This thesis is dedicated to all the people who never stop believing in me and who along

with god, have been my 'footprints in the sand'

My mother

My father

Lola & Dakshina

Neil

Shabana & Lee

Paul Hardiman- I remain forever in your debts

Andrew Leonard- thank you for being a terrific friend

And lastly to my Grandmother, who taught me to get up after a fall and start again.

AKNOWLEDGMENTS:

PUBLICATIONS

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CONTENTS page

CD pocket with cd containing microarray GeneSPring raw data: back cover.

ABBREVIATIONS

AB- Applied bioscience

ACTH- Adenocorticotrophic hormone

APES- 3-aminopropyltriethoxylane

ASGPR- Asiologlycoprotein-1

BMI- Body mass index

CAH- Congenital adrenal hyperplasia

COCP- Combined oral contraceptive pill

DAB- Diaminobenzedene

d-ATP- Deoxyadeninetriphosphate

d-CTP- Deoxycytidinetriphosphate

d-GTP- Deoxyguanidinetriphosphate

DHEAS- Dihydroepiandrostenedione sulphate

DHT- Dihydrotestosterone

DLG 7- Disc-large homologue 7

DNA/cDNA- Deoxyribonucleic acid/ copy deoxyribonucleic acid

d-NTP- Deoxyribonucleotide triphosphate

E/ E2- Oestrogen

EAC- Endometrial adenocarcinoma

EC- Endometrial carcinoma

ECM- Extracellular matrix

EIN- Endometrial intraepithelial neoplasia

FAI- Free androgen index

FSH- Follicle stimulating hormone

GnRH- Gonadotrophin releasing hormone

hCG- human chorionic gonadotrophin

H- Hyperplasia

IMP2A- Integral membrane protein 2A

Ig- Immunoglobulin

IGF- Insulin growth factor

LC- Long cycle

LDL- Low density lipoprotein

LWTF- Dr Lan Wong Te Fong

KCL- Potassium chloride

LH- Luteinising hormone

MMR- Mismatched repair

m-RNA- Messenger RNA

MSI- Microsatellite instability

$NaPO_4$- sodium phosphate

OCP- Ouma Chedumbarum Pillay

OGP- Oviduct specific glycoprotein

OPN/OP- Osteopontin

P- Progesterone

PBS- PhoSPhate buffer saline

PCOS- Polycystic ovary syndrome

PCO- Polycystic Ovary (ovaries)

PCR/ RTPCR- Polymerase chain reaction/real time polymerase chain reaction

PL- Long proliferative

PTEN- Phosphatase Tensin Homologue Delete on Chromosome 10

RC- Regular cycle

RFH- Royal Free Hospital

RNA- Ribonucleic acid

SHBG- Sex hormone binding globulin

SMR- Standard mortality rate

PS- Short proliferative

SSC- Sodium chloride/sodium citraein buffer

T- Testosterone

TBS- Tris phosphate buffer saline

TGF- Transforming growth factor

Tris-HCL- tris- hydrochloric acid buffer

UCL- University College London

UCLH - University College London Hospital

WHO- World Health Organisation

The association between Polycystic Ovary Syndrome and Endometrial cancer.

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Presented to University College London
for the degree of
MD (Res)

February 2009

Abstract

Title: The association between polycystic ovary syndrome and endometrial carcinoma.

Hypothesis: Women suffering from polycystic ovary syndrome have an increased risk of developing endometrial carcinoma.

Aim: To determine whether people with polycystic ovary syndrome have an increased risk of developing endometrial carcinoma.

Background:

Endometrial cancer is one of the commonest cancers to occur in women in the Western World and unopposed oestrogen stimulation of the uterus is amongst one of the aetiologies postulated for this condition. It is generally assumed that women with polycystic ovary syndrome are more likely to develop endometrial hyperplasia and carcinoma for the reason mentioned above. The validity of this association has however never been tested. This relationship is investigated within this thesis.

Methods:

1. A case- control study comparing the ovarian morphology of women diagnosed with endometrial carcinoma and those with benign gynaecological conditions.

2. An immunohistochemical study to assess expression of cell cycle and apoptotic proteins (surrogate markers of prognosis) in cases of endometrial carcinoma from women with PCO or from women with normal ovaries.

3. A microarray study of gene expression in the endometrium of cycling and non-cycling women with polycystic ovary syndrome.

Results:

We found no significant difference in the prevalence of PCO (using this as a marker of PCOS in the absence of information on biochemistry) in the ovaries of patients diagnosed with endometrial cancer compared to women with benign gynaecology disease. Cyclin D1 expression was significantly increased in the endometrial cancers associated with PCO whilst the expression of ki67, Bcl2 and p53 was not significantly different.

A total of 101 genes were differentially expressed in the endometrium of women with PCOS with regular cycles, long cycles and simple endometrial hyperplasia.

Conclusion:

Although our numbers were small, these results challenge the assumption that PCOS is a risk factor for endometrial cancer. The raised expression of Cyclin D1 in endometrial cancers associated with PCO suggests that the prognosis for women with PCO is not necessarily better than those without PCO. There were alterations in the expression of genes which affect endometrial function in women with PCOS including those with regular menstrual cycle women. This suggests that the altered hormonal environment has an effect on gene expression at a very early stage which may have an implication on endometrial carcinogenesis.

Chapter 1

INTRODUCTION

1.1   Outline

This thesis investigates the hypothesis that polycystic ovary syndrome (PCOS) is a risk factor for endometrial carcinoma (EC).

This is, in general, the currently accepted view [1] however many clinicians accept it is based on scanty evidence, while seeming eminently logical. Specifically, menstrual periods are absent in many women with PCOS thus endometrial tissue remains exposed to proliferative estrogenic stimulation for abnormally long periods, without the differentiation stimulating effects of Progesterone[2]. Endometrial re-differentiation and over proliferation in women with PCOS would be expected to increase the incidence of EC.

The introduction to this thesis includes a description of the anatomy, physiology and histology of the ovary and endometrium, in addition to the changes in these organs in the normal menstrual cycle. The nature of PCOS and EAC are then discussed, and evidence relevant to assumed association between the two conditions is reviewed. Finally the studies used to test the proposed hypothesis are outlined.

1.2   The ovary

1.2.1 Ovarian development.

The ovaries appear in the 5th week of foetal development in the human female, as swellings on the right and left medial and caudal aspects of the urogenital ridge [3]. These

gonadal ridges are produced by proliferation of the mesothelial coelomic epithelium and condensation of the underlying mesenchyme. Germ cells appear within the gonadal ridges in the 6th week, and in the 7th, the surface epithelium gives rise to the cortical cords, which penetrate the underlying mesenchyme [3]. In the 4th month of development, the cords split into isolated cell clusters surrounding one or more germ cells, which subsequently differentiate into oogonia while the cord epithelial cells form follicular cells[3]. The oogonia undergo a number of mitotic divisions and differentiate to produce primary oocytes, which replicate their DNA then stall at prophase I of meiosis. By the 5th month, there are *ca*. 7 million germ cells per ovary, but a wave of apoptotic cell death occurs and many oogonia and primary oocytes become atretic[3]. In the 7th month, primordial follicles form consisting of primary oocytes surrounded by flattened follicular epithelial cells [3].

Ovarian development then remains static until puberty, when 5 - 15 primordial follicles begin to mature with each ovarian cycle. The primary oocytes enlarge and the surrounding follicular cells proliferate and differentiate into a one-cell thick layer of granulosa epithelial cells, forming primary follicles. Granulosa cells secrete a layer of glycoprotein onto the oocyte - the zona pellucida. As the follicle continues to grow through granulosa cell proliferation, it acquires a fluid-filled cavity - the antrum - and is referred to as an antral secondary follicle. Generally only one follicle reaches maturity in each ovarian cycle, the Graafian follicle. The primary oocyte it contains resumes its first meiotic division to produce the secondary oocyte and the 1st polar body. The second meiotic division then starts, ovulation generally occurring when the oocyte has

14

progressed to prophase II. Oocyte meiosis arrests at metaphase II and further maturation occurs only if the oocyte is fertilized.

1.2.2 Ovarian anatomy

Normally two ovaries are found in the human female. Each is ovoid, measuring approximately 3 x 1.5 x 1 cm, and composed of dense fibrous tissue, in which primary and developing follicles are embedded. They are attached by the mesovarium to the posterior leaf of the broad ligament and lie on the peritoneum of the side wall of the pelvis, in the ovarian fossa between the angle of the internal and external iliac vessels and the ureter, just superior to the obturator nerve. The ovaries can sometimes be palpated through the vagina on digital examination, and are in close proximity to the sigmoid colon and the ileum within the rectouterine pouch (Pouch of Douglas). Each ovary usually lies with its long axis oblique and its tubal extremity uppermost and medial, attached to the upper angle of the uterus by the ovarian ligament, a mass of fibrous tissue and smooth muscle which is continuous with the round ligament and lies between the two sheets of the broad ligament [4]

Figure 1.1- The pelvic anatomy with a cross-section of an ovary courtesy of Kellogg foundation. (www.academic.kellogg.cc.mi.us/herbrandsonc/bio201). Permission required for reproduction.

The ovaries derive arterial blood from the right and left ovarian arteries, which leave the abdominal aorta just below the renal arteries. Venous drainage occurs *via* a plexus of veins (the pampiniform plexus) which drain into the ovarian veins, which lie alongside the ovarian arteries. The right ovarian vein terminates in the inferior vena cava whereas the left ovarian vein terminates in the left renal vein. Lymphatic drainage occurs *via* the paraortic nodes, adjacent to the junctions of the ovarian arteries and the dorsal aorta.

Some drainage also occurs through the inguinal nodes, via the round ligament and the inguinal canal, and through the contralateral ovary via the uterine fundus.

Sympathetic autonomic fibres from the aortic plexus reach the ovaries along their vascular supply. These fibres have their pre-ganglionic cell bodies in the spinal cord at the T10 and T11 level. Parasympathetic autonomic fibres from the hypogastric plexus supply the ovaries via the uterine artery.

1.2.3 Ovarian histology

Macroscopically, the each ovary consists of a capsule, a cortex and a medullar region. The capsule consists of an outer germinal epithelium - a single layer of cuboidal or squamous cells - with the fibrous tunica albuginea lying below. The central medullary region contains connective tissue and the main blood vessels. The peripheral portion surrounding the medulla, the cortex, contains ovarian follicles embedded in connective tissue. The cortex is interrupted at the hilus

Several types of ovarian follicles can be identified in the adult ovarian cortex; primordial, growing (primary and secondary) and Graafian. Primordial follicles occur just beneath the tunica albuginea, but move deeper in the cortex as they mature to primary then secondary follicles, such that the fully mature Graafian follicle may extend the full thickness of the ovarian cortex, and bulge from the ovarian surface. The granulosa epithelial cells, and the theca interna cells which surround them in the maturing follicle, produce steroid hormones essential for follicular maturation and reproductive control.
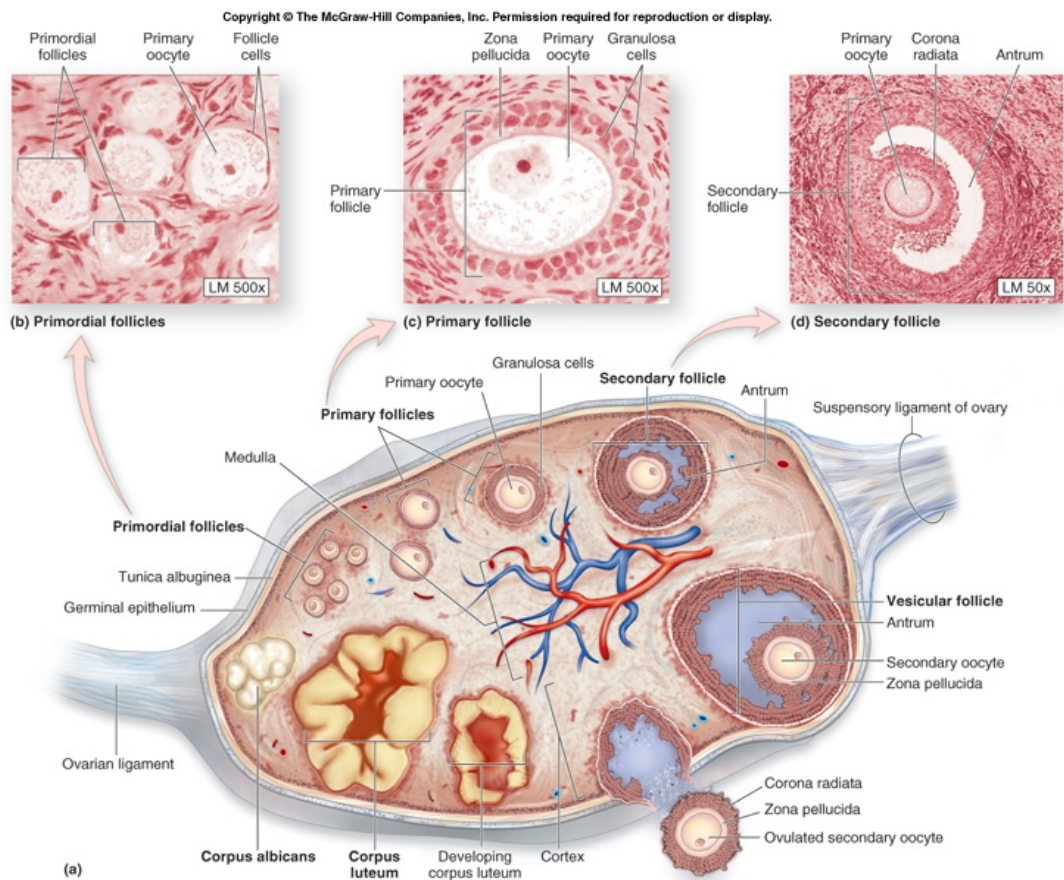
Figure 1.2- The pelvic anatomy with a cross-section of an ovary courtesy of Kellogg foundation. (www.academic.kellogg.cc.mi.us/herbrandsonc/bio201)

1.3 The endometrium

1.3.1 Endometrial anatomy and histology

The endometrium, endodermal of origin[3], is a thin layer of mucous membrane lining the interior of the uterus. Throughout reproductive life, the endometrium undergoes changes during each menstrual cycle in structure and function, which prepare it for blastocyst implantation and to support the growing embryo in the early phase of pregnancy. These

changes are in general driven by the ovarian steroid hormones, which again vary with the menstrual cycle, and are coordinated with the growth and maturation of ovarian follicles. The end of each menstrual cycle is marked by menstruation, during which the thickened endometrium sloughs off and is lost in the menstrual blood flow.

Figure 1.3- Haematoxalin and eosin (H&E) staining of histological specimen of the endometrium magnified x100. http://www.ansci.wisc.edu/jjp1/ansci_repro/index.html. No copyright application needed.

The endometrium consists of a basal layer and stoma covered by ciliated columnar epithelial cells, which extend into the stoma to form the endometrial glands. Regeneration of the epithelium after each menstrual cycle takes place from epithelial stem cells present at the base of the endometrial gland, which remain after menstruation. The endometrium

has also been characterised as being composed of two layers which differ in structure and function, the thicker *stratum functionale*, i.e. functional layer, is sloughed off at menstruation, while the *stratum basale* is retained and provides cells for the cyclic regeneration of the *stratum functionale*. The stratum functionale is apposed on the stratum basale.

1.3.2 The endometrium during the menstrual cycle.

During the menstrual cycle the endometrium varies from 1 to 6 mm in thickness. The 3 recognisable endometrial phases during the menstrual cycle are the proliferative, the secretary and the menstrual phases. At the end of the menstrual phase (day 5 of the cycle), oestrogen levels start to rise and this stimulates the ciliated epithelial cells at the base of the endometrial glands to proliferate rapidly and migrate to cover the surface of the endometrium. The stoma also proliferates and the spiral arteries, which supply blood to the endometrium, lengthen. The proliferative phase continues until *ca*. 24 hours after ovulation (i.e. *ca.* Day 15 of cycle), by which time the endometrium has reached a thickness of *ca*. 3 mm.

The secretary phase then begins, lasting until *ca.* Day 28 of the cycle. Under the influence of P from the corpus lutea, the endometrium becomes oedematous and can grow to *ca*. 6 mm in thickness. The endometrial glands enlarge and become tortuous, and the ciliated epithelial cells within the glands differentiate to a secretary phenotype and produce copious amount of mucoid product. This process occurs in preparation to implantation of a fertilized egg.
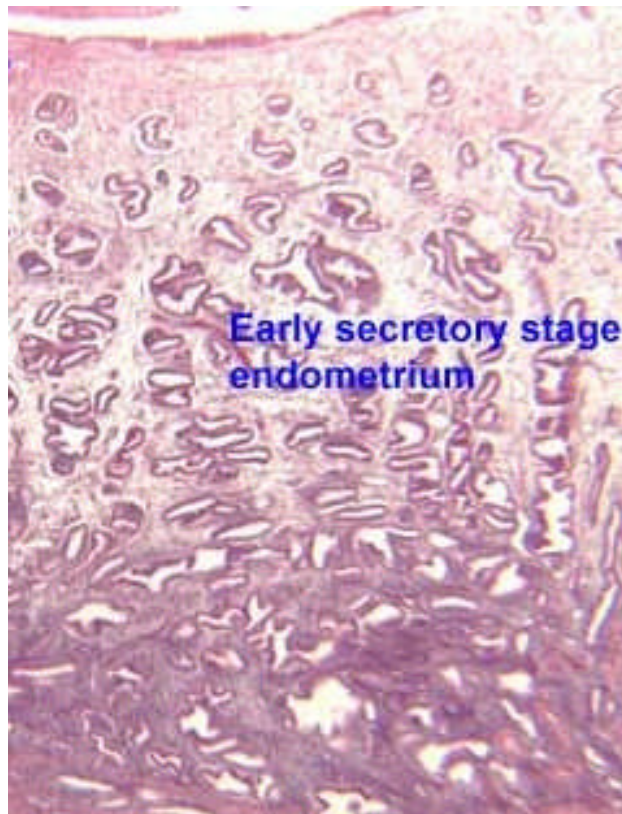
Figure 1.4- H&E staining of endometrium in the secretary phase.

http://www.ansci.wisc.edu/jjp1/ansci_repro/index.html. No copyright permission needed

The menstrual phase occurs if the ovum released at ovulation fails to fertilise. After the corpus lutea degenerates, there is a rapid decline in hormone levels, particularly P, levels. The spiral arteries contract, the endometrial glands stop secreting and the endometrium shrinks. P withdrawal and the reduction in spiral artery blood flow cause the *stratum functionale* to degenerate and slough off, until only the *stratum basale* remains. Spiral artery blood flow still occurs however and it carries the degenerating endometrial lining out of the body *via* the vagina (menses). Indeed, clotting is inhibited during this period of menstrual flow, albeit blood loss is normally restricted except during brief periods when

the walls of the spiral arteries are relaxed. The start of menses is conventionally considered as Day 1 of the new menstrual cycle and the start of the menstrual phase, which normally lasts for 5 days. The average blood loss in each menstrual phase is between 35 and 50 millilitres (mls). Blood flow through the straight arteries maintains the stratum basale.

In the absence of fertilization, a cessation of bleeding accompanies the growth and maturation of a new ovarian follicle. The epithelial cells proliferate quickly and migrate out to restore the surface epithelium as the proliferative phase of the next cycle begins.

In the absence of ovulation (as in anovulatory cycles) a corpus luteum does not form and P is not produced. Thus the endometrium does not enter the secretory phase and continues in the proliferative phase until menstruation.

1.3.3 Apoptosis in the endometrium

As discussed above, the endometrium is in a constant state of flux, developmentally cycling between the proliferative, secretory and menstrual phases. Changes in hormone levels are reflected at the cytological level, but overall endometrial homeostasis is achieved by maintaining a balance between proliferation and apoptosis. Endometrial cell behaviour is primarily governed by the ovarian steroids [5], although other molecules, such as inhibin and activin, play key role. In animal models, proliferation and survival of the endometrial glandular epithelium is dependent on E2, acting via the oestrogen receptor (α and/or β isoform)[6-8]. In contrast, P induces epithelial cell differentiation to the secretory

phenotype, by down-regulating oestrogen receptor expression and by stimulating the conversion of biologically active 17-β-oestradiol into the less active metabolite oestrone[9] [10]in cell culture. Subsequent reduction in the P concentration, or application of P antagonists such as RU486, precipitates apoptotic degeneration of the glandular epithelium[11]. Dysregulated endometrial epithelial cell proliferation may lead to hyperplasia and endometrial carcinoma, although there are thought to be several pathways *via* which endometrial carcinogenesis occur and this will be discussed later. Progesterone acts as the gate-keeper of the endometrium per say with regards to inducing differentiation and apoptosis and halting uncontrolled proliferation.

Apoptosis is a process by which cells undergo 'programmed' cell death, *i.e.* through a controlled sequence of intracellular events occurring in a regulated pathway. Apoptosis was first noted 100 years ago, as cell loss (histolysis) occurring during embryonic development. Events during apoptosis broadly comprise cell shrinkage, nuclear reorganization, cell membrane blebbing and finally nuclear fragmentation to produce apoptotic bodies which are phagocytosed by neighbouring cells or macrophages [12]. Apoptosis plays a significant role in the maintenance of cellular balance in differentiated tissues which show a high cell turnover, such as the intestine and endometrium.

Although apoptosis has for some time been suspected to occur during the menstrual cycle, it is only recently that this process has been studied in each menstrual phase. One of the most studied proteins is bcl2, an inhibitor of apoptosis, which was originally characterised as a 'loss of function' mutated form in a B cell lymphoma[13]. Mertens et al

investigated the endometrial expression of bcl2 during normal ovulatory menstrual cycles and found expression was increased in the proliferative phase and decreased in the secretory phase, especially in glandular epithelial cells [14]. Ki67, a marker of proliferation showed a similar pattern but with a later onset in the proliferative phase and a later decline in the secretory phase than bcl2[15]. It was concluded that bcl2 promotes cell survival by preventing apoptosis. Certainly increases in E2 concentration and oestrogen receptor expression, stimulate bcl2 and Ki67 expression as well as cell proliferation. P on the other hand decreased the levels of bcl2, Ki67 and androgen receptor. Androgen receptor levels remain high in PCOS endometrium and are more highly stimulated by the elevated serum testosterone – this could modify endometrial epithelial cell behaviour considerably. The expression of bcl2 persisted throughout the menstrual cycle in the basal lamina and regenerative epithelial cells of the base of the endometrial glands, presumably preventing apoptosis in these tissues, thus maintaining a source of epithelial cells and a substrate for their growth, allowing reconstruction of the functional endometrium after menstruation.

In a further study, Dahmoun et al noted a rapid increase in the apoptotic index, determined by Bcl2 levels in the endometrial stroma and epithelium during the secretory phase/menstrual phase transition, with a maximum on the second day of menstruation [16]. This occurred in parallel with a decline in the Ki67 index, indicative of reduced proliferation, up to the second day of menstruation. The decrease in the Ki67 proliferation index appeared greater in the endometrial epithelium than the stroma, suggesting that net cell loss is greater in the former. These changes are probably hormone

dependant, as discussed above. Indeed, the late secretory phase decline in serum E2 and P preceded the onset of apoptosis in the endometrial glands and stoma by 2 days, leading to the conclusion that apoptosis may be of importance in hormone withdrawal-induced endometrial gland involution during menstruation[17,18]. It is possible therefore that endometrial glandular hyperplasia may be induced by a reduction in, or absence of, apoptosis, under conditions in which cyclical hormonal withdrawal is infrequent or absent, for example in amenorrhoea or oligomenorrhoea, as it is known that anti-apoptotic agents such as bcl2 are expressed in abundance in endometrial hyperplasia[19].

1.3.4 Inhibin and activin and the endometrium.

There other molecules that are also thought to be involved with homeostasis in the endometrium. Inhibin and activin are such molecules.

Inhibins are dimeric glycoproteins composed of and α subunit and one of two β subunits. They were initially isolated from the gonads and identified as modulators of FSH production from the anterior pituitary gland [20]. Activin, like inhibin consists of an α-subunit but only one of the β subunits. The inhibin/activin subunits are homologues of each other and belong to the transforming factor-beta (TGF-β) family of proteins. In the regulation of the endometrial menstrual cycle inhibin and E2 seem to be the negative controls of FSH secretion that disappear at the time of the luteal regression [21]. The precise role of inhibin and activin in the endometrium are still unknown, however they are thought to play an important role in the regulation of endometrial cell function, regulating endometrial maturation and implantation process [22] . It has also been found that inhibin

an activin are also synthesised in different compartments in the endometrium. Inhibin being predominantly synthesized in the epithelium whilst activin, mostly in the stroma. Activin A, inhibin A and betaglycan (inhibin binding molecule, which is a membrane bound glycoprotein and operates as an inhibin receptor) are expressed and secreted by healthy endometrium[23] and endometrial adenocarcinoma[24,24]. However their exact role in tumorigenesis has not yet been determined. It has been suggested that inhibin may act as a tumour suppressor in the endometrium its expression gradually declines from hyperplastic to malignant tissue.

1.3.5 Investigation of gene expression by microarray

Two main types of gene array exist: large generic arrays and small tailored arrays. The former are available commercially or *via* collaboration with dedicated microarray research laboratories. One well-established large generic technology is the Affymetrix Genechip System[25], in which two-dimensional arrays of synthetic oligonucleotides (ca 20 mers) are synthesized using a combination of photolithography and solid phase DNA synthesis. Affymetrix gene chips allow thousands of transcripts to be analyzed in a single hybridization, and are ideal tools with which to search for unexpectedly regulated genes. Indeed, the recently introduced Affymetrix Human Genome U133 Plus 2.0 Array is considered by the company to be the 'First and most comprehensive whole human genome expression array' and to probe the expression of 47,000 transcripts – virtually the entire set of expressed transcripts. Specificity during hybridization is however dependent on fragment length. Affymetrix circumvent this issue by having oligonucleotide spots at very high density, allowing the expression of a target gene to be assessed by multiple

spots representing ten or more sequences within the gene. spots of DNA containing single base mismatches are also included to account for non-specific binding. Due to the high number of spots and the complex computation required to provide gene expression data, such microarrays are themselves expensive and are supplied within an even more expensive system which includes a fluidics workstation and microarray scanner/detector, as well as a computer with the required proprietary software for data analysis. While it was possible to access an Affymetrix platform for this study, the cost of the microarrays proved beyond the limited budget available. In addition, at the time of the study the gene lists used to compile commercial arrays were inflexible and may have omitted genes of interest. Such considerations are now less likely since the introduction of the very large arrays such as the Affymetrix U133 Plus 2.0 array, which cover the vast majority of possible transcripts produced by the human genome.

An alternative approach is for individual research laboratories to devise and produce relatively small tailored gene arrays focused on their individual interests and needs. These have the advantage of being cost-effective, which allows for sufficient replicates to maximize experimental power. They also tend to be produced by arraying cDNAs of ca. 100 - 1000 bp, rather than smaller oligonucleotides, and therefore exhibit a greater specificity of interaction between the target and probe cDNAs. Thus fewer spots are required to determine the expression of a gene accurately and such microarrays tend to have a greater sensitivity of detection. Tailored gene arrays encourage researchers to perform well-planned, hypothesis driven experiments investigating the expression of a focused set of transcripts. They also allow the use of radio- or fluorophor-labelled cDNA

probes, which can determine gene expression changes in as little as 3-5 µg of total RNA[26]. The ability to use small amounts of starting material is useful when dealing with the female reproductive tract as sometimes only small samples of tissue are available. In our study, the endometrial harvesting method provided, at a maximum, one gram of tissue. A reliable quantitative amplification method was used (as described in Chapter 2, section 2.3.4., page 104) to generate fluorophor-labelled probe for microarray analysis, to minimize usage of endometrial RNA.

As for many techniques used in molecular biology, microarrays rely on the complimentarity of the DNA duplex for specificity, i.e. that the two strands of a DNA duplex, if separated, will reassemble (hybridize) with base-pairing A to T and C to G between the strands. In microarray analysis, multiple single-stranded DNA molecules of defined sequences (targets) are each spotted on a solid support to form a grid array, where they are available for hybridization with any complementary single stranded DNA molecules (probes) in sample applied to them. Commonly, spots are 20 – 500 μm in diameter and arrays contain of the order of $10^2 – 10^5$ spots. Microarrays are generally synthesized on nylon or glass[25] since single stranded DNA binds strongly and is immobilized on these supports. Glass has several advantages over nylon however, in that glass: has a surface which can be chemically altered enabling covalently attachment of DNA; can sustain high temperatures and washes at high ionic strength and is structural tougher; is non-porous allowing small hybridization volumes to be loaded; Has low background fluorescence, hence does not contribute significantly to background 'noise' during detection of probes labelled with fluorophores. The main advantage of glass over

nylon however is that two different probes (commonly one a test sample and one an internal control) can be labelled with different fluorophores and simultaneously incubated on a glass microarray in a single reaction, making data analysis easier and the results more accurate and reliable. In contrast, Nylon arrays cannot be used with fluorescently labelled probes thus can only be used to analyze a single sample, normally radio labelled, at a time. The same array can be subsequently stripped and reprobed, however the conditions used to strip the array often reduce the density of target DNA molecules and thus the run-run comparability becomes unreliable. Effectively, Nylon microarrays are more difficult to control for.

When using microarrays, the manipulation of samples prior to and during experimentation, need to be rigorously controlled. For example, microarray analysis is so sensitive that during gene expression analysis, responses to microenvironment (*e.g.* position of culture dishes in an, or between, incubators) can be detected. Furthermore, microarray processing is also prone to variability due, for example, to slight differences in microarray preparation, sample probe preparation and labelling, wash efficiencies and/or hybridization temperatures. Such variability can cloud data analysis of the effect on gene expression of the primary modified parameter in the experiment, especially when one is searching through thousands of genes, for a small subset of genes that may be variant[27]. The requirement to use suitable controls during microarray hybridization was one reason why this study used glass microarrays hybridized simultaneously to test and common internal standard samples, labelled with the fluorophores Cy3 and Cy5 respectively.

A significant challenge when performing microarray analysis is in the primary and downstream analysis of data. Each gene is likely to be variably expressed in each samples and standardization becomes very difficult. Computational scientists have devised a number of techniques for standardization of gene expression and there is still no recognized best-practice method of data analysis.

1.3.6 Gene expression in the endometrium

As we have previously demonstrated, endometrial carcinogenesis is a multistep process which is under genetic control. It thus seems reasonable to investigate the effects of the altered hormonal environment, as is the case in PCOS, on the genetic profile in the endometrium, in the context of carcinogenesis.

The development of gene microarrays has revolutionised molecular biology in recent years. They allow simultaneous assessment of expression of thousands of genes [28]. Whether it is used to investigate genes responsible for a specific clinicopathological feature or phenotype or genomic classification of tissue, they provide data which has the potential to improve clinical outcome by adapting therapy based on the molecular characteristics of a tumour[29]. They provide information on a large catalogue of genes that may be individually or collectively be targeted for research and also has the advantage of revealing candidate genes that may be missed on a gene by gene approach.

Microarrays have been used in some recent studies to investigate endometrial carcinogenesis and the results suggest that genes are differentially expressed depending on the type and the aetiology of endometrial cancer.

It is also clear that gene expression in the endometrium varies according to the type of tumour[30]. In endometrioid endometrial carcinoma, the type mostly associated with oestrogen stimulation and accounting for more than 80% of all tumours, the loss of the protein receptor protein expression is still unclear. However a study using medroxyprogesterone acetate (MPA) –treated endometrial cancer cell-lines, demonstrated that progestogens regulate the expression of certain proteins which are known to be involved in tumorigenesis. These proteins are CD44, CPSG/Versican, Tenasein and fibronectin[31].

CD44 is expressed on the cell surface and is involved in cancer cell matrix interaction[32,33]. CD44 can promote tumour growth and its increased expression is also associated with increased myometrial invasion[34] as is CPSG /Versican[35], a proteoglycan. Tenasein-C plays a role in tissue remodelling during embryonic development and is re-expressed in many different tumours[36]. It can stimulate proliferation and may be correlated with increased invasion thus suggestively being viewed as a prognostic factor for metastasis in certain cancers[37]. Fibronectin-1, which is a major component of basal lamina, is involved in proliferation, adhesion and migration [38]. It is also known to mediate cell-matrix adhesion by interacting with a wide variety of membrane and matrix component such as fibronectin-1 receptor, integrin α5β1, collagen and CPSG.

Fibronectin-1 is known to be down-regulated by progesterone in poorly differentiated endometrial cancer cell line. These present observations in this study that progestogens such as MPA down-regulates expression of CD44, CPSG, Tenasein-C and Fibronectin-1, suggests that progesterone could play an inhibiting role in tumour cell invasion[39].

Other array studies have suggested increased endometrial expression of genes that are involved cell secretion namely, Mammoglobin 2 (MGB2), Lactotransferrin (LTF), Endothelin 1 & 3(END 1&3). Other genes are concerned with cell adhesion (CTNNA1), transcription (NFYC, HOXB5, CHD3, REST), or even extra-cellular matrix remodelling (HPSG2, MMP11).

MGB2 is over-expressed during implantation and is involved in glandular secretion in hormone responsive tissues. It is also over-expressed in primary breast, stomach and colon cancer[40].

Lactotransferrin is an iron-binding glycoprotein present in most exocrine secretions and its m-RNA and protein are expressed in the uterus and over expressed in endometrial cancer. Its role in carcinogenesis of the endometrium is still undetermined but in vitro studies suggest that it promotes cell proliferation of normal and neoplastic endometrial cells[41].

Endothelin 1 and its m-RNA are present in the human endometrium throughout the menstrual cycle (maximally in the late secretory phase) and are known to provoke

smooth muscle contraction[42]. It is also known to be mitogenic on vascular smooth muscle[43]. Human endometrial carcinoma cells are also known to express endothelin-1[44]. Little is known about the function of endothelin-3 in the endometrium but is has been found to be down-regulated during the window of implantation[45].

A more recent study of the endometrium has also revealed Oviduct-specific glycoprotein to be differentially expressed as the endometrium changes from a benign state to a neoplastic one[46]. It was first identified in oviduct epithelium and has been thought to be an early indicator for malignant transformation[47]. OGP expression begins under conditions of unopposed oestrogen exposure, which is a known risk factor for endometrioid EC. It has recently been identified in the stem cells of the basalis layer of the endometrium[46]. It has been proposed that genetic alterations that induce endometrial neoplasia are acquired sequentially by the non-shedding stem cells. Woo et al demonstrated a correlation between loss of PTEN and gain in OGP expression along. They also demonstrated a gain in staining in tissue where there has been prolonged oestrogen stimulation such as is the case in non-typical and atypical hyperplasia.

Gene expression in endometrial tissue, whether benign or malignant is still a relatively new area of study. As part of this research project we plan to explore this area further by studying gene expression in the endometrium of women with PCOS and to determine whether this changes in the presence of disturbed metabolic and hormonal environment.

1.4 The menstrual cycle and ovulation

The menstrual cycle results in the preparation of the endometrium for implantation, the production of a ripe fertilization-competent ovum at ovulation, and either the endometrial implantation and support of the developing zygote if fertilization occurs, or the shedding of the endometrial lining and unfertilized ovum in the menstrual blood flow if it does not. The menstrual cycle is normally approximately one month long, although cycle length may vary between 21 to 35 days, without associated pathology. It is regulated at the endocrine level by the cyclical release of follicle stimulating hormone (FSH), luteinizing hormone (LH) and prolactin from the anterior pituitary. FSH and LH govern the ovarian biosynthesis of steroid hormones, primarily E2 and P.

The menstrual cycle is divided into a proliferative or follicular phase, commonly of 7 - 21 days (depending on the time required for follicular maturation), a secretory phase of relatively constant in length (14 days), and a menstrual phase of 5 – 7 days. The ovaries, uterus and cervix all undergo substantial changes during the cycle, as detailed below for a typical 28 day cycle.

Day 1: Onset of vaginal menstrual bleeding during which the secretory endometrial mucosa degenerates, sloughs off and is shed in the menstrual blood flow.

Day 5 – 14: The follicular phase begins at the end of menstruation and continues until ovulation takes place. Increasing levels of pituitary FSH stimulate the development and maturation of a cohort of follicles. One dominant follicle then acquire granulosa cells which then response to the LH surge. The dominant follicle itself synthesizes increasing

amounts of E2. E2 production rises steeply from day 10 and reaches a peak on day 13, the concurrent rise in LH leading to ovulation. The uterine epithelium proliferates throughout the follicular phase under the influence of E2, to provide a highly vascularised surface receptive to the fertilized ovum. The cervix remains small and closed by highly viscous mucus.

Day 14: Ovulation occurs and the ovum (egg) is released from its follicle and enters the fimbria of the fallopian tube. During ovulation the cervical mucus becomes less viscous and the os cervix enlarges, allowing sperm to pass through.

Day 14 – 28: The luteal phase begins as the corpus luteum develops from the now empty Graafian follicle and starts to produce P. In response, the endometrium differentiates to perform a secretory function, as discussed in Section 1.3. The effect of P on the endometrium is maximal around day 22, when the embryo, if fertilization occurs, arrives in the uterus from the fallopian tube. If fertilization has not occurred, E2 and P begin to inhibit the release of hypothalamic gonadotrophin releasing hormone (Gn-RH) which leads to degeneration of the corpus luteum. The resulting rapid drop in serum E2 and P levels cause constriction of the endometrial blood vessels and endometrial ischaemia; the uterine mucosa is shed and this begins another menstrual bleed. (See diagram below)
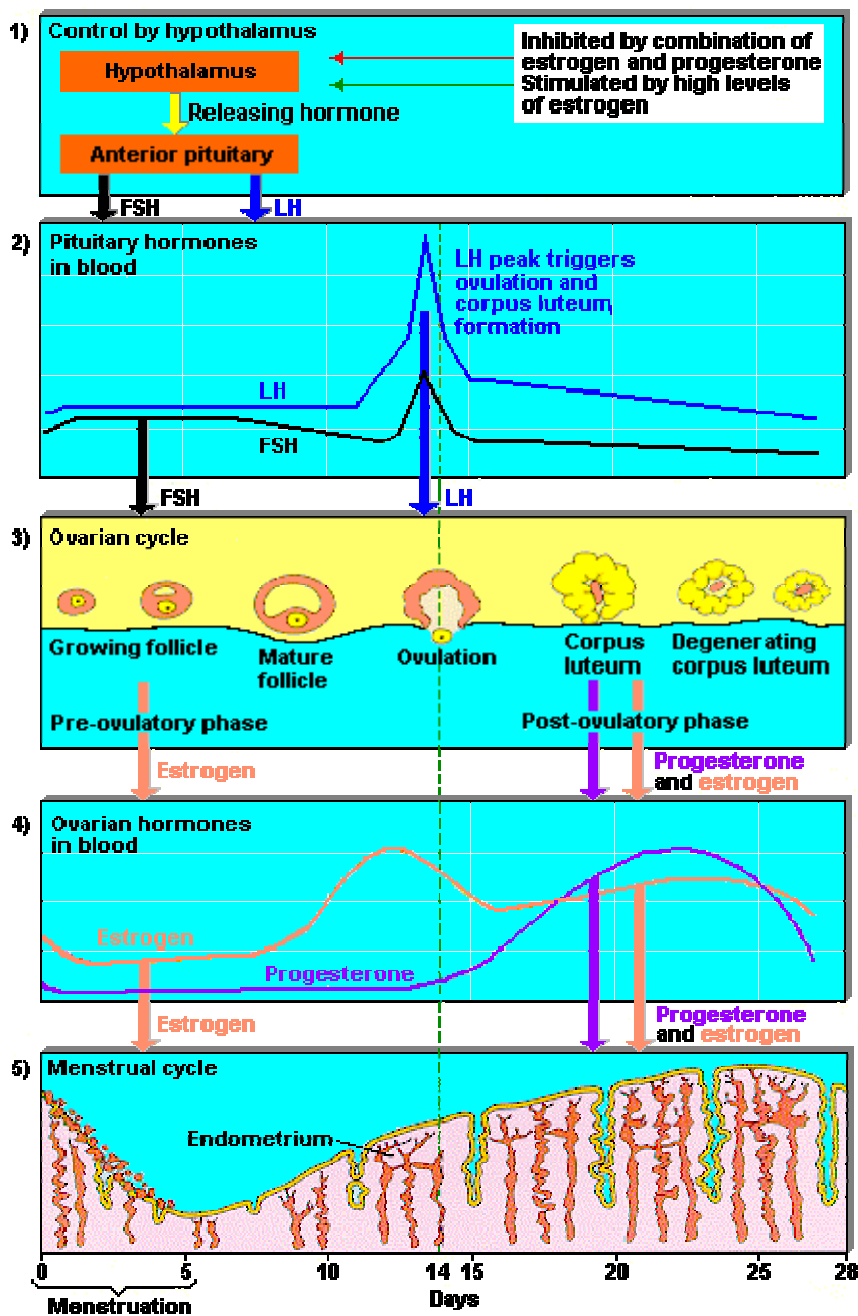
Figure 1.5– Illustrate the menstrual cycle and the various endocrine, morphological and histological changes that take place during this period. Reproduced courtesy of Dr Lam.

http://www.lammd.com/A3R_brief_in_doc_format/print/Estrogen_Dominance.htm

1.4.1 Hormonal control of ovulation

GnRH, produced in the hypothalamus, stimulates the release of FSH and LH from the anterior lobe of the pituitary gland. GnRH is released in a pulsatile fashion with a frequency of 1-1.5 pulse/hour before ovulation and then up to 4 hours thereafter the release of LH and FSH is also under the influence of other factors, such as the central nervous system and E2 and P levels, by positive and negative feed-back. . A faster frequency of GnRH release greatly reduces the amount of LH and FSH secreted by the pituitary and is associated with anovulation. Slower GnRH release allows the development of E2 synthesizing follicles, but ovulation cannot occur as the pituitary requires several days exposure to GnRH pulses at 1 – 1.5 pulses/hour to allow it to respond to the mid cycle increase in E2 with the pre-ovulatory LH surge.

During the follicular phase, the secretion of LH remains low, but on day 12 - 13, a positive feed-back loop develops in which FSH-driven ovarian E2 production stimulates further release of FSH and LH from the pituitary, which in turn stimulates greater follicular E2 and P synthesis and release. This positive feedback induces a surge in serum LH, FSH, E2 and P, the rapid increase in LH having a key role in initiating ovulation.

In the luteal phase of the menstrual cycle, negative feedback of E2 and P on FSH and LH is established, thus E2 and P inhibit FSH and LH secretion. The rapid decline in FSH and LH after ovulation prevents the maturation of further follicles, and also leads to a decline in ovarian E2 and P production. Indeed E2 and P drop drastically by day 26 of the cycle, resulting in the beginning of the menstrual bleed.

1.5. Polycystic Ovary Syndrome

Descriptions of what is now described as Polycystic Ovary Syndrome (PCOS) can be traced as far back as 1844, when Chereau described sclerocystic changes in the human ovary[48]. It was not until 1935, that Stein and Leventhal published their classic paper formally describing PCOS in a group of infertile women[49].

PCOS is one of the most common endocrine conditions, present in 5-10% of women of reproductive ages, although its aetiology is still unknown[50,51]. It is a heterogeneous disorder whose expression may vary from one individual to another and also temporally within the same individual. The signs and symptoms (as classified in table 1), associated with this condition are menstrual cycle disturbances, obesity, infertility, acne, hirsutism and other signs of hyperandrogenism, and polycystic ovary (PCO) morphology on transvaginal ultrasound scan[50,52]. Metabolic disturbances such as elevated serum concentrations of LH, testosterone (T), insulin and prolactin are often present and these may have profound implications on the long-term health of women with PCOS[53].

1.5.1 Aetiology

The causes of PCOS would appear to have a genetic component as there is a weak tendency for it to cluster in families [54]. Hyperandrogenaemia appears to be the strongest inherited characteristic in familial cases. Genetic studies in PCOS are hampered by several factors; PCOS is associated with infertility and this make linkage analysis of a large pedigree, difficult. Furthermore, as PCOS is a heterogeneous condition, familial studies cannot be easily compared because of the use of differing diagnostic criteria. In

addition to this a male phenotype for the syndrome has not yet been clearly identified[55]. However despite all this, all studies seem to suggest that PCOS has a dominantly inherited trait of low penetrance and variable expressivity[56].

Symptoms (% patients affected)	Associated endocrine manifestations	Possible late sequelae (%)
Obesity (38) Menstrual disturbance (66) Hyperandrogenism (48) Infertility (73-anovulatory) Asymptomatic (20)	↑Androgens ↑Luteinizing hormone ↑LH: FSH ratio ↑Free Oestradiol ↑Fasting Insulin ↑ Prolactin ↓ Sex hormone binding Globulin	Diabetes mellitus (11) Cardiovascular disease Hyperinsulinaemia High LDL Endometrial hyperplasia

Table 1.6 - shows the spectrum of clinical features of PCOS

The most studied genes are those involved in steroidogenesis and those involved in the metabolic pathways involved in insulin action.

CYP17 (cytochrome P450 17-hydroxylase/17, 20-desmolase) - this encodes for 17-hydroxylase/17, 20-lyase. Initial studies showed an association with PCOS[57] though subsequent ones failed to confirm this[58].

CYP11A (cytochrome P450 side-chain cleavage enzyme), encodes for cholesterol side-chain cleavage enzyme and one study found a link with hyperandrogenaemia in women with PCOS[59].

CYP21 (cytochrome P450 21-hydroxylase) - encodes for 21-hydroxylase, the enzyme responsible for most cases of congenital adrenal hyperplasia (CAH). Recent studies have

found a significant prevalence of CYP21 mutations in women with PCOS with a normal 17-hydroxyprogesterone response to adrenocorticotrophic hormone (ATCH)[54].

Androgen receptor- The short trinucleotide repeat receptor CAG is known to be inversely associated with androgen levels. The longer CAG alleles and biallelic means in exon 1 of the androgen receptor is significantly more expressed in women with PCOS compare to normal women[60].

Insulin receptor – A number of studies examining the insulin receptor for gene sequence major mutations have been inconclusive so far[61].

Recently two studies have found linkage between a marker (D19S884 at chromosome 19p13.3) located near the insulin receptor gene and PCOS[61]. Although the putative PCOS gene in this region remain to be identified, likely involvement in signal transduction mechanisms, leading to altered expression of a family of genes involved in steroidogenesis and /or insulin action.

CaPLain-10- CaPLain-10 is a cysteine protease that has been shown to be associated with susceptibility to type II diabetes. A recent study showed the 112/121-haplotype was associated with higher insulin levels in African- American women and a higher risk of PCOS in both African-American and Caucasian women.

As we mentioned , it is believed that multiple genes are disturbed in PCOS, due to the heterogeneity of symptoms seen between patients, and that the expression of some of

those functionally compromised genes are environmentally sensitive, for example to nutrition and exercise.

While PCOS is now thought to be manifested biochemically from the foetal stages onwards, the syndrome normally becomes apparent in adolescence. At this time it is associated with increased weight gain at puberty, although it is not known whether this is a result of the syndrome, or a feature which exacerbates the expression of other PCOS[62].

In the past, it was difficult to evaluate studies on PCOS, due to the lack of consensus over diagnosis of the syndrome. In Europe, a combination of all the above-mentioned symptoms was considered before making a diagnosis[2,63] , whilst in America, the syndrome was diagnosed on findings of biochemical hyperandrogenism and ovulatory dysfunction, in the absence of non-classical adrenal hyperplasia; the presence of PCO morphology was not required[64]. Recently however, agreed diagnostic criteria have been produced by the ASRM/ESHRE (American Society Of Reproductive Medicine/ European Society for Human Reproduction and Embryology), where by two of the following support a diagnosis of PCOS: chronic anovulation\oligo-ovulation; clinical or biochemical evidence of hyperandrogenism; PCO on trans-vaginal ultrasound scan.

A number of interlinking factors are thought to affect the symptoms seen in PCOS. A gain in weight worsens symptoms, whilst weight loss usually brings about an improvement in the endocrine and metabolic features[65]. It is clear that the feed-back mechanism from the polycystic ovary to the pituitary and the hypothalamus is disturbed,

41

secondary to abnormalities in the secretion of ovarian steroid hormones and also other hormones such as leptin or inhibin[66].

One of the most common features of PCOS is hyperandrogenaemia and its ovarian source (theca cells) is important in understanding the pathogenesis of the syndrome and its sequelae. PCOS patients have an exaggerated ovarian response to gonadotrophin (both LH and FSH) stimulation[67], resulting in hypersecretion of androgens (such as androstenedione, testosterone, and DHT) by the ovary[67]. It has been proposed that this dysregulation in steroid production is due to an excess of LH secretion in the pituitary or increased sensitivity to LH of the thecal cells of the ovary[68]. Insulin and other growth factors may also be implicated in this process. LH hypersecretion is found in ca. 40% of women with PCOS and LH probably induces ovarian steroidogenesis whilst also negatively influencing ovulation and oocyte maturation; hence the deleterious effect on reproductive health[69]. It is also believed that there is a defect in the ovarian-pituitary negative feed-back mechanism whereby a still unidentified non-steroidal factor that inhibits LH secretion, is absent in women with PCOS.

The action of LH is thought to be further enhanced by insulin and insulin growth factors (IGF's)[68]. In fact, in addition to this, insulin and IGF's are also known to increase androgenesis via the theca cells and augment FSH action on the granulosa cells of the ovaries[70]. The action of insulin and IGF's on the ovaries to promote steroidogenesis is still not well known but it is thought to encompass both transcriptional and post transcriptional actions including the stability of specific m-RNA. Insulin is known to

rapidly increase m-RNA translation but there is no data to suggest that insulin or IGF's increase m-RNA translation of protein encoding for steroidogenesis. Marked ovarian hyperandrogenaemia is associated with severe insulin resistance due to mutations that interfere with binding or signalling as well as stimulating auto antibodies to the insulin receptor[71]. The resulting hyperinsulinaemia is thought to activate ovarian IGF type I receptors and consequently increase androgen production via a synergistic effect of LH and IGF. There seem to thus be a paradox where there is insulin resistance and yet insulin seems to increase ovarian androgen production. This is explained by the fact that insulin and IGF's- have multiple actions and their actions are mediated via multiple intracellular pathways and finally, insulin can regulate ovarian androgenesis via its cognate receptor[72,73]. Insulin mitogenic and metabolic actions are known to be mediated via distinct post receptor signalling pathways[74]. Experimentally, selective insulin resistance can be produced whilst its mitogenic effect can remain intact[71]. Therefore a defect in insulin clearance of glucose in muscle leading to a reduced disposal of glucose may not disrupt or even enhance steroidogenesis in theca cells and suppress sex hormone binding globulin production by hepatocytes and consequently increase androgen levels [75]. This hypothesis, of tissue specific actions of insulin, is supported is supported by several observations. Insulin can modulate gonadotrophin-driven steroidogenesis by PCOS granulosa cells in vitro[70]. Insulin is also known to stimulate thecal cells androgen synthesis via other mediators like inositolglycan rather than the standard pathway involving GLUT-4 translocation. Finally there is some evidence to show that there may be metabolic but not mitogenic alterations in insulin action[76]. Evidence on the mechanism of insulin resistance in PCOS is still not known but there is a belief that there is serine

phosphorylation of the insulin receptor in the ovarian cells of women with PCOS with selective induction of the 17,20-lyase activity, thus producing more androgen[77].

The ovary in women with PCOS also contains an abnormal accumulation of multiple, small antral follicles, which are neither atretic nor apoptotic, but are arrested in their development [78]. The mechanism, by which a dominant follicle is selected, seems to be disturbed. These small follicles seem to be under the influence of insulin-like growth factor (IGF; which isoform), sex hormone binding globulin (SHBG), plasminogen activator type 1 and leptin. Insulin is also known to directly stimulate ovarian stromal cells androgen synthesis and to inhibit hepatic SHBG synthesis, thus increasing circulating levels of free active testosterone[67].

Metformin, an insulin sensitizer, seems to decrease serum insulin concentrations in women with PCOS, thus directly counteracting these effects of insulin and controlling the abnormal biochemical profile commonly found in PCOS[79].

1.5.2 The treatment of PCOS

The treatment of PCOS consists mainly of controlling the symptoms of the syndrome in an attempt to achieve short- and long-term goals. Short-term goals include regulation of menses, treatment for infertility if required, control of hirsutism and acne, and stabilization or reduction of weight. Long-term goals include the prevention of endometrial hyperplasia and diabetes, avoiding obesity and the control of biochemical risk factors associated with heart disease. Multiple concomitant therapies are often

necessary in order to address the variety of symptoms exhibited in PCOS, but they may also confer additive benefits with regards to the efficacy of treatment.

Lifestyle modifications such as calorific restriction and exercise can improve overall weight, waist circumference, insulin sensitivity and fasting insulin levels in anovulatory women with PCOS. Indeed, an intensive program of calorie restriction, exercise and lifestyle changes for 6 months, resulted in the re-establishment of ovulatory cycles in 90% of previously amenorrhoeic anovulatory patients[80], while in another study, a mean weight loss of 11.5% significantly improved insulin sensitivity in obese women with PCOS[81].

Hirsutism and acne can be managed by a multidisciplinary approach involving the gynaecologist, the endocrinologist and the dermatologist. Hirsutism can be treated by mechanical hair removal, by shaving, plucking, depilation, electrolysis, intense pulse light (IPL) or laser hair removal; however treatment is most effective when used in combination with hormonal treatment in the form of an anti-androgen to decrease the rate of hair growth. Recently, topical eflornithine which reduces the rate of hair growth, was found to be most effective when used in combination to laser or IPL[82]

The combined oral contraceptive pill (COCP) effectively controls many symptoms of PCOS[83,84]. In addition to regulating menstrual cycles, endometrial hyperplasia is prevented and the incidence of acne and hirsutism is reduced, presumably because COCP

suppresses pituitary gonadotrophin production by negative feed-back mechanism, thus decreasing ovarian steroidogenesis.

The effects of hyperandrogenaemia can be controlled using anti-androgen therapy (with or without COCP)[85]. Such therapies either block the interaction between androgens and the androgen receptor, thus preventing effects at the levels of gene expression, or inhibit 5α-reductase[86], the enzyme that converts the less active testosterone into its more active metabolite androgen dihydrotestosterone, for example within the hair follicle.

Metformin, as mentioned above, is an insulin sensitizer widely used as a treatment for PCOS [87]. As such, it inhibits hepatic glucose production and increases peripheral tissue sensitivity to insulin. In doses of 1.5 – 2 g per day, metformin decreases ovarian androgen production, serum androgen and insulin levels. It also improves ovulatory rates and menstrual cyclicity, in women who were previously suffering from ovulatory dysfunctions[79].

In anovulatory women with PCOS, the first-line treatment to improve fertility is the administration of clomiphene citrate, an antagonist of E2 followed by chronic low-dose FSH, if the former alone is unsuccessful. GnRH agonists such Bursereline acetate, are also used for ovulation induction in patients with high tonic LH levels, to improve pregnancy and decrease miscarriage rates[88]. Laparoscopic ovarian drilling is also used in normal-weight patients with high LH tonic concentrations [89], since it decreases ovarian

androgen production and improves ovulation rates. The biochemical alterations brought about by this procedure are only temporary however.

Further investigation of the causes of PCOS will ultimately lead to a better ability to direct therapy to manage the variety of symptoms encountered, or even to cure the syndrome itself. Traditionally, treatment of PCOS has focused on controlling the overt symptoms of androgen excess, correcting anovulation if fertility is an issue, and trying to minimize the risk of endometrial hyperplasia. Recent therapies concentrate more on correcting the metabolic disturbances dyslipidaemia or impaired glucose metabolism. More randomized controlled studies are required to determine whether the traditional or more recent approaches are better.

1.5.3 Polycystic ovarian morphology

A clear distinction has to be made between the presence of polycystic ovaries (PCO) and PCOS. The former can be observed in women with regular menstrual cycles and in whom there are no symptoms or signs of hyperandrogenism[90], thus a diagnosis of PCOS is not supported. Historically, PCO were observed visually at laparotomy and confirmed by histological analysis of an ovarian biopsy. Further studies identified consistent clinical and endocrine abnormalities in some women with PCO, and the concept of PCOS was developed. With the advent of ultrasonographic ovarian scanning it became clear that some women with PCO do not display any of the other common symptoms of PCOS[91]. Indeed, the prevalence of PCO in the general population has been found to be as high as 25%[92].
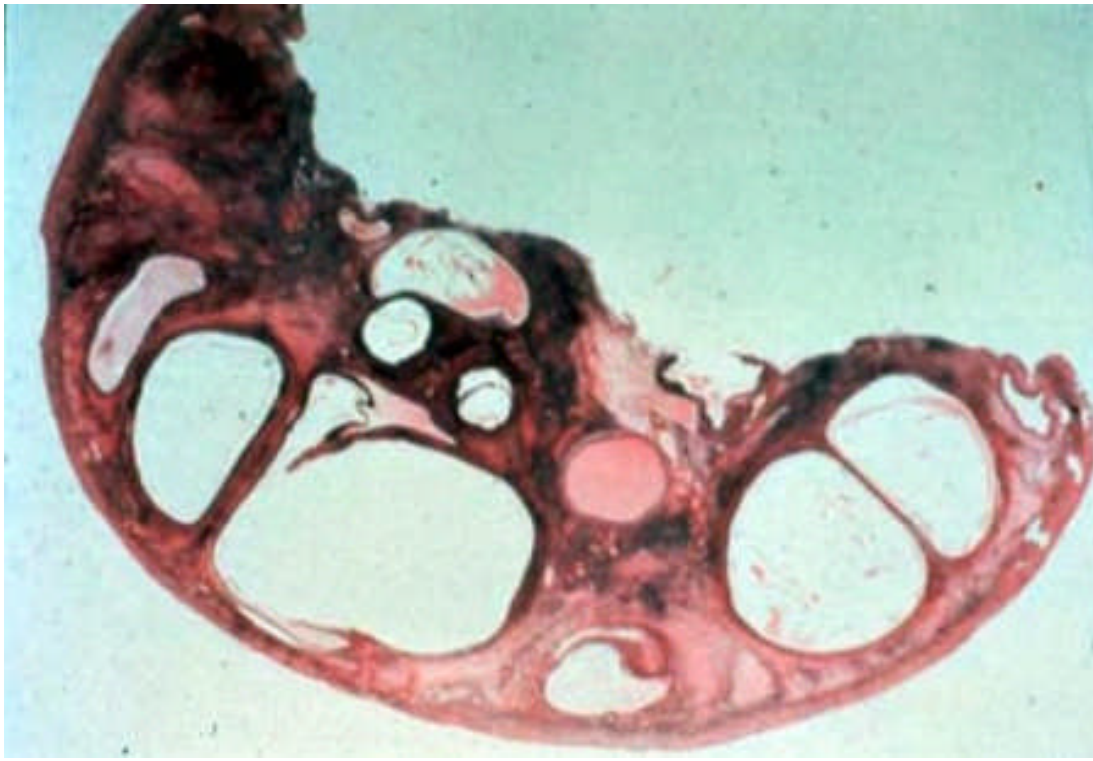
Fig 1.7 -shows a cross-section through a polycystic ovary. This picture was reproduced courtesy of http://www.ansci.wisc.edu/jjp1/ansci_repro/index.html.

On histology, a PCO exhibits prominent theca, thickening of the tunica albuginea, increased stromal volume and multiple cystic follicles. Good correlation has been shown between ultrasound imaging and histopathological assessment when applied to the diagnosis of PCO morphology [93]. Indeed, histological diagnosis of PCO has on the whole been superseded by ultrasonographic assessment, due to the relative non-invasiveness, and ease and quality of imaging, provided by the latter technique. The most widely quoted paper regarding ultrasonographic assessment of PCO morphology was published in 1985 by Adams et al. An ovary was considered polycystic if it contained 10 or more

follicles between 2 and 8 mm in diameter in one imaging plane, arranged peripherally around a dense core of ovarian stroma, or scattered throughout an increased amount of stroma. Ultrasound was original performed trans-abdominally, however this mode of operation has largely been superseded by trans-vaginal scanning, which provides better spatial resolution since the ovaries are closer to the vaginal wall, albeit at the cost of increased invasiveness to the patient and decreased examination depth. Trans-vaginal scanning also confers the advantage that the patient does not have to have a full bladder - which is more comfortable and saves time.

While the definition of PCO morphology developed by Adams et al [94] has been of some utility in PCOS research, recent international consensus has been achieved regarding simplified uniform criteria to define PCO morphology[95]. With modern high resolution ultrasound machines operated by skilled personnel, and taking a trans-vaginal approach especially in obese patients, a PCO is characterised by 12 or more follicles measuring 2 – 9 mm diameter and/or increased ovarian volume (>10 ml), calculated as 0.5 x ovarian length x width x thickness. Follicle number must be estimated in longitudinal, transverse and antero-posterior ovarian cross-sections and follicle size should be expressed as the mean of the diameters measured in these three axes. If a dominant follicle (>10 mm diameter) or corpus luteum is present, the scan must be repeated during the next cycle, while any indications of an ovarian cyst necessitate further investigation.

In contrast to earlier definitions, follicle distribution and stromal echogenicity and volume are no longer criteria for diagnosis of PCO; follicle distribution is variable

between different PCO, and stromal echogenicity is an operator-dependent and a subjective assessment criterion; stromal volume increases are though considered specific to the PCO. Measurement of ovarian volume is however a good surrogate for stromal volume in clinical practice. It should be noted that this definition does not apply to women on the OCP, which is known to reduce ovarian volume in women with PCO, although 'polycystic' features may persist. Furthermore, it requires that women with regular menstrual cycles should be scanned in the early follicular phase (days 3-5 of cycle), while oligo/amenorrhoeic women should either be scanned at random or after a progestogen-induced bleed.

Although PCO are found in a significant number of patients with PCOS (nearly 70%)[63], some women exhibit PCO in the absence of typical features of PCOS, such as oligomenorrhoea and hyperandrogenaemia [90]. These women are considered normal, according to the consensus ESHRE/ASRM definition of PCOS, yet there is evidence to suggest they also exhibit elevated levels of cardiovascular risk markers[96],disturbances in insulin action and glucose homeostasis[97]. The endometrium in such women may also be less receptive to implantation [98].

Fig. 1.8 a) - Ultrasound image of a polycystic ovary

Fig. 1.8 b) Laparoscopic appearance of a polycystic ovary. The ovary is enlarged and has a

smooth opaque appearance.

Both images were reproduced with permission of Dr Samuel Marcus – Director of the IVF-

fertility center, Chicago.

1.6   Endometrial cancer

1.6.1 Epidemiology

Endometrial cancer (EC) is the most common gynaecological cancer in the Western World. The United Kingdom incidence in 2005[99,100] was 6821 cases (2% of all reported cancers). EC resulted in 1% of all cancer deaths in that year, though the overall 5-year survival rate for EC is greater than 70%. EC occurs during reproductive and menopausal life, with a median age of 61 years. Although the majority of patients are post-menopausal at diagnosis, 5% present with the condition before the age of 40 and 20 - 25% will be diagnosed before the menopause. The incidence of EC has increased over the last 3 decades igniting new debate as to whether it is linked to the use of exogenous oestrogens or other environmental factors.

1.6.2 Aetiology

The OCP is thought to offer some protection against the development of EC, especially in nulliparous women as, surprisingly, does cigarette smoking[101]. Multiple risk factors for EC have been described and may be classified into 3 main groups: variants of normal anatomy and physiology; frank abnormality or disease(endocrine or metabolic); and exposure to carcinogen [102]. Obesity, nulliparity and late menopause can all be included under the first group, with upper body or central obesity conferring the greatest risk[103]. Serum SHBG is depressed in obese women, especially those with upper body fat localization[104], and it is possible such women have higher levels of free oestrogens

(unbound to SHBG) available to stimulate the proliferation of normal endometrial epithelial cells, as well as oestrogen-sensitive endometrial tumour cells.

Within the second group , diabetes mellitus (type II) is prominent, increasing the risk of EC 2.8-fold, even after adjusting for weight, age and socio-economic status [105]. The relationship between unopposed oestrogenic stimulation of the endometrium in anovulatory women and EC is also well-documented, such women having an increased risk of EC which is counteracted by progestogen therapy[106]. In some patients with EC, a previous history of complex endometrial hyperplasia with atypia can be demonstrated. An increased incidence of EC has also been found in association with tamoxifen treatment of breast cancer, perhaps related to the oestrogenic effect of tamoxifen on the endometrium[107].

A high index of suspicion of EC should be present when a post-menopausal woman complains of vaginal bleeding, or pre-menopausal women with associated risk factors present with persistent, inter-menstrual bleeding. Investigation, after the taking of a careful patient history, involves a full blood count, a trans-vaginal ultrasound, an endometrial biopsy and a hysteroscopy.

1.6.3 EC morphology

The most common EC cell type (75 – 80% of case) is endometrioid adenocarcinoma, which is composed of malignant endometrial glandular epithelial elements, which can be morphologically divided into the subtypes: ciliated adenocarcinoma; secretory

adenocarcinoma; papillary or villoglandular; adenocarcinoma with squamous differentiation (either adenoacanthoma or adenosquamous). Clear cell (4% of cases) and papillary serous carcinoma (<10% of cases) of the endometrium are tumours that are histologically similar to those noted in the ovary and the fallopian tube, and carry are a worse prognosis than does endometrioid EC. Mucinous, squamous and undifferentiated endometrial tumours are rarely encountered (each <1% of cases), while mixed tumour morphology is noted in 10% of cases.

1.6.4 EC staging

A hysterectomy is required to determine the degree of myometrial and pelvic invasion. Surgical staging criteria have been adopted by the International Federation of Gynaecology and Obstetrics (FIGO) and by the American Joint Committee on Cancer (AJCC) [REF; 30-32]. Stage I EC is confined to the corpus uteri, either within the endometrium itself (Stage IA) or to less than (Stage 1B) or greater than (Stage 1C) half the myometrial thickness. Stage II EC has not extended beyond the uterus but does involve the corpus uteri and the cervix, either limited to endocervical glandular involvement (Stage IIA) or with cervical stromal invasion (Stage IIB). Stage III EC extends outside of the uterus but is confined to the true pelvis, either having invaded the serosa and/or adnexa and/or peritoneum (Stage IIIA), the vagina (Stage IIIB) or the pelvic and/or para-aortic lymph nodes (Stage IIIC). Stage IV EC exhibits more widespread dissemination, either to the bladder and/or bowel mucosa (Stage IVA) or to distant metastases including the intra-abdominal and/or inguinal lymph nodes (Stage IVB).

EC can also be grouped with regard to the degree of differentiation of the adenocarcinoma; either ≤5% (G1), 6 – 50% (G2) or 50%≤ (G3) of cells exhibiting a non-squamous or solid growth pattern. By mixing the FIGO staging criteria with this differentiation based grouping, EC can be relatively precisely described with respect to degree of spread and morphology/invasive potential[108].

1.6.5 The pathology of EC

Clinicopathological findings indicate that EC arises through a series of precursor lesions, namely simple and complex hyperplasia, with or without atypia[109]. These lesions are thought to develop, survive and proliferate in response to unopposed and prolonged oestrogenic stimulation of the endometrium, with the assistance of various growth factors and cytokines[110]. It is however an over-simplification to suggest that endometrial tumours can be divided into oestrogen-dependent and -independent.

Recent evidence suggests that endometrial carcinogenesis occurs *via* several pathways, namely hyperplasia, metaplasia, and *de novo*[110]. This has lead to reclassification of the histopathological criteria regarding endometrial carcinogenesis endometrial intra-epithelial neoplasia (EIN) is proposed as the primary precursor lesion for EC[111]. Multistep genetic alterations are thought to take place to produce malignant tumours, and three steps have been suggested to be important in endometrial tumorigenesis. In the first step - initiation - a limited number of genetic disturbances are thought to be required to produce EIN. In the second stage - promotion -a further tumour promoting events occur

which amplify the effects of those earlier events, leading to immortalization and uncontrolled proliferation. In the third stage – Tumour progression- further genetic deficits occur to facilitate progression to a highly proliferative, metastatic phenotype[110]

1.6.5.1 Initiation of EC

Initiation can occur *via* activation of proto-oncogene, inactivation of tumour suppressor genes or by the effects of microsatellite instability on gene structure and expression.

Expression of the *K-ras* proto-oncogene has been detected in 10 - 37% of EC and 6 - 16% of atypical endometrial hyperplasia, whereas *K-ras* expression is undetectable in simple and complex hyperplasia[112]. The activated *K-ras* gene product, a 21 kD protein (p21), produces an unregulated intracellular signal that induces cell proliferation [113]. Its activation is thought to be an early initiating event in the development of EC. *K-ras* mutations are much more frequent in the well differentiated endometrioid EC type than in any other type of endometrial tumour [114]. Other oncogenes are also involved in initiation, such as *myb*, *fos, myc*, *fms* and *HER-2/neu*, and their expression is associated with a poorer prognosis when observed in EC[110].

DNA microsatellites are scattered throughout the genome, consisting of multiple repeats of simple one to six nucleotide units. Microsatellites based on the CA dinucleotide repeat are the most common in humans, occurring at >10,000 sites throughout the genome [115]. Microsatellite instability (MSI) is characterized by changes in microsatellite length due to variation in the number of repeat units within the microsatellite. This type of alteration

can be detected only if many cells are affected by the same change, thus it is an indicator of clonal expansion, e.g. in neoplasia. MSI is believed to be caused by defects in genes coding enzymes involved in DNA-mismatched repair (MMR). Several genes, such as *MSH2*, *MLH1*, *PMS1, PMS2, GTBP*, are involved in DNA mis-matched repair in the human, but the loss of *MLH1* expression due to hypermethylation of its promoter, specifically leads to deficiencies in endometrial MMR capacity. Indeed, *MLH1* - promoter methylation seems to be a key early event in endometrial carcinogenesis[116].

Tumour suppressor genes encode proteins that normally inhibit or modulate cell proliferation. Perhaps the most investigated tumour suppressor gene is p53, which has been found to be mutated in a variety of tumours including EC[117]. Mutations impacting on p53 tumour-suppressor function are more common in poorly differentiated endometrioid type EC, and p53 over expression (the mutated or wild type of p53 still binds to antibody when investigated with immunohistochemistry) is associated with advanced clinical stage, myometrial invasion, and distant metastatic PSread[118]. Therefore, inactivation of p53 is likely to be a late event in carcinogenesis[119].

PTEN (Phosphatase and Tensin Homolog Deleted on Chromosome 10), on the other hand, seems to be inactivated early in the carcinogenic pathway in EC [120]; it was identified as a candidate tumour suppressor gene by a number of study groups [111]. PTEN is often mutated in many human tumour types such as endometrial, prostate, glioblastomas and breast cancers[121]. PTEN encodes a 403-amino acid protein, which is a member of the tyrosine phosphatase family. It inhibits the phosphatidylinositol 3-kinase

/Akt signalling pathway by removing the phosphate in D3-phoPShate group of phoPShoinositide-3, 4, 5-triphoPShate (PIP3)[122]. Dephosphorylation of PIP3 is a critical determinant for controlling cell growth, proliferation, and survival. Inhibition of PIP3 causes blocking of Akt signalling, which in turn, ends up with an increased activity on some pro-apoptotic molecules. Therefore, PTEN plays an important role in cell survival and apoptosis because it inhibits cell cycle progression by down-regulating Cyclin D1 and activates pro-apoptotic molecules through Akt-dependent and independent pathways[123]. PTEN also inhibits the integrins, a large family of specific cell surface receptors for extracellular matrix proteins, which have a major role in cell adhesion, migration and invasion. Integrins also have intracellular functions, in that they integrate the extracellular environment into intracellular signal transduction pathways, and also link that environment to the cytoskeleton. Thus integrins initiate intracellular signals, which modulate cell morphology, migration and proliferation[124].

1.6.5.2 Promotion of EC

Promotion is the second step in EC carcinogenesis. It involves the dysregulation of cellular proliferation, ultimately resulting in the clonal expansion of an initiated cell under the influence of tumour promoters, and also leads to immortalization [110]. Several studies have found that cells are generally monoclonal in endometrial carcinoma and complex hyperplasia with atypia, whereas they are generally polyclonal in simple and complex hyperplasia without atypia, as well as in normal tissue[125]. This suggests that most simple and complex hyperplasia without atypia constitute a general proliferative reaction in a tissue, hence the polyclonality, which can be referred to as true hyperplasia.

The tissues exhibiting monoclonality, *i.e.* many complex atypical hyperplasia and some simple/complex hyperplasia without atypia, are in fact precancerous neoplasia[125].

Cellular transformation or immortalisation is also required for promotion since a normal cell stops proliferating after *ca* 70 – 100 cycles of cell replication and becomes senescent. This process is thought to be regulated by telomere length at the tips of chromosomes. DNA polymerase is unable to duplicate the tips of chromosomes, so a small amount of DNA is lost with each cell division. To prevent the loss of important genes, chromosomal ends consist of long, non-coding, repetitive sequences known as telomeres. After 70 -100 cell cycles however, the telomeres in somatic cells have eroded below a critical limit, which shuts down cell replication. The reverse transcriptase telomerase elongates telomeres, which must occur at some stage in development otherwise the chromosomes derived at fertilization would be eroded such that they could not support the cell replication required for subsequent development. Indeed, telomerase is active during gametogenesis but is undetectable in the vast majority of adult somatic tissues. Telomerase is however activated in most malignant tumours, including EC, where it is assumed to support cellular immortalisation[126]. Telomerase activity is also pronounced in highly regenerative tissue, including the endometrium, presumably to prevent stem cells 'burn out'. Endometrial telomerase is most active in the proliferative phase of the menstrual cycle and is under the influence of sex hormones. Thus telomerase activity correlates with cell proliferation, and it is probable that telomerase has critical role in tumour promotion – via its induction of cell immortalization.

Another aspect of promotion is that apoptosis become inhibited. Apoptosis is a process of controlled cell death following a defined physiological pathway [127]. When apoptotic mechanisms are disturbed, the cell does not die and can continue to grow with a malignant phenotype [128]. Mutations in oncogenes such as *p53* and *bcl2* can inhibit apoptosis allowing the survival of uncontrollably proliferating transformed cells[117].

1.6.5.3 Metastasis of EC

The last step in carcinogenesis within the endometrium - metastasis - involves progressive genetic events which influence cell proliferation, proteolysis, adhesion, migration and angiogenesis[110]. After promotion, there is local invasion by tumour cells into the surrounding tissues. Degradation of the epithelial basement membrane and underlying extracellular matrix (ECM) is a critical histological marker for the transition of carcinoma from *in situ* to invasive. The ECM is composed of proteins and carbohydrates including collagen, fibronectin, elastin and proteoglycans. During invasion, cancer cells activate fibroblasts and recruit macrophages, and produce or stimulate the production of, crucial enzymes required for basement membrane and ECM destruction.

Matrix metalloproteinases (MMP) constitute a family of endopeptidases composed of at least 16 gene products that are involved in the degradation of ECM macromolecules and subsequent tissue destruction in a variety of pathologic conditions. MMP-2 (gelatinase) and MMP-9 are both secreted in human endometrial carcinoma. MMP-7 is significantly

activated in advanced EC and may be a key proteolytic enzyme in its invasion and metastasis, proliferation, proteolysis, adhesion, migration and angiogenesis[129].

1.7   Endometrial hyperplasia as a precursor of EC

The course of endometrial hyperplasia is similar to dysplasia of the cervix. Some of these lesions spontaneously revert to normal, or do so after medical therapy, whilst many persist as hyperplasia; some may however progress to carcinoma[130]. A diagnosis of endometrial hyperplasia requires histopathological analysis of an endometrial Pipelle biopsy, usually obtained from symptomatic women who present with dysfunctional uterine bleeding. Unlike the cervix, there is no reliable screening method to detect changes in the endometrial architecture (an endometrial biopsy is taken on clinical indication basis) Most endometrial hyperplasia is thought to arise from persistent, prolonged oestrogenic stimulation of the endometrium and the most common cause of this is a succession of anovulatory cycles[131]. Hyperplasia may also however result from hyperoestrogenaemia, as a result of excessive endogenous oestrogen synthesis or exogeneously administered oestrogen[132].

1.7.1 Histology of endometrial hyperplasia

The gross appearance of an endometrial cavity which contains hyperplastic tissue varies. Often the endometrium is thickened or polyploid, and large quantities of tissue are obtained at curettage. In other patients however, particularly post-menopausal women, the curettings are usually scanty and only small foci of hyperplasia can be found. In

general, hyperplasia involves excess proliferation of both stroma and endometrial glands. The glands are tubular or slightly convoluted and of variable size and shape; occasionally some of the glands may be cystic and dilated. Secretory activity is usually absent or sporadic. Mitotic activity is not as prominent as in the proliferative endometrium. Nucleoli are prominent and there is little budding of the glandular epithelium into the lumen

In 1984, the World Health Organization (WHO) adopted a new classification based on the study of Kurman et al (1984)[133].

Histological diagnosis	Cytological atypia	Architectural pattern
Simple	Absent	Regular
Complex	Absent	Irregular: glands crowded back to back
Simple atypical	Present	Regular
Complex atypical	Present	Irregular: glands crowded back to back

Table 1.9: Criteria for histological diagnosis of endometrial hyperplasia[132].

In endometrial hyperplasia, there is an increase in the gland-stroma ratio, and various abnormal patterns of tissue architecture have been noted. Glands may vary in shape and size and may also become irregular in outline. The most important distinction in endometrial hyperplasia is whether or not nuclear atypia are present. Cells showing atypia have an increase nuclear-cytoplasmic ratio, due to enlargement of the nucleus [134,135]. Often such cells have lost their normal polarity and are irregular in outline.

Hyperchromatic changes also occur due to nucleolar clumping. Atypia is an important factor with regard to the malignant potential of the lesion [136].

The WHO classification of endometrial hyperplasia has been challenged recently, as it is subject to unacceptably high inter- and intra-observer variability, for a number of reasons[137]. Firstly, morphological changes in normal endometrium during the menstrual cycle are extremely variable, and are influenced by numerous factors, such as age and hormone therapy [138]. Consequently, normal and hyperplasic endometrial biopsies have a large number of overlapping morphological features, making it difficult to discern between the two[139]. Secondly morphology may vary greatly within the same endometrium: areas with normal appearance are found adjacent to areas with simple cystic abnormality and/or complex proliferation. Furthermore, the WHO criteria give the impression that non-atypical simple and complex hyperplasias are easily distinguished from each other; in practice this is not the case. There is evidence that both these forms of hyperplasia have minimal risk of progression to carcinoma[140] and that both require similar treatment in the form of progestogens[141]. It would therefore seem more appropriate to group non-atypical simple and complex hyperplasia under one category, that of non-atypical endometrial hyperplasia [142].

According to the WHO criteria, atypical hyperplasia can also be subdivided into simple and complex categories. However it is argued that simple atypical hyperplasia is rare[132] and difficult to separate histologically from complex atypical hyperplasia, since features

such as crowding of the glands, stratification of the epithelium and proliferation of the stroma are often intermixed in one specimen.

The risk of an atypical hyperplasia within this classification scheme progressing to a low-grade carcinoma ranges from 15 to 40%. The wide variation reflects different diagnostic criteria and interpretations of what constitutes atypical hyperplasia, however this type of lesion should always be considered as an early form of cancer [140,143].

More recently, there has been a shift to re-name atypical hyperplasia as endometrial intraepithelial neoplasia (EIN)[110]. This term is used to describe monoclonal endometrial pre-cancers, characterized by morphological features that have been documented to increase the risk of cancer (discussed in section 1.6.5.2.). These features include volume percentage stroma <55%, cytological demarcation, exceeding 1mm minimum in diameter (at the exclusion of differential diagnoses for example, cancer, polyps secretory endometrium or artefacts).

Immunohistochemical biomarkers have further improved the detection limit of pre-malignant endometrial disease. The tumour suppressor gene PTEN is mutated in 83% of endometrial adenocarcinomas[144]. As this process is thought to occur at, or near the time of, initiation of carcinogenesis, pre-malignant and malignant glands can be distinguished by the loss of this marker. Thus immunohistochemical analysis of PTEN protein expression in EIN showed lesion-wide loss of PTEN protein in 63% of lesions studied, suggesting that these glands are monoclonal outgrowths from an abnormal PTEN-null

cell. PTEN-null glands can also be identified by immunohistochemistry in overtly 'normal' endometrial tissue, and such glands may represent an early stage of carcinogenic initiation. It is likely that the PTEN gene acts as a gatekeeper for endometrial carcinogenesis, being (at present) the initial genetic change observed in the endometrial epithelium which appears to predict the development of EC.

1.8   Is PCOS associated with EC?

Women with PCOS may have several months or more between menstrual periods. Thus the endometrium is exposed to oestrogen and in the proliferative phase for abnormally long periods of time, even permanently in amenorrhoeic women, before the progesterone surge around ovulation causes endometrial differentiation and entry into the secretory phase. Progesterone levels fall if fertilization does not occur and the endometrium degenerates and is lost in the menstrual blood flow. In women with PCOS therefore, the endometrium is subjected to unopposed oestrogenic stimulation, and remains within the uterus, for abnormally long periods of time.

The treatment of women with polycystic ovary syndrome (PCOS) has been influenced for more than 50 years by the belief that PCOS is associated with an increased risk of endometrial cancer (EC). It was theorized that unopposed oestrogen stimulation, and thus endometrial proliferation, was likely to cause endometrial hyperplasia which could lead to EC. EC is age-related, 5% of patients being aged <40 years, 20% aged 40 - 50 years and 75% >50 years.   A reproductive lifetime of unopposed oestrogen stimulation may have a role in EC in post-menopausal women; it may also have aetiological significance

in pre-menopausal patients. Legros and colleagues [145], after studying a group of self-selected women with PCOS, concluded that they should receive progesterone therapy to reduce their EC risk. Experimental and epidemiological evidence for an association is sparse however, as discussed below.

When an association between PCOS and EC was first proposed, a primary characteristic of cancers was understood to be their uncontrolled proliferation. Thus it seemed logical that if excessive proliferation occurred in the endometrium due to unopposed oestrogen action, it could induce EC. Understanding of cancer has progressed and the logic of this hypothesis is less certain, however, the assumption that EC is a risk in women with PCOS still underpins treatment strategies quoted in the Royal College of Obstetricians and Gynaecologists Guidelines for Good Clinical Practice [146] (Disorders of the Menstrual Cycle), the Health Information website of the National Library of Medicine, USA, Medline Plus, and in internationally recognized textbooks of gynaecologic oncology.

Approximately 10% of women suffer from PCOS so it is important that correct treatment is provided if an associated risk of EC is present, as substantial numbers of women could be affected. Equally, it is medically and economically important that treatment is provided only to those at risk. Thus the presence and severity of the risk of EC in women with PCOS, and the characteristics of the at risk population, need to be accurately determined. As discussed below, it is apparent that such risk assessment has not been adequately performed and though EC has a number of aetiological factors, evidence for the link between PCOS and EC is at best circumstantial.

Over the years, various publications have investigated the possible effects of unopposed oestrogenic stimulation on the risk of EC in women with PCOS. To investigate the research relevant to this proposed association, and to obtain evidence allowing an assessment of risk and at-risk group characteristics to be made, a literature search was performed on Medline (covering biological/medical science articles from 1957 to 2002), the Cochrane database and EMBASE. In addition, searches were performed in the archives of the Royal College of Obstetricians and Gynaecologists and on all theses submitted at the Royal Free Hospital Medical School in the last five years. These searches were performed using the terms polycystic ovarian disease, polycystic ovary syndrome, polycystic ovaries, endometrial hyperplasia, endometrial carcinoma and EC. The search included case reports, clinical trials and all other publications dealing with one or all of the above subjects

PCOS was first described by Stein and Leventhal in 1935[147], yet there are relatively few publications thereafter, reporting on single instances or small series of patients, which link PCOS and EC. The first of such publications, by Speert et al. in 1949[148](15 years after the first description of what is now known as PCOS by S and L), described 14 young women with EC, the majority of whom may have suffered from PCOS, since they exhibited primary infertility and ovaries described as cystic, sclerotic or 'hyaline', with no histological evidence of ovulation. Speert concluded that endometrial carcinoma in young women was not as rare as previously thought, and that abnormal bleeding in non-pregnant women warranted investigation with endometrial curettage.

It 1957, Jackson et al. specifically linked EC and Stein-Leventhal syndrome [1]. Clinical and pathological data were reported on 43 patients with Stein-Leventhal syndrome, the majority being obese, hirsute, hypertensive and infertile, and presumably suffering from PCOS. Sixteen of these cases were identified retrospectively, from among 'several thousand patients' with EC, by examining surgical ovarian biopsies. The other 27 were prospectively diagnosed with Stein-Leventhal syndrome by examination and ovarian biopsy. Endometrial tissue from 15 of the 27 cases was examined, 13 showed 'thickening' of the endometrium, 2 were atrophic and none were cancerous. Nevertheless, the authors concluded that 'our most important observation in Stein-Leventhal syndrome concerns the complication of endometrial carcinoma'. They also suggested that ovarian wedge resection be evaluated in preventing endometrial carcinoma, since it improved the other signs and symptoms encountered in Stein-Leventhal syndrome. The main conclusion has not been challenged over the following 45 years, and the Jackson et al. study continues to be described as showing a 37% prevalence of endometrial malignancy in women with polycystic ovaries.

It is now apparent that the study of Jackson et al. has serious irregularities. Two patient groups were described. In one group, 16 of 'several thousand' EC cases had ovaries consistent with Stein-Leventhal syndrome on retrospective analysis. Assuming 'several thousand' to be 2000 cases, the best case scenario for the author's conclusions, this suggests 0.8% of EC cases have PCO. Recent research indicates however PCO morphology is seen in 10 – 20% of women. Thus the data presented by Jackson et al. has

an inordinately low incidence of PCO in EC patients, and could even be taken to suggest that PCO morphology has a protective influence on the risk of EC, rather than being a positive risk factor for EC as suggested by the authors. In fact, the observation of only 16 cases of PCO in several thousand EC cases must raise suspicions regarding the accuracy of the histopathological analysis in this study. Alternatively, it raises the possibility, though slight, that EC ameliorates PCO morphology. More likely is that only 16 cases were found because of the difficulty in noting PCO morphology in ovaries from post-menopausal women, as seen in this study (Chapter 4), and the likelihood that the 'several thousand' EC cases investigated were predominantly post-menopausal, since even today the median age of EC diagnosis is 61 years.

Another point is that in the prospectively assessed Stein-Leventhal group of 27 cases, even though Stein-Leventhal is concluded to be a risk factor for EC, no EC cases were noted in the 15 patients assessed by endometrial biopsy. Thickening was noted in 13, but the relevance to EC of this observation is, at best, open to question. The statistical approach used is also suspect. Adding the 16 patients with PCO and EC to the 27 with PCO but not EC, to give an EC prevalence in PCO sufferers of 16/43 (37%) is invalid. If 'blonde hair' is substituted for PCO as the patient characteristic in this approach, the faults in this approach are manifest. Jackson et al. also did not attempt to explain the Liedig-like and Sertoli-like cells in the ovaries. In addition, the study may have been subject to selection bias as it was done retrospectively on surgical biopsies.

Coulam et al[149], in1983 studied a cohort of 1270 women with chronic anovulation and assessed their risk factors for developing neoplasia. The observed number of malignancies was similar to that expected in the general population (30 and 29.8, respectively). When the site of neoplasia was considered however, the endometrium was the only site at increased risk. It was concluded that a risk of EC had to be considered in patients with chronic anovulation. Only some of these patients would have had PCOS however, thus this study only provides circumstantial evidence linking PCOS and EC. The possibility that women with PCOS may have other disturbances that are unique to them that may predispose them or protect them from EC was not considered. For example patients with PCOS are insulin resistant[150] and tend to have high circulating levels of insulin in the body. Insulin in turn is a potent endometrial mitogen[151].

In 1984, Gallup et al [152] studied EC patients with respect to their age and potential risk factors. It was found that 14.4% of 111 patients with EC were 40 years or younger. These younger EC patients tended to have well differentiated tumours compared to patients in the older age group. They were also more obese and likely to be nulliparous, and 31.2% had PCO morphology, albeit the prevalence of PCO was not reported in the older EC patient group. This study seems to confirm an association between EC and aspects of Stein-Leventhal/PCOS, such as infertility, obesity etc. However the prevalence of PCO morphology in the young EC patient group (31.2%) is greater than the estimated prevalence of PCO in the general population. It is certainly greater than noted in the study by Jackson et al., perhaps because PCO morphology was more easily recognized in the younger women. Indeed, this may be why the prevalence in the older patients was not

described. The sample size in this study is relatively small however – 111 patients of which 16 were <40 years, and 5 were <40years with PCO morphology.

In 1991, Dahlgren[153] retrospectively studied 70 women with EC in Sweden, aged between 31 - 45 years. Subsequently, the same authors recruited 99 women aged 46 - 65 years, suffering from the same condition. Both groups were compared to 1746 control women, aged between 39 - 65 years, randomly selected from a population-based study performed in the city of Goteborg. All cases were studied by examining hospital records and a questionnaire. Hirsutism, high body mass index and hypertension were more common in both groups of EC patients, compared to the age-matched control groups. Nulliparity and infertility were more common in the younger EC patients. The controls used the combined oral contraceptive pill more often (more prevalent usage) than women with EC (there was however no difference in duration of use by either groups). There was a negative correlation with cigarette smoking. These findings seem to indicate that untreated ovarian dysfunction similar to that present in PCOS, with unopposed oestrogen stimulation of the endometrium, is associated with EC in young women. Cigarette smoking seemed to have a protective effect.

It could be argued from this study that there was potential selection bias since the cases came from a large area with mixed urban and rural background whereas the controls all came from only one large city. There also may have been information bias; for example, information about hirsutism can be very subjective. What is perceived, as excess hair

growth by one person may not necessarily be so for another. There was also no mention of the measures taken to reduce recall bias.

Lastly in 1991, Escobedo et al [154] studied 399 women with EC and compared them to 3040 controls. They found that the former group had more 'ovarian factor' infertility than in the control group. However they did not specifically look at the incidence of PCOS in those patients.

Most publications otherwise have been in the form of literature reviews and case reports but little work concerning case studies or randomized trial is documented. It is also surprising to find that most of the studies that are quoted as providing evidence for an association between PCOS and EC, did not included controls. This omission brings the conclusions of such studies into question.

1.8.1: Proposed mechanism by which PCOS may predispose to EC

There are a number of mechanisms by which patients with PCOS are thought to develop endometrial carcinoma. The oldest and perhaps the most established is that prolonged unopposed oestrogen stimulation of the endometrium can induce oncogenic changes, as first postulated by Jackson[1] in 1957. Since this time however, other studies have been performed and their results used in support of additional mechanisms.

For example, Jafari et al[155] in 1978 studied six cases of adenocarcinoma of the endometrium associated with Stein-Leventhal syndrome (PCOS), of average age 27.8

years. They found that adenocarcinoma associated with PCOS was well differentiated and had a good prognosis. They proposed that the elevated levels of LH seen in PCOS increased production of androstenedione and testosterone, and that the effect of these androgens may be a causal factor in the development of well differentiated adenocarcinoma in oestrogen hyperstimulated endometrium. Jafari et al also noted that progesterone, is deficient in Stein-Leventhal syndrome and that progesterone receptors are expressed in endometrial tissue, and when occupied, may modulated oestrogen receptor activity and reduce endometrial cell growth potential. Hence they proposed that the depressed progesterone levels in PCOS may be permissive to the development of adenocarcinoma in the oestrogen hyperstimulated endometrium. They also suggested that although unopposed oestrogen stimulation seems to be the predominant factor in the development of endometrial carcinoma, Stein-Leventhal syndrome and adenocarcinoma of the endometrium both were associated with metabolic and endocrine effects. This fact suggested some potential hypothalamic and pituitary roles in the development of the two disorders.

In a study by Konishi et al[156] in 1996, LH and hCG receptor expression was assessed by immunohistochemistry in endometrial biopsies (20 normal and 24 abnormal, the latter subdivided into 9 exhibiting simple endometrial hyperplasia, 6 complex hyperplasia, 6 atypical hyperplasia and 3 overt endometrial carcinoma). Fifteen of the 24 patients were 40 years old or younger and all were anovulatory. Serum levels of LH, FSH, prolactin, Oestradiol and testosterone were measured by radioimmunoassay. LH and HCG receptor expression was detected in 19 of the 21 biopsies exhibiting hyperplasia. Expression

appeared stronger in the glandular cells of complex and atypical hyperplasia, relative to simple hyperplasia and normal endometrial glands. The 3 carcinoma specimens showed strong expression of LH and HCG receptors compared with normal endometrium, as expected. They concluded that LH and HCG receptor over expression is a feature of complex and atypical endometrial hyperplasia and endometrial carcinoma in younger anovulatory women, many of whom were PCOS sufferers.

Ho et al, in 1997[157], conducted a study in Singapore to determine the incidence of EC in endometrial hyperplasia and try to identify patients at risk of EC. They retrospectively studied 116 patients who had simple, complex or mixed (simple with complex focus) endometrial hyperplasia, with or without atypia and without coexisting endometrial carcinoma, between January 1991 to December 1994, selected by screening the database of the histopathological laboratory at a teaching hospital. The selected patients' notes were reviewed and a second endometrial biopsy performed five years later. They found that 29 patients had endometrial hyperplasia with atypia and 87 without atypia on the first biopsy. The incidence of endometrial carcinoma in the second biopsy was 27.6% in those with endometrial hyperplasia with atypia and 3.4% in those without atypia; all carcinoma cases were found to be stage 1 adenocarcinoma. The incidence of PCOS and sub fertility was elevated in case of endometrial hyperplasia with atypia, but not in cases of carcinoma. After reviewing patient records, no predictive factors for endometrial carcinoma were apparent.

This study assessed whether endometrial hyperplasia with atypia was a precursor for the development of endometrial carcinoma. The fact that many patients with PCOS tend to develop atypical hyperplasia, which was associated with an increased probability of endometrial adenocarcinoma, was used to conclude that women with PCOS people are at increased risk of developing EC.

More recently, Elliot at al [151], in May 2001, presented two case reports and a short literature review. They write that multiple mechanisms may be responsible for the increased risk of EC. In the context of PCOS, endometrial carcinoma need not be associated with abnormally high levels of oestrogen or insulin alone. Yet the chronic anovulatory or oligo-ovulatory state of PCOS is characterised by high oestrogen levels and hyperinsulinaemia but little or no progestogenic activity. They postulate that this ultimately results in endometrial hyperplasia, which is thought to be a precursor of malignancy. Tonically elevated serum insulin levels up-regulate aromatase expression and oestrogen synthesis in the endometrial glands and stroma. This has an additive and deleterious effect in woman who are hyperinsulinaemic and anovulatory, as is the case in many women with PCOS, since chronic oestrogen hyperstimulation of the endometrium is exacerbated. So for a non-hysterectomised woman with PCOS, endometrial hyperplasia represents a common end-point of two distinct pathophysiological processes.

They also mention that in vitro, insulin and insulin growth factors seem to act as potent mitogens in specific cell subpopulations. EC cell lines demonstrate such activity. This

may explain why, once present, atypical endometrial hyperplasia advances to frank carcinoma in as many as 30% of cases.

There have been subsequent studies linking endometrial hyperplasia and EC. Studies investigating the link between p53 expression and endometrial hyperplasia, e.g. Kaku et al [158], have characterised two classes of endometrial carcinoma. In Type 1 EC, which is associated with prior endometrial hyperplasia, unopposed oestrogen secretion is thought to be an important determining factor. Such tumours tend to occur in younger patients, and the tumours exhibit less myometrial invasion and limited up-regulation in p53 expression, and have better prognosis. In contrast, type 2 EC tumours are not associated with prior endometrial hyperplasia. They tend to occur in older patients, to exhibit p53 over expression and to have poorer prognosis.

Fabjani et al[159], in 2000, investigated genetic alterations in endometrial hyperplasia and cancer. They found that some genetic microsatellite markers, such as D1S518, D3S2387 and D8S1992, were predictive of the ability of endometrial hyperplasia to progress to endometrial carcinoma. They also found that a deletion from chromosome 8p was frequently found in endometrial hyperplasia, as well as EC. This finding supports the hypothesis that chromosome 8 contains tumour suppressor genes and that their deletion occurs early during endometrial tumorigenesis.

The literature review performed has however also revealed studies that do not support the view that PCOS predisposes to EC. In 1978, Ramzy et al [160] assessed the histological

appearance of ovaries from 15 patients in whom endometrial adenocarcinoma had developed before the age of 40 years, from 21 age-matched controls and from 25 patients diagnosed with Stein-Leventhal syndrome (i.e. PCOS). The features assessed included type and size of cysts, presence and number of primary follicles, corpora lutea, corpora albicantia, and the presence of stromal luteinization and sclerosis. It was found that the ovaries of patients less than 40 years old who have endometrial carcinoma were more similar to normal ovaries than to polycystic ovaries. The authors concluded that abnormal ovarian function may be a predisposing factor for EC in young women, however if such a defect of ovarian function exists, it is not apparent on visual examination of the ovary.

This study seems to contradict the hypothesis that women with polycystic ovaries (an ultrasound criterion for diagnosing PCOS) are at increased risk of developing endometrial carcinoma. However more recent findings suggest that there is a proportion of women with polycystic ovarian morphology, but without the associated endocrine disturbances. Similarly, according to the recent ARSM/ESHRE consensus definition for PCOS, it is possible for patients to exhibit endocrine and ovulatory disturbances characteristic of PCOS (i.e. Stein Leventhal syndrome), in the absence of polycystic ovarian morphology.

In 1998, Pierpont et al[161] published the results of a long-term follow-up study (average 30 years) of the mortality of 786 women in the UK with PCOS diagnosed between 1930 and 1979. The authors calculated standardized mortality ratios (SMR) to compare the death rates, from a variety of causes, in these women with national rates. Breast cancer was the

commonest cause of death in women with PCOS; the standardized mortality ratio indicated the death rate was higher than in the national rate. The overall death rate from cancer in women with PCOS was however lower than the national rate. Endometrial carcinoma was not separately discussed, and it must be assumed that it was included in the "other" category, which represented only a small fraction of the total number of deaths before 75 years of age. If PCOS was a strong predisposing factor for endometrial carcinoma we would expect the standardized mortality rate to be higher for this condition when compared with national rates.

The literature study performed reveals how little evidence there is linking PCOS per se with endometrial carcinoma. To attempt to assess the strength of this linkage, a case control study involving patients with endometrial carcinoma and age-matched controls was performed in which the proportion of patients from each category who had features suggestive of PCOS was assessed. This study is discussed in Chapter 4.

Another potential line of study is to examine endometrial biopsies from patients with PCOS and endometrial carcinoma using microarray analysis to assess gene expression. If PCOS and EC are strongly linked, similarities in gene expression between the two classes should exist, especially for genes known to be involved in oncogenesis. Such a correlation with in gene expression with women with EC, might not be seen in all women with PCOS, but may only occur in a subgroup of women with PCOS at-risk of EC. If identifiable using microarrays however, analysis of the patient histories in such a group may allow identification of easily characterised risk-factors or markers for the at-risk

group. Such a finding will allow targeting of screen strategies and treatment to this group, which will save lives and money. This approach and investigation is discussed in Chapter 6.

PCOS and chronic anovulation seem to be treated synonymously in most of the literature available. Though the two conditions overlap, insulin resistance and its repercussions characterize many cases of PCOS but are not found in non-PCOS chronic anovulation. Hence it is not correct to consider PCOS and chronic anovulation as interchangeable conditions; although PCOS is the commonest cause of anovulation, there are numerous other aetiologies which have very different endocrine and metabolic profiles.

One of the dilemmas faced by PCOS patients and their physicians is when to intervene if amenorrhoea is present. Such women may not have periods for months and they are the ones who are thought to be at greatest risk of developing EC due to unopposed oestrogen stimulation and failure of the endometrium to be shed and regenerated again. Currently doctors treat women with the oral contraceptive pill or progestagens every 3 months to ensure a withdrawal bleed, believing that this is the 'safest' options to reduce the risk of EC. This treatment regime is not base on any concrete evidence and more research is needed in this area so that guidelines may be implemented.

In conclusion, this literature review has shown there is not enough evidence on which to design management guidelines for treatment of the risk of endometrial carcinoma in women with PCOS.

1.9 Hypothesis

For more than fifty years clinicians have been advising and treating women with PCOS in the belief that they have a high risk of developing EC. Having reviewed the relevant literature, it is clear that the association between PCOS and endometrial carcinoma has never been clearly demonstrated in elaborate well-controlled studies.

PCOS is responsible for creating a hyperoestrogenic state[162], an endocrine environment known to favour the development of endometrial malignancies. PCOS is also strongly associated with endometrial hyperplasia, a variant of which - atypical hyperplasia - is known to be a precursor of EC. Insulin resistance and hyperinsulinaemia, which are also commonly seen in PCOS, are also risk factors for the development of endometrial carcinoma, insulin having a potent mitogenic effect on the endometrium, at least in vitro.

It is however incorrect to assume that PCOS is a significant risk factor for endometrial carcinoma on the basis that both conditions share similar features in their pathophysiology. PCOS is a heterogeneous condition, the aetiology of which is still not fully understood. It may be that some women with PCOS have innate protection from EC and/or do not exhibit some risk factor symptoms, while others are more susceptible and/or have risk factor symptoms. Certainly, this is suggested by the contrasting incidence rates of the two diseases. EC is the most common gynaecological cancer in the Western world - its incidence in the UK being approximately 6891 cases per year - yet this rate is small in comparison to 750000 women with PCOS. If there was a direct

association between the two conditions, the incidence of EC should be higher. Obviously the proportion of women with and without PCOS who develop EC is important in determining this risk- one justification for doing the study.

It is also generally assumed that women with EC arising in a hyperoestrogenic environment have a better prognosis, as tumours tend to be well-differentiated and less aggressive [155]. Again, the evidence for such an association is not clear. As discussed previously endometrial carcinogenesis has a distinct pathogenesis and it has also been observed that some EC tumours arising in a hyperoestrogenic environment, may display aggressive behaviour, depending on whether they arise in an atrophic or hyperplastic background.

Clinicians have a responsibility to treat patients according to the best possible evidence. Women with PCOS, who are not trying to conceive often present to their general practitioners, complaining of secondary amenorrhoea or oligomenorrhoea. Some women may have menstrual cycles of six to eight weeks duration, whilst others may only have one menstrual bleed per year or none at all. In order to minimize the development of endometrial hyperplasia in these women, the treatment options are usually the oral contraceptive pill or progestogens to induce withdrawal bleeds. Although the contraceptive pill is a good option, it is not without risk, especially when patients are obese - as is often the case in PCOS. This leaves the administration of progestogens and although there is little evidence regarding the efficacious frequency of administration, most clinicians in the United Kingdom believe it is "safe" to induce withdrawal bleeds

every three months in amenorrhoeic women to protect them from the risk of EC. Again, this belief has not been validated by in-depth, well controlled research investigations. Thus patients are treated at some expense and risk, with limited evidence to support the necessity of treatment and its efficacy. This thesis attempts to provide such evidence.

Women with PCOS exhibit several characteristics which are considered to be risk factors for EC - obesity, hyperinsulinaemia, hyperoestrogenaemia and lengthened menstrual cycles. These characteristics alone are enough to increase an individual's likelihood of developing EC regardless of the fact that they have PCOS or not. It is still unclear whether PCOS confers a greater risk to women where EC is concerned. The studies described in this thesis were also designed to explore this area of uncertainty.

The hypotheses to be investigated are that:

A) PCOS is associated with EC.

B) The type of endometrial carcinoma arising in women with PCOS carries a better prognosis.

C) The risk of EC is confined to a subgroup of women with PCOS.

To test these hypotheses, the following studies were performed.

To test hypothesis A, this thesis aims to investigate the degree of association between PCOS and EC. To facilitate this, a retrospective study was performed in which the

ovaries of patients who had had a total abdominal hysterectomy and bilateral salpingoopherectomy for EC were histologically assessed for evidence of PCOS. As a control group, ovaries from women who had had the same operations for benign gynaecological problems were also assessed. This investigation allowed an assessment to be made as to whether the incidence of PCO morphology, a marker of PCOS, is more common in patients with EC than in controls, and thus whether PCO morphology is associated with EC.

To test hypothesis B, this thesis aims to ascertain whether women with PCOS have a worst prognosis when they have developed EC. To achieve this aim, a study was performed in which immunohistochemistry was used to assess the expression of p53, bcl2, ki67 and Cyclin D1, as surrogate markers for tumour prognosis, in endometrial tumours from women with and without PCO morphology.

To test hypothesis C, this thesis aims to determine whether a specific subgroup of women with PCOS are at increased risk of developing endometrial carcinoma. To achieve this, a second study was designed to investigate gene expression using microarray analysis, in the endometrium of women with PCOS, well characterised with regard to serum biochemistry, patient history and endometrial histopathology were used to characterize gene expression Particular attention was focused on genes implicated in oncogenesis, particular in the endometrium. The interaction between gene expression and characteristics defining PCOS in each patient was investigated to attempt to define a

group of patients, exhibiting certain serum, histological, or biometric characteristics, with

endometrial gene expression suggestive of endometrial dysplasia and risk of EC.

Chapter 2

METHODS

2.1 The prevalence of polycystic ovarian morphology in women with endometrial cancer

This study was designed to assess the incidence of PCO morphology in women with endometrial cancer compared with a control group. If endometrial cancer is associated with PCOS, a greater proportion of women with endometrial carcinoma should exhibit PCO morphology than in the control group.

2.1.1 Study design

This retrospective cross-sectional study was carried out between December 2002 and December 2003 at the Royal Free Hospital, London (RFH). Archived sections of both whole ovaries from each subject were evaluated for the presence of PCO morphology. Ethical approval for this study was obtained from the RFH and UCH Research Ethics Committees (Ethics no. 5252, valid from April 2002 to April 2003, then extended to April 2004).

2.1.2 Subjects

Patients diagnosed with endometrial cancer who had had total hysterectomy with bilateral salpingoopherectomy at the RFH between 1986 and 2001 (N = 42), or at UCH between 2000 and 2003 (N = 81), were included. The control group consisted of 83 patients who had the same operative procedure, or a bilateral salpingoopherectomy, at the RFH or UCH, between 1990 and 2003, for reasons other than gynaecological malignancies.

The SNOMED computer database is kept by the Pathology Department, RFH. It stores the location of archived samples and histopathological results for all tissue biopsies collected from patients since 1990. Results for biopsies prior to 1990 are stored on microfiche. A SNOMED-like database was also available at the Department of Pathology, UCH. These resources were used to access histopathology reports for ovarian sections from the selected patients and to allow retrieval of archived sample sections from both ovaries from each subject for further investigation. All slides were H&E slides that had been stored at room temperature in the Pathology Departments at each hospital.

2.1.3 Histological characterization

Originally, it was thought that the stored histological assessments, performed and recorded directly after the biopsies were taken, could be used to determine whether PCO morphology was present. It was found however that these assessments often failed to state clearly whether an ovary was not polycystic, nor did they contain the requisite detailed description which would allow PCO morphology to be deduced. Indeed, this was a common problem encountered several times during the course of the work in this thesis - histopathologists in the past have tended not to report PCO morphology if this was not mentioned by the clinician who completed the request form. This was perhaps because the detrimental effects of PCOS on general health were not as well recognized as they are now.

It was necessary therefore to reanalyze the ovarian sections from each patient and to assess them microscopically for features of PCO morphology. Ovaries were classified as

polycystic if they showed a three-fold increase in capsular width (the capsule is considered to be normal if it is one-cell thick, and this was a histopathology criteria before the ESHRE\ARSM agreed diagnostic criteria was published), peripherally (this study was devised and started before the agreed ESHRE/ARSM ultrasound diagnostic criteria was published hence peripherally arranged cysts were included rather than multifollicular ovaries) arranged regular follicular cysts (>8 per whole ovary section), stromal thickening and an absence or decreased abundance of corpora albicantia (<5 per whole ovary section). All sections from the RFH archives were assessed independently by the author, Dr P. Menon and Dr J. Crow (both consultant histopathologists at RFH), while slides from UCH were assessed by the author, Dr E. Benjamin (consultant histopathologist at UCH) and Dr J. Crow. Ovarian sections from endometrial cancer cases and controls were obtained and examined at the same time, the examiners being blinded as to which category any slide belonged. The slides were examined using an electric light microscope (Nikon phase contrast, 1.25, Japan), first at x100 magnification, to estimate the number of follicular cysts and corpora albicantia, then at x200 magnification to determine the presence of stroma thickening and to estimate the capsular width.

2.1.4 Statistics

The medical statistician at the Royal Free Hospital was consulted for statistical input.

More details about the statistical test are provided in Chapter3, section 3.6, page 124.

2.2 The expression of cell-cycle and apoptotic proteins in the endometrium of women displaying PCO morphology

This study was designed to assess whether endometrial tumours arising in women with PCO, carry a better or worse prognosis than endometrial tumours arising in women with no evidence of PCO.

2.2.1 Study design

This study was conducted at the RFH between November 2001 and July 2003 and at UCH between November 2000 and July 2003. Ethical approval for this study was obtained from the RFH and UCH Research Ethics Committees (Ethics no. 5858, obtained March 2002 and extended through to December 2003). Endometrial tumour biopsies were obtained from patients diagnosed with endometrial cancer between these dates, one group exhibiting PCO morphology and one group normal ovarian morphology. Endometrial tumours sections were analyzed by immunohistochemistry for expression of the cell cycle regulatory proteins Cyclin D1 and Ki67, and the apoptotic proteins p53 and Bcl2. These proteins were selected as evidence suggests that their expression is related to tumour prognosis. The intensity of staining for these proteins between the PCO and non

PCO associated endometrial tumours was then compared to assess whether there was any difference in these prognostic indicators between the groups.

2.2.2 Subjects

A sub-group of the subject cohort detailed in Section 2.1.2 was used in this investigation. This sub-group was comprised of patients diagnosed at the time of the study (2000 – 2003), from whom endometrial tumours biopsies could be obtained which were suitable for immunohistochemical staining for the selected prognostic indicators. The sub-group consisted of 27 patients diagnosed with endometrial carcinoma, but no other gynaecological cancers. Analysis of ovarian morphology showed that 11 subjects exhibited PCO morphology, and 16 (controls) did not. The 16 controls were age-matched with the 11 subjects exhibiting PCO morphology.

2.2.3 Sample preparation

Immediately after retrieval, endometrial tumours were paraffin embedded and were stored in the Pathology Department of the host hospital at room temperature. When required, endometrial tumour blocks were retrieved from storage and 5 µm sections were cut by a microtome and mounted on 3-aminopropyltriethoxysilane (APES) coated glass microscope slides.

Immunohistochemistry

Standard immunohistochemical techniques were applied to the slide mounted paraffin-embedded 5 µm endometrial tumour sections and sections of normal ovary, as an

appropriate positive control tissue. Slides were normally manipulated whilst in standard 18 slide racks and all washes were performed in standard 500 ml glass troughs.

Sections were deparaffinized in xylene (3 x 5 min), rehydrated in methanol (3 x 5 min), incubated with 3% (v/v) hydrogen peroxide for 10 min to block endogenous peroxidase activity, then twice washed in tris-buffered saline (TBS; pH 7.4) for 5 min. Antigen retrieval was performed by microwaving the sections in 400 ml 10 mM citrate buffer (pH 6.0) for 10 min at full power (800 W) then 5 min with no heating [163]. Cooling was allowed to take place at room temperature for 30 min in distilled water, then sections were washed in phosphate buffered saline (PBS; pH 7.4) for 2 x 5 min. Non-specific protein binding capacity was blocked by incubating section for 15 min with either normal rabbit serum (Dako, Ely, UK) diluted x10 in PBS (Cyclin D1, p53, Bcl2) or normal goat serum (Dako) diluted x10 in TBS (Ki67) [163-165]. After washing in PBS, sections were incubated with appropriately diluted primary antibodies, which were replaced by PBS in negative controls (Table 2.1).

Antigen	Source	Clone	dilution	Antigen retrieval
Cyclin D1	Novocastra	DCS-6	1: 100	Yes
Bcl2	Dako M0887	124	1: 100	Yes
p 53	Dako M7001	DO-7	1: 100	Yes
Ki67	Immunotech IM0505	MIB-1	1 : 25	Yes

Table 2.1: Primary antibodies used for immunohistochemical staining of cell cycle (Ki67, Cyclin D1) and apoptotic (p53 and Bcl2) proteins.

Unbound primary antibody was then removed by washing sections in PBS for 3 x 10 min, after which they were treated for 45 min with either biotinylated rabbit anti-mouse IgG (Dako) diluted x200 in PBS (Cyclin D1, p53, Bcl2) or with goat anti-mouse IgG (Dako) diluted x200 in TBS (Ki67). Again unbound antibody was removed by washing in PBS for 3 x 10 min, after which sections were incubated with streptavidin-biotin-horseradish peroxidase complex (Dako) diluted x200 in TBS for 30 minutes. Sections were developed with 3, 3'-diaminobenzidine solution (Sigma-Aldrich, Poole, Dorset, UK), counterstained with Mayer's haemalum (Merck, Lutterworth, Leics, UK), dehydrated in methanol, cleared in xylene and mounted in DPX.
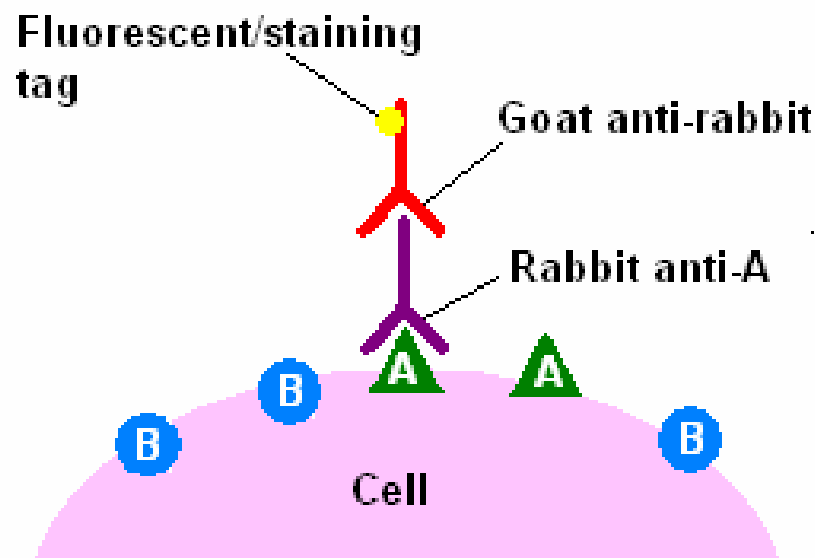
Figure 2.2 illustrates the principles of immunohistochemistry- Free reproduction from http://en.wikipedia.org/wiki/Immunohistochemistry.

2.2.5 Assessment of immunostaining intensity

Two sections from each biopsy block were assessed. The slides were selected by Dr Crow as the most representative of that block. The sections were viewed under a light microscope at x100 magnification and scored by two independent observers (Ouma C.Pillay, L. Wong Te Fong). Sections were scored on two separate occasions by each observer to ensure reproducibility. A mean of the 3 readings were obtained. (O.C. Pillay, scoring only was taken into consideration but they were all verified blindly first by L. Wong Te Fong. Intra-observer and intra-section variability, the latter assessed on adjacent sections, was less than 5%.

Sections were scored positive for Cyclin D1, Bcl2 and p53 if >10% of tumour cell nuclei were stained; positively scored structures appeared brown on a blue background. This value was chosen as a firm cut-off in the staining just above the negative control background level. The Ki67 staining index was expressed as the percentage of positively stained tumour cell nuclei relative to the total number of tumour cell nuclei in 4/5 randomly selected fields on a section[163-165]. (The biopsies were all derived from solid blocks of tumour). The staining index for a biopsy was then calculated as the mean of the staining indices of each section from the biopsy assessed (a mean of 3 readings was calculated).

2.2.6 Statistics

Differences in immunostaining were tested by Fishers Exact Test (Cyclin D1, Bcl2, p53) or Student t test (Ki67). $P < 0.05$ was taken as the threshold level of significance.

2.3 Differential gene expression in the endometrium of women with PCOS.

This study was designed to determine whether a specific subgroup of women with PCOS is at increased risk of developing endometrial carcinoma. Gene expression was analyzed in endometrial biopsies from a well characterised group of women with PCOS. The interaction between gene expression, particularly the expression of genes implicated in oncogenesis, and characteristics defining PCOS in each patient was investigated, to attempt to define a sub-group of patients at risk of endometrial dysplasia and EC.

2.3.1 Study design

This prospective study was conducted at the RFH between September 2003 and July 2004. Ethical approval for the study was obtained from the Royal Free & University College Medical School Ethics Committees (ethics no.6219, March 2003-March 2004; extended for another 12 months to March 2005).

Endometrial biopsies were obtained from women with PCOS, the subjects being well characterised with regard to serum biochemistry, patient history, biometric data and endometrial histopathology. RNA was isolated and microarray analysis, performed in

collaboration with the Department of Pathology, University of Cambridge, was used to assess gene expression in the endometrial biopsies. Particular attention was focused on genes implicated in oncogenesis, particular in the endometrium. The interaction between gene expression and characteristics defining PCOS in each patient was investigated to attempt to define a group of patients, exhibiting certain serum, histological, or biometric characteristics, with endometrial gene expression suggestive of endometrial dysplasia and risk of EC.

2.3.2 Subjects

Fifty patients, diagnosed with PCOS according to the ESHRE consensus diagnostic criteria[95], were recruited. To make this diagnosis, all subjects had assessment of: ovarian morphology by transvaginal ultrasound; menstrual cyclicity and evidence of hyperandrogenaemia (acne, hirsutism, balding) by clinical inspection and patient history; biochemical status by serum analysis for testosterone, sex hormone binding globulin (SHBG), LH and FSH.

During recruitment, all the subjects received an information sheet regarding the study and gave informed consent. At the next consultation, held in the morning after an overnight fast, a detailed medical history was taken, and their weight, height, waist/hip ratio and systolic and diastolic blood pressure were measured. A blood sample was also taken, serum immediately prepared by centrifugation at 1000gf for 15 min, and comprehensively analyzed using standard laboratory methods by the Department of Chemical Pathology, RFH, for LH, FSH, testosterone, SHBG, free androgen index,

androstenedione, Oestradiol, progesterone, 17-hydroxyprogesterone, insulin, cholesterol and fasted glucose. A glucose tolerance test was then performed in which the patient drank 498 mls of "Lucozade" containing 75 g of glucose. After 2 hr a repeat blood sample was taken and processed for serum glucose estimation as for the fasted blood sample.

The patients subsequently attended clinic for a Pipelle (Laboratoire C.C.D, Paris, France) endometrial biopsy, on days 4 – 7 of their menstrual cycle if normally cycling or mildly oligomenorrhoeic (i.e. cycling >4 times/year, or at a random time if cycling <4 times/year. Patients who were amenorrhoeic were seen at any time. Two endometrial samples were taken, one was snap frozen in a sterile tube on dry ice within 5 minutes and stored at -80°C for later RNA isolation, the other was fixed in formalin and sent to the Department of Histopathology, RFH, for histopathological assessment. All patients received 1 gram of azithromycin to protect against ascending infection related to the biopsy.

2.3.3 Isolation of endometrial RNA

Total RNA was isolated from the endometrial biopsies using Qiazol according to the manufacturer's instructions (Qiagen Ltd., Crawley, UK). This method is essentially a commercial modification of the acid guanidinium thiocyanate-phenol-chloroform RNA extraction method[166]. Because microarray analysis is expensive, time consuming and highly sensitive to the quality of the RNA, the isolated endometrial RNA was repurified,

by DNase I digestion and subsequent RNeasy MinElute column. This provides RNA suitable for microarray analysis

2.3.3.1 Primary isolation of endometrial total RNA

Endometrial biopsies were retrieved from the -80°C freezer onto dry ice, but immediately prior to homogenization, each was allowed to warm at room temperature for 1 min. Biopsies (50 – 100 mg) were however still frozen when added to a pre-weighed 13 ml test tube (Sterilin Ltd, Hounslow, UK) containing 1 ml of Qiazol reagent, thus RNA degradation would have been limited. After reweighing, to allow determination of the amount of tissue used, tissues were immediately dispersed using a rotary homogeniser (Model X120, Status Ltd, Germany) at maximum setting for 20 s. Samples were left on the bench for 5 min, then combined with 0.2 ml chloroform in an autoclaved eppendorf tube, shaken vigorously for 30 s, then left on the bench for 3 min. Samples were centrifuged at 12000 g for 15 min, and the overlying RNA containing clear aqueous phase was collected into a fresh eppendorf tube, taking care to avoid contamination with the DNA interface. An equal volume (ca. 0.6 ml) of isopropanol was added and, after vigorous mixing, the tubes were incubated for 2 hr at -20 °C to allow RNA to precipitate. After re-centrifugation (as above), the RNA pellet was twice washed by resuspension in 1 ml 75% (v/v) ethanol and centrifugation at 12000g for 5 min. The RNA pellet was air-dried for 20 min, dissolved in 50 nml nuclease-free water (Ambion, Applied Biosystem, Warrington, UK) at 65°C for 10 min with vortexing, and then stored at -20 °C.

2.3.3.2 Spectrophotometric determination of RNA concentration

RNA samples were diluted with nuclease-free water (normally 2 µl RNA in 800 µl water) and the absorbance read at 260 and 280 nm, in a quartz cuvette, against a blank consisting of nuclease-free water. RNA concentration was estimated based on the assumption that a solution of 40 µg/ml RNA has an absorbance of 1 at 260 nm. An A260/A280 ratio of 1.8 - 2.0 indicates reasonably pure RNA, whereas a ratio much below 1.7 is indicative of protein contamination. Only RNA with an A260/A280 ratio of >1.4 was selected for re-purification in this study.

2.3.3.3 Analysis of RNA integrity by agarose gel electrophoresis

RNA (2µl, containing 2 - 10 µg) was mixed with 8 µl RNA sample buffer (16 µl; Table 2.2), heated at 65 °C for 15 min, and then immediately chilled on ice. Samples were mixed with RNA loading buffer (2.0 µl; Table 2.2), before loading onto gels (1% w/v agarose, 18% v/v formaldehyde in 1X MAE; Table 2.2). Gels were run in 1X MAE at 100 - 120 V for 0.5 - 1 h, until the bromophenol blue tracking dye had migrated 80% of the lane length. Gels were then visualized under UV transillumination, revealing ethidium bromide chelated to the RNA, and photographed using a Kodak DC40 digital camera. A 28S/18S rRNA ratio of approximately 2:1 is indicative of reasonably intact RNA. RNA degradation is indicated by a decline in this ratio, and also by excess smearing in the region of the gel below the 18S rRNA band. Only intact RNA was used for downstream analysis.
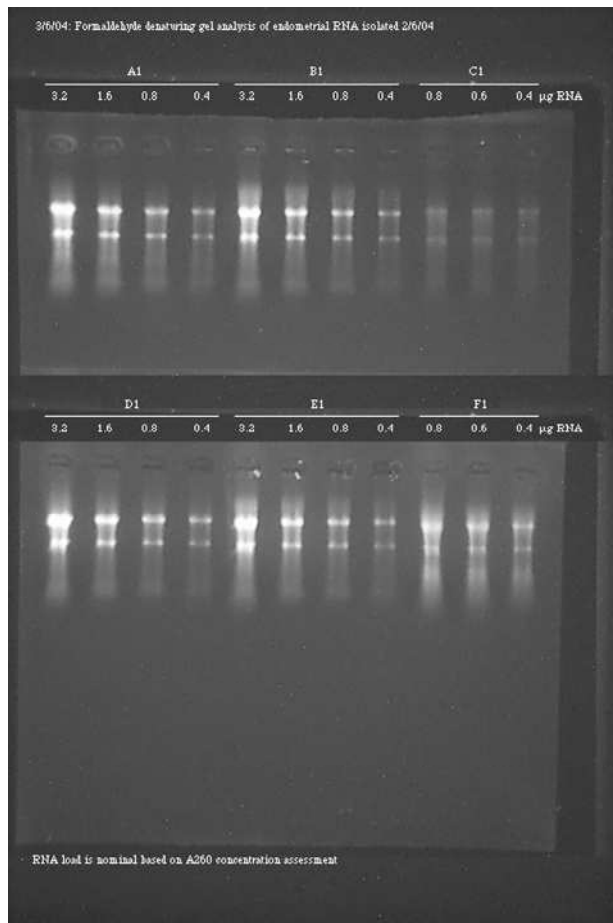
Figure 2.3: shows a photograph of analysis of RNA integrity by agarose gel electrophoresis.

2.3.3.4   RNA repurification

It was apparent that the initial RNA isolates required repurification. RNA is prone to degradation by nucleases (RNases) which are present in tissue and in the environment; Spectrophotometric analysis (Section 2.3.4) indicated some RNA samples were contaminated with protein, perhaps including RNases. Furthermore, reagent contaminants from the isolation procedure can interfere with cDNA synthesis and other downsteam

processes. Gel analysis (Section 2.3.4) also suggested some RNA samples were contaminated with genomic DNA, which would interfere with microarray analysis. RNA was therefore treated with RNase-free DNase I (Qiagen, Crawley, UK) to remove genomic contamination and repurified using the RNeasy MiniElute kit (Sigma-Aldrich Ltd, Poole, UK), both according to the manufacturer's instructions, to remove DNase I, RNases, other proteins and organic contaminants.

DNase I treatment of RNA samples was performed in 100 µl reactions, containing 50 µg RNA, 2.5 µl RNase-free DNase I (2.7 Kunitz units/µl; Qiagen, Crawley, UK), 10 µl x10 reaction buffer and nuclease-free water to 100 µl, incubated for 15 min at room temperature (25°C). Samples were then purified with the RNeasy MiniElute kit (Sigma-Aldrich Ltd, Poole, UK). After DNase I treatment, 350 µl of guanidinium-containing buffer RLT and 250 µl of 100% (v/v) ethanol were added with mixing to each reaction. Reactions were then applied to an RNeasy MinElute column in a 2 ml collection tube and centrifuged at 12,000 g for 15 s. The flow-through was discarded and the column and bound RNA washed in 500 µl buffer RPE, to remove protein, and centrifuged at 12,000 g for 15 s. The column was further washed in 500 µl 80% (v/v) ethanol, centrifuged at 12,000 g for 2 min, transferred to a fresh collection tube and centrifuged at 12,000 for 5 min to dry it. The column was transferred to a fresh collection tube and purified RNA was eluted in 30 µl nuclease-free water by centrifugation at 12,000 g for 2 min.

2.3.3.5 Analysis of purified RNA integrity and concentration

Reverse transcription and subsequent microarray analysis of RNA samples is a time consuming and expensive process. To avoid analyzing defective RNA samples, the quantity and quality of the repurified RNA samples was analyzed prior to these procedures using the Agilent 2100 bioanalyser

The Agilent 2100 bioanalyser consists of a single use cartridge containing a gel column through which up to 12 RNA samples, diluted to approximately 200 ng/µl, can be sequentially electrophoresed. The output from the cartridge passes through a Spectrophotometer cell, tuned to 260 nm, capable of detecting RNA and DNA. Electrophoresis is performed once the samples are loaded, along with a set of RNA size markers, and controlled via the PC-based Agilent bioanalyser software interface set on the Eukaryote Total RNA Nano protocol. This protocol provides an output for each sample consisting of an electropherogram, the total RNA concentration, the abundance of 28S and 18S rRNA relative to the total and the 28S/18S rRNA ratio. Samples which appeared partially degraded or overly dilute were not further investigated.

2.3.4 Probe generation for gene microarray analysis

To generate probe from the endometrial RNA for microarray analysis, it was necessary to reverse transcribe the RNA to complementary DNA (cDNA), this being less prone to degradation. In many studies, cDNA has been directly labelled with fluorophores or biotin, and applied to microarrays. This however requires large amounts of RNA to ensure a reasonable level of sensitivity on the microarray. To ensure multiple arrays

could be performed if required, and to use a methodology which provides unparalleled sensitivity for genes expressed at low levels, it was decided to use the SMART PCR cDNA synthesis kit (Clontech, St Germain-en-Lahaye, France) allied to Cy3-dCTP and Cy5-dCTP cDNA labelling in this study.

The Clontech SMART (Switch Mechanism At the 5' end of Reverse Transcript) PCR cDNA synthesis method, first reverse transcribes RNA to cDNA from a specialized primer (SMART CDS IIA primer), which anneals at the 3' polyA tail of mRNAs. The reverse transcriptase used also has terminal transferase activity and it adds several non-complementary cytidine deoxyribonucleotides at the 3' end of the first strand cDNA product. A second primer (SMART TS IIA primer) anneals to them, since it has a 3' rGrGrG sequence, providing a template for continuation of first strand synthesis, tagging the cDNA with a 3' extension complementary to the SMART IIA primer. Both primers contain a common sequence motif of 25 bases; one motif is therefore present in the cDNA in the incorporated 5' SMART CDS IIA primer, and a complementary motif is present at the 3' end in the SMART TS IIA primer complementary sequence. Thus all cDNA species can be amplified by polymerase chain reaction (PCR) with a common primer made up of the first 23 bases of the common motif (the PCR primer IIA).

In the method used in this thesis, the cDNA first strand product was amplified by PCR for a limited number of cycles, to ensure that proportional representation of each mRNA species was maintained in the cDNA mixture (REFS). This provided substantial amounts of endometrial cDNA suitable for highly sensitive microarray analysis. This cDNA was

102

labelled with dCTP-Cy3 using random octomer primers and Klenow DNA polymerase I fragment extension. Normal proliferative endometrial RNA was used as a control and was labelled in the same manner as the test endometrial samples, except with dCTP-Cy5.

After purification, each Cy3 labelled cDNA sample was hybridized to a microarray with an aliquot of the Cy5 labelled control. After incubation and washing, probe signals on the arrays were detected using a laser scanner, capable of independently detecting fluorescence from Cy3 and Cy5 labelled probe at 575 nm and 670 nm, respectively, after excitation with green (532 nm) laser and red (653 nm) laser light, respectively. Signals from the Cy5 labelled controls were used to standardise the Cy3 signals on each array, since variation in the Cy5 intensities would be related to differences in hybridization and detection efficiencies between arrays. Corrected Cy3 signals on each array provided measures of the expression of multiple genes in the original endometrial RNA sample from which the cDNA applied to the array was generated. Statistical analysis was performed to compare gene expression between arrays and to correlate differences with endometrial histopathology and PCOS-related serum biochemical and biometric parameters.

2.3.4.1   Reverse transcription

Re-purified DNase I-treated total RNA (0.5 µg) was mixed with 1 µl each of 10 µM SMART CDS IIA primer (5'-AAGCAGTGGTATCAACGCAGAGTACT$_{30}$VN-3', N = A, C, G, or T; V = A, G, or C) and 10 µM SMART TS IIA primer (5'-AAGCAGTGGTATC AACGCAGAGTACGCrGrGrG-3', rG = (guanosine

ribonucleotide), and made up to 5µl with RNase-free water. The mixture was incubated at 72°C for 2 min, and placed on ice. 2 µl first-strand buffer (5x; 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 30 mM MgCl$_2$; Clontech), 1 µl 100 mM dithiothreitol (DTT; Clontech), 1 µl 10mM dNTPs (Promega) and 1µl Powerscript reverse transcriptase (Clontech) were added as a mastermix to the RNA/primer mixture, giving a total reaction volume of 10 µl. Reactions were incubated at 42°C for 60 min to allow reverse transcription, then stored at -20°C.

2.3.4.2   cDNA amplification by PCR

PCR for each cDNA sample was performed in 100 µl reactions, containing 75 µl of RNase-free water; 10 µl of x10 PCR buffer; 2 µl of 10 mM dNTP; 5 µl of 25 mM MgCl$_2$; 4 µl of PCR primer IIA (5'-AAGCAGTGGTATCAACGCAGAGT-3') and 2 µl Amplitaq DNA polymerase (5 U/µl; Applied Biosystems, Foster City, USA). These were all prepared and added as a mastermix to improve pipetting accuracy. Each reaction also contained 2µl of cDNA, produced in Section 2.3.4.1, to make a final volume of 100 µl. PCR was performed on a SMART A62 PCR machine. The block was pre-warmed to 95°C, and the heating profile consisted of a 95°C/1 min step to denature nucleic acid secondary structure and non-Specific interactions, followed by 15 cycles of 95°C/10 s; 65°C/10 s; 68°C/6 minutes, after which samples were kept at 4°C until unloaded and stored at -20°C.

2.3.4.3 Light sensitivity – a warning

The Cy-3 and Cy-5 fluorophores are light-sensitive, light absorption causing molecular breakdown and fluorescence quenching. Microarrays are also light sensitive as they contain Cy-3 labelled pGEM bacterial plasmid dots for orientation. Light may also damage the DNA arrays directly, and the Cy-3/Cy-5 labelled probes applied to them. *Therefore when using Cy-dyes, their derivative products and microarrays, exposure to light was kept to a minimum. All were stored in light-proof boxes, manipulated in low light conditions, and/or pipette into light-proof tubes.*

2.3.4.4 Cy3 and Cy5 labelling of probe cDNA

Cy3 or Cy5-dCTP labelling was performed using the BioPrime DNA labelling system (Invitrogen, Paisley, UK). A Cy5-dCTP labelling control was included for each Cy3-dCTP labelled sample. 21 μl of PCR amplified cDNA from Section 2.3.4.2 was aliquoted into a sterile thin-walled 0.2 ml tube. To this was added 20 μl of 2.5x random prime buffer. The sample was mixed, pulse spun and incubated at 95°C for 5 min, then placed on ice. 5 μl of 10x Low C-dNTPs (2 mM dCTP, 5 mM dATP, dCTP, dGTP), 2 μl of 1 mM Cy3 or Cy5-dCTP (Amersham Pharmacia Biosciences, Little Chalfont, UK) and 1 μl of DNA polymerase I Klenow fragment D (40 U/μl), were then added. After mixing, the 50 μl reactions were incubated for 2 hours at 37°C. 5 μl of STOP buffer was then added to quench the reaction.

2.3.4.5 Cy3 and Cy5 labelled probe purification

Labelled probes were purified using Autos G50 resin columns (Amersham Pharmacia Biosciences, Little Chalfont, UK) which operate on the size exclusion principle. The resin was resuspended by vortexing, the bottom closure snapped off, and excess column buffer was removed by centrifugation at 2000g for 1 min. The column was then transferred to another collection tube and the whole Cy3 or Cy5 labelling reaction from Section 2.3.4.3 was applied to the centre of the top of the column. Columns were spun at 2000g for 1 min, the eluate contained Cy3 and Cy5 labelled cDNA, unincorporated nucleotides were retained in the column resin.

2.3.4.6 Estimation of Cy3 and Cy5 incorporation

1 μl of each column eluate was analyzed using a nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc, Delaware, USA), which performs spectral analysis between 250 and 750 nm on 1 – 2 μl samples. The associated software analyses absorbance data, providing estimates of cDNA and Cy3 or Cy5 label concentrations, allowing label incorporation to be calculated. The machine was zeroed when required using nuclease-free water.

2.3.4.7 Precipitation of purified Cy3 and Cy5 labelled probes

Prior to precipitation, all purified Cy5 labelled controls were pooled and mixed to ensure the internal standard applied to each microarray was uniform. Each Cy3 labelled product was then combined with an equal aliquot of Cy5 labelled product pool (*ca.* 100 μl total volume) in a light-tight container. To this was added with mixing 5 μl of human Cot-1

106

DNA (1 mg/ml; Invitrogen, Paisley, UK), 10 µl of 3 M sodium acetate (pH 5.2), and 250 µl of 100% ethanol. Probe was precipitated overnight at -20°C (or for >2 hours), pelleted by centrifugation at 12000g for 15 min, washed with 75% ethanol, centrifuged again at 12000g for 5 min and left to air dry for 15 min after discarding the supernatant. Thus each Cy3 labelled test sample was in a minute volume with an equal volume of Cy5 labelled control, ready to be dissolved and applied to a microarray.

2.3.5 Performing microarray analysis

2.3.5.1   The microarrays

The arrays were custom-made cDNA arrays, with ~8000 genes spotted in duplicate. This included controls and housekeeping genes spotted in each of the blocks for QC purpose. The genes included those known to be important in cell adhesion, apoptosis, signalling, cell cycle regulation, ECM remodelling and angiogenesis. The arrays were provided courtesy of the department of Pathology Cambridge University and funded by the BBRSC.

2.3.5.2   Microarray prehybridization

All washes of cover-slips and microarrays throughout the prehybridization and hybridization procedure were in 50 ml in Falcon 50 tubes, with rocking at room temperature, unless otherwise noted.

Ridged cover slips were stored in 70% ethanol and were washed twice in ultrapure water, generated using a MilliQ (Millipore, Watford, Hertfordshire) system, once in isopropanol and then air-dried. A cover slip was gently placed over each microarray, with the cover slip

edge 1 mm from the bottom of the array and the ridges toward the array, thus producing a glass sandwich with a pocket for fluid samples over the array of *ca*. 50 μl volume.

Hybridisation buffer (40% v/v deionised formamide; 5x SSC; 5x Denhardt's solution; 1 mM sodium pyrophosphate; 50 mM Tris-HCl (pH 7.4); 0.1% w/v sodium dodecyl sulphate) was warmed to 50°C, mixed and 50 μl slowly applied to the pocket at labelled end of the array, such that the buffer was slowly drawn into the pocket by capillary action without forming bubbles within the pocket or a fluid droplet which touched the label. Each array was immediately placed in a hybridization chamber, with 10 ml humidifying solution (40% v/v deionised formamide; 5x SSC) on a flat pad of tissue in the bottom, pre-equilibrated in a water bath at 50°C. Pre-hybridisation was allowed to take place for 2 hr.

Cover slips were then removed by soaking the microarrays in x2 SSC. The cover-slips were soaked in 70% v/v ethanol, washed as above and air-dried. The prehybridized microarrays were washed once in 2x SSC, with rocking, for 5 min, then twice in ultrapure water, once in isopropanol and air-dried. Prior to application of probe solution and hybridization (Section 2.3.5.3), cover slip-microarray sandwiches were prepared in the same way as for pre-hybridization.

2.3.5.3 Microarray hybridization

Air-dried pellets of Cy3-labelled sample cDNA and Cy5-labelled control cDNA, from Section 2.3.4.7, were each dissolved in 54 μl solution containing 50 μl hybridization buffer,

2 µl human Cot-1 DNA (1 mg/ml; Invitrogen, Paisley, UK), 1 µl polydeoxyadenosine 5'
phosphate (polyA, 8 µg/µl; Amersham Pharmacia Biosciences, Little Chalfont, UK) and 1
µl yeast tRNA (4 µg/µl), for 5 min at room temperature, then 2 min at 95°C, with mixing.
Dissolved probes were then centrifuged at 12000g for 5 min and kept at 50 °C until loaded
on to microarrays.

Once prepared, 50 µl of probe solution was loaded onto each array as quickly as possible, in
the same manner as for pre-hybridization (Section 2.3.5.2). After loading, microarrays were
immediately placed in a hybridization chamber, with 10 ml humidifying solution (40% v/v
deionised formamide; 5x SSC) on a flat pad of tissue in the bottom, pre-equilibrated in a
water bath at 50°C. Hybridisation was allowed to proceed for 16 - 18 hr.

2.3.5.4   Post-hybridization microarray washing

After hybridization, the cover slips were removed by immersing microarrays in 2x SSC.
Cover-slips were washed in 70% ethanol and stored in 70% ethanol for re-use. Microarrays
were washed twice for 5 min in 2x SSC, twice for 5 min in 0.1x SSC with 0.1% w/v SDS,
and twice for 5 min in 0.1x SSC. The microarrays were then dip-rinsed successively in 0.1x
SSC, ultrapure water and isopropanol, before being clamped in a plastic frame to aid
manipulation, placed in an empty 50 ml test tube and centrifuged at 250g for 2 min to dry.
Care was taken to ensure the identification label was at the bottom of the array, to prevent
contamination of the microarray surface with gum from the label. The slides were stored in
the dark at room temperature and scanned as soon as possible, certainly within 7 days.

2.3.6 Microarray scanning

Microarrays were scanned to obtain Cy3- and Cy5-labelled probe intensities (at 532 nm and 635 nm, respectively) hybridized at each target DNA spot, using an Axon Instruments 4100A scanner (Molecular Devices Corporation, 1311 Orleans Drive, Sunnyvale, CA 94089-1136, U.S.A.) with GenePix pro 4.1 software, according to the manufacturers' instructions. The microarray slide was first insert into the scanner with the microarray downwards and identifier barcode at the front. Filters appropriate for Cy3 and Cy5 detection were selected (575DF35 and 670DF40, respectively), as were the green (532 nm, excitation of Cy3) and red (653 nm, excitation of Cy5) lasers. A preview scan was performed at 40 μm resolution to allow selection of the appropriate array area for high resolution scanning, and adjustment of the photomultiplier tube gain settings so that the total array signal through the Cy3 and Cy5 detection channels was broadly equal (i.e. the count ratio field approximates 1.0). A high resolution scan at 10 μm resolution was performed for primary data acquisition, and the images acquired through each filter were saved in uncompressed TIFF file image format.

2.3.7 Image analysis

Image analysis was performed using the program GenePix version 3.1. The Cy 3 and Cy5 filtered TIFF images were opened and amalgamated. A grid tool was overlaid over the image, automatically aligned then manually adjusted, so that each array spot was contained within an individual circular element on the grid (diameter *ca* 130 μm). Intensity estimates for Cy3 and Cy5-fluorescence from each array spot were then

110

collected and saved as a spreadsheet amenable to manipulation in the software packages Excel for Windows and Genespring 4, which listed the array 'address' and associated Cy3 and Cy5 fluorescence intensities

The hypothesis underlying microarray analysis is that the measured probe fluorescence intensity on each arrayed gene spot is proportional to the expression level of that gene. Biologically relevant patterns of expression are identified by comparing measured mean expression levels for each sample type on a gene-by-gene basis. Before the levels can be compared, data has to be processed to eliminate questionable or low-quality measurements and to adjust the measured intensities using factors provided by the common internal standard (Cy5-labelled), to compensate for bias and variability and thus facilitate effective comparisons.

Most microarray experiments investigate the relationships between related biological samples based on patterns of gene expression, and the simplest approach looks for genes that are differentially expressed. If we have an array that has N gene-spots, and compare an experimental and a reference sample (E and R, respectively), then the expression ratio (T) for a gene x is

$$T_x \quad = \quad E_x/R_x$$

Expression ratios provide an intuitive measure of expression changes but they also have the disadvantage of treating up- and down- regulated genes differently. Genes up-regulated by a factor of 2 have an expression ratio of 2 whereas those down-regulated by the same factor, have an expression ratio of 0.5. The commonest used transformation of

the ratio in these situations is the logarithm base 2, which has the advantage of producing a continuous spectrum of values and treating up- and down-regulated genes in the same way.

Typically, the first transformation applied to expression data is referred to as normalization and this adjusts the individual hybridization intensities, to balance them appropriately so that meaningful biological comparisons can be made. Data has to be normalized for a number of reasons such as differences in: The quantity and/or quality of sample RNA; Reverse transcription efficiency; Probe labelling efficiency; Microarray hybridization and washing efficiency; Detection efficiencies between the fluorescent dyes; Systematic biases in measured gene expression levels. Conceptually, normalization is similar to adjusting the expression level of a target gene measured by northern blotting or RT-PCR, relative to the expression of one or more reference genes whose levels are assumed to be constant between samples.

There are a number of normalization processes that may be used but the Lowess normalization was used in this study. Lowess normalization has the advantage of removing intensity dependent effects which can arise as a result of transformation of data. The starting point for Lowess normalization is an intensity/ratio plot. Lowess detects systematic deviations in the ratio/intensity (R-I) plot and corrects then by carrying out a local weighted linear regression as a function of the intensity and subtracting the calculated best-fit average ratio from the experimentally observed ratio for each data

point. Lowess uses a weight function that de-emphasies the contributions of data from array elements that are far (on the R-I plot) from each point.
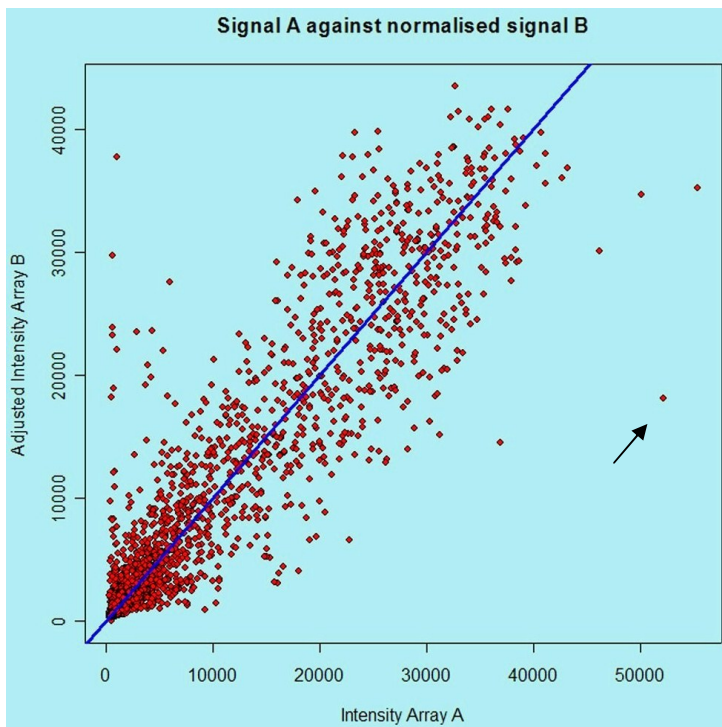
Figure 2.4 shows a plot of intensity data after lowess normalization. (courtesy of www.obgyn.cam.ac.uk/.../loess) -normalisation.htm. The arrow points to a significantly expressed gene.

2.3.8 Statistics

Primary fluorescent intensity data was analysed using the software package Genespring which has been specifically designed for analysis of microarray data.

A Spearman correlation (non-parametric data) was performed between hormones and genes.

113

Array analysis method -

1. The primary intensity results files (*.gpr files) were filtered by removing data associated with empty spots and internal calibration spots which contained pGEM plasmid dilutions.

2. The arrays were normalised per spot and per chip using an intensity dependent Lowess procedure.

3. Array data was filtered by removing data from 'flagged' gene-spots. If a gene was flagged in 15 of the 20 arrays, all data for that gene was omitted.

4. Array data was also filtered on the basis of the expression intensity of the internal standard at each gene-spot, *i.e.* the Cy5 intensity value. Expression data for a gene-spot was omitted if it was below an intensity threshold, set at the mean background on all microarrays plus 1 standard deviation interval. This threshold was calculated to be 950, the maximum intensity being 63,999, since the background on the arrays was somewhat high. If the expression intensity of the internal standard at a gene-spot was below this level in 7 of the 20 arrays, all data for that gene was omitted. Filtering in steps 4) and 5) left a gene list of 3975 genes, having removed genes the expression of which was at a low intensity level barely above background which could not be accurately quantified.

5. On the basis of the expression of these 3975 genes, cluster analysis was performed using a condition tree with a Spearman correlation.

6. Selection of genes was then performed, to find those the mean expression level of which were modified more than 1.75-fold between the different samples types, at a p value <0.05 by two-tailed Students t test. This produced a list of 15 genes for the H vs.

PL comparison, 63 genes for the H vs. PS comparison and 75 genes for the PL vs. PS comparison.

7. To view which genes are common or different between the sample groups, a Venn diagram was constructed, showing for the 100 genes in total varying between the samples sets, the numbers of genes different between each samples set comparison in a colour coded format. These genes are then listed in the same colour coding, along with clone ID, gene description and for each sample type, the mean normalised ratio (values for duplicate), mean internal control (Cy5) intensity value (values for duplicate) and the mean experimental sample (Cy3) intensity value (values for duplicate).

8. Gene lists were also prepared for each comparison main sample type comparison, i.e. H vs. PL, H vs. PS and PL vs. PS, including fold change, significance level and gene function. The ratio values here represent the mean normalised ratio of the biological replicates (the range of the normalised ratio in all samples in that group)

9. Finally, cluster analysis was performed using a condition tree with a Spearman correlation based on the expression of the 100 genes defined after filtering in steps 1) – 6)

2.4.0 Validation of data By Real-Time PCR

RT-PCR quantifies the initial amount of the template specifically, sensitively and reproducibly and seems to be preferable to other forms of quantitative RT-PCR that detect the amount of final amplified product at the end-point. Real-Time PCR monitors

the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e. in real time) as opposed to the end-point detection.

Quantitative real-time RT-PCR was performed to validate microarray data, using TaqMan methodology on an ABI prism 7900 analyzer, according to the manufacturer's instructions (Applied Biosystems, Warrington, UK). 4 genes were targeted which were differently expressed between groups, 2 genes (osteopontin, OPN, and Drosophila disc-large 1 tumour-suppressor like-7, DLG7) had high signal intensity on the microarrays and 2 (asialoglycoprotein receptor 1, ASGPR1, and cell division kinase 8, cdk8) had signal intensity below the Cy5 signal intensity threshold applied. cDNA was first produced from each endometrial RNA sample by reverse transcription with random hexamers using 2 µg of total RNA with 200 IU Superscript RT (Invitrogen Life Technologies, Applied Biosystem, Warrington, UK).
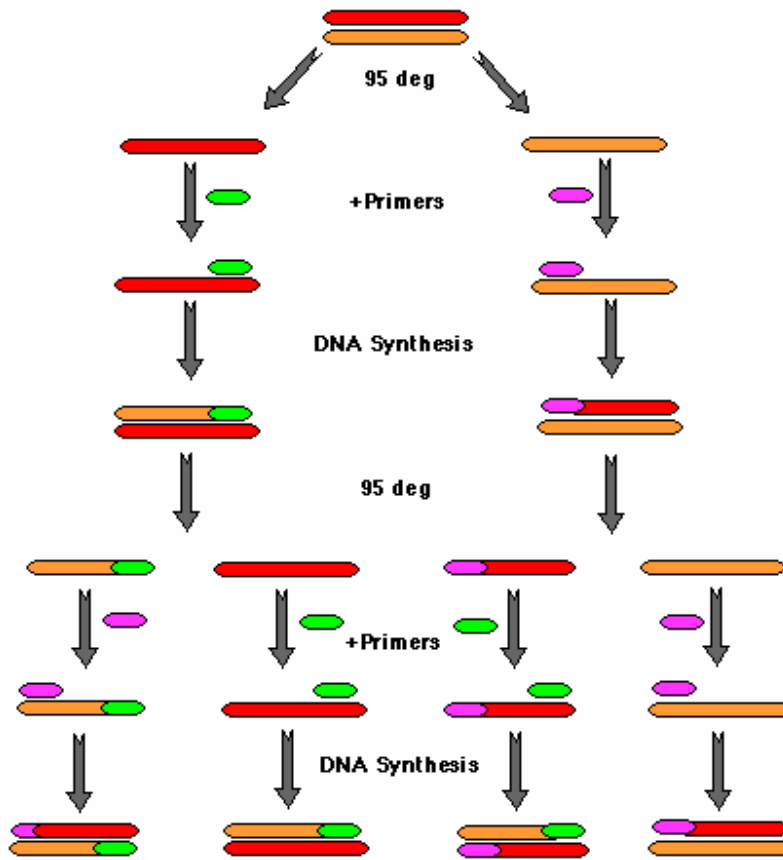
Figure 2.5 showing the principle of RT-PCR- courtesy of Dr Frank Orme. An electronic tutorial. Merritt College, Oakland, USA. Free reproduction.

Taqman gene expression assays consist of a 20X mix of unlabelled PCR primers and Taqman MGB probe (FAM dye-labelled). These assays are designed for the detection and quantification of specific genetic sequences in RNA samples converted to cDNA. Gene expression using Taqman Gene expression assays is performed as the second step in a two-step reverse transcription- polymerase chain-reaction (RT-PCR) protocol on any ABI prism Sequence Detection System Instrument. All Taqman Gene expression assays

are optimized to work with either Taqman Universal PCR Master Mix (P/N 4304437) and with cDNA.

Specific real-time PCR assays were performed for each gene with simultaneous in tube estimation of 18S rRNA as internal standard, the specific primers and probes being supplied as assays on demand, target gene probes being TAMRA labelled and 18S probe FAM labelled (Applied Biosystems, Warrington, UK). The mix was then run through a thermal cycler. Denaturing was allowed to happen for 40 cycles of 15 seconds at 95˚C then annealing and extension occurred in another 40 cycles of 60 seconds at 60˚C.

Master mix for 95 runs was prepared and consisted of 12.5 μm of master mix, 1.25 μm of 18s rRNA, 1.25 μm of probe and 5 μm of RNAse –free water.

5 μl of cDNA was then added.

Expression levels in each endometrial RNA sample were calculated by the ddCt method, and mean expression levels in the three groups were compared by ANOVA.

We also correlated the ratio of expression for each gene to the value for the different parameters tested for such as cycle length, insulin levels, and testosterone levels.

Chapter 3

The prevalence of polycystic ovarian morphology in women with endometrial cancer

3. Prevalence of Polycystic Ovaries in women with endometrial carcinoma.

3.1 Introduction

For a number of decades, there has been a general assumption that women with PCOS are at increased risk of developing endometrial carcinoma. This is thought to be due to the fact that these women are subjected to high oestrogen and insulin levels.

However as was discussed in Chapter 1, section 1.8, conclusive evidence for such an association is lacking and the results of some studies contradict this assumption. For example Ramzy found that ovarian morphology in women with endometrial cancer was similar to normal women than to women with PCOS. Similarly, a study was undertaken to look at the prevalence of PCO in patients with endometrial cancer.

3.2 Hypothesis

Polycystic ovarian morphology is more prevalent in patients with endometrial carcinoma than in controls.

3.3 Aim

To demonstrate whether patients with endometrial carcinoma show histological evidence of polycystic ovaries.

3.4   Ethics

Ethics approval was first granted for the study from the Royal Free Hospital Medical School ethics committee for the study to be performed between June 2002 to June 2003 and this was later extended for another year by chairman's action. Ethics approval for the same study was also sought from the University College Hospital (UCL) Medical School and was granted for a year stating August 2003

3.5   Methods

Study design, population and case selection

The experimental group (n = 128) comprised all patients who had been diagnosed with EC, for whom archived ovaries and endometrial tissue were available, following total hysterectomy and bilateral salpingoopherectomy at the Royal Free Hospital (RFH), London between July 1987 and July 2003 or University College Hospital (UCH), London, between July 2000 and July 2003. The control subjects (n = 83) were age-matched (± 5 years) to the experimental subjects, and were derived from a larger group of patients with benign gynaecological conditions operated on in the hospitals above over the same time periods, for whom archived ovaries and, in most cases, endometrium were available. Controls had also undergone total hysterectomy and bilateral salpingoopherectomy or bilateral oopherectomy. No other selection criteria were applied and patients with gynaecological cancers other than EC were excluded.

The Royal Free Hospital Histopathology department keeps a data base, known as "Snomed". This contains the details of all patients who have had surgical specimen examined for histology from 1990 up to date. A similar database is kept at UCL.

The ovaries of each patient were independently assessed by the main investigator and the histopathologists at respective centres.

Assessment of ovaries

The prevalence of PCO morphology in subjects operated on for EC or for benign gynaecological conditions was assessed by examination of archived Haematoxalin-eosin stained 5 µm ovary sections from each patient. In clinical histopathological practice, PCO morphology is often diagnosed using a qualitative assessment[167,168]. The criteria used to diagnose PCO were those described in 2.13.

3.6   Statistics

The difference in PCO prevalence between the EC and control group was tested using Chi-squared analysis, with a Mantel-Haenszel correction to adjust for differences in age distribution, and using Fishers Exact Test in age-restricted groups.

3.7 Results

	subjects	controls
No. of patients	128	83
Mean age	63.9	51.6
No. PCO	11	7
% PCO	8.59	8.75

Table 3.1 shows the patients demographics.

Investigation of patient records revealed there was no significant difference in the ethnicity profile between subjects with EC or benign gynaecological conditions (Table 3.2). It also showed that patient records could not be used to determine consistently and without bias whether patients had PCOS. Archived ovarian sections were therefore investigated from each subject, to determine the prevalence of features consistent with PCO morphology as a surrogate for PCOS.

Overall the prevalence of PCO was comparable in women with EC or benign gynaecological conditions (11 of 128 [8.59%] *versus* 7 of 83 [8.75%], respectively, $p = 1.00$). P*ost hoc* Power analysis indicated the experiment had a Power of 0.834 and 0.999 to detect a 2- or 3-fold increase, respectively, in PCO prevalence in the EC set at $p < 0.05$; no significant difference was however noted. Ethnicity appeared not to influence PCO prevalence as in the EC set, 10 women with PCO were Caucasian and one was of unknown ethnicity, while in the benign controls with PCO, 6 were Caucasian and one was of Indian-Asian origin. When subjects were subdivided by age (Fig. 3.1), there were again no differences in the prevalence of PCO between the EC patients and benign controls, albeit non-significant effects were

noted in women aged 20 – 39 years and 40 – 49 years. When subjects were divided into two groups aged <50 years and ≥50 years (50 years being the average age of menopause), the prevalence of PCO in patients aged <50 years was greater in those with EC than in controls (10 of 16 [62.5%] *vs.* 6 of 22 [27.3%], respectively, **p = 0.033**; β = 0.153 by *post hoc* Power analysis). No difference was noted in PCO prevalence in patients aged ≥50 years.

Data reported for each ethnic group are the number of subjects (N), the % of total cases and the % of cases for which ethnicity data was reported. Ethnicity data was not reported in some cases, primarily from the early years of the study. The proportion of patients in each ethnic group did not differ significantly between EC and control subjects; neither did the proportion of unreported cases.

Ethnicity	EC cases			Controls		
	N	% of total	% of reported	N	% of total	% of reported
Caucasian	91	71.1	95.8	48	57.8	88.9
Asian Indian	2	1.6	2.1	3	3.6	5.6
Asian Other	1	0.8	1.1	0	0.0	0.0
African-Caribbean	1	0.8	1.1	3	3.6	5.6
Unreported	33	25.8		29	34.9	
Total	128			83		

Table 3.2:   Ethnicity data for subjects with endometrial cancer (EC) and benign gynaecological disease (Controls), obtained retrospectively from patient records.

patients with endometrial carcinoma

8.59%

PCO +ve
PCO -ve

91.41%
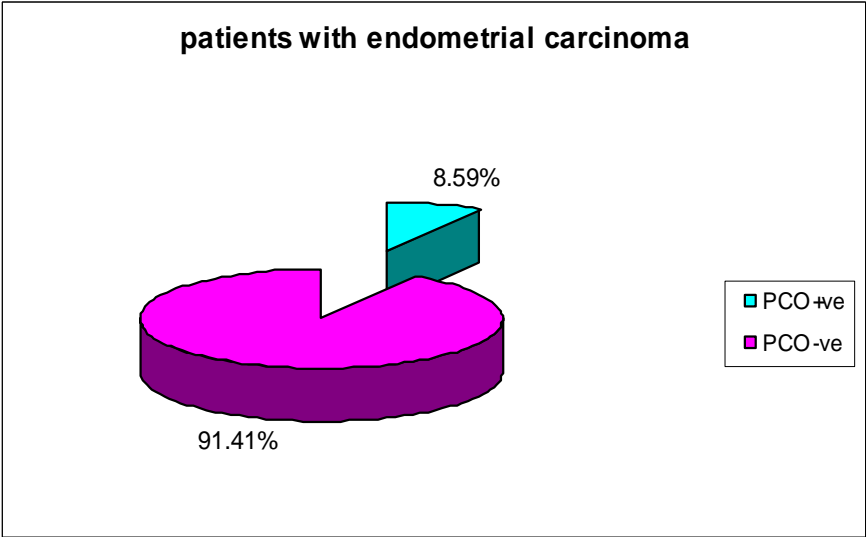
Fig 3.3- A pie chart representing the proportion of cases with PCO morphology.

patients without endometrial carcinoma

8.75%

PCO +ve
PCO -ve

91.25%
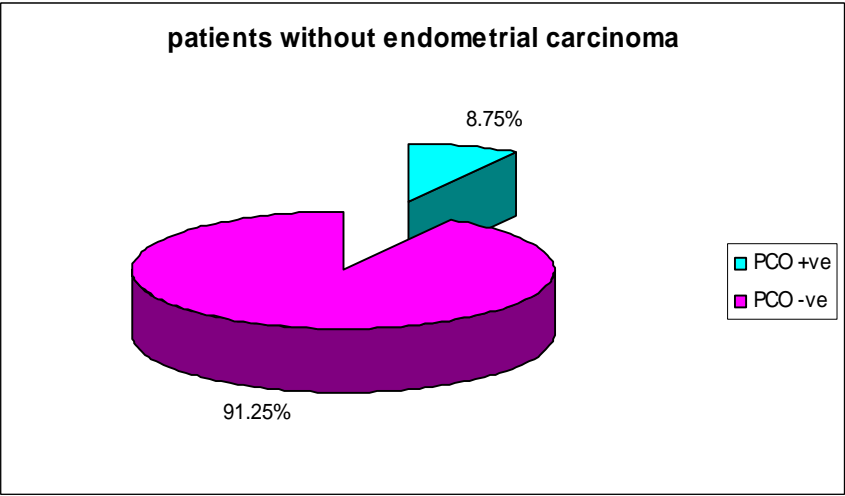
Fig 3.4-A pie chart representing the proportion of controls with PCO morphology.

However we did find that there was a significant difference (Chi-squared test) in the prevalence of PCO in the younger patients with endometrial cancer when compared to controls of a similar age-group, as illustrated in figure 1.
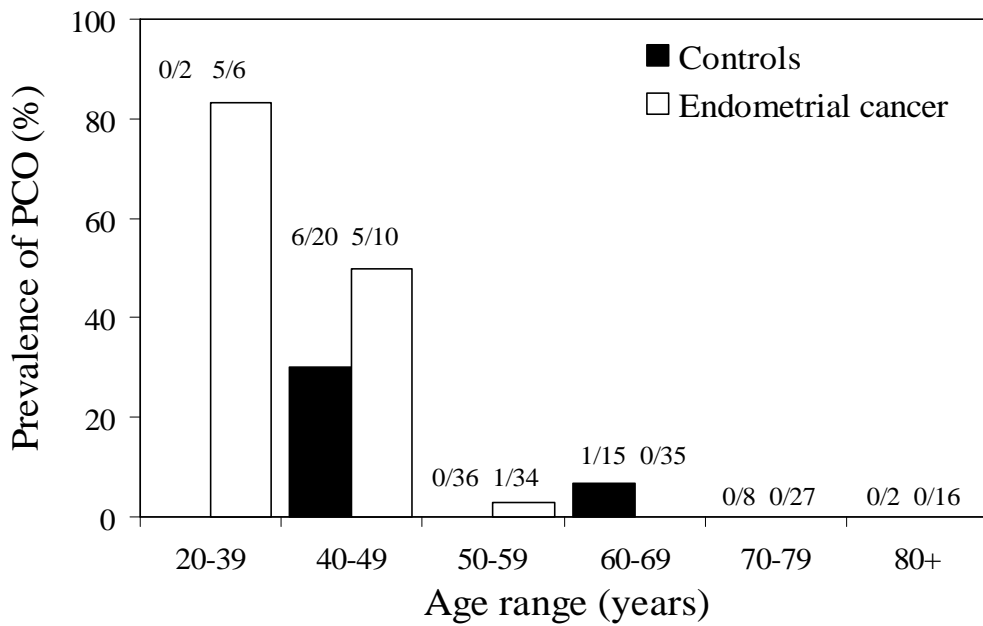
Figure 3.5- A bar chart representing the prevalence of PCO morphology in women with endometrial cancer and in controls, as a function of patient age (data = patients with PCO/total number of patients).

3.8   Discussion

The results obtained for this prevalence study questions the relationship between PCOS and endometrial cancer. PCO as a marker of PCOS as it is a known fact that a great percentage of people diagnosed with PCOS will also exhibit PCO morphology[169]. . The

number of women with PCOS in the UK is around one million (affecting 10 % of the reproductive population aged 20-50). If we arbitrarily assign the risk of EC to be as low as 1% in PCOS women, this would give an incidence of EC of 10,000 each year. However according to the Office for National Statistics, in 2005, in the UK, the incidence of endometrial cancer was 6891. (5% of all female cancers [99,100,170,171]). This raises doubt as to whether the two conditions are really linked. It could be that only a sub-group of PCOS women only are at risk of developing EC.

As discussed in the introduction to this thesis, endometrial carcinoma arises from a number of pathways and originates from a number of different endometrial environments. Some tumours arise from a background of unopposed stimulation of high levels of oestrogen whilst other tumours do not depend on high levels of oestrogen to develop. Whilst PCOS is associated with high levels of circulating oestrogens, it may be that the condition also confers a protective effect on the endometrium. Hyperoestrogenism is not the only characteristic of PCOS and it may be that there yet unknown biochemical or molecular factor that offers some immunity to the endometrium in the presence of hyperoestrogenism. As mentioned before, there are a number of key steps in endometrial carcinogenesis. Mutation is one key step and is involved as an early change in microsatellite instability[116,172]. After the menopause, when the endometrium is no more subjected to the high oestrogen levels, it may be that the hormonal environment in PCOS women becomes similar to that found in women without PCOS. This would explain why after adjusting for age-differences in the study that was conducted, no significant difference was found in the presence of polycystic ovarian morphology. However

Winters et al, in a cohort of 84, pre, peri and post menopausal women with PCOS, found that there were differences in the levels of serum testosterone. Pre and post-menopausal women with PCOS were found to have increased levels of testosterone compared to PCOS women who were perimenopausal and (ages between 42 and 47). This finding was supported further by a study in which post-menopausal women undergoing coronary angiography were scanned and up to 45% of them were found to have pco in addition to biochemical disturbances similar to those found in PCOS[173]. These studies confirm that post-menopausal women with PCOS will continue to be subjected to an altered biochemical environment and a large number of them will also display PCO morphology.

Another possible explanation for these findings may be that this study centred entirely on detecting ovarian morphology (PCO) as a marker of PCOS. Consequently, it is likely that some women with endometrial carcinoma with no evidence of PCO but biochemical evidence of the condition will have been allocated to the control group and missed altogether. Absence or reduction in the number of corpora albicantia was an essential criterion for an ovary to be scored positive for polycystic ovarian morphology. This feature denotes that the ovary has had impaired ovulation. This was taken as one of the essential markers to score an ovary as polycystic in attempt to minimize the problem that some patients will not necessarily have ovarian features of PCOS. . However the consensus definition of PCOS allows this diagnosis in women with regular menstrual cycles (if they have symptoms of hyperandrogenaemia) and in such cases the number of corpora albicanta would not necessarily be reduced. It can also be argued that the ultrasound features of PCO are a transient finding in the ovary and that at the time that

the ovaries were removed there was no evidence of PCO but on histological examination, features such as capsular thickening, stromal hyperthecosis and absence of hallmark of ovulation, are not transient. Elting et al [174], followed women ageing women with PCOS and found that those who developed regular cycles towards the end of their menstrual life tended to have fewer follicular cysts that those with irregular cycles. Again that may explain why PCO was not found in some of the older patients, as some of them may have developed regular cycles.

According to the ESHRE/ARSM diagnostic criteria for the diagnosis of PCOS, although PCO on ultrasound is one of the criteria necessary, it is not a prerequisite. There are subgroups of women who will exhibit the symptoms of hyperandrogenaemia, hyperinsulinaemia and may not necessarily have suffered from chronic anovulation. An attempt was made to address this point by trying to retrieve the patients records for examination but this had to be abandoned as some of these patients had been seen at an early time and their records had already been destroyed or sent to archive record keeping and it was impossible to retrieve these within the time frame in which this study was conducted. Another problem that was faced was that record keeping by medical staff was unreliable. Many patients were not asked specific questions about their menstrual cycles, fertility or symptoms of PCOS (or possibly documentation was lacking) However some patients were being seen for the first time when they had been menopausal for at least a decade and their recall of menstrual and subfertility history was likely to be inaccurate in some cases. Similarly, as most doctors believed that PCOS is linked to endometrial

cancer, it probably had been considered unnecessary and inappropriate to investigate patients with endometrial cancer for PCOS.

For the purpose of this study, polycystic ovarian morphology was diagnosed using criteria usually applicable to pre-menopausal ovaries where oestrogen levels and action on the architecture are intact. However it is not well documented what happens to a post-menopausal ovary. The ovary is known to atrophy after the menopause, but less is known about the faith of the capsule, the follicular cyst, the stroma and the corpora albicantia after the drop in oestrogen levels. It is possible that we have missed some polycystic ovaries due to the fact that the architecture has change in these patients. Previously, histopathologists would often fail to report polycystic ovarian morphology as its significance was overlooked. Our finding of no increase prevalence in the post menopausal women with endometrial carcinoma could in fact result from a failure to identify the altered ovarian morphology in this group. However Birdsall et al published their work in 1998 where they found that polycystic ovaries could be diagnosed in up to 50% of postmenopausal women on ultrasound scan[175]. Alternatively PCO and endometrial cancer may be unassociated conditions in post menopausal women.

Another interesting finding was that polycystic ovarian morphology was significantly more common in younger patients (20-40 years old) with endometrial carcinoma. It is possible that polycystic ovaries may constitute a risk factor for endometrial cancer in women aged 20 – 49, (the reproductive years). After this, the hormonal changes occurring in the menopause may remove the putative endometrial cancer stimulating

effect of the presence of the polycystic ovary. One of the mechanisms by which patients with PCOS are thought to develop cancer is *via* unopposed oestrogenic stimulation of the endometrium resulting from chronic anovulation. Indeed, it has been proposed that agonist-bound progesterone receptor-A inhibits the transcriptional effects of oestrogen receptor in the human reproductive system [176]. Thus the lack of a luteal phase progesterone surge in anovulatory women with PCOS may leave the oestrogen receptor unduly active at an abnormal time in the menstrual cycle, with the risk only removed after the menopause, which would explain our finding of more PCO in young women with endometrial cancer. Women with PCOS suffer from a high incidence fibromyoma, myohypertrophy and endometrial hyperplasia[177], perhaps due to unopposed oestrogen stimulation of the endometrium. PCOS is associated with hyperinsulinaemia and insulin resistance[178], worsening with increasing age[179]. Aside from exacerbating the hyperandrogenaemia by enhancing aromatase activity[180], these endocrine factors may underlie the association between PCO and EC, since insulin and IGF-I stimulate EC cells in vitro[181].

Hyperandrogenaemia has been associated with EC in post-menopausal women on the basis that those with testosterone levels in the upper quartile, have an approximate three-fold increased risk of EC[182]. The lack of association between PCO and EC in post-menopausal women in the present study may reflect the hypoestrogenic environment in post-menopausal women. Alternatively, the absence of an increased prevalence of PCO morphology in women aged ≥50 years with EC, may be because it is difficult to identify such morphology in this age-group. PCO can however be reliably identified in post-

menopausal women by ultrasonography[183] and it is unlikely that the histological analysis used in this study would be less sensitive. Thus it is probable that elements of PCO morphology remain after the menopause and, if present, would have been detected in this study. Indeed, hyperandrogenaemia commonly persists in post-menopausal women with PCOS[183].

A limitation of this study is that subjects were characterised as having PCO rather than PCOS. It was impossible to identify women with PCOS from patient records, consistently and without bias, because the clinical and endocrine details required were infrequently recorded for these subjects, who were being treated either for EC (many of whom were post-menopausal) or other gynaecological disorders unrelated to PCOS. Subject follow-up could also not be performed as many were untraceable or deceased. Between 25 and 50% of women with PCO morphology exhibit symptoms supportive of PCOS[92,184]. In the present study the concordance between PCO morphology and PCOS was likely to be higher, since the definition of PCO morphology used included the criterion that a reduced number of corpora albicantia were present, suggestive of impaired ovulation. Such a criterion has not been previously used and, if satisfied in association with the presence of more than 8 cystic follicles, is supportive of a retrospective diagnosis of PCOS. It is possible that a few subjects with normal ovaries may have had symptoms of hyperandrogenaemia (part of the recently agreed diagnostic criteria) and ovulatory dysfunction supportive of a diagnosis of PCOS[95]. Indeed, an ovary was classed as normal if <8 follicles were present, even if ovulatory dysfunction was indicated by the absence of corpora albicantia. This group of patient with no

histological evidence but possible biochemical disturbances would have been missed and could have influenced the results. This situation seldom occurred however; few ovaries classed as normal had a limited prevalence of corpora albicantia. It is also possible that some of the controls operated on for benign gynaecological conditions may have had occult endometrial tumours. The level of contamination of the control group was probably insignificant however since the subjects had been assessed by a gynaecologist and endometrial histopathological assessment was made in most cases as hysterectomy was performed. Finally, no endometrial cancers were noted in post-operative records when hysterectomy was not performed, albeit these records were sometimes limited in the length of follow-up.

3.9   CONCLUSION

This study was performed to compare the prevalence of polycystic ovaries in women with endometrial cancer with women without endometrial cancer. We did not find the prevalence of pco to be raised overall in women with endometrial cancer. However, when we stratified women into different age groups, we found that women younger women with endometrial cancer seem to exhibit polycystic ovaries. We also found that there are no set criteria to assess polycystic ovaries in women and the condition in post menopausal women is even less described. This potentially would be an area of future research.

These results challenge the assumption that women with PCOS have a greater risk of developing endometrial cancer.

Chapter 4

THE EXPRESSION OF PROGNOSTIC
MARKERS OF ENDOMETRIAL
CANCER IN THE ENDOMETRIUM OF
WOMEN WITH POLYCYSTIC OVARIES

4.0 The expression of prognostic markers of endometrial cancer in the endometrium of women with Polycystic Ovaries.

4.1 Introduction

The incidence of endometrial cancer is on the increase in the UK. It is the fifth most common cancer in women in the UK with around 6,891 cases diagnosed in 2005 alone, accounting for around 5% of all female cancers. The clinicopathologic features vary, including age at diagnosis, histologic characteristic, DNA ploidy, hormonal receptor status and clinical course. This is likely to reflect a complex aetiology involving both endogenous and exogenous factors[129]. Various factors that seem to influence the risk of endometrial cancer and the prognosis. For example, there is clearly a genetic component as women with the gene for hereditary non-polyposis colorectal cancer carry a 10-fold increased risk of developing endometrial cancer compared to the general population[185]. Environmental factors are also important and the increase in frequency of endometrial carcinoma has been attributed to dietary and hormonal factors as well as an ageing female population[186]. Other factors considered to have an influence in the prevalence and prognosis of EC include increased BMI, exogenous oestrogen treatment , especially when unopposed by cyclic progestogens, and hormonal disturbances as seen in diabetes mellitus or hormone producing tumours of the ovary[186]. It is however, generally believed that tumours that arise in women with PCOS, tend to be well differentiated and carry a better prognosis. Jafari et al[155] carried out a prospective study involving 6 patients with known PCOS who had been diagnosed with endometrial cancer. The subjects had an age range of 23 to 33 years and all had a total abdominal hysterectomy and bilateral

135

salpingoopherectomy. 5 patients were found to have well differentiated tumours and 1 had a moderately differentiated tumours. After 15 years of follow-up, they were all found to be disease-free.

To investigate the hypothesis that women with PCOS and EC exhibit well differentiated tumours which carry a good prognosis, a second study was performed. The expression of p53, Cyclin DI, Ki 67 and bcl2 was assessed in the tumours of women with polycystic ovaries. These proteins were chosen as specific prognostic markers for endometrial cancer as we discuss below.

P53 – The nuclear protein encoded by the p53 gene acts as a tumour suppressor. It is essential in the modulation of cellular responses to cytotoxic stress by contributing both to cell cycle arrest and programmed cell-death [12]. Mutations of the p53 tumour suppressor gene have been found in about 10-20% of endometrial carcinomas[187]. As p53 mutations rarely occur in cases of endometrial hyperplasia, it is thought to be a relatively late event in carcinogenesis. p53 mutations often result in overexpression of mutant p53 protein, which is conformationally altered and functionally defective with a longer half-life than the normal wild- type counterpart, thus permitting in immunohistochemical detection of the protein product[188].

Cyclin D1 is a cell cycle protein whose over-expression may be associated with gene amplification or transcriptional dysregulation in cancers. This over-expression in endometrial glands increases progressively in intensity and extent from normal to

neoplastic endometrium[189]. The pattern of Cyclin D1 over- expression also suggests that this is an early event in carcinogenesis. Cyclins, cyclin-dependent kinases, and tumour suppressor gene products interact and regulate the normal cell cycle[190]. Cyclin D1 and cyclin-dependent kinases are required for completion of the G1/S transition in normal mammalian cells[190]. Cyclin D1 dysregulation in endometrial carcinoma contributes to an increase in the proportion of cells in transition from G1 to S phase. Cyclin D1 overexpression may be one of several mechanisms involved in endometrial neoplasia, and proliferative endometrial glands and stroma, even when actively mitotic do not over express Cyclin D1[191]. Cyclin D1, in in-vitro studies has been seen to be rendered insensitive to degradation by threonine 286 mutation and this Cyclin D1 mutant induces transformation in murine fibroblasts[192]. Moreno-Bueno et al[193] found similar mutations in proline 287, which is required for a number of enzymatic functions. The authors proposed that a combination of the mutations in threonine 286 and proline 287 probably cause conformational changes that would impact on enzyme function.

Bcl2 is an anti apoptotic protein associated with a 14; 18 chromosomal translocation and is primarily a mitochondrial membrane protein. It is thought that increased bcl2 staining correlates with a good prognosis. When the expression of this protein is decreased, programmed cell death is up-regulated and this in turn stimulates proliferation[13]. We have discussed Bcl2 in more details in chapter 1, section 1.3.3.

Ki67 is a recognized indicator of cell mitotic activity and an increased expression of this protein indicates an increased in cell mitotic activity and proliferation. Ki67 expression is

normally elevated in the proliferative phase of the menstrual cycle with glandular epithelium showing the strongest expression. Stromal staining of Ki67 is more apparent in the secretory phase but is still lower than that of the endometrial glands in the proliferative phase[194].

4.2 Hypothesis

Women with polycystic ovary syndrome and EC have tumours with a better prognosis than women with EC who do not have PCOS.

4.3 Aim

The aim of the study was to assess the expression of Cyclin D1, Bcl2, P53 and Ki67 (as surrogate markers of prognosis, in endometrial tumours of women with and without polycystic ovaries).

4.4 Ethics

Ethics approval was granted for the study to be undertaken from July 2002 to July 2003, by the Royal Free & University College Medical School. An extension was subsequently granted for another year.

4.5 Materials and Methods

In the study detailed in Chapter 3, patients with endometrial cancer were selected and assessed the prevalence of polycystic ovarian morphology. 11 patients were identified and the endometrial tumours of the patients were then selected for immunohistochemistry. Controls were patients who had endometrial cancer but did not exhibit pco morphology. Controls were selected if they fell in the same age-range as the subjects.

Immunohistochemistry

Standard immunohistochemical techniques were applied to paraffin-embedded 5 µm endometrial tumour sections and ovary sections (as an appropriate positive control tissue; [165]), mounted on 3-aminopropyltriethoxysilane-coated slides as described in 2.2.4.

The sections were viewed under a light microscope at x100 magnification and scored by two independent observers (OCP, LWTF) as described in 2.2 .5.

4.6 Statistics

Differences in immunostaining were tested by Fishers Exact Test (Cyclin D1, Bcl2, p53) or Student t test (Ki67). $P < 0.05$ was taken as the threshold level of significance.

4.6 Results

Endometrial tumours which stained positively for Cyclin D1 tended to be more prevalent in women with PCO than in those with normal ovaries (4 of 11 [36.4%] *versus* 1 of 16 [6.25%], respectively, p = 0.071; Table 4.1, Figure 4.1). No differences were noted in the prevalence of Bcl2, p53 or Ki67 staining tumours. (In all cases, endometrial tumours from women with PCO or normal ovaries expressed p53 at a moderate to strong level. The prevalence of Bcl2 positive endometrial tumours was also comparable in women with PCO or normal ovaries (*ca.* 75%), as was the Ki67 staining index (*ca.* 35%).

In all cases p53 expression was moderate to strong. Endometrial cancer Bcl-2 expression was similarly unaffected by ovarian morphology with 8 from 11 positive (75%) in women with pco compared with 12 from 16 positive (72.5%) in normal ovaries. Similarly the endometrial tumour Ki67 staining index in women with polycystic ovaries (39% was not significantly different from that in women with normal ovaries (30%).

	subjects	controls
No. tumours tested	11	16
%p53 +ve	82	100
%cyclin D1+ve*	36.4	6.25
%bcl2+ve	75	72.5
%Ki67+ve	39	30

Table 4.1 shows the results obtained in each group, after immunohistochemistry.
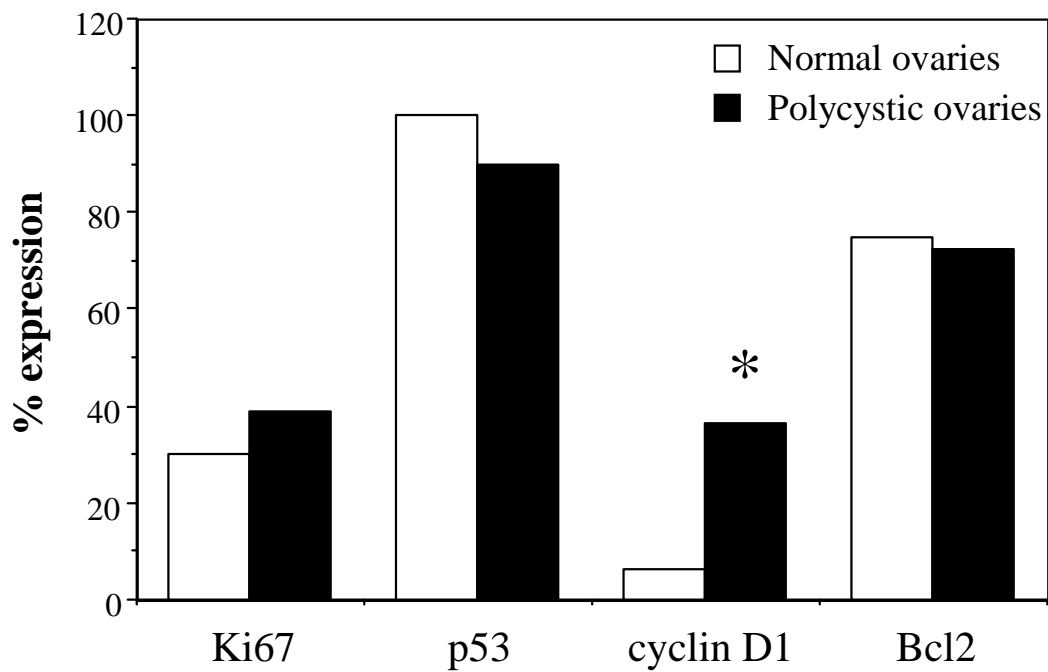
Figure 4.2- Cell cycle (Ki67, cyclin D1) and apoptotic (p53 and Bcl2) protein expression in endometrial tumours from women with normal or polycystic ovaries (*P = 0.071).
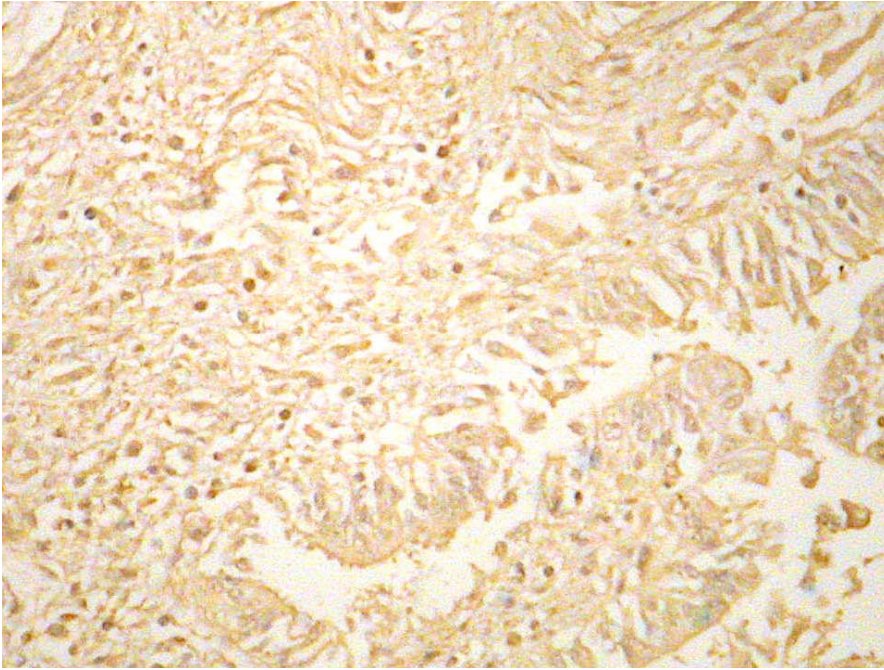
Figure 4.3- Immunohistochemistry showing expression of p53 in the endometrium of women with PCO.
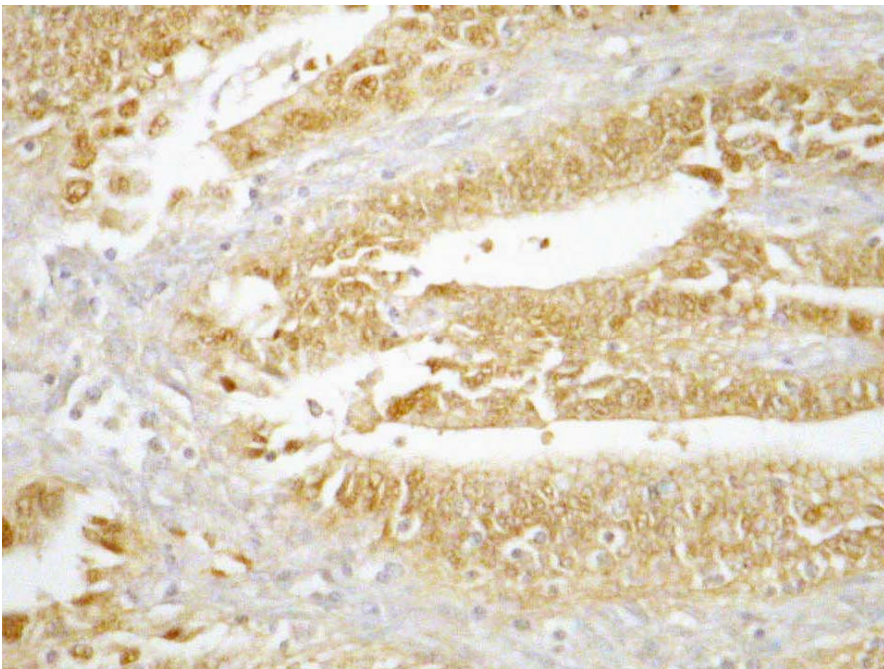
Figure 4.4- Expression of Bcl2 in the endometrium of women with PCO.
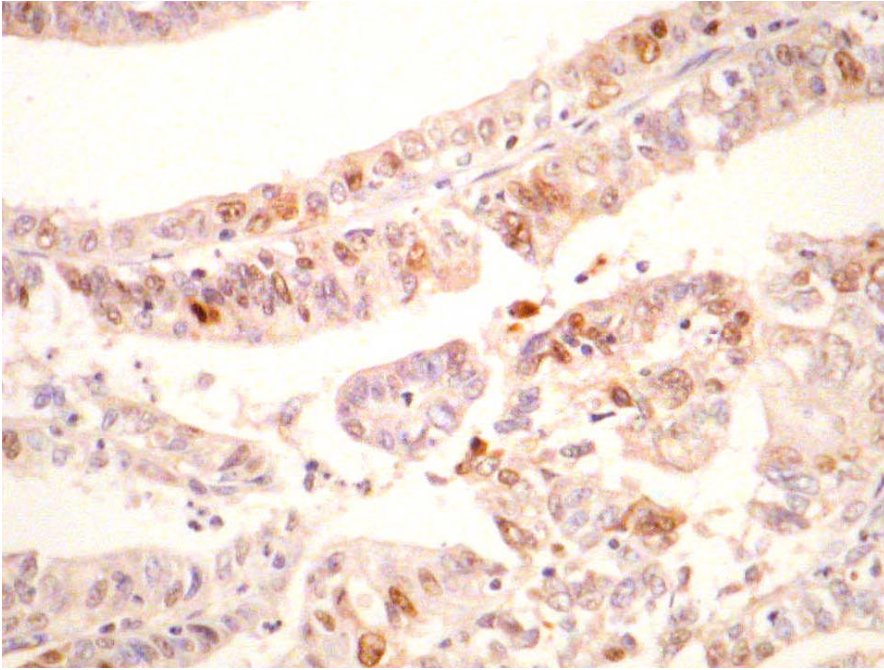
Figure 4.5- Expression of cyclin D1 in the endometrium of women with PCO.
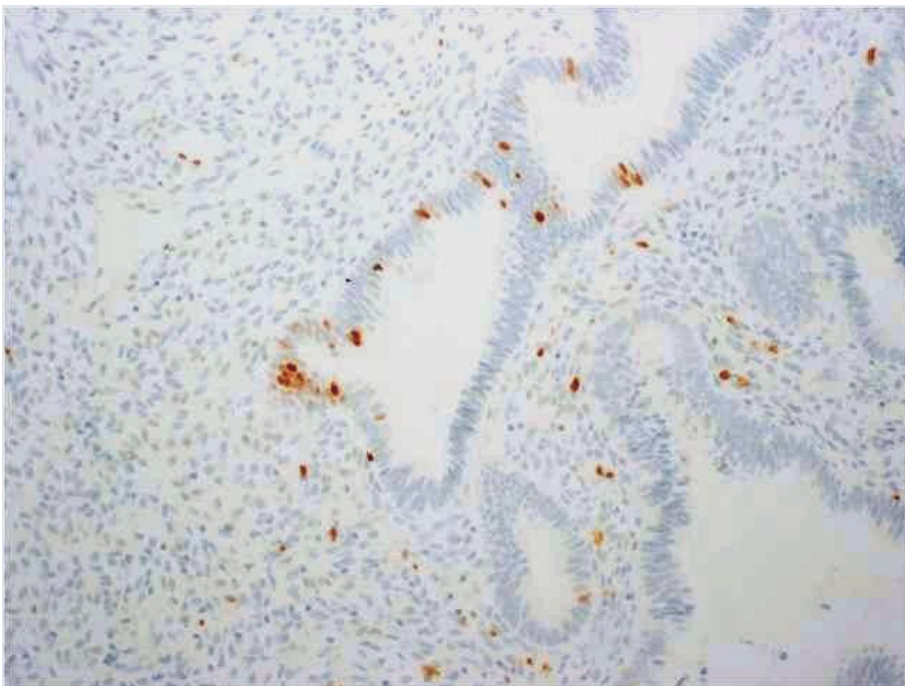
Figure 4.6: Expression of Ki67 in the endometrium of women with PCO.

4.7: Discussion

This study showed that Cyclin D1 expression (a marker of tumour prognosis) was greater in endometrial cancers from women with PCO. However the expression of Bcl2, Ki67 and p53 in endometrial tumours was not different in this subgroup of women when compared to women without PCO.

Our results challenge the belief that endometrial tumours in patients with PCOS are less aggressive and have a better prognosis. The implications of this may involve greater vigilance and aggression in treating PCOS women who develop endometrial cancer.

Although the expression of cell cycle and apoptotic proteins bcl2, p53 and Ki67 in endometrial tumours was unaffected by ovarian morphology, cyclin D1 expression was elevated in endometrial tumours from women with polycystic ovaries. The significance of this change is unclear but it may suggest however that raised cyclin D1 expression is involved in the pathogenesis of endometrial tumours in women with polycystic ovaries. It was previously thought that endometrial carcinogenesis occurs by two pathways, type 1 tumours arising from unopposed oestrogen stimulation on a background of endometrial hyperplasia (discussed in Chapter 1, section 1.6.), while type 2 tumours arose in atrophic endometrium, usually in older patients. Recent studies suggest greater complexity involving perturbation of multiple processes such as ras proto-oncogene expression, microsatellite instability, apoptosis, tumour immortalization (via telomerase activation), tumour suppressor gene expression, proteolysis, adhesion, angiogenesis and clonal

expansion[110]. It may be that patients with PCOS develop endometrial cancer via a pathway that involves cyclin D1, perhaps as a response to the modified endocrine environment in these women.

Cyclin D1 is a cell cycle protein, member of the G1 Cyclin family that regulates the G1/S transition in the cell cycle, whose over-expression may be associated with actual gene amplification or transcriptional dysregulation in cancers[190]. Over-expression in endometrial glands increases progressively in intensity and extent from normal to carcinomatous endometrium[189]. The pattern of over- expression also suggests that this is an early event in carcinogenesis. Oestrogen promotes endometrial growth and progesterone inhibits its proliferation and progesterone will strongly inhibit c-Jun (a member of the activating proteins family AP-1 involved in endometrial growth) recruitment to the cyclin D1 promoter[195]. This modulation of AP-1 activity and cyclin D1 expression via c-Jun may be a potential pathway of progesterone-induced growth inhibition in endometrial cancer cells in women with PCO who have defective progesterone production[196].

One limitation of this study is that the sample size of endometrial cancer available for staining was very small. The limitations of the previous study had an impact on this study. We selected endometrial cancers which were associated with polycystic ovarian morphology, using this a marker of PCOS, however, according to the Rotterdam diagnostic criteria, patients with a history of oligo/anovulation and or clinical or biochemical signs and symptoms of hyperandrogenaemia can also be diagnosed as

having pcos. It was impossible to determine from the patients notes whether they had showed signs and symptoms of pcos due to a lack of documentation at the time by clinicians (some of the patients were seen at a time where the links between pcos and endometrial cancer were not being investigated). This study can be considered as a pilot study for a much larger one as we nowadays have stricter diagnostic criteria for pcos and most cancer cases are treated in tertiary designated cancer centre which will make access to a greater number of endometrial cancer tissue samples.

4.8 Conclusion

This is the first study of its kind in the sense that it directly looks at markers of prognosis in tumours associated with PCO and those not associated with PCO. We found that there was no significant difference in the expressions of p53, ki67and bcl2 in the two groups, Furthermore, cyclin D1 expressing endometrial tumours tended to be more prevalent in women with PCO rather than normal ovaries, albeit this difference was of marginal significance. It may though suggest that cyclin D1 over-expression is involved in the pathogenesis of endometrial tumours in women with PCO. Certainly, these results provide no support for the contention that the prognosis of EC is better in women with PCOS. The significance of this finding is open to many interpretations as the role of cyclin D1 in endometrial carcinogenesis is still unclear. For more than 50 years after an association between PCOS and endometrial carcinoma was first suggested, the nature of this association remains unclear. The hypothesis that women with PCOS tend to develop a better prognosis tumour is not necessarily disproven but on the contrary doubts are being raised about this statement. The results do not directly assess survival with

146

endometrial carcinoma but are indicative of cell-cycle dysregulation when this tumour

changes occur in association with PCO.

Chapter 5

GENE EXPRESSION IN THE ENDOMETRIUM OF WOMEN WITH POLYCYSTIC OVARY SYNDROME

5.1 Introduction

In Chapters 3 and 4, an association between PCOS (using PCO as a marker of PCOS) and EC was not demonstrated, albeit the small number of women with EC who were less than 50 years of age exhibited an increased abundance of PCO morphology relative to age-matched controls. This suggests that a specific group of women with PCOS – those who are pre-menopausal - are at increased risk of developing endometrial cancer.

As discussed previously, there is no single treatment which alleviates the wide range of symptoms associated with PCOS. For women whose main concern is fertility, effective treatment with clomiphene citrate, metformin, ovarian drilling or FSH, is available. Treatment is also available for the cutaneous sequelae of PCOS associated hyperandrogenaemia, such as acne and hirsutism (roaccutane, Spironolactone, laser therapy, vaniqua). To reduce the risks of developing type 2 diabetes and cardiovascular disease, PCOS patients are advised about lifestyle changes such as diet and exercise. There is however, surprisingly little evidence and data regarding the treatment options available for PCOS women suffering from oligo/amenorrhoea, who are concerned about their risk of developing endometrial hyperplasia and carcinoma.

It is likely that unopposed oestrogenic stimulation of the endometrium is an important risk factor for EC in women with PCOS. The observation in Chapter 3 that EC and PCO morphology are associated in women less than 50 years of age (i.e. pre-menopausal) is consistent with this proposal. To investigate this possibility, gene expression was assessed by microarray analysis in endometrium from women with PCOS who

demonstrated either: normal menstrual cycles and proliferative phase endometrium; oligo/amenorrhoea and proliferative endometrium; or oligo/amenorrhoea and endometrial pathology exhibiting both proliferative and hyperplasic characteristics. Endometrial hyperplasia is consider a precancerous lesion predisposing to EC, thus changes in gene expression related to hyperplasia might also be expected to occur in proliferative phase endometrium from women with PCOS who are oligo/amenorrhoeic but not when they are normally cycling.

5.2   Hypothesis

The hypothesis investigated in this study is that in women with PCOS, the endometrium of those who are oligo/amenorrhoeic expresses genes that are related to carcinogenesis and are seen in endometrial hyperplasia, but not in the endometrium of those who are normally cycling.

5.3   Aim

I. To investigate endometrial gene expression in women with PCOS.

II. To investigate which types of histological state of the endometrium of women with PCOS express more genes that are associated with carcinogenesis.

5.4   Ethics

Ethical approval for the study (Project number 6719) was granted by the Royal Free & University College Medical School Ethics Committee in July 2003.   Patients were recruited over a period of twelve months between July 2003 and July 2004.

5.5   Methods

5.5.1 Subject selection

Mr. Paul Hardiman heads weekly infertility and joint gynaecology/endocrinology clinics where patients with PCOS are referred.   All referral letters from General Practitioners were vetted and patients with a history suggestive of PCOS were contacted regarding enrolment into the study.

Chapter 2 describes in detail the selection criteria and the various investigations requested. Strict inclusion criteria were applied to select endometrial samples for microarray investigation.

-All endometrial biopsies exhibited simple hyperplasic(no atypical hyperplasia was found) and/or proliferative morphology

-All included subjects exhibited data for biochemical tests (the tests are discussed in Chapter 2, section 2.3.2, page 96), blood pressure, BMI, age and Ferriman and Galway score within 3 standard deviations from the mean value for these parameters. The mean value for each parameter was calculated for all the patients and only patients, whose data set fell within 3 standard deviations or less from the mean, were included.

Finally, 20 subjects were selected for endometrial microarray analysis on the basis of whether they exhibited normal menstrual cycles and proliferative phase endometrial morphology (n = 10; PS), oligo/amenorrhoea and proliferative phase morphology (n = 5; PL) or oligo/amenorrhoea and hyperplasic morphology on a proliferative phase background (n = 5; H). The twenty subjects selected also had good quality RNA extracted from their endometrial biopsy and this was confirmed by analysis on Agilent RNA analyser. These tissue comparisons were selected on the basis that they best represented the early transition from normal proliferative tissue to an abnormally dividing tissue such as hyperplasia where cells were likely to have been exposed to deranged hormonal environment. Although the normally cycling women were under the influence of insulin and androgen (both known to be to influence gene expression), the influence of these hormones was likely to be less than in the other two groups. This is because the normally cycling women's endometrium would also have been under the influence of progesterone. Hence, the three tissue groups were selected on the basis that all the groups would be under the influence of hormones to varying degree (which in turn will affect gene mutations or silencing in their endometrium). These three groups were also thought to best represent the natural progression of the endometrium when it undergoes changes associated with growth. Group 1- Women with PCOS, regular cycles (< 32 days- PS, these women were having regular cycles 6 month prior to having biopsy and blood tests) and proliferative endometrium- As these women will be cycling, they are expected to be influenced to a lesser extent by increased levels of insulin and androgen and will also be under the influence of progesterone. The genetic mutations/silencing expected will be associated with PCOS itself.

a) Group 1- Women with PCOS, regular cycles (< 32 days - PS, these women were having regular cycles 6 month prior to having biopsy and blood tests) and proliferative endometrium- As these women will be cycling, they are expected to be influenced to a lesser extent by increased levels of insulin and androgen and will also be under the influence of progesterone. The genetic mutations/silencing expected will be associated with PCOS itself.

b) Group 2- Women with PCOS, prolonged cycles (>32 days- referred as PL for long cycles.) and proliferative endometrium- The endometrium of these women will be exposed to the effect of increased levels of insulin and androgen and unopposed oestrogen (lack of progesterone).

c) Group 3- Women with PCOS, irregular cycles (>32 days- H) and hyperplastic endometrium- These women will exhibit the changes in Group 2 (PL group) along with gene expression associated with hyperplasia.

PCOS is a heterogeneous condition where the signs of symptoms may be manifest at one time and very circumspect at other times. When the syndrome starts to express itself in the form of irregular menstrual cycles, the endometrium becomes exposed to various hormonal imbalances which may or may not affect growth.

Comparing gene expression from the endometrium in each group will give valuable information on genetic changes that occur as a result of prolonged oestrogen stimulation. This is thought to be the mechanism by which women with PCOS develop EC; however this study will be looking at this effect at a very early stage at the molecular level-

153

If group a (PS) and b (PL) are compared, we hope to get information about the direct effect of prolonged cycles, androgen and insulin levels on the endometrium.

If group b (PL) and c (H) are compared, this study aims to demonstrate the genetic changes associated with the morphological shift from proliferative endometrium to hyperplasia

Comparing group a (PS) and c (H), we hope to assess the combined effect of both prolonged unopposed oestrogen stimulation, increased androgen and insulin levels and morphological tissue change from a benign to an early neoplastic state.

The endometrial biopsies were snap-frozen and stored at -80°C, then processed as described in Chapter 2, section 2.3.3.1-2.3.3.3, in order to extract RNA for microarray analysis. Extracted RNA was purified and processed to prepare probe for microarray analysis as described in Chapter 2, section 2.3.3.4. The reference RNA applied on all microarrays comprised of pooled endometrial RNA isolated on day 7 of the menstrual cycle from 3 subjects. These subjects were selected on the basis that they had regular menstrual cycles and did not suffer from PCOS and had not used any form of hormonal supplements in the six months before they underwent endometrial biopsy. Sample pooling is often required when RNA is in limited supply and to minimize biological variation. Reference RNA was provided by Dr Andrew Sharkey, Department of Pathology, Cambridge University.

Sample	Age (years)	Days since last period	class	Age at menarche (years)	BMI Kg/m^2	Ferryman and Galway score	Acne?	PCO on scan?	PCOS Diagnosed?
1	24	250	PL	13	26.45	28	y	y	y
2	27	90	H	12	22.86	17	y	y	y
3	23	7	PS	12	23.03	15	y	y	y
4	30	6	PS	11	20.70	Not given	n	y	y
5	28	7	PS	16	16.53	35	y	y	y
6	26	7	PS	12	24.54	7	y	y	y
7	33	90	H	15	20.94	22	y	y	y
8	22	7	PS	11	34.53	15	y	y	y
9	18	18	PL	13	25.10	20	y	y	y
10	31	5	PS	15	22.31	Not given	n	y	y
11	25	7	PS	13	24.97	3.8	y	y	y
12	28	6	PS	10	23.71	20	y	y	y
13	20	30	PL	13	35.28	20	y	y	y
14	35	7	PS	14	21.45	10	y	y	y
15	32	60	PL	11	21.67	15	y	y	y
16	30	250	H	11	24.00	22	y	y	y
17	29	70	PL	12	37.18	20	y	y	y
18	27	7	H	14	19.72	22	y	y	y
19	31	122	H	11	29.14	21	n	y	y
20	34	6	PS	11	35.09	15	y	y	y

Table 5.1 showing patient demographics and timing of biopsy.

Sample	LH (nM)	FSH (nM)	TESTOSTERONE (nM)	SHBG (nM)	FAI	ANDROSTENEDIONE (nM)	PROG (nM)	ESTRADIOL (nM)	INSULIN (nM)	CHOL (mM)	Fasting Glucose (mM)	2 hr glucose (mM)	Histology
1	13.4	5.5	4.4	13	33.8	11	3.1	161	126	6	5.2	6.8	proliferative
2	14.2	4.8	3.3	19	17.4	17.6	2.6	186	111	6.4	3.4	3.6	hyperplasia
3	7.8	5.5	2	29.5	6.8	12.2	2.1		19	5	4.3	5.9	proliferative
4	9.2	6.7	2.7	94	2.9	16	2.6	293	11		4.7	5.8	proliferative
5	13	7.7	2	16	12.5	11.3	3	110	19	5.8	4.6	5.9	proliferative
6	6.1	6	1.7	91	1.9	8	1.2			5.4	4.8	4.4	proliferative
7	10.2	5.4	1.6	40.8	3.9	11.2	2	208	43	5.4	3.7	4.8	hyperplasia
8	5.4	6.1	2.3	27	8.5		3	144	58	5.8	5.2	5.8	proliferative
9	11.7	6.9	2.3	17	13.5	13.2	1.2	159	116	6	4.1	6.5	proliferative
10	18.5	5.8	4	44.2	9	12.5	3		10		3.7	4.6	proliferative
11	7.1	6.4	2.9	49.3	5.9	10.4	2.6	229	23	3.8	5.1	4.8	proliferative
12	7.3	9.7	2.4	34	7.1	10.8	1.4	139	132	3.8	4.9	6.1	proliferative
13	27.8	6.9	3	14.9	20.1	1.3	1.5	446	30	4.1	4.9	6.9	proliferative
14	8.5	4.8	2.3	63.4	3.6	9.4	2	116	17	5.9	4.8	5.1	proliferative
15	16.9	5.3	3.1	29	10.7	11.8	2.1	170	120	5.5	4.6	4.3	proliferative
16	6.4	2.2	1.6	59	2.7	11.6	36.4	563	97	4.4	4.6	4.6	hyperplasia
17	0.4	1.7	1.2	19	6.3	12.2	1.2	180	114	5.6	4.4	6.3	proliferative
18	22	4.6	5.5	59	9.3	13	2.6	317	10	5.4	4.4	5.4	hyperplasia
19	12.2	5.7	3.7	45.5	8.1	15	2.1	214	61	6.6	4.3	5.3	hyperplasia
20	4	9.1	2.1	42.6	4.9		1.8	243	76	4.8			proliferative

Table 5.2- shows all the biochemical profiles and histological report of each patient in the study

	PS n= 10	PL n= 5	H N=5
Progesterone (nM) Mean- 3.875	2.27	1.82	9.14
Testosterone (nM) Mean- 2.705	2.44	2.8	3.14
FAI Mean – 9.45	6.31	16.88	8.28
Insulin (nM) Mean- 62.8	40.6	101.2	64.4
Oestrogen (nM) Mean- 228.1	183	223.2	297.6

Table 5.3- shows the mean values of Progesterone, testosterone, free androgen index, insulin and oestrogen in the three groups studied (short cycle proliferative - PS, long cycle proliferative- PL and the hyperplasia- H).

Because of the non-normality of distribution in T, progesterone, E2 and Insulin, a nonparametric test of significance was utilised. This Kruskall-Wallis test showed that two hormones varied between the subject groups: mean E2 was significantly higher in the hyperplasic (297.6 nM) than in the PL (long cycle) (223.2 nM) or the PS (short cycle) (183 nM) groups (Chi Square = 5.99, df=2, p<.05), and mean insulin was significantly higher in the PL (101.2 nM) than the PS group (40.6 nM) (Chi Square = 6.04, df=2, p<.049).

There was a significant difference in FAI level between long cycling (PL), regular cycling (PS), and hyperplasic (H) patients (F=4.74, df=2, 17, p<.023). Post hoc comparisons using the LSD test show that the long cycling PL patients had significantly higher FAI than both the regular cycling PS (p<.007) and hyperplasic patients (p<.047). However using a Bonferroni post-hoc test the difference remained significant only between the long cycling and regular cycling patients (p<.022).

5.5.2 Choice and preparation of microarray

The HMN2 microarrays used in this study were produced by the Center for Microarray Resources at the Department of Pathology, Cambridge University (Tennis Court Road, Cambridge, CB2 1QP, UK). The composition of the arrays makes them an invaluable tool for the analysis of 'novel' gene involvement in endometrial function. Each array is constructed from ~1500 Mammalian Gene Collection full-length cDNAs, ~1000 hand-selected clones of angiogenesis related genes and ~5000 Soares Normalised Human Testis library clones. The ~7500 clones, together with a collection of control clones, are available duplicated as a ~18,000 feature array built on the Corning GAPSII surface. 900 genes are known to be specifically involved in endometrial function, such as apoptosis and extracellular matrix remodelling, as well as house-keeping genes. More information about the arrays can be obtained at

http://www.path.cam.ac.uk/resources/microarray/microarrays/.

These arrays were chosen because they have been used in the Cambridge Laboratories to generate good results. They also contained genes that are known to be activated during proliferation processes such as carcinogenesis such as apoptosis, cell-matrix remodelling, growth and cell repair. The microarrays were hybridized to probe cDNA in a manner similar to that used in Southern blotting [197].

Array generation

The arrays were generated by Dr A. Brown and his team in the Molecular Biology Department, Cambridge University using the following method: The arrays were made by first generating PCR-amplified cDNAs which were then suspended in 150 mM $NaPO_4$, (pH 8.5)/0.001% SDS. These were spotted onto bar-coded CMT-GAPSII surfaces (Corning). The spotter used was a Biorobotics MicroGrid (Genomic Solutions Ltd), using microspot 2500 pins (Genomic Solutions Ltd). After spotting was completed, the slides were baked at 80°C for 2 hours, and then washed in 1% BSA/3x SSC/0.1% SDS at 55°C for 20 minutes. The slides were subsequently incubated in boiling MilliQ purified water for 2 minutes to denature the cDNAs; washed for 5 minutes in MilliQ purified water; rinsed in propan-2-ol and finally dried by centrifugation.

RNA preparation

Chapter 2, section 2.3.3.1-2.3.3.5, pages 98-103, describes in great detail the method of RNA extraction.

Good labelling and optimal hybridization of probe material is dependent upon high quality RNA. Several issues need to be considered from the moment that tissue is collected to facilitate the preparation of high quality RNA. In this study, tissue was snap-frozen in dry ice within seconds of collection, minimizing RNA degradation. Labelling of degraded or contaminated RNA will invariably produce misleading results.

The amount of tissue available was limited; hence a method of RNA extraction which would produce high yields was chosen. Of a variety of method tested, QIAZOL (Qiagen, Hilden, Germany) produced a good RNA yield with acceptable levels of purity. The RNA obtained was then purified by column purification using the Rneasy Minikit (Qiagen, Hilden, Germany).

The RNA yields were determined by spectophotometry at 280 and 260 nm. The integrity and purity were then determined by using an Agilent Technologies 2100 Bioanalyser (Agilent technologies UK limited, Cheshire, UK) (as described in Chapter 2, section 2.3.3.8).

Production of labelled cDNA from RNA samples

Reverse Transcription is used to produce cDNA from total cellular RNA. To ensure multiple arrays could be performed if required, and to use a methodology which provides unparalleled sensitivity for genes expressed at low levels, it was decided to use the SMART PCR cDNA synthesis kit (Clontech, St Germain-en-Lahaye). cDNA samples were then subjected to Cy3-dCTP and Cy5-dCTP cDNA labelling to produce probe for

use in microarray analysis. (Details of the procedure are outlined in Chapter2, section2.3.4, page 102)

Hybridization

Hybridisation buffer (40% v/v deionised formamide; 5x SSC; 5x Denhardt's solution; 1 mM sodium pyrophosphate; 50 mM Tris-HCl (pH 7.4); 0.1% w/v sodium dodecyl sulphate) was used and this has been used repeatedly in Cambridge with good results. Hybridisation was allowed to occur in a water-bath at 50°C for 16-18 hours. Optimal hybridization conditions ensured that the maximum microarray sensitivity and data of high quality are achieved. It also allows even very low abundance genes to be detected and differential expression determined.

Background agents such as Cot-1 DNA and polyadenylic acid are used to minimize background. (Details outlined in Chapter 2, section 2.3.5.2, page 108)

Data normalization and analysis

 After image acquisition and conversion of the image into spot intensity, the large volume of data obtained were stored onto a spreadsheet for further analysis.

Before analysis took place the, the raw intensity data was log transformed and any values attributed to background were eliminated. A scatter plot was then created to further identify spot artefacts and display the effects of normalization on the data.

When extracting biologically relevant data from gene arrays, the main problem is making comparisons between arrays. Numerous factors influence the signal generated for any

particular spot; for example, the quality of the specimen (protein contamination, RNA degradation), arrays quality and image processing (efficiently quantifying spot signals and background correction). Finally there is variation between samples which may be attributed to reverse transcription efficiency, hybridization method efficiency and label incorporation into probe cDNA.
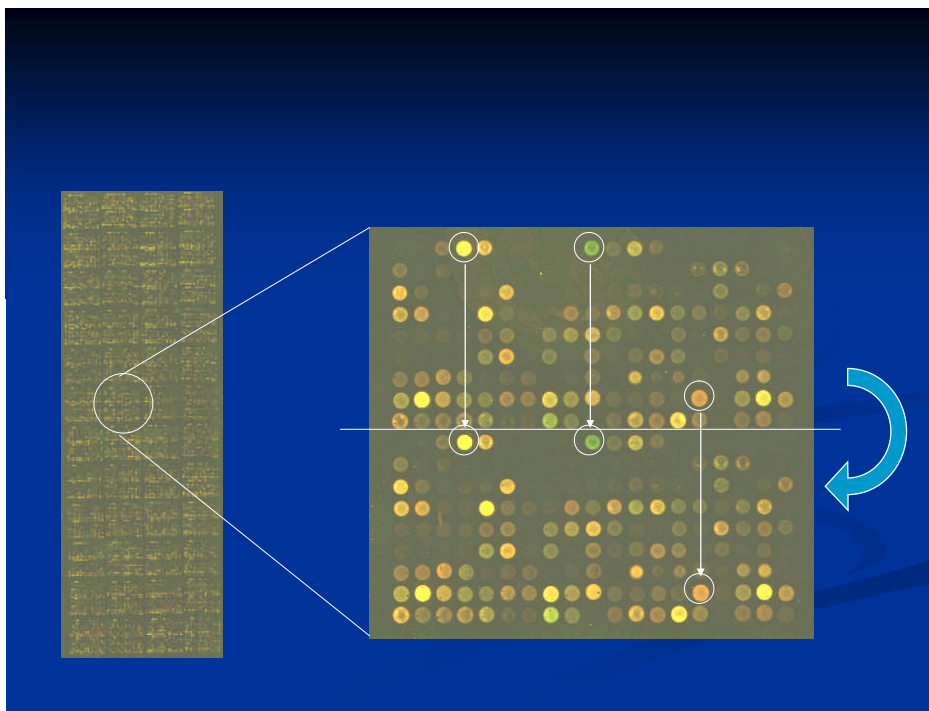

Figure 5.4 shows a hybridized and labelled microarray chip as seen through an image analyzer. A green spot represents an over-expressed gene whilst the red spot represent an under-expressed gene, relative to the control endometrial RNA run in parallel with each experimental sample on each chip.

In this study, some mathematical adjustments were made to the datasets to allow meaningful comparisons between different samples on different arrays. This is termed normalisation and the method used in this study for analysis was the internal reference method, where a constant internal standard was used on all arrays and a set of predetermined genes (housekeeping genes) which were expected to give consistent signal intensities between different samples, was used[26]. Normalisation is explained in greater depth in Chapter 2, section 2.3.7, page 112.

In the majority of microarray experiments, the expression of only a few genes will be altered by the condition under investigation; whilst the rest of the transcripts probed by the array should be unchanged. When plotting an intensity graph of signal generated by internal standard against signal generated by experimental sample at each spot on an array, ideally, all constantly expressed genes should fall on the diagonal line y = x.

The aim of any microarray experiment is usually to identify differentially expressed genes between two sets of samples treated in two different ways , with a measure of statistical significance[26]. The most common statistical testing used is the paired t-test (where there is only one categorical factor, but an anova test is used when a number of categorical factors). The t-test works on the null hypothesis of equal mean expression levels between the two samples (controls and reference). Various other tests may be applied. In this study, Genespring analysis was also used.

5.6 <u>Statistical analysis using t-testing:</u>

The mean intensity for the duplicate spot for each gene, Chapter 2 section 2.3.8, page 115, details the preliminary handling of data. After Loess correction and screening for genes expressed at low level, microarray expression data for 3975 genes was investigated by cluster analysis with a Spearman correlation (Figure 1). Cluster analysis tended to group the endometrial gene expression patterns from normally cycling women (PS samples) together, and the patterns from oligomenorrhoeic women with (H samples) and without (PL samples) hyperplasia together, albeit there were deviations from this pattern. At a simple level this suggested endometrial gene expression varied with menstrual cyclicity, however good group definition could not be obtained probably because the effects of the relatively few differentially expressed genes were masked by the many genes which were not differentially expressed. (Chapter 2, section 2.3.7, 112)

Primary fluorescent intensity data was analysed using the software package Genespring which has been specifically designed for analysis of microarray data.

A Spearman correlation (non-parametric data) was performed between hormones and gene

5.7 Results

After Genespring analysis of the normalized data, a total of 100 genes showed significant gene expression changes between the various tissue types:

15 genes were altered in expression when comparing the hyperplastic to the long proliferative endometrial samples. (H versus PL; Venn diagram)-Comparison 1

75 genes were found to be differentially expressed when comparing the long proliferative to the short proliferative endometrium. (PL versus PS; Venn diagram)-Comparison 2

63 genes were found to be differentially expressed when comparing the hyperplastic to the short proliferative endometrium. (H versus PS; Venn diagram)- Comparison 3

5 genes were differentially expressed in comparison 1 and not in comparison 2 or 3.

23 genes were found to be altered only in comparison 2 and not in 1 or 3.

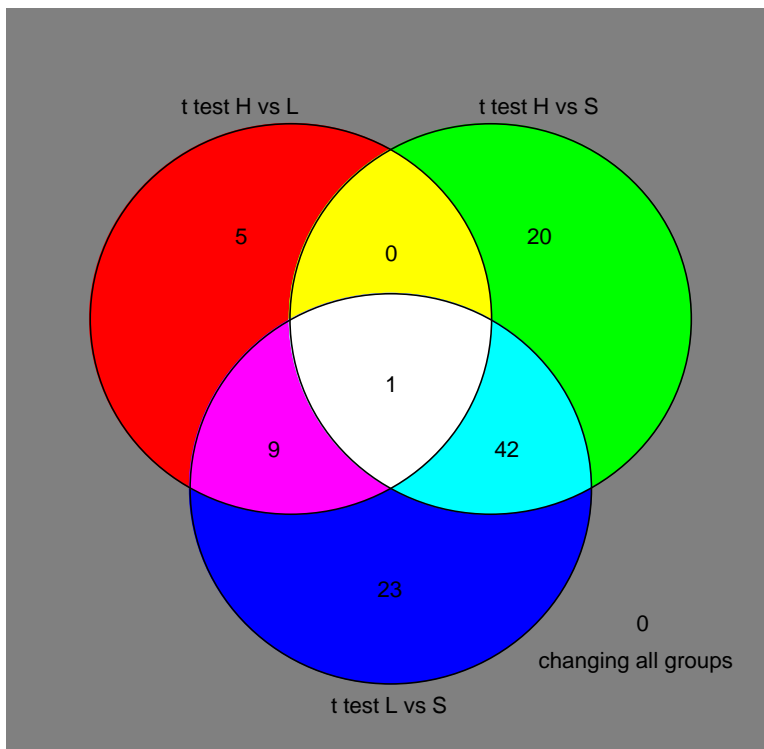20 genes were found to be altered in only comparison 3 and not in 1 or 2

Figure 5.5- A Venn diagram showing the three tissue comparisons and the altered gene distribution between the three groups. (Please refer to table in CD provided with file name PCO gene list to see gene name and expression level)

White- All 3 tissue-type comparisons- only 1 gene was common.

Yellow- genes variably expressed in the H versus PL and H versus PS comparison.

Purple- genes expressed variably H versus PL and PL versus PS comparison.

Turquoise- genes expressed variably PL versus PS and H versus PS comparison

Red- genes variably expressed only in the H versus PL comparison.

Green- genes variably expressed only in the H versus PS comparison

Blue- genes variably expressed only in the PL versus PS comparison
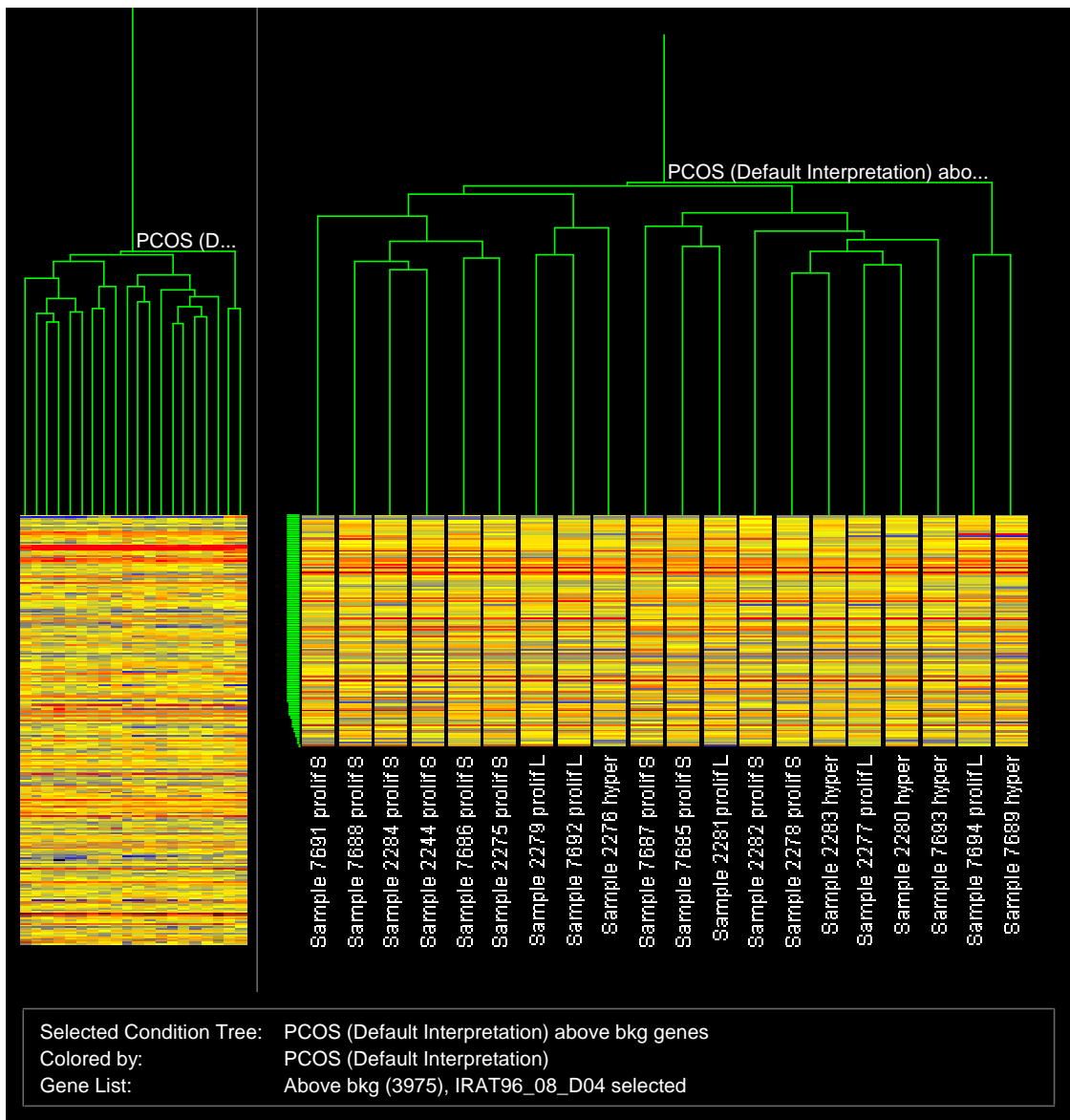
Figure 5.6- A Spearman correlation test was used to a correlate gene expression to biochemical markers and cycle length and a Bonferroni correction was also used where data was skewed. As can be seen this does a good job in dividing the samples into three

groups, there are some PS with PL, but the H have grouped as a four with one outlier but even this is more similar to the hyper than the PL or PS.

Figure 5.7- This second correlation tree was obtained after further sieving out genes which were unqualified.

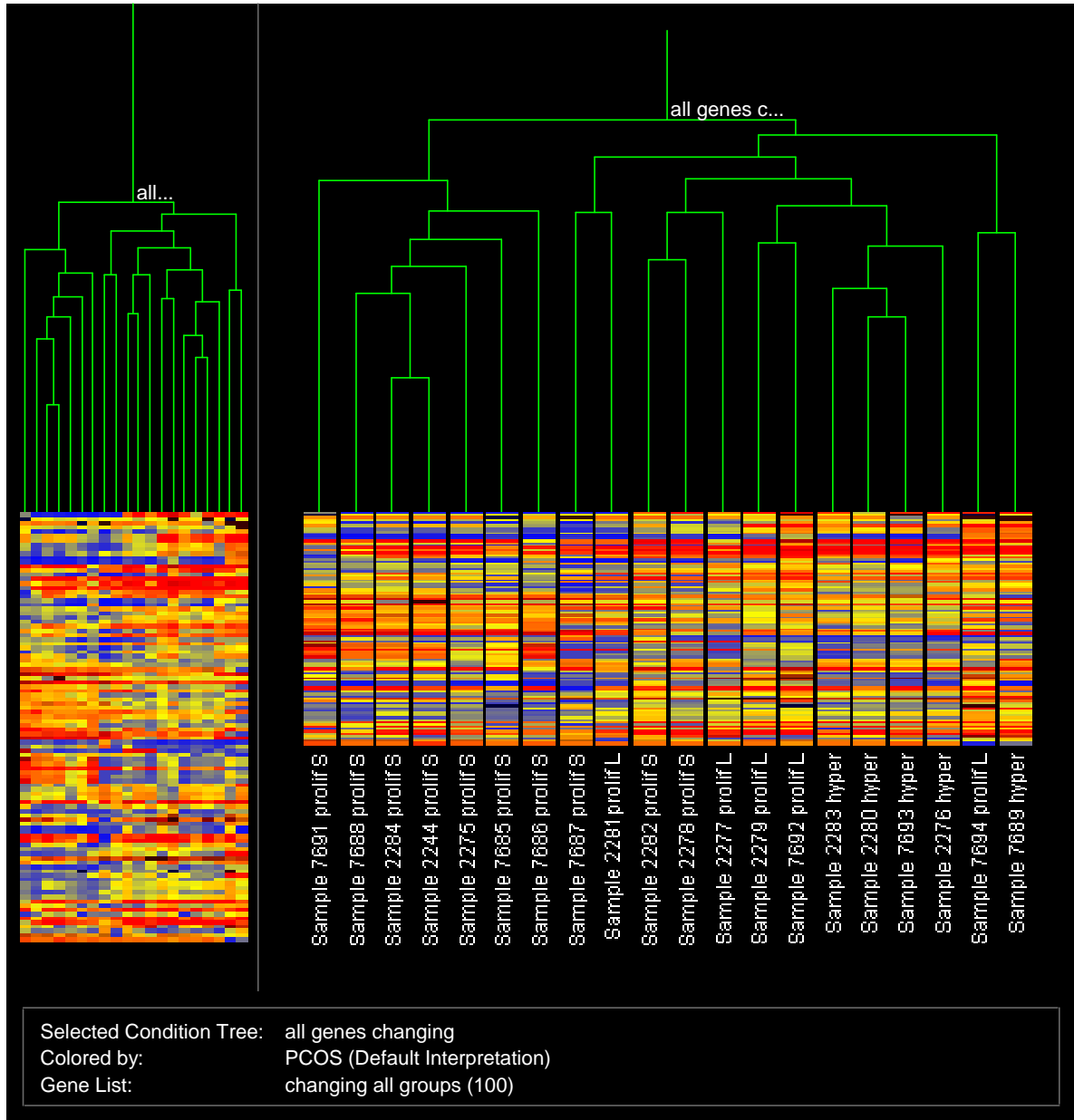| GENE	Accession Number	Expression ratio with p value by t-test			
H/PL		**H/PL**	**'p'**	**H/PS**	**'p'**
TPX2, microtubule-associated protein homolog (Xenopus laevis)	AI024647	**0.488**	**0.066**	0.657	0.138
Discs, large homolog 7 (Drosophila)	BC010658	**0.503**	**0.085**	0.636	0.158
Immunoglobulin lambda constant 1 (Mcg marker)	BC012876	**2.543**	**0.070**		
H/PS		**H/PL**	**'p'**	**H/PS**	**'p'**
Human placental protein 14 (PP14) gene, complete cds.	M34046	0.167	0.391	**0.052**	**0.012**
Glutathione peroxidase 3 (plasma)	AI040990	0.404	0.281	**0.224**	**0.053**
IGFBP 3	AI032308	0.685	0.581	**0.320**	**0.040**
IGFBP 3	NM_000598	0.668	0.545	**0.336**	**0.041**
H19, imprinted maternally expressed untranslated mRNA	BC006831	0.714	0.393	**0.519**	**0.034**
Homo sapiens BAC clone RP11-20N16 from 2, complete sequence.	AC009469	0.705	0.407	**0.552**	**0.040**
NDRG family member 2	BC011240	1.307	0.390	**1.770**	**0.056**
NDRG family member 2	BC010458	1.289	0.405	**1.850**	**0.029**
IGFBP 5	BC011453	1.437	0.362	**1.929**	**0.022**
IGFBP 5	L27560	1.516	0.249	**2.054**	**0.009**
Hypothetical protein LOC128344	AI041071	1.188	0.639	**2.213**	**0.048**
Hypothetical protein MGC35194	AI027239	1.455	0.164	**2.411**	**0.024**
PL/PS		**PL/PS**	**'p'**	**H/PS**	**'p'**
Osteopontin, bone sialoprotein I	X13694	**0.285**	**0.071**	*3.531*	*0.181*
Osteopontin, bone sialoprotein I	J04765	**0.297**	**0.073**	*3.500*	*0.186*
Integral membrane protein 2A	AI028165	**0.336**	**0.024**	*1.773*	*0.043*
Chemokine (C-C motif) ligand 3	M23452	**0.353**	**0.051**	1.314	0.157
Hypothetical protein DKFZp761D221	AI041262	**0.362**	**0.060**	1.171	0.705
Apolipoprotein C-I	BC009698	**0.416**	**0.038**	1.554	0.218
ov39c10.x1 IMAGE:1639698 3', mRNA sequence.	AI024974	**0.497**	**0.032**	1.424	0.123
Major histocompatibilty complex, class II, DQ alpha 1	BC008585	**0.554**	**0.046**	1.680	0.179
Deiodinase, iodothyronine, type II	U53506	**1.846**	**0.019**	0.798	0.495
Glycoprotein hormones, alpha polypeptide	V00518	**3.387**	**0.075**	0.491	0.374
PL/PS; H/PS		**PL/PS**	**'p'**	**H/PS**	**'p'**
ov40b04.x1 STN_Hs_cDNA IMAGE:1639759 3' similar to gb:X12451 Cathepsin L precursor mRNA	AI025084	**0.490**	**0.031**	**0.533**	**0.044**
Cathepsin L	NM_001912	**0.512**	**0.021**	**0.544**	**0.027**
ov60b08.x1 IMAGE:1641687 3' similar to gb:X12451 Cathepsin L precursor mRNA.	AI024426	**0.495**	**0.018**	**0.558**	**0.031**
TYRO protein tyrosine kinase binding protein	BC011175	**0.473**	**0.019**	**0.591**	**0.068**

TGF beta 3	J03241	**0.447**	**0.005**	**0.602**	**0.027**
KIAA1210 protein	AI024396	**1.985**	**0.010**	**2.079**	**0.000**
Ras homolog gene family, member U	AI018035	**1.997**	**0.002**	**2.080**	**0.021**
Extracellular matrix protein 1	AI066584	**2.076**	**0.009**	**2.368**	**0.011**
Human prostaglandin D synthase precursor, mRNA, complete cds.	M61900	**2.111**	**0.028**	**3.161**	**0.031**
PL/PS; H/PL		**PL/PS**	**'p'**	**H/PL**	**'p'**
ov90a05.x1 IMAGE:1644560 3' similar to TR:Q61500 Integral membrane protein 2 mRNA.	AI028165	**0.34**	**0.024**	**1.77**	**0.043**

Table 5.8: Genes differently expressed between proliferative endometrium from normally cycling women with PCOS (PS) and proliferative (PL) and hyperplasic (H) endometrium from oligomenorrhoeic women with PCOS. .

A Spearman correlation (non-parametric data) was performed between hormones and genes. There was a positive correlation between Insulin and Integral membrane protein 2A (N=19, p<.000, 2-tailed).

There was a positive correlation between Insulin and Transforming growth factor, beta 3 (N=19, p<.001, 2-tailed).

There was a negative correlation between LH and Insulin-like growth factor (N=20, p<.003, 2-tailed).

After Bonferroni correction, the other correlations were non-significant.

Average cycle length

Average cycle length was positively correlated with Major Histocompatibilty Complex, class II, DQ alpha (r=.511, n=20, p<.021, 2-tailed).

5.8　Data validation by Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR does not detect the size the size of the amplicon and thus does not allow differentiation between DNA and cDNA. Real-time PCR quantification eliminates post-PCR processing of PCR products. This helps to increase yield and reduce the chances of carryover contamination. It also offers a wider dynamic range, which means that a wide range of ratios of target and normaliser can be assayed with equal sensitivity and specificity. (Dynamic range of an assay determines how much target concentration can vary and still be quantified.)

Real-time PCR is based on the detection and quantitation of a fluorescent reporter which is released from a primer only when that primer molecule is used to extend cDNA in amplification cycle. This signal increases proportional to the amount of PCR products in the reaction. The higher the starting copy of the nucleic acid target, the sooner a significant increase in fluorescence is noted. (After ~3-15 cycles). A fixed fluorescence threshold is set significantly above the baseline on the real-time cycler detector. The parameter Ct (threshold cycle) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold. Ct is related by a known mathematical formula to the starting concentration of target templates.

Real-time PCR quantitation eliminates post-PCR processing of PCR products as signal is only generated when an amplification cycle is initiated from a target specific primer. This

helps to increase yield and reduce the chances of carryover contamination. Real-time PCR also offers a wider dynamic range, *i.e.* a wider range of ratios of target and normaliser can be assayed with equal sensitivity and specificity. (Dynamic range of an assay determines how much target concentration can vary and still be quantified.)

There are three main fluorescence-monitoring systems for DNA amplification-1) hydrolysis probes 2) hybridizing probes and 3) DNA-binding agents. Hydrolysis probes include TaqMan probes as used in this study. TaqMan uses the fluorogenic 5'exonuclease activity of Taq polymerase to release a fluorophore from a target specific primer during amplification, to allow measurement of the amount of target sequences in cDNA samples[198]. TaqMan was chosen as it has a greater specificity to annealing to PCR products. TaqMan probes are oligonucleotides longer than primers (20-30 bases long) that contain a fluorescent dye usually at the 5' end and a quenching dye at the 3' end. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing. There is no fluorescence if the probe is intact; however when the polymerase replicates a template to which the TaqMan probed is annealed, its 5' exonuclease activity cleaves the fluorescent probe, which separated from the quenching dye, fluoresces. Accumulation of PCR products is detected by monitoring the increase in fluorescence as the fluorescent probes are released by Taq polymerase when it extends from a primer. As cleavage occurs only if the probe hybridizes to the target and is extended from the same target by Taq polymerase, the origin of the detected fluorescence is measured in specific amplification.

To evaluate the microarray data, 4 genes were selected which were differentially expressed, these being OPN (osteopontin), DLG 7 (Disc-large homologue 7), APSRG-1 (asiologlycoprotein-1), CDK8 (cell division kinase). OPN and DLG 7 were selected because of their high signal intensity in the microarray whilst APSRG-1 and CDK 8 had signal intensity below the Cy5 signal intensity threshold applied. Thus only OPN and DLG7 expression levels, as assessed by microarray, would be expected to be comparable when assessed by real-time RT-PCR.

The real-time RT-PCR data replicates the findings obtained by microarray for the high intensity genes OPN and DLG-7. OPN expression was approximately 3-fold greater in the PS and H tissue compared to PL, when assessed by microarray. This pattern of expression was replicated when assessed by real-time RT-PCR, as shown in figure y. For DLG-7, the pattern of expression is not dissimilar in the three tissue type and although the expression is higher in PL this is very marginally different.
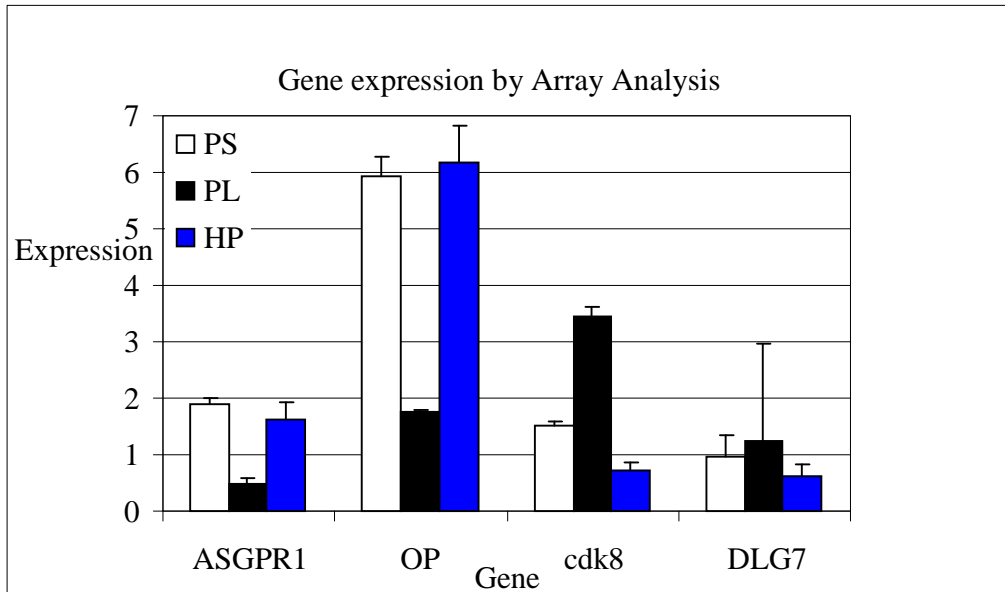
Figure 5.9 shows mean transcript expression levels in each tissue type, relative to the internal control sample, as estimated by microarray analysis.
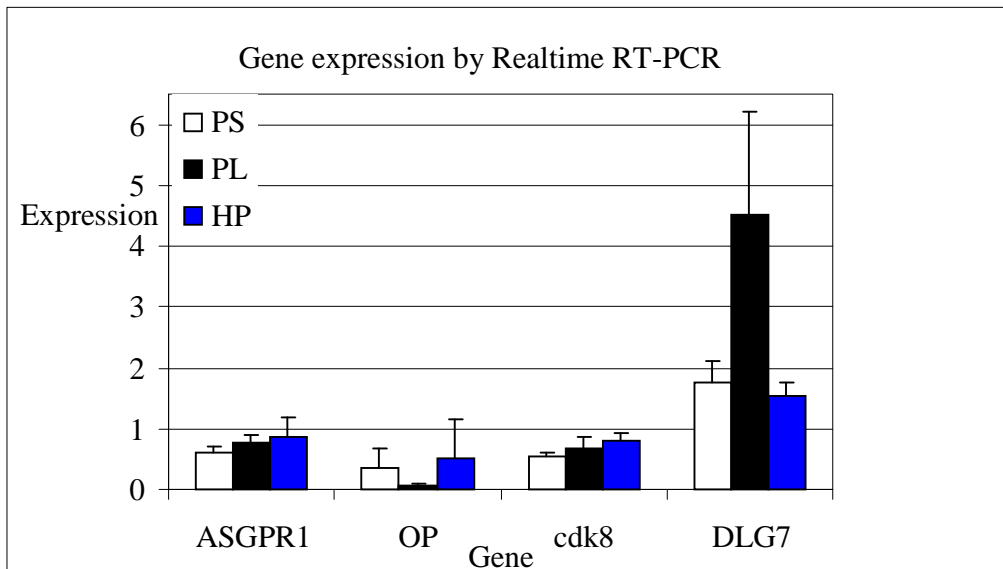

Figure 5.10 shows mean transcript expression levels in each tissue type, relative to a standard endometrial cDNA sample, as estimated by real-time RT-PCR analysis.

5.9 Discussion

In the normal menstrual cycle, endometrium stays in the proliferative state for the first 14 days, whereas in patients with cycles longer than 28 days, the endometrium remains under the influence of oestrogen in the proliferative state for considerably longer than 14 days. Finally the hyperplastic endometrium is the result of the endometrium remaining under the influence of oestrogen for a prolonged period and having morphological changes indicative of a precancerous state. This discussion will focus on variant gene expression when the three endometrial types are compared and attention will be paid to genes that are known to play a role in carcinogenesis.

These specific tissue comparisons (H versus PL, H versus S, PL versus S), give us important information on gene expression changes as the endometrium changes from a normal proliferative type to a hyperplastic one, in women with PCOS. The short proliferative tissue biopsies were from women who had normal 28-32 days menstrual cycles and the long proliferative tissues were from women who had oligomenorrhoea. The hyperplastic tissues were taken from women who were suffering both from oligomenorrhoea and their endometrium had also started to undergo neoplastic transformation.

As shown by the Venn diagram in page 172 when comparing H versus PL and PL versus PS, there 26 variant genes expressed. The difference between these tissue type lies in the fact that in the PL versus PS comparison (23 genes, see table xx where only qualified genes have been tabulated), page175 , the gene expression may be altered because of the

175

prolonged exposure to hormones such as oestrogen, insulin, and androgen whereas in the H versus PL (5 genes) comparison the variation in gene expression may be accounted for by the prolonged exposure to insulin and sex steroid in addition to neoplastic transformation or altered growth. In the H versus S tissue comparison and PL versus PS tissue comparison, the genes variably expressed only in the first tissue comparison (20 genes), may be a direct result of altered growth in the endometrium and may be independent of hormonal exposure.

This discussion will concentrate mainly on the proteins that are known to have a role in carcinogenesis and all the other various processes associated with it. One such protein is **Osteopontin**. This protein was found to be variably expressed in all tissue comparison. This raises the possibility that the alteration in the expression of osteopontin must be an early occurrence as the endometrium starts to be affected by the altered hormonal environment present in PCOS patients as they become oligo/amenorrhoeic. The expression ratio was higher in the H versus PS (expression ratio: 3.531) than in the PL versus PS (expression ratio: 0.285) comparison, raising the possibility that this may be hormone independent. **Osteopontin** is a secreted non-collagenous, sialic-acid rich, chemokine-like protein playing an important role in determining the oncogenic potential of various cancers[199]. Overexpression of OPN results in an increase in the malignant potential. OPN is thought to exert its pro-metastatic effects by interacting with various Integrins and CD44 receptors regulating the cell signalling events that ultimately lead to tumour progression[200]. OPN regulates the bioavailability of MMP's (matrix metalloproteinases) and potentially provides the molecular link between degradation of

176

extracellular matrix, tumour progression, and vascularisation. There is evidence to indicate that OPN might be considered as a candidate target for the treatment of cancer[201]. It is being hail as a prognostic marker in various cancers such as breast, prostate and melanoma. OPN is also thought to promote cancer progression by regulating the tumour-surveillance mechanism and inhibiting apoptosis of neoplastic cells [202] and is thought to do so via antiapoptotic protein Bcl-xl[203]. One of the key steps involved in the process of tumorigenesis and metastasis is the degradation of the basement membrane and interstitial matrix which is dependent on the activity of MMPs and there has been evidence to demonstrate that OPN can induce MMP activity in tumour cells[204]. Finally OPN is known to belong to a family of proteins collectively known as SIBLING. Their mode of action is via the regulation of MMP activity and they are known to be over expressed in several cancer cells and their abundance directly correlates with tumour grade[202].

Matrix metalloproteinase-3 (MMP-3) is a member of a family of proteolytic enzymes whose function include degrading constituents of the extra-cellular matrix surrounding some carcinomas[205] and some regulatory genes such as cytokines, growth factors, cell-surface receptors and adhesion molecules. They are classified in 4 groups: 1) interstitial collageneases 2) gelatinases, 3) stromelysins (MMP-3, belonging to this group) and 4) membrane type. Although MMP's are expressed by tissue at various stage of development, they are typically absent from cells of the adult organism. Furthermore they have been implicated in the establishment growth, invasion and/or metastasis of tumours owing to the high frequency with which they of are found in invasive

tumours[206]. MMP activity is regulated at least at 3 levels: transcription/translation, proteolytic activation of the zymogen and inhibition of the active enzyme by up-regulation of each of these associated with pathological events. MMP3 has broad substrate specificity, degrading type IV collagen, laminin, fibronectin and proteoglycans. MMP3 is expressed in areas of tissue growth[207], focally expressed around invasive cells in the stromal component of breast tumours and expressed in both benign and malignant breast cancer phenotypes[208].

MHC class-II- Tumour formation in immunocompetent hosts is believed to be dependent on the ability of tumour cells to evade the immune system. This is reflected by the alterations in the expression of the major histocompatibilty complex (MHC) and related molecules in a number of cancers. Tumour cells are thought to exhibit reduced surface expression of MHC class-II proteins thus reducing immunogenicity and favouring tumour growth. MHC class-II proteins will associate with peptides, which are derived from antigens, for presentation to T4 lymphocytes. CD4 molecules have been shown to interact with MHC class-II molecules, leading to enhanced responses of T4 cells. This balance seems to be affected in endometrial cancer, as in many other cancers. In endometrial stromal cells (antigen presenting cells), high expressions of MHC class-II molecules may be used as an as a lymphocyte activation marker in endometrial carcinoma and this seems to be an early event in invasive cancers[209].

Disc-large homologue 7 (drosophila- dlg 7)- this is not a well-characterised gene but it is known to be a novel-cell-cycle regulated gene and is a homologue of the drosophila

melanogaster disc large -1.The latter is known to be a tumour suppressor gene[210]. It has also independently been shown to be over expressed in hepatocellular carcinoma[211] and transitional cell carcinoma of the bladder[212]. The function of dlg7 in mammalian cells is currently unknown but in the mouse, its expression is tightly regulated during cell cycle [213]. It is closely associated with Ubiquitin to which it binds and then becomes available for proteolysis. It has recently been associated with Aurora-A which is expressed in many human tumours. It is also postulated to have a function in cell-differentiation in haemopoietic stem cells[214]. It is also thought to be involved in cell differentiation (especially haemopoietic stem cells).

Transforming growth factor beta 3- this cytokine is both a tumour suppressor (at early stages) and a tumour promoter (at later stages) in carcinogenesis. TGF beta acts by binding to intracellular type I and type II receptors, and these in turn cause the phosphorylation of Smad receptors. This initiates a chain of reaction, involving gene expression, with end results anti-proliferation and apoptosis. There is evidence that the TGF beta/ smad pathway is responsible for early tumour suppression, however in late stage tumours, this suppressive role is reversed into a promoting one where there is stimulation of invasion and metastasis. Moreover it has also been shown that in tumours like breast cancer, TGF beta, favours metastasis to bone and it has been suggested that this protein should be targeted for therapy to reduce metastatic bone disease in breast carcinoma. [215]

Integral membrane protein 2A (IMP2A) - This cell-membrane protein is known to be involved in cell proliferation and cell destruction. In some tumours, e.g. Burkitt Lymphoma, the absence of integral membrane protein 2A, will lead to tumour cells being recognized and destroyed by natural killer and cytotoxic T-cells. In their presence or over-expression of IMP2A the reverse is observed, i.e. a slowdown in apoptosis. IMP2A has also been found to inhibit transforming growth factor B 1- induced apoptosis through phosphatidylinositol 3-K/Akt pathway[216].

Insulin-like growth factor binding protein 3- Insulin growth factors (IGF) are known to increase the risk for the development of certain tumours whilst the reverse is true for insulin-like growth factor binding protein-3 (IGFB-3)[217,218]. IGFB-3 is known to suppress the mitogenic activity of IGF-1. IGFBP-3, like IGF-1, is a protease which is manufactured in almost every cell of the body and is found mainly in extracellular fluid or on the cell surface. IGFB-3 determines the bioavailability of IGF as it bounds 99% of the latter[219]. IGF expression is enhanced by oestrogen and they are known to increase cell proliferation and apoptosis. A recent study by Oh et al ,looking at IGF-1, IGF-2 and IGFBP-3 and their relationship with endometrial cancer risk, found that increased levels of IGF-2 was associated with an increase risk of obesity and endometrial cancer whilst a lower level of IGFBP-3 was directly associated with an increased risk of endometrial cancer[220].

BCL2-related protein A1- Bcl2A1 is a member of the anti-apoptotic Bcl2 family whose mechanism of action is through the inhibition of mitochondrial membrane release of

cytochrome c. Bcl2 A1 was first identified as a tissue specific Bcl-2 related factor which was induced during myeloid cell differentiation[221]. It has later been found to inhibit apoptosis triggered by various factors such as anti-cancer drugs or antigen receptor ligation. Bcl2, more specifically, exerts an anti-apoptotic action by inhibiting mitochondrial depolarization and release of cytochrome c by sequestering Bid (an apoptotic protein) and preventing the latter's association with Bax[222].

Human placental protein 14 (pp14) - This protein is also known as Glycodelin A, which is secreted in by human endometrium in the late secretory phase of the menstrual cycle and in the early phase of pregnancy under the regulation of progesterone[223]. It is known to induce immunosuppression[224] and has the ability to induce apoptosis via T-cells[225]. The role of pp14 is better characterised in the regulation of implantation however it has also been implicated in endometrial carcinoma. Pp14 levels are higher in the endometrial flushings of women with endometrial cancer. It was even proposed as a potential marker of endometrial cancer.

As shown in table 5.3, there is a significant increase of FAI in patients who are oligomenorrhoeic as compared to the regular cycle and hyperplasia patients. The altered hormonal environment has been postulated to be the reason for a decreased endometrial receptivity in women with PCOS. Could it also explain the switching on of important oncogenes, such as osteopontin at a very early time in such patients? This study is small but it is a known fact that PCOS patients have unopposed oestrogen as well as increased androgen and insulin levels. Fugimoto et al[226] found that high oestrogen levels increase

the number of androgen receptors in the endometrium. Apparao et al[98] later found that there was an increased number of androgen receptors in the epithelial and stromal compartment of the endometrium in the fertile window in women with PCOS. This is a direct consequence of chronic hyperoestrogenism and hyperandrogenism was considered responsible for the poor endometrial receptivity found in these patients. Often in the reproductive world, women with PCOS are thought to have 'poor quality' endometrium. This abnormal milieu acts on $\alpha v\beta 3$ integrin and modifying the endometrial implantation window. The study raises a few similar issues and it is possible that this same chronic altered milieu switches on oncogenes in the endometrium. For example, this study has determined that one such oncogene is osteopontin.

Insulin was also found to be associated with altered expression of transforming growth factor beta3, bcl2-A1, integral membrane protein 2A and major histocompatibilty complex II and DQ aPLha1. Insulin is known to be a potent mitogen in the endometrium [227]. It is possible that through binding to it receptors, insulin sets off a series of reactions involving all these different protein which affect apoptosis, cell differentiation and re-modelling.

Although the number of cases investigated in this study was small, a correlation was found between cycle length and the expression of major histocompatibilty complex class II. The longer the cycle length, the stronger the correlation. Clinicians, up to this date, have been treating patients with PCOS with progestogens to induce a withdrawal bleed in the presence of oligomenorrhoea. This study seems to support this view and more

182

research is warranted to establish firm guidelines on when to offer therapy and what seems to be the acceptable length of amenorrhoea.

Finally, the RT-PCR data confirms the microarray findings for OP and DLG-7. For OP the expression is greater in PS and H compared to PL after microarray and this pattern of expression is mirrored by the RT-PCR findings. DLG-7 expression was found to be raised in PL tissue at both microarray and DLG-7. For the low signal intensity genes, CDK 8 and APSRG-1, the expression after RT-PCR does not correlate well with that found after microarray, raising doubts as to whether these two proteins were variably expressed. It would have been preferable to confirm these findings with RT-PCR for more than 4 proteins, but unfortunately due to time constraints and availability of material, it was impossible to do so.

5.10 Conclusion

This is the first study of gene expression in the endometrium of women with PCOS. As we have demonstrated even in the mildest form of morphological changes seen in simple hyperplasia, there are gene alterations which are involved in carcinogenesis in other studies. Furthermore, genes expression changes involved in carcinogenesis are seen in seemingly normal endometrium in women who do not cycle regularly. Osteopontin, an oncogene involved in carcinogenesis in other hormone sensitive cancers, is variably expressed in the endometrium of the cohort of PCOS women studied. In addition other oncogenes involved in cell growth, matrix remodelling and apoptosis, all processes involved in carcinogenesis, were found to be variably expressed.

To this date, there is no evidence to guide clinicians in manageing PCOS women with oligomenorrhoea, to reduce the risk of developing endometrial hyperplasia. Some medical practitioners firmly believe that these women are at high risk of endometrial cancer and empirically prescribe progestogens every 3 months to induce withdrawal bleed. In our study, some the women in the PL group had cycle length that were shorter than 90 days and yet this subgroup of women were found to have genes expression alterations in their endometrium which some studies have associated with carcinogenesis (including endometrial carcinogenesis). The next step for this study is to treat women with progestogens and then again study the endometrium for gene expression. This would give valuable information to clinicians who would in turn be able to counsel and treat women with PCOS accordingly. If these changes occur at such an early stage, these oncogenes may be a target for screening. Osteopontin in prostate cancer can give valuable information on prognosis. Similarly in the presence of endometrial cancer, these genes could give information about prognosis.

It would also be of interest to do similar microarray studies on PCOS patients with high insulin levels and then repeat the studies after they have been on a six months course of insulin sensitizers such as metformin. Insulin is known to be mitogenic to the endometrium. It is possible that the altered endometrial gene expression seen in the H and PL groups maybe under higher hormonal influence such as insulin. There is still reluctance for clinicians in the UK to prescribe metformin for PCOS patients for other reason than impaired glucose metabolism. If insulin is also demonstrated to be the

184

intrinsic stimuli for switching on oncogenes then undoubtedly there should be a greater emphasis on metformin use.

This study helps shed more light on the once assumed association between PCOS and endometrial cancer. However there is still more work to be done to target patients who are more at risk as it is known that PCOS is a heterogeneous condition and patients can present to clinicians with many phenotypes of the condition.

Chapter 6

Conclusion

6.1 Conclusion

In 1949, Speert reported that young patients with EC demonstrated ovaries which were hypertrophied or cystic and subsequently Jackson et al published a study where they found a strong association between women with Stein-Leventhal syndrome and endometrial cancer. Though it has been demonstrated that the second study was incorrect[228], many clinicians believe that there is a strong association between PCOS and EC. In fact, this belief is so strong that across the world patients with PCOS are warned about the risk of developing endometrial cancer. When a literature review on the subject was performed, we found that most of the studies were poorly designed and inconclusive and hence the evidence for such an association was lacking.

For a number of years, there also has been a theory that endometrial cancer has two different aetiologies. The first is from a background of unopposed oestrogen stimulation of the endometrium, as is the case in PCOS or in a background of atrophic endometrium. In the former, EC was thought to develop mainly in relatively young obese women with type 2 diabetes and carried a good prognosis, whilst the latter, EC was thought to be found mainly in elderly women and carried a worst prognosis. However, endometrial carcinogenesis is now known to involve more complex pathways where altered hormone environments may play an active role. Oestrogen and insulin are known to be potent stimulants of carcinogenesis [106,151] whilst and progesterone has been shown to have a negative effect on the mitogenic potential of cells of the endometrium. Women with PCOS are known to have high levels of oestrogen and insulin and as they tend to have anovulatory menstrual cycles, they have less progestogenic effects on the endometrium.

The aim of this study was to demonstrate whether the belief that has been held for more than fifty years (and that has remained unquestioned), that PCOS women are more at risk of developing endometrial cancer was true.

The hypothesis that -

1) PCOS is associated with EC

2) The type of endometrial carcinoma arising in women with PCOS carries a better prognosis.

3) The risk of EC is confined to a subgroup of women with PCOS.

Hypothesis 1: PCOS is associated with endometrial cancer.

A retrospective study was undertaken on the ovaries of women diagnosed with endometrial carcinoma and these were compared to the ovaries of age-matched controls that had had oopherectomy for benign gynaecological conditions. This retrospective study looked at the ovaries of a total of 211 women. 128 of these women were diagnosed with endometrial cancer from 1987 to 2003, 83 age-matched controls were also selected. Ovarian morphology was studied as a surrogate marker of PCOS. Semi-quantitative criteria for the diagnosis of PCO morphology were devised in this study, these being the presence of >8 peripherally arranged follicular cysts per section and <5 corpora albicantia per section, the latter as evidence of reduced ovulation rate. In borderline cases (6 - 8 follicular cysts and 5 - 7 corpora albicantia), a diagnosis of PCO morphology was made if

capsular thickening and/or stromal hyperplasia/hyperthecosis were also apparent. Sections which failed to achieve these criteria were judged as normal.

The prevalence of PCO morphology in the women diagnosed with endometrial cancer was found to not differ from those of women without EC. Thus, the first hypothesis was proven to be incorrect.

Another point of interest that was revealed by this study was that on stratifying patients according age, PCO morphology was significantly more prevalent in women aged less than 50 years (51 years old being the average age of menopause). A criticism of the study is that PCO was used as a marker of PCOS. It is known that 50- 70% of women with PCOS will exhibit PCO morphology [169]. However women with PCO only are also known to exhibit metabolic and endocrine disorders similar to those exhibited in women with PCOS, but to a lesser degree.

Hypothesis 2: The type of endometrial carcinoma arising in women with PCOS carries a better prognosis.

Although evidence is limited, it is believed that the type of endometrial tumours arising in the endometrium of women with PCOS tend to carry a better prognosis [155]. Jafari et al found that endometrial carcinoma in young PCOS patients tended to be well-differentiated and carried a good prognosis. The assumption also stems from the fact that endometrial cancers that originate from a background of unopposed oestrogen stimulation

189

(as is the case in PCOS) tend to be well differentiated, responsive to treatment and carrying a good prognosis.

To test the hypothesis, immunohistochemistry was performed on the endometrial tumours of women with PCO morphology of the ovaries and compared to the endometrial cancers of women without PCO morphology. p53, ki67, bcl2and cyclin d1 expression were used as surrogate markers of prognosis. We found that there was no significant difference in the expression of p53, ki67 and bcl2these proteins in both groups of tumours. The prevalence of cyclin D1 was in increased in the tumours associated with PCO. This study fails to prove the second hypothesis that endometrial cancer arising in women with PCOS carries a better prognosis. This puts into question the previously held belief and adds weight to our third hypothesis, since it would be expected that the expression of these proteins would be significantly higher in the tumours not associated with PCO morphology; the action and expression of p53 being well documented and it is usually found in aggressive tumour types. The expression of cyclin D1 has been shown to be up-regulated in endometrium subjected to oestrogen[229].

One would expect to see a vast difference in the expression of tumour markers such as p53 or even Bcl2 in the two types of tumours if the prognosis of EC in women with PCOS was different from that in women without PCOS. It thus appears that the second hypothesis is correct, i.e., that the type of endometrial cancer arising in women with PCOS not carries a better prognosis. Although cyclin D1 suggested a worse prognosis for PCOS related tumours this was not supported by the bcl2 data. Bcl2, however, does not

work in isolation but is opposed by bax and the ratio of bcl2: bax maybe more relevant. Unfortunately, this was not investigated. It is possible that bcl2: bax expression may add more weight to the findings with cyclin D1.

<u>Hypothesis 3: The risk of EC is confined to a subgroup of women with PCOS.</u>

A microarray study was undertaken which investigated the gene expression in the proliferative endometrium of women with PCOS. Our subjects were classified into 3 groups: The H group which consisted of endometrial tissue obtained from women with cycle length of greater than 40 days displaying simple endometrial hyperplasia, the PL group consisted of endometrium from women who displayed proliferative endometrium but were known to have cycle length greater than 40 days and the PS group consisted of endometrium from women with cycle lengths less than 40 days. RNA was extracted and Cy3 labelled c-DNA was generated which was hybridized onto microarray, along with Cy5 labelled cDNA obtained from pooled RNA from the proliferative endometrium of normal women with regular menstrual cycles.

 This study revealed that a number of genes were differentially expressed across all 3 groups. One such example is the level of osteopontin, which was invariably altered in the comparison between PS and PL, PL and H and finally H and PS. This indicates that gene expression changes occur at an early stage when a PCOS patient starts becoming oligo/amenorrhoeic. The results suggest say that, oligomenorrhoeic patients with PCOS have altered expression of several genes which are linked to carcinogenesis, hence

proving strong evidence in support of the third hypothesis that a subgroup of PCOS patients are at risk of developing endometrial cancer.

As mentioned previously, the entire hyperplasia samples collected were of the simple category. It was unfortunately not possible to obtain any atypical hyperplasia sample in such a small sample group. It would have been very in formative to obtain samples with histological subtype as well as it known that if left untreated, up to 40-45% of cases can progress to endometrial cancer. On a molecular level, this tissue subtype has undergone ore transformation towards becoming cancerous. It would be expected that gene expression in atypical endometrial hyperplasia would be similar or overlap gene expression in endometrial cancer. If the gene expression from the endometrium of women with PCOS with atypical endometrial hyperplasia were compared to that in PCOS with simple hyperplasia, more information would be obtained about oncogenes involved in converting simple hyperplasia into atypical hyperplasia. If these genes are also overexpressed in a subgroup of PCOS, then this may shed more evidence of a stronger link between PCOS and cancer. Obviously this was outside the scope of this study and can be a potential source for future work.

This thesis was first devised after it became clear that the evidence was lacking in support of the assumption that endometrial cancer and PCOS are linked. I set out to prove this assumption and in fact found that if there is a link between the two conditions, it seems to be confined to younger women with long menstrual cycles, raised androgen levels and increased insulin levels. These risk factors remain increased in the women whose

endometrium demonstrated endometrial hyperplasia (even the mildest form of endometrial hyperplasia - simple hyperplasia).

This study could be considered as a pilot study and larger numbers would be helpful in confirming the findings.

6.2 Future work

As previously mentioned, this is the first time that endometrial gene expression has been specifically investigated in women with PCOS. It would be interesting to confirm the findings of Chapter 5 by performing immunohistochemical study to localize the sites of gene expression differences. For example, hyperplasia may be focal and in chapter five hyperplasia tissues on a background of proliferation was used. Immunohistochemistry may show exactly the site at which the variable expressed genes were operating. It may be when the whole tissue is considered, immunohistochemistry may not show a difference in expression between the three tissue types. However, the changes may be confined to the small hyperplasic section and this in itself poses certain dilemmas; The small proportion of hyperplasia, in a sea of proliferative tissue, was found in the microarray study to be driving gene expression changes 1.7 fold or more, for the whole endometrium from which the sample was taken this difference must be greater as it is very likely that there are several foci of hyperplasia.

The National Cervical Screening Programme has greatly improved the detection of pre-cancerous conditions of the cervix and in fact the prevalence of cervical cancer in the

western world has fallen as a result. A similar screening programme may help in reducing the incidence of endometrial cancer as it is the most common gynaecological tumour in the Western World. Women with PCOS may well constitute such an 'at risk' group and more specifically those who are oligo/amenorrhoeic, may benefit from regular endometrial biopsy to detect pre-malignant pathology. Those found to be suffering from hyperplasia may be given progestogens therapy.

Prevention should also involve primary care where PCOS patients should actively be encouraged to maintain a healthy lifestyle and be counselled about having regular menstrual cycles. (As the various gene alterations note in this thesis seem to happen early as a patient becomes oligomenorrhoeic, clinicians may have to be more aggressive in inducing withdrawal bleeds with progestogens.) There is still little evidence about the frequency of use of progestogens. Clinicians have arbitrarily chosen to give progestogens on a 3-monthly period to reduce the risk of endometrial hyperplasia in PCOS women with oligo/amenorrhoea. A future study could possibly utilize microarray as used in this study to analyze gene expression in endometrium from patient before progestogens use and then repeated after progestogens use to determine whether gene expressions is altered by the withdrawal bleed.

Metformin is a drug now widely used in the management of PCOS. It has been shown to act on insulin metabolism and has been known to restore ovulation in oligo/amenorrhoeic PCOS patients. This is due to its effect on the peripheral action of insulin which in turn acts on the ovarian stroma, hence androgen production. Another potential study could

194

involve using microarray analysis to investigate gene expression in the endometrium of women with PCOS before and after they have been on metformin for a specific period.

Risk factors for endometrial cancer include obesity, type II diabetes, hyperinsulineamia and unopposed oestrogen stimulation of the endometrium. These are also features of PCOS. It would be of value to find out whether there is a specific factor in PCOS alone which increases the risk of endometrial cancer or whether it is the presence of the confounding factors mentioned that increases that. One such factor such as obesity could be investigated by microarray studies in the endometrium of oligomenorrhoeic PCOS patients and that of oligomenorrhoeic obese non-PCOS patients.

This thesis sheds some light on the association between PCOS and endometrial carcinoma. Currently it is advisable to counsel women about their risk of endometrial hyperplasia when they are diagnosed with PCOS but it may be somehow felt premature to counsel them about their risk of developing endometrial cancer. My study has shown that there are significant oncogenic alterations even in simple hyperplasia, the mildest form of neoplasia found in the endometrium. It is clear that it is important to treat PCOS as a very serious condition with many health implications in later life, including a higher risk of developing endometrial cancer. Women with PCOS and irregular cycles should be tested for insulin resistance and hyperinsulineamia as my study shows that these women's endometrium are more at risk of switching on oncogenes which may play a role in endometrial carcinogenesis. Every effort should be made by their general practitioner or the gynaecologist to regularise their menstrual cycle. This could be in the form of

195

lifestyle management with diet and regular exercise, progestogen (or the COCP) on a regular basis if they are oligo or amenorrhoeic. In addition, if found to be insulin resistant and/or hyperinsulinaemic, PCOS women with oligo/amenorrhoea, could be managed as part of a multidisciplinary team, involving a gynaecologist, a dietician and an endocrinologist. Insulin sensitizing agents may be started if they are found to be hyperinsulinaemic and they should be carefully monitored with regular transvaginal scan (endometrial screening) and glucose tolerance tests.

APPENDIX

SCORING SHEET

Name: D.O.B:

Hospital no.

                                        L   **R**

Capsular thickness

Presence of peripheral cyst

Number

Stromal thickening

Number of corpora albicantia

Polycystic ovary:…………………………………………….

IMMUNOHISTOCHEMISTRY: REAGENTS AND BUFFERS

<u>1% (v/v) Acid-alcohol</u>
> 1400 ml ethanol
> 580 ml distilled water
> 20 ml concentrated HCl

<u>Acetone</u> (BDH)

<u>3-Aminopropyltriethoxysilane solution -APES</u> (Sigma-Aldrich A3648)
> 294 ml of acetone with 6ml APES
> Keep APES at 4°C and dilute in acetone just before use.
> <u>APES coating for slides</u>
> 1. Wash in detergent for 20 minutes.
> 2. Rinse in distilled water and dehydrate in methanol.
> 3. Dry at room temperature.
> 4. Drop in APES solution in fume cupboard, rinse in distilled water.
> 5. Dry overnight at 37°C.

<u>Blueing solution</u>
> 1. Add 5g disodium tetraborate to 1 litre distilled water. 0.5% (w/v) final concentration.

<u>Bovine serum albumin</u> (Fraction V, Sigma A-2153)

<u>Citric acid monohydrate</u> (Merck, 'Analar' 100813M)

<u>DPX mountant</u> (Merck 360294H)

<u>Diaminobenzidine–DAB</u> (Sigma-Aldrich, D-5637)

<u>Disodium hydrogen phosphate anhydrous</u> (Sigma-Aldrich, S0876)

<u>Disodium tetraborate</u> (Merck, 102674E)

<u>Ethanol</u> (Hayman 'Absolute alcohol 100')

<u>Formaldehyde</u>
> <u>Formol saline</u>
> 40% formaldehyde 100ml
> Sodium chloride 9g
> Water 900ml
> <u>Neutral buffered formaldehyde</u>
> 40% formaldehyde 100ml
> Distilled water 900ml
> Sodium dihydrogen phosphate monohydrate 4g
> Disodium hydrogen phosphate anhydrous 6.5g

<u>Haematoxylin and Eosin</u>

Hydrocholoric acid-concentrated (Merck, 'Analar' 101250D)

Hydrogen peroxide solution (Merck, 'Analar' 101284N)
1. Add 10ml 30% hydrogen peroxide to 90 ml distilled water.

Imidazole Solution (Merck, GPR 285466K)
1. Add 0.681g imidazole to 100ml distilled water.
2. Store at 4°C.
3. 0.1M imidazole solution.

Industrial methylated spirit

Mayers Haemalun 'Gurr' (BDH 350604T), (Merck, Lutterworth, Leics, UK)

Methanol (Merck, 'Analar' 101586B)

NaCl (Merck, 'Analar' 1913833X)

Normal Goat Serum (Dako X0907)

Normal Rabbit Serum (Dako, X0902)

Fresh Phosphate Buffered Saline (Sigma Aldrich, P4417)
1. Dissolve 1 tablet in 200ml distilled water.

Phosphate Buffered saline (ICN 17-604-20)
1. Dissolve 5 PBS powder sachets in 5l distilled water. pH 7.4

Protease XXIV (Sigma Aldrich P8038)
Protease solution for protease digestion:
1. Dissolve 37.5mg of protease XXIV in 200ml fresh PBS at 37°C.
2. Leave tissue sections for 7 minutes.

Sodium Citrate Buffer
1. Weigh out 2.1g citric acid monohydrate
2. Add 950 ml distilled water
3. Add 13ml 2M NaOH. Make up to 1l.
4. Adjust pH 6.0. Store at 4°C.
5. 0.01M Sodium citrate (pH 6.0 at 25°C).

Streptavidin-biotin complex duet kit (Dako K0492)

Tris [Tris (hydroxymethy) methylamine] (Merck 'Aristar' 452054C)
Tris buffered saline (TBS)
1. Weigh out 43.83g NaCl and 30.6g Tris base.
2. Make up to 5l distilled water.
3. Add ~35ml of concentrated HCl, ensure pH is at 7.6.
4. Store at 4°C.
5. 0.05M Tris-HCl, 0.15M NaCl, pH 7.6.

Xylene (Merck 'Analar'102936H)

Composition of buffers used in RNA analysis

Buffer	Composition
10X MOPS-acetate-EDTA (MAE)	0.8 M sodium acetate, 0.05 M EDTA in 0.5 M 3-(*N*-morpholino) propanesulphonic acid
RNA loading buffer	0.4% (w/v) bromophenol blue, 1 mM EDTA in 50% (v/v) glycerol
RNA sample buffer	8% (v/v) formaldehyde, 65% (v/v) formamide, 20 μg/ml ethidium bromide in 0.6X MAE

List of figures and tables.

Fig 1.7 -shows a cross-section through a polycystic ovary. This picture was reproduced courtesy of http://www.ansci.wisc.edu/jjp1/ansci_repro/index.html.

Fig. 1.8 a) - Ultrasound image of a polycystic ovary

Figure 2.2- shows a photograph of analysis of RNA integrity by agarose gel electrophoresis.

Fig. 1.8 b) Laparoscopic appearance of a polycystic ovary.

Figure 2.2 illustrates the principles of immunohistochemistry- Free reproduction from http://en.wikipedia.org/wiki/Immunohistochemistry.

Figure 2.3: shows a photograph of analysis of RNA integrity by agarose gel electrophoresis.

Figure 2.4 shows a plot of intensity data after lowess normalization. (courtesy of www.obgyn.cam.ac.uk/.../loess) -normalisation.htm.

Figure 2.5- showing the principle of RT-PCR- courtesy of Dr Frank Orme. An electronic tutorial. Merritt College, Oakland, USA. Free reproduction.

Fig 3.4- A pie chart representing the proportion of controls with PCO morphology

Figure 3.4- A bar chart representing the prevalence of PCO morphology in women with endometrial cancer and in controls, as a function of patient age.

Figure 3.5- A bar chart representing the prevalence of PCO morphology in women with endometrial cancer and in controls, as a function of patient age.

Figure 4.2- Cell cycle (Ki67, cyclin D1) and apoptotic (p53 and Bcl2) protein expression in endometrial tumors from women with normal or polycystic ovaries (*P = 0.071).

Table 3.1: shows the patients demographics.

Table 3.2: Ethnicity data for subjects with endometrial cancer (EC) and benign gynaecological disease (Controls), obtained retrospectively from patient records.

Table 4.1 shows the results obtained in each group, after immunohistochemistry.

Table 5.1 showing patient demographics and timing of biopsy.

Table 5.2- shows all the biochemical profiles and histological report of each patient in the study

Table 5.3- shows the mean values of Progesterone, testosterone, free androgen index, insulin and oestrogen in the three groups studied (short cycle proliferative - PS, long cycle proliferative- PL and the hyperplasia- H).

Table 5.4: Genes differently expressed between proliferative endometrium from normally cycling women with PCOS (PS) and proliferative (PL) and hyperplasic (H) endometrium from oligomenorrhoeic women with PCOS.

Figure 5.5 shows mean transcript expression levels in each tissue type, relative to the internal control sample, as estimated by microarray analysis.

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