Endometrial Carcinoma
Steroid Hormones and Receptors in Relation to Proliferation

Karin Boman

University of Umeå 1993
Endometrial Carcinoma
Steroid Hormones and Receptors in Relation to Proliferation

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av Rektorsämbetet vid Umeå Universitet för avläggande av doktorsexamen i medicinsk vetenskap vid Umeå Universitet kommer att offentligen försvaras i Onkologiska Klinikens föreläsningssal, JK-byggnaden, 2 tr, Norrlands Universitetssjukhus, den 11 juni kl 09.00

av
Karin Boman, leg. läk.

Fakultetsopponent: Docent Bo Frankendal

From the Departments of Oncology and Obstetrics and Gynaecology, University of Umeå, Sweden
Editor: the Dean of the Faculty of Medicine
Abstract

The significance of the hormonal milieu for endometrial changes is as well-known as its link with endometrial carcinoma. Unopposed oestradiol treatment is shown to increase the incidence for this cancer. Obesity leads to elevated levels of oestrogens and is a risk factor for endometrial carcinoma. An association between high tumour proliferation and prognosis is a general feature of human cancer. Tumour growth can be expressed as proliferation rate and flow cytometry (FCM) is a sensitive and reproducible method to estimate S-phase fraction (SPF) and ploidy level. Both parameters have been shown to correlate with prognosis. Sex steroid hormone levels were analysed together with clinical parameters, SPF, and receptors in established endometrial carcinoma.

The study consisted of postmenopausal women with endometrial adenocarcinoma. Hormones were analysed in 127 patients, 99 were analysed for FCM and 60 for oestrogen and progesterone receptors. RIA technique was used for hormone assay of oestrone, oestradiol, progesterone, androstenedione and testosterone plasma levels. The receptors were analysed with an immunohistochemical method, and SPF and ploidy level by flow cytometry.

A wide range of oestrogen concentrations was found. Some patients had levels comparable to fertile women. Strong correlations were found between body mass index, weight and depth of uterine cavity. No relations were found between receptors and SPF, apart from oestrogen-receptor positive tumours having a lower SPF when compared with receptor negative tumours. The influence of oestradiol on tumour proliferation expressed as SPF was ambiguous. SPF was increased with higher oestradiol levels in the group of peri-diploid, well-differentiated tumours, while a negative correlation was found for the peri-diploid, moderately differentiated tumours. The aneuploid and poorly differentiated tumours had a high SPF regardless of oestradiol concentration. The association between progesterone concentration and SPF was of a more general nature. Progesterone above a threshold level was related to a lower SPF in well-differentiated and moderately differentiated tumours. Thus endogenous progesterone seems to play a role in controlling the tumour's proliferation activity, in contrast to oestradiol, that had a role which did not appear to relate to proliferation activity in any specific direction. The only stimulative association was seen in well-differentiated tumours, but SPF was still below the mean value for all diploid tumours.
Endometrial Carcinoma
Steroid Hormones and Receptors in Relation to Proliferation

Karin Boman

From the Departments of Oncology and Obstetrics and Gynaecology
University of Umeå, Sweden
Editor: the Dean of the Faculty of Medicine
To

with best regards from the author
Abstract
The significance of the hormonal milieu for endometrial changes is as well-known as its link with endometrial carcinoma. Unopposed oestradiol treatment is shown to increase the incidence for this cancer. Obesity leads to elevated levels of oestrogens and is a risk factor for endometrial carcinoma. An association between high tumour proliferation and prognosis is a general feature of human cancer. Tumour growth can be expressed as proliferation rate and flow cytometry (FCM) is a sensitive and reproducible method to estimate S-phase fraction (SPF) and ploidy level. Both parameters have been shown to correlate with prognosis. Sex steroid hormone levels were analysed together with clinical parameters, SPF, and receptors in established endometrial carcinoma.

The study consisted of postmenopausal women with endometrial adenocarcinoma. Hormones were analysed in 127 patients, 99 were analysed for FCM and 60 for oestrogen and progesterone receptors. RIA technique was used for hormone assay of oestrone, oestradiol, progesterone, androstenedione and testosterone plasma levels. The receptors were analysed with an immunohistochemical method, and SPF and ploidy level by flow cytometry.

A wide range of oestrogen concentrations was found. Some patients had levels comparable to fertile women. Strong correlations were found between body mass index, weight and depth of uterine cavity. No relations were found between receptors and SPF, apart from oestrogen-receptor positive tumours having a lower SPF when compared with receptor negative tumours. The influence of oestradiol on tumour proliferation expressed as SPF was ambiguous. SPF was increased with higher oestradiol levels in the group of peri-diploid, well-differentiated tumours, while a negative correlation was found for the peri-diploid, moderately differentiated tumours. The aneuploid and poorly differentiated tumours had a high SPF regardless of oestradiol concentration. The association between progesterone concentration and SPF was of a more general nature. Progesterone above a threshold level was related to a lower SPF in well-differentiated and moderately differentiated tumours. Thus endogenous progesterone seems to play a role in controlling the tumour's proliferation activity, in contrast to oestradiol, that had a roll which did not appear to relate to proliferation activity in any specific direction. The only stimulative association was seen in well-differentiated tumours, but SPF was still below the mean value for all diploid tumours.

KEY WORDS: Endometrial carcinoma, oestrogens and progesterone hormones and receptors, S-phase fraction, ploidy level.
Papers

This thesis is based on the following papers which will be referred to by their Roman numerals:


V. Boman K., Mäentausta O., Bäckström T., Strang P. and Stendahl U.: Sex steroid hormones and receptors in relation to S-phase fraction and ploidy level in endometrial carcinoma. (Manuscript)

Reprints were made with the permission of the publishers.
## Contents

Abbreviations .......................................................................................................8

Introduction. ..........................................................................................................9

THE NORMAL ENDOMETRIUM ........................................................................9
Postmenopausal Endocrinology ................................................................. 9
The fertile endometrium ...............................................................................10
The postmenopausal endometrium ............................................................11
ENDOMETRIAL CARCINOMA .......................................................................11
Epidemiology .....................................................................................................11
Endocrinology ...................................................................................................12
Prognostic factors ..............................................................................................13
Ploidy and proliferation: background .............................................................13
Chromosomes and ploidy level ......................................................................15
Flow cytometry and ploidy .............................................................................15
Flow cytometry and S-Phase fraction (SPF) ....................................................15
Other Methods ................................................................................................16
Receptors ............................................................................................................17
Aims of the study. ..............................................................................................18

Material and methods. .......................................................................................19

Patients ................................................................................................................19
Clinical parameters ..........................................................................................19
Hormone assays ...............................................................................................20
Flow cytometry ..................................................................................................21
Steroid receptors ...............................................................................................22
Data analysis ......................................................................................................22

Results. ................................................................................................................23

Hormone concentrations and clinical parameters
(Papers I and III) .................................................................................................23
Hormone concentrations and flow cytometric analysis (FCM)
(Papers II and IV) ...............................................................................................26
Hormone concentrations, FCM and sex steroid receptors
(Paper V) .............................................................................................................29

General Discussion. ..........................................................................................31

Conclusions. ........................................................................................................35

Acknowledgements. ..........................................................................................36

References. ..........................................................................................................37

Papers I-V. ............................................................................................................47

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviations</td>
<td>8</td>
</tr>
<tr>
<td>Introduction.</td>
<td>9</td>
</tr>
<tr>
<td>THE NORMAL ENDOMETRIUM</td>
<td>9</td>
</tr>
<tr>
<td>Postmenopausal Endocrinology</td>
<td>9</td>
</tr>
<tr>
<td>The fertile endometrium</td>
<td>10</td>
</tr>
<tr>
<td>The postmenopausal endometrium</td>
<td>11</td>
</tr>
<tr>
<td>ENDOMETRIAL CARCINOMA</td>
<td>11</td>
</tr>
<tr>
<td>Epidemiology</td>
<td>11</td>
</tr>
<tr>
<td>Endocrinology</td>
<td>12</td>
</tr>
<tr>
<td>Prognostic factors</td>
<td>13</td>
</tr>
<tr>
<td>Ploidy and proliferation: background</td>
<td>13</td>
</tr>
<tr>
<td>Chromosomes and ploidy level</td>
<td>15</td>
</tr>
<tr>
<td>Flow cytometry and ploidy</td>
<td>15</td>
</tr>
<tr>
<td>Flow cytometry and S-Phase fraction (SPF)</td>
<td>15</td>
</tr>
<tr>
<td>Other Methods</td>
<td>16</td>
</tr>
<tr>
<td>Receptors</td>
<td>17</td>
</tr>
<tr>
<td>Aims of the study</td>
<td>18</td>
</tr>
<tr>
<td>Material and methods.</td>
<td>19</td>
</tr>
<tr>
<td>Patients</td>
<td>19</td>
</tr>
<tr>
<td>Clinical parameters</td>
<td>19</td>
</tr>
<tr>
<td>Hormone assays</td>
<td>20</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>21</td>
</tr>
<tr>
<td>Steroid receptors</td>
<td>22</td>
</tr>
<tr>
<td>Data analysis</td>
<td>22</td>
</tr>
<tr>
<td>Results.</td>
<td>23</td>
</tr>
<tr>
<td>Hormone concentrations and clinical parameters</td>
<td>23</td>
</tr>
<tr>
<td>Hormone concentrations and flow cytometric analysis (FCM)</td>
<td>26</td>
</tr>
<tr>
<td>Hormone concentrations, FCM and sex steroid receptors</td>
<td>29</td>
</tr>
<tr>
<td>General Discussion.</td>
<td>31</td>
</tr>
<tr>
<td>Conclusions.</td>
<td>35</td>
</tr>
<tr>
<td>Acknowledgements.</td>
<td>36</td>
</tr>
<tr>
<td>References.</td>
<td>37</td>
</tr>
<tr>
<td>Papers I-V.</td>
<td>47</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>FCM</td>
<td>flow cytometry</td>
</tr>
<tr>
<td>SPF</td>
<td>S-phase fraction</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>LH</td>
<td>luteineizing hormone</td>
</tr>
<tr>
<td>LI</td>
<td>labelling index</td>
</tr>
<tr>
<td>MI</td>
<td>mitotic index</td>
</tr>
<tr>
<td>E1</td>
<td>oestrone</td>
</tr>
<tr>
<td>E2</td>
<td>oestradiol</td>
</tr>
<tr>
<td>T</td>
<td>testosterone</td>
</tr>
<tr>
<td>A</td>
<td>androstenedione</td>
</tr>
<tr>
<td>P</td>
<td>progesterone</td>
</tr>
<tr>
<td>ER</td>
<td>oestrogen receptor</td>
</tr>
<tr>
<td>ER+</td>
<td>oestrogen receptor positive</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PR+</td>
<td>progesterone receptor positive</td>
</tr>
<tr>
<td>M</td>
<td>mitosis</td>
</tr>
</tbody>
</table>
THE NORMAL ENDOMETRIUM

Postmenopausal Endocrinology

The mean age for menopause varies in different parts of the world. The current estimate in Sweden is 51 years.\(^1\) With a life expectancy of 80 years,\(^2\) women can expect to live a substantial part of their life as postmenopausal.

The phases and the function of the normal fertile endometrium are directly connected with hormone production of the ovaries that takes place in the theca and granulosa cells of the maturing follicle and in corpus luteum. These, in turn, are regulated by two pituitarian hormones, follicle stimulating hormone (FSH) and luteineizing hormone (LH). Oestradiol dominates during the proliferative phase, and progesterone during the secretory phase.

Deficit of ovarian hormones, secondary to the nearly complete depletion of ovarian follicles, leads to menopause and associated systemic effects. Simply stated, the endocrine profile of the menopause is that of hypoestrogenism with relative androgen excess.

In postmenopausal women there is a major decrease in both oestradiol and oestrone concentrations. During the reproductive years more than 90 per cent of these hormones is produced by the ovaries,\(^3\) but after menopause the ability to synthesise oestradiol from pregnenolone in the ovaries is decreased as a result of the aromatase system disappearing with the granulosa cells. Oestradiol drops to less than 10 per cent of the premenopausal level and the concentration of circulating oestradiol is less than 50 pmol/l.\(^4\)\(^-\)\(^6\) The major source of the remaining oestradiol is peripheral conversion of oestrone to oestradiol.\(^7\)

The decrease of oestrone to approximately thirty per cent of premenopausal levels is significantly smaller than that for oestradiol. Oestrone thus becomes the predominant oestrogen, with a mean concentration of approximately 130 pmol/l.\(^4\)\(^-\)\(^6\)\(^,\)\(^8\) Oestrone is primarily derived from peripheral conversion of androstenedione,\(^9\)\(^,\)\(^10\) and the mean conversion rate is more than doubled after menopause, from 1.2 to 2.8 per cent.\(^11\)\(^,\)\(^12\) The rate is also correlated to body mass index (BMI), as the most efficient conversion of androstenedione to oestrone takes place in adipose tissue.\(^13\) In postmenopausal obese women this rate rises to 3.9 per cent.\(^10\) In some reports chronological age has been considered to be an additional factor for an increased conversion rate,\(^10\)\(^,\)\(^14\) but this is contradicted in others.\(^15\)

Androstenedione is reduced to about 50 per cent of premenopausal levels, and is mainly produced by the adrenal glands, with an ovarian contribution of approximately 20-30 per cent.\(^12\)\(^,\)\(^16\)

With the postmenopausal decrease of oestradiol level, the negative feedback on the gonadotrophins disappears. High levels of LH stimulate the remaining ovarian stroma to produce androgens, but in contrast to androstenedione, testosterone is only marginally lowered after menopause. The ovarian secretion
of testosterone increases and contributes with approximately 50 per cent, while peripheral contribution decreases because androstenedione, as a source of peripheral conversion, is reduced.\textsuperscript{12, 16}

Progesterone synthesis diminishes during the perimenopausal period, manifested by corpus luteum insufficiency. Postmenopausal concentrations are equal to levels seen in the follicular phase (0.6-3.5 nmol/l).\textsuperscript{16, 17} Progesterone is now mainly secreted from the adrenals, but there is still some production from the ovaries.\textsuperscript{18}

The fertile endometrium

Studies of the fertile endometrium have demonstrated that the hormone profile of the follicular phase is characterised by an average oestradiol level between 150 and 370 pmol/l, and with a pre-ovulatory peak value higher than 690 pmol/l. Progesterone values during the follicular phase are 1.0 - 4.4 nmol/l, with a minor pre-ovulatory rise. The LH surge induces the luteal phase with the next rise of progesterone. The concentration reaches 15-42 nmol/l during this phase.\textsuperscript{19-21}

Proliferation rate can partly be estimated by using morphometrical or biochemical methods to estimate, e.g., mitosis activity, glandular size, or amount of DNA and RNA.\textsuperscript{22} During the secretory phase a decrease in proliferation can be seen from the number of mitosis and from the amount of DNA + RNA. This starts already two days before the LH-peak, thus simultaneously with the pre-ovulatory progesterone rise, and before the substantial rise in the progesterone concentration that occurs two days after the LH-peak.\textsuperscript{20, 22} Ferenczy has reported a similar development; between days 8-10 and 11-14 in the menstrual cycle there is a decrease in DNA synthesis.\textsuperscript{23} The proliferative activity starts early in the menstrual cycle, and is shown to be at a maximum level before oestradiol reaches its preovulatory peak.\textsuperscript{24} The onset of proliferation differs in different cells and layers, and is first seen in the glandular epithelium in the functional layer.\textsuperscript{23}

In the normal menstrual cycle, the concentrations of the oestrogen and progesterone receptors undergo characteristic variations. The expression is regulated by oestradiol and progesterone.\textsuperscript{25-27} Oestradiol stimulates the synthesis of both receptors, whereas progesterone depletes them.\textsuperscript{28}

The highest content of endometrial oestrogen receptors occurs in the epithelial cells during the proliferative phase, and persists until the mid to late secretory phase, when it decreases.\textsuperscript{25, 27, 29} Progesterone receptor concentration remains low in the early proliferation phase and reaches a maximum level in the late proliferation phase,\textsuperscript{26} and then gradually disappears during the late secretory phase.\textsuperscript{27} Flow cytometric studies on the normal fertile endometrium have shown that the mean S-phase fraction (SPF) for proliferative endometrium is significantly higher than for secretory endometrium. In hyperplastic endometrium, the SPF mean value falls in between those values, while carcinomatous endometrium has the highest SPF value.\textsuperscript{30, 31}
The postmenopausal endometrium

After menopause the endometrium is senile atrophic due to the lack of oestrogen, but might also be weakly proliferative. It is very rarely secretory. Cystic, adenomatous or atypical hyperplasia and cancer in situ may develop, partly depending upon the endocrine milieu, and will be discussed.

ENDOMETRIAL CARCINOMA

Epidemiology

Endometrial cancer rates are highest in Northern Europe, North America; intermediate in Southern Europe and Latin America; and low in Africa and Asia.32

In Sweden, approximately 950 new cases are diagnosed every year, and endometrial cancer is second to ovarian cancer in frequency among gynaecological cancers. Eighty-five percent are diagnosed after 55 years of age when 98 per cent of the women are postmenopausal. The median age of incidence is 66 years.2

There are some well-known endogenous risk factors identified with endometrial adenocarcinoma: Obesity, nulliparity, late menarche, late menopause are examples of variation of normal physiology,53-37 and polycystic ovaries and granulosa- theca cell tumours are examples of diseases.38-40

Both diabetes mellitus and hypertension are associated with endometrial cancer, but it is unclear whether the association is independent of the effects of obesity and age.33, 35, 36, 41 However, remaining correlations have been shown when corrected for these two factors.35

Among exogenous risk factors, unopposed oestrogen has received most attention. In epidemiological studies, Persson has shown that oestrogen treatment involves an increased risk for endometrial carcinoma unless opposed by progesterone.42, 43 Numerous retrospective studies have correlated the use of exogenous oestrogen to a 2- to 12-fold increased risk of developing endometrial carcinoma.41, 44-47 Some of these studies have received methodological criticism, but the consensus is that there is a true causal relationship between oestrogen treatment and an increased risk of endometrial carcinoma. The risk is dependent on duration, dose and habitus. Non obese, non diabetic and normotensive women are at higher risk, suggesting that the additional oestrogen effect is less crucial to obese, diabetic and hypertensive women.44 Whether this depends on a threshold effect or a different metabolism has not yet been answered.

Exogenous hormones administered as combination oral contraceptives are associated with a 50 per cent reduction in incidence compared with non-users.47-49 The protective effect seems to be higher with increasing doses of progesterone and decreases with years of discontinuation.49 The protective effect was also higher among nulliparous.49

First degree relatives more often have endometrial cancer. There is also an over representation of breast cancer among those women. Thus specific inherited factors, including adipositas, might exist but this can also be due to coincidences associated with environmental risk factors clustered in families.50
Endocrinology

Unopposed oestrogen causes endometrial proliferation and hyperplasia, including atypical hyperplasia.\textsuperscript{51} This condition is a premalignant stage for progression to endometrial adenocarcinoma.\textsuperscript{52-54}

Conditions with unopposed oestrogen are anovulatory cycles in the polycystic ovarian syndrome, during climacterium when the cyclical progesterone fails, and oestrogen substitution without sufficient addition of progesterone.

Oestrogen can be considered as a cocarcinogen, probably necessary but not by itself enough to cause development of cancer.\textsuperscript{55} Yet, endometrial adenocarcinoma occasionally occurs in ovariectomized women,\textsuperscript{56} exemplifying that cancer can develop under hypoestrogenic conditions.

Several studies have focused upon the increased risk for endometrial carcinoma after unopposed oestrogen therapy,\textsuperscript{45, 46, 57} and also upon whether patients with cancerous endometrium have an inadequate endogenous hormone balance that might predispose for malignant changes. The results are contradictory. Some early studies found elevated levels of oestrogens.\textsuperscript{58-60} When patients and controls were matched for weight and age, two conditions that affect the conversion rate of androstenedione to oestrone,\textsuperscript{12} the difference disappeared.\textsuperscript{10, 61-63} However this result has been contradicted by others.\textsuperscript{64} Enhanced aromatase activity in vitro in adipose tissue from endometrial cancer patients has been demonstrated.\textsuperscript{13, 65, 66}

The relationship between androgens and endometrial adenocarcinoma has also been difficult to evaluate since C-19 steroids can be converted either to oestrogens or steroids with an androgenic profile through different steroid pathways. A study of androgen concentrations where endometrial cancer was compared with a control group did not show any significant difference.\textsuperscript{67}

Obesity is associated with decreased levels of sex hormone binding globulin (SHBG),\textsuperscript{68, 69} resulting in more unbound oestrogen, mainly oestradiol, which is the most potent oestrogen. Simultaneously, there is, per se, an increased oestrogen pressure due to the increased conversion rate.

No difference in progesterone concentration has been shown between endometrial cancer and control groups and low concentration has not been associated with an increased risk of cancer.\textsuperscript{60, 70-72} After menopause, progesterone concentrations are comparable with the low levels of the follicle phase in fertile women and elevated oestrogen levels causes a constant "anovulatory state". The importance of having a progesterone concentration in balance with oestrogen has been shown when studying hormone substitution therapy. In studies with cyclic high-dose therapy, 8 of 22 developed hyperplasia, and 1 of 24 during sequential low-dose therapy.\textsuperscript{73}

The influence of endogenous hormones on the growth of endometrial carcinoma in vivo is one of the topics of this thesis.
Prognostic factors

The prognosis for a patient with endometrial adenocarcinoma is dependent on the malignancy of the tumour and duration of disease and can be expressed in the classical prognostic parameters: clinical stage, histopathological grade and myometrial invasion. These factors have been the base for choice of therapy. Clinical stage (used in these papers) is a strong and independent factor. However, as 90 % of the patients are in stages I and II the majority of deaths are in this stage. Myometrial invasion has thereby become very important for choice of therapy in early stages. In many studies histopathological grade has been correlated to survival, \textsuperscript{129, 139} but the estimation is subjective.

There is a strong need for objective reproducible methods of compiling a prognosis. As a result, different methods for measurements have been developed to express proliferation rate. During the last decade, interest has been focused upon flow cytometric measurements, since the method is sensitive and reproducible. Ploidy level,\textsuperscript{74-77} and S-phase fraction\textsuperscript{77-79} have been shown in several studies to be of prognostic value and in multivariate analysis even to be superior to traditional parameters, except stage\textsuperscript{77} or grade.\textsuperscript{79} The background and its relation to other methods are discussed below.

Sex steroid receptors also contain prognostic information and have been of clinical interest for hormone treatment with gestagen. This is discussed later.

Ploidy and proliferation: background

An association between a high tumour proliferation rate and prognosis is a general feature of human cancer.\textsuperscript{77, 96, 98} In order to estimate proliferation, several morphometric and biochemical methods have been developed.

The first histological base for development of the different measuring methods was laid when Miescher isolated "nuclein" from the cell nuclei.\textsuperscript{80} This later led to the discovery of DNA as the main constituent of the chromosomes and as carrier of the genetic code information. In 1936, it was found that the amount of DNA doubled during the cell cycle.\textsuperscript{81}

The cell cycle is the name given to the series of events that occur in a proliferating cell (Fig. 1):

1. G1-phase represents the time between the end of mitosis and the beginning of the S-phase. Most of the specialised functions of the cell are carried out during G1 (and G0).
2. S-phase: The time during which the DNA is duplicated.
3. G2-phase: The phase while the cell prepares itself for mitosis, with the number of chromosomes doubled.
4. Mitosis (M) or cell division.
Figure 1. The cell cycle with its different phases and DNA content.

This occurs provided that the mother cell has doubled its DNA contents. In a normal cell the doubling is from 2c to 4c (1c = 1 set of chromosomes, n=23). Immediately before division the chromatin condenses into chromosomes, each of which divides in two for distribution to each daughter cell. This phase is usually easy to identify in a light microscope. After the mitosis, the cell continues the next cycle or enters a resting phase, G0.

Individual cells of human solid tumours have a mean duration in the cell cycle of 2 days, but there is a wide variety. The cell can chose to re-enter the cycle or rest in G0. A recent theory is that the cell may also rest in the S-phase, thus enter a S0 phase. The explanation would be nutrient deprivation and/or cell-crowding phenomena.

The growth rate of a tumour depends upon three main factors: proportion of cells in the active cell cycle, the duration of the cell cycle and the amount of cell loss, i.e. necrosis and apoptosis.
Chromosomes and Ploidy level

The chromosomal changes can be characterised as numerical and structural. Numerical changes indicate the gain or loss of chromosomes. Structural changes with rearrangements of the chromatin may be of several varieties, e.g. translocation and deletion. Chromosome studies on hyperplastic and atypical endometrium seldom show aneuploidy, and studies on tumourous endometrium show that a fairly large part, 42%, retains a diploid chromosome set. The pattern is related to histopathologic differentiation, and deviations are more common in the undifferentiated tumours.

Ploidy measurements, regardless of method (chromosomal, single cell or FCM) show that the majority of endometrial tumours are near diploid, and their generally favourable prognosis may be associated with this characteristic. Ploidy also relates, to some extent, to the degree of differentiation.

Flow cytometry and ploidy

In the FCM measurement, it is the deviation of DNA content that can be estimated. When the cell has the DNA content of a normal set of chromosomes, 2c, it is diploid, and divergence is regarded as aneuploidy. Lindahl et al. evaluated 166 endometrial cancers, stages I and II, and showed that DNA ploidy had a much more marked prognostic value than the degree of differentiation, myometrial invasion and receptor content concentration. Britton et al. evaluated 256 endometrial cancers. The recurrence rate was 10% in the diploid tumours compared to 34% in the aneuploid, and in their multivariate analysis only histological subtypes and DNA ploidy remained significant.

Flow cytometry and S-Phase fraction (SPF)

Cytometric investigations are of importance both for prognostic and descriptive purposes to increase the understanding of basic tumour biology. FCM is one of the methods for studying cell proliferation and today probably the one used most. The method reveals the ploidy level and the proportions of cells in the different phases, G1, S and G2 + M. It is a static measurement of the number of cells in S-phase and described in a histogram. FCM has the advantage of being an objective and rapid technique. FCM also has good reproducibility, given that the conditions are optimal.

After staining the cell suspension with a DNA binding fluorescence the DNA content in each cell is measurable. The SPF is calculated from the number of cells between the G1 and G2 peaks. This calculation is easily done in the diploid case, but might be more difficult in certain aneuploid tumours as there may be overlapping aneuploid peaks. SPF is known to have prognostic value in a variety of tumours: Urinary bladder, breast cancer, cervical, ovarian and endometrial carcinoma.

The impact of SPF as a prognostic independent factor has been shown. Stendahl et al. found SPF to be, next to clinical stages III and IV, the strongest
predictor of outcome. Rosenberg et al. found that only SPF/ploidy and FIGO grade (considering the histological subgroup uterine papillary serous carcinoma) were independent prognostic variables. Geisinger et al. also found SPF to carry prognostic information.

Other Methods

Other methods for evaluation of proliferative properties are described below.

Thymidine labelling index (LI).

The description of the thymidine labelling index in the 1950s by Taylor et al. provided a new approach to the study of growth kinetics. The added tritiated thymidin (³H-TdR) is incorporated in the newly synthesised DNA and detected either by autoradiography or by scintigraphy. The method is still in use and measures both the duration of all the phases in the cell cycle and the proportion of cells in S-phase and mitosis. Disadvantages are the multistep technique and the time required. Good correlation has been reported between LI and FCM, LI and MI. Several studies have shown the method’s value in describing the biological course of events.

Mitotic index (MI).

The condensation of the chromatin directly prior to mitosis enables us to visualize this part of the cell cycle. The method depends on the investigator's capability to recognise the mitosis. Contradictory results on reproducibility, both intra- and interindividually, have been reported. The mitotic index relies on the fixation of the cells to make the mitosis last and prevent the fission from taking place. Measurements of MI are facilitated by adding a dye, which better visualises mitosis. Several studies have reported MI to be an important predictor for survival in different malignancies.

Bromodeoxyuridine labelling index

Bromodeoxyuridine is a pyrimidine analogue of thymidine which is incorporated into DNA by competitive antagonism with thymidine. It can be detected by monoclonal antibodies, and used both in vivo and in vitro. The method is able to identify different cell-cycle phases, cell kinetic parameters such as S-phase duration time, and the potential tumour doubling time (T_{pot}) can be calculated. It is a faster technique than LI, and does not need radioactive labelling, thus being more attractive for in vivo use. A good correspondence between this technique and other FCM methods has been described.

Proliferation-associated antigens

Proliferating cell nuclear antigen (PCNA) is a protein identical to cyclin which plays a critical roll in the initiation of cell proliferation as an auxiliary protein for DNA polymerase δ.

Ki-67 is a nuclear antigen which is expressed in cycling cells but not in quiescent cells. Correlation between Ki-67 and SPF has been demonstrated.
Receptors

An abundance of receptors could be another reason for increased hormonal stress. Compared to the normal endometrium, the hyperplastic one has a denser representation of progesterone receptors. Others found levels comparable to those of the normal proliferative endometrium. Usually there is a decreased receptor representation in the endometrial adenocarcinoma tissue, and also a tendency for a lower receptor content with decreased differentiation.

The receptor might also be low or non-functioning even when present, leading to inadequate response to present hormone levels. Thus tumours may be receptor positive but non-responding. Primary endometrial resistance to progesterone has been described, but is probably rare.

Receptors as a prognostic factor are not as frequently used in endometrial cancer as in breast or prostate cancer, but receptor presence seems to be favourable for prognosis. Some studies indicate that the progesterone receptor has a higher degree of prognostic value than oestradiol receptors, or is perhaps the only receptor with a sufficient prognostic value. However, the results are not unambiguous.
Aims of the study

To describe the relation between clinical parameters, associated with endometrial adenocarcinoma, and plasma levels of the sex steroid hormones: oestrone, oestradiol, progesterone, androstenedione and testosterone.

To study the distribution and interrelationship between the plasma concentration of oestrone, oestradiol, progesterone, androstenedione and testosterone in endometrial adenocarcinoma patients.

To investigate the endogenous concentration and balance of oestradiol and progesterone and their relation to flow cytometric SPF and ploidy in endometrial adenocarcinoma.

To investigate the SPF, ploidy level and receptor content in endometrial carcinoma tissue in relation to oestradiol and progesterone plasma concentration.
Material and methods

Patients

The patient material in Papers I and III consisted of 128 consecutive postmenopausal women with adenocarcinoma of the endometrium, admitted to the Department of Gynaecologic Oncology, University Hospital, Umeå between July 1984 and July 1986. The median age was 67 years (range 50-94). To be defined as postmenopausal, two years of amenorrhea before clinical symptoms of malignancies was required.

All patients were examined under anaesthesia and fractional uterine curettage was performed. According to the clinical FIGO system, the clinical staging of the patients was as follows: 111 (87%) of the patients were in stage I, 10 (8%) patients in stage II, 3 (2%) patients in stage III and 4 (3%) in stage IV. According to the WHO classification, 129 42 (33%) tumours were well differentiated, 67 (52%) moderately differentiated and 19 (15%) poorly differentiated. All specimens were reviewed by the same pathologist, who confirmed the diagnosis of endometrial adenocarcinoma.

The patient material in Papers II and IV consisted of the same 128 patients as discussed in Papers I and III. Ninety-nine tumour samples were available for FCM analysis. The difference was due either to necrotic or insufficient samples or to prior surgery.

The patient material in Paper V, where also receptor analyses were performed, consisted of 60 patients taken from the later part of the bigger consecutive material from Papers I and III.

The patient material analysed for FCM (n=99) and for receptors (n=60) was compared with the consecutive group (n=128) as regards hormone concentrations, grade, age, BMI, SPF and ploidy level. No significant differences were found between the groups (Table 1).

Clinical parameters

The clinical parameters investigated were: Clinical stage of disease, histopathology, body mass index (BMI) weight, depth of uterine cavity, age, pregnancy, parity, years of menstruation, menopausal age and years, hypertension and diabetes mellitus.
Table 1. Mean hormone levels and clinical parameters in the patient groups from paper I+III, II+IV and V. No significant differences between materials were found.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Hormone</th>
<th>FCM</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>127</td>
<td>99</td>
<td>60</td>
</tr>
<tr>
<td>Oestrone (pmol/l)</td>
<td>247</td>
<td>263</td>
<td>284</td>
</tr>
<tr>
<td>Oestradiol (pmol/l)</td>
<td>85†</td>
<td>87</td>
<td>98</td>
</tr>
<tr>
<td>Progesterone (nmol/l)</td>
<td>0.51</td>
<td>0.51</td>
<td>0.55</td>
</tr>
<tr>
<td>Androstenedione (nmol/l)</td>
<td>2.9</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Peridiploid (%)</td>
<td>-</td>
<td>80</td>
<td>77</td>
</tr>
<tr>
<td>Aneuploid (%)</td>
<td>-</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>S-phase fraction (%)</td>
<td>-</td>
<td>11.7 †</td>
<td>12.1</td>
</tr>
<tr>
<td>Depth of uterine cavity (cm)</td>
<td>7.7</td>
<td>7.7</td>
<td>8.0</td>
</tr>
<tr>
<td>Age (years, median)</td>
<td>67</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>73</td>
<td>74</td>
<td>73</td>
</tr>
<tr>
<td>Stage I (%)</td>
<td>87</td>
<td>87</td>
<td>85</td>
</tr>
<tr>
<td>Grade 1 (%)</td>
<td>33</td>
<td>27</td>
<td>32</td>
</tr>
<tr>
<td>Grade 2 (%)</td>
<td>52</td>
<td>56</td>
<td>47</td>
</tr>
<tr>
<td>Grade 3 (%)</td>
<td>15</td>
<td>17</td>
<td>22</td>
</tr>
</tbody>
</table>

† n=126 patients. †† n=78 patients

Hormone assays

Pretreatment blood samples for oestrone, oestradiol, progesterone, androstenedione and testosterone assays were collected between 10 a.m. and 1 p.m. After centrifugation, the plasma was frozen directly in small aliquots and stored at -70°C until analysed. Plasma levels of oestrone, oestradiol, testosterone, androstenedione and progesterone were measured in duplicate samples by radioimmunoassay (RIA) after celite chromatography for each patient. The RIA method used has been described elsewhere. All samples from the same patient were assayed in duplicate in the same run. The accuracy of each method is expressed as the coefficient of the intra-assay variation, which has been calculated as the variation between duplicates of the same sample within the same assay. If the mean value differed more than 30% between the duplicates, the sample was reassayed before
it was accepted. In order to express the accuracy of the method in terms of coefficient of inter-assay variation, the same sample was analysed in triplicate for each assay run. The middle sample in the assay had only 50% of the volume compared with the other two, thereby enabling parallelism to be controlled.

Antisera for oestrone used were obtained from Bio Mérieux (Charbonnierers, France) and for oestradiol from Miles-Yeda (Rehvat, Israel). Interassay coefficients of variation were 12.7% and 13% and intraassay 10.5 and 10.0% for oestrone and oestradiol, respectively. Antisera used for progesterone and androstenedione were obtained from Endocrine Sciences, Tarzana, California and for testosterone from Chemical Laboratory, The Faculty of Veterinary Medicine, University of Agricultural Sciences, Uppsala, Sweden). Interassay coefficients of variation were 7.5%, 9.9% and 10.4% and intraassay 5.5%, 8.0% and 8.4% for progesterone, androstenedione and testosterone, respectively.

Hormone levels from postmenopausal healthy women were used as reference to define dividing points between "low" and "high" plasma hormone concentration. A concentration below the mean of the reference value (60 pmol/l) was considered as low and concentration equal and above the mean +2 SD was considered as high (120 pmol/l).

Flow cytometry

Samples for flow cytometry were taken at the second curetage. The specimens were preserved in a 12% DMSO (dimethylsulphoxide) solution and instantly frozen to -70°C. Before preparation the biopsies were thawed at room temperature for 60-90 minutes.

A suspension of cell nuclei was obtained using a combined mechanical and enzymatic technique, described by Tribukait et al. Cells were fixed in absolute ethanol and digested with RNAse and pepsin. The suspended nuclei were washed and stained with ethidium bromide. The DNA content of a mean number of 30 000 cell nuclei was analysed from each tumour biopsy. For the analysis an ICP 11 flow cytometer, with a flow rate of up to 1000 cells/sec was used. The excitation and emission wavelengths were 455-490 nm and 590-630 nm, respectively.

The tumour DNA values were expressed as relative values, where the DNA content of normal diploid G0/G1 cells was given the value 2.0c. Human lymphocytes with a diploid DNA content (2.0c) and with a coefficient of variation of 2-3%, were used as an external standard. The modal DNA value of each sample was defined by the most prominent G0/G1 peak. Ploidy levels of 1.8-2.2c were regarded as near diploid (peridiploid) tumours, with other values considered to be aneuploid.

Diploid and peridiploid tumours in this study were considered as one group, since these groups together have a better prognosis than tumours outside this range.

When possible, SPF was calculated as the proportion of cells between the G1 and G2 peaks, corrected for background fluorescence, according to the simplified method described by Baisch et al.
Steroid receptors

An immunohistochemical localisation of oestrogen and progesterone receptors was performed using commercial reagent sets (Abbott Laboratories, Diagnostic Division, North Chicago, IL) according to the instructions of the manufacturer. The results were evaluated as specific staining: not present (-), weak (+), moderate (++) and strong (+++). All three grades of staining have been interpreted as receptor-positive.

Data analysis

The Mann-Whitney U-test or the Kruskal-Wallis test was used when non-continuous parameters, such as histopathology, clinical stage, ploidy level and receptor content were analysed for differences in hormone concentration and other parameters. Continuous clinical parameters, such as hormone concentrations, SPF and age, were studied by using Spearman’s rank correlation or normal correlation coefficient analysis. Chi-square and Fisher’s exact tests were used for comparing frequencies.

Hormone concentrations of all patients were studied and described. The distribution patterns were described in histograms, in skewness to reflect the degree of symmetry in the distribution, and kurtosis to measure the amount of data in the tail.

Mean, ±SE, was used if not otherwise stated.

Table 2. Statistical measurements of hormone concentrations.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>±SE</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 (pmol/l)</td>
<td>247</td>
<td>17</td>
<td>191</td>
<td>18</td>
<td>1196</td>
<td>2.1</td>
<td>5.5</td>
</tr>
<tr>
<td>E2 (pmol/l)</td>
<td>85</td>
<td>8.3</td>
<td>59.5</td>
<td>5</td>
<td>628</td>
<td>3.5</td>
<td>15</td>
</tr>
<tr>
<td>T (nmol/l)</td>
<td>1.2</td>
<td>0.046</td>
<td>1.04</td>
<td>0.17</td>
<td>3.3</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>A (nmol/l)</td>
<td>2.9</td>
<td>0.13</td>
<td>2.52</td>
<td>0.9</td>
<td>9.8</td>
<td>1.8</td>
<td>4.6</td>
</tr>
<tr>
<td>P (nmol/l)</td>
<td>0.51</td>
<td>0.024</td>
<td>0.50</td>
<td>0.03</td>
<td>1.8</td>
<td>0.83</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Results

Hormone concentrations and clinical parameters (Papers I and III)

From the 128 patients, 126 oestradiol assays were obtained together with 127 assays from each of oestrone, progesterone, androstenedione and testosterone. Values for mean, ±SE, median, range, skewness and kurtosis are given in Table 2. The distributions of the hormones are shown in Figs. 2-6. Oestradiol and oestrone had a skewed distribution, both with a tail towards high concentrations. The same tendency was noted for androstenedione.

A wide range of values was obtained for the oestrogens: oestrone 118-1196 and oestradiol 5-628 pmol/l. Eighteen patients (14%) had an oestradiol concentration above 120 pmol/l, a value corresponding to levels seen in proliferation phase in fertile women. This was arbitrarily regarded as a high value in our analysis. Progesterone had a range between 0.03-1.75 nmol/l. The concentrations (>1 nmol/l) are comparable to values seen during the proliferative phase.

The correlations between and p-values for different hormone concentrations are shown in Table 3 and Fig. 7. Oestrone correlated with oestradiol, testosterone, androstenedione and progesterone. Oestradiol correlated with oestrone, testosterone and androstenedione. Progesterone correlated with oestrone. Androstenedione correlated with oestrone, oestradiol and testosterone. Testosterone correlated with androstenedione, oestrone and oestradiol.

Table 3. Interrelation between the plasma hormone concentrations.

<table>
<thead>
<tr>
<th></th>
<th>E1</th>
<th>E2</th>
<th>T</th>
<th>A</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>-</td>
<td>p=0.0001</td>
<td>p=0.002</td>
<td>p=0.03</td>
<td>p=0.01</td>
</tr>
<tr>
<td>E2</td>
<td>rs=0.63</td>
<td>-</td>
<td>p=0.003</td>
<td>p=0.02</td>
<td>ns</td>
</tr>
<tr>
<td>T</td>
<td>rs=0.28</td>
<td>rs=0.27</td>
<td>-</td>
<td>p=0.02</td>
<td>ns</td>
</tr>
<tr>
<td>A</td>
<td>rs=0.19</td>
<td>rs=0.20</td>
<td>rs=0.20</td>
<td>-</td>
<td>ns</td>
</tr>
<tr>
<td>P</td>
<td>rs=0.22</td>
<td>rs=0.04</td>
<td>rs=0.06</td>
<td>rs=0.08</td>
<td>-</td>
</tr>
</tbody>
</table>

rs = Spearman rank correlation coefficient. ns = not significant.

The hormone concentrations were analysed against the clinical parameters mentioned in the section on Material and Methods. The results are summed up in Table 4. BMI, weight and depth of uterine cavity were the features which most consistently correlated with oestrone and oestradiol. A negative correlation between androstenedione and age was found. There were significant relations between oestrogens and diabetes mellitus and oestradiol and hypertension, respectively.
Figures 2-6. Frequency distribution of sex steroid hormone plasma concentrations. For values see Table 2.
Figure 7. A schematic figure of the steroid synthesis, with arrows showing the pathways, and dotted lines showing the significances found between the hormone assays of P, A, T, E1 and E2. Significance values are shown in Table 3.
Table 4. Correlations and significances between investigated clinical parameters and hormone concentrations.

<table>
<thead>
<tr>
<th></th>
<th>E1</th>
<th>E2</th>
<th>P</th>
<th>A</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1-3</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Stage I-IV</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Depth of uterine cavity</td>
<td>0.43</td>
<td>0.31</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Residual tumour</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Age</td>
<td>0.18</td>
<td>ns</td>
<td>ns</td>
<td>0.22</td>
<td>ns</td>
</tr>
<tr>
<td>BMI</td>
<td>0.43</td>
<td>0.52</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Weight</td>
<td>0.36</td>
<td>0.44</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>No. of pregnancies</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>No. of parities</td>
<td>ns</td>
<td>p&lt;0.05</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Age of menarche</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Age of menopause</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Years of menstruation</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>-0.20</td>
</tr>
<tr>
<td>Menopausal years</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>-0.25</td>
<td>0.20</td>
</tr>
<tr>
<td>Hypertension</td>
<td>ns</td>
<td>p&lt;0.05</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Diabetes mellitus†</td>
<td>p&lt;0.001</td>
<td>p&lt;0.01</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

E1: Oestrone. E2: Oestradiol. T: Testosterone. A: Androstenedione. P: Progesterone. p<0.05: rs=0.18-0.20. p<0.01: rs=0.22-0.25. p<0.001: rs=0.31-0.52.

†The diabetes mellitus group had a significantly higher BMI, p<0.05.

Hormone concentrations and flow cytometric analysis (FCM) (Papers II and IV)

Ninety-nine tumours were available for flow cytometric analysis. Seventy-nine tumours (80%) were peridiploid and 20 (20%) were grossly aneuploid. DNA values between 1.7 c and 4.0 c were found. S-phase fraction (SPF) was evaluable in 78 (79%) of the samples. The mean value was 11.7%; range 4.0% -34.9%. Aneuploid tumours had a higher mean SPF than peridiploid tumours, 16.4% and 10.5%, respectively (p< 0.001).

There was no difference between peridiploid and aneuploid tumours in mean hormone levels of oestrone, oestradiol, progesterone, androstenedione and testosterone, and no correlation was found between hormone concentrations and SPF when the whole group was studied.

The material was further studied in subgroups stratified for tumour grading and ploidy level.
Table 5. Definition of cutoff levels that produced significant differences between subgroups, defined according to ploidy level and tumour grading.

<table>
<thead>
<tr>
<th>nmol/l</th>
<th>Diploid &amp; Aneuploid</th>
<th>Diploid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>W+M+P</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>W+M</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>W</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>M</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>P</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

W: well differentiated; M: moderately differentiated; P: poorly differentiated.

The subgroups W, M and P were subjected to stepwise analysis at the cutoff levels of progesterone shown in the table. Where few observations were made, no statistical value is given.

There was no linear correlation, but when progesterone concentration exceeded 0.8 nmol/l a lower S-phase fraction was demonstrated in the subgroups of: (1) well (2) well and moderately (3) diploid: well, (4) diploid: well and moderately and (5) diploid: well, moderately and poorly differentiated tumours (Table 5 and Figs. 8-9). When poorly differentiated and/or aneuploid tumours were excluded, the correlation between higher progesterone concentration and lower SPF became more obvious.

The following four subgroups were analysed for oestradiol: (1) aneuploid: well, moderately and poorly differentiated, n=16 (20%), (2) diploid: well-differentiated, n=20 (26%), (3) diploid: moderately differentiated, n=34 (44%) and (4) diploid: poorly differentiated tumours, n=8 (10%). The aneuploid group showed a high SPF which did not relate to oestradiol concentration, and neither did the group diploid: poorly differentiated tumours. The diploid: well-differentiated tumours analysed as a whole group showed no significant linear correlation, but the subgroup with low oestradiol concentration, <60 pmol/l, had lower SPF than the group with oestradiol concentration above 60 pmol/l, p<0.01. Still the SPF was below the mean of the peridiploid group (9.6% and 10.5% respectively). Diploid: moderately differentiated tumours showed a negative correlation between oestradiol concentration and SPF, p<0.05, rs= 0.40 (Fig. 10).

The SPF, on one hand, was similar in the subgroup consisting of patients with high oestradiol (>120 pmol/l) and low progesterone (<0.5 nmol/l, 6 of 78 patients) compared with the complementary subgroup. The subgroup with low oestradiol (<60 pmol/l) and high progesterone (>0.8 nmol/l, 6 of 78 patients) showed, on the other hand, a significantly lower S-phase fraction, 7.3% and 12.1%, p<0.005).
Figures 8-9. Mean S-phase fraction values at a progesterone concentration exceeding the presented level. Each observation includes the cumulative number of patients, shown inside the figure. The X axis shows the lowest progesterone concentration included. The Y axis shows the mean S-phase fraction. Fig. 8 includes all tumors. Fig. 9 shows the values when only well-differentiated and moderately differentiated peridiploid tumors are included.
Figure 10. SPF (±SE) in the groups defined according to ploidy level, tumor grading and oestradiol level. The oestradiol concentration in the left bar is <60 pmol/l and in the right bar ≥60 pmol/l. A: aneuploid; D:W diploid: well differentiated, D:M diploid: moderately differentiated and D:P diploid: poorly differentiated. (⁎)=p<0.05 (⁎⁎)=p<0.01.

Oestrone, androstenedione and testosterone were analysed in relation to SPF in the same manner, without finding any correlations or differences between the groups.

**Hormone concentrations, FCM and sex steroid receptors (Paper V)**

Sixty tumours were analysed for oestrogen and progesterone receptors. Fifteen out of the 60 tumour samples were receptor positive, and 45 were receptor negative. Nine were positive for oestrogen receptors (ER+) and 12 for progesterone receptors (PR+). Six tumours were stained for both ER+ and PR+. Six biopsies showed only PR+ and thus lacked ER+ and 3 were only ER+ (Fig. 11).

The following parameters were analysed in the different receptor positive subgroups against the receptor negative one: Ploidy level, SPF and plasma concentrations of oestrone, oestradiol, androstenedione, progesterone and testosterone. The results of ploidy level, SPF, and levels of oestradiol and progesterone are shown in Table 6.
Figure 11. The distribution of oestrogen and progesterone receptor positivity in the 15 tumours stained for receptor presence.

Table 6. SPF, ploidy level, estradiol and progesterone plasma concentration in the different receptor groups. The receptor positive groups were compared with the receptor negative group.

<table>
<thead>
<tr>
<th>S-Phase Fraction</th>
<th>Ploidy level</th>
<th>Hormones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SPF</td>
<td>Aneu</td>
</tr>
<tr>
<td></td>
<td>No.  SPF %</td>
<td>ploid</td>
</tr>
<tr>
<td>Rec neg†</td>
<td>37 13</td>
<td>42 11</td>
</tr>
<tr>
<td>ER+ or PR+</td>
<td>13 9.3*</td>
<td>14 2</td>
</tr>
<tr>
<td>ER+ and PR+</td>
<td>4 9.1</td>
<td>5 0</td>
</tr>
<tr>
<td>ER+</td>
<td>7 8.1*</td>
<td>8 0</td>
</tr>
<tr>
<td>PR+</td>
<td>10 10.1</td>
<td>11 2</td>
</tr>
<tr>
<td>ER+ PR-</td>
<td>3 6.7*</td>
<td>3 0</td>
</tr>
<tr>
<td>PR+ ER-</td>
<td>6 10.7</td>
<td>6 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†The receptor negative group was used as a control group.

SPF was evaluable in 50 tumours and ploidy level in 56 tumours.

E2: oestradiol; P: progesterone.

(♦)=p<0.05.

Fifty-six tumours were evaluated for ploidy level and 50 for SPF. A low SPF was seen in the groups with oestrogen receptors, p<0.05 (Table 6). No other differences in SPF were noted. There was no significant difference in ploidy level between the different receptor subgroups. Several groups were small. It is noteworthy that all ER+ tumours were peridiploid, while in the group (PR+ER-), 2 of 6 were aneuploid.

Among the tumours positive for progesterone receptors, only (PR+ER-) showed a significantly higher concentration of oestradiol than the receptor-negative group (p<0.05). As to the oestrogens, this was the only difference found between the groups. There were no significant differences seen for progesterone (Table 6).
General Discussion

In Papers I and III the clinical parameters, analysed against the hormone concentrations, demonstrated that BMI and depth of the uterine cavity were the parameters that most consistently correlated to both oestrone and oestradiol. BMI and its correlation to the oestrogens is reported elsewhere,\(^\text{15, 63, 134}\) and further discussed below.

Oestrone levels correlated with age, otherwise there was no association between oestrogens and age. This result is consistent with some previous findings,\(^\text{15, 61, 134}\) but contradicts others.\(^\text{135}\) The negative correlation between androstenedione and age is in agreement with what has earlier been shown.\(^\text{134}\) The reason why the concentrations of oestrogens do not decrease, while that of androstenedione does, could be related to the increase in aromatase activity during ageing.\(^\text{14, 136}\) There was no significant change of BMI with age.

Parity is of interest in endometrial carcinoma and it has been found that nulliparous and low parity women have a higher frequency of endometrial carcinoma.\(^\text{54, 157}\) In this study, both oestrone and oestradiol showed significant positive correlation with the total number of pregnancies but the number of children born showed only a significant positive correlation with oestradiol. No obvious explanation for this result was found.

Endometrial carcinoma is known to be more common in nulliparous and low parity women.\(^\text{34, 137}\) There were significantly higher values of both oestrone and oestradiol in the diabetic patients, but BMI was also significantly higher in this group. Similar correlations have been shown earlier.\(^\text{65}\) The hypertensive patients demonstrated significantly higher oestradiol levels than normotensive ones. In this case, BMI was comparable between groups. There was no evident explanation of these relations and it would appear that they require further study.

Previous investigations have reported higher levels of oestrogens in well differentiated tumours.\(^\text{138}\) The present investigation was unable to support this. The difference might depend upon the materials used. The present study includes only postmenopausal women and the oestradiol concentration was less than half of that in the other study.

Progesterone concentration was investigated in relation to the same parameters and no correlations were found. In the etiology of endometrial cancer, progesterone has not been connected with any of the risk factors mentioned. Theoretically a deficit could be as crucial as an abundance of oestrogen, but no differences concerning progesterone concentrations between patients and controls have been shown.\(^\text{60, 70-72}\)

Oestrogen has long been considered as an important risk factor for developing endometrial carcinoma.\(^\text{42}\) Both oestrone and oestradiol values were distributed over a wide range, with a considerable proportion in the range of premenopausal ovulating women. There were also patients with low concentrations, which supports the opinion that hormone concentrations cannot be the only factor for development of endometrial carcinoma.\(^\text{56}\) The highest progesterone concentra-
tions in these patients were well below the levels seen during the secretory phase of fertile women, where progesterone exerts its main function.

Non-ovarian sources assume major roles in oestrogen production in menopause. Adipose tissue has been identified and described as an important source of oestrone. The presence of aromatase enzymes in adipose tissue has previously been shown in several publications, and thus aromatase enzymatic activity is dependent on the amount of adipose tissue. This indicates the presence of a rate-limiting step. The finding of a strong positive correlation between BMI and oestrone concentration gives further credence to this statement.

In Paper III, the analysis of different hormone concentrations showed several interrelations. The correlation between androstenedione and oestrone (p<0.05) is in agreement with earlier observations, but there are also reports where no correlation was found. The correlation between progesterone and oestrone appears to have attracted less interest.

There was a highly significant correlation between oestrone and BMI but none between BMI and androstenedione or progesterone. That oestrone depends on BMI is understandable, as the synthetic enzymes are situated in the adipose tissue, especially if the aromatase enzyme is a rate-limiting step. If so, a relation between the precursors and the product will become weaker and might not even be seen in plasma. This is in contrast to a situation where aromatase has unlimited capacity, when a strong relation between precursors and products would be expected. However, if there is a correlation between a precursor and the product (progesterone and androstenedione versus oestrone), as was the case in this study, it is likely that this correlation indicates the importance of the precursor. The correlation between progesterone and oestrone was even stronger than between androstenedione and oestrone, which might indicate that the peripheral synthesis of oestrone also has progesterone as a precursor. That would further stress the importance of an increased BMI.

In Paper II, the material was analysed on the basis of endogenous hormones and their known stimulative and antiproliferative effect on the normal endometrium. The proliferative activity was evaluated by FCM, a technique that measures the number of cells in the synthesis phase, i.e. the S-phase fraction (SPF). Several studies have shown the prognostic value of SPF for endometrial adenocarcinoma.

The stimulative effect of oestrogen was only seen in the diploid well-differentiated tumour group and with a threshold effect. The response is similar to that of the fertile endometrium to oestradiol, in which a certain threshold gives the maximum effect on proliferative parameters such as the number of glandular mitosis and the amount of DNA + RNA. Diploid and well-differentiated tumours with an oestradiol concentration ≥60 pmol/l, still had an SPF-value below the mean value of the whole diploid group. SPF in the poorly differentiated and aneuploid tumours was high, regardless of the oestradiol concentration.

Uterine depth and size probably have minor or neglectable prognostic value. The uterine depth of cavity correlated with oestradiol concentration which, by itself, influences uterine growth. There was no correlation between SPF and depth of the uterine cavity in this material. In previous studies, multivariate analysis did not demonstrate any correlation between depth and survival, while SPF was
highly correlated to survival.77, 140 Depth of uterine cavity still influences the subdivision of clinical stage, but recent information indicates that uterine size is not an independent risk factor, but rather relates to cell type, grade and myometrial invasion.141 Delayed diagnosis and a tumour location preventing drainage of the cavity are other reasons for uterine enlargement.

In the association between progesterone concentration and SPF a more general influence could be seen in the different subgroups (Paper IV). SPF decreased significantly when progesterone reached a concentration of 0.8 nmol/l if the aneuploid or poorly differentiated tumours were excluded. Progesterone thus related to SPF even in groups where the tumours have more malignant characteristics, while oestradiol only related to the diploid, well-differentiated group. The diploid, moderately differentiated tumours, showed a negative correlation with oestradiol and SPF. The receptor content did not differ between groups and does not explain the negative correlation. The mean SPF was equal to the mean of all tumours, 11.7%.

The association between progesterone concentration and SPF was also seen in the endogenous hormone balance. The group with low oestradiol and high progesterone had a lower SPF than the others, while there was no difference between high oestradiol and low progesterone and the complementary group. Thus also in the hormone balance, the antiproliferative effect of the progesterone can be seen through lower SPF. It is known from previous studies that the SPF, as measured by FCM, is correlated to survival.77

In Paper V, it is shown that SPF was significantly lower in the oestrogen receptor positive groups (ER+), (ER+ or PR+) and (ER+PR-), than in the receptor negative group. These groups display a combination of favourable factors, as they are also receptor-positive and mainly peridiploid; all in the groups of (ER+) and (ER+PR). But no significant difference concerning ploidy and receptor content was shown. The groups were throughout small.

Of the receptor positive specimens, 6 were positive only for progesterone receptors. This (PR+ER-) representation has also been found by others.120, 142 This pattern deviates from the normal endometrium, containing both oestrogen and progesterone receptors, but with a later decrease for PR during the secretory phase.27, 112, 143 The explanation could be that PR is less sensitive to tumour transformation and better resists histological dedifferentiation than ER. It has been shown that poorly differentiated tumours were more likely to be (PR+ER-), than well-differentiated ones.144

Results on the prognostic value of receptors in the endometrial carcinoma have been contradictory, but some reports show that PR per se has better prediction value.117, 120, 126-128 It seems reasonable to conclude that progesterone receptor positivity by itself indicates a favourable prognosis. The receptor may persist even in the dedifferentiated tumours and consequently the tumour is still adjustable to endogenous antiproliferative monitoring through progesterone. In contrast to oestradiol, the progesterone concentration showed a more consistent effect on the growth rate of endometrial adenocarcinoma. Even in (ER+) tumours, SPF was significantly lower than in the receptor negative tumours.

An immunohistochemical technique was used for receptor analysis. The use of this technique results in a lower number of endometrial cancer specimens being
classified as receptor-positive. This might be due to different sensitivities of the biochemical and immunohistochemical assays. Alternatively, tissue homogenates used for biochemical assays may contain a substantial amount of benign tissue, which may elevate the apparent receptor concentration. The different results concerning the prognostic value of receptors could depend on these differences.

Oestrogens can be regarded as cocarcinogenous because of their effect on developing hyperplasia which, in turn, may be a premalignant stage to endometrial carcinoma. In this investigation on invasive carcinoma, oestradiol did not influence the proliferative rate (Paper II). It might be critical only during tumour induction or in a fairly intact endocrine target, as discussed above.

A hormone-poor state might possibly be more precarious. Progesterone above a threshold level was associated with decreased SPF (Paper IV). Experiments on nude mice have shown that growth under oestrogen-poor conditions leads to development of less differentiated tumours and a tendency for enhanced tumour growth. Oestradiol, within physiological levels or opposed, might no longer be a main risk or driving factor in established cancer, and low hormone levels, including both low oestrogen and progesterone, might be more critical for continued tumour development. If so, a restrictive attitude towards combined substitution treatment is unnecessary, and to some extent it has already changed.

Patients treated for stage I endometrial adenocarcinoma were given substitution therapy because of severe postmenopausal symptoms. The recurrence rate was lower in the substituted group, and the conclusion was that postoperative oestrogen replacement therapy was not contraindicated in selected low-risk patients.

Two important questions remain: Should there be a substitution-free period after treatment and should progestagen be added? There are no reports in favour of an interval between treatment and replacement therapy. Progesterone relates to decreased growth rate and a supplement in physiological doses ought to be beneficial in the event of occult metastasis.
Conclusions

Oestradiol and oestrone concentrations are strongly correlated with body mass index, weight and depth of uterine cavity. Increased oestradiol concentration is correlated to both diabetes mellitus and hypertension, both diseases being known to covariate with endometrial adenocarcinoma. The hormone concentrations were not correlated to histopathological grade or clinical stage of disease, two of the most established prognostic factors.

A wide range of oestrone and oestradiol plasma hormone concentrations was seen in the patients. A considerable number had oestradiol concentrations in the same range as seen in the proliferative phase of the fertile endometrium. Interrelations were found between the two oestrogens, between oestrogens and androstenedione, and between oestrone and progesterone. The latter correlation could indicate a wider peripheral conversion, giving further importance to the amount of adipose tissue.

The influence of endogenous oestradiol on proliferation was ambiguous. Increased SPF was seen only in well-differentiated, peridiploid tumours, while moderately differentiated, peridiploid tumours had a negative correlation. The association between progesterone concentration and SPF was of a more general nature. Endogenous progesterone above a certain level related to lower SPF. The combination of high progesterone and low oestradiol was related to a decreased SPF, while high oestradiol and low progesterone did not affect SPF.

Presence of oestrogen receptors did not increase proliferation, but SPF was significantly lower in oestrogen receptor positive than in the receptor negative tumours. SPF did not differ between progesterone receptor positive tumours and receptor negative tumours.
Acknowledgements

I wish to express my sincere gratitude to:

Professor Torbjörn Bäckström for his inspiring attitude and constructive tutorship in leading me through the sex steroid jungle. His creative and enthusiastic approach to research makes me proud to have had him as supervisor.

Professor Ulf Stendahl, for his strong support and absolute conviction that this was something I could manage. Without his interest and encouragement this thesis would never have been completed.

Peter Strang for his generous way of introducing me to, and sharing his deep knowledge of, flow cytometric measurements — and for being a good friend!

Professor Olle Kjellgren for his curt but interested way of commenting the manuscripts — and for generously sharing his vast knowledge.

The Department of Physiology and Center for Reproductive Biology are acknowledged for providing laboratory facilities for hormone assays, and especially Mrs. Agneta Andersson for her skilful work.

My co-authors, Olli Mäentausta and Ulf Gerdes, for stimulating and positive collaboration.

The staff of the Department of Gynaecological Oncology for continuous support and for always being ready to help. And my colleagues: Helena Persson, my tutor in clinical work, who is always willing to share her experience; Mona Ridderheim for her stimulating and interesting cooperation; and Göran Edbom for his friendship and always being ready to start an intense discussion.

Monica Forsgren and Karin Gladh, for everyday help with things big and small.

Khama Rogo for valuable comments and for revising the English text in my first manuscripts.

Bengt Hållberg for his statistical advice and stimulating teaching.

Nigel Rollison for skilful help in revising the English text.

Rune Hagberg for his art that always gives me pleasure and which adorns my papers in this thesis.

Claes for all the time he saved me through his Mac assistance. His patience with my work and with me was even more impressive than his computer skills.

Christofer and Karolina for their understanding and all the times they took care of themselves and my part of the housework. Sometimes I am almost astonished by the fact that I am the mother of these three wonderful people.

This work has been supported financially by Lions Research Foundation, University of Umeå and the Swedish Cancer Research Foundation. I am most grateful for this support.
References


84. Drewinko B, Li Ying Yang, Barlogie B, Trujillo JM Cultured human tumour cells may be arrested in all stages of the cycle during stationary phase: demonstration of quiescent cells in G1, S and G2 phase. Cell Tissue Kinet. 198417:453.


