.28	0.27	0.21	-	-	-	0.32	0.44
DHT								
MEAN	0.39	0.23	0.21	-	-	-	0.27	0.44
±SEM	0.03	0.02	0.02	-	-	-	0.04	0.06
DHAS								
MEAN	9.1	9.1	9.4	-	-	-	9.2	7.4
±SEM	1.1	0.96	1.1	-	-	-	1.7	1.3
% FREE T								
MEAN	1.85	4.29	4.36	-	-	-	4.59	1.90
±SEM	0.25	0.37	0.27	-	-	-	0.44	0.26
CONC FREE T								
MEAN	0.019	0.030	0.030	-	-	-	0.029	0.018
±SEM	0.003	0.006	0.005	-	-	-	0.006	0.003

* See TABLE 3.5 for key to units

TABLE 3.2: SUMMARY OF THE EFFECTS OF DANAZOL ON MEAN ±SEM SERUM CONCENTRATION OF HORMONES (NORMAL DISTRIBUTION)

	TIME ON TREATMENT							
HORMONE	PRE	M1	M2	M3	M4	M5	M6	M7
FSH								
MEAN	3.8	5.2	4.9	4.6	4.2	5.2	5.4	4.7
±SEM	0.52	0.38	0.37	0.38	0.44	0.38	0.50	0.79
SHBG								
MEAN	61	8.5	4.6	4.5	4.4	4.3	2.9	30
±SEM	5.4	2.3	0.49	0.45	0.60	0.54	0.60	5.2
A4								
MEAN	3.5	2.4	2.1	-	-	-	2.6	2.9
±SEM	0.39	0.20	0.19	-	-	-	0.25	0.40
DHT								
MEAN	0.40	0.18	0.17	-	-	-	0.17	0.24
±SEM	0.04	0.02	0.03	-	-	-	0.03	0.02
DHAS								
MEAN	6.8	7.9	7.5	-	-	-	9.4	6.8
±SEM	0.68	0.72	0.64	-	-	-	2.7	0.83
% FREE T								
MEAN	1.64	5.22	5.40	-	-	-	4.59	1.80
±SEM	0.17	0.38	0.37	-	-	-	0.74	0.18
CONC FREE T								
MEAN	0.015	0.023	0.021	-	-	-	0.023	0.012
±SEM	0.002	0.003	0.002	-	-	-	0.005	0.002

* See Table 3.5 for key to units

TABLE 3.3: SUMMARY OF THE EFFECTS OF GESTRINONE ON GEOMETRIC MEAN SERUM CONCENTRATION OF HORMONES (WITH UPPER AND LOWER 95% CONFIDENCE INTERVALS)

	TIME ON TREATMENT							
HORMONE	PRE	M1	M2	M3	M4	M5	M6	M7
LH								
GM	7.3	8.2	6.4	6.3	6.5	7.8	6.3	10.7
UCI	9.2	10.5	8.2	8.2	8.5	10.3	9.1	14.4
LCI	5.8	6.5	4.1	4.9	5.0	5.9	4.3	8.0
PRL								
GM	289	245	212	210	238	225	206	276
UCI	334	285	247	246	279	267	259	332
LCI	250	212	182	179	203	190	165	229
E2								
GM	347	269	215	265	251	283	261	360
UCI	435	337	268	335	317	362	358	477
LCI	277	214	172	210	199	221	190	272
TOTAL T								
GM	1.04	0.55	0.52	-	-	-	0.52	0.78
UCI	1.21	0.64	0.61	-	-	-	0.66	0.94
LCI	0.90	0.47	0.44	-	-	-	0.42	0.65

GM = GEOMETRIC MEAN
UCI AND LCI = UPPER AND LOWER 95% CONFIDENCE INTERVALS

*See Table 3.5 for key to units

TABLE 3.4: SUMMARY OF THE EFFECTS OF DANAZOL ON GEOMETRIC MEAN SERUM CONCENTRATIONS OF HORMONES (WITH UPPER AND LOWER 95% CONFIDENCE INTERVALS)

	TIME ON TREATMENT							
HORMONE	PRE	M1	M2	M3	M4	M5	M6	M7
LH								
GM	8.0	8.1	7.3	6.9	5.5	6.3	6.5	7.4
UCI	10.0	10.4	9.5	8.9	7.2	8.2	9.6	10.0
LCI	6.3	6.4	5.6	5.3	4.2	4.8	4.4	5.5
PRL								
GM	300	242	214	240	209	189	197	260
UCI	346	280	251	281	246	222	250	311
LCI	260	209	183	205	177	161	155	217
E2								
GM	427	194	216	243	219	200	246	356
UCI	527	241	277	307	280	254	365	464
LCI	346	156	168	193	172	158	166	273
TOTAL T								
GM	0.98	0.39	0.39	-	-	-	0.44	0.73
UCI	1.14	0.45	0.46	-	-	-	0.58	0.89
LCI	0.84	0.33	0.33	-	-	-	0.34	0.60

GM = GEOMETRIC MEAN
UCI AND LCI = UPPER AND LOWER 95% CONFIDENCE INTERVALS

*See Table 3.5 for key to units

TABLE 3.5: KEY TO UNITS AND NORMAL RANGES FOR HORMONE VALUES SHOWN IN TABLES 3.1 - 3.4

HORMONE	UNIT	NORMAL RANGE
LH	IU/L	3.0 - 11
FSH	IU/L	0.5 - 5.0
PRL	mIU/L	100 - 620
E2	pmol/L	100 - 1400
SHBG	nmol/L	40 - 90
TOTAL T	nmol/L	0.7 - 2.7
A4	nmol/L	3.1 - 10.1
DHT	nmol/L	0.2 - 1.0
DHAS	µmol/L	3.0 - 13
CONC FREE T	nmol/L	Not available

were any month 6 samples taken after completion of the drugs, which involved 5 gestrinone and 2 danazol samples.

In the repeated measures analysis of variance, there was no statistically significant difference between the drugs in their effects on any of the hormones examined. For the hormones in which there was no drug/time difference (i.e. a significant difference between the effects of the drugs at any individual time point), the effect of time was considered for the results from the two drugs together. However for the hormones in which a significant drug/time difference occurred, the effects of each drug with time were considered separately.

All mean values mentioned in the text are described together with their respective SEMs. All geometric means are described with upper and lower 95% confidence intervals.

SERUM LUTEINIZING HORMONE (Figure 3.1, Table - Appendix A.1, A.2)

No significant change from pre-treatment LH levels was seen at any month during gestrinone or danazol treatment. There was also no significant drug/time difference between the effects of the 2 drugs.

SERUM FOLLICLE STIMULATING HORMONE (Figure 3.2, Tables - Appendix A.3, A.4)

There was no significant change in FSH levels during treatment with gestrinone or danazol at any time.

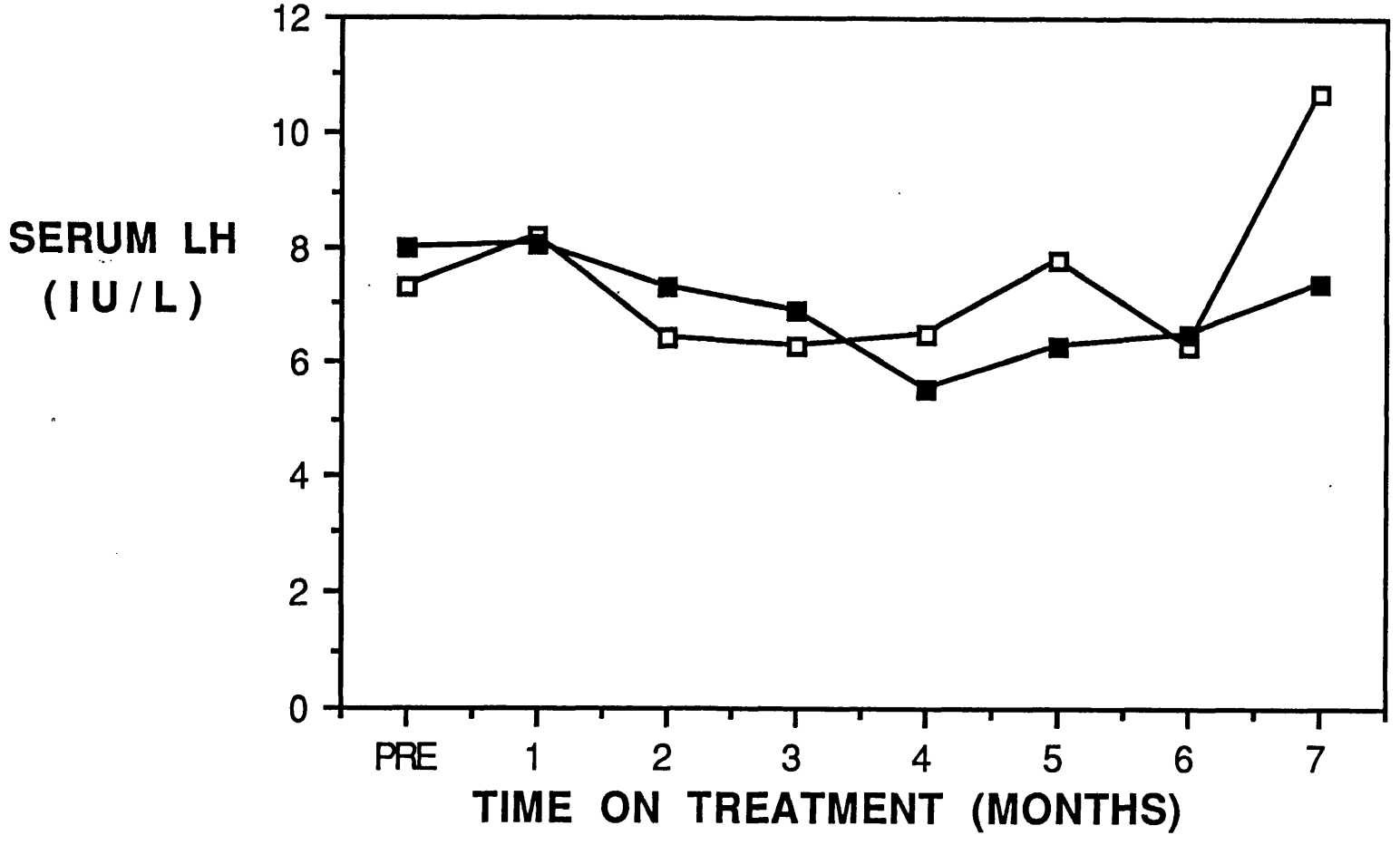


FIGURE 3.1: The effect of Gestrinone □ and Danazol ■ on geometric mean serum concentration of LH
(normal range: 3 - 11IU/L)

NB: Month 7 is post-treatment

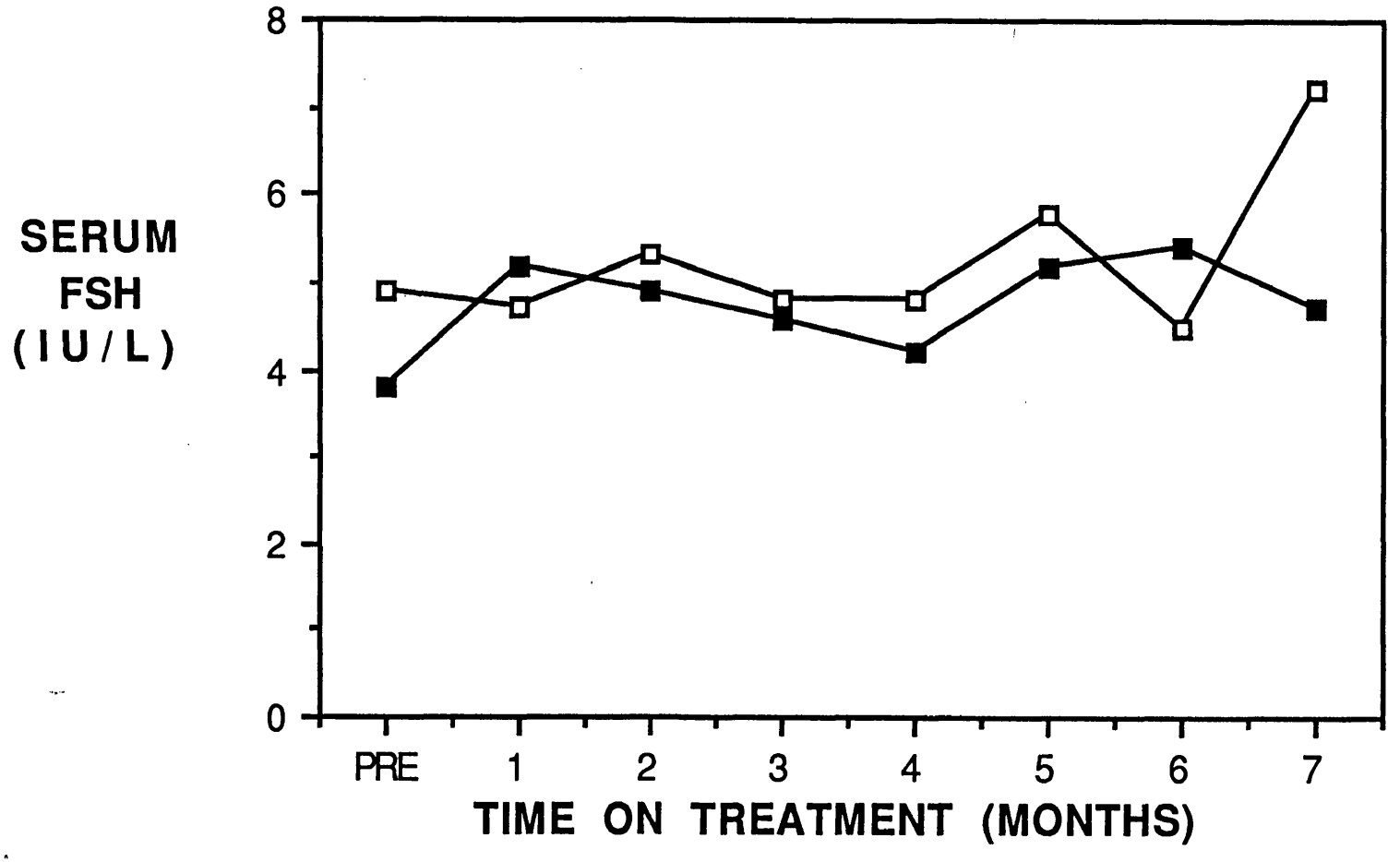


FIGURE 3.2: The effect of Gestrinone □ and Danazol ■ on mean serum concentration of FSH
(normal range: 0.5 - 5IU/L)

NB: Month 7 is post-treatment

SERUM PROLACTIN (Figure 3.3, Tables - Appendix A.5, A.6)

There was a significant fall in serum prolactin levels at all times during treatment with both gestrinone and danazol (p=0.0001). However, there was no significant difference in the effect of the two drugs (p=0.52). The pretreatment geometric mean value of 289 (95% C.I. 334 -250) mIU/L fell to the lowest level of 206 (95% C.I. 259-165) mIU/L at month 6 in gestrinone treated patients, and from 300 (95% C.I. 346-260) mIU/L to the lowest level of 189 (95% C.I. 222-161) mIU/L at month 5 in the danazol treated patients. One month post-treatment the levels had returned to the pre-treatment range, 276 (95% C.I. 332-229) mIU/L and 260 (95% C.I. 311-217) mIU/L in the gestrinone and danazol groups respectively.

SERUM OESTRADIOL (Figure 3.4, Tables - Appendix A.7, A.8)

Serum oestradiol levels fell from a geometric mean of 347 (95% C.I. 435-277) pmol/L to a minimum level of 215 (95% C.I. (268-172) pmol/L at month 2 in gestrinone treated patients, and from 427 (95% C.I. 527-346) pmol/L to the lowest mean value of 194 (95% C.I. 241-156) pmol/L at month 1 in the danazol group. At all times during treatment there was a significant decrease in E2 levels, (p=0.0001), but no difference in the treatment effects. The geometric mean E2 value returned to a level similar to that before treatment by one month after the completion of therapy, 360 (95% C.I 477-272) pmol/L and 356 (95% C.I. 464-273) pmol/L, for gestrinone and danazol respectively. Although the analysis

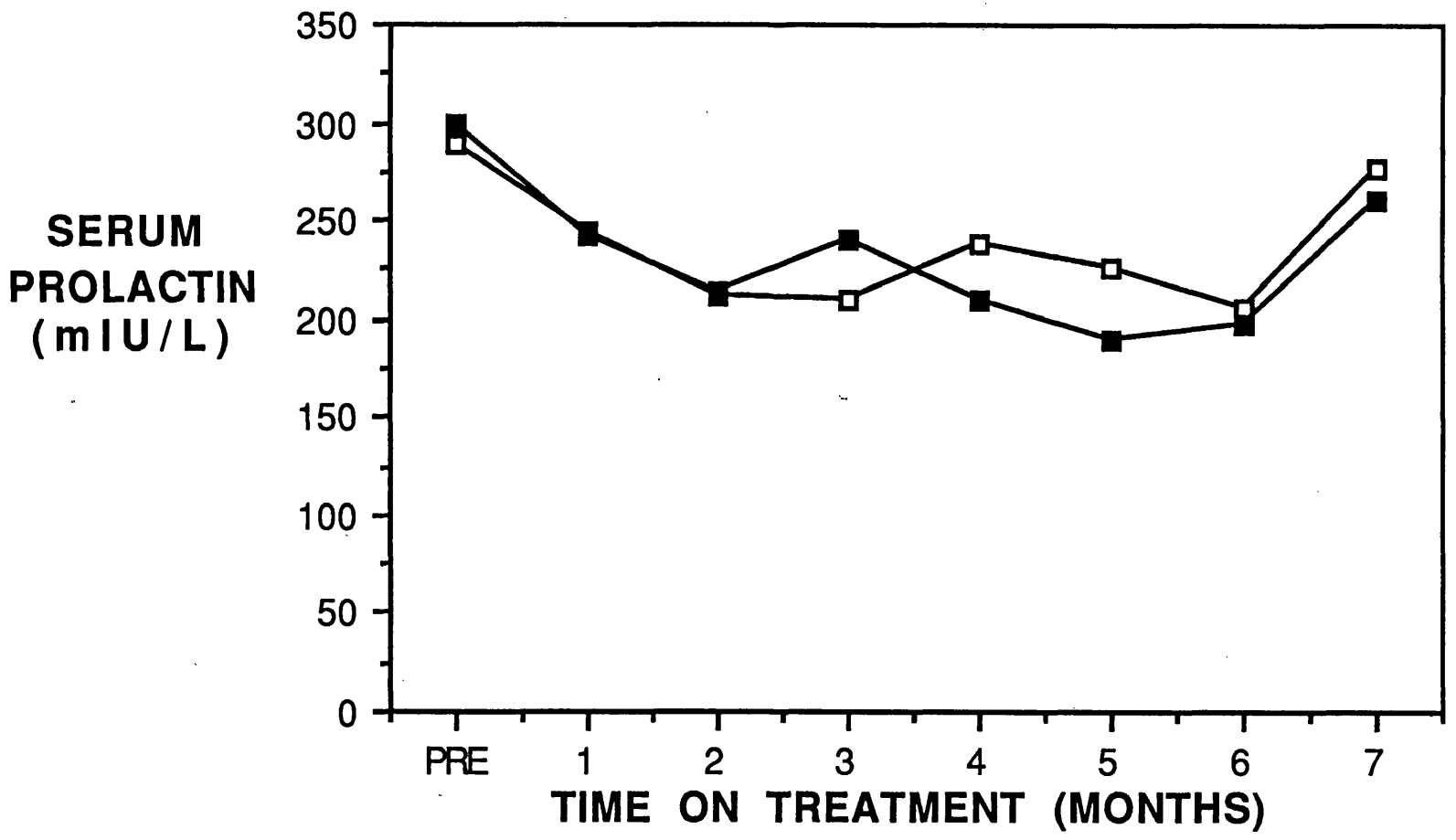


FIGURE 3.3: The effect of Gestrinone □ and Danazol ■ on geometric mean serum concentration of Prolactin (normal range: 100 - 620mIU/L)

NB: Month 7 is post-treatment

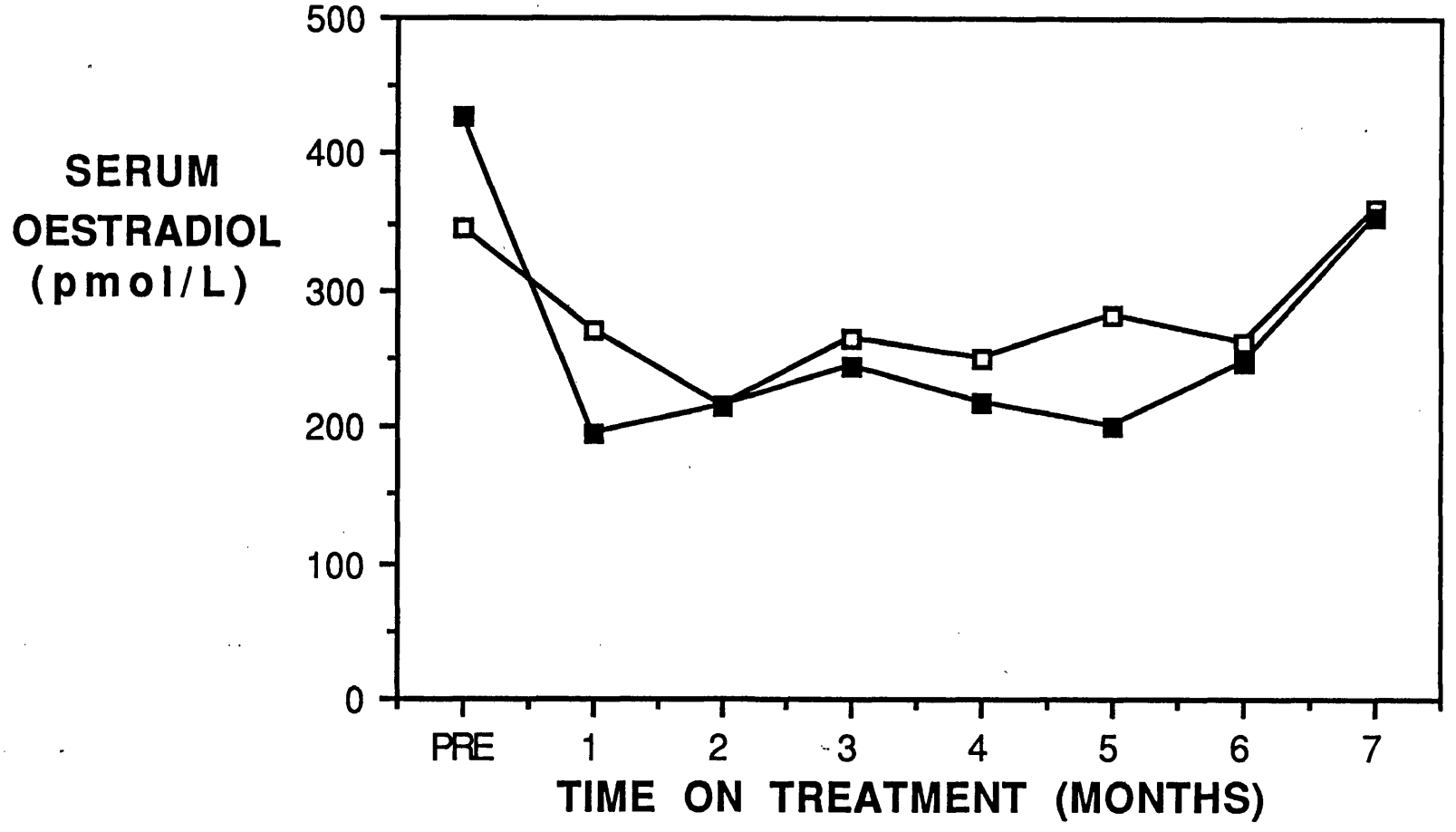


FIGURE 3.4: The effect of Gestrinone □ and Danazol ■ on geometric mean serum concentration of Oestradiol (normal range: 100 - 1400pmol/L)

NB: Month 7 is post-treatment

did not show any drug/time difference between gestrinone and danazol, both the individual means and the p values suggest a more consistent suppression of E2 concentration with danazol and a greater variability in levels during gestrinone treatment. Despite the decrease in E2 compared with those in the pre-treatment cycle, menopausal levels (<90 pmol/L) were rarely seen, indicating that some ovarian E2 production occurred despite the inhibition of ovulation.

PROGESTERONE

Pre-treatment progesterone levels indicated ovulation in 80% of gestrinone and 72% of danazol treated patients. However, the remaining anovulatory levels may have resulted from samples not being taken in the mid-luteal phase of the cycle. In all patients treated with gestrinone and danazol, serum P levels were anovulatory at month 1. They were not therefore assayed again during treatment. The post-treatment concentration of P (i.e. 3 weeks after stopping medication) confirmed ovulation in only 2 patients in the gestrinone group and 4 in the danazol group.

SEX HORMONE BINDING GLOBULIN (Figure 3.5, Tables - Appendix A.9, A.10)

Both drugs caused a highly significant fall in SHBG at month 1, for gestrinone from 52.3 ± 5.1 nmol/L to 9.0 ± 1.2 nmol/L and for danazol from 60.9 ± 5.4 nmol/L to 8.5 ± 1.2 nmol/L, (p=0.0001). At all times during treatment the levels remained significantly suppressed compared with

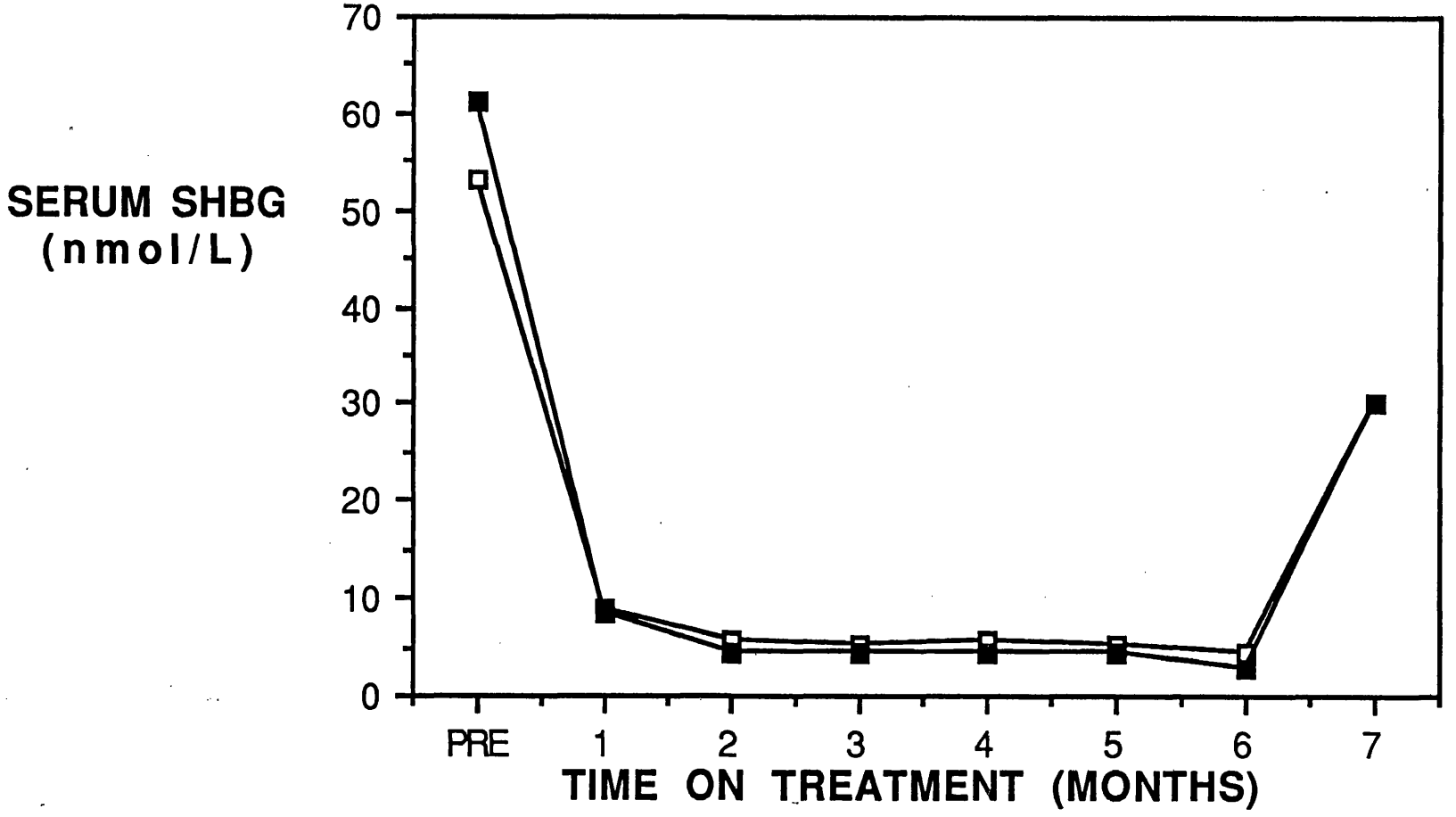


FIGURE 3.5: The effect of Gestrinone □ and Danazol ■ on mean serum concentration of SHBG (normal range: 40 - 90nmol/L)

NB: Month 7 is post-treatment

pre-treatment levels, but there was no further change with time during treatment. One month post treatment, the levels returned to 30.5 ± 3.4 nmol/L for gestrinone and 30.1 ± 5.2 nmol/L for danazol which were both significantly different from the pre-treatment values, (p=0.0001). A drug/time interaction was therefore seen because the pre-treatment levels were significantly different, (p=0.005), but there was no difference between the overall effects of the drugs.

The effect of the drugs added on the measurement of SHBG binding capacity is shown in Table 3.6. Gestrinone (3 X 10^{-7} mol/L), danazol (3 X 10^{-6} mol/L) and 2OHME (10^{-5} mol/L) had no significant effect, but E at 3 X 10^{-7} mol/L and 10^{-6} mol/L reduced the measured SHBG binding capacity by 11% and 38% respectively. Charcoal treatment had no significant effect on the mean value of SHBG binding capacity in samples taken before treatment or in those patients on gestrinone treatment. However, in samples from danazol treated patients, levels after four weeks treatment were a mean of 35% lower before charcoal treatment.

SERUM TESTOSTERONE (Figure 3.6, Tables - Appendix A.11, A.12)

There was no significant difference between the effect of gestrinone and danazol on total T, but there was a highly significant effect with time, (p=0.0001). The geometric mean T for gestrinone fell from 1.04 (95% C.I. 1.21-0.90) nmol/L pre-treatment to 0.55 (95% C.I. 0.64-0.47) nmol/L and for danazol from 0.98 (95% C.I. 1.14-0.84) nmol/L

TABLE 3.6: THE EFFECT OF DRUGS ON THE MEASUREMENT OF SHBG BINDING CAPACITY IN A POOL OF NORMAL FEMALE SERUM

DRUG	CONCENTRATION	ESTIMATED SHBG BINDING CAPACITY (NMOL/L) MEAN ±SEM
CONTROL	-	73.7 ± 0.6
GESTRINONE	3×10^{-7}	72.0 ± 1.0
DANAZOL	3×10^{-6}	72.1 ± 1.0
2-HYDROXYMETHYL ETHISTERONE	10^{-5}	69.0 ± 2.0
ETHISTERONE	3×10^{-7}	65.8 ± 2.1
ETHISTERONE	10^{-6}	45.9 ± 0.6

(N = 3 in each case)

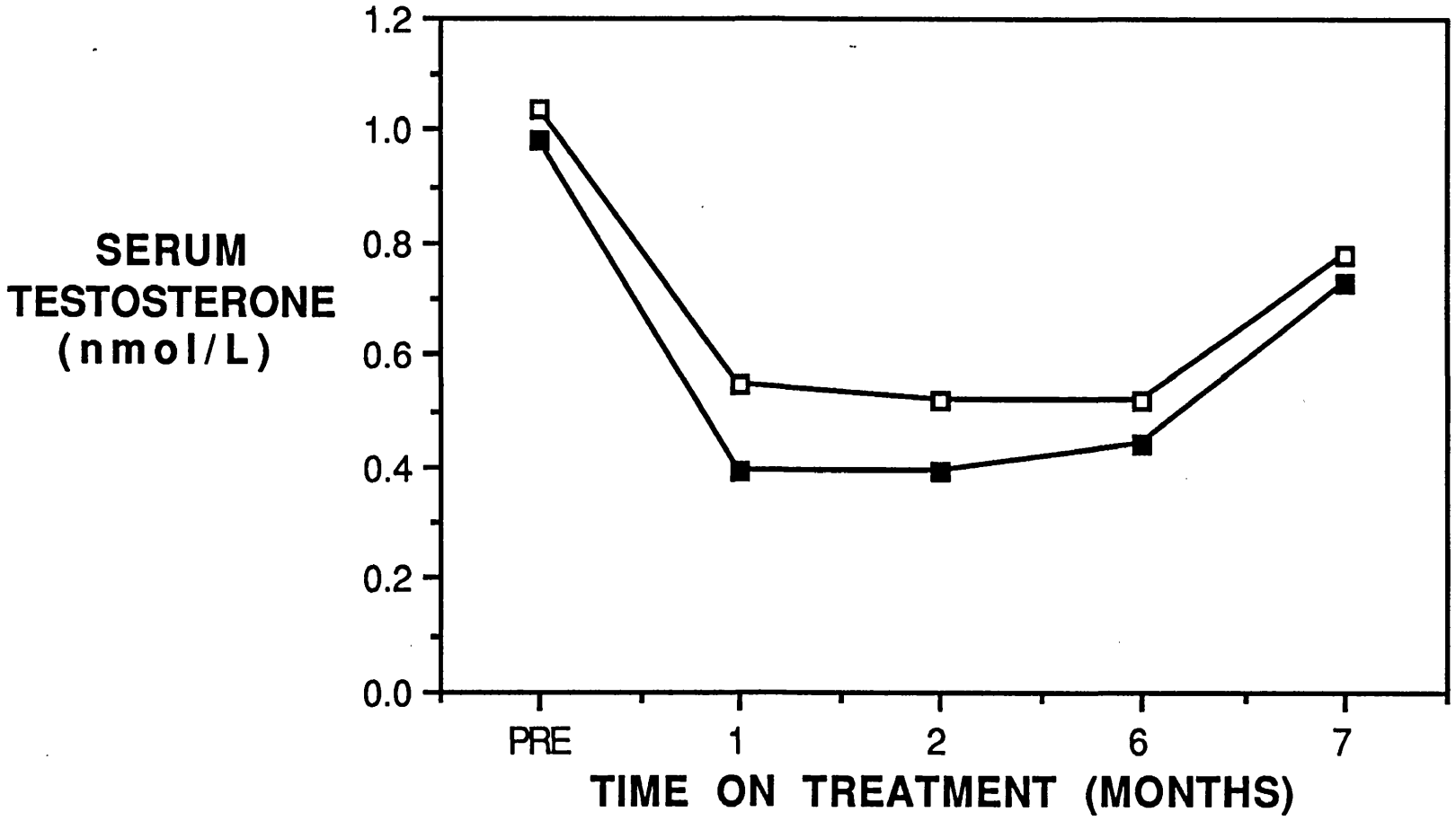


FIGURE 3.6: The effect of Gestrinone □ and Danazol ■ on geometric mean serum concentration of Testosterone (normal range: 0.7 - 2.7nmol/L)

NB: Month 7 is post-treatment

to 0.39 (95% C.I. 0.45-0.33) nmol/L at month 1. There was no further significant change in T levels during treatment, which remained suppressed at month 2 and 6. However the post treatment T increased only to 0.78 (95% C.I. 0.94-0.65) nmol/L and 0.73 (95% C.I. 0.89-0.60) nmol/L for gestrinone and danazol respectively, which were both significantly less than the pre-treatment values (p=0.02).

On examining the individual results for gestrinone and danazol, it is apparent that the mean T is lower for patients treated with danazol than with gestrinone, but the pre-treatment T was also lower for the danazol group and hence the overall effect of the 2 groups was comparable.

SERUM ANDROSTENEDIONE (Figure 3.7, Tables - Appendix A.13, A.14)

There was no significant difference between the effect of gestrinone or danazol on A4. Overall, there was a highly significant fall in A4 from 3.36 ± 0.23 nmol/L pre-treatment to 2.56 ± 0.17 nmol/L at month 1, (p=0.0001). Although A4 levels fell again at month 2 to 2.25 ± 0.14 nmol/L, this further decrease was not significant, (p=0.17), and levels remained suppressed at month 6. Post-treatment levels increased to 2.88 ± 0.29 nmol/L which was lower than the pre-treatment value, but this was not a significant difference, (p=0.06).

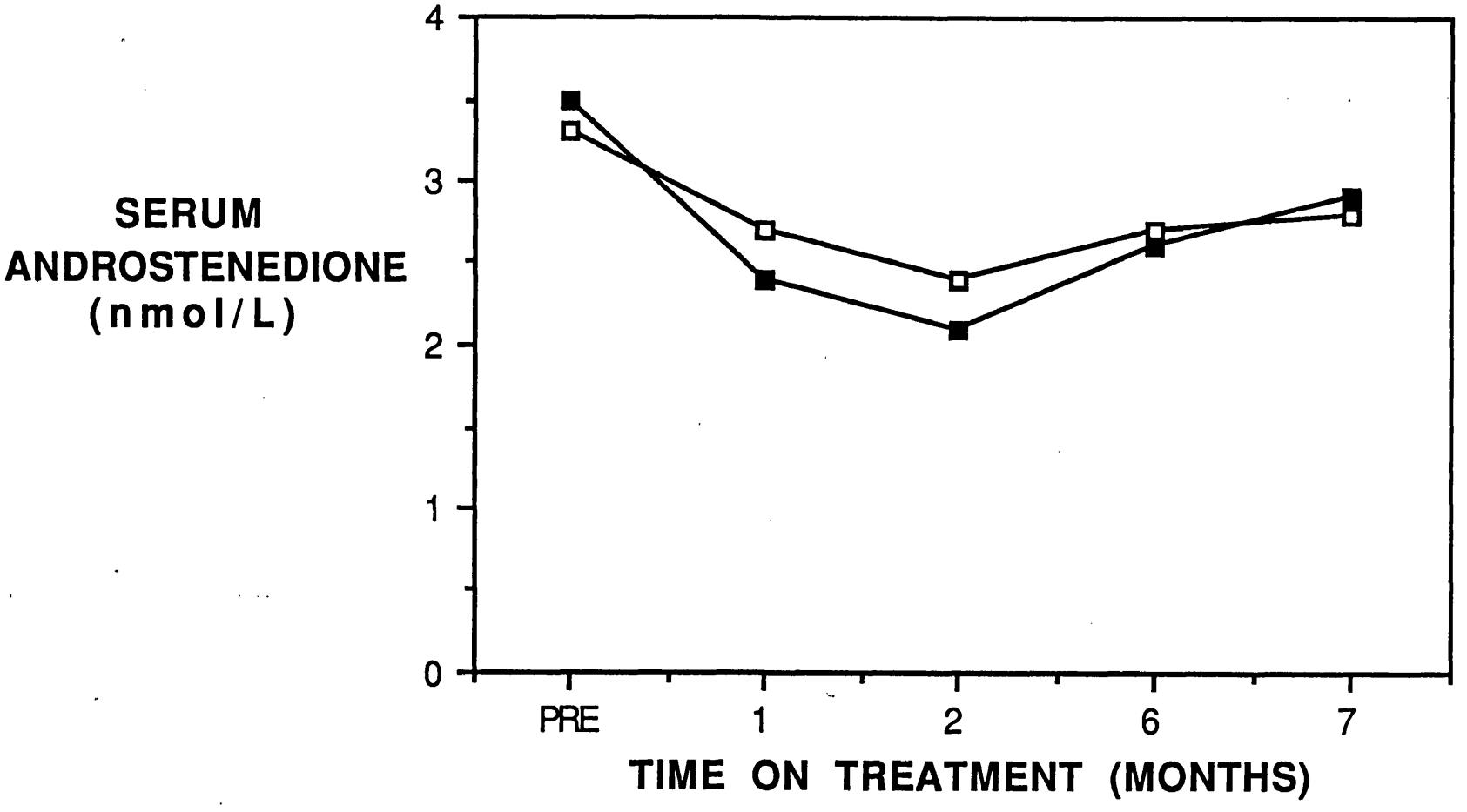


FIGURE 3.7: The effect of Gestrinone □ and Danazol ■ on mean serum concentration of Androstenedione (normal range: 3.1 - 10.1nmol/L)

NB: Month 7 is post-treatment

PLASMA 5αDIHYDROTESTOSTERONE (Figure 3.8, Tables - Appendix A.15, A.16)

Although there was no significant difference in the effect of gestrinone and danazol on DHT, there was a difference in the drug/time effect, (p=0.01). This was due to the difference at month 7. Both drugs caused a significant decrease in DHT at month 1, for gestrinone from 0.39 ± 0.03 nmol/L to 0.23 ±0.02 nmol/L and for danazol from 0.40 ± 0.04 nmol/L to 0.18 ± 0.02 nmol/L, (p=0.0001). The DHT remained suppressed during treatment with no further significant changes occuring in either group. However, the DHT in the gestrinone group returned to 0.44 ± 0.06 nmol/L at month 7, whereas DHT rose only to 0.24 ± 0.02 nmol/L in the danazol group, and although not significantly different from the pre-treatment danazol level, this varied significantly from the gestrinone value at this time, (p=0.0003).

PLASMA DEHYDROEPIANDROSTENDIONE SULPHATE (Figures 3.9, Tables - Appendix A.17, A.18)

The analysis of variance detected no difference in the effect of gestrinone or danazol on plasma DHAS levels, neither of which caused a change in DHAS during treatment.

PERCENT FREE TESTOSTERONE (Figure 3.10, Tables - Appendix A.19, A.20)

Gestrinone and danazol both produced a significant increase in % free T during treatment, (p=0.0001) for both,

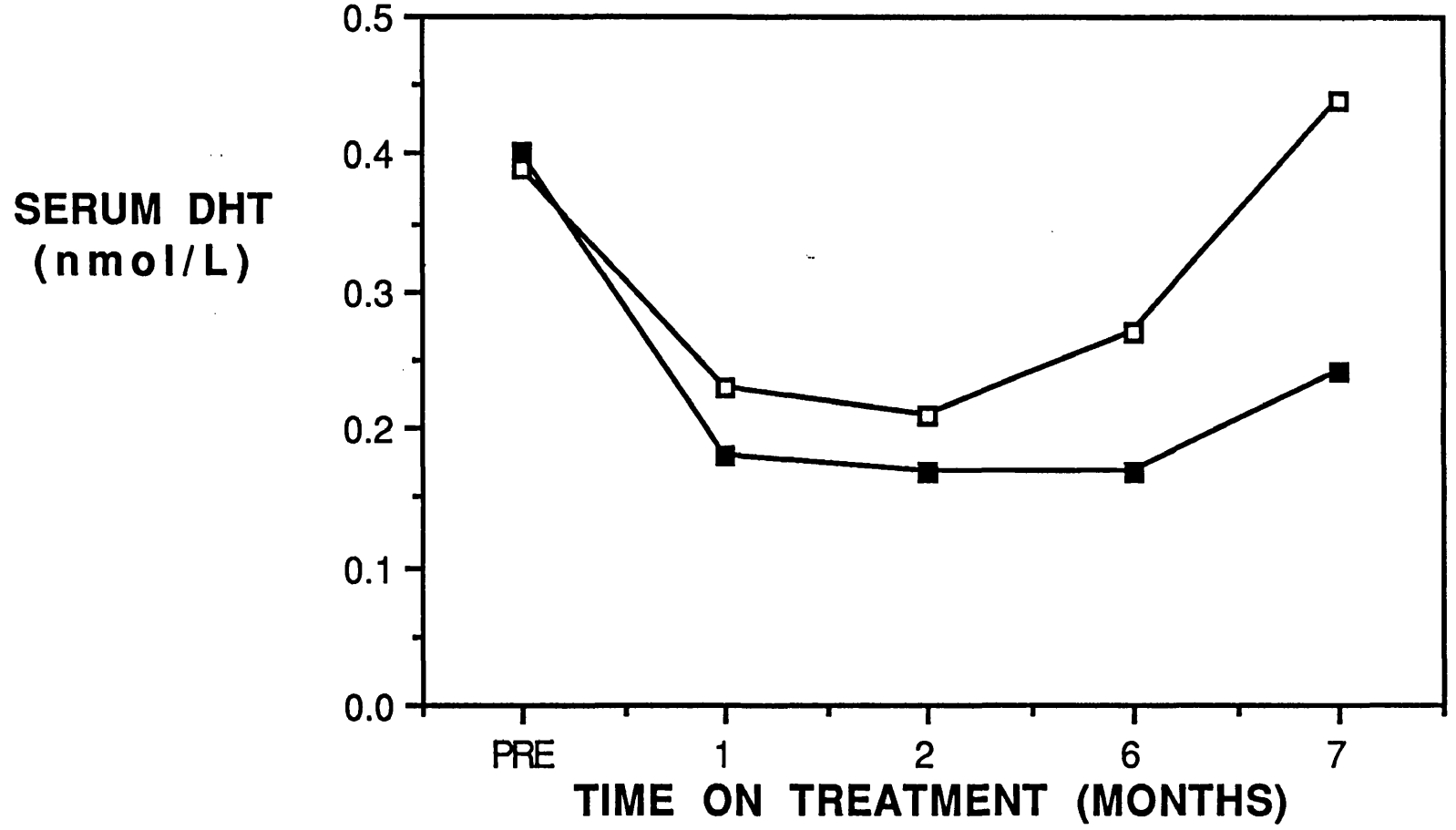


FIGURE 3.8: The effect of Gestrinone □ and Danazol ■ on mean serum concentration of Dihydrotestosterone
(normal range: 0.2 - 1.0nmol/L)

NB: Month 7 is post-treatment

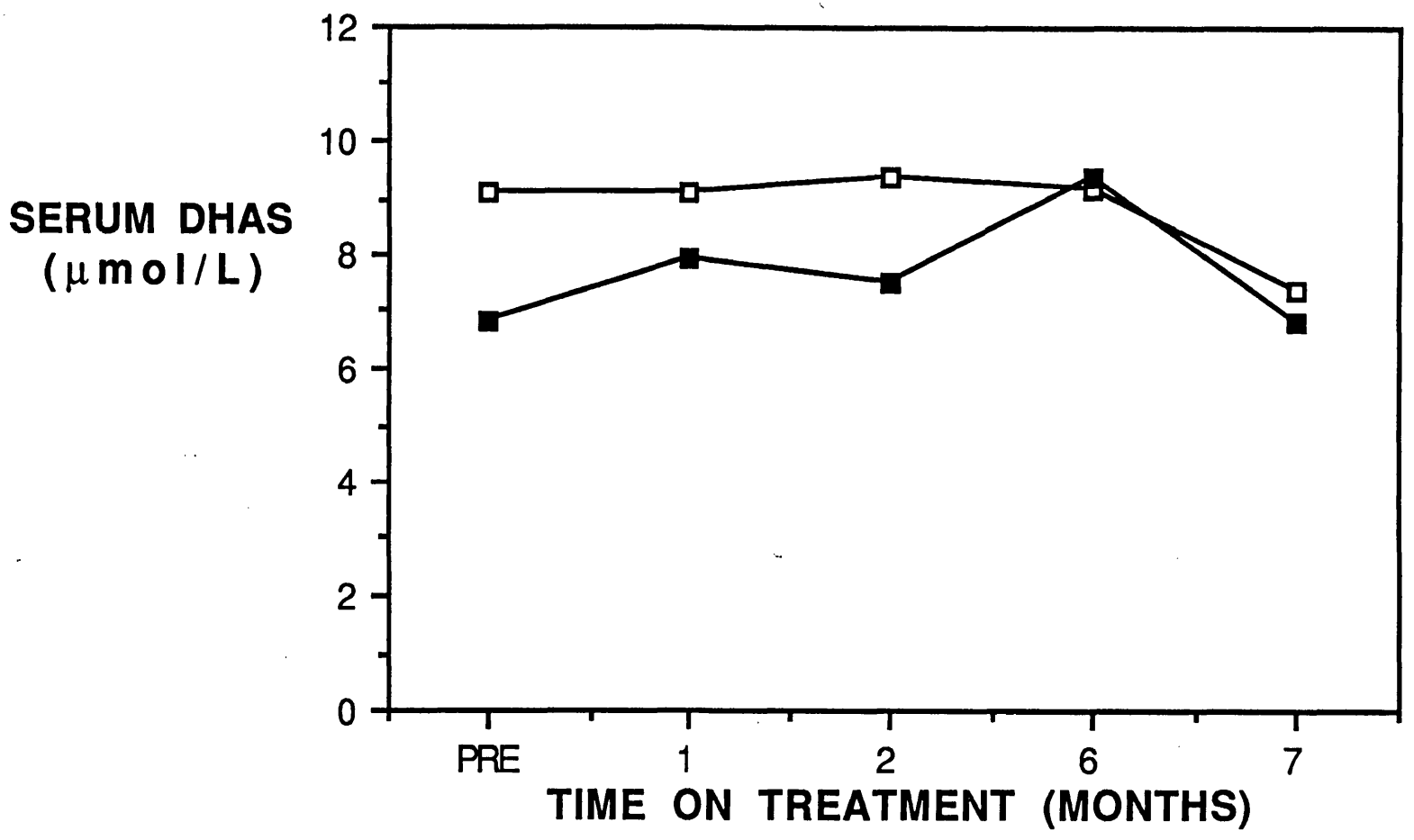


FIGURE 3.9: The effect of Gestrinone □ and Danazol ■ on mean serum concentration of DHAS
(normal range: 3.0 - 13.0 umol/L)

NB: Month 7 is post-treatment

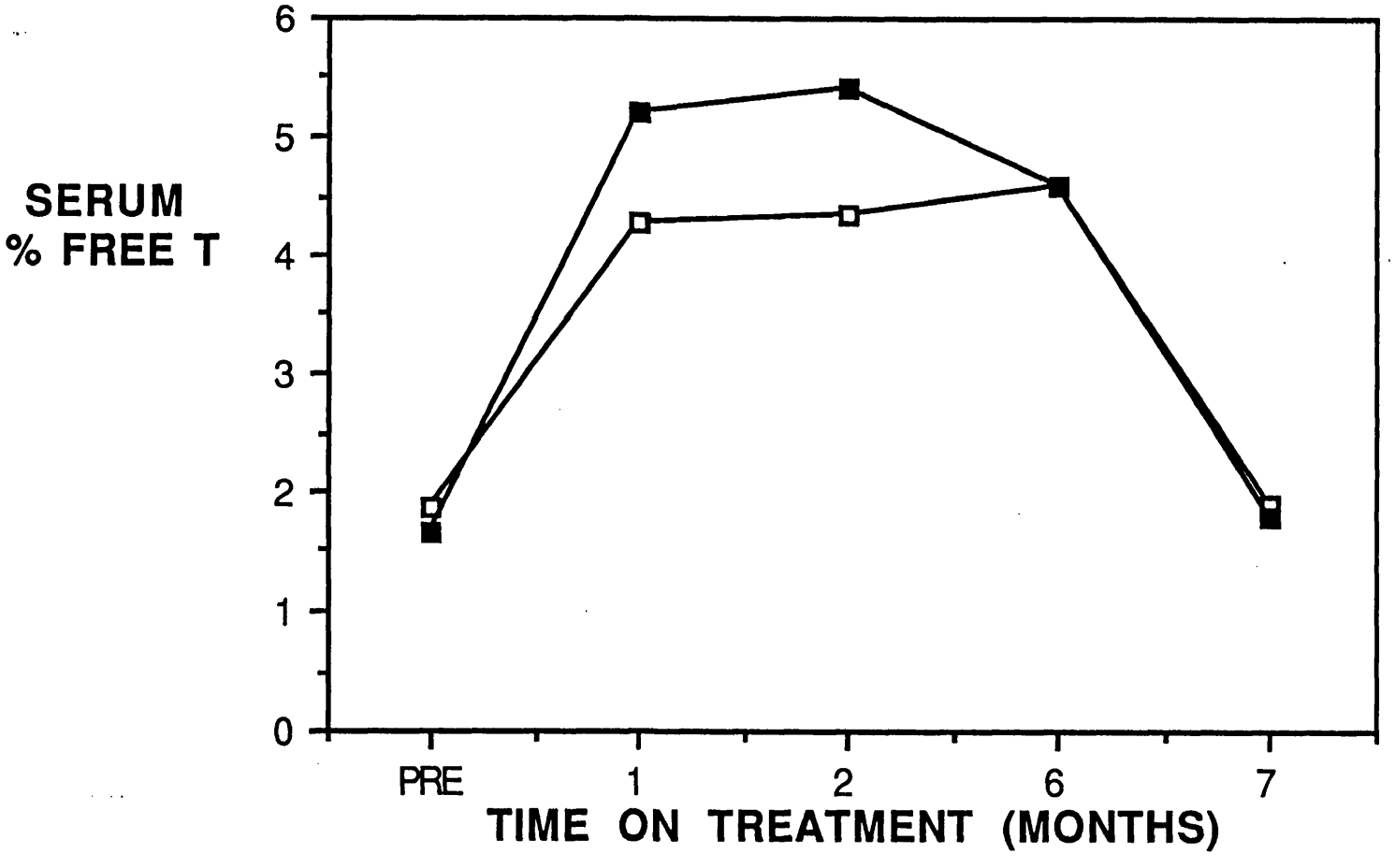


FIGURE 3.10: The effect of Gestrinone □ and Danazol ■ on mean serum concentration of % Free Testosterone

NB: Month 7 is post-treatment

but there was a drug/time difference in their effects. Gestrinone increased % free T from 1.85 ± 0.25 to 4.29 ± 0.37 at month 1 and 4.36 ± 0.27 at month 2. Danazol increased % free T from 1.64 ± 0.17 to 5.22 ± 0.38 at month 1 and 5.40 ± 0.37 at month 2. However, the increase produced by danazol at month 1 was significantly greater than that caused by gestrinone, (p=0.003), and this also was the case at month 2, (p=0.0001). At all other times, there was no significant difference in the % free T levels produced by the drugs, and post-treatment levels were unchanged from pre-treatment, with gestrinone returning to 1.9 ± 0.26 and danazol to 1.8 ± 0.74.

The effect of the drugs on % free T in vitro is shown for the normal serum pool (SHBG binding capacity of 76 nmol/L) in Figure 3.11 and for the same pool after heating (SHBG binding capacity of 12 nmol/L) in figure 3.12. The percent free T in the unheated and heated pools before addition of the drugs was 1.45 ± 0.09 (SD) (n=8) and 4.48 ± 0.66 (n=6), respectively. All four compounds increased % free T in a dose dependent manner in both pools. The order of potency in the unheated pool was E > gestrinone > danazol > 2OHME. In the heated pool the curves for danazol and gestrinone were virtually identical but the order of potency was otherwise similar. At concentrations within the therapeutic range, gestrinone had no significant effect on % free T in either of the pools, whilst both E and 2OHME caused a greater increase than danazol. At concentrations between 3×10^{-7} mol/L and 3×10^{-6} mol/L, E showed a plateau

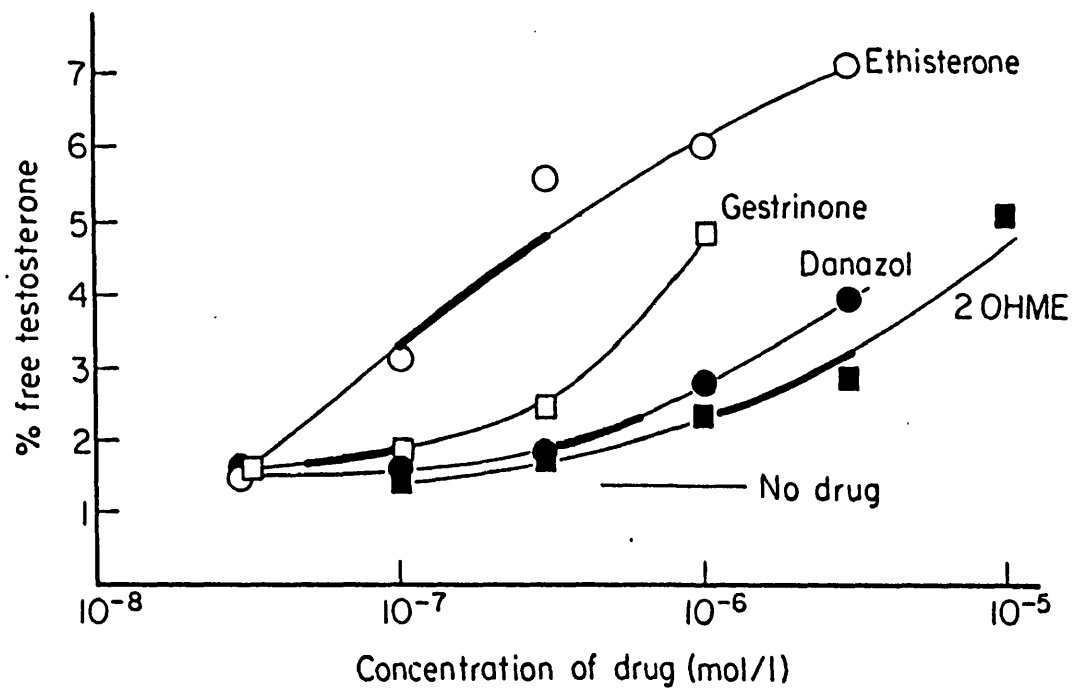


FIGURE 3.11: The effect of drugs on % free Testosterone in a pool of normal female serum (SHBG binding capacity = 76nmol/L). Each point is the mean of two estimates. The bold line represents in each case the therapeutic range of the compounds as determined by the drug manufacturers

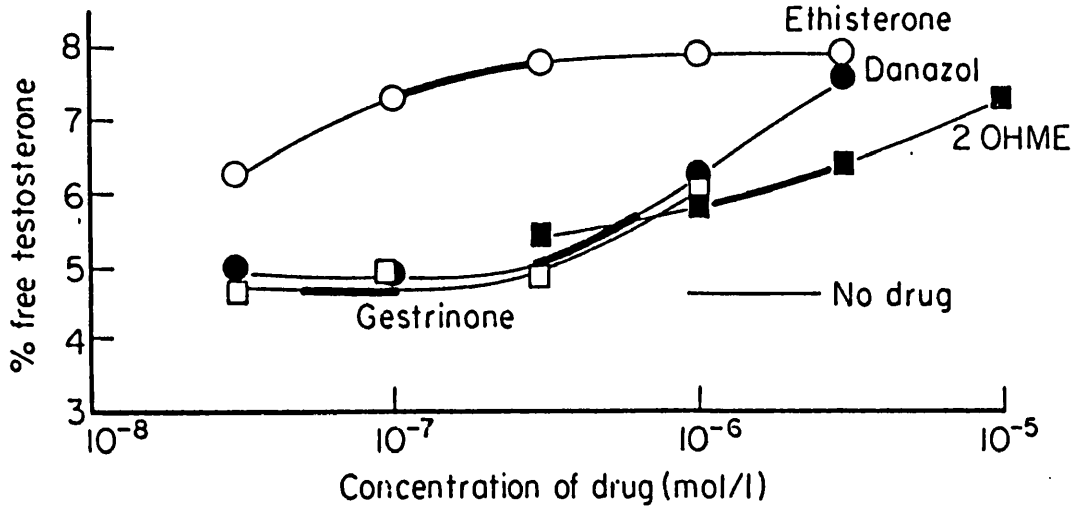


FIGURE 3.12: The effect of drugs on % free Testosterone in a heat-treated pool of normal female serum (SHBG binding capacity = 12nmol/L) in vitro

in the level of % free T.

The relationship between % free T and SHBG binding capacity in samples achieved by mixing portions of a heated and non-heated pool is shown in Figure 3.13. An inverse relationship was found and the curve was fitted by eye.

CONCENTRATION OF FREE TESTOSTERONE (Figure 3.14, Tables - Appendix A.21, A.22)

Concentration of free T was calculated as the product of total T and % free T divided by 100. There was no significant difference between the effect of either drug. The drugs caused an increase in concentration of free T from 0.017 $\pm$ 0.002 nmol/L to 0.026 $\pm$ 0.003 nmol/L after 1 month, (p=0.004). The concentration of free T remained elevated throughout treatment and returned to pre-treatment levels at month 7.

CORRELATIONS BETWEEN CLINICAL AND ENDOCRINE DATA

In order to identify which of the changes in the endocrine parameters may have been related to clinical improvement in endometriosis, linear regression analyses were performed on the changes in the following:

1) endometriosis score and oestradiol

2) endometriosis score and number of bleeding days (including spotting but excluding the first 28 days of treatment)

3) endometriosis score and % free T

4) endometriosis score and concentration of free T

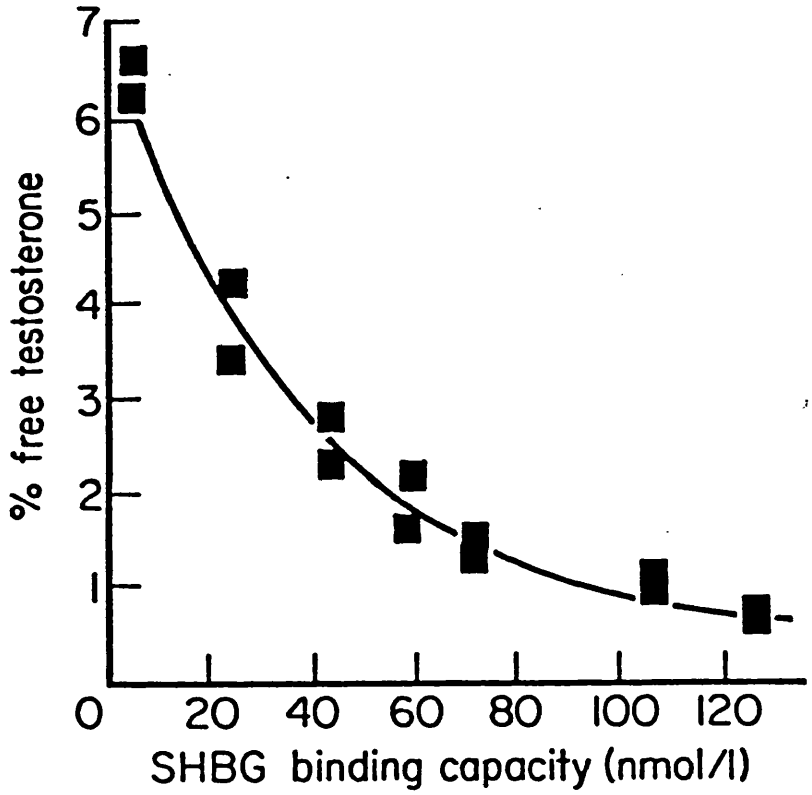


FIGURE 3.13: The relationship between SHBG binding capacity and % free Testosterone. The seven serum pools for analysis were formed by mixing heated (SHBG = 4nmol/L) and unheated (SHBG = 124nmol/L) portions of a single serum pool

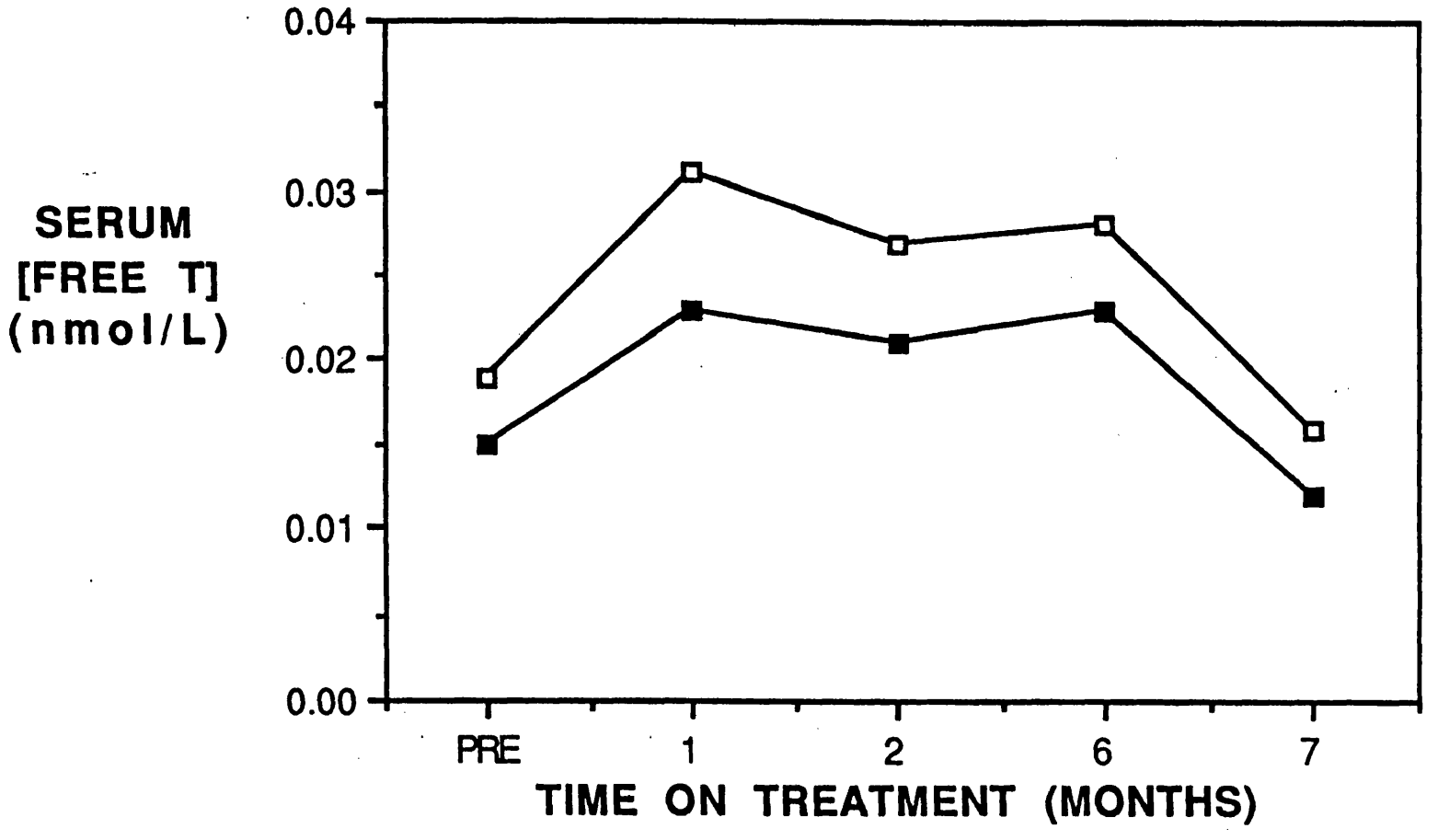


FIGURE 3.14: The effect of Gestrinone □ and Danazol ■ on mean serum concentration of Free Testosterone

NB: Month 7 is post-treatment

The endocrine levels at month 2 were selected for comparison as all the significant changes in the hormone levels had occurred by this time.

Although there was no relationship between oestradiol, bleeding days or % free T and the change in endometriosis scores, there was a significant correlation between concentration of free T and pure endometriosis scores when the results for the 2 drugs were considered together, ($r=0.392$, $0.02<p<0.05$). This relationship is shown in Figure 3.15. When the results for the drugs were analysed separately, there was no association for danazol, ($p>0.5$), but statistical significance was approached for the relationship with gestrinone, ($r=0.525$, $0.05<p<0.1$).

A linear regression analysis (Figure 3.16) was also performed on the mean level of E2 during treatment and the number of bleeding days occurring (excluding the first 28 days), which showed a statistically significant relationship, ($0.01<p<0.001$, $r=0.43$)

In view of the increases seen in % free T and the concentration of free T and the androgenic side effects developed during treatment with gestrinone and danazol, linear regression analyses were performed on the changes in the following:

1) % free T and the hirsutism score for both drugs together

2) % free T and hirsutism score for gestrinone patients (danazol alone was not performed as no change in hirsutism scores was seen in this group)

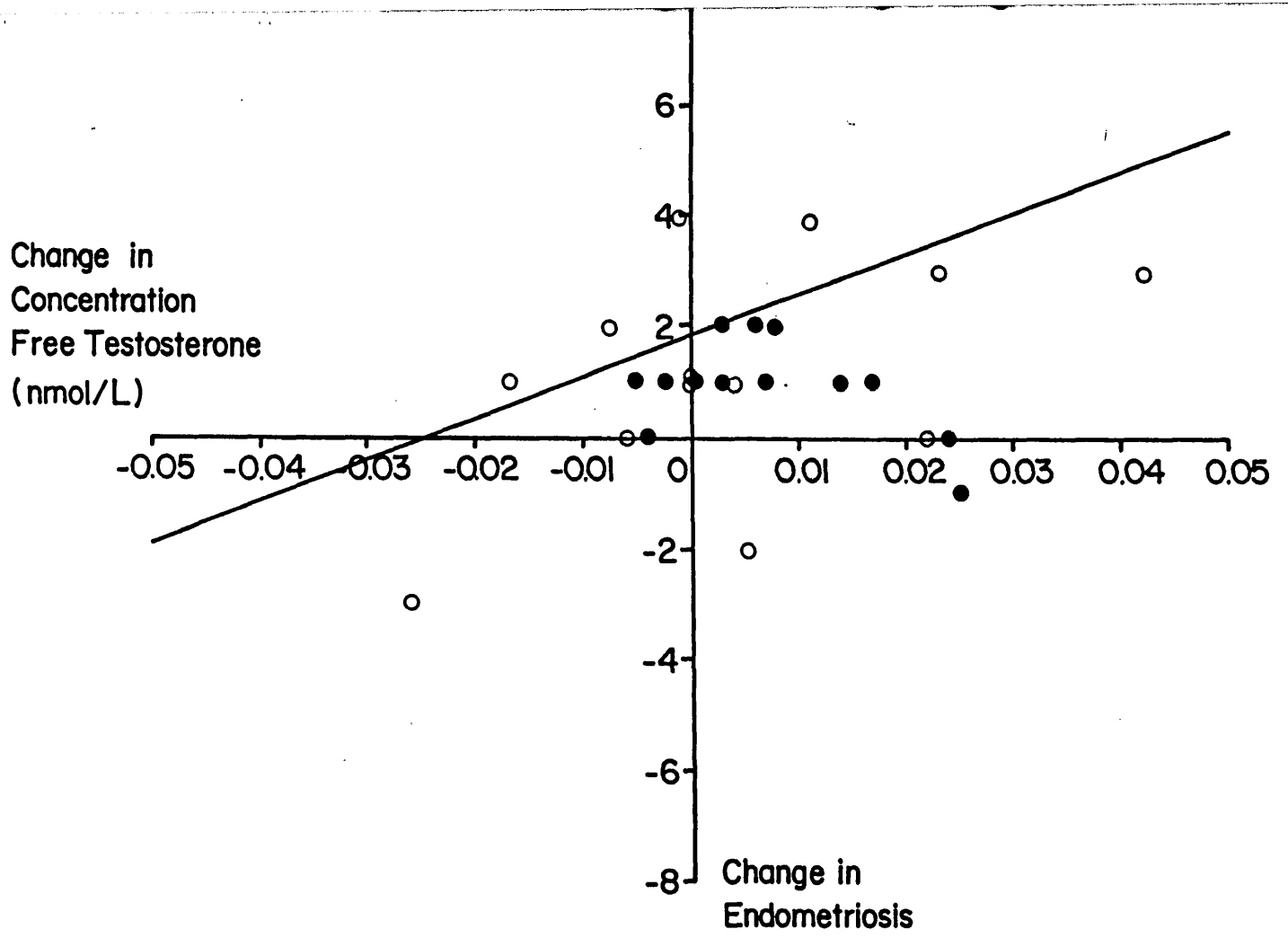


FIGURE 3.15: The relationship between change in endometriosis score and change in Conc. Free Testosterone: Y-1.79 = 74.79 (x-0.001) ($r = 0.392$, $0.02<p<0.05$) (O Gestrinone, ● Danazol)

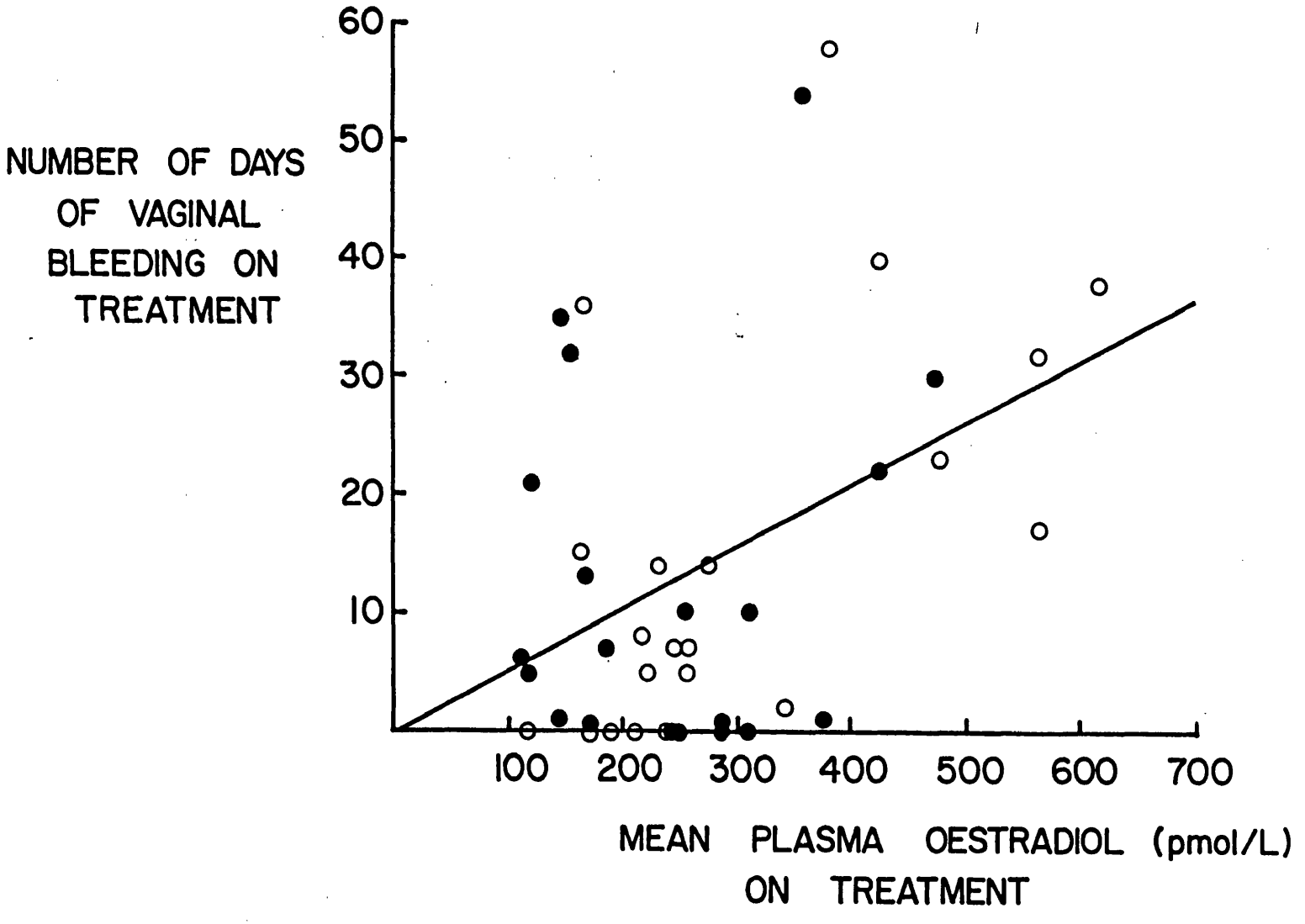


FIGURE 3.16: The relationship between vaginal bleeding (number of days) and mean plasma Oestradiol during treatment with Gestrinone O and Danazol ●
Y-13.9 = 0.0522 (x-273), r = 0.43, $0.01<p<0.001$

3) concentration of free T and hirsutism scores for both drugs together

4) concentration of free T and hirsutism score for the gestrinone group

5) % free T and acne scores for the combined groups

6) change in concentration of free T and acne scores for the combined groups

There was no significant relationship demonstrated between any of these, although the relationship between concentration of free T and acne scores approached significance, ($0.1<p<0.05$).

SUMMARY

1) There was no qualitative difference in the endocrinological changes produced by treatment with gestrinone and danazol.

2) The effect of treatment with gestrinone and danazol resulted in:

a) no significant change in LH, FSH and DHAS.

b) a significant fall in prolactin, E2, P, SHBG, T, A, DHT.

c) a significant rise in % free T and concentration of free T.

3) There was a significant correlation between the change in the endometriosis scores and concentration of free T. Oestradiol and the number of days of vaginal bleeding during treatment were also significantly related.

CHAPTER 10

DISCUSSION

Danazol and gestrinone have both been shown to effect endocrine changes, particularly in androgens and it has been suggested that the androgenic activity has a major role to play in the efficacy of these drugs in treating endometriosis. The major change is the elevation of % free T which may be due to either increased production, a decrease in SHBG binding capacity (Dowsett et al, 1986) or a decreased clearance rate. As the total T level also decreases, the latter explanation is not likely, and in fact the increased clearance rate of free T (Vermeulen et al, 1969) probably explains the diminution of total T. This study analysed various endocrine changes in an attempt to clarify the literature.

In all studies, including this one, the timing of the samples in relation to the menstrual cycle has been given inadequate emphasis. In sampling patients before treatment commenced, it should have been mandatory to withdraw blood during the time of the menses. This would have enabled all patients basal levels to have been estimated. The same criticism must apply to the post-treatment sampling. Unfortunately, the logistics involved precluded this being possible and cycle variation will have led to the data representing levels above basal. Comparisons of pre-, post- and intra-treatment data

therefore become more difficult to justify and interpret. In this study, the levels of E2 in the pre- and post- treatment samples may be physiologically elevated and may not reflect the early follicular phase levels which should have been used.

The conditions of collection of samples, separation of serum from cells and subsequent storage are those which were standard in the Department of Biochemical Endocrinology at the Chelsea Hospital for Women, and were based upon extensive experience in the analysis of these hormones in clinical samples. There is no evidence to suggest that the hormones are unstable under these conditions (Supraregional Assay Service Handbook of Assays for Steroids, Polypeptides, Proteins, Tumour markers and Genetic Enzymes, 1989, Jeffcoate, 1989).

Basal gonadotrophin levels have been shown to be unaltered by gestrinone or danazol in this study and no increase in gonadotrophins was seen with time in spite of the amenorrhoea, supporting the hypothesis that danazol and gestrinone may have some effect at the level of the hypothalamus. There are a number of ways in which this may occur. They could block the feedback of E2 by occupying the E2 receptor in the hypothalamus, but danazol and gestrinone bind only very weakly to oestrogen receptors (Barbieri et al, 1979, Chamness, 1980) and thus, this direct mechanism is unlikely. However, Musich et al (1981) and Tamaya et al (1983) have suggested that the action of danazol on the progestin and/or androgen receptors may inhibit oestrogen

receptor production, thus influencing the sensitivity of the feedback. It is possible that LHRH release may be directly inhibited, and the responsiveness of the pituitary to exogenous LHRH (Fraser et al, 1982, Braun et al, 1983) would suggest this as the likely explanation. Whether the suggested decrease in pulse frequency and increase in amplitude (Dmowski et al, 1983, Braun et al, 1983) will be confirmed awaits further studies, but the end result if this is substantiated would be an alteration in ovarian response and a decrease in follicular activity.

Such a change in response was confirmed in the results of E2 levels. These remained in the low early follicular phase range, indicating an absence of active granulosa cell activity. However, the inhibitory effect of danazol on steroidogenesis (Barbieri et al, 1977) may play a role within the granulosa cell, and Olsson et al (1988) reported that danazol is found to accumulate in follicular fluid at concentrations equivalent to that required to inhibit steroidogenesis in vitro (Olsson et al, 1986)

The reduction in the level of A4 may also reflect the suppression of steroidogenesis. Androstenedione is the precursor of T and about 60% of circulating levels are derived from the ovary (James and Goodall, 1982). Androstenedione is produced as a result of the conversion of either DHEA by 3β-hydroxysteroid dehydrogenase, or 17 hydroxyprogesterone by 17-20 desmolase. Both of these enzymes are inhibited by danazol (Barbieri et al, 1977).

The explanation of these changes in E2 and A4 for

gestrinone treated patients must be different. Gestrinone has no direct effect on steroidogenesis and thus, the decreased levels of E2 reflecting ovarian inactivity must be either due to the influence on gonadotrophin release or due to a receptor blocking effect. Gestrinone binds strongly to P receptors but no data exist as to whether it binds to FSH receptors, although it is unlikely that a steroid will bind to a peptide receptor. It is most likely that an indirect effect is seen with the change in T levels leading to inhibition of granulosa cell activity.

The effect of danazol and gestrinone on SHBG and T is well described (Laurell and Rannevik, 1979, Bevan et al, 1984, Dowsett et al, 1986, Forbes et al, 1986). These findings are confirmed by the results of this study, lending further support to the theory that the reduced levels of SHBG and the displacement of bound T result from the effect of the drugs.

The SHBG levels fell dramatically with both drugs. By 1 month, the concentration of SHBG had fallen to 15% of the pretreatment level and by 6 months, it was reduced to 6%. This concurs with the findings of Forbes et al (1986), where the degree of fall in SHBG was also shown to be dose dependent. The change in levels of SHBG could have been due to increased breakdown but Laurell and Rannevik (1979) showed that other plasma proteins remain unchanged and it is unlikely that such an effect would be limited to an individual protein. The alternative explanation is that SHBG synthesis is inhibited. Most plasma proteins are hepatic in

origin and steroids have been shown to effect the production rates. As amenorrhoeic women with similarly low levels of E2 have unchanged SHBG concentration, the fall must be due to an inhibitory effect of danazol on the hepatocyte (Laurell and Rannevik, 1979). As this would seem to reflect androgen receptor blocking capacity, gestrinone, E and 2OHME must act in a similar manner. It is unlikely that the rise in free T would be responsible for the fall in SHBG as the suppression is considerably greater than that seen in hirsute women or men.

The changes in total T and % free T and the correlation with free T concentration again support the existing literature. The increase in % free T results from the decreased levels of SHBG and from previous evidence, in the first week, also from competition for the binding sites on SHBG by danazol, E and to a lesser extent 2OHME (Dowsett et al, 1986). The increase in free T should lead to an increase in the metabolic clearance rate (Vermeulen et al, 1969) and a resultant fall in total T. These expectations are confirmed by this study where total T levels fell significantly. The variously reported effects of danazol on total T in the past can be explained by the lack of recognition of the cross-reactivity of danazol, E and 2OHME with the T assay. The use of a chromatographic method to separate danazol and its metabolites and the subsequent similar levels in gestrinone treated patients would suggest that this technique has overcome the cross-reactivity. However, the biological activity of T is not well

represented by % free T if total T levels fall, and thus total free T concentration was calculated. This showed a lesser, but significant rise in the concentration of free T on both gestrinone and danazol treatment. Thus, the rise in biologically active T may well explain the side effects which are described.

The explanation of lower total T levels being a result of increased metabolic clearance rates may not be the complete story. This study showed a significant fall in levels of A4 which is the T precursor. This may indicate that decreased synthesis of T makes a contribution to the overall fall in total T.

A decrease in mean DHT was found in patients treated with gestrinone and danazol. This concurs with the data of Forbes et al (1986) and may be explained in two ways. The decrease in SHBG leads to a decrease in DHT binding and a subsequent increase in free DHT. Vermeulen et al (1969) has shown that this change leads to an increased metabolic clearance rate and thus a decrease in total DHT. The other possibility is that as DHT is a product of T conversion, a reduction in total T may mean a decrease in total DHT. It is unlikely that the changes affect the bound/free ratio as DHT has a very high affinity for SHBG and the changes in the level of DHT mimic those of SHBG.

The circulating levels of DHEAS were not found to change in this study. These data are in accordance with the previous study for gestrinone (Robyn et al, 1984), but not for danazol. The major publications indicate a rise in DHEAS

during danazol therapy and this has been thought to be due to inhibition of hepatic sulphatase activity (Floyd, 1980, Luciano et al, 1981, Carlstrom et al, 1984). Whilst hepatic sulphatase activity may be inhibited by danazol, this only accounts for a small fraction of DHEA that is converted to DHEAS, the major metabolic pathway occurring in the adrenal. The unchanged levels of DHEAS in this study are consistent with this axiom.

The effect of gestrinone and danazol on prolactin shows a significant fall with time. This agrees with Braun et al (1983) and Rannevik and Thorell (1984) and may reflect the decreased E2 levels. However, the lower levels of E2 do not fall into the post menopausal range and therefore this is an unlikely explanation. The effect could be mediated at the level of the hypothalamus through an increase in dopamine production and a subsequent decrease in prolactin, or if the pituitary lactotrope was inhibited by these drugs, then there could be an increased sensitivity to dopamine. The data in this study cannot be used to answer these possibilities.

A further explanation may be a change in psychological stress. There are data from Miyabo et al (1977) that neurotic women have some elevation in mean prolactin levels. It may be that the relief of psychological stress in patients, either from a diagnosis being made and treatment commenced or from the alleviation of pain, may lead to the fall in prolactin levels.

The correlation between the androgenic changes

associated with gestrinone and danazol therapy has been claimed to explain the side effects of the drugs. It was, therefore, important to ascertain whether this was so in this study.

The described changes in % free T and concentration of free T would suggest that biologically active T was increased and that this could effect a response. There was, however, no significant association with either of these with acne or hirsutism, but this may reflect that too few patients were studied. The incidence of hirsutism is only 5-10% of patients on danazol therapy (Spooner, 1977, Barbieri et al, 1982) and whilst this study failed to support this change, larger numbers would be required to prove a statistical association. Of interest is the significant incidence of hirsutism seen in the gestrinone treated patients. Although a definite case can be made for circulating androgen being the effector, the hirsutism may be explained in two ways. The pilo-sebaceous unit is activated by DHT. Testosterone is converted to DHT by 5α reductase and although danazol and/or gestrinone may stimulate this enzyme's activity and induce increased tissue levels of DHT, it is unlikely that this is the mode of action. Alternatively, as the concentration of biologically active T is elevated, it may be that an increased conversion of T to DHT occurs and the androgenic action is induced as a result. The DHT normally produced attaches to the androgen receptor and is transferred to the nucleus of the cell where it influences protein synthesis. Danazol and gestrinone bind

to androgen receptors and thus it may be that the hirsutism results from a direct effect on the pilo-sebaceous unit. Gestrinone has 5-10 times the affinity for the androgen receptor than danazol, (Azadian-Boulanger et al, 1984, Moguilewsky and Philibert, 1984) and in spite of the attempt to equate the dose/response concentrations by using a twice weekly preparation, the intermittent high levels of the drug at the time of ingestion may explain the increased hair growth recorded.

It has been claimed that the production of a hypo-oestrogenic state is essential to the successful treatment of endometriosis (Barbieri et al, 1985). Dickey et al (1984) demonstrated that the clinical response to danazol was correlated to the suppression of E2 but the inverse relationship quoted ($p < 0.001$) must be interpreted with caution as the numbers of patients in each of their groups were very small (range 2-8). However, the hypo-oestrogenic induction of atrophy of the endometriotic lesion is the basis of gonadotrophin releasing hormone agonist therapy (Meldrum et al, 1982), and certainly, response rates have been impressive. When comparing E2 and endometriosis in this study however, no correlation could be demonstrated and it is probable that the absolute level of E2 is much less important than the production of an acyclic environment. Similarly, when comparing the number of bleeding days (as an index of "failed" amenorrhoea) to endometriosis response, there was no significant relationship, a finding also seen by Dickey et al (1984). As would be expected, there was a

relationship between E2 levels and bleeding days, where higher mean E2 levels during treatment were associated with more intermittent endometrial activity. Thus, the commonly held belief that amenorrhoea is an absolute necessity to effective treatment of endometriosis does not appear to be correct.

The effective medical treatment of endometriosis revolves around the principle of creating an androgenic environment which leads to binding of androgen receptors by either the drugs or the free T and subsequent atrophy of endometriotic lesions. It would follow, therefore, that changes in circulating levels of free T should be correlated to the improvement in endometriosis scores. This indeed was the case when the changes in the concentration of free T for the two drugs together were compared to the change in endometriosis scores. The lack of a significant relationship for the individual drug results may reflect the small numbers of patients in each group.

In the next chapter, the results are described of in vitro experiments performed in an attempt to distinguish between a direct effect by danazol and/or gestrinone, or an effect mediated through the rise in free T that they produce.

SECTION 4

THE INVESTIGATION OF DANAZOL, DANAZOL METABOLITES, GESTRINONE AND TESTOSTERONE ON THE GROWTH OF HUMAN ENDOMETRIAL CELLS IN VITRO

CHAPTER 11

PREVIOUS EXPERIENCE IN ENDOMETRIAL CELL CULTURE: A REVIEW

Despite the large number of publications in the literature, discussing the possible mechanism(s) of action of danazol and gestrinone, this remains uncertain. In this chapter, a possible direct effect of these drugs is further investigated by examining their effect on the growth of human endometrial cells in vitro.

TISSUE CULTURE OF HUMAN ENDOMETRIUM

Endometrium in vivo is known to respond histochemically and morphologically to cyclical endocrine stimulation, (Noyes et al, 1950, Page et al, 1951, McKay et al, 1956). The growth of human endometrial cells in vitro provides a method of studying the response to steroids and drugs at a cellular level. It further enables a distinction to be made between direct and indirect effects, which cannot be made in vivo.

Human endometrial cells were first cultured in vitro by Mayer and Heim, in 1926. In 1927, Cron and Gey, attempted to culture epithelium from a menstruating uterus, as it had previously been the general opinion that epithelial tissue in menstrual flow was non-viable. This was of particular interest at that time, as Sampson (1925, 1927), had just described the clinical condition, termed endometriosis, and had suggested retrograde transportation of menstrual

fragments through the tube with implantation and growth in the peritoneal cavity as an explanation for its aetiology. Cron and Gey were able to culture this menstrual tissue, as were Keettel and Stein, in 1951, and recognise both stromal and epithelial cells. In 1933, using staining techniques with neutral red in 95% absolute alcohol and a solution of Janis Green B, Geist (1933) confirmed that shed endometrial cells were living.

Certain features of the growth of endometrial cells have been further characterised in culture by Papanicolaou and Maddi (1958, 1959), Figge (1960), Notake (1963) and Hiratsu (1968), who all observed:

1. Two morphologic cell types in culture; epitheloid and stromal (fibroblast-like).

2. The growth of epithelial type cells is more active during the first 10 days of incubation and thereafter they are replaced by the fibroblastic cell type.

3. Endometrial cells obtained from the proliferative phase of the menstrual cycle grow better than secretory endometrial cells.

4. Epithelial cells tend to grow by spreading out from explants in the form of flattened cells with round or oval nuclei.

5. Premenopausal cells grow better than postmenopausal cells

Figge (1960) further observed that cultured endometrium fails to maintain the expected responsiveness of tissue in vivo. This was shown by the absence of the

documented variation in glycogen and nuclear protein concentrations seen during the changing phase of in vivo endometrium, and that all cell cultures demonstrated comparable patterns of nuclear protein concentrations irrespective of the phase of the cycle the parent tissue was derived from. This suggests that changes observed may be dependent on environmental influences rather than triggered by an intracellular response, adding further to the use of tissue culture models for examining responses to steroids.

Only Hiratsu (1968) has been able to develop a cell line, which could be maintained for 31 passages and 386 days.

More recent techniques, using different enzymatic separation procedures , have purified the epithelial and stromal cells from endometrium for separate cultures, (Kirk et al, 1978; Liu and Tseng, 1979; Satyaswaroop et al, 1979; Shapiro et al, 1980; Tseng, 1984). The epithelial and stromal cells can then be identified morphologically at both the cellular and ultrastructural level. This enabled further studies into the precise mechanism by which ovarian hormones regulate growth, differentiation and metabolic activities of the endometrium with regard to cell type. It was also developed in order to overcome the limitations of the short-term nature of mixed endometrial primary cultures. When separated, the epithelial cells, however, still only survived a relatively short-time, i.e. 10-15 days, although the stromal cultures showed a life span in vitro equivalent to that of human diploid fibroblasts, (Kirk et al, 1978).

The short life span of endometrial epithelial cultures is thought likely to be related to the rapid accumulation of polyploid cells, a process not observed in stromal cultures, (Kirk and King, 1979).

Endometrial tissue cultures, both mixed and separated, have been used to investigate direct effects of steroidal hormones on parameters such as cell growth, steroid receptor levels, aromatase activity and glycogen content. The results, however, have been extremely varied and no consensus has been formed in the literature.

Figge (1963) found no effect on endometrial growth activity or glycogen concentration from oestradiol, conjugated oestrogens or progesterone in mixed endometrial cultures. Liszczak (1977) and Kirk et al (1978) confirmed no increase in epithelial cell proliferation with oestrogen and progesterone, although they did find an increase in stromal cell proliferation with progesterone. In separated epithelial and stromal cultures Liu and Tseng (1979) found the growth rate of both cultures was accelerated by the addition of oestradiol and medroxyprogesterone into the culture medium. However, in an earlier study by Hiratsu (1968), he observed that oestradiol at a concentration of 0.1ug/ml stimulated human endometrial cellular growth significantly, whilst the same hormone at 1 and 10ug/ml concentrations showed growth inhibitory effects. He further reported that premarin had a slight growth promoting effect at a dose of 0.01ug/ml, but inhibitory results at higher doses and that progesterone, testosterone and

androstenedione displayed growth inhibitory effects within a range of concentrations from 0.01 to 50ug/ml. Pavlik and Katzenellenbogen (1978), confirmed a dose dependent stimulatory effect on the rate of cell proliferation with not only 17β-oestadiol, but also with diethylstilboestrol.

Experimental work using immature rat uterine tissue in culture (Kassis et al, 1984a), found uterine cells to contain oestrogen receptors, and to be oestrogen responsive in terms of increased progesterone receptor levels but not in terms of cell growth. However, Kassis et al (1984b) later showed that oestrogen responsiveness and binding capacity were dramatically affected by the frequency of culture medium changes.

Tseng (1984), examined the effect of oestradiol and progesterone on aromatase activity in isolated endometrial stromal cells. She found that oestradiol alone did not change aromatase activity, but that progesterone caused a significant increase as compared to control values and that this activity was further increased in the presence of oestradiol. Much smaller effects of oestradiol and progesterone were noted on the aromatase activity in endometrial epithelial glands.

Using glycogen content as a marker of in vitro responsiveness to steroids, Shapiro et al (1980), showed an increase of 13 fold in the glycogen content in proliferative endometrial tissue in media containing progesterone. This change is analogous to that produced in vivo, and the response seen in vitro occurred at approximately the same

concentration of hormone in the medium as that found in post-ovulatory serum (Ross et al, 1970).

Despite the varying reports on the effect of oestrogen on endometrial tissue in culture, this may in fact be due to the important observation made by Berthois et al (1986) about phenol red, which is commonly used as a pH indicator in tissue culture media. They first noticed that anti-oestrogens suppressed growth in oestrogen responsive human breast cancer cells below that of control cells, in the apparent absence of oestrogens. Whilst searching for the potential source of this oestrogenic activity they noticed that phenol red bore some structural resemblance to certain non-steroidal oestrogens (e.g. cyclofenil, chlorotrianisene). Further investigations revealed that phenol red bound to oestrogen receptors of MCF-7 human breast cancer cells with an affinity 0.001% that of oestradiol, and stimulated the proliferation of oestrogen receptor-positive MCF-7 breast cancer cells in a dose dependent manner, but had no effect on the growth of oestrogen-receptor negative MDA-MB 231 breast cancer cells. Phenol red is included in tissue culture media at a dose of 15-45uM, at which concentrations it shows significant oestrogenic activity. This, therefore, may contribute to the confusion of described effects in the literature of the effect of oestrogen on endometrial tissue in culture.

In summary, it would seem that endometrial cells in culture require oestrogen support in order to maintain growth. The conflicting results may be due to the presence

of phenol red in the culture medium which will have confused the necessity for the addition of further exogenous oestrogen.

Although most workers obtained endometrial tissue for culture directly from the uterine cavity, Willemsem et al (1985), performed cultures using uterine/tubal flushes obtained from the peritoneal cavity of women undergoing diagnostic laparoscopy for infertility, and recognised epithelial "tadpole-like" cells in 77 of 115 cultures. They also recognised these cells in the cultures of laparoscopic biopsy specimens of active endometriosis nodules, but were unable to identify them in cultures from peritoneal washings taken from patients prior to uterine/tubal flushing. These "tadpole-like" cells were identified as arising from the lining and glandular epithelium of part of the genital tract. Although the endometriotic tissue was cultured and the "tadpole-like" cells recognised, the explanted nodules were found to contain relatively large amounts of fibrous connective tissue and consequently only a small number of cells grew out and attached to the bottom of the culture vessels. Further, cultures from peritoneal washings from patients with endometriosis did not show the presence of this cell type, presumably because endometriosis nodules are encapsulated by fibrous connective tissue, hampering a migration of the columnar cells. The poor cell growth obtained from endometriotic tissue may explain the lack of any other evidence in the literature of culture of this cell type.

COMPARISONS BETWEEN ENDOMETRIUM AND ENDOMETRIOTIC TISSUE

A) HISTOLOGICAL

In 1960, Roddick et al, retrospectively examined 88 endometrial and endometriotic tissue samples, taken in each case from the same patient. They found a correlation between the phase of the menstrual cycle in the two tissues in 72 out of the 88 (82%) cases. In three cases the endometrium was secretory and the corresponding endometriotic tissue proliferative, and the opposite occurred in 13 cases. However, a further 520 samples they examined were unsuitable for interpretation as the endometriosis had been destroyed, presumably by bleeding, pressure and necrosis. Berqvist et al (1985) found a distinct menstrual pattern in 82% of endometriotic samples, of which 70% were synchronous with the endometrium from the same patient, but 30% varied inconsistently and in 18% there was no identifiable menstrual phase pattern.

This group also observed that:

1) although they were able to recognise a menstrual phase pattern in endometriotic samples, there was considerable variability in the glandular and epithelial cell morphologies compared to eutopic endometrial tissue.

2) endometriotic lesions were frequently surrounded by a mantle of fibrosis, with well preserved epithelium within, that showed no phase pattern development.

3) different endometriotic samples taken from one patient had the same menstrual phase pattern.

This group also performed experiments involving the grafting of endometrial and endometriotic tissue to athymic "nude" mice. Examination of the tissues 10 weeks later revealed no histological difference in the two tissues, although the endometriotic grafts contained fewer glands. Both tissues showed similar responses to oestrogen, medroxyprogesterone and danazol treatment and they concluded that differences observed in vivo may be due to environmental factors.

B) ULTRASTRUCTURAL

Schweppe et al (1984) examining the ultrastructural features of endometriotic tissue found proliferative and secretory changes in 75% of their specimens, but that these were delayed and not in phase with the menstrual cycle, suggesting an incomplete reaction to hormonal stimuli. The remaining 25% showed poorly differentiated glands indicating no hormonal response. In a further study (Schweppe and Wynn, 1984), they comment that ectopic endometrium showed a wide range of morphological development, with significantly different patterns occurring within the same specimen, ranging from poorly to highly differentiated glands. These investigators do not speculate whether these dissimilarities were due to an inherent difference in maturation and differentiation of endometriotic tissue or due to local influences.

3) STEROID RECEPTORS

As reviewed in Chapter 1 in the Introduction

(steroid receptors), numerous comparisons have been made between the ER, PR and AR content of endometriotic tissue. Whilst there is considerable debate, it would appear from the available literature that there is either slightly lower or equivalent receptor levels in endometriotic as compared with endometrial tissue. However, the cyclical change in receptors seen in uterine endometrium is not mimicked by endometriotic lesions. Sternfeld et al (1988) were unable to demonstrate any change in the mean percentage of ER positive cells or the total ER content in the endometriotic tissue of rhesus monkeys during the menstrual cycle. Similarly in the human, ER concentrations remained constant throughout the cycle in endometriotic tissue, whereas no oestrogen binding sites have been found in the endometrium during the luteal phase (Gould et al, 1983, Kauppila et al, 1984, Vierikko et al, 1985). The latter groups however also described a lower mean concentration of PR during the luteal compared to the follicular phase of the cycle, as seen in endometrial tissue. In conclusion, it is likely that endometriotic cells are under hormonal control, but that cell growth regulation may be different from that of endometrium in situ, due to the altered receptor concentration and response.

4) HISTOCHEMICAL AND BIOCHEMICAL

Based on the premise that hormonal control of tissues is mediated via alterations in cellular metabolism, Prakash et al (1963) compared the enzymatic activity occuring in endometrial and endometriotic tissue. Alkaline

phosphatase activity in endometriotic tissue did not show the same cyclical variations seen in endometrial tissue, but the degree of activity was equivalent to that seen in proliferative or early secretory endometrium. Acid phosphatase activity however was equal to or slightly less intense than that seen in corresponding endometrium. Lactic dehydrogenase activity was less in endometriotic tissue and isocitric dehydrogenase more, whereas glucose-6-phosphatase and succinic dehydrogenase activity were the same in both tissues. Thus, the cellular metabolic activity in endometriotic tissue reflects the changes in receptor response between the two tissues.

In normal-oestrogen primed endometrium, P results in the accumulation of glycogen in the glandular epithelium during the early secretory phase, and later, mucopolysaccharides are secreted into the glandular lumen. Berqvist and Myre (1986) examined glycogen and mucopolysaccharide synthesis in endometriotic tissue, and although they found both of these present, they were unable to demonstrate any cyclical differences. They concluded that P must be biologically active in endometriotic tissue, but assumed a disturbance in the cyclical expression of this hormone.

The metabolism occuring in endometriotic tissue was further investigated by Carlstrom et al (1988) who found that oestrone sulphatase activity and oestrogen formation were similar in endometriotic compared to endometrial tissue. In both tissues the rate of oestrogen production was

higher in the secretory phase, but this cycle variation was greater for endometrium.

The findings of all the above authors suggest histological and functional similarities between endometrial and endometriotic tissue. However, there are also differences which appear mainly to be a failure to respond to hormonal stimulation in the expected cyclical manner. It may be that this is a result of a compromised blood supply secondary to the fibrosis often seen surrounding endometriotic lesions rather than a receptor problem. Endometrium is easily obtainable by dilatation and curettage. In view of the similarities between endometrium and endometiotic tissue, and the difficulties in obtaining sufficient cells from endometriotic biopsies for culture, endometrium was chosen in this study as a model for endometriotic tissue.

DANAZOL, DANAZOL METABOLITES AND GESTRINONE BINDING TO STEROID RECEPTORS

Gestrinone and danazol have both been shown to bind to PR and AR (chapter 2) and a possible direct effect on endometrial and endometriotic tissue must therefore be considered in the attempt to understand their therapeutic efficacy in the treatment of endometriosis. In view of the available data on the two major metabolites of danazol, E

and 2OHME, in terms of a) progestational and androgenic activity (for E) and b) binding to SHBG, it was thought possible that these compounds may also have a direct effect on these two tissues.

TESTOSTERONE BINDING

In view of the historic effectiveness of androgen therapy in the treatment of endometriosis and the increase in free testosterone produced by danazol and gestrinone, it seemed important and appropriate to investigate a possible direct effect of testosterone itself on endometrial tissue.

The aim of this part of the study, therefore, was to use an endometrial tissue culture system to investigate a possible direct effect on endometrial growth of the drugs, danazol and gestrinone, the two major metabolites of danazol, E and 2OHME, and the hormone, T. The endometrium was used in vitro as a model for endometriotic tissue.

CHAPTER 12

MATERIALS AND METHODS

PATIENTS

Endometrial tissue was obtained from 13 patients who had normal regular menstrual cycles, defined as 24-35 days in length, by dilatation and currettage, for example at the time of laparoscopic sterilisation. The day in the cycle on which surgery was performed and cycle length were noted.

Endometrial tissue was not obtained from any patients with the following exclusion criteria:

1. Any patients taking hormonal treatment either concurrently or during the two months preceding surgery.
2. Any patients with a history suggestive of a uterine pathology such as intermenstrual bleeding, menorrhagia etc.
3. Any patients with a known pathology such as a polyp.
4. Patients with an indwelling intrauterine device were also excluded from sample collection because of the risk of infection and subsequent contamination of tissue cultures.

The endometrial tissue samples were collected using sterile technique and immediately placed into 0.015M, pH 7.3 phosphate buffered saline (PBS), warmed to 37 C, for transport to the tissue culture laboratory. Tissue preparation was commenced as soon as possible, but always within two hours of collection.

TISSUE PREPARATION

All manipulations of the tissue were conducted under sterile conditions using a laminar flow hood. After first removing any obvious blood clots, the endometrial tissue was repeatedly divided using two number 11 scalpel blades for five minutes in a 60mm X 15mm diameter petri dish. The tissue was then incubated on an orbital shaker at 37° C for one hour, in 20ml of PBS containing 6.4mg collagenase type 1A and 2.3mg DNAse type 1 (both Sigma, Poole, UK). The flask containing the tissue was then firmly shaken manually two or three times, and a largely single cell suspension resulted. This suspension was then centrifuged at 40g for 10 minutes at 20°C. The supernatant was decanted and the cells in the pellet resuspended in 10mls Medium 199, containing penicillin and streptomycin sulphate antibiotics, L-Glutamine and 10% horse serum (Gibco, Paisley, Scotland) filtered through a 0.22um millipore filter (Millipore, Molshern, France). The cell number in the suspension was estimated using a microscope and haemocytometer, after the addition of trypan blue (0.4%), to ensure only live cells were counted. A known number of cells were then plated in 1ml of culture medium into each well (2.4cm deep X 1.7cm diameter) of a 3 X 4 multiwell dish. Four mls of medium were added to each well and the dishes were incubated at 37°C in a automatically regulated humidified atmosphere of 5% carbon dioxide in air. After 24 hours in culture, the cells were washed and the medium changed (see below for details).

TRYPSINISATION AND CELL COUNTING

On conclusion of an experiment, the culture medium was removed and the cells were washed twice with PBS. The cells growing on the bottom of each well were removed with 2 X 1ml of 0.05% trypsin (Sigma, Poole, Dorset, UK) and 0.025% ethylenediaminetetraacetic acid (Sigma, Poole) in PBS, by repeated flushing of this solution over the cells, under direct microscopic observation, at room temperature. This ensured that very few cells remained attached to the base of the well. In early experiments, when the post trypsinisation cells were recounted using a haemocytometer, large clumps of endometrial cells were revealed (Figure 4.1) making it impossible to count cell number. Therefore immediately after trypsinisation, the cell suspension was gently flushed through a 23 gauge needle, and the separated single cells (Figure 4.2) were fixed by the addition of 2ml of 10% formaldehyde. During development of the technique, these cells were counted on a haemocytometer, but once established and validated, all subsequent counts were performed on 100ul aliquots, on a coulter counter, at amplification X16 and aperture current 1amp. The individual tubes containing the cells from each experiment were counted in a random order.

PLATING

After 24 hours in culture, the culture medium was removed and the cells were washed twice with PBS. At this time the characteristic whorled pattern of endometrial cells in culture could be seen, with cells growing out from

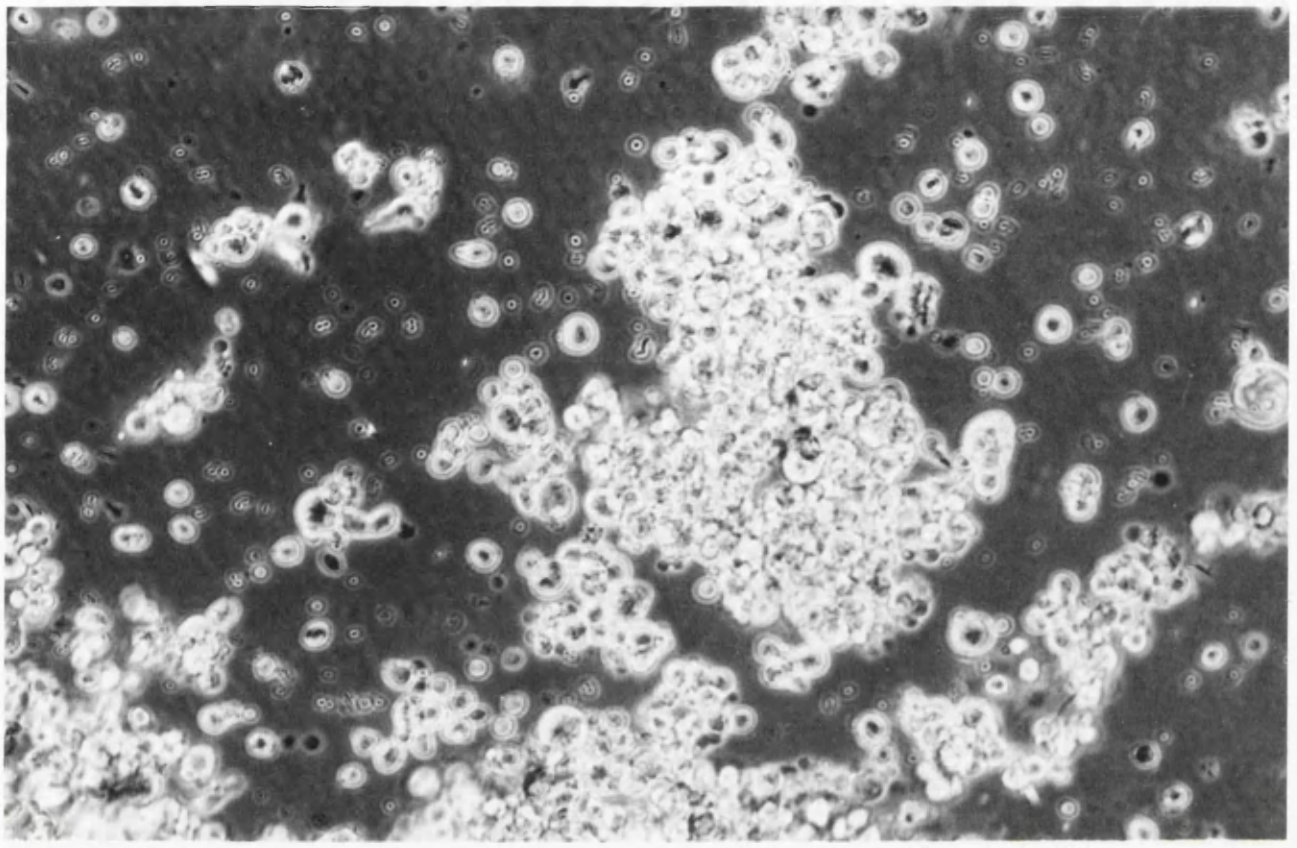


FIG 4.1

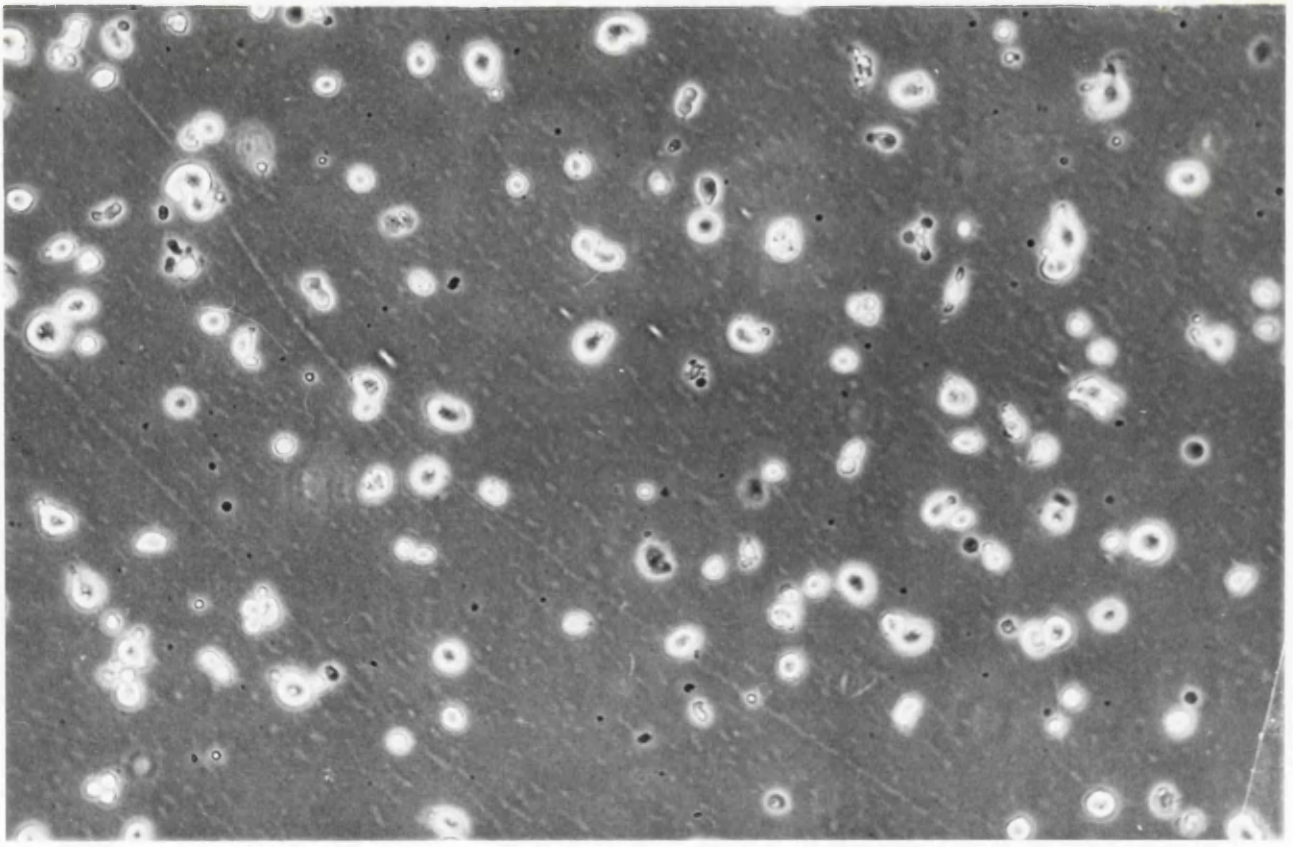


FIG 4.2

FIG 4.1: Clumps of endometrial cells immediately following trypsinisation

FIG 4.2: Single endometrial cells in suspension after flushing through a 23 gauge needle

implanted fragments of tissue (Figure 4.3).

Initial experiments were performed to establish the most suitable number of cells to plate and the plating efficiency. To determine the actual number of cells in each well that became attached and grew (the plating efficiency), cells were trysinised after 24hrs in culture. 425,000, 660,000, and 850,000 cells were plated in multiple wells and left for 24hrs before being washed and trypsinised as described above. The results are shown in Table 4.1, and indicate an aproximately 10% plating efficiency at 24hrs for all three cell concentrations.

Cultures which had previously been plated with 250,000, 500,000 and 1,000,000 cells per well, were washed as described at 24hrs and 5ml fresh culture medium was added to each well. These were then reincubated undisturbed for a further 72hrs. When viewed at this time under a microscope, it could be seen that in the 1,000,000 cell cultures, many cells had died and floated free from the base, which was completely covered with cells (confluent). In the 500,000 plated well, confluence had been acheived, with few remaining uncovered areas on the base of the well, but in the wells plated with 250,000 cells, many bare areas remained. In experiments where less than 250,000 cells were plated (i.e. 50,000, 100,000, 150,000 and 200,000), growth of endometrial cells was rarely established, and only a few isolated cell growth areas were seen.

As a result of these observations, 500,000 cells per well was chosen for all experiments in which drug tests were

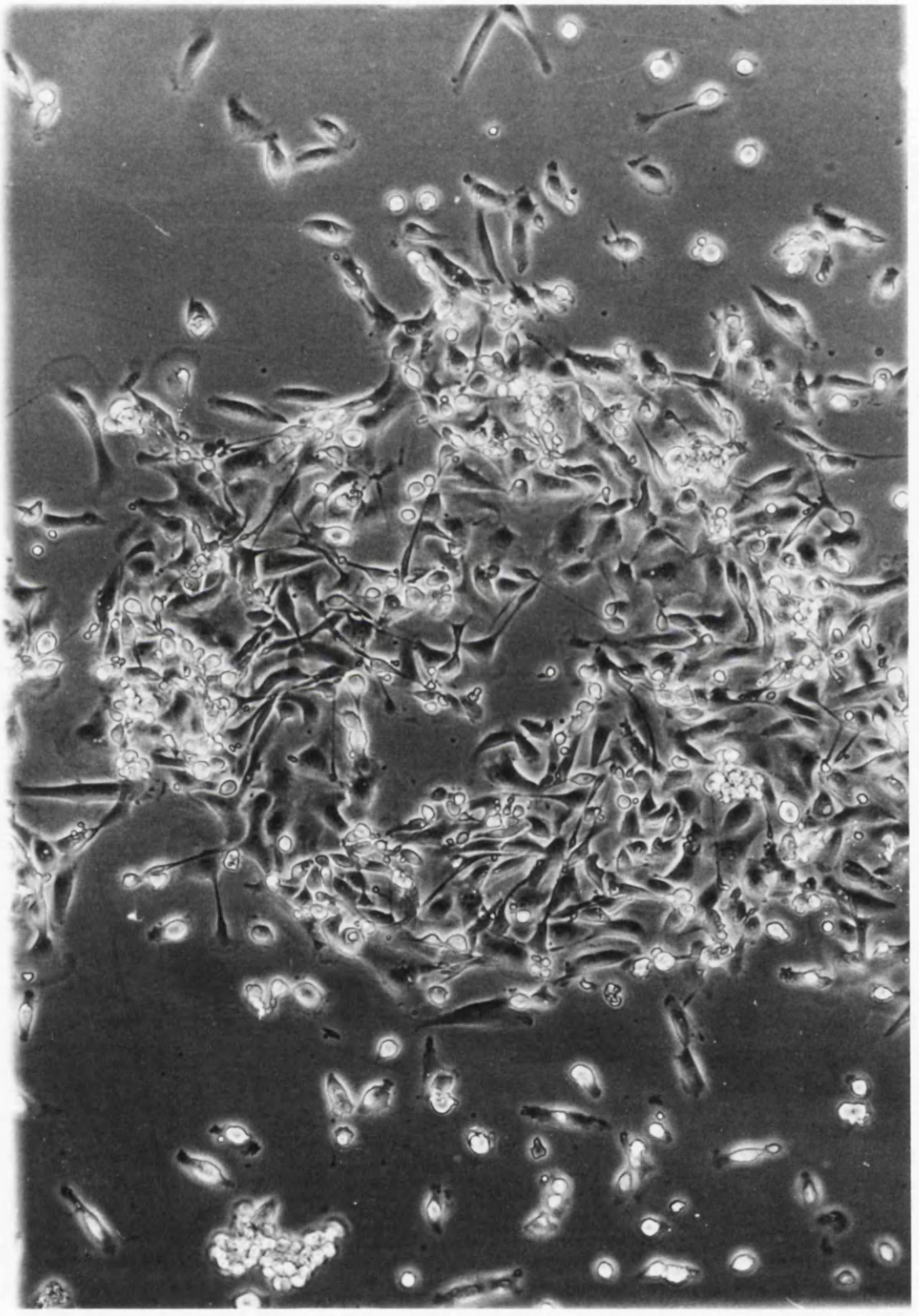


FIG 4.3: Endometrial cells in culture demonstrating the characteristic whorled pattern

TABLE 4.1: PLATING EFFICIENCY OF ENDOMETRIAL CELLS AFTER 24 HOURS IN CULTURE

NO OF CELLS PLATED	CELL COUNT AFTER 24 HOURS	MEAN ± SD CELL COUNT AFTER 24 HOURS	MEAN CELL COUNT AFTER 24 HOURS % OF PLATED
425,000	52,250 41,250 52,250 38,500 38,500	44,550 ±7,120	10.5%
660,000	79,750 52,250 49,500 68,750 68,750 46,750	60,958 ±13,300	9.2%
850,000	88,000 60,500 96,250 77,000 90,750 88,000	83,417 ±12,900	9.8%

made, and the suspension of cells obtained prior to plating was diluted to provide this concentration.

HORMONES AND DRUGS

The drugs were prepared in ethanol, at a concentration that resulted in a final ethanol concentration of 0.2% in the culture medium. All control cultures had ethanol added to the culture medium at this concentration.

After 24hrs in culture, each well plated with 500,000 cells was washed as described in sterile conditions, and the drugs in 5ml of culture medium were added in the following concentrations:

Danazol	1×10^{-6} M, 1×10^{-5} M
Ethisterone	2.5×10^{-7} M
2OHME	3×10^{-6} M
Gestrinone	3×10^{-8} M, 3×10^{-7} M
Testosterone	2×10^{-9} M, 2×10^{-8} M

Danazol and 2OHME were supplied by Sterling Winthrop (Guildford, Surrey, UK) and Gestrinone by Roussel Laboratories Ltd (Uxbridge, Middlesex, UK). E and T were purchased from Sigma (Poole, Dorset, UK).

The doses selected for danazol, E and 2OHME were one and ten times (for danazol) the plasma concentrations expected in vivo during therapy (Creange and Potts, 1974, Davison et al, 1976 and Potts, 1977). For gestrinone, as steady state levels are not acheived during treatment with the usual regime of 2.5mg, twice weekly, one and ten times the expected maximum concentration after a single 2.5mg dose

was tested (Salmon et al, 1984). Testosterone was tested at one and ten times the mean normal female plasma concentration. The drugs were added sequentially, i.e. control, G, D, E, 2OHME, T, control, G, D, E, 2OHME, T, etc, to ensure the plating order did not influence the results.

For each multiwell dish, there were always three control wells containing culture medium with ethanol only, and each drug being investigated was also added in triplicate. The multiwell dishes were then replaced in the humidified automatic carbon dioxide incubators and left undisturbed for a further 72hrs. At that time they were removed, washed, trypsinised and counted in a coulter counter, as desribed above.

STATISTICS

Results were compared using either paired or unpaired Student's t-tests as appropriate, on an Apple II computer. In no cases were multiple comparisons made which required Bonferroni correction.

CHAPTER 13

RESULTS

In early experiments, it was determined that only endometrial tissue obtained during the postmenstrual, proliferative or early secretory phases showed satisfactory growth, and such samples were selected thereafter.

The day of the menstrual cycle at the time of tissue collection ranged from day 6-23, (median day 9), and the usual cycle length was 28 days for all but 2 patients whose cycle lengths were 24 and 33 days (Table 4.2).

The mean number of cells in control cultures ranged from 2.2×10^5 to 4.8×10^5 after the 4-day culture period, the overall mean being 3.2×10^5. The mean coefficient of variation for the cell counts in the triplicate control cultures was 6.4% ranging from 2% to 19%, and for the triplicate drug-treated cultures was 5.0% ranging from 0.8% to 12%.

In each experiment the mean cell count for control wells was calculated for an individual multiwell dish, even if several multiwell dishes were set up from one patient sample, and comparisons with drug-containing wells only made to controls in the same multiwell dish. For each test well, the cell count was calculated as a percentage of the control mean, and the mean, standard deviation (SD) and standard error of the mean (s.e.m.) for the three triplicates were calculated. In all figures the counts from test wells are

TABLE 4.2: RELATIONSHIP BETWEEN CYCLE LENGTH AND DAY OF ENDOMETRIAL SAMPLING

PATIENT NUMBER	DAY OF CYCLE	LENGTH OF CYCLE (DAYS)
1	6	33
2	9	28
3	7	28
4	6	28
5	7	28
6	15	24
7	16	28
8	8	28
9	14	28
10	7	28
11	18	28
12	11	28
13	23	28

expressed as a percentage of the control count with s.e.m.s. An example of the results from one experiment is shown in Table 4.3.

The results for each drug or steroid are shown for the X1 dose in Table 4.4, and for the X10 dose in Table 4.5, and are summarised graphically in Figure 4.4. For gestrinone at the X1 dose (n=8) there was a 9.1% ± 4.9 inhibition, and at the X10 dose (n=9) a 3.8% ± 2.4 inhibition, neither of these being statistically significant. The metabolites of danazol, E and 2OHME were only examined at the X1 dose because of limited tissue availability and priority was given to the drugs themselves and T. The inhibition of endometrial cell growth at the X1 dose by E (n=4) was 7.2% ± 4.4, and by 2OHME (n=4) 8.2% ± 10.5. Again, neither of these results was significantly different from control cultures. However, danazol at the X1 dose (n=8) suppressed cell growth by 20.8% ± 5.8 ($p<0.008$), and at the X10 dose (n=10) by 26.9% ± 5.0 ($p<0.001$). The most marked effect was found with testosterone which at the X1 (n=7) and X10 (n=8) dose suppressed growth by 25.0% ± 5.9 ($p<0.009$) and 35.5% ± 6.6 ($p<0.002$), respectively.

The effects of gestrinone, danazol and testosterone are demonstrated individually in Figures 4.5, 4.6 and 4.7, respectively. In these graphs, each point represents the mean result of the triplicate wells for each drug, for the X1 and X10 dose. The line between two points, (i.e. the X1 and X10 dose result), indicates results which have been derived from the same patient sample.

TABLE 4.3: RESULTS FROM CELLS CULTURED FROM PATIENT NO 6
THE EFFECT OF GESTRINONE, DANAZOL AND TESTOSTERONE AT x 1 AND x 10. DATA COLLECTED AND CALCULATION OF % INHIBITION OF CELL GROWTH IN A SINGLE PATIENT SAMPLE.

		RESULTS OF CELL COUNTS					
DRUG	DOSE	INDIVIDUAL WELL COUNTS	MEAN WELL COUNT	INDIVIDUAL COUNT AS % OF CONTROL	MEAN %	SD OF % RESULT	SEM OF % RESULT
C		330,960 337,040 326,520	331,507		100		
G	x 1	297,920 291,600 292,840		89.9 88.0 88.3	88.7	1.0	0.6
D	x 1	170,880 190,520 172,840		51.6 57.5 52.1	53.7	3.3	1.9
T	x 1	159,440 150,760 148,160		48.1 45.5 44.7	46.1	1.8	1.0
C		333,360 319,440 325,160	325,987		100		
G	x 10	300,840 304,840 303,840		92.3 93.5 93.2	93.0	0.6	0.4
D	x 10	213,720 209,400 205,120		65.6 64.2 62.9	64.2	1.3	0.8
T	x 10	195,480 196,840 185,040		60.0 60.4 56.8	59.0	2.0	1.1

TABLE 4.4: THE EFFECT OF DRUG TREATMENT AT x 1 DOSE ON ENDOMETRIAL CELL GROWTH IN VITRO. THE RESULTS ARE EXPRESSED AS A PERCENTAGE OF THE MEAN CONTROL CELL NUMBER, AND SHOW THE MEAN ±SEM OF THREE WELLS

PATIENT NUMBER	GESTRINONE	DANAZOL	ETHISTERONE	20HME	TESTOSTERONE
1	70.6 ±6.5	61.1 ±2.4	-	-	-
2	88.2 ±3.9	76.1 ±3.6	95.1 ±3.6	107.4 ±4.2	83.7 ±3.4
3	99.8 ±7.7	96.9 ±7.5	101.1 ±11.0	107.1 ±0.6	93.4 ±15.2
4	110.5 ±4.2	100.9 ±3.2	94.8 ±4.9	89.9 ±5.0	79.3 ±5.3
5	79.5 ±8.0	81.5 ±4.1	80.2 ±5.5	62.8 ±7.1	69.6 ±1.8
6	88.7 ±0.6	53.7 ±1.9	-	-	46.1 ±1.0
7	107.6 ±3.4	89.2 ±3.2	-	-	85.2 ±2.4
8	82.4 ±1.8	74.1 ±5.1	-	-	67.5 ±4.3
N	8	8	4	4	7
MEAN	90.9	79.2	92.8	91.8	75.0
SD	14.0	16.5	8.9	21.0	15.6
SEM	4.9	5.8	4.4	10.5	5.9

TABLE 4.5: THE EFFECT OF DRUG TREATMENT AT x10 DOSE ON ENDOMETRIAL CELL GROWTH IN VITRO. THE RESULTS ARE EXPRESSED AS A PERCENTAGE OF THE MEAN CONTROL CELL NUMBER, AND SHOW THE MEAN ± SEM OF THREE WELLS

PATIENT NO	GESTRINONE	DANAZOL	TESTOSTERONE
4	100 ±5.0	85.7 ±4.3	80.0 ±11.6
5	107.9 ±11.6	50.9 ±3.4	40.7 ±3.4
6	93.0 ±0.4	64.2 ±0.8	59.0 ±1.1
7	88.8 ±0.5	61.7 ±2.5	52.0 ±2.75
8	95.4 ±0.5	76.6 ±2.4	82.8 ±1.1
9	99.1 ±4.1	98.5 ±9.5	-
10	102.6 ±0.9	94.6 ±5.0	-
11	-	73.5 ±7.5	92.6 ±9.1
12	95.3 ±2.0	66.9 ±2.3	61.4 ±1.3
13	83.8 ±0.8	58.5 ±2.6	47.8 ±0.4
N	9	10	8
MEAN	96.2	73.1	64.5
SD	7.3	15.8	18.5
SEM	2.4	5.0	6.6

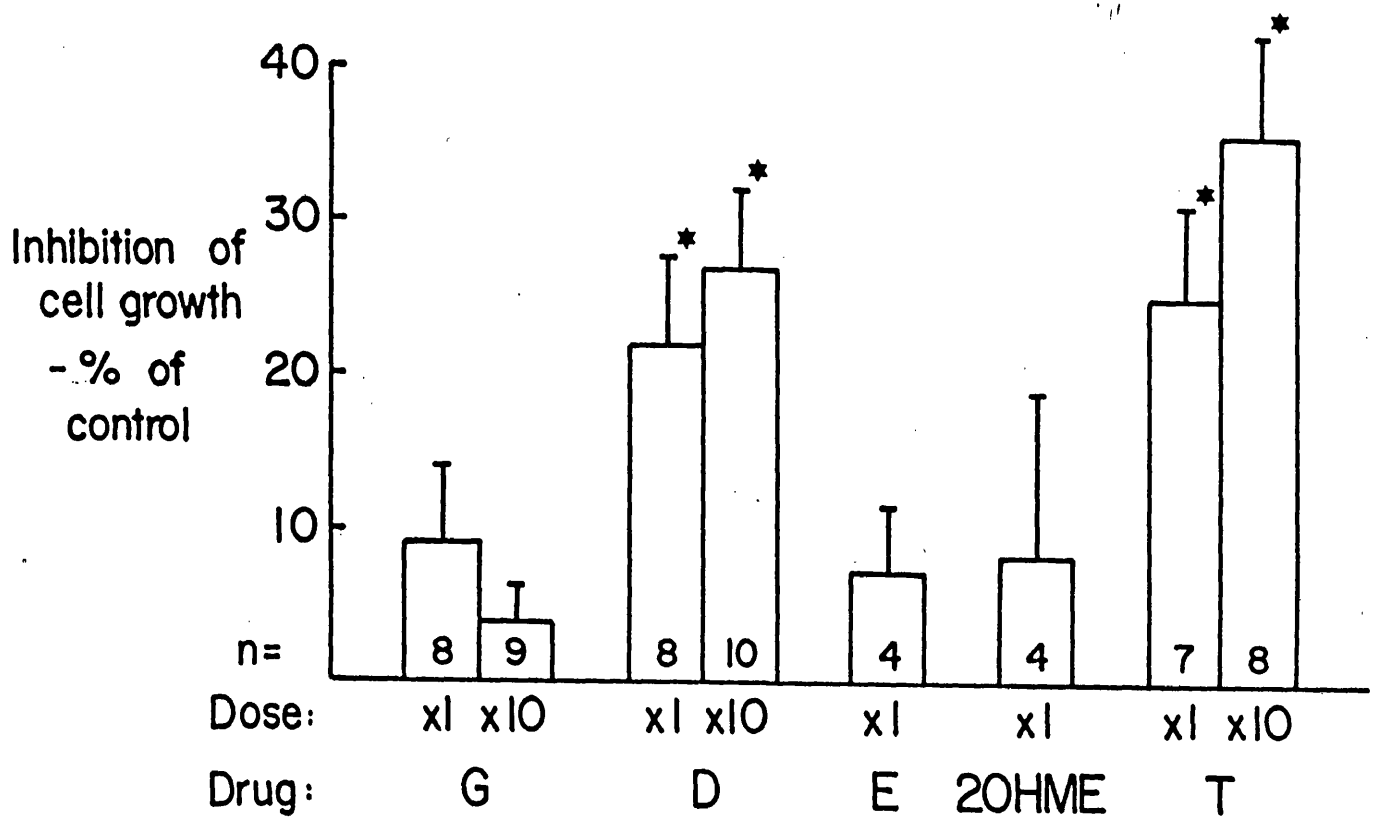


FIGURE 4.4: Drug-induced inhibition of the growth of normal human endometrial cells in culture. The bar in each case represents the mean ± SEM of N values

* p <0.01 versus control

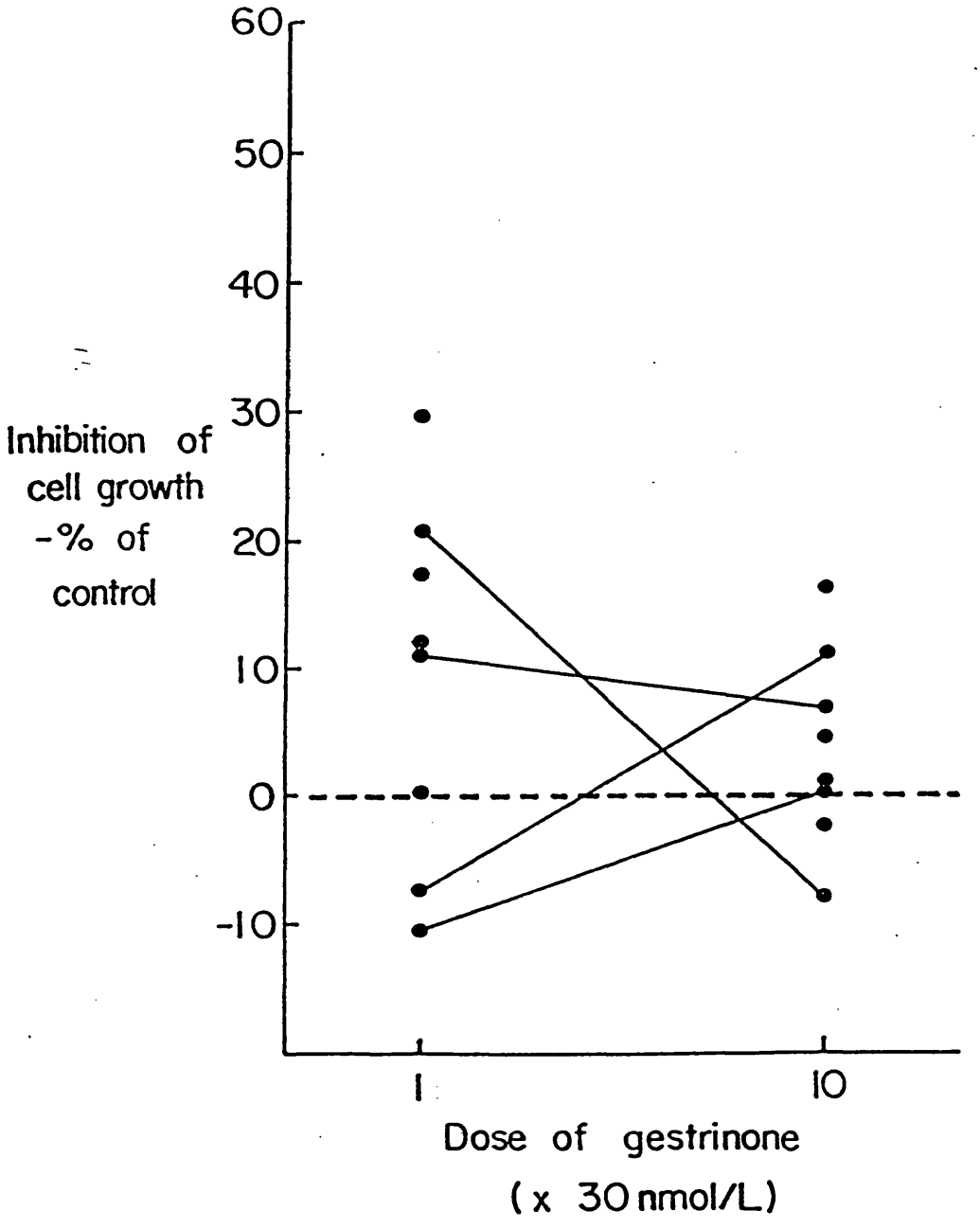


FIGURE 4.5: The effect of Gestrinone on the growth of normal human endometrial cells in culture.

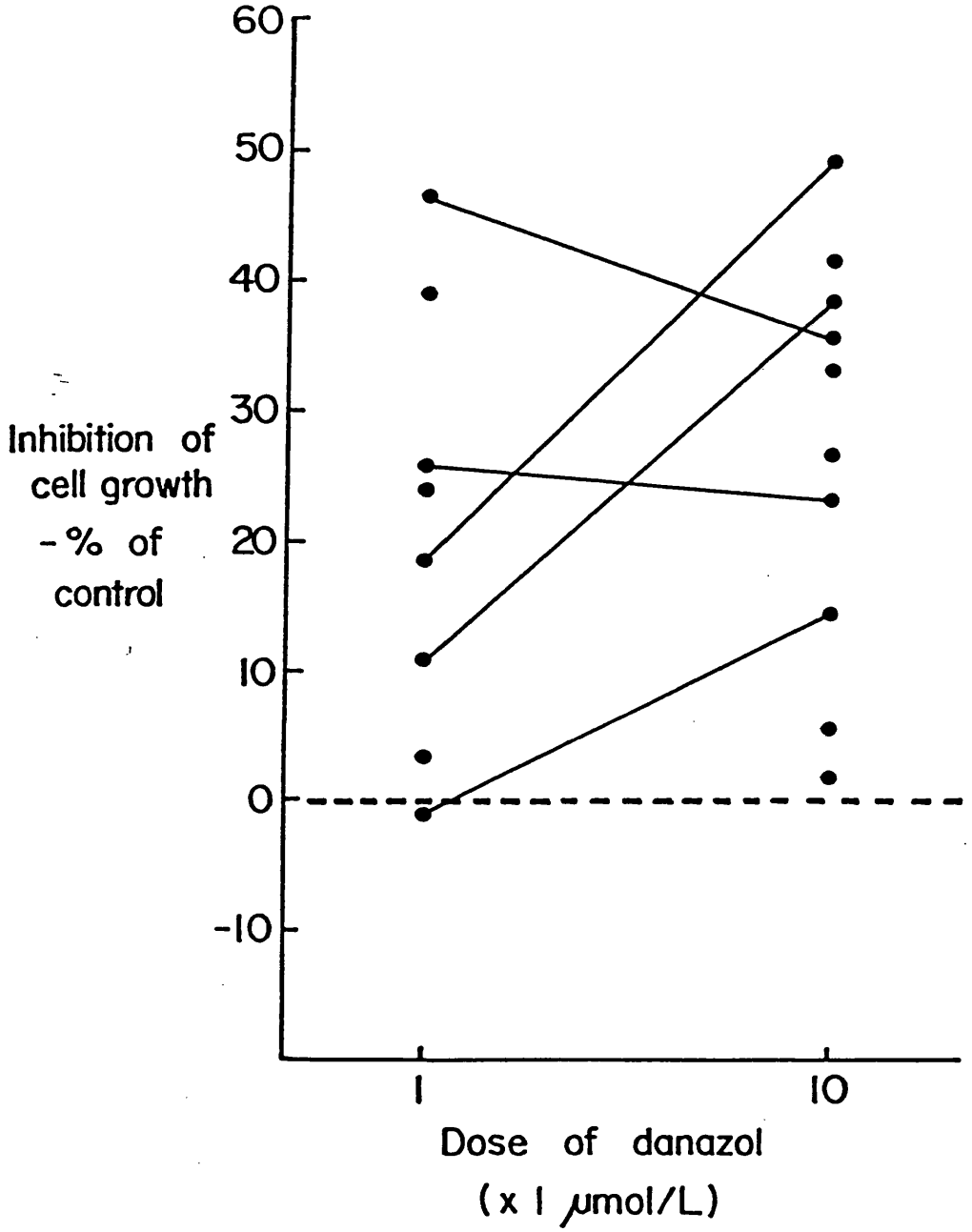


FIGURE 4.6: The effect of Danazol on the growth of normal human endometrial cells in culture

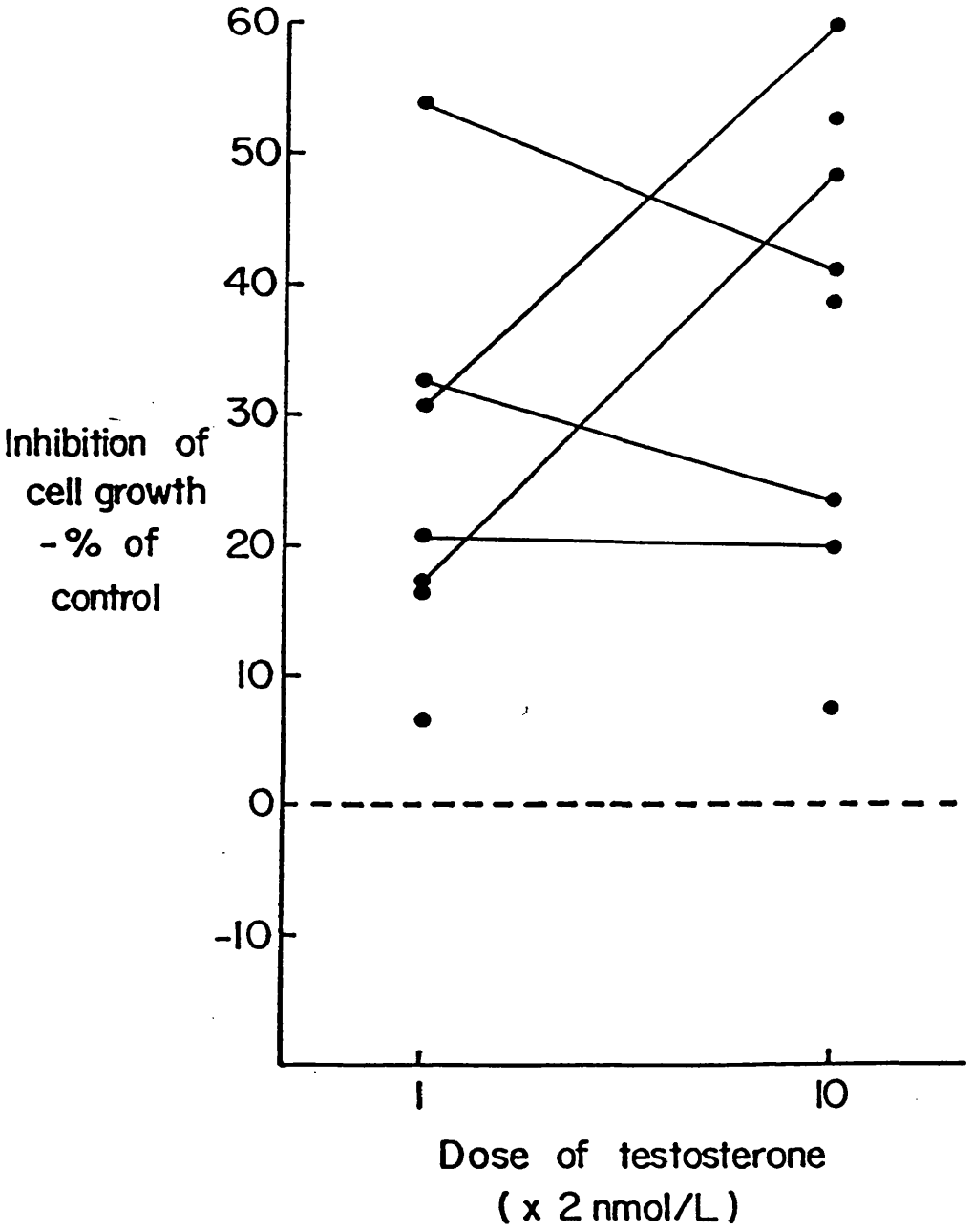


FIGURE 4.7: The effect of Testosterone on the growth of normal human endometrial cells in culture

For gestrinone, there is the suggestion of a minor suppressive effect on endometrial cell growth, but this was neither consistent nor significant. The graph for danazol shows an effect at both doses, in all but one sample , and an obviously greater effect than that seen in the gestrinone samples. In 3 of the 5 samples there appears to be a dose relationship, with an increased inhibition of growth at the higher dose, but the two other samples do not support this finding. Testosterone caused suppression of growth in all samples at both doses, with a more marked effect at the X10 dose. Again, two samples suggest a dose relationship, but this effect was not reproduced in all the samples.

In view of these findings, a further experiment was performed on a sample providing an especially high yield of endometrial cells, comparing the X1 and X10 dose as previously described, and also including a X3 dose, for G, D, and T. The X3 doses were:

Gestrinone 9×10^{-8} M

Danazol 3×10^{-6} M

Testosterone 6×10^{-9} M

The results of this experiment are shown in full in Table 4.6, and are plotted graphically in Figure 4.8. For each steroid there is an increase in percent inhibition with each increment in dose. The control value in the X1 and X10 dishes is extremely similar, and therefore a possible difference in cell number plated would not readily explain any differences between doses.

The relationship between the inhibitory effects of D

TABLE 4.6: RESULTS FROM CELLS CULTURED FROM PATIENT NO 7
THE EFFECT OF GESTRINONE, DANAZOL AND TESTOSTERONE AT x 1, x 3, x 10 DOSE, IN A SINGLE PATIENT SAMPLE

DRUG	DOSE	INDIVIDUAL WELL COUNTS	MEAN WELL COUNT	INDIVIDUAL COUNT AS % OF CONTROL	MEAN %	SD OF % RESULT	SEM OF % RESULT
C		458,360 444,520 399,600	434,160		100		
G	x 1	462,160 444,240 495,200		106.5 102.3 114.1	107.6	6.0	3.4
D	x 1	397,040 360,040 450,000		91.5 82.9 93.3	89.2	5.5	3.2
T	x 1	356,840 362,440 390,640		82.2 83.5 90.0	85.2	4.2	2.4
C		443,520 520,120 538,360	500,667		100		
G	x 3	447,760 524,200 499,760		89.4 104.7 99.4	97.9	7.8	4.5
D	x 3	399,480 399,280 347,160		70.5 70.5 69.3	70.1	0.7	0.4
T	x 3	344,840 317,040 337,200		68.9 63.3 67.4	66.5	2.9	1.7
C		434,680 460,000 435,480	443,387		100		
G	x 10	397,680 394,280 388,960		89.7 88.9 87.7	88.8	1.0	0.6
D	x 10	252,840 277,120 290,840		57.0 62.5 65.6	61.7	4.3	2.5
T	x 10	206,880 248,560 235,720		46.7 56.1 53.2	60.0	4.8	2.8

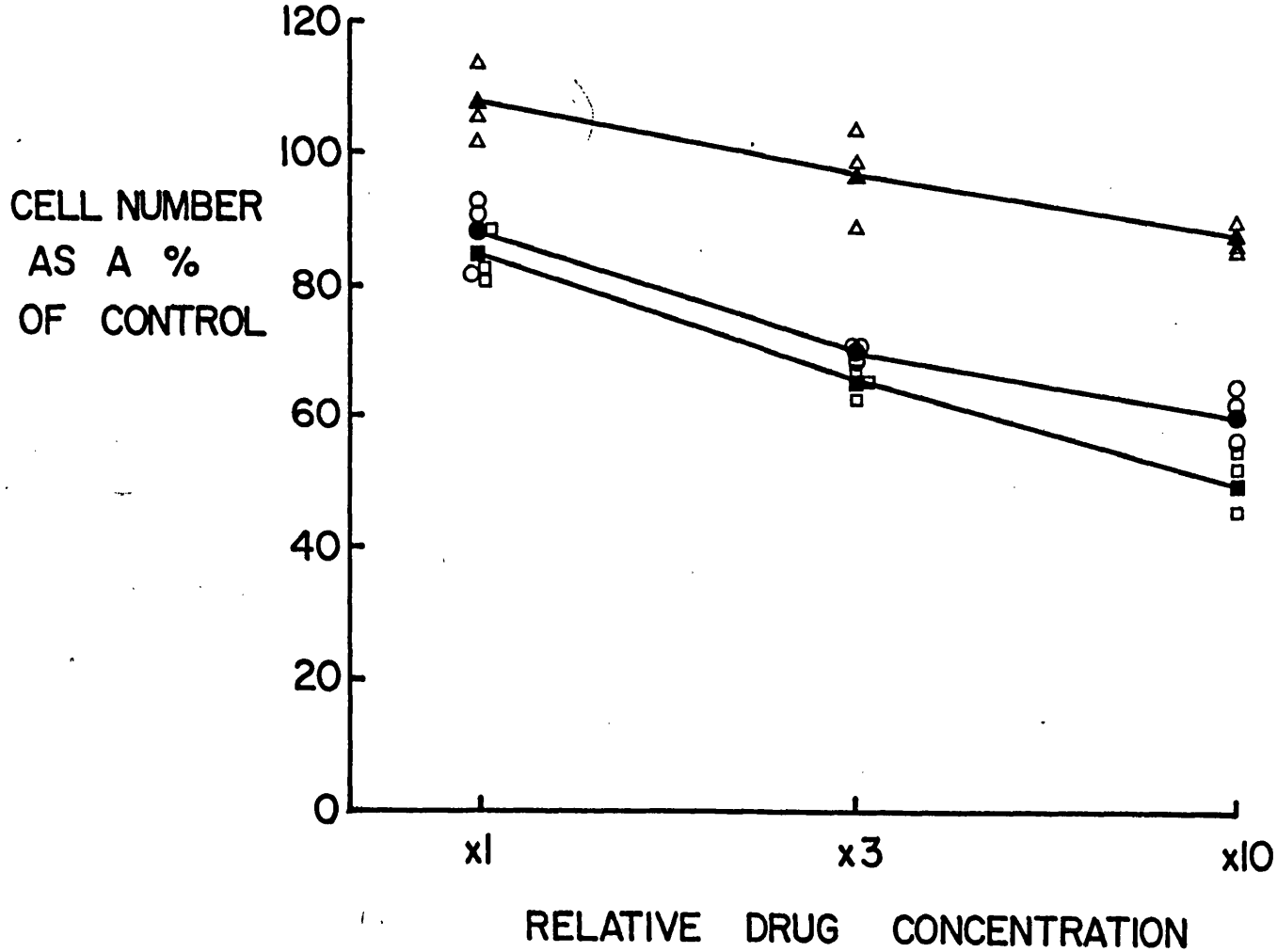


FIGURE 4.8: The effect of Gestrinone ▲ , Danazol ● and Testosterone ■ at the x1, x3 and x10 dose on the growth of human endometrial cells in an individual patient

individual well △ mean of 3 wells ▲

and T in the same samples was examined by linear regression analysis for the X1, X10 and all samples together. A highly significant correlation was found for all points disregarding dose (Figure 4.9) with a regression equation: $y = 0.72x + 3.8$, ($r = 0.83$, $p<0.001$). In addition, there was a statistically significant correlation for both dosages when analysed individually: for the lower doses $r = 0.843$, ($p<0.002$); for the tenfold higher doses, $r = 0.877$, ($p<0.01$).

There was no relationship between the day in the menstrual cycle on which the tissue sample was obtained and the effect of the drugs on cell growth (figure 4.10, 4.11).

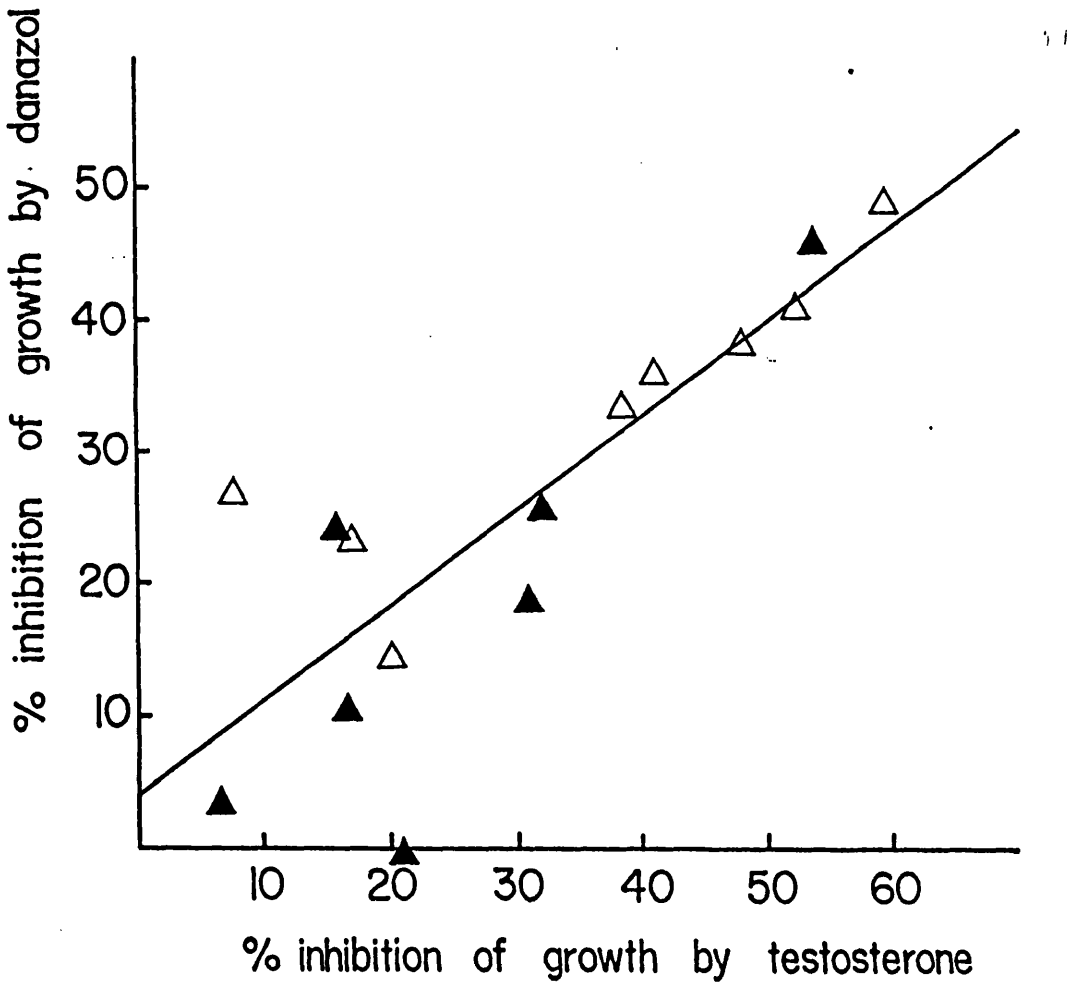


FIGURE 4.9: The correlation between the growth inhibitory effects of Danazol and Testosterone in the same tissue sample ▲ Danazol 1 x 10^{-6}M, T 2 x 10^{-9}M
△ Danazol 1 x 10^{-5}M, T 2 x 10^{-8}M

Regression equation, Y = 0.72 x + 3.8, r = 0.83, p <0.001

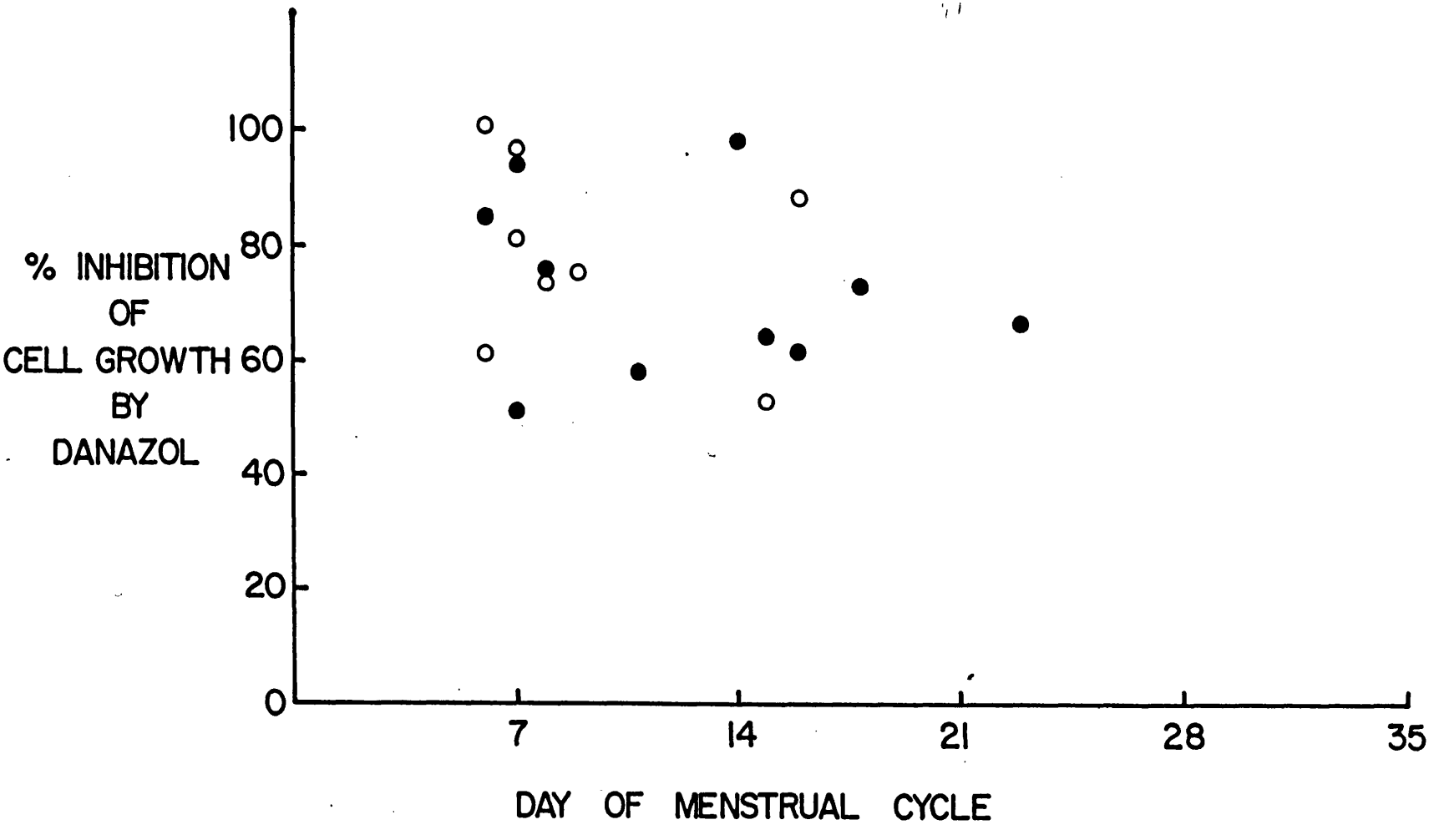


FIGURE 4.10: Relationship of % inhibition of endometrial cell growth by Danazol and day of menstrual cycle ○ x1 dose ● x10 dose

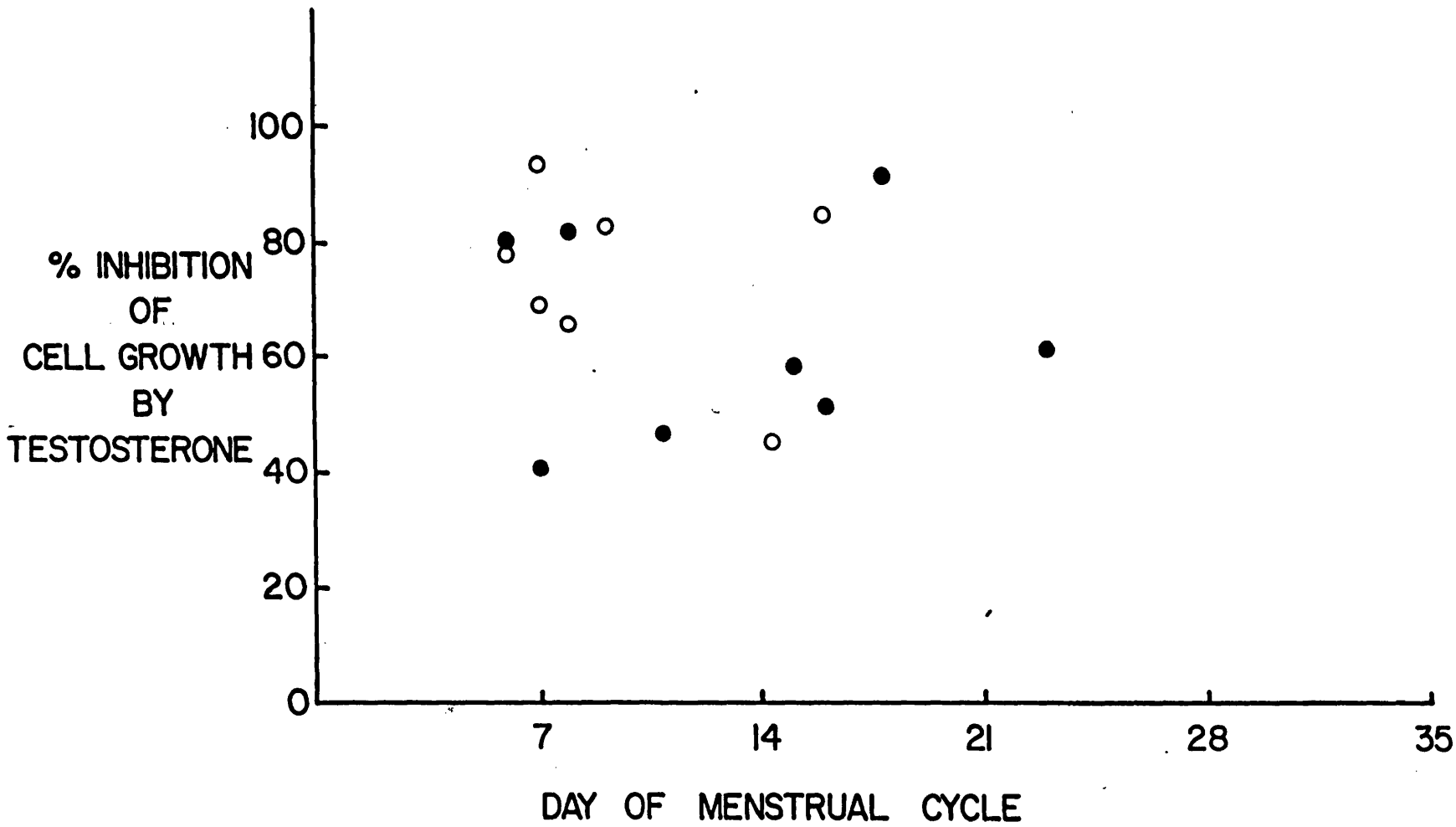


FIGURE 4.11: Relationship of % inhibition of endometrial cell growth by Testosterone and day of menstrual cycle ○ x1 dose ● x10 dose

CHAPTER 14

DISCUSSION

In this study the possibility that danazol and gestrinone have direct inhibitory effects on the growth of human endometrium has been examined in vitro. It has been suggested that such an effect may explain their clinical effectiveness in the treatment of endometriosis, (Barbieri et al, 1979, Chamness et al, 1980) and the possibility that the major metabolites of danazol may themselves inhibit endometrial growth has also been examined, as their biological activity at therapeutic dosages in the displacement of T from SHBG has been demonstrated (Dowsett et al, 1986). Since both danazol and gestrinone increase the biologically active fraction of T, and it has been shown that androgens such as methyl testosterone are effective in the treatment of endometriosis, it is possible that these drugs act through this mechanism and that endogenous T is the biological effector. For this reason the activity of T on endometrial cell growth was also examined.

In early experiments it was determined that endometrial samples from the proliferative and early secretory phases of the menstrual cycle showed the most successful growth, and only samples from this part of the cycle were sought thereafter. Tissue was only collected from women with regular menstrual cycles. However, ovulation during the cycle of collection was not proven by either

endometrial histology or luteal phase progesterone measurement. This may have been relevant as endogenous oestrogen priming of endometrium may influence subsequent in vitro growth.

The endometrial cells in culture demonstrated the typical whorled appearance as described by Kirk et al, 1978. The gross morphology of the cultures was also confirmed as being typical of primary endometrial cell cultures by members of Dr R.J.King's team at the Imperial Cancer Research Fund. More definitive methods of identification of human endometrial cells have recently been reported. Kawanami, 1986, described the combined use immunohistochemistry to identify and tritiated thymidine autoradiography to quantify endometrial stromal cells and Thornton et al, 1989, further developed this technique by double labelling with bromodeoxyuridine and tritiated thymidine. This latter technique allows separate recognition of both the glandular and the stromal elements of endometrium and application of this method would have allowed an estimate of the proportional growth of these two tissue types in the study culture system.

In certain circumstances of particularly poor tissue availability, luteal phase samples were accepted for culture. One of these (patient 13) showed adequate growth and has been included in the results. This finding supported the earlier observations reported on endometrial tissue culture by Papanicolaou and Maddi (1958, 1959), Figge (1960), Notake (1963) and Hiratsu (1968). No attempt was

made to separate the endometrial tissue into the stroma and epithelial components, as it was initially felt important to maintain maximal integrity of the tissue in the culture system. Despite this, it would be interesting to investigate the responses occurring in the separated cellular elements.

The four day culture period was chosen since Kirk and King (1979) had demonstrated that the rate of endometrial growth diminished thereafter due to a gradual increase in the number of polyploid nuclei, although they were unable to explain the specific mechanism for this polyploidization.

Despite the findings of Hiratsu (1968) and Pavlik and Katzenellenbogen (1978) who suggested that oestradiol can stimulate the growth of human endometrial cells in culture, and of Liu and Tseng (1979) who suggested both oestradiol (E2) and progesterone (P) produced a stimulatory effect, it was not found necessary to add either steroid to the culture medium to maintain adequate growth. It was considered possible that the horse serum might have contained significant amounts of E2 or P, and the culture medium (Medium 199 with 10% added horse serum) was therefore analysed by the routine assays available in the Endocrine Department (see Chapter 8), but no detectable levels were found (E2 < 100pmol/l and P < 2nmol/l). The medium used did contain phenol red, however, at the concentration of 50umol/l. Phenol red has been shown to have significant (dose dependent) oestrogenic activity on breast cancer cells

in vitro (Berthois et al, 1986). This concentration of phenol red would be expected to be equivalent to approximately 500pmol/l E2 and consequently it may be considered that oestrogenic stimulation was present in all the culture wells.

A degree of inter-well variation will have been introduced at the various steps of the tissue culture technique, and this may have contributed to the changes noted between test and control cultures. However, the inhibitory effects produced by D and T, at both the X1 and X10 dose were significantly greater than this. Great care was taken at each step to attempt to minimise this possible variation. This included:

1) During cell plating, the cell solution was thoroughly mixed between each aspiration of 1ml of medium containing the 500,000 cells.

2) The drugs and steroids were always added to the multiwell dishes in sequential pattern, so that the plating order could not influence the results.

3) The counting of cells on the coulter counter was performed in a random order.

4) If several multiwell dishes were obtained from one patient sample, and multiple experiments performed (i.e. X1 and X10 dose), the drug/steroid response was compared to the control triplicate of wells in the same multiwell dish on all occasions. Although in most instances the control wells in different multiwell dishes from the same patient were similar, small differences did occur. This may have been due

to minor variations in the culture conditions or differences in the time taken to change the medium and inevitable temperature changes.

5) Horse serum was always filtered prior to addition to Medium 199, to remove any particles which may have affected the coulter counter reading.

Initially, it was considered possible that the coulter counter may be counting cell fragments or debris, or interpreting changes in cell size, rather than cell number. However, the comparability of counts found using the haemocytometer excluded this. The coulter counter was chosen as the method of cell counting because of ease and speed of use. Also the small number of cells actually counted with the haemocytometer may have led to greater error between sample counts. An attempt was made to confirm the findings of an inhibition of cell growth using the DNA assay modified from Karsten and Wollenberger (1972). However, the DNA content in the cells obtained from culturing 500,000 cells was insufficient to detect significant amounts for interpretation using this assay. This technique was therefore discarded at an early stage of the study.

Ideally, time course and further dose relationship experiments would also have been conducted, but the number of experiments was severely limited by the small amounts of tissue available. Priority was given to examining the effect of the drugs and steroids at their therapeutic and X10 doses. A more extensive investigation of a possible dose relationship was conducted on one occasion (Table 4.6 and

Figure 4.8). The trend was to confirm a dose response for G, D and T, but clearly this should be repeated before any conclusions can be drawn.

Overall, the results demonstrated that at therapeutic doses, danazol but not gestrinone had a direct inhibitory effect on the growth of human endometrial cells in culture. If the culture system is representative of the behaviour of endometriotic tissue in vivo, this may be taken as evidence that danazol exerts at least part of its therapeutic effectiveness by a direct action. There was no evidence of a similar effect by the metabolites of danazol, E and 2OHME. However, the potential effects of these compounds was only examined on 4 samples, due to the small amounts of tissue available, and ideally a larger number of tests would have been conducted. Minor effects of the metabolites may not have been detected because of experimental variability. Quantitatively, no metabolite of gestrinone is more important than intact gestrinone. Also found in appreciable amounts are the two monohydroxy derivatives of gestrinone, 16β and 16α hydroxy gestrinone (Salmon et al, 1984). However, as these have been synthesized and their biological activity found to be less than that of gestrinone, the effect of these was not investigated on the endometrial cultures.

The additional observation that T also inhibits endometrial cell growth supports the previous suggestion (Section 3) that the increase in the biologically active fraction of T elicited by both gestrinone and danazol may

also contribute to their mechanism of action. Previous experiments to examine the effects of steroids on endometrium in culture have been performed by Neulen et al (1987), who used ^{3}H-thymidine uptake rather than cell counting to evaluate their responses. They also found a significant reduction in endometrial cell growth with T and danazol, but using higher doses, e.g 10^{-4} for each. At this dose they found that danazol caused a 66% reduction of ^{3}H-thymidine incorporation, being less effective than T (90%) at the same dose. In their study, at the doses used in these experiments, they obtained very similar results for danazol, but did not observe suppression at the lower doses of T. It is difficult to explain the lack of effect described by these authors at the lower concentrations of T. However, Hiratsu (1968) also showed inhibition of endometrial cell growth at all concentrations of testosterone, in the range of 0.0001 to 50 ug/ml.

The lack of effect of gestrinone as compared to danazol might be dose-related but this seems unlikely because the higher dose of gestrinone was only 3-fold less than the lower dose of danazol. The observation that the effects of danazol and T on cell growth were closely correlated suggests that there is an inherent sensitivity to these two compounds and that the mechanism by which they exert their direct inhibitory effect may be through a common pathway, possibly via androgen receptors. It is known that danazol binds to androgen receptors with a relative affinity of 35% that of T (Azadian-Boulanger et al, 1984). Further

evidence for this being a common pathway might be sought by adding an anti-androgen (e.g. flutamide or anandron) to the endometrial tissue cultures to see if the effect of D and T were blocked.

Theoretically steroid binding proteins present in the horse serum used in the medium might have influenced the free concentration of the hormones added to the cultures and thereby affected their biological activities. The proportion of testosterone in the protein free form was measured and found to be between 50-60%. This was not increased by heating of the medium (with horse serum) to 60 C for one hour which would be expected to abolish any binding by high affinity binding proteins which are generally heat-sensitive. This therefore indicates that the binding in the horse serum is exclusively non-specific and probably largely to albumin. The low affinity of such binding would not be expected to affect the biological availability to the cells in culture of the administered steroids.

It would have been preferable to have cultured endometriotic rather than endometrial tissue in these experiments, but suitable, adequate samples were rarely available, and further it appeared as Willemsen et al (1985) had previously shown, that only small numbers of cells were obtained for culture from endometriotic samples. Similar receptor contents have been described for endometrium and endometriotic tissue (Berqvist et al, 1984, Saraglu et al, 1985), or slightly lower for the latter tissue (Berqvist et

al, 1981, Kaupilla et al, 1984), which may suggest corresponding hormone sensitivity. However, the evidence in the literature suggests that endometriotic tissue does not show the same cyclical variations in receptor content in vivo as that seen in eutopic endometrium. Vierikko et al (1985) gave danazol to patients for a range of 1-30 days and found no change in ER and PR receptor concentrations, and ER content was also constant throughout the cycle in endometriotic tissue in rhesus monkeys (Stenfeld et al, 1988). In immature rats, short term (4 days) treatment caused an increase in ER concentration and binding, but longer term (14 days) therapy a marked decrease in ER binding capacity (Musich et al, 1981). However, these authors concede that this may not necessarily have been a direct effect of the drug on receptor content, and Tamaya et al (1984) conversely found in women, that danazol given in the luteal phase caused decreased endometrial ER and PR and long term (more than one month) therapy resulted in increased levels. As the endometrium after one month of danazol was atrophic and the samples they obtained were consequently small, there must be doubt as to the accuracy of their results.

As previously described, both histological and ultrastructural examination of endometriotic lesions reveals some differences in the morphology of the tissue, between patients and in some cases between samples. Schweppe and Wynn (1984) further differentiated the ultrastructural pattern of endometriotic tissue in relation to its response

to danazol i.e.

1) poorly differentiated endometriosis does not respond to danazol

2) highly differentiated endometriosis responds to danazol in 80% of cases

3) endometriosis with mixed areas of differentiation are eliminated or arrested in the proliferative phase.

It would appear that endometriotic tissue is comprised of endometrial tissue of variable differentiation and that the most highly differentiated most closely resembles uterine endometrium in terms of morphology, steroid receptor content and hormonal response. It may be therefore, that the observations made on the growth of endometrial tissue in culture in response to danazol and T can only be used as a model for well differentiated endometriotic tissue. However, the lack of cyclical and hormonal response seen in poorly differentiated tissue may result from bleeding, necrosis and fibrotic scarring which interferes with the normal blood supply and consequently the availability of hormones to the tissue. This may explain the variation seen in the clinical response to medical treatment and the failure of approximately 20% of patients to improve with this form of therapy.

SUMMARY

This study provides further evidence that danazol, though not gestrinone, acts by a direct effect on endometrial tissue. If endometriotic tissue exhibits the same sensitivity, such a direct effect may partially explain the clinical efficacy of danazol in the inhibition of the growth of endometriotic tissue .

Danazol and gestrinone have both been shown (Section 3) to effect testosterone binding, and to increase the biological availability of T in vivo. Since T was also found to have a direct growth inhibitory effect on endometrial tissue in vitro, these changes in protein binding of testosterone may also contribute to the mechanism of action of these drugs.

SECTION 5

CONCLUSIONS AND SUMMARY

CHAPTER 15

OVERALL CONCLUSIONS AND THE IMPLICATIONS FOR FUTURE RESEARCH

The basic aims of medical treatment of endometriosis are to produce atrophy of endometriotic lesions. It is plain that the mode of action by which danazol and gestrinone achieve any response is complex.

Danazol would seem to function in a number of ways:

1) There is evidence to support the fact that gonadotrophin control is influenced by danazol. It is probable that this is an interference with the feedback mechanism on the hypothalamus, disturbing the release of LHRH and thus, LH and FSH release. Basal levels of LH and FSH remain unchanged, but pulsatile release is probably altered. As a result folliculogenesis in the ovary is inhibited.

2) Danazol has been demonstrated to inhibit steroidogenesis and in combination with inhibited folliculogenesis, E2 levels are basal. However, the concept that there is a hypo-oestrogenic state is questionable. There are no studies which have recorded E2 levels in the post-menopausal range, merely in the anovulatory range. It is more plausible that the loss of folliculogenesis and the creation of an acyclic environment is the essence of the "hypo-oestrogenism". Whilst Dickey et al (1984) demonstrated the relationship of clinical response to E2 levels, it is most probable that those patients with higher E2 levels were

inadequately treated.

3) There is unequivocal evidence that danazol suppresses SHBG levels. This results from an inhibition of synthesis and as it is hepatic in origin, a suppression of other protein synthesis would be expected. However, not all plasma proteins are affected in this way and so the exact hepatic influence awaits clarification. The overall effect of lowered levels of SHBG is a decrease in androgen binding capacity. The circulating androgens and danazol, E and 2OHME therefore, compete for a reduced number of binding sites and a resultant increase in % free T and concentration of free T has been demonstrated and supported by this thesis. An androgenic environment is thus created, and further androgenic activity is manifest by the direct action of danazol and its metabolites, E and 2OHME.

4) This thesis further elucidates the mode of action of danazol at a cellular level. It has been demonstrated that danazol and T, but not E or 2OHME, directly inhibit endometrial cell growth in vitro, and that this is dose-related. This work confirms the previously held theory that the androgenic environment is fundamental to the efficacy of the drug.

5) Danazol and T are known to occupy the PR and AR in the endometrium which reduces E2 receptor production. Thus danazol indirectly creates a local anti-oestrogenic effect with resultant disturbance of cellular oestrogen metabolism.

6) Finally, danazol may manifest some effect on the immune system. One of the current theories of the aetiology of

endometriosis is that it is an auto-immune disease, and preliminary evidence exists to suggest that danazol does inhibit lymphocyte function, in a similar manner to dexamethasone. Whether danazol will be shown to effect peritoneal macrophage activity remains to be seen.

Danazol is therefore, a drug with multiple modes of action and the complexity of the endocrine changes and the local effects make it difficult to suggest which mechanisms are the most important. It is probable that the effects are synergistic.

Gestrinone is a drug with some similarities to danazol although the data in this thesis demonstrate some fundamental differences. Gestrinone has some actions which are comparable:

1) There is an interference with gonadotrophin release in a manner which is probably the same as danazol. The resultant anovulation ensues in the same way.

2) Gestrinone binds to the androgen receptor with a greater affinity than danazol.

3) Progesterone receptor binding seems to be similar to danazol and thus the local antioestrogenic effects outlined for danazol, apply to gestrinone.

4) Gestrinone inhibits SHBG production and thus the circulating % free T and concentration of free T levels are the same as with danazol. However, there is evidence that the increasing free T is only a result of decreasing SHBG

and that there is no direct displacement of T from SHBG. It is apparent that this difference is due to the metabolites of danazol, especially E which displaces T from SHBG during the first few days of therapy, rather than danazol or gestrinone.

However:

5) There is no evidence that gestrinone has a significant effect on steroidogenesis.

6) This study has failed to demonstrate a direct action of gestrinone on endometrial cell growth in vitro.

This suggests that the action of the drug is manifested indirectly through the changes in the androgenic state and a loss of cyclicity, rather than by any direct cellular effect (Figure 5.1).

The efficacy of danazol and gestrinone is difficult to assess as the end-point is so variable. The effect of treatment on infertility and subsequent pregnancy rate is an inadequate parameter because of the multiple factors involved in the failure to conceive and the relatively poor methods of defining the problems of the female and the male, mean that randomized studies of "pure" populations are almost impossible to mount. Pain is the other major symptom but again the use of a subjective end point is difficult to evaluate. The possiblity of other psychosomatic and functional disease processes manifesting themselves through pelvic pain is highly likely and thus complicate this index. A further problem is the poor correlation between symptoms

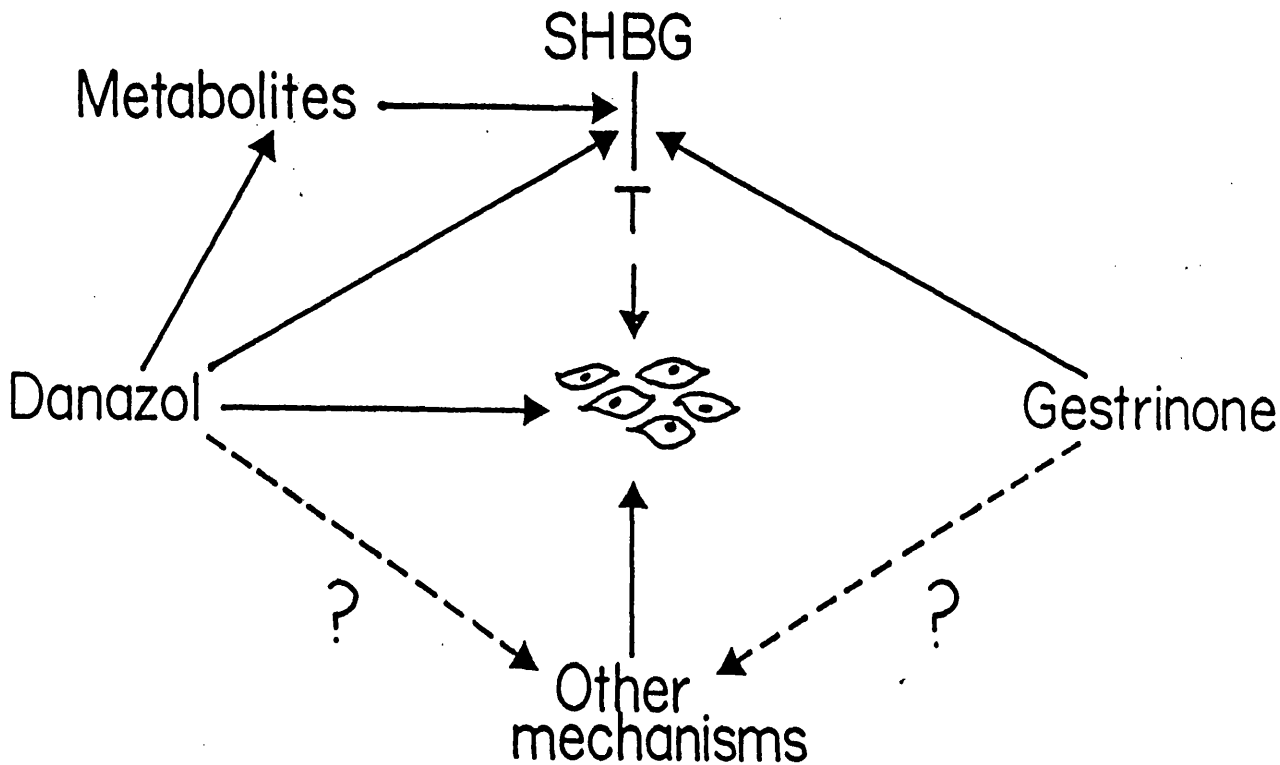


FIGURE 5.1: Diagrammatic representation of possible mechanisms of action of Danazol and Gestrinone

of pain and the severity of the endometriosis. This disparity makes even the evaluation of endometriosis visually difficult to assess.

Some hope was generated by the reports of the measurement of the antigen, CA 125 (Barbieri et al, 1986, Barbieri, 1986, Patton et al, 1986, Pittaway and Fayez, 1986) in patients with endometriosis. The initial suggestion of an association of CA 125 with the severity of the disease has subsequently not been substantiated and, thus, we still await a finite denominator of response.

As outlined earlier, there is a paucity of randomized controlled trials assessing drug therapy in endometriosis and the published data of most non-randomized studies has led to an unrealistic appreciation of the efficacy of the drugs, and also the patients in whom they may be considered valuable. The lack of evidence that medical therapy is of value for infertile women with minimal endometriosis is the most obvious example.

The reported recurrence rates of endometriosis following medical therapy may be fallacious. It might be suggested that "recurrence" is a misnomer as evidence from this study demonstrates that it is more likely to be a failure to respond. Why do some endometriotic deposits not respond to therapy? There are a number of possibilities:

1) There are some patients whose AFS scores improve on therapy, but "cure" is not effected. Perhaps the treatment may not have been administered for long enough. At present, it is accepted practice to treat for 6 months, but this

length of time seems to have been somewhat arbitrarily selected and probably based on the "pseudopregnancy" data of the past. Individual patients may require less than 6 months therapy, and some evidence exists to support this (Brosens et al, 1987). Further studies need to be performed to elucidate these points.

2) Some endometriotic lesions do not undergo histological changes during hormone stimulation and thus some endometriotic cells may be relatively independent of E2 and P control. This may result from the lower concentration of E2 and P receptors in some endometriotic tissue. Data also exist to show that there are morphologically heterogeneous populations of endometriotic cells and some resemble endometrium more than others. It may be that the less well differentiated the cell pattern, the poorer the response to medical therapy.

The implications for future research are:

1) the clinical assessment of the natural history of endometriosis still requires clarification. Although the study reported by Thomas and Cooke (1987a) suggests that AFS scores increase over a short period of time (six months), there is no epidemiological information to describe the long term natural course of the disease. Thus the implications for preventative measures or therapeutic regimes remain speculative.

2) Further studies are required to determine the effect of danazol and gestrinone on biologically active

gonadotrophins, as this may reveal an underlying effect of the drugs on gonadotrophin release and folliculogenesis.

3) The necessity for a hypo-oestrogenic environment seems to be debatable in danazol and gestrinone therapy, but the effect of the menopause and LHRH analogues would support the notion that true hypo-oestrogenism is effective in eradicating endometriotic lesions. Thus, it is likely that the combination of effective hypo-oestrogenisation and the use of danazol or gestrinone may improve response rates and a study would seem to be warranted.

4) The cell culture work presented has demonstrated an inhibitory effect of danazol and T, but not gestrinone, on endometrial cell growth. However, the culture system did not differentiate the stromal and epithelial elements. Cunha et al (1983) hypothesised that steroids regulate endometrial epithelial function by first causing changes in stromal secretion of paracrine growth factors which might subsequently control epithelial events. It may be that endometriotic lesions are under the same regulatory control or they may be different. This work is awaited with interest and the definition of the control of endometriotic cell growth may lead to the development of new therapeutic regimes.

5) Finally, the immunological theories of endometriosis require investigation and are potentially the most exciting areas of research. If these hypotheses finally explain the aetiology of endometriosis, the mystery of this disease will be revealed, and new therapies directed towards the cell

mediated immune response may be developed.

The drugs danazol and gestrinone seem to work in a wide variety of ways and they have been demonstrated to have some similar actions. The creation of an androgenic environment is a major common factor. Evidence is presented to support the direct action of danazol, but not its metabolites or gestrinone, on endometrial tissue. This thesis provides further clarification of the mechanism of action of danazol and gestrinone, and hopefully dispels some of the erroneous endocrine anomalies, leading to an improved understanding of the drugs and their limited role in the treatment of endometriosis.

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APPENDIX

TABLE A.1: THE EFFECT OF GESTRINONE ON SERUM CONCENTRATION OF LH (NORMAL RANGE: 3 - 11 IU/L)

PATIENT	PRE	M1	M2	M3	M4	M5	M6	M7
2	8.4	3.9	3.3	4.3	2.0	5.9	-	6.7
3	2.6	9.9	1.7	7.1	1.3	3.2	-	1.7
7	-	-	-	-	-	-	-	-
8	4.3	5.5	6.2	16	18	10	9.8	3.4
10	8.4	6.0	6.3	3.8	13	6.2	4.6	46
11	17	7.2	2.5	6.5	6.4	4.5	-	45
12	5.5	3.6	5.0	2.3	4.7	-	4.5	6.2
14	4.9	1.3	3.5	-	-	-	-	-
17	13	12	5.3	11	7.4	-	-	-
18	21	9.6	7.5	4.4	-	6.6	13	>50
23	>50	7.0	8.4	8.4	4.5	5.0	-	-
25	6.9	9.6	12	-	15	7.4	-	-
28	5.7	8.0	4.7	8.3	14	3.6	7.6	11
29	7.6	8.5	7.9	14	7.4	13	-	3.8
30	3.6	24	8.2	2.1	8.1	21	-	-
31	21	8.4	7.2	7.0	4.7	20	2.7	>50
35	6.8	15	-	-	-	-	-	-
36	7.7	15	6.2	4.1	9.9	9.9	4.7	9.0
39	7.1	7.6	7.1	6.5	10	7.1	-	6.4
40	4.3	5.2	4.0	6.7	5.8	5.2	4.4	5.1
41	5.2	4.0	8.7	4.8	4.9	6.6	2.5	3.2
43	2.2	4.3	2.1	2.9	2.0	-	-	6.3
44	14	-	-	-	-	-	-	-
48	4.5	22	18	4.0	11	14	16	7.8
49	5.0	>50	21	32	5.5	30	39	46
321	5.7	6.8	13	8.3	7.2	12	-	-
N	25	24	23	21	21	19	11	17
GM	7.3	8.2	6.4	6.3	6.5	7.8	6.3	10.7
UCI	9.2	10.5	8.2	8.2	8.5	10.3	9.1	14.4
LCI	5.8	6.5	4.1	4.9	5.0	5.9	4.3	8.0

GM = GEOMETRIC MEAN;
UCI AND LCI = UPPER AND LOWER 95% CONFIDENCE INTERVALS

TABLE A.2: THE EFFECT OF DANAZOL ON SERUM CONCENTRATION OF LH
(NORMAL RANGE: 3 - 11 IU/L)

PATIENT	PRE	M1	M2	M3	M4	M5	M6	M7
1	4.9	4.3	3.3	6.0	2.7	3.6	-	7.5
4	12	13	8.8	5.3	4.9	5.1	7.1	1.6
5	2.1	11	6.2	10	-	8.8	6.2	3.5
6	6.5	10	12	12	12	7.7	-	4.5
9	7.7	8.9	5.1	4.6	2.6	8.8	3.1	12
13	5.3	13	10	14	8.6	4.6	-	13
15	3.5	4.1	11	5.1	2.5	3.1	-	2.5
16	8.2	10	3.4	4.0	4.5	4.6	5.4	4.8
19	22	32	-	-	-	-	-	-
20	7.8	7.7	5.7	7.6	2.7	3.4	-	>50
21	12	5.6	5.0	5.2	12	10	-	12
22	4.6	8.2	8.2	9.8	5.1	7.8	-	-
24	6.0	3.6	4.2	3.3	3.4	-	-	3.4
26	5.1	15	7.7	10	12	10	6.5	-
27	40	8.5	4.4	3.0	-	9.7	3.9	-
32	8.7	7.3	12	8.4	5.3	20	22	23
33	5.2	7.9	9.1	10	5.7	2.3	-	-
34	9.6	14	11	13	9.5	11	-	8.8
37	8.1	12	3.6	3.5	3.6	4.6	8.3	6.9
38	20	1.7	-	-	-	-	-	-
42	7.5	2.9	5.6	2.9	3.6	5.3	5.0	3.8
45	21	-	-	-	-	-	-	-
46	4.8	12	8.4	11	5.3	4.2	-	5.6
47	1.7	4.5	7.7	3.1	5.5	4.9	-	-
50	35	7.1	-	6.4	4.4	3.7	4.3	6.7
N	25	24	21	22	20	21	10	17
GM	8.0	8.1	7.3	6.9	5.5	6.3	6.5	7.4
UCI	10.0	10.4	9.5	8.9	7.2	8.2	9.6	10.0
LCI	6.3	6.4	5.6	5.3	4.2	4.8	4.4	5.5

GM = GEOMETRIC MEAN
UCI AND LCI = UPPER AND LOWER 95% CONFIDENCE INTERVALS

TABLE A.3: THE EFFECT OF GESTRINONE ON SERUM CONCENTRATION OF FSH (NORMAL RANGE: 0.5 - 5.0 IU/L)

PATIENT	PRE	M1	M2	M3	M4	M5	M6	M7
2	5.2	10	6.0	7.0	0.6	8.2	-	7.7
3	0.6	3.2	3.0	4.6	0.7	0.6	-	0.7
7	-	-	-	-	-	-	-	-
8	3.6	4.4	8.7	6.8	9.2	7.6	4.8	3.3
10	2.6	3.1	4.6	4.4	5.5	7.2	3.6	14
11	4.1	1.3	1.3	4.7	1.5	4.4	-	10
12	7.9	4.3	7.8	0.9	6.4	-	2.2	1.8
14	3.1	0.9	4.7	-	-	-	-	-
17	4.3	2.8	6.9	3.5	3.3	-	-	-
18	2.9	6.0	6.6	4.3	-	4.8	4.8	13
23	14	3.9	3.0	3.3	3.1	3.4	-	-
25	4.7	4.2	4.4	-	2.1	2.2	-	-
28	3.7	2.4	1.8	2.8	8.1	1.8	4.5	7.7
29	1.8	4.4	4.5	5.4	5.2	4.9	-	0.6
30	1.8	8.0	6.4	1.1	6.4	7.7	-	-
31	13	7.0	5.2	13	8.2	16	2.3	>20
35	6.0	3.8	-	-	-	-	-	-
36	4.0	6.7	5.0	2.8	6.6	5.4	2.3	4.1
39	7.1	5.5	5.2	5.5	4.9	5.5	-	7.9
40	5.8	5.8	5.6	8.0	6.3	3.4	6.2	8.6
41	3.4	1.8	5.3	3.9	3.9	4.4	4.3	5.2
43	1.7	5.0	3.5	2.9	2.8	-	-	5.2
44	1.3	-	-	-	-	-	-	-
48	2.6	7.5	6.2	6.8	2.8	8.8	7.1	6.4
49	16	6.8	9.5	2.2	7.9	7.7	7.2	5.6
321	2.2	3.7	6.2	5.8	5.2	5.3	-	-
N	25	24	23	21	21	19	11	17
MEAN	4.9	4.7	5.3	4.8	4.8	5.8	4.5	7.2
STD	4.0	2.2	2.0	2.7	2.5	3.4	1.8	5.3
SEM	0.80	0.46	0.41	0.59	0.55	0.77	0.54	1.2

TABLE A.4: THE EFFECT OF DANAZOL ON SERUM CONCENTRATION OF FSH
(NORMAL RANGE: 0.5 - 5.0 IU/L)

PATIENT	PRE	M1	M2	M3	M4	M5	M6	M7
1	12	6.3	3.0	6.0	3.8	7.0	-	8.5
4	5.0	8.1	7.6	5.6	5.2	5.9	5.7	1.3
5	1.4	7.2	8.0	8.4	-	7.4	6.4	2.1
6	6.9	5.7	7.8	8.6	7.9	6.1	-	5.6
9	1.7	3.9	5.3	4.6	0.6	5.9	4.5	2.3
13	1.8	5.5	5.0	6.0	4.4	5.0	-	2.9
15	1.4	5.4	6.0	3.5	3.0	6.6	-	0.8
16	3.2	9.2	4.3	3.7	6.5	5.7	5.8	4.3
19	2.7	6.7	-	-	-	-	-	-
20	6.0	4.8	3.0	4.2	1.8	2.8	-	14
21	7.0	3.5	2.5	2.6	3.9	6.0	-	8.9
22	1.7	4.7	3.8	4.6	4.6	6.4	-	-
24	0.9	2.4	2.9	2.4	2.9	-	-	2.2
26	1.4	4.6	4.7	4.5	4.9	4.1	4.1	-
27	2.5	5.6	4.4	3.6	-	4.8	4.4	-
32	4.3	2.2	5.7	5.8	1.5	7.7	6.0	5.4
33	2.4	6.5	6.6	3.7	7.6	1.5	-	-
34	4.9	6.3	5.7	6.0	6.2	4.6	-	5.0
37	2.7	6.5	4.6	2.9	3.0	5.1	9.0	5.7
38	4.9	2.3	-	-	-	-	-	-
42	2.1	2.3	2.2	1.4	2.8	2.1	3.3	0.7
45	4.4	-	-	-	-	-	-	-
46	4.2	6.6	4.4	4.6	3.1	3.9	-	4.0
47	2.1	3.9	6.1	4.6	5.8	7.3	-	6.8
50	7.9	3.9	-	3.6	3.7	3.5	5.0	3.6
N	25	24	21	22	20	21	10	18
MEAN	3.8	5.2	4.9	4.6	4.2	5.2	5.4	4.7
STD	2.6	1.9	1.7	1.8	2.0	1.7	1.6	3.4
SEM	0.52	0.38	0.37	0.38	0.44	0.38	0.50	0.79

TABLE A.5: THE EFFECT OF GESTRINONE ON SERUM CONCENTRATION OF PROLACTIN (NORMAL RANGE: 100 - 620 mIU/L)

PATIENT	PRE	M1	M2	M3	M4	M5	M6	M7
2	470	80	70	140	240	130	-	-
3	220	=50	80	100	140	130	-	180
7	-	-	-	-	-	-	-	-
8	400	520	680	770	490	510	800	270
10	450	150	140	270	170	250	350	730
11	80	100	80	90	80	60	-	130
12	320	250	260	370	220	-	300	230
14	130	230	100	-	-	-	-	-
17	180	290	210	140	270	-	-	-
18	2000	980	1200	450	-	660	680	3500
23	630	190	110	80	110	90	-	-
25	260	300	350	-	640	360	260	-
28	-	310	320	290	210	340	210	240
29	230	210	190	210	220	230	-	260
30	220	190	170	220	170	160	-	-
31	370	570	350	360	490	350	390	640
35	160	430	-	-	-	-	-	-
36	390	310	280	240	380	380	240	330
39	280	430	210	150	210	290	-	170
40	170	150	210	210	250	230	-	170
41	190	180	170	170	170	180	150	160
43	100	100	90	90	100	-	-	120
44	290	-	-	-	-	-	-	-
48	540	770	760	420	490	510	310	600
49	820	380	300	350	360	280	300	340
321	200	250	200	170	140	270	-	-
N	24	24	23	21	21	19	11	16
GM	289	245	212	210	238	225	206	276
UCI	334	285	247	246	279	267	259	332
LCI	250	212	182	179	203	190	165	229

GM = GEOMETRIC MEAN
UCI AND LCI = UPPER AND LOWER 95% CONFIDENCE INTERVALS

TABLE A.6: THE EFFECT OF DANAZOL ON SERUM CONCENTRATION OF PROLACTIN (NORMAL RANGE: 100 - 620 mIU/L)

PATIENT	PRE	M1	M2	M3	M4	M5	M6	M7
1	120	130	<50	80	170	90	-	210
4	310	200	320	180	220	220	250	80
5	130	140	90	180	-	160	150	330
6	320	410	280	320	240	200	-	210
9	140	100	120	80	240	60	90	140
13	750	480	440	350	230	210	-	540
15	120	100	140	130	90	110	-	170
16	290	270	160	200	170	190	260	250
19	180	190	-	-	-	-	-	-
20	130	390	530	560	170	170	-	460
21	320	330	280	360	380	280	-	430
22	560	230	260	180	230	270	-	-
24	350	160	150	160	150	-	-	210
26	940	460	230	300	460	340	230	-
27	480	170	110	210	-	100	120	-
32	870	940	1200	880	1200	780	920	980
33	270	190	160	190	160	150	-	-
34	790	650	600	1100	360	760	-	680
37	390	160	150	270	160	160	210	270
38	220	230	-	-	-	-	-	-
42	750	280	440	690	330	330	230	330
45	250	-	-	-	-	-	-	-
46	200	280	260	270	150	190	-	170
47	220	280	290	270	270	300	-	-
50	170	150	-	<90	<90	<90	<90	110
N	25	24	21	22	20	21	10	17
GM	300	242	214	240	209	189	197	260
UCI	346	280	251	281	246	222	250	311
LCI	260	209	183	205	177	161	155	217

GM = GEOMETRIC MEAN
UCI AND LCI = UPPER AND LOWER 95% CONFIDENCE INTERVALS

TABLE A.7: THE EFFECT OF GESTRINONE ON SERUM CONCENTRATION OF OESTRADIOL (NORMAL RANGE: 100 - 1400 pmol/L)

PATIENT	PRE	M1	M2	M3	M4	M5	M6	M7
2	430	310	350	590	1180	650	-	1080
3	520	360	280	280	220	1670	-	730
7	-	-	-	-	-	-	-	-
8	540	310	160	150	210	210	340	210
10	260	170	130	110	560	140	360	360
11	140	920	<90	1080	630	110	-	350
12	100	110	170	520	160	-	740	310
14	780	720	150	-	-	-	-	-
17	280	920	120	910	890	-	-	-
18	570	170	210	140	-	190	140	840
23	1100	170	300	210	230	190	-	-
25	160	-	230	-	520	990	170	-
28	200	240	640	460	170	700	350	270
29	640	180	250	180	220	210	-	680
30	530	310	410	530	300	480	250	-
31	420	180	380	210	130	500	230	-
35	240	340	-	-	-	-	-	-
36	410	180	320	190	270	250	330	550
39	350	290	220	300	260	210	-	220
40	-	-	260	200	310	180	-	-
41	380	430	200	120	110	250	180	180
43	-	240	190	190	130	-	-	220
44	-	-	-	-	-	-	-	-
48	350	250	230	130	100	160	=90	130
49	740	190	120	220	130	160	150	190
321	130	110	120	160	100	=90	-	-
N	22	22	23	21	21	19	12	15
GM	347	269	215	265	251	283	261	360
UCI	435	337	268	335	317	362	358	477
LCI	277	214	172	210	199	221	190	272

GM = GEOMETRIC MEAN
UCI AND LCI = UPPER AND LOWER 95% CONFIDENCE INTERVALS

(WHEN OESTRADIOL = <90, 90 WAS USED FOR THE CALCULATION OF THE MEAN)

TABLE A.8: THE EFFECT OF DANAZOL ON SERUM CONCENTRATION OF OESTRADIOL (NORMAL RANGE: 100 - 1400 pmol/L)

PATIENT	PRE	M1	M2	M3	M4	M5	M6	M7
1	200	260	380	370	180	240	-	1540
4	420	160	130	150	180	150	140	250
5	290	110	<90	100	-	110	150	<90
6	230	190	180	160	170	150	-	140
9	750	160	<90	200	170	210	160	420
13	790	160	350	210	140	230	-	660
15	270	220	190	120	90	110	-	350
16	280	280	240	370	230	270	860	470
19	360	<90	-	-	-	-	-	-
20	170	100	120	320	<90	<90	-	760
21	340	120	430	520	390	<90	-	180
22	1300	130	<90	140	160	120	-	-
24	310	190	230	270	310	-	-	240
26	250	220	200	200	120	200	160	-
27	580	120	120	<90	-	170	100	-
32	480	700	620	270	690	230	340	340
33	280	260	260	410	210	310	-	-
34	320	160	150	250	190	480	-	340
37	730	350	390	590	520	270	-	340
38	1500	130	-	-	-	-	-	-
42	760	200	-	290	670	270	-	-
45	660	-	-	-	-	-	-	-
46	430	250	-	270	250	240	-	420
47	460	240	270	270	360	280	-	1100
50	410	260	-	330	310	310	320	170
N	25	24	19	22	20	21	8	17
GM	427	194	216	243	219	200	246	356
UCI	527	241	277	307	280	254	365	464
LCI	346	156	168	193	172	158	166	273

GM = GEOMETRIC MEAN
UCI AND LCI = UPPER AND LOWER 95% CONFIDENCE INTERVALS

(WHEN OESTRADIOL = <90, 90 WAS USED FOR THE CALCULATION OF THE MEAN)

TABLE A.9: THE EFFECT OF GESTRINONE ON SERUM CONCENTRATION OF SHBG (NORMAL RANGE: 40 - 90 nmol/L)

PATIENT	PRE	M1	M2	M3	M4	M5	M6	M7
2	47	8.0	6.0	8.0	9.0	7.0	-	28
3	19	4.0	4.0	3.0	3.0	4.0	-	24
7	-	-	-	-	-	-	-	-
8	71	8.0	5.0	4.0	4.0	2.0	3.0	31
10	23	5.6	1.8	3.6	3.4	3.1	3.6	18
11	32	4.9	1.4	2.0	1.4	1.0	-	33
12	23	1.4	1.8	<0.5	<0.5	-	<0.5	27
14	109	11	4.1	-	-	-	-	-
17	65	31	13	10	11	-	-	-
18	28	3.0	3.0	2.0	-	3.0	1.4	8.5
23	46	5.4	3.4	3.2	3.6	3.0	-	-
25	48	5.8	5.5	-	5.6	6.4	5.6	-
28	66	8.8	6.1	5.6	6.6	6.4	5.7	36
29	85	11	9.0	7.0	7.2	7.7	-	35
30	49	11	7.6	7.0	9.0	9.1	4.8	-
31	33	7.9	3.2	4.2	4.2	3.1	1.5	28
35	71	16	-	-	-	-	-	-
36	64	7.5	12	6.6	6.2	6.2	8.0	42
39	10	5.1	5.9	4.1	5.4	4.8	-	26
40	50	5.6	4.1	3.6	4.3	2.9	<0.5	<0.5
41	93	21.2	13	14	13	12	12	63
43	-	6.0	5.0	3.9	5.3	-	-	43
44	-	-	-	-	-	-	-	-
48	60	10	6.0	5.5	5.2	5.9	7.2	33.2
49	58	9.2	6.5	5.2	5.5	5.1	5.3	42
321	53	8.6	4.9	5.7	6.0	5.2	11	-
N	23	24	23	21	21	19	13	17
MEAN	53	9.0	5.8	5.2	5.7	5.2	4.6	30
STD	25	6.3	3.3	3.0	3.0	2.7	3.3	14
SEM	5.1	1.3	0.69	0.65	0.65	0.61	0.92	3.4

TABLE A.10: THE EFFECT OF DANAZOL ON SERUM CONCENTRATION OF SHBG
(NORMAL RANGE: 40 - 90 nmol/L)

PATIENT	PRE	M1	M2	M3	M4	M5	M6	M7
1	37	6.0	6.0	4.0	5.0	4.0	-	-
4	49	3.0	2.0	2.0	2.0	<0.5	<0.5	16
5	52	8.0	5.0	5.0	-	6.0	4.0	34
6	48	9.0	3.0	3.0	2.0	2.0	-	20
9	44	5.0	3.0	2.0	1.0	2.5	4.2	32
13	118	8.4	2.0	1.3	0.7	2.6	-	-
15	50	<0.5	<0.5	<0.5	<0.5	<0.5	-	26
16	116	5.7	4.8	5.3	4.7	6.3	4.0	62
19	58	8.0	-	-	-	-	-	-
20	74	11	7.0	4.7	6.6	5.6	-	22
21	75	6.1	6.6	6.9	10	10	-	86
22	52	7.8	5.2	6.7	7.1	5.9	-	-
24	69	33	3.1	2.9	3.7	-	-	3.3
26	83	14	5.7	5.5	5.0	5.6	5.2	-
27	58	6.8	6.7	8.9	-	7.2	4.1	-
32	38	6.7	7.0	5.6	7.2	6.2	<0.5	20
33	104	11	7.3	6.8	7.0	6.4	-	-
34	98	9.2	7.4	6.6	7.0	1.5	-	44
37	47	4.1	6.2	4.2	0.8	1.3	1.6	22
38	47	11	-	-	-	-	-	-
42	77	6.3	<0.5	4.6	5.2	4.8	<0.5	41
45	14	-	-	-	-	-	-	-
46	41	11	3.7	4.5	4.8	3.5	-	27
47	21	4.2	2.8	2.2	2.5	3.6	-	2.7
50	52	8.8	-	4.7	4.3	3.8	4.6	23
N	25	24	21	22	20	21	10	16
MEAN	61	8.5	4.6	4.5	4.4	4.3	2.9	30
STD	27	6.0	2.2	2.1	2.7	2.5	1.9	21
SEM	5.4	1.2	0.49	0.45	0.60	0.54	0.60	5.2

TABLE A.11: THE EFFECT OF GESTRINONE ON SERUM CONCENTRATION OF TESTOSTERONE (NORMAL RANGE: 0.7 - 2.7 nmol/L)

PATIENT	PRE	M1	M2	M6	M7
2	0.9	-	0.3	-	0.7
3	1.1	0.6	0.6	-	-
7	0.9	-	-	-	-
8	1.3	0.3	0.4	0.7	1.0
10	1.1	0.3	0.4	0.3	0.9
11	0.6	0.4	-	-	0.6
12	0.6	0.5	1.3	0.6	0.4
14	1.2	1.2	1.6	-	-
17	0.6	0.6	0.4	-	-
18	1.1	0.7	0.9	0.7	0.6
23	1.8	0.5	0.5	-	-
25	2.7	1.9	1.8	1.2	1.8
28	-	0.3	-	0.2	0.9
29	1.3	0.9	0.7	-	0.9
30	0.7	0.7	0.4	-	-
31	-	0.2	0.3	0.4	0.3
35	2.4	0.5	-	-	-
36	0.6	0.3	0.2	0.2	0.3
39	1.6	0.8	0.5	-	1.1
40	0.9	0.4	0.4	0.5	0.9
41	0.7	0.5	0.5	0.5	0.4
43	1.1	0.6	0.4	-	1.4
44	-	-	-	-	-
48	1.1	0.5	0.4	0.4	0.7
49	2.5	1.8	0.9	1.1	1.5
321	1.2	0.7	0.4	-	-
N	23	23	21	12	17
GM	1.04	0.55	0.52	0.52	0.78
UCI	1.21	0.64	0.61	0.66	0.94
LCI	0.90	0.47	0.44	0.42	0.65

GM = GEOMETRIC MEAN
UCI AND LCI = UPPER AND LOWER 95% CONFIDENCE LIMITS

TABLE A.12: THE EFFECT OF DANAZOL ON SERUM CONCENTRATION OF TESTOSTERONE (NORMAL RANGE: 0.7 - 2.7 nmol/L)

PATIENT	PRE	M1	M2	M6	M7
1	1.0	0.1	0.3	-	0.4
4	1.2	0.6	0.6	0.5	0.4
5	1.5	0.4	0.4	0.4	0.9
6	1.3	0.6	0.3	-	1.0
9	1.5	0.4	0.4	0.5	1.0
13	0.8	0.5	0.4	-	1.1
15	0.2	0.2	0.2	-	0.2
16	0.6	0.4	0.4	0.4	0.8
19	0.7	0.7	-	-	-
20	0.9	0.1	0.2	-	0.9
21	1.1	0.5	0.2	-	0.7
22	1.0	0.4	0.3	-	-
24	0.7	0.3	0.4	-	0.5
26	1.5	0.6	0.7	0.7	-
27	0.4	0.5	0.5	0.4	-
32	0.6	0.2	0.4	0.6	0.5
33	0.9	0.2	0.2	-	-
34	1.7	0.6	0.4	-	1
37	1.2	0.6	0.4	0.4	0.6
38	1.8	0.2	-	-	-
42	0.7	0.5	0.4	0.4	0.6
45	1.3	-	-	-	-
46	0.7	0.3	0.3	-	0.7
47	-	-	0.6	-	-
50	-	1.3	-	-	-
N	23	23	21	9	16
GM	0.98	0.39	0.39	0.44	0.73
UCI	1.14	0.45	0.46	0.58	0.89
LCI	0.84	0.33	0.33	0.34	0.60

GM = GEOMETRIC MEAN
UCI AND LCI = UPPER AND LOWER 95% CONFIDENCE INTERVALS

TABLE A.13: THE EFFECT OF GESTRINONE ON SERUM CONCENTRATION OF ANDROSTENEDIONE (NORMAL RANGE: 3.1 - 10.1 nmol/L)

PATIENT	PRE	M1	M2	M6	M7
2	1.1	-	1.6	-	2.4
3	4.3	3.7	3.1	-	-
7	1.1	-	-	-	-
8	3.1	1.3	2.1	2.8	2.0
10	3.1	1.4	1.8	-	-
11	1.7	2.2	-	-	2.5
12	2.2	1.5	1.5	3.6	1.9
14	3.7	2.0	1.8	-	-
17	2.6	2.1	2.7	-	-
18	5.2	3.8	4.5	4.5	4.6
23	3.3	2.3	2.4	-	-
25	2.7	3.9	3.3	2.9	2.9
28	-	0.7	-	1.9	2.0
29	4.5	2.5	2.2	-	1.7
30	3.8	5.2	3.4	-	-
31	-	1.9	1.4	2.4	3.8
35	3.3	2.6	-	-	-
36	3.0	2.4	1.4	1.3	1.6
39	6.4	5.0	4.3	-	4.5
40	2.8	2.7	0.8	1.5	2.3
41	3.3	2.3	1.7	1.6	1.6
43	5.4	4.6	2.8	-	8.2
44	-	-	-	-	-
48	3.0	3.3	2.9	3.3	2.3
49	4.3	4.4	2.5	4.0	1.0
321	1.4	0.9	1.6	-	-
N	23	23	21	11	16
MEAN	3.3	2.7	2.4	2.7	2.8
STD	1.4	1.3	0.97	1.1	1.8
SEM	0.28	0.27	0.21	0.32	0.44

TABLE A.14: THE EFFECT OF DANAZOL ON SERUM CONCENTRATION OF ANDROSTENEDIONE (NORMAL RANGE: 3.1 - 10.1 nmol/L)

PATIENT	PRE	M1	M2	M6	M7
1	2.4	3.4	1.6	-	2.5
4	5.4	4.0	3.4	3.5	1.6
5	4.2	3.3	3.9	2.3	2.2
6	2.2	2.1	1.2	-	3.8
9	5.7	4.2	3.9	3.7	7.0
13	3.5	2.2	2.2	-	5.2
15	1.6	1.9	1.4	-	0.9
16	2.1	2.6	2.7	3.2	2.3
19	3.6	1.8	-	-	-
20	0.2	0.9	1.9	-	2.1
21	1.2	0.5	0.8	-	0.8
22	1.5	1.8	2.7	-	-
24	2.8	1.8	1.4	-	2.1
26	3.5	3.4	2.7	1.8	-
27	2.4	1.9	1.5	2.2	-
32	3.5	2.1	2.6	2.4	3.8
33	3.1	1.8	2.0	-	-
34	3.7	3.8	2.3	-	2.2
37	5.0	3.4	2.0	2.6	3.2
38	9.0	1.9	-	-	-
42	3.6	2.5	1.2	1.5	4.4
45	5.3	-	-	-	-
46	3.9	1.2	1.2	-	2.9
47	-	2.4	1.9	-	-
50	-	3.3	-	-	-
N	23	24	21	9.0	16
MEAN	3.5	2.4	2.1	2.6	2.9
STD	1.9	0.98	0.88	0.75	1.6
SEM	0.39	0.20	0.19	0.25	0.40

TABLE A.15: THE EFFECT OF GESTRINONE ON SERUM CONCENTRATION OF DIHYDROTESTOSTERONE (NORMAL RANGE: 0.2 - 1.0 nmol/L)

PATIENT	PRE	M1	M2	M6	M7
2	0.39	-	0.11	-	0.26
3	0.39	0.19	0.30	-	-
7	0.51	-	-	-	-
8	0.42	0.33	0.17	0.15	0.32
10	0.33	0.28	0.16	0.22	-
11	0.24	0.15	-	-	0.33
12	0.50	0.19	0.25	0.22	0.31
14	0.51	0.18	0.26	-	-
17	0.30	0.28	0.22	-	-
18	0.53	0.27	0.28	0.22	0.41
23	0.17	0.11	0.13	-	-
25	0.29	0.14	0.14	0.12	0.83
28	-	0.16	-	0.53	0.25
29	0.27	0.22	0.18	-	0.17
30	-	-	0.19	-	-
31	-	-	0.17	0.37	0.83
35	0.47	0.23	-	-	-
36	0.29	0.12	0.12	0.09	0.10
39	0.47	0.14	0.31	-	0.80
40	0.26	0.17	0.05	0.10	0.26
41	0.18	0.21	0.17	0.31	0.25
43	0.59	0.23	0.09	-	0.70
44	-	-	-	-	-
48	0.44	0.35	0.43	0.46	0.66
49	0.66	0.32	0.44	0.47	0.59
321	0.32	0.61	0.26	-	-
N	22	21	21	12	16
MEAN	0.39	0.23	0.21	0.27	0.44
STD	0.13	0.11	0.10	0.15	0.25
SEM	0.03	0.02	0.02	0.04	0.06

TABLE A.16: THE EFFECT OF DANAZOL ON SERUM CONCENTRATION OF DIHYDROTESTOSTERONE (NORMAL RANGE: 0.2 - 1.0 nmol/L)

PATIENT	PRE	M1	M2	M6	M7
1	0.31	0.11	0.11	-	0.15
4	0.36	0.23	0.19	0.17	0.11
5	0.59	0.29	0.16	0.14	0.26
6	0.45	0.46	0.16	-	0.31
9	0.38	0.16	0.14	0.31	0.38
13	0.22	0.09	0.09	-	0.31
15	0.22	0.36	0.12	-	0.19
16	0.26	0.26	0.16	0.10	0.25
19	0.21	0.06	-	-	-
20	0.29	0.15	0.13	-	0.18
21	0.37	0.14	0.10	-	0.31
22	0.15	0.05	0.10	-	-
24	0.14	0.04	0.03	-	0.13
26	0.46	0.22	0.21	0.27	-
27	0.90	0.10	0.19	0.15	-
32	0.24	0.09	0.28	0.18	0.23
33	0.32	0.28	0.20	-	-
34	-	0.24	0.23	-	0.35
37	0.69	0.16	0.12	0.19	0.24
38	0.81	0.19	-	-	-
42	0.58	0.09	0.07	0.03	0.16
45	0.54	-	-	-	-
46	0.27	0.19	0.20	-	0.22
47	-	-	0.66	-	-
50	-	0.26	-	-	-
N	22	23	21	9	16
MEAN	0.40	0.18	0.17	0.17	0.24
STD	0.21	0.11	0.13	0.08	0.08
SEM	0.04	0.02	0.03	0.03	0.02

TABLE A.17: THE EFFECT OF GESTRINONE ON SERUM CONCENTRATION OF DHAS (NORMAL RANGE: 3.0 - 13.0 µmol/L)

PATIENT	PRE	M1	M2	M6	M7
2	8.8	10	6.5	-	5.2
3	15	12	10	-	-
7	-	-	-	-	-
8	12.7	12.2	12.3	12.4	8.8
10	13.3	11.5	12.6	11.5	9.8
11	7.2	15.5	-	-	10
12	19.3	19	15.3	16.9	10.9
14	8.9	15	8.0	-	-
17	8.0	13	12	-	-
18	19	-	24	18	24
23	5.7	7.9	10	-	-
25	21	13	18	18	11
28	6.3	4.2	9.6	6.3	4.0
29	2.7	3.1	4.6	-	3.0
30	1.8	4.5	10.4	2.9	-
31	7.2	13	11	3.9	4.6
35	5.4	6.1	-	-	-
36	3.3	6.8	1.9	5.5	3.1
39	14	7.1	8.1	-	5.7
40	9.7	-	4.5	-	-
41	2.4	2.6	2.0	2.1	1.3
43	8.5	5.8	6.6	-	6.8
44	-	-	-	-	-
48	5.8	7.1	7.4	6.4	5.9
49	5.5	6.6	6.5	6.3	4.3
321	5.9	4.9	4.9	-	-
N	24	22	22	12	16
MEAN	9.1	9.1	9.4	9.2	7.4
STD	5.5	4.5	5.2	5.9	5.4
SEM	1.1	0.96	1.1	1.7	1.3

TABLE A.18: THE EFFECT OF DANAZOL ON SERUM CONCENTRATION OF DHAS (NORMAL RANGE: 3.0 - 13.0 µmol/L)

PATIENT	PRE	M1	M2	M6	M7
1	3.1	8.3	6.8	-	4.7
4	6	6.1	5.7	-	3
5	8.5	8.7	10	9	6.7
6	4.7	4.3	4	-	3.6
9	17.3	15.7	12.3	22.8	13.5
13	9.4	12	13	-	10
15	5.7	6.3	5.9	-	7
16	6.3	16	9.3	15	14
19	10	11	-	-	-
20	5.2	9.9	10	-	9.5
21	7.7	6.8	10	-	10
22	8.4	9.8	7.5	-	-
24	8.5	8.6	7.2	-	5.2
26	3.5	6.8	7.3	-	-
27	6.2	3.2	2.8	3.2	-
32	7.6	6.2	6.4	4.3	3.6
33	14	12	10	-	-
34	4	8.1	8.8	-	3.8
37	2.6	2.2	3.3	5.1	4.9
38	6.9	5.5	-	-	-
42	4.4	6.1	-	-	5.2
45	6	-	-	-	-
46	5.8	6.3	6.7	-	5.1
47	2.6	3.6	3.9	-	-
50	5.8	6.5	-	6.1	5.2
N	25	24	20	7	17
MEAN	6.81	7.92	7.54	9.36	6.76
STD	3.38	3.55	2.87	7.13	3.42
SEM	0.68	0.72	0.64	2.69	0.83

TABLE A.19: THE EFFECT OF GESTRINONE ON SERUM % FREE TESTOSTERONE

PATIENT	PRE	M1	M2	M6	M7
2	1.62	-	4.94	-	1.70
3	1.89	4.41	5.34	-	-
7	1.00	4.16	-	-	-
8	-	5.18	5.02	4.78	2.42
10	2.99	5.48	4.08	-	-
11	2.40	-	-	-	2.36
12	5.08	8.13	5.61	4.99	-
14	-	-	-	-	-
17	1.54	3.37	3.67	-	-
18	1.80	-	4.80	5.53	4.08
23	2.87	-	5.25	-	-
25	-	5.88	5.30	6.31	-
28	2.01	4.20	5.93	6.78	3.06
29	1.12	5.01	5.28	-	2.04
30	1.70	4.19	5.67	5.56	-
31	-	-	2.41	-	0.70
35	-	1.51	-	-	-
36	0.80	-	1.86	2.33	0.66
39	2.19	5.01	5.69	-	2.20
40	-	3.26	2.73	2.63	1.11
41	1.09	3.14	3.73	3.35	1.08
43	-	4.62	4.91	-	2.45
44	-	-	-	-	-
48	0.88	2.47	3.84	4.76	1.56
49	0.86	3.01	2.86	3.44	1.21
321	1.45	-	2.73	-	-
N	18	17	21	11	14
MEAN	1.85	4.29	4.36	4.59	1.90
STD	1.04	1.51	1.25	1.47	0.96
SEM	0.25	0.37	0.27	0.44	0.26

TABLE A.20: THE EFFECT OF DANAZOL ON SERUM % FREE TESTOSTERONE

PATIENT	PRE	M1	M2	M6	M7
1	2.64	7.34	7.00	-	-
4	0.97	4.89	6.15	6.48	1.84
5	0.85	5.18	4.12	4.31	1.82
6	0.78	6.21	6.09	-	-
9	1.54	4.97	4.52	6.55	1.99
13	0.89	0.82	6.08	-	1.40
15	2.45	8.87	6.19	-	2.40
16	0.72	5.83	5.50	-	-
19	2.78	5.06	-	-	-
20	1.21	5.56	7.00	-	2.57
21	1.63	5.48	7.12	-	1.43
22	2.21	5.65	-	-	-
24	2.97	6.13	7.97	-	2.54
26	1.47	7.88	7.28	6.55	-
27	1.51	5.41	5.91	7.13	-
32	-	2.48	2.35	2.57	0.78
33	-	-	2.68	-	-
34	-	3.20	3.05	-	0.69
37	0.98	3.27	3.12	3.11	1.03
38	-	-	-	-	-
42	1.43	6.49	5.99	0.81	1.89
45	3.23	-	-	-	-
46	1.40	5.56	5.24	-	2.27
47	1.81	5.46	4.60	-	3.16
50	1.07	3.02	-	3.80	1.23
N	21	22	20	9	15
MEAN	1.64	5.22	5.40	4.59	1.80
STD	0.77	1.81	1.65	2.21	0.71
SEM	0.17	0.38	0.37	0.74	0.18

TABLE A.21: THE EFFECT OF GESTRINONE ON SERUM CONCENTRATION OF FREE TESTOSTERONE

PATIENT	PRE	M1	M2	M6	M7
2	0.015	-	0.015	-	0.012
3	0.021	0.026	0.032	-	-
7	0.009	-	-	-	-
8	-	0.016	0.020	0.033	0.024
10	0.033	0.016	0.016	-	-
11	0.014	-	-	-	0.014
12	0.030	0.041	0.073	0.030	-
14	-	-	-	-	-
17	0.009	0.020	0.015	-	-
18	0.020	-	0.043	0.039	0.024
23	0.052	-	0.026	-	-
25	-	0.112	0.095	0.076	-
28	-	0.013	-	0.014	0.028
29	0.015	0.045	0.037	-	0.018
30	0.012	0.029	0.023	-	-
31	-	-	0.007	-	0.002
35	-	0.008	-	-	-
36	0.005	-	0.004	0.005	0.002
39	0.035	0.040	0.028	-	0.024
40	-	0.013	0.011	0.013	0.010
41	0.008	0.016	0.019	0.017	0.004
43	-	0.028	0.020	-	0.034
44	-	-	-	-	-
48	0.010	0.012	0.015	0.019	0.011
49	0.021	0.054	0.026	0.038	0.018
321	0.017	-	0.011	-	-
N	17	16	20	10	14
MEAN	0.019	0.031	0.027	0.028	0.016
STD	0.012	0.026	0.024	0.020	0.011
SEM	0.003	0.006	0.005	0.006	0.003

(UNITS: nmol/L)

TABLE A.22: THE EFFECT OF DANAZOL ON SERUM CONCENTRATION OF FREE TESTOSTERONE

PATIENT	PRE	M1	M2	M6	M7
1	0.026	0.007	0.021	-	-
4	0.012	0.029	0.037	0.032	0.007
5	0.013	0.021	0.016	0.017	0.016
6	0.010	0.037	0.018	-	-
9	0.023	0.020	0.018	0.033	0.020
13	0.007	0.004	0.024	-	0.015
15	0.005	0.018	0.012	-	0.005
16	0.004	0.023	0.022	-	-
19	0.019	0.035	-	-	-
20	0.011	0.006	0.014	-	0.023
21	0.018	0.027	0.014	-	0.010
22	0.022	0.023	-	-	-
24	0.021	0.018	0.032	-	0.013
26	0.022	0.047	0.051	0.046	-
27	0.006	0.027	0.030	0.029	-
32	-	0.005	0.009	0.015	0.004
33	-	-	0.005	-	-
34	-	0.019	0.012	-	0.007
37	0.012	0.020	0.012	0.012	0.006
38	-	-	-	-	-
42	0.010	0.032	0.024	0.003	0.011
45	0.042	-	-	-	-
46	0.010	0.017	0.016	-	0.016
47	-	-	0.028	-	-
50	-	0.039	-	-	-
N	19	21	20	8	13
MEAN	0.015	0.023	0.021	0.023	0.012
STD	0.009	0.012	0.011	0.014	0.006
SEM	0.002	0.003	0.002	0.005	0.002

(UNITS: nmol/L)