THE IMPACT OF OBESITY AND WEIGHT LOSS ON THE MALIGNANT POTENTIAL OF ENDOMETRIUM

A thesis submitted to The University of Manchester for the degree of Doctor of Medicine MD in the Faculty of Medical and Human Sciences

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LIST OF ABBREVIATIONS

AdipoR	Adiponectin receptor
AEH	Atypical endometrial hyperplasia
AKT	Protein kinase B
AMPK	5’ AMP-activated protein kinase
AR	Absolute risk
ATP	Adenosine triphosphate
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma 2 protein
BMI	Body mass index
BRCA	Breast cancer susceptibility gene
BSO	Bilateral salpingo-oophorectomy
CA125	Tumour marker
CAH	Complex atypical hyperplasia
CC	Cell conditioner
CD3	Cluster of differentiation 3, T-cell co-receptor
CD20	B lymphocyte antigen
CI	Confidence interval
CMFT	Central Manchester Foundation Trust
COC	Combined oral contraceptive
COPD	Chronic obstructive pulmonary disease

CPAP	Continuous positive airway pressure
CPP32	Active caspase 3
CRF	Case report form
CRP	C reactive protein
CT	Computed tomography
DAB	3,3' diaminobenzidine, chromogen used in immunohistochemistry
DFS	Disease free survival
DHEAS	Dihydroepiandrosterone
DNA	Deoxyribonucleic acid
EEC	Endometrioid endometrial cancer
EDTA	Ethylenediaminetetraacetic acid, anticoagulant for blood samples
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Oestrogen receptor
ERK	Extracellular signal regulated kinases (aka MAPK)
ESHRE/ASRM	European Society of Human reproduction & Embryology/American Society for Reproductive Medicine
ET	Endometrial thickness
FAI	Free androgen index
Fas	Membrane protein of TNF family
FFA	Free fatty acids

FFE	Fresh frozen endometrium
FFPE	Formalin fixed paraffin embedded
FIGO	International Federation of Gynecology & Obstetrics
FSH	Follicle stimulating hormone
G1, 2, 3	Grade of disease
G1-S	Gap 1 and Synthesis phases of cell cycle, cells grow and DNA replicates
GDP	Guanosine diphosphate
GH	Growth hormone
GIP	Gastric Inhibitory Polypeptide
GLP1	Glucagon-like peptide-1, an incretin
GnRH	Gonadotropin Releasing Hormone
GOR	Gastro-oesophageal reflux
GP	General Practitioner
GPRD	General Practice Research Database
GTP	Guanosine triphosphate
H&E	Haematoxylin & eosin
HbA1c	Glycosylated haemoglobin
hCG	Human chorionic gonadotrophin
Her2	Human epidermal growth factor receptor 2 (aka Neu)
HMB	Heavy menstrual bleeding

HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HOMA-IR	Homeostatic Model Assessment of insulin resistance
HPF	High powered field
HR	Hazard ratio
HRT	Hormone replacement therapy
HTN	Hypertension
ICC	Interobserver intraclass correlation
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IHC	Immunohistochemistry
IL-6	Interleukin 6
IMB	Intermenstrual bleeding
IMS	Industrial methylated spirit
IQR	Interquartile range
IR	Insulin receptor
IRS	Insulin receptor substrate
ISRCTN	International Standard Randomised Controlled Trial Number
IUD	Intra-uterine device
IVF	In vitro fertilisation
Ki-67	Nuclear protein, cellular marker of proliferation

K-ras	Proto-oncogene
LH	Luteinising hormone
LKB1	Liver kinase B1, protein kinase, tumour suppressor
LMP	Last menstrual period
LNG-IUS	Levonorgestrel releasing intra-uterine system
M	Mitosis phase of cell cycle
MAPK	Mitogen activated protein kinase (aka ERK)
MDT	Multidisciplinary team
MEK	Mitogen activated protein kinase enzymes
MLH1	Gene located on chromosome 3
MMP	Matrix metalloproteinases
MPA	Medroxyprogesterone
MRC	Medical Research Council
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSH2	Gene located on chromosome 2
MSH6	Gene that codes DNA mismatch repair protein
MSI	Microsatellite instability
MTOR	Mammalian target of rapamycin
Neu	See LKB1

NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICE	National Institute for Health and Care Excellence
NIHR	National Institute for Health Research
NNS	Number needed to screen
ObR	Leptin receptor
OCP	Oral contraceptive pill
OR	Odds ratio
P53	Protein 53, tumour suppressor protein, regulates cell cycle
PAF	Population attributable fraction
pAKT	Phosphorylated AKT
PARP	Poly ADP ribose polymerase
PCOS	Polycystic ovary syndrome
PCB	Postcoital bleeding
PCT	Primary care trust
pERK	Phosphorylated ERK
p-H3	Phosphorylated histone
PI	Proliferation Index (% Ki-67 positivity)
PI3K	Phosphatidylinositol 3 Kinase
PIK3CA	Serine/Threonine protein kinase, PI3K catalytic alpha-polypeptide
PIS	Patient information sheet

PMB	Post-menopausal bleeding
PMS2	Gene which encodes mismatch repair endonuclease
POLE	Gene which encodes subunit of DNA polymerase epsilon, involved in DNA repair and replication
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
PTEN	Phosphatase & tensin Homologue
PYY	Peptide YY, reduces appetite
QC	Quality control
R&D	Research & development
Ras	Family of small GTPase proteins involved in cellular signal transduction
RCOG	Royal College of Obstetricians and Gynaecologists
RCT	Randomised controlled trial
REC	Research Ethics Committee
RNA	Ribonucleic acid
RR	Relative risk
RTU	Ready to use
SAE	Serious adverse event
SD	Standard deviation
SEER	Surveillance, Epidemiology and End Results Program
SERM	Selective oestrogen receptor modulator

SF-36	Medical Outcomes Trust Short Form 36
SGO	Society of Gynecologic Oncologists
SHBG	Sex hormone binding globulin
SOP	Standard operating procedure
SRFT	Salford Royal Foundation Trust
STMN1	Stathmin
T0	Baseline
T1	2 months post weight loss
T2	12 months post weight loss
T2DM	Type 2 diabetes mellitus
TBS	Tris buffered saline
TCRE	Trans-cervical resection of endometrium
TLH	Total laparoscopic hysterectomy
TMA	Tissue microarray
TNF	Tumour necrosis factor
TSC	Tuberous sclerosis complex
TVUS	Trans-vaginal ultrasound
UKCRN	UK Cancer Research Network
UKCTOCS	United Kingdom Collaborative Trial of Ovarian Cancer Screening
UKMEC	UK Medical Eligibility Criteria for contraceptive use

VBG	Vertical banded gastroplasty
VBT	Vaginal brachytherapy
VEGF	Vascular endothelial growth factor
VTE	Venous thromboembolism
WHO	World Health Organisation
WHR	Waist hip ratio
Wnt	Group of signalling pathways involved in carcinogenesis and embryology

ABSTRACT

The University of Manchester
Michelle Louise MacKintosh
Doctor of Medicine MD
The impact of obesity and weight loss on the malignant potential of endometrium
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Introduction
The incidence of endometrial cancer is rising steeply, with the obesity epidemic believed to be the cause. Women with a BMI > 42kg/m^2 have a 9-fold increase in their relative risk of endometrial cancer. Few studies have investigated the endometrial effects of obesity or weight loss. I hypothesised that morbidly obese women had a high prevalence of undiagnosed endometrial cancer and pre-cancer, and that major weight loss would result in measurable systemic and endometrial effects.

Methods
118 morbidly obese women undergoing weight loss surgery or non-surgical weight management were recruited into a prospective cohort study. Blood and endometrial samples were taken at baseline, 2 and 12 months.

Results
80 women have undergone baseline assessment (mean age 44 years, median BMI 52kg/m^2). Menstrual and reproductive dysfunction was common (15% pre-menopausal amenorrhoea, 31% oligomenorrhoea) and less than one third reported regular menstrual cycles. Four cases of endometrial cancer and six of atypical endometrial hyperplasia were detected at baseline (prevalence 12.5%, 95% CI 6.2-21.8), and women with abnormal endometrium had significantly higher HbA1c and pAKT levels. Undiagnosed diabetes was found in 6%, and overall more than 38% were diabetic and up to 40% more had raised HOMA-IR levels.

Significant serial improvements were seen in insulin resistance, adipokines, inflammation and androgens after bariatric surgery. In endometrium significant reductions were seen in Ki-67, pAKT, ER and PR expression. In samples matched for cycle timing and not affected by exogenous hormone treatment Ki-67 reduced by 11% and 17% at 2 and 12 months post-surgery. AEH resolved with weight loss alone in 3/6 patients and with weight loss and LNG-IUS in 2/6 women. Ki-67 expression correlated weakly with pAKT, serum oestradiol, HOMA-IR, FAI and adipokines.

Conclusions
Such a high prevalence of endometrial cancer and pre-cancer in morbidly obese women supports targeted screening in this high-risk group and highlights the importance of diagnosing and managing insulin resistance. Reduction in proliferation appears to be mediated by the PI3K/AKT pathway and through changes in insulin resistance, reproductive hormones and inflammation. Ki-67 may have a use as a marker of the 'high-risk' endometrium or in the future surveillance of endometrial abnormality being managed by fertility-sparing means.

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

COPYRIGHT STATEMENT

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DEDICATION

To my parents, Andrew and Erica MacKintosh, for your unconditional support and understanding, and for teaching me that anything is achievable if you work hard enough.

To my husband, Bil Kirmani, for getting me here. For being my most ardent advocate and my harshest critic, for all that you have taught me and for knowing more about endometrial cancer than any cardiac surgeon ever should. Thank you for sharing this amazing journey with me.

PREFACE

In 2005 I graduated from The University of Manchester with an MBChB, and in 2007 commenced training in Obstetrics & Gynaecology on the North West rotation. In 2011 I obtained membership of the Royal College of Obstetricians and Gynaecologists at the first attempt. In February 2012 I enrolled at The University of Manchester on a four-year part time MD programme whilst also working at St. Mary's Hospital, Manchester as a Clinical Research Fellow in Gynaecological Oncology. I returned to training in February 2015 and in September 2015 was appointed as Subspecialty Trainee in Gynaecological Oncology in Manchester and commenced in post at The Christie Hospital in November 2015. Earlier this year I was elected to the role of Deputy Trainee Representative on the Subspecialty Committee of the RCOG.

My commitment to clinical research has seen me attend events such as the Annual Academic Meeting at the RCOG where I have presented my work, as well as workshops at the Royal College of Surgeons of England which promote and support successful clinical and surgical studies in surgical oncology. The first of these, a Tomorrow's Leaders workshop, brought about the development of a gynaecological oncology trainee research collaborative – the Surgical Gynaecological Oncology Research Network, of which I am Secretary. In December 2014 I was appointed to the NCRI Gynaecological Oncology Clinical Studies Group as a trainee member.

1. Introduction

1.1 Endometrial Cancer

Endometrial cancer is the most common gynaecological malignancy in Europe and North America, and the fourth most common female cancer in the UK after breast, lung and colorectal cancer. Over the past twenty years the incidence of endometrial cancer has risen dramatically, by over 40%. Currently more than 2000 British women die from endometrial cancer each year, compared to less than 1500 just over a decade ago (1). The ageing population, uterine sparing treatments for menstrual dysfunction and tamoxifen use for breast cancer have all contributed to this rise, but the greatest culprit appears to be the evolving obesity epidemic. Throughout Europe it has been estimated that 60% of new endometrial cancer cases each year may be attributable to excess weight (2).

Worldwide the prevalence of obesity has doubled in the last three decades. Each year 2.8 million people around the world die as a result of being overweight or obese, and in the UK alone one in three adults are overweight and one in four are obese (3). Obesity is accountable for 44% of the disease burden of diabetes mellitus and 23% of that of ischaemic heart disease (4). The World Health Organisation (WHO) defines obesity as a person having a Body Mass Index (BMI) greater than 30kg/m^2. Whilst once being regarded as a problem limited to high income countries, prevalence in low income countries is on the rise, particularly in urban areas (4). As obesity continues to rise it would seem to be likely that the incidence of endometrial cancer, and in all probability its mortality, will continue to increase also.

1.1.1 Epidemiology

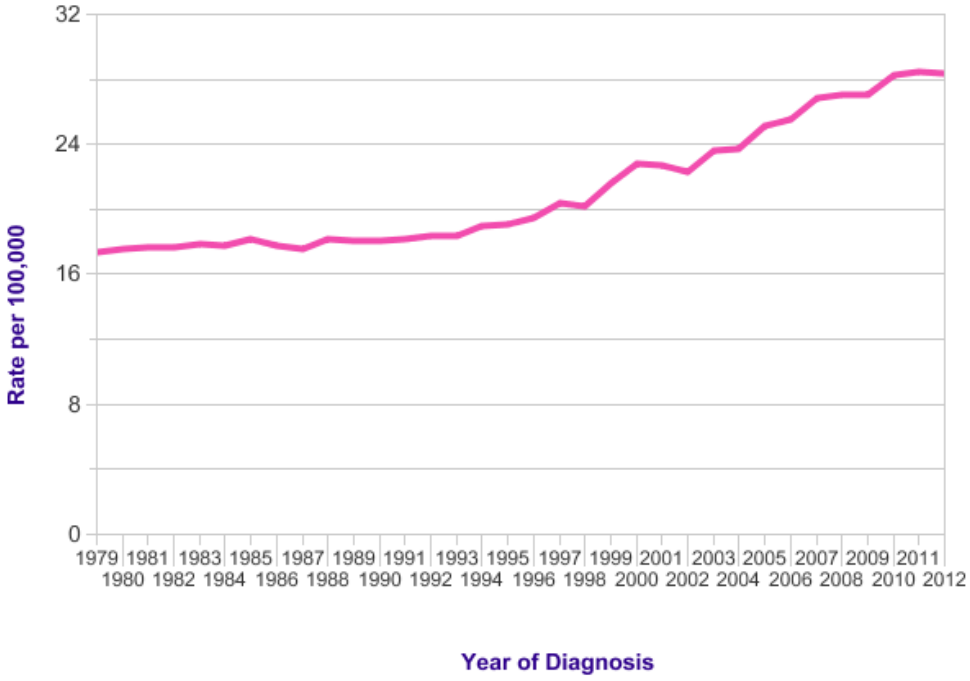
1.1.1.1 Incidence & Survival

Despite improving survival rates, deaths from endometrial cancer have increased by almost 20% in the last decade. The incidence has increased by 40% since 1993 (Figure 1) and now stands at 19.6/100,000. In 2013 9022 new cases were diagnosed in the UK, predominantly in postmenopausal women, with many diagnosed at an early stage. Across all stages 5 year survival reaches 79%, although in advanced disease or in the presence of adverse prognostic indicators 5 year survival can be less than 20% (5).

1.1.1.2 AETIOLOGY

The endometrium is the epithelial lining of the uterus and is comprised of epithelial glands and specialised stroma. Endometrial adenocarcinomas, which arise from epithelial cells, comprise 95% of malignancies of the uterine body. The majority of endometrial cancers are thought to be sporadic occurrences, although 2 to 5% of women show a genetic predisposition to the disease (6). The most common of these is HNPCC (Hereditary Non-Polyposis Colorectal Cancer) now more commonly known as Lynch syndrome, an autosomal dominant syndrome of cancer-susceptibility. The lifetime risk of endometrial cancer in these women is 40-60%, compared with 2-3% in the general population.

FIGURE 1: EUROPEAN AGE STANDARDISED INCIDENCE RATES FOR UTERINE CANCER PER 100,000 POPULATION, BETWEEN 1975 AND 2012 (1)



1.1.1.3 DUALLISTIC MODEL

Endometrial cancer has frequently been described in terms of a "duallistic model" of oestrogen driven carcinogenesis (type I) versus non-oestrogen related carcinogenesis (type II) (Table 1). Endometrioid adenocarcinomas account for 80% of endometrial tumours. Most endometrioid adenocarcinomas are type I tumours, where low-grade cancer develops in a hyperplastic endometrium, in women who often have risk factors such as obesity and diabetes mellitus. By comparison type II cancers, such as serous or clear cell tumours, arise from malignant transformation of atrophic endometrium. Type II tumours are more aggressive, have a poorer prognosis and a higher rate of recurrence. Whilst serous tumours account for only 10% of endometrial cancers they constitute 50% of cases of recurrent endometrial cancer (7). By virtue of their prevalence type I tumours remain responsible for the majority of deaths (8).

TABLE 1: THE DUALLISTIC MODEL OF ENDOMETRIAL CARCINOGENESIS

Type I	Type II
Endometrioid (G1, G2)	G3 endometrioid, non-endometrioid histological subtypes
Risk factor related, younger age at diagnosis	Associated with increasing age
ER & PR positive	ER & PR negative
Associated with endometrial hyperplasia	Arise on background of endometrial atrophy
Good prognosis	Poor prognosis, prone to recurrence

More recently Setiawan et al have proposed that the aetiology of type II tumours may not be entirely oestrogen independent, as had previously been thought (9). In their pooled analysis of 10 cohort and 14 case control studies they included 13,707 cases of endometrial cancer (854 type II and 12853 type I) and 35,312 controls. The mean age at diagnosis was higher amongst patients with serous endometrial tumours and lowest in

women with endometrioid tumours. All histological subtypes demonstrated a higher mean BMI than controls and the expected associations between type I tumours and age at menarche, parity, use of combined oral contraceptives and diabetes were also seen in type II tumours with the exception of clear cell tumours. BMI appeared to have less of an effect on type II tumours than on type I (Per 2kg/m^2 increase in BMI OR 1.12 for type II compared with 1.2 for type I, $p<0.0001$).

Molecular profiling studies have also suggested that the traditional assumptions of a duallistic model can be refined for the purposes of targeting adjuvant treatment and accurately assessing prognosis (10).

1.1.1.4 Risk factors

Type I endometrial cancers are risk factor related. Risk factors implicated in non-inherited endometrial carcinogenesis include obesity, nulliparity, exogenous unopposed oestrogen therapy, tamoxifen, diabetes, polycystic ovarian syndrome, early menarche and late menopause. Many of these risk factors are believed to act through pathways that reflect a greater lifetime exposure to oestrogen.

Obesity

The mechanisms underpinning the association of excess body weight and cancer risk are incompletely understood, and the relationship is thought to be only partially explained by excess exogenous or endogenous oestrogen exposure (11). Meta-analyses have supported a relationship between the incidence of endometrial cancer and obesity, with women who have a BMI ≥42kg/m^2 having a relative risk (RR) of endometrial cancer of 9.11 (95% CI 7.26-11.51) when compared with a woman of normal BMI. Obesity will be discussed in further detail throughout this thesis.

Diabetes

Diabetes has been shown to increase the risk of endometrial cancer by a relative risk of 2.1 (95% CI 1.75-2.53) in a meta-analysis of 25 studies (13 case-control, 12 cohort)(12) which included 7,596 endometrial cancer cases and more than 96,000 women overall. Such analyses are often plagued by the confounders of physical inactivity and obesity as alternative, and often co-existent, independent risk factors for endometrial cancer.

After multivariate adjustment studies have estimated the increase in relative risk of endometrial cancer with diabetes to be between 1.42 and 4.1 (13).

This increase in risk is also seen in insulin resistance, commonly occurring in conjunction with obesity. In their cohort study of 541 endometrial cancer cases and 961 age matched controls Freidenreich et al demonstrated an increase in endometrial cancer risk in women who had the greatest degree of insulin resistance, as measured by insulin levels and HOMA-IR (highest quartile of insulin and HOMA-IR associated with increased risk of 64% (95% CI 1.12-2.4) and 72% (95% CI 1.17-2.53) respectively, when compared to the lowest quartile) after adjusting for multiple variables including weight, age and hormone use (14).

It has been suggested that whilst diabetes is associated with endometrial cancer in women who are normal weight to moderately obese, it is unrelated to endometrial cancer risk in severely obese women (BMI > 35kg/m^2, OR 1.1 95% CI 0.6-2). This may reflect a higher level of underlying occult diabetes or insulin resistance in these women, or a smaller contribution of insulin resistance to the aetiology of the disease in the context of severe obesity (15).

TAMOXIFEN

Tamoxifen is a Selective Estrogen Receptor Modulator (SERM) frequently prescribed as adjuvant therapy in the prevention and control of breast cancer. For many years the use of Tamoxifen for 5 years was standard adjuvant endocrine therapy after treatment for breast cancer. Recent updated guidelines now recommend up to 10 years of adjuvant endocrine therapy in the case of hormone receptor positive breast cancer (16) (17). Hormone receptor positive breast cancer is the most common type of breast cancer, and adjuvant endocrine therapy is both effective and appropriate for most women with ER and/or PR positive tumours.

It has been associated with an increased risk of endometrial polyps, hyperplasia and cancer. The National Surgical Adjuvant Breast and Bowel Project P-1 trial reported a doubling of endometrial cancer risk (RR 2.53 cf. placebo, 95% CI 1.35-4.97) amongst all women. Premenopausal women treated with tamoxifen did not have an increased risk

of endometrial cancer (RR 1.21, 95% CI 0.41-3.6), the increase was in women aged 50 or over (RR 4.01 95% CI 1.7-10.9) (18). More recent data suggests a greater risk increase than this. The precise mechanism of action is unclear (19).

Since 2014 NICE guidelines have recommended offering 5 years of tamoxifen or raloxifene in the context of prevention to women who are at a high risk of breast cancer, unless they have a past history of bilateral mastectomy or may be at increased risk of thrombosis or endometrial cancer (20)(21). It remains to be seen whether this increased use of tamoxifen will impact further upon endometrial cancer or hyperplasia incidence.

Polycystic Ovary Syndrome

Polycystic Ovary Syndrome (PCOS) is a condition affecting 6-8% of women of reproductive age, characterised by anovulatory cycles, androgen excess, morphologically polycystic ovaries, insulin resistance and, in 30-70%, obesity. Diagnosis is based on exhibiting at least 2 of 3 Rotterdam ESHRE/ASRM criteria, with other endocrine causes of oligo or anovulation having been excluded:

1. Oligo/anovulation
2. Clinical or biochemical evidence of androgen excess
3. Polycystic ovaries

Excess androgen production and reduced SHBG levels result in a higher free androgen index and androgenic symptoms such as hirsutism and acne. Premenopausal obese women with PCOS may be chronically anovulatory, and as such be exposed to prolonged periods of unopposed endogenous oestrogen. Women with PCOS have been shown to express lower levels of progesterone receptors in the endometrium than women without PCOS (22). PCOS is more common in morbidly obese women than in the general population.

PCOS is over-represented in young endometrial cancer patients (23). An Australian case control study of women under 50 years reported a 4 fold increase in the risk of endometrial cancer in women with PCOS, and more than a 2 fold increase in risk even when BMI was corrected for. A more recent meta-analysis puts the lifetime risk of

endometrial cancer in women with PCOS in the region of 9% (OR 2.89) (24). In women with PCOS who are obese and hyperinsulinaemic this may be higher. Rosato et al (25) estimated the odds ratio for developing endometrial cancer to be as high as 8.4. Furthermore, 35.7% of women with PCOS may have endometrial hyperplasia, with one quarter of these being precancerous atypical lesions (26). A wide range of estimates are reported and the actual prevalence of atypical endometrial hyperplasia in asymptomatic women with PCOS is unknown, which in part reflects the heterogeneity of PCOS and the varying diagnostic criteria employed (27).

1.1.1.5 Precursors to malignancy

Atypical endometrial hyperplasia (AEH) is a non-obligate precursor of endometrioid endometrial cancer (EEC), a pre-malignant phase which can be detected in the endometrium. It may produce similar symptoms to EEC, and is associated with the same risk factors (28). Endometrial hyperplasia has an incidence of 133 per 100,000 woman years. A recent review concluded that in asymptomatic postmenopausal women the presence of atypical hyperplasia increased the risk of developing endometrial cancer by a relative risk of 14.2 (95% CI 5.3-38) (29). A study of 229 cases of endometrial hyperplasia, and 413 controls found that 20 years after first diagnosis of endometrial hyperplasia <5% of those with non-atypical hyperplasia had progressed to endometrial cancer, compared with approximately 30% of those with atypical hyperplasia (30).

The pathological appearance of AEH and EEC are similar (Figure 2), as are their immunohistochemical profiles and molecular markers. Endometrial hyperplasia is characterised by a proliferation of endometrial glands, causing an increase in the gland to stroma ratio when compared to that observed in normal endometrium (31). The proliferative glands vary in size and shape, and are crowded. Cells display cytological/nuclear atypia such as nuclear enlargement or clumping of chromatin. There is still residual stroma separating the glands, and it is this that differentiates AEH from grade one endometrial cancer. The revised 2014 WHO classification is the system recommended for use in the UK (32,33), which divides hyperplasia into hyperplasia

without atypia and hyperplasia with atypia. Other classifications such as the EIN classification are used, but not widely in the UK (34).

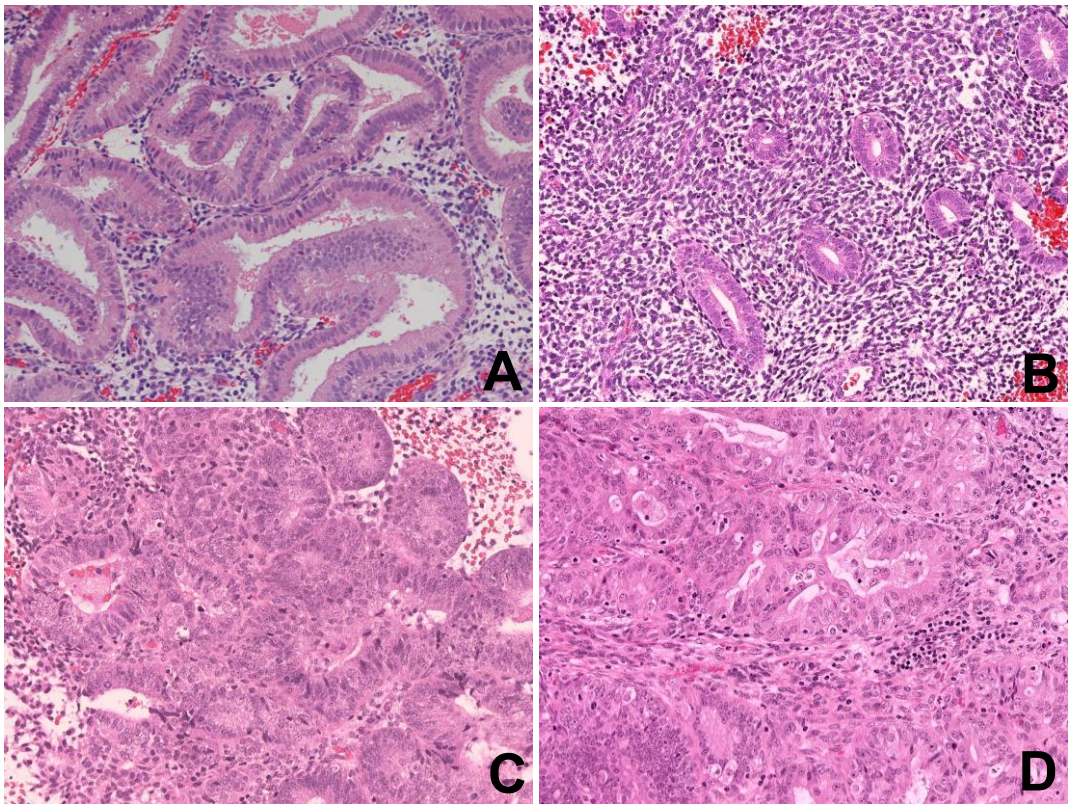
AEH and EEC are often found concurrently, and in topographic proximity. Up to 50% of women with atypical hyperplasia may have coexistent endometrial carcinoma in the hysterectomy specimen. Until February 2016 there were no standard treatment guidelines in use in the UK, and endometrial hyperplasia with atypia, like endometrial cancer, has tended to be treated surgically. Recent RCOG guidelines recommend hysterectomy for atypical hyperplasia, unless fertility preservation is required in which case treatment with intrauterine progestagen and 3 monthly biopsies are advised, followed by hysterectomy when family complete (35). The majority of women with AEH, up to 80%, will have surgical treatment. Hysterectomy and removal of both ovaries and fallopian tubes is the recommended approach, due to the possibility of coexistent cancer.

High dose oral or intra-uterine progestagen treatment and serial follow up biopsies of the endometrium may be an alternative in women who are not fit for surgery, decline surgery, or wish to preserve fertility. Endometrial hyperplasia without atypia can be treated with oral or intrauterine progesterone therapy. In young women endometrial hyperplasia is usually highly reversible, albeit not indefinitely, with progesterone therapy. In older women this response to exogenous progesterone is dampened and it may be that a relative lack of endogenous progesterone in the presence of excess oestrogen may account for this, or it may reflect differential endometrial receptor expression or variation in the disease process between pre and postmenopausal women.

A meta-analysis published in 2010 assessed 24 observational studies including 1001 women, and found that lower rates of disease regression were seen with oral progestagens than with the levonorgestrel intrauterine system (LNG-IUS), in complex and atypical endometrial hyperplasia (36). The lack of high quality studies was noted. A Cochrane review has since looked at six randomised controlled trials (RCT) that examined the use of LNG-IUS in high-risk women (women on oral oestrogen only

hormone replacement therapy (HRT), taking tamoxifen or with endometrial hyperplasia). Only two of these RCTs have looked at the benefits of LNG-IUS in women with endometrial hyperplasia. They reported that regression of non-atypical endometrial hyperplasia was seen in all women treated with LNG-IUS (37).

FIGURE 2: HAEMATOXYLIN AND EOSIN STAINED SECTIONS OF NORMAL, HYPERPLASTIC AND MALIGNANT ENDOMETRIUM



Key: Images of H&E sections from study participants of A) early secretory endometrium, B) proliferative endometrium, C) atypical endometrial hyperplasia and D) G1 endometrioid adenocarcinoma.

1.1.2 PRIMARY PREVENTION AND SCREENING

1.1.2.1 PRIMARY PREVENTION

There is convincing evidence that use of combined oral contraception (COC) is associated with a significant and enduring reduction in the lifetime risk of endometrial cancer, particularly in women using COC for longer than 12 months. Epidemiological data demonstrates that the use of COC for three years or more reduces a woman's risk of endometrial cancer by more than 50%. The protective effect increases in proportion with duration of use, with an average 10% reduction in risk per year of use after three years of use, and even 20 years post exposure the protective effect remains statistically significant (38). Some studies suggest a reduction in the efficacy of the contraceptive effect in obese women, which may translate to a reduction in the protective benefit they receive. The endometrial protection afforded by COC is presumed to be a function of its progestagen content, but it is unclear what changes take place at a cellular level to explain the reduction in cancer risk, which persists decades after COC exposure. Of prime consideration should be the risk of venous thromboembolic disease associated with these treatments, which limits the clinical utility of combined oral contraception as chemoprophylaxis in an obese population (39,40). The Faculty of Sexual and Reproductive Health advises that at a BMI > 35kg/m^2 the risks of COC are likely to outweigh the benefits (UK Medical Eligibility Criteria (UKMEC) 3 recommendation) (40). This probably precludes the use of COC as a primary chemo-preventative strategy in the majority of obese women.

Oral, injectable and intra-uterine progestagen use has been shown to reduce the risk of endometrial cancer, although the evidence is less convincing than that for COC use as the numbers of studies are small. Cyclical oral progestagens are recommended to induce four withdrawal bleeds per year in women with chronic anovulation (41). The LNG-IUS may provide primary prevention against endometrial polyp formation and endometrial hyperplasia in women using tamoxifen (42). A protective effect of non-hormone releasing intra-uterine contraceptive devices has also been demonstrated (43).

The World Cancer Research Fund concluded that endometrial cancer risk was reduced by moderate physical activity and maintaining a healthy weight. Their report

recommended that to prevent cancer individuals should be lean but not underweight, perform at least 30 minutes of physical activity per day, increase fruit, vegetable, grain and legume intake and limit sugar and fat intake (44). Moderate physical activity has been associated with a decrease in endometrial cancer risk of more than one third, and weight loss has been shown to improve various surrogate markers (inflammatory markers, oestrogen, measures of insulin resistance) that have been implicated in endometrial carcinogenesis (45). In addition to this bariatric surgery has been reported to reduce the incidence of endometrial cancer 7-fold over 12 years (HR 0.22, $p<0.0001$) (46).

Risk-reducing surgery has a place in primary prevention of endometrial cancer in women with Lynch Syndrome, who have a 40-60% lifetime risk of endometrial cancer and a 10-12% lifetime risk of ovarian cancer. Prophylactic hysterectomy and bilateral salpingo-oophorectomy once fertility is no longer desired is an effective means of preventing endometrial and ovarian cancer in these women (47).

Aspirin and anti-inflammatory medications have been reported to reduce cancer risk in colorectal and other cancers, and given the proven association of type I endometrial cancer and inflammation, a similar effect in modifying endometrial cancer risk would be biologically plausible. No such effect was demonstrated in the Women's Health Study, a primary prevention study of almost 40,000 women using placebo or 100mg aspirin per day (RR 1.22, 95% CI 0.94-1.58, $p=0.14$) (48) or in higher doses of 600mg per day over 4 years in Lynch syndrome patients, in a randomised study which demonstrated a reduction in risk of colon cancer and other Lynch associated cancers (49). A meta-analysis of case-control and cohort studies demonstrated a weak beneficial effect of aspirin on endometrial cancer risk, and suggested this effect may be more pronounced in obese women, however this study was fraught with limitations due to the studies included within it and their inability to control for multiple confounders (50).

Whilst we know that insulin resistance, diabetes and PCOS are key risk factors for endometrial cancer; it has not been proven that improvement of insulin resistance

lowers the risk of cancer. The potential of metformin, which inhibits gluconeogenesis and increases peripheral glucose uptake, as primary prevention for endometrial cancer is as yet unproven and under-researched, but would at first glance seem a logical strategy. Studies to date have assessed surrogate markers rather than clinical end-points, and a definitive chemo-prevention study would be difficult to run as it would require huge numbers of women being followed up over many years (51).

1.1.2.2 Population screening

Currently there is no population-based screening for endometrial cancer and so our knowledge of the natural history of the disease is limited. Aetiologic and mechanistic studies have largely been limited to a selected proportion of cases with persistent hyperplasia and clinical symptoms. In fact, symptomatic hyperplasia may only be the tip of the iceberg. The natural history of occult endometrial pathology is largely unknown.

In the early 1990's Korhonen et al carried out the CHART 2 study of HRT and as part of this performed baseline endometrial biopsies with a Vabra aspiration curette on 2964 postmenopausal women, mean age 52 years (range 40-66), mean weight 65kg. Two cases of well-differentiated adenocarcinoma of the endometrium were detected (0.07%), and no atypical hyperplasia. 196 biopsies (6.6%) were insufficient for diagnosis. Clearly much will have changed in our demographic in the intervening 30 years and these were asymptomatic, postmenopausal, normal weight women. Data from Jacobs et al, from a nested case control cohort in the UKCTOCS study (a trial of population-based ovarian cancer screening in postmenopausal women), gives important prevalence data as the largest study of its kind (n=37,038). During the follow up period (median 5.11 years) 125 women (0.34%) were found to have endometrial cancer and 11 (0.03%) to have atypical endometrial hyperplasia. Of these women 112/136 (82.4%) were asymptomatic at the time of diagnosis.

There is no evidence that screening asymptomatic women in the general population with transvaginal ultrasound (TVUS) or endometrial sampling reduces the mortality from endometrial cancer. As such, an effective screening programme for endometrial cancer has not been identified, and the Jungner & Wilson criteria adopted by the

national screening committee have not been fully evaluated for this condition (Table 2). As a result, it is not possible to calculate Number Needed to Screen (NNS) or the reduction in Absolute Risk (AR) that could be anticipated with screening for endometrial cancer. Whilst the NNS for the general population may be too great as to be realistically implementable, in selected high risk populations it may be in the region of existing screening programmes such as faecal occult blood testing, cervical screening or mammography which have NNS of between 400-1500. More data is needed before a clinically meaningful approach to screening for endometrial cancer can be considered.

Most cases of endometrial cancer are detected at an early stage as 85% are symptomatic, and 5-year survival across all stages is 77%. Crucially, no survival benefit of screening has been demonstrated thus far, largely due to a paucity of evidence. In their retrospective study, Gerber et al (8) found that there was no prognostic advantage for screen detected endometrial cancer compared to women with a short (< 8 week) history of postmenopausal bleeding (52). Screening increased iatrogenic morbidity and cost.

Studies have primarily evaluated endometrial sampling and TVUS as prospective screening tools for endometrial cancer, as these are frequently used in the investigation of abnormal uterine bleeding. In view of the relatively low incidence of the disease however, and the investment and service delivery required to provide screening at this level, introducing population screening may be neither feasible nor cost effective.

Whilst TVUS in symptomatic postmenopausal women using an endometrial thickness (ET) cutoff of ≤4mm has high sensitivity and reduces the post test probability of endometrial cancer to <1% (53), there remain many contentious issues around cost, impact on health and acceptability of screening. In women without abnormal vaginal bleeding, the same ET thresholds have unacceptably high false positive rates and poor sensitivity. Jacobs et al (54) and Smith-Bindman et al (55) have published data suggesting improved sensitivity of TVUS using alternative ET cut-offs than those used in symptomatic postmenopausal women, however in the absence of mortality data or a

consensus on thresholds for further investigation, this cannot yet be extrapolated to justify the adoption of ultrasound screening of asymptomatic women.

Endometrial sampling, e.g. with a Pipelle (Pipelle de Cornier, Paris, France), is indicated in symptomatic women with a thickened endometrium on TVUS, however its use in asymptomatic women may be limited by a perceived lack of acceptability. Endometrial biopsy can result in discomfort, bleeding, infection and rarely uterine perforation. In asymptomatic women, up to 23% of endometrial biopsies may yield insufficient tissue for diagnosis (56).

Other tests that have been evaluated in the literature include cervical cytology, endometrial brush cytology, sonohysterography and serum markers such as CA125. Cervical cytology may occasionally detect abnormalities suggestive of endometrial cancer (e.g. endometrial cells of uncertain significance) but is too insensitive to be used as a screening tool (57). Endometrial brush cytology may be equivalent to endometrial sampling in the presence of endometrial abnormalities, but its use as a screening tool has not been evaluated (58,59).

Evaluations of minimally invasive sampling techniques are uncommon in the literature. As many women who develop endometrial cancer are known to or thought to have progressed from a precursor lesion over a period of 1 to 20 years, there is theoretically a window of opportunity for early detection and intervention. Optimisation of diagnostic markers and collection techniques will need our attention. Women undergoing hysterectomy for endometrial cancer (n=37) or AEH (n=1) and a control group of women undergoing hysterectomy for benign indications (n=37) were recruited to a prospective cohort study at the Mayo Clinic, USA (60). Sampling of vaginal secretions via tampon at least 30 minutes prior to surgery, as well as endometrial brushing under anaesthesia and biopsy of primary tumour was performed. Data analysis showed that endometrial cancer specific DNA methylation markers were detectable in vaginal secretions obtained via tampon, a potentially self administered and widely acceptable method of sampling which may have a future application in screening or in research studies. It is not known whether shedding of cancer cells is

TABLE 2: UK NATIONAL SCREENING COMMITTEE SCREENING PROGRAMME CRITERIA IN THE CONTEXT OF ENDOMETRIAL CANCER

The condition: Endometrial Cancer	**Clear evidence criterion met for endometrial cancer**
Important health problem	Yes
Natural history well understood. Detectable risk factor, disease marker, latent period or early symptomatic stage	Partly
Natural history of genetic mutation carrier status known	Partly
All cost effective preventative measures should be in place	No
Test: ultrasound/endometrial sampling	
Safe, simple, reliable and validated	Yes
Normal distribution known and agreed cutoffs preset	No
Acceptable to patients	Partly
Agreed policy regarding the management of positive results	Partly
Treatment: e.g. hysterectomy	
Effective treatment, and evidence that early treatment improves outcome	No
Adequate health service provision for the extra workload	No
Screening programme	
High quality evidence that screening reduces morbidity or mortality	No
Benefit from screening should outweigh physical and psychological harm	Unknown
Cost implications must be proportional to the benefits of the screening test	Unknown

affected by prior endometrial biopsy as had taken place for diagnosis in the cancer cohort of this study.

1.1.2.3 Screening of high-risk groups

With the exception of patients with Lynch syndrome, there are currently no recommendations for selective screening of high-risk groups (Table 3).

Lynch syndrome

Between 2 and 5% of cases of endometrial cancer are inherited rather than sporadic. Lynch syndrome is associated with a significantly increased risk of endometrial cancer (both type 1 and 2 tumours) compared to the general population, with up to a 60% lifetime risk (cf. 2-3% general population). Lynch syndrome is caused by an autosomal dominant inherited mutation in DNA mismatch repair genes that promotes tumour development affecting the colon, endometrium, ovary and other sites. The risk differs depending upon the germline mutation. The mean age at diagnosis is 47 years, compared to 60 years for non-inherited endometrial cancer, however in the limited comparison data available it appears that prognosis and survival are similar (61).

The high risk of endometrial cancer in Lynch Syndrome and an earlier age at onset, together with a detectable and treatable premalignant or early malignant stage, is justification for screening in these women (62). There is no evidence that screening reduces their mortality from endometrial cancer. Screening does not take the place of risk reducing hysterectomy once family is complete, and there are concerns that should screening reduce the uptake of hysterectomy the incidence of endometrial cancer in this population may increase. It is debatable whether a TVUS is of benefit in a premenopausal woman, due to lack of clarity regarding thresholds of suspicion. Equally, if the ET in a postmenopausal woman is within normal limits it is unclear what additional benefit would be derived from an endometrial biopsy.

Tamoxifen

Routine screening with TVUS, endometrial biopsy or both has not been shown to be effective in women taking tamoxifen. Postmenopausal women using the drug should be routinely questioned at breast cancer follow up appointments about symptoms of vaginal bleeding or discharge and made aware of the risks, and that these clearly outweigh the potential benefits. Tamoxifen use should be limited to 10 years and use

should be reassessed if hyperplasia is identified. Symptomatic women should be investigated with hysteroscopy as well as ultrasound and endometrial biopsy (63). Ultrasound measurements of ET are poorly correlated with endometrial pathology in asymptomatic women using tamoxifen due to tamoxifen induced sub-epithelial stromal hypertrophy. Ultrasound has a high false positive rate, even at an ET cutoff of 10mm, and a low predictive value in this group (64)(65).

OBESITY

In asymptomatic overweight or obese premenopausal women (mean BMI 35.5kg/m^2, range 26.7-66.3) the incidence of hyperplasia may be 5.8%, with 1% having endometrial cancer. In the equivalent postmenopausal population (mean BMI 37.65kg/m^2) up to 12.1% may have undiagnosed hyperplasia and 3% may have occult endometrial cancer (56). However, these figures report hyperplasia without atypia as well as AEH, and so include abnormalities that are less likely to progress to cancer and are probably not strictly pre-malignant as they may reverse spontaneously or will regress with progesterone treatment. When further examining the data it is evident that the rate of AEH or endometrial cancer in this cohort is in the region of 2.2%. Logistic regression demonstrated an increased risk of endometrial abnormality being detected in overweight and obese postmenopausal women, but not in pre-menopausal women.

A recent study of women undergoing bariatric surgery (n=59, median age 39 years, median BMI 46kg/m^2) found no cases of occult atypical hyperplasia or endometrial cancer on endometrial sampling prior to gastric bypass surgery (66). Whilst this study quoted a 6.8% prevalence of endometrial pathology in asymptomatic morbidly obese women, the abnormalities they found were also non-atypical (3 cases of simple hyperplasia without atypia, 1 case of complex hyperplasia without atypia). These findings are likely to be an underestimate of the actual risk, as the cohort was highly selected (any abnormal bleeding in the preceding 6 months excluded) and had a high rate of progestin use for contraception or treatment of menstrual dysfunction (30%). In a smaller asymptomatic bariatric surgery cohort who underwent endometrial sampling (n=30, mean BMI 50.9 kg/m^2) one case of atypical hyperplasia was detected (3.3%). Insufficient samples were obtained from 8 women (27%) (67). Kaiyrlykyzy et al

attempted to sample the endometrium of 47 bariatric surgery patients (mean BMI 46kg/m^2); 38% were unsuccessful. Endometrial abnormality was seen in 28% but this included polyps, metaplasia and hyperplasia. Only 3% demonstrated atypical hyperplasia. Abnormal endometrium was found to be significantly associated with higher BMI compared with normal endometrium (mean BMI 55 kg/m^2 cf. 45 kg/m^2, $p<0.01$) (68).

There is no evidence to support the implementation of a screening programme in women who are obese or, for that matter, women with diabetes or PCOS. There is a lack of evidence that detecting occult disease improves outcomes. Women and clinicians should be aware of the symptoms of concern and the increase in their risk, and of the risk of earlier age at presentation.

TABLE 3: ENDOMETRIAL CANCER: HIGH RISK GROUPS AND EVIDENCE FOR SELECTIVE SCREENING

Risk Factor	Increase in endometrial cancer risk	Evidence	Recommendation
Lynch syndrome (61)	40-60% lifetime risk	Earlier age of onset (mean 47 years) Prognosis and survival appear similar No evidence of reduction in mortality with screening	Offer annual screening (TVUS or outpatient hysteroscopy and endometrial biopsy +/- CA125) from age 35 years. No formalised programme – provision varies Offer risk reducing hysterectomy when family complete
Tamoxifen (18,63)	Postmenopausal women: RR 4.01 (95% CI 1.7-10.9)	Routine screening has not been shown to be effective TVUS sensitivity and specificity low in this group	Patient education and routine questioning at breast cancer follow up regarding symptoms of concern Investigate symptoms with hysteroscopy as well as TVUS and biopsy
PCOS (24)	OR 2.89 (95% CI 1.52-5.48)	No evidence to support screening	Clinicians should be aware of the increased risk and younger age at presentation Induce 4 withdrawal bleeds per year if amenorrhoeic
Diabetes (12)	RR 2.1 (95% CI 1.75-2.53)	No evidence to support screening	Clinicians should be aware of the increased risk
Obesity (69)	Non-linear, dose dependent relationship RR 1.59 per 5kg/m^2 increase in BMI BMI > 42kg/m^2: RR 9.11 (95% CI 7.26-11.51)	No evidence to support screening	Clinicians should be aware of the increased risk

1.1.3 Diagnosis

The most common presentation of endometrial cancer is with post-menopausal bleeding (PMB) and the pre-test probability of endometrial cancer in a woman with PMB is approximately 10%. In women who are obese the pre-test probability is 18%, and in obese women who are diabetic it may reach 29% (70).

Transvaginal ultrasound is used to measure endometrial thickness and can be used to identify women with a low risk of endometrial cancer (71). A large meta-analysis including 5892 women showed that when endometrial thickness on TVUS was ≤5mm, the negative predictive value for detecting endometrial cancer was 96% (53). They concluded that an endometrial thickness of ≤4mm reduced the probability of underlying endometrial cancer to less than 1%.

When the postmenopausal endometrium is thickened an endometrial biopsy is indicated. Dilatation and curettage has been superseded by the use of outpatient aspiration biopsies with devices such as the Pipelle sampler or directed biopsy at outpatient hysteroscopy. Pipelle and similar devices have been shown to be sensitive for detecting endometrial cancer (72). In some women hysteroscopy may be performed to visualise the uterine cavity, and a directed endometrial biopsy may be taken. Hysteroscopy may be indicated in women with additional risk factors, e.g. tamoxifen use, or if an inadequate specimen for analysis was obtained from aspiration biopsy, or if TVUS suggests polyps or irregularities of the uterine cavity. Once histopathological examination of the biopsy has confirmed the histological subtype and grade of the endometrial cancer, further investigations in the form of Magnetic Resonance Imaging (MRI) or Computed Tomography (CT) may be requested for preoperative staging and planning.

Diagnosis can be more difficult in pre-menopausal women when symptoms can often be assumed to be dysfunctional uterine bleeding approaching the menopause. NICE guidelines for Heavy Menstrual Bleeding advise further investigation for women with inter-menstrual bleeding or in women aged over 45 years with heavy menstrual bleeding who had not responded to treatment (73). Current NICE guidelines only mandate referral on a suspected cancer pathway for women with symptoms of concern

who are over the age of 55 years (74). Diagnosis can also be complicated by the absence of data on what constitutes an abnormally thickened endometrium on TVUS in pre-menopausal women, and the huge variation seen amongst this group.

1.1.4 MANAGEMENT

Pre-operative assessment will involve clinical examination as well as either an MRI or CT scan depending upon suspected stage of disease. Final staging however is surgical (Table 4).

TABLE 4: 2009 FIGO STAGING OF ENDOMETRIAL CANCER AND STAGE RELATED 5-YEAR SURVIVAL (75)

Stage	Spread	5YS (%)
I	**Confined to uterus**	
IA	<50% myometrial invasion	97.4
IB	≥50% myometrial invasion	
II	**Invades cervical stroma but no extension beyond uterus**	80.2
III	**Local/regional spread**	
IIIA	Invades uterine serosa +/- adnexae	
IIIB	Vaginal involvement +/- parametrial extension	59.6
IIIC1	Positive pelvic lymph nodes	
IIIC2	Positive para-aortic lymph nodes	
IV	**Bladder or bowel involvement or distant spread**	
IVA	Invades bladder/bowel mucosa	28.6
IVB	Distant metastases incl. intra-abdominal disease/inguinal nodes	

1.1.4.1 SURGERY

The mainstay of treatment is surgery, either open or laparoscopic, to remove the uterus, fallopian tubes and ovaries. Following the publication of several studies and most recently a meta-analysis that included 9 RCT's and 1263 patients, surgery is increasingly being performed laparoscopically, and indeed has become the standard of care, as it appears to reduce complication rates and improves recovery time (major complications with total laparoscopic hysterectomy (TLH) RR 0.53, 95% CI 0.29-0.98) (76). Laparoscopic hysterectomy has also been shown to improve patient-reported outcome measures in the short term. In cases where cervical involvement is suspected radical hysterectomy and bilateral salpingo-oophorectomy (BSO) is preferred. Full inspection of the abdominal cavity with omentectomy and lymphadenectomy may also be indicated.

The role of pelvic and para-aortic lymphadenectomy as part of surgery remains controversial, and practice varies between centres and internationally. A Cochrane review which examined 2 RCT's including 1945 women found that there was no evidence that lymphadenectomy reduced the risk of death or disease recurrence in presumed stage 1 disease, but did increase morbidity (77). The MRC ASTEC study concluded that pelvic lymphadenectomy did not confer an improvement in overall survival or progression-free survival (i.e. a therapeutic benefit) in women with early endometrial cancer. Whilst lymphadenectomy does not appear to improve survival, improved staging of women who are suspected to have more advanced disease may reduce the numbers of women undergoing adjuvant chemoradiotherapy (78). Controversy in this area persists and practice varies between surgeons and internationally. STATEC is an international RCT that will be the largest randomised trial in endometrial cancer to date and aims to determine whether or not lymph node assessment (lymphadenectomy or sentinel node biopsy) should form part of the standard treatment of endometrial cancer.

1.1.4.2 ADJUVANT TREATMENT

Poor prognostic indicators include lymph node involvement, high grade or advanced stage, more aggressive non-endometrioid subtypes, cervical involvement, more than 50% myometrial involvement and lymphovascular space invasion and these women

receive adjuvant treatment (16). High-intermediate risk cases may receive adjuvant vaginal brachytherapy (VBT), which reduces the risk of vault recurrence and increases relapse-free survival, but not overall survival. External beam radiotherapy (EBRT) reduces pelvic relapse but is now limited to a small number of patients who have high risk or advanced disease, as its benefits relative to its morbidity are unclear due to the high levels of toxicity (79–81). A Cochrane analysis of 5 trials (n=2965 women) found that whilst EBRT reduced locoregional recurrence there was no significant impact upon cancer related mortality or overall survival (HR 0.99, 95% CI 0.82-1.2), and that for intermediate to intermediate/high risk cases VBT alone was adequate (79). The PORTEC 2 study found that VBT was effective in ensuring vaginal control of disease but had less GI toxicity than EBRT (81).

Chemotherapy also has a role in advanced stage disease (82) and, although the evidence is not strong, is being used increasingly as adjuvant therapy in high risk disease. A meta-analysis of 3 observational studies and 3 RCT's suggested there may be a benefit of chemoradiotherapy in reducing disease progression and overall mortality in advanced stage disease (83). The PORTEC 3 study is trying to help us understand more clearly who should and should not receive adjuvant chemotherapy, and was on target to complete primary data collection in December 2015.

1.1.4.3 Non-standard treatment

Many patients with endometrial cancer are elderly and may have multiple comorbidities so surgery and adjuvant treatments must be tailored to take account of this. In some cases primary radical radiotherapy may be used instead of surgery, although this should be considered a suboptimal treatment option. Progestagen treatment has also been used in this setting for disease control.

Approximately 20% of women diagnosed with endometrial cancer are premenopausal, and 5% are below the age of 40, meaning fertility preservation may be desired. Fertility sparing treatment with high-dose progestagens may be an option in stage 1a well differentiated endometrioid tumours, however the optimal type and duration of treatment is unknown and recurrence is common (84,85). Ovarian preservation to

avoid surgical menopause may also be requested although patients remain at risk of synchronous ovarian disease (86,87) and will be suboptimally staged.

A systematic review conducted in 2012 included 45 studies, and 391 women, and examined the oncologic and reproductive outcomes of hormonal therapy for atypical hyperplasia and grade 1 endometrial cancer (88). In this review 19% of women were treated with a LNG-IUS and 74% with oral medroxyprogesterone or oral megestrol. A complete response was seen in 77.7%, with a complete response and no evidence of recurrence demonstrated in 53.2%. Response was better in endometrial hyperplasia (65.8%) than for endometrial cancer (48.2%), suggesting important biological differences between the two entities, however in clinical practice they are frequently treated the same (with surgery) due to concerns about coexistent carcinoma.

Better responses and recurrence rates have been seen with levonorgestrel-releasing intrauterine systems (LNG-IUS) (Mirena, Schering Health Care, Burgess Hill, U.K.). Known to be associated with a reduced incidence of endometrial cancer, their use in the treatment of endometrial hyperplasia and cancer has more recently been described in several small prospective studies. Regression rates at 12 months of over 85% have been reported in AEH, with recurrence rates comparable to those of complex or simple hyperplasia which has been treated with intrauterine progestagen (89,90).

One U.K. study used LNG-IUS as treatment for AEH in 19 women who declined or were not fit for hysterectomy (90). At 12 months regression of disease was seen in 16/19 (84.2%) women. One woman underwent trans-cervical resection of the endometrium (TCRE) for persistent complex hyperplasia without atypia. The remaining two women had hysterectomy within three months of diagnosis; one had persistent atypia and one had a well-differentiated stage 1b endometrial adenocarcinoma. At seven years of follow up there were no cases of recurrence and no further cancers detected. Larger prospective studies are needed to reliably comment on the rates of disease progression and risk of occult cancer in this group.

Ørbo et al enrolled 170 women aged 30-70 with low-medium risk endometrial hyperplasia into a RCT to compare LNG-IUS with cyclic or continuous

medroxyprogesterone (MPA). In the women with atypical hyperplasia regression of disease was seen in 100%, 88% and 60% of those on LNG-IUS, continuous oral MPA and cyclic oral MPA respectively (91). The 135 women in this study who responded to progestagen treatment underwent 24-month follow up. 41% relapsed during the follow up period and the risk of relapse appeared independent of BMI and treatment arm (92).

Six months was the median time needed to see a complete response, but other studies have suggested longer may be required (93), particularly perhaps in women who are obese and/or anovulatory. The median time to recurrence was 24 months. Pregnancy data was available for 38 studies (n=315). It is unclear how the results were calculated but it appears that 41% of women with AEH (28/111) and 34.8% of women with endometrial cancer (86/280) became pregnant either spontaneously or through IVF. Not all women who chose fertility sparing treatment attempted to conceive.

A prospective phase II trial, which has closed to recruitment but not yet reported, aims to evaluate the use of the LNG-IUS to treat atypical hyperplasia and grade 1 EEC. Initial results showed a 58% overall 12 month response rate (85% CAH and 33% endometrial cancer), although at 12 months 5/26 (19.2%) patients had progressive disease (4 endometrial cancer patients and 1 complex atypical hyperplasia patient) (94). A phase II RCT of LNG-IUS +/- metformin +/- non-surgical weight loss in patients with early stage endometrial cancer or atypical hyperplasia (the feMMe trial) is open to recruitment for women who have a BMI ≥ 30kg/m^2 who choose fertility sparing treatment or who are unfit for surgical management due to obesity or comorbidity (95).

1.1.4.4 Patient selection for fertility sparing treatment

The notion of patient selection for non-surgical treatment of atypical hyperplasia or well-differentiated endometrial cancer is in fact two separate issues: can we identify women who are more likely to have coexistent endometrial cancer and can we identify up front women who will or will not respond to progestin treatment. Within our group the MIRENA study is underway, which aims to identify an endometrial signature of response to LNG-IUS, which may assist the triage of women to hysterectomy at diagnosis if they are unlikely to respond to LNG-IUS.

In their retrospective review of 46 patients (median age 35, median BMI 37 kg/m^2) treated with oral or intrauterine progesterone for atypical hyperplasia (37%) or well-differentiated endometrial cancer (63%) Gunderson et al reported that pre-treatment ER and PR expression did not predict response to treatment (96). Lower ER and PR levels have been reported in patients with concurrent endometrial cancer, as typically hormone receptor expression is preserved in precursor lesions (97).

Stathmin is an oncogene that has been validated as a surrogate marker for PTEN loss and PI3K dysregulation. It has been found to be frequently overexpressed in preoperatively undetected EEC in women undergoing hysterectomy for atypical hyperplasia and may be a significant indicator of risk of coexistent occult carcinoma (97).

1.2 Normal physiology of the endometrium

1.2.1 Hypothalamic-pituitary axis

Gonadotropin Releasing Hormone (GnRH) is secreted by the hypothalamus in a pulsatile manner and via the hypophyseal portal system acts upon the anterior pituitary gland, stimulating the production of the gonadotropic hormones, Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH). FSH and LH stimulate gonadal secretion of the sex steroids, oestrogen and progesterone in females and testosterone in males. They also regulate germ cell development.

1.2.2 Ovarian cycle

This elevation in FSH level stimulates an antral follicle to continue to grow, beyond the stage it reached independent of FSH secretion. The inner granulosa cell layer enables oestrogen production, and the secretion of oestradiol into the circulation exerts negative feedback control over FSH, preventing further follicles from being stimulated by FSH in that cycle. The rising oestradiol level triggers an LH surge from the anterior pituitary, bringing about final maturation of the oocyte and leading to follicle rupture and ovulation. Following ovulation progesterone is produced from the ruptured follicle (corpus luteum), which then facilitates the secretory phase of the menstrual cycle. If pregnancy does not occur, and hCG secretion does not begin, the corpus luteum involutes leading to reduction in progesterone levels and menstruation.

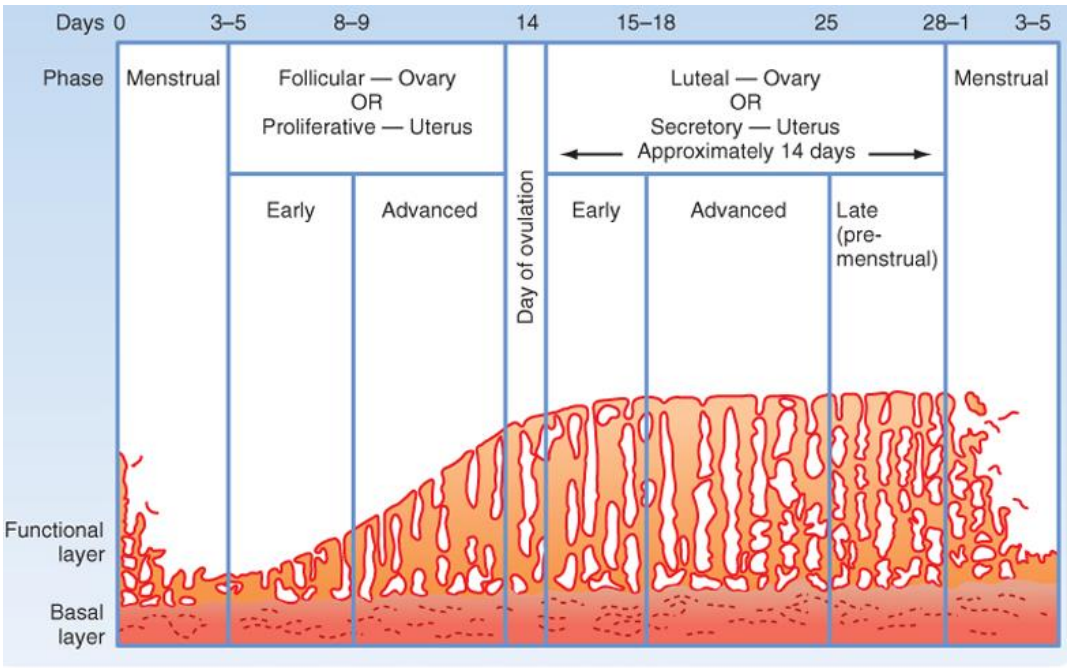
1.2.3 Menstrual cycle

The endometrial cycle describes a series of repeating changes in the endometrium in response to hormonal stimulation by the ovaries (Figure 2). The menstrual phase sees patchy degeneration of the functional zone of the endometrium, due to constriction of spiral arteries, and results in bleeding into the functional zone and sloughing of the endometrium. After this the epithelial cells of the glands of the basilar zone proliferate and restore the integrity of the endometrium, stimulated by the ovarian follicles secreting oestrogen. This proliferation continues up until ovulation, by which point the functional zone is highly vascularised, with prominent glands filled with glycogen-rich mucus. Following ovulation the secretory phase is maintained by progesterone from the corpus luteum, and sees enlargement of glands, increasing secretion and vascularisation of the entire functional zone. If fertilisation does not occur the corpus

luteum collapses at around 12-14 days post ovulation, and the reduction in oestrogen and progesterone levels cause the endometrial cycle to begin again as menstruation occurs.

After the menopause oestrogen and progesterone levels fall and this loss of negative feedback sees a sustained rise in GnRH secretion and FSH and LH levels rise. The endometrium is often described as atrophic, and the presence of proliferating glands is unusual. Stromal fibrosis may be seen due to aging or parity.

FIGURE 2: THE ENDOMETRIAL CYCLE (98)



Koeppen & Stanton: Berne and Levy Physiology, 6th Edition.

1.2.4 Effect of obesity on normal physiology

Oligomenorrhoea is more common in obese premenopausal women than women of normal bodyweight (26% and 14% respectively in a cross-sectional study of 6,840 women) and both body composition and menstrual irregularity have been associated with high testosterone and FAI and, to a greater extent, raised insulin and low SHBG levels (99). Compared with normal weight women, obese women had a more than two fold increase in irregular menstrual cycles when defined by BMI (OR 2.61, 95% CI 1.28-

5.35) or by waist hip circumference (OR 2.27, 95% CI 1.09-4.72) (99). Although difficult to objectively assess, heavy menstrual bleeding is recognised as being more common in obese women also (100).

In women the pattern of GnRH secretion controls the menstrual cycle. Abnormalities in GnRH secretion are associated with a variety of reproductive endocrine disorders. In PCOS LH pulse frequency and amplitude are higher throughout the menstrual cycle than in healthy women, which is thought to contribute to chronic anovulation and amenorrhoea/oligomenorrhoea. Hypothalamic-pituitary-gonadal axis dysfunction due to polycystic ovary syndrome is the most common cause of ovulatory disorders. There is often difficulty separating out PCOS from obesity related reproductive effects as there is often overlap in the clinical picture and androgens may be raised due to obesity alone. Obesity has also been associated with increased LH pulse frequency in women who are oligomenorrhoeic.

Obesity can also lead to a picture of hypopituitarism; wherein high estradiol levels suppress FSH and LH levels. Decreased SHBG levels and insulin stimulation of androgen production in ovarian stroma can all lead to significant disturbance of normal ovulation and menstruation.

Higher rates of hysterectomy for benign disease are seen in obese women. There may be a variety of reasons for this, in addition to their increased incidence of menstrual dysfunction. Obese women have been shown to have a longer time to achieve amenorrhoea with the LNG-IUS (6-12 months), and when taken orally obese women demonstrated lower peak levonorgestrel levels than their normal weight counterparts. The COC is likely to be contraindicated, if BMI is > 35kg/m2. A reduction in the availability and efficacy of uterine sparing treatments for benign indications probably explains the observed increase in hysterectomy rates seen in obese women. Indeed, current estimates of endometrial cancer risk do not adjust for hysterectomy rates and are therefore likely to underestimate the association between obesity and cancer risk, if hysterectomised women are not excluded from the denominator population (101).

1.3 Physiological alterations implicated in endometrial carcinogenesis

1.3.1 Endometrial proliferation

The proliferative phase of the endometrial cycle sees oestrogen acting to promote cell proliferation and endometrial regeneration, characterised by rapid epithelial growth and programmed angiogenesis.

Ki-67 is a nuclear antigen, a protein expressed in all active phases of the cell cycle, and acts as an indicator of the proliferative activity of a cell and a marker of its mitotic index. Development of antibodies for Ki-67 allows the detection of proliferating cells in FFPE sections of endometrium. It has been extensively used to measure proliferation, including in histologically normal endometrium. Ki-67 expression varies across the menstrual cycle, and is highly expressed in the proliferative phase. Expression is reduced or absent in the secretory phase and in post-menopausal endometrium. In histologically normal endometrium Villavicencio et al found Ki-67 and phosphorylated histone (p-H3) levels correlated positively with increases in BMI, serum oestrogen, leptin and insulin levels, albeit in three small groups of normal, overweight and obese women (n=10, 9 and 12 respectively) and it is unclear if the oestradiol levels were trough or random levels in terms of menstrual cycle timing (102). Phosphorylated histone 3 reinforces the information Ki-67 gives regarding proliferation, as it is only expressed during the M phase of the cell cycle and is a complementary measurement of proliferation rate (102).

Increased cellular proliferation is one of the “hallmarks” of cancer, where cells increase in number unchecked. In endometrial cancer Ki-67 expression is positively correlated with grade, FIGO stage and histological subtype (Table 5) (103). Increased expression is independently associated with a poorer prognosis (104), although such studies are relatively old and current staining and scoring protocols can differ significantly. Specifically, “hot spots” of staining intensity were selected for scoring, rather than representative fields, which better account for the heterogeneity of staining one would expect to see.

TABLE 5: VARIATION IN KI-67 EXPRESSION ACROSS THE SPECTRUM OF ENDOMETRIAL CANCER (103)

Prognostic indicator		**Median Ki-67 expression**
Stage	I/II	17%
	III/IV	37%
Histological subtype	Endometrioid	20%
	Serous/clear cell	39%
Grade	1	10%
	2	16%
	3	27%

High levels of proliferation have been described in terms of an "obesity phenotype". When proliferative phase endometrial biopsies from normal weight and obese controls were compared to biopsies from obese postmenopausal women with type I EEC, two groups were identified in the obese controls. High proliferating women had Ki-67 levels similar to EEC cases and low proliferating women had Ki-67 levels similar to normal weight controls. No significant differences were found between the high and low proliferating groups in BMI, SHBG or androgen levels. The high proliferating group had significantly higher circulating oestradiol and ERα expression than both the low proliferating controls and the EEC cases (although they were postmenopausal) (105). Ki-67 positivity was found to be increased 9.9 and 12.6 fold in overweight (mean BMI 27kg/m^2) and obese (mean BMI 36kg/m^2) pre-menopausal women respectively when compared with normal weight pre-menopausal women (mean BMI 22kg/m^2). Phosphorylated histone 3 was also increased in obesity (102).

1.3.2 ENDOMETRIAL APOPTOSIS

Apoptosis, or programmed cell death, is a process by which cells are eliminated in the absence of an inflammatory response. It is characterised by distinct morphological and biochemical events (Table 6). It plays a critical part in embryological development and

tissue homeostasis, as well as serving an immunological function. It can be induced by many stimuli, proceeding through a regulatory pathway before cells undergo structural alterations.

TABLE 6: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF APOPTOSIS

Morphological changes	Biochemical changes
Membrane blebbing	ATP dependent process
Chromatin aggregation	Non-random DNA fragmentation
Cytoplasm shrinkage	Cytochrome c release (& other factors)
Nuclear condensation	Activation of caspase cascade
Cell fragmentation	Altered membrane biochemistry
Formation of apoptotic bodies	
Pore formation in mitochondrial membrane	

Adapted from Harada et al (106)

Apoptosis eliminates cells from the functional endometrial layer during the late secretory and menstrual phases. Minimal apoptosis is seen in proliferative or early secretory endometrium, and it is probable that oestrogen and progesterone regulate the induction of apoptosis in endometrium given the cyclical nature of apoptosis in normal endometrium and a demonstrable response to exogenous progestagen (107).

Apoptotic control has been linked to two key pathways. B cell lymphoma 2 (Bcl-2) and Bax proteins control an intrinsic mitochondria dependent pathway, and a further Fas mediated extrinsic pathway, associated with tumour necrosis factor (TNF) group receptors and adaptor proteins, has been described. These pathways direct cells through a final common pathway wherein a proteolytic cascade of caspase proteins is initiated (107) and matrix metalloproteinase (MMP) expression peaks. Caspase enzymes degrade structural and regulatory proteins, causing DNA fragmentation and cell re-organisation.

Overexpression of the Bcl-2 gene is associated with neoplasia, and correlates with treatment response and outcome in non-endometrial tumours. Reduced cytoplasmic and increased nuclear expression of Bcl-2 has been shown to correlate with a poorer outcome in endometrial cancer (108). This increase in Bcl-2/Bax ratio promotes cell proliferation and tumour genesis (109). In the endometrium of women with PCOS increased Bcl-2/Bax ratio is evident, irrespective of the phase of the endometrial cycle. The ratio is proportionately higher in PCOS with AEH than PCOS with normal or non-AEH endometrium. Unopposed oestrogen has been identified as a Bcl-2/Bax inducer, and activation of oestrogen receptors has been shown to cause Bax suppression and consequent Bcl-2 up-regulation (109). Aberrant Bcl-2/Bax expression is reported in many other cancers, including breast, prostate and pancreatic tumours (110).

1.3.3 OESTROGEN AND PROGESTERONE

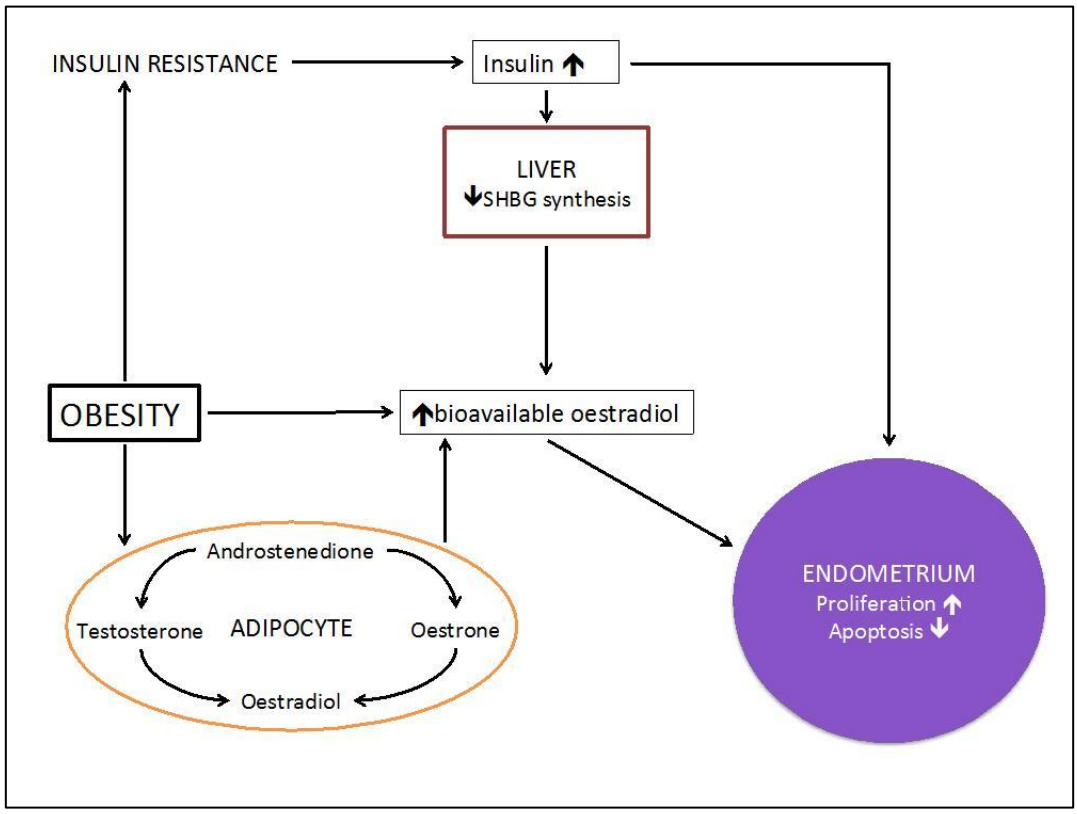
1.3.3.1 HYPEROESTROGENAEMIA

Oestrogen is a growth factor and can promote cell proliferation and inhibit apoptosis, in contrast to the ability of progesterone to inhibit proliferation and promote apoptosis, and to down-regulate expression of the oestrogen receptor. Increased proliferation can increase the likelihood of random mutations occurring, and through promoting proliferation oestrogen confers a selective advantage on mutated cells that have escaped the usual eradicative checkpoints.

Obesity is a hyper-oestrogenic state as androgens are converted to oestrone and oestradiol by aromatase in adipose tissue. Peripheral aromatisation of androgenic precursors replaces the ovaries as the primary source of oestrogen production in post-menopausal women. Aromatase levels increase correspondingly with both age and obesity (111) and can be seen along with an increase in circulating oestrogen levels with rising BMI in post-menopausal women. However, oestrogen levels in postmenopausal women are unlikely to reach supra-normal levels even in obese women with high levels of endogenous hormones, therefore it is postulated that the development of endometrial cancer is potentiated by another stimulant, such as hyperinsulinaemia (29).

Furthermore, levels of sex hormone binding globulin (SHBG) are reduced in obese subjects. SHBG is a glycoprotein which binds testosterone and oestrogen, reducing their free 'active' fractions in the serum and thereby diminishing their activity (22). The reduction in SHBG with obesity is thought to be partly related to high insulin levels (Figure 3). Oestrogens bind directly to DNA to increase transcription, and interact with PI3K and MAPK signalling pathways favouring tumorigenesis. These pathways are frequently hyperactivated in the presence of cancer.

FIGURE 3: THE SYSTEMIC EFFECTS OF OBESITY AND HORMONES



1.3.3.2 THE UNOPPOSED OESTROGEN EFFECT

Excessive or 'unopposed' oestrogenic stimulation of the endometrium, for example in postmenopausal women or chronic anovulatory states (e.g. PCOS), drives tumour genesis. Progestagens, naturally released during the second half of the menstrual cycle in ovulating women, or delivered exogenously, 'protect' the endometrium from the proliferative effects of oestrogen (112).

A prolonged absence of progesterone with continuous exposure to oestrogen therefore promotes endometrial proliferation and may eventually result in hyperplasia and/or neoplasia. After the menopause the ovarian production of oestrogen and progesterone ceases, however peripheral oestrogen generation continues unabated in overweight and obese women. As a result, there is inadequate progesterone to counteract the proliferative and anti-apoptotic effects of oestrogen (22). It is hypothesised that endometrial cancer risk is particularly increased in women with a high serum oestrogen to progesterone ratio. Epidemiological studies would seem to support this as it has been shown that risk is increased in women taking oestrogen only HRT or combined oral contraceptives with less than 10 days of progestin per approximately 16 days of oestrogen. This is in contrast to the use of combined HRT, which does not increase endometrial cancer risk (69), or combined oral contraceptives that deliver constant levels of oestrogen and progestagen, which are protective against endometrial cancer. Some have suggested that the use of combined oestrogen/progesterone HRT ameliorates the increase in endometrial cancer seen with increasing BMI (69,113).

1.3.3.3 Oestrogen and progesterone receptor expression

Both oestrogen and progesterone exert their effects through intra and extra-nuclear hormone receptors. As previously stated, endometrium is a hormone responsive tissue and as such it variably expresses both oestrogen (ER) and progesterone receptors (PR) throughout the menstrual cycle. Oestrogen receptors (ERα and ERβ) are encoded by different genes and have distinct patterns of expression and physiological roles, ERα drives endometrial proliferation and ERβ is thought to exert anti-proliferative effects. Oestrogen receptors mediate oestrogenic actions, regulating cell growth and differentiation of normal tissues and hormone-responsive tumours. Hormone receptor complexes bind directly to DNA, or work indirectly by interacting with co-regulator proteins to modulate transcription.

Progesterone receptors exist in two isoforms, PR-A and PR-B, which also mediate distinct physiological effects. Their expression varies across the menstrual cycle, both peaking immediately prior to ovulation and gradually declining thereafter; PR may be absent from the glandular epithelium in the late luteal phase. Expression is thought to

be maintained in the early menopause (114). PR-A expression correlates with ERα, and given that the transcription of the PR gene is oestrogen induced and often inhibited by progesterone, the two are considered to be interlinked. Postmenopausal obese females have been reported to have higher levels of PR expression in endometrial polyps than normal weight postmenopausal females. No difference has been reported in ER expression in polyps (115). Both ER and PR levels have been shown to be increased in the endometrium of obese women (116) and ER expression has been implicated in the development of metabolic dysfunction (117). ERα has been shown to have an influence on glucose metabolism and adiposity and in murine models ERα-null mice demonstrate pronounced adiposity and insulin resistance and, in wild type mice with no ovaries on a high fat diet, sustained exogenous oestrogen therapy prevents fat acquisition and insulin resistance (118).

Grade 1 and 2 endometrial cancers have been shown to have reduced ERα and PR-A expression, both superficial and stromal, when compared to non-malignant controls (superficial ERα 71.7% ±25.6 cf. 90.7% ± 29; superficial PR-A 93.7% ±13.9 cf. 74.7% ±29) (119). Whilst expression of ER and PR is usually maintained in type I tumours, loss of ER or PR expression is often seen in advanced stage, poor prognosis endometrial tumours. Loss of PR-A expression has been reported in both well-differentiated and poorly differentiated endometrial cancers, and over-expression of PR-B has been reported in poor prognosis endometrial, cervical and ovarian cancers. Decreased PR expression has been correlated with myometrial invasion (119) and with increased proliferative activity (Ki-67) (103). The mechanism by which ERα and ERβ interact with endometrial cancer progression is unclear. Cell line work using Ishikawa cells (ERα and ERβ positive) and KLE cells (ERβ only) has demonstrated that overexpression of ERα activates the PI3K signalling pathway. Both ERα and ERβ enhanced cell migration, invasion and proliferation when up regulated, which would seem to contradict loss of ER expression in advanced cancer but may be an early event which precedes receptor loss (120).

1.3.4 Insulin resistance & hyperinsulinaemia

1.3.4.1 Insulin resistance

Insulin resistance is a modifiable precursor to diabetes mellitus and describes a condition where target tissues have reduced sensitivity to insulin, leading to elevated blood glucose and insulin levels. It is thought to contribute to the development of breast, prostate, colon, endometrial and pancreatic cancers (22). These cancers are also strongly linked with obesity, and it can prove difficult to separate out the individual impact of these two risk factors (29). Insulin resistance is thought to be a poor prognostic feature in association with breast, colon and prostate cancer survival (121).

Obesity, diabetes and PCOS are all associated with insulin resistance and are known risk factors for endometrial cancer (11,22). Type II diabetes has been linked to colorectal, pancreatic, endometrial and renal cancers, in particular recently diagnosed type II diabetes which would coincide with the observation that early type II diabetes is characterised by a compensatory hyperinsulinaemia. There is some evidence to suggest that insulin treatment for diabetes may itself increase cancer risk, in the case of colorectal cancer (122).

A prospective, multi-institutional study from the MD Anderson Cancer Centre in Houston examined the incidence of insulin resistance in a cohort of 99 newly diagnosed endometrial cancer patients (11). Of these, two thirds were insulin resistant. On multivariate analysis, high BMI, low socioeconomic status and nulliparity were significantly associated with insulin resistance in women with endometrial cancer. Tumour characteristics did not show any variation between insulin resistant and non-insulin resistant women. In both groups endometrioid tumours prevailed and stage and grade were equivalent. Other prospective studies have found similar levels of insulin resistance in endometrial cancer patients (123).

It is thought that the increased risk of endometrial cancer with PCOS is due to insulin resistance. A prospective cross sectional single centre study compared endometrium from women with PCOS without endometrial cancer, women with endometrial cancer without PCOS and normal controls (n=34 in each group, age 18-45) and found that women with endometrial cancer and women with PCOS have increased expression of

genes involved in insulin signalling in the endometrium compared to controls, independent of BMI, oestradiol and androgen levels and HOMA-IR. IGF1 and IGFBP1 genes are significantly overexpressed in endometrial cancer and in PCOS endometrium compared with controls (124).

1.3.4.2 Hyperinsulinaemia

Beta cells of the pancreas secrete insulin, a two-chain peptide, to regulate fat and carbohydrate metabolism. Hyperinsulinaemia is an independent risk factor for endometrial cancer development, in both obese and non-obese individuals (22). The bulk of evidence so far is retrospective and insulin resistance is difficult to assess retrospectively as it requires fasting blood samples.

Direct activation of the insulin receptor triggers intracellular signalling cascades. Ishikawa 3-H-12 endometrial cancer cells express insulin receptors, and the administration of insulin results in dose dependent induction of proliferation and inhibition of apoptosis (22). Receptor mediated phosphorylation of the insulin receptor substrate 1 (IRS-1) scaffold protein activates both the PI3K/AKT/mTOR and Ras/MAPK pathways. The PI3K/AKT/mTOR pathway is inhibited by AMPK. AMPK is frequently inactivated in association with obesity and insulin resistance, possibly by leptin (125). Chronically elevated serum insulin levels have both mitogenic and anti-apoptotic effects (39). However, it is thought that physiological levels of insulin, even the elevated levels seen in obese and insulin resistant individuals, are insufficient to have directly mitogenic effects.

Indirectly insulin inhibits the hepatic synthesis of SHBG, which is responsible for the regulation of the levels of bioavailable sex hormones. Thereby hyperinsulinaemia causes increased circulating oestrogen. In addition, insulin promotes ovarian production of androgens (22).

The insulin-cancer hypothesis speculated that prolonged hyperinsulinaemia reduced the production of Insulin-like Growth Factor Binding Proteins 1 and 2 (IGFBP-1, IGFBP-2) increasing the levels of bio-available IGF-1, favouring tumour development (126). Obese individuals have lower levels of IGFBP-1 and IGFBP-2, and these are inversely

related to insulin levels. IGF-1 is mitogenic, promotes angiogenesis and inhibits apoptosis, conditions which favour tumour development. However, more recent data has failed to substantiate these associations and highlighted flaws in this hypothesis, which would seem now to be an over-simplification of events, and problems such as heterogeneity of study design are thought to contribute to this (127).

Unfortunately the literature on insulin and the IGF system in relation to cancer risk, and biomarker studies in general, are likely to suffer from reporting or publication bias due to the skew towards the reporting and publication of positive results (128).

1.3.5 INFLAMMATION

Inflammation has a central role in the normal regulation and remodelling of the endometrium during the menstrual cycle, where cycles of rapid growth, differentiation and shedding induce inflammation, the response of the body to tissue damage and its subsequent cascade of inflammatory events. Cytokines are synthesised and released by the endometrium under the control of sex steroids.

Adipose tissue is a complex endocrine organ in its own right, secreting an assortment of pro- and anti-inflammatory cytokines and hormones, which create a state of chronic systemic inflammation related to obesity. Secretion of tumour necrosis factor alpha (TNFα) induces phosphorylation of IRS proteins, activating the PI3K/AKT/mTOR and MAPK signalling pathways (Figure 10). In addition, other pro-inflammatory adipokines are secreted which have been linked to endometrial cancer development, namely interleukin-6 (IL-6), resistin and leptin. High serum levels of C reactive protein (CRP) have also been found in endometrial cancer, and obesity has been described as a "chronic sub-clinical inflammatory state" (121). Pro-inflammatory markers have been implicated in carcinogenesis via direct and indirect effects on innate and adaptive immunity, altered tissue homeostasis and increased oxidative stress.

Many of the risk factors described in endometrial cancer are associated with chronic inflammation (obesity, diabetes, inactivity) and inflammation has been linked to the promotion and progression of cancer. Potential mechanisms for this could be direct, via alteration of the nuclear factor kappa-light-chain enhancer of activated B cells (NFκB)

pathway, or indirect, resulting from the development of insulin resistance or modulation of aromatase activity by the cytokines in adipose tissue.

Endometrial cancer patients have altered inflammatory cytokine expression, with higher levels of CRP, TNFα and IL-6 being described, amongst others. These inflammatory markers also correlate with BMI, and after adjusting for BMI only TNFα remains a significant predictor of endometrial cancer risk (TNFα > 1.33pg/mL, OR 1.73) (129). Expansion of white adipose tissue is thought to lead to the secretion of pro and anti-inflammatory cytokines resulting in higher than normal levels of IL-6, TNFα, leptin and VEGF and lower levels of adiponectin.

The NFκB pathway regulates apoptosis, proliferation and growth arrest, enhances angiogenesis via VEGF and is a second messenger system for inflammatory cytokine signalling. Aberrant NFκB activity has been described in many cancers, including oestrogen associated breast cancer due to mutation or upregulation of upstream signalling kinases. TNFα activates NFκB and IL-6 directly stimulates endometrial cancer cell proliferation and results in local oestrogen biosynthesis. Inhibition of NFκB - ERK signalling blocks this (130).

Progesterone and the progesterone receptor have anti-inflammatory effects in the endometrium, as they mediate the NFκB activation normally seen in the proliferative phase. Consequently, anovulatory cycles or unopposed oestrogen exposure can be perceived as promoting inflammation in the endometrium.

It has been suggested that the link between inflammation and cancer risk has been overstated, also due to publication bias affecting those studies that produced statistically significant results, and particularly in relation to circulating CRP. Data from the Women's Health Study did not substantiate previously reported positive associations between CRP and colorectal cancer, and whilst low-grade colorectal cancer may well be in part inflammatory in its aetiology, there is little evidence to suggest local and systemic markers of inflammation correlate (128).

1.3.6 ADIPOKINES

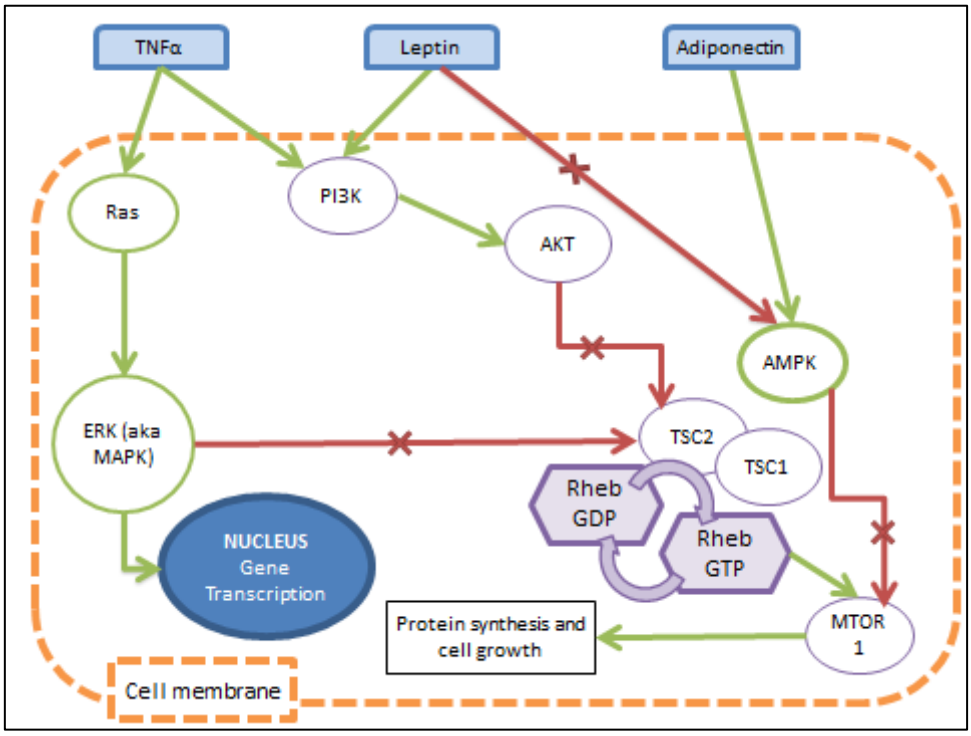
Leptin is secreted by adipocytes in white adipose tissue and is under the positive feedback control of insulin (131) via the Leptin *ob* gene to signal suppression of appetite. Leptin levels are chronically increased in obese individuals, with levels being proportional to the amount of body fat, and in obese subjects a degree of central leptin resistance is assumed (132). Leptin deficient mice overfeed and rapidly become hyperinsulinaemic and develop diabetes. Leptin is pro-angiogenic, anti-apoptotic and pro-inflammatory and has been shown to promote the proliferation and invasiveness of endometrial cancer cells (22,131). It activates PI3K and MAPK transcription signalling pathways which are essential for cell persistence, proliferation and differentiation (121) and is thought to inactivate AMPK (Figure 4). Leptin has both central and gonadal actions on the hypothalamic-pituitary-ovarian axis, and is thought to contribute to the abnormalities seen along this axis in obese women.

Diurnal variation in leptin levels has been described, and fasting also reduces leptin levels even in the absence of weight loss (133). In rodents reductions in leptin levels as a product of caloric restriction reduces tumour growth and increases cancer remission (134). In oestrogen related breast cancer serum leptin is found in higher levels than in breast cancer associated with BRCA1 mutations, and leptin has been found to stimulate the proliferation of cancer cells by activation of the MAPK pathway (131). Binding of leptin to the leptin receptor, ObR, stimulates cell growth and survival via the PI3K and AKT pathways, as well as increasing cell migration and invasion via the Rac/Rho pathways. Women with endometrial cancer have been shown to have higher levels of leptin and increased expression of the ObR receptor. ObR expression is increased in carcinoma, compared to normal tissue, however no correlation with overall or disease free survival has been demonstrated (135).

Adiponectin is predominantly secreted by adipocytes and serum levels are inversely correlated with central obesity and BMI. It is thought to improve insulin resistance in peripheral tissues and may exert anti-neoplastic activity via suppression of proliferation and angiogenesis and induction of apoptosis. Low levels have been associated with hyperinsulinaemia, type 2 diabetes, atherosclerosis, metabolic syndrome and fatty liver disease.

Adiponectin levels have also been shown to be independently and inversely related to endometrial cancer risk in a European prospective study and several retrospective studies (136). However, the prospective Nurses' Health Study published in 2011 did not confirm this. This may reflect the seven year time period between serum testing and development of endometrial cancer in this study (137).

FIGURE 4: THE INTERACTIONS OF ADIPOKINES WITH PRO-CARCINOGENIC SIGNAL TRANSDUCTION PATHWAYS



It is unlikely that adiponectin acts purely through its effects on insulin resistance for several reasons (138):

- LKB1 knockdown has been shown to negate adiponectin-mediated inhibition of proliferation, colony formation, adhesion and invasion of endometrial cancer cell lines and the tumour suppressor function of LKB1 results from its ability to activate AMPK.
- AKT phosphorylation is down regulated by adiponectin in PTEN competent cell lines but not in PTEN deficient cells.

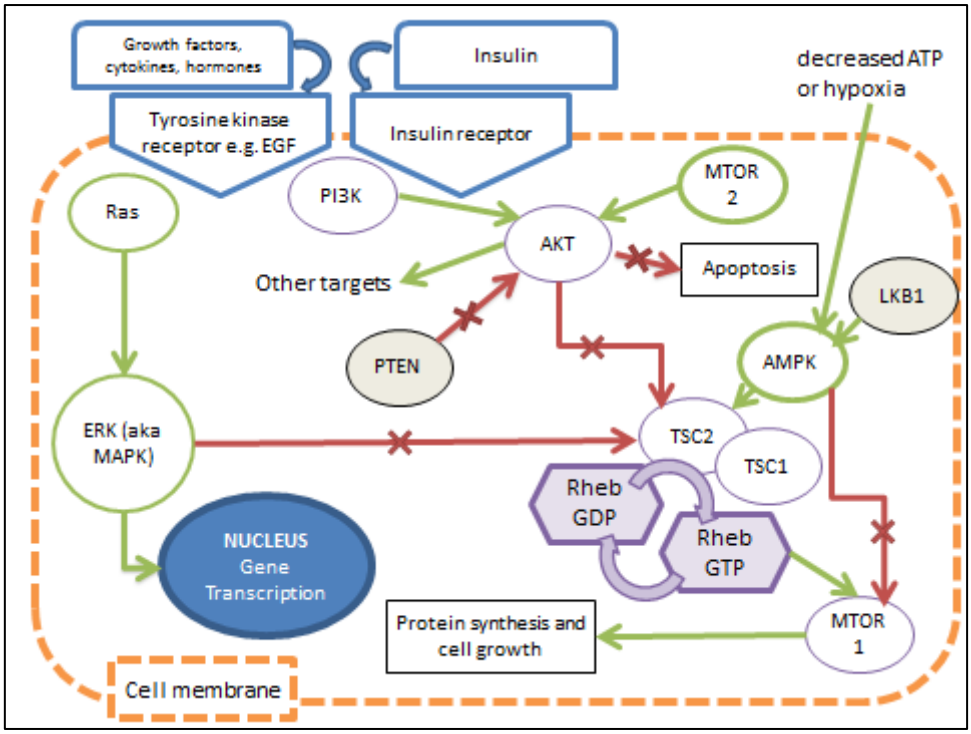
- Adiponectin reduces phosphorylation of ERK 1,2 in PTEN deficient endometrial cancer cells, but does not affect ERK 1,2 phosphorylation in PTEN competent cell lines.
- Expression of cyclin D1 is reduced by adiponectin. Cyclin D1 is a key element in cell cycle progression from G1 to S, and is overexpressed in endometrial cancer wherein it correlates with poor prognosis.

Two types of adiponectin receptor, AdipoR1 and AdipoR2, mediate peripheral effects. AdipoR1 are abundant in the liver and skeletal muscle, and through these adiponectin suppresses proliferation by phosphorylation of AMPK. AdipoR2 are mostly expressed in the liver and mediate PPAR pathways. Both types of receptor are a feature of both normal secretory, proliferative and neoplastic endometrium, and expression of AdipoR1 in endometrial cancer correlates with increased progression free and overall survival (135).

1.3.7 Signalling pathways

A predominant feature of cancer is the alteration of signal transduction pathways, in part due to gene mutations. Type I endometrial cancer is closely related to abnormal signalling of PI3K/AKT and MAPK/ERK1, 2 pro-proliferative pathways, which have been implicated in carcinogenesis (Figure 5).

FIGURE 5: AN OVERVIEW OF THE SIGNALLING PATHWAYS IMPLICATED IN ENDOMETRIAL CARCINOGENESIS



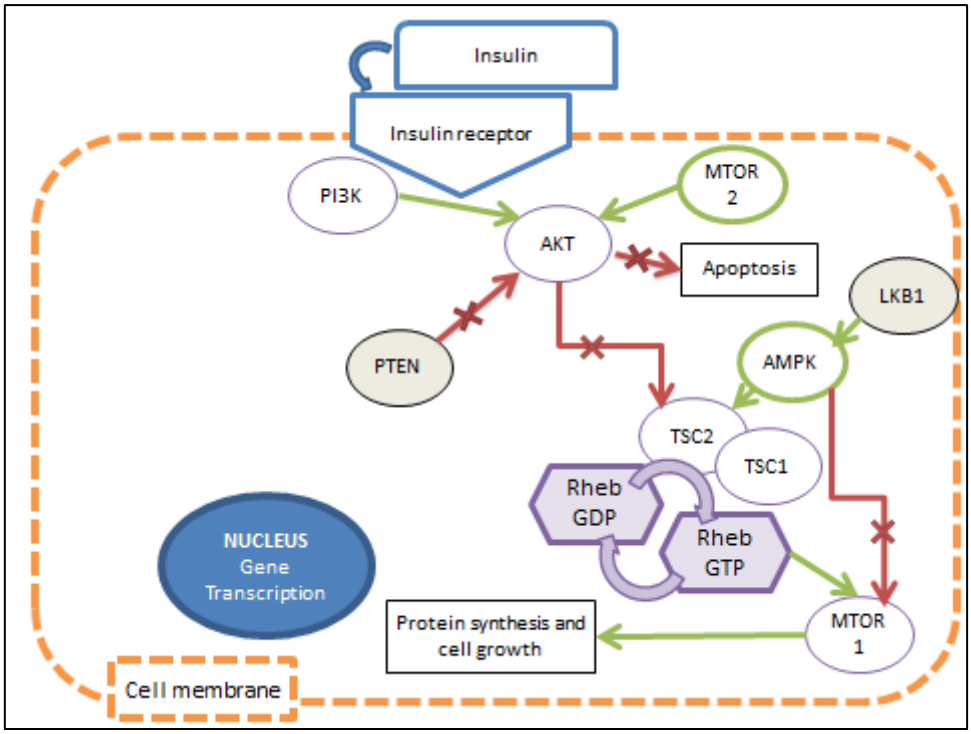
1.3.7.1 PI3K/AKT/mTOR

As one of the most critical pathways in the promotion of carcinogenesis, Phosphatidylinositol-3 Kinase (PI3K) activation initiates a signal transduction cascade, which promotes cell growth and persistence via AKT (also known as Protein Kinase B), which inhibits apoptosis (Figure 6). AKT is a serine-threonine kinase that stimulates activation of mammalian target of rapamycin (mTOR) complex 1 downstream. mTOR signalling impacts upon most cellular behaviours and coordinates protein translation, cell growth and survival. mTOR deregulation has been linked to cancer, diabetes mellitus and obesity and attempts to develop drugs that modify this pathway are ongoing. The PI3K/AKT/mTOR pathway is the most frequently altered biochemical pathway in EEC and alterations are thought to occur early in the process of carcinogenesis (139).

The activation of this pathway, measured by the proportion of phosphorylated AKT (pAKT) in endometrium, is enhanced in premenopausal obese and overweight women (1.6 fold increase) in a study which examined 31 premenopausal women of varying weight/BMI categories with normal endometrium and 10 obese women with endometrial cancer (102). This is seen alongside increased proliferation indices in this group. Opinion is divided as to whether pAKT expression is increased in hyperplastic or neoplastic endometrium, compared with normal endometrium (102,140,141).

The increased activation of this pathway associated with obesity may reflect leptin-induced phosphorylation of the pathway, elevated serum insulin or oestradiol levels, or the higher expression of ligand activated oestrogen receptors in the endometria of obese and overweight women. There is evidence to suggest endometrial nuclear pAKT expression correlates with ERα expression, and in vitro oestrogen leads to translocation of pAKT from cytoplasm to nucleus and inhibition of apoptosis (141). PTEN is the foremost inhibitory regulator of this pathway and PTEN function is lost in between 35 and 80% of EEC (139). A significant inverse correlation has been demonstrated between pAKT levels and PTEN expression in EEC (142).

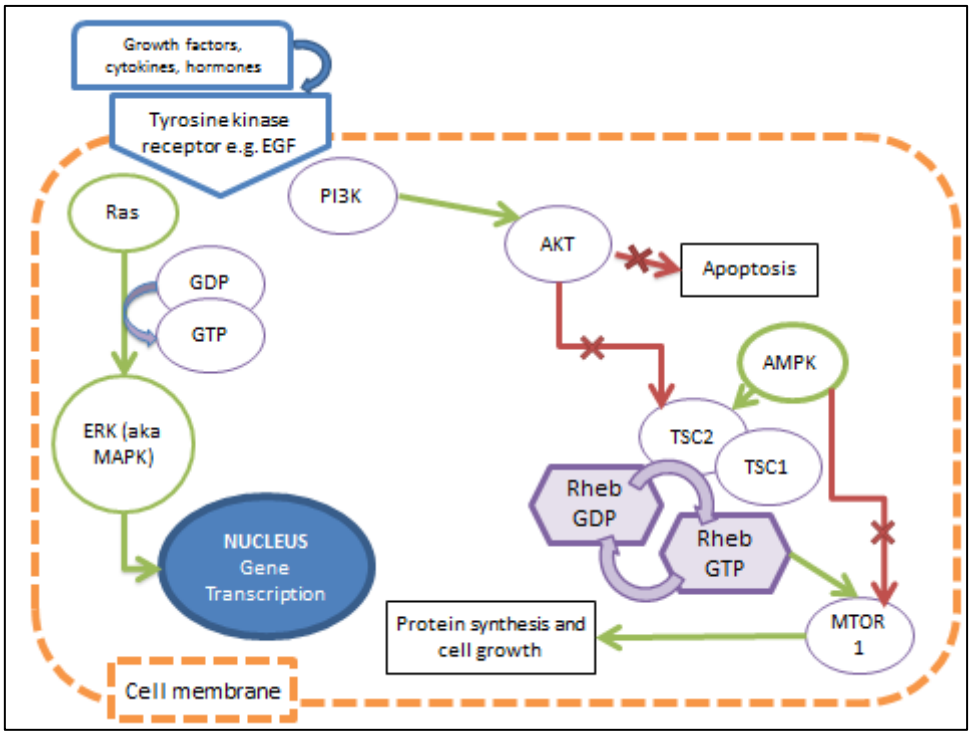
FIGURE 6: THE PI3K/AKT/MTOR PATHWAY



1.3.7.2 RAS/MAPK/ERK1, 2

Tyrosine kinase receptors in the cell membrane are activated by extracellular ligands, e.g. epidermal growth factor (EGF) binding to the IR specific receptor (e.g. EGFR). This allows the Ras protein to exchange its guanosine diphosphate (GDP) for a guanosine triphosphate (GTP). In doing so it becomes active and triggers a cascade of events which seek to activate a mitogen activated protein kinase (MAPK), previously known as extra-cellular signal-regulated kinases (ERK 1, 2). MAPK has an important role in translation of mRNA to proteins and transcription of genes involved in the cell cycle. Defective signalling within the pathway can lead to uncontrolled cell growth, a prerequisite for the development of cancers (Figure 7). Ras/MAPK also interacts with the PI3K pathway via RAS proteins. Somatic mutations of the K-ras gene are found in 15-30% of EEC and can coexist with mutations in PTEN and PIK3CA.

FIGURE 7: THE RAS/MAPK/ERK 1,2 PATHWAY



Higher levels of activation of the RAS/ERK1, 2 pathway, in the form of elevated phosphorylated ERK1, 2 (pERK1, 2), are seen in premenopausal overweight and obese women (8.7 fold increase). Higher still are the proportions of phosphorylated ERK 1,2 seen in the endometria of women with type I endometrial tumours.

Immunohistochemical analysis of 63 endometrial cancer specimens by Mizumoto et al demonstrated pERK 1,2 expression in 63% (40/63), to varying degrees. No correlation was seen between pERK 1,2 and total ERK 1,2 expression suggesting increasing pERK 1,2 is related to increased pathway activation rather than increased expression of ERK 1,2 (143). Hyperinsulinaemia may stimulate this pathway, and oestradiol and leptin induce phosphorylation of ERK 1,2 in endometrial cancer cells (102).

1.3.8 Genetic dysregulation

Type I endometrial tumours are often found to have inactivation of the tumour suppressor PTEN, as well as mutations in K-ras, β-catenin, PIK3CA and PIK3. Sometimes abnormalities are seen which are associated with microsatellite instability (MSI) such as mutated MSH6. The genetic profile of type II endometrial cancers differ (Figure 8), in that they are often found in association with p53 and p16 mutations, reduced E-cadherin expression and increased Her-2/Neu expression (144) (Table 7). The gene mutations most often encountered in type I endometrial cancers are involved in the regulation of signal transduction, a fundamental process in normal cell replication and cell-cell adhesion (Figure 9). Alterations in these pathways lead inevitably to abnormal cell proliferation and an alteration in the propensity of cells to invade or metastasise.

1.3.8.1 PTEN

The most common genetic defect in endometrial cancer is inactivation of the phosphatase and tensin homologue tumour suppressor gene (PTEN) located on chromosome 10 (145). PTEN codes for a lipid phosphatase that dephosphorylates PI3K substrates, antagonising PI3K, maintaining cell cycle arrest at G1-S and facilitating apoptosis. Loss of these effects upon the cell cycle, together with downregulation of IGF1 receptors, promote hyperactivity of the PI3K/AKT/mTOR pathway, and tumour genesis. PTEN loss appears to inhibit the downregulation of AKT phosphorylation and also demonstrates protein phosphatase activity, inhibiting cell spread and migration.

PTEN mutation and inactivation is found in 20-55% of endometrial hyperplasia, and between 40 and 80% of type I endometrial cancers (but only 10% of non-endometrioid endometrial cancer) which has led to the conclusion that loss of PTEN function is an early event in type I endometrial carcinogenesis (144,145). PTEN can be inactivated by

gene mutation, promoter methylation or protein degradation, leading to loss of expression or heterozygosity.

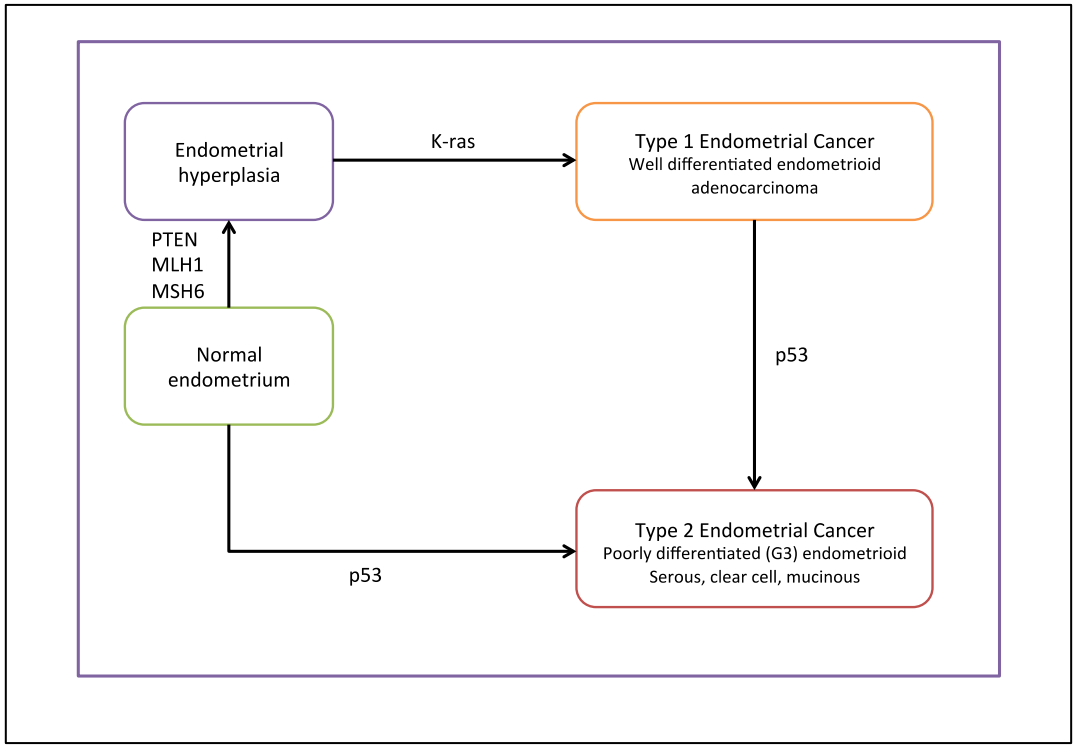
Loss of PTEN can be reversible, as described by Ørbo et al in their RCT comparing LNG-IUS to oral progesterone as treatment for endometrial hyperplasia (n=153, 11% atypical, 89% non-atypical). Clearance of PTEN null glands was significantly more effective when treated with LNG-IUS than with oral progesterone (58% regression and 4% persistence of PTEN null glands with LNG-IUS cf. 35% regression and 31% persistence with cyclic oral progesterone and 52% regression and 14% persistence with continuous oral progesterone, p=0.008). Clearance of PTEN null glands was significantly related to regression of disease (100% LNG-IUS, 69% cyclic MPA, 96% continuous MPA, p=0.002) (146). Progesterone treatment of cultured endometrial stromal cells has been shown to up-regulate PTEN (142).

Normal endometrium expresses PTEN in both stromal and glandular epithelium albeit not at constant levels in cycling endometrium (147), but when PTEN is inactivated the glands no longer express PTEN on immunohistochemistry. Inactivation of PTEN on IHC of endometrial carcinoma is inversely correlated with pAKT expression (r=-0.796) (148) which would be in keeping with a loss of control of the pro-proliferative PI3K/AKT pathway with PTEN loss. PI3K pathway alteration also happens early in endometrial carcinogenesis. The potential for PTEN-null glands to be seen in histologically normal endometrium means PTEN loss has low sensitivity and specificity for use as a marker of malignant change (149) and of future cancer risk on an individual basis (150). Isolated PTEN deficient clones do not usually persist but are replaced by other PTEN null clones over time (146).

The impact of PTEN loss on tumour biology and patient outcomes should be interpreted in the context of BMI as there is evidence that PTEN loss in endometrial cancer is associated with improved progression free survival (PFS) in obese patients (HR 0.15, 95% CI 0.03-0.9, p=0.038) and retained PTEN in obese women is associated with reduced PFS. The two groups also appear to demonstrate different protein changes as a result of PTEN loss. In the non-obese EEC group canonical PI3K pathway activation

was seen, in the obese EEC group PTEN loss was associated with decreased expression of β-catenin and pFOXO3A which is a target of AKT (116).

FIGURE 8: GENETIC DYSREGULATION LEADING TO TYPE 1 AND TYPE 2 ENDOMETRIAL TUMOURS



Adapted from Banno et al (144)

1.3.8.2 K-RAS

Mutations of the K-ras gene are found in 10 to 30% of type I endometrial cancers (144,145) and 16% of hyperplastic endometria. The K-ras proto-oncogene encodes an inner plasma cellular membrane GTPase involved in cell signalling for growth and differentiation. Mutations in this gene also facilitate mTOR kinase activity downstream increasing cell survival, angiogenesis and proliferation. K-ras and PTEN mutations do not tend to coexist.

Studies on human endometrial epithelial cell lines suggest that the tumourigenic effects of K-ras mutations are independent of key pro-cancer ERK and AKT pathways. Oncogenic K-ras facilitates DNA binding of NFκB to its target sequences in endometrial cancer cells, as well as enhancing the ability of NFκB to transactivate the target gene promoter in endometrial cancer cells. In vitro upregulation of NFκB transcriptional

activity was not inhibited by the addition of a MEK inhibitor, which inhibits the ERK pathway. In addition to this inhibition of NFκB activity abrogates the tumourigenic effect of K-ras mutant endometrial cancer cells (151).

In gene expression studies of fresh frozen samples of endometrium from patients with atypical hyperplasia, key differences emerged between obese and non-obese patients. Non-obese patients exhibited overexpression of the oncogene Stathmin (STMN1) mRNA, whereas K-ras expression was significantly higher in obese women alongside an increase in pro-inflammatory gene sets. This would be consistent with our understanding that obesity induced inflammatory processes are involved in obesity related endometrial carcinogenesis (97).

TABLE 7: THE INCIDENCE OF GENETIC ABERRATIONS IN ENDOMETRIAL CANCER

Alteration	Type 1 (%)	Type 2 (%)
PTEN inactivation	50-80	10
K-ras mutation	15-30	0-5
β-catenin mutation	20-40	0-3
MSI	20-40	0-5
p53 mutation	10-20	80-90
HER2/Neu mutation	10-30	40-80
p16 inactivation	10	40
E-cadherin overexpression	10-20	60-90

Adapted from Banno et al (144)

1.3.8.3 PIK3CA

The PIK3CA gene encodes the alpha subunit of PI3K, resulting in downstream activation of targets including AKT and mTOR. Mutations of PIK3CA are seen in 36% of

endometrial cancer patients, and 26% have associated PTEN mutations. PIK3CA mutations are strongly linked to endometrioid type tumours and particularly advanced stage disease (152). PIK3CA mutations are uncommon in AEH and are seen to increase with de-differentiation, suggesting they are a later event (97).

1.3.8.4 LKB1

LKB_1 encodes a protein kinase that regulates the 5' adenosine monophosphate activated protein kinase (AMPK)-mTOR signalling pathway. LKB_1 activates AMPK, an enzyme involved in cellular homeostasis. AMPK monitors intracellular ATP levels and controls protein synthesis by phosphorylating TSC2 (tuberin) and regulating mTOR. Loss of LKB_1 activity relaxes control of protein synthesis, promoting growth, proliferation and consequently tumour genesis. While this explains the role of LKB_1 as a tumour suppressor gene, its promotion of invasion is probably mediated by one of the other myriad proliferation mechanisms LKB_1 controls (153). Loss of LKB_1 expression has been documented in up to 65% of endometrial cancers. It has also been associated with higher grade or more advanced tumours.

1.3.8.5 MICROSATELLITE INSTABILITY

Microsatellites are brief segments of repeated DNA base pairs found throughout the genome, mainly in non-coding DNA. Due to the nature of these multiple repeated sequences microsatellites are prone to replication errors, which ordinarily are corrected by the DNA mismatch repair system. Genetic mutations that cause a loss of function in this system cause persistence of replication errors in microsatellites, and a propensity for tumour genesis.

In the case of endometrial cancer the most common mismatch repair gene to be inactivated through mutation is MLH1. Inactivation is achieved through hypermethylation of particular elements of the gene promoter, a process described as epigenetic silencing (145). MSH6 is also a DNA mismatch repair gene commonly mutated in endometrial cancers; both sporadic and inherited. Lynch syndrome is often a result of inherited mutations in DNA mismatch repair genes, MLH1, MSH2, MSH6 or PMS2. Both microsatellite instability (MSI) and abnormal methylation of MLH1 are early events in the process of endometrial carcinogenesis, and have been reported in

precancerous lesions. MSI is rare in type II tumours (<5%) but occurs in approximately 20% of sporadic endometrioid endometrial cancers. MLH1 inactivation has also been documented in 33% of cases of atypical hyperplasia (6). In type II tumours genetic instability is more often derived from p53 gene mutations than from defective mismatch repair mechanisms. This manifests as aneuploidy, i.e. instability at a chromosomal rather than at a microsatellite level.

1.3.8.6 B-CATENIN & E-CADHERIN

Approximately 20% of endometrioid carcinomas display β-catenin mutation, which can also be seen in atypical hyperplasia. β-catenin is essential for forming a complex with E-cadherin in the process of cell-cell adhesion, and also plays a key role in the Wnt signal transduction pathway involved in tissue maintenance (154). In 5 to 40% of endometrioid tumours reduced E-cadherin expression results in loss of cell-cell cohesion. This may precede tumour cell motility, a feature of tumour cell lines with a high potential for metastasis (154).

1.3.8.7 P53

p53 is a nuclear phosphoprotein that arrests proliferation or induces autophagy or apoptosis in response to cellular stress. In approximately 5% of endometrioid tumours (albeit grade 3, type II endometrioid tumours) abnormally elevated levels of inactivated p53 are seen, although this is far more common in serous or clear cell tumours where 80-90% levels of p53 inactivation are reported (145). High levels of p53 are correlated with high tumour grade and stage and in itself is a poor prognostic indicator (154).

1.3.8.8 HER2/NEU

Her2/Neu is an oncogene involved in cell signalling. It is more commonly overexpressed in grade 2 and 3 endometrioid tumours, and may be a late event in the development of endometrial cancer.

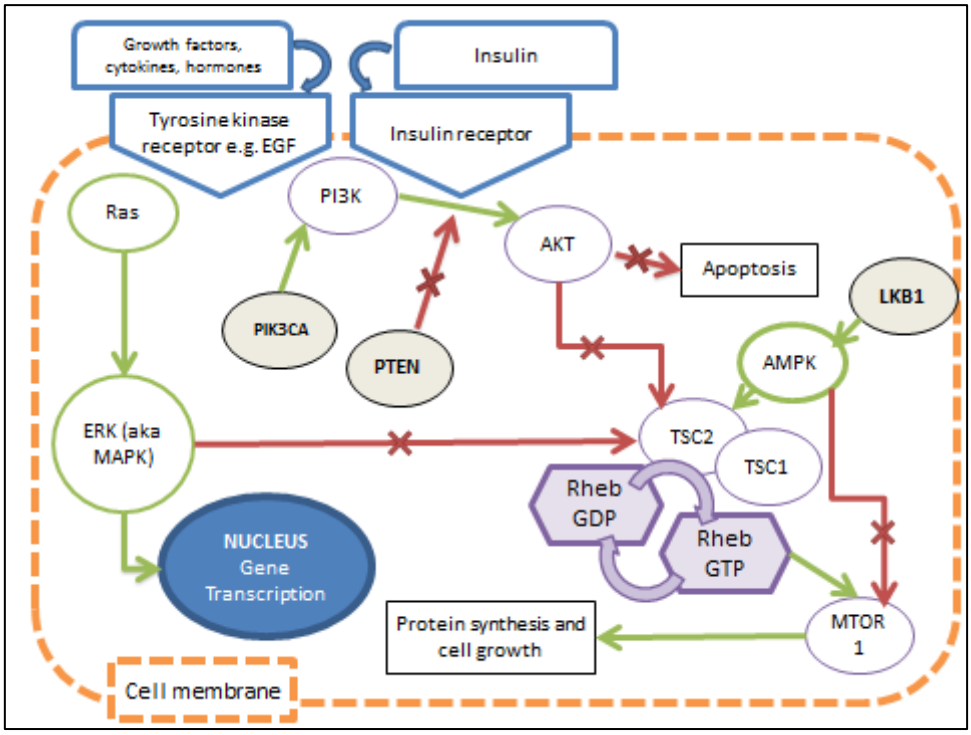
1.3.9 MOLECULAR PROFILING OF ENDOMETRIAL CANCERS

Four prognostically significant molecular subgroups have been proposed by the Cancer Genome Atlas Research Network (155). Tumour samples and germline DNA from 373 patients were analysed (307 endometrioid, 53 serous, 11 mixed) and 4 new groups of tumours were identified: copy-number low, copy-number high, POLE (DNA polymerase

epsilon) ultramutated and microsatellite instability mutated. The group found that endometrial cancers have more alterations of the PI3K/AKT pathway than any other tumour group studied. Whilst endometrioid tumours requiring adjuvant therapy are often treated with radiotherapy, serous tumours are likely to be treated with chemotherapy. High grade serous and endometrioid endometrial tumours are difficult to subtype and intra-observer agreement between pathologists may be variable. This data suggested that around 25% of high grade endometrioid tumours had a molecular profile more akin to serous tumours, and genome based decision making around adjuvant treatment may better serve these patients.

Translational data from samples from PORTEC 3 of all histological subtypes have also shown that even amongst endometrial tumours currently classified as high-risk there is a great deal of heterogeneity in terms of molecular abnormalities and patient outcome, knowledge of which could inform the risk-assessment and treatment. Importantly, tumours with microsatellite instability (MSI) and POLE-mutant tumours may have a better prognosis than current risk assessment would attribute (10).

FIGURE 9: THE EFFECT OF GENETIC DYSREGULATION ON PATHWAYS IMPLICATED IN CARCINOGENESIS



1.4 Obesity & Cancer

1.4.1 Effect of obesity on cancer risk

Obesity is a well-established risk factor for multiple types of cancer, including colorectal cancer, adenocarcinoma of the oesophagus and cancers of the kidney, prostate, pancreas and breast (156). Obesity may also increase the risk of multiple myeloma, leukaemia and non-Hodgkin's lymphoma. Variations have been demonstrated between men and women, pre- and post-menopausal women and histological subtypes. In post-menopausal women in the UK it is estimated that 5% of all cancers, approximately 6000 cancers each year, are attributable to being overweight or obese. Both current adiposity and adult weight gain have been linked to the increase in risk (28). Classification and nomenclature applied to obesity are documented in Table 8.

TABLE 8: CLASSIFICATION OF OBESITY (157)

BMI	WHO Classification of obesity	Terminology used in literature
BMI 30-34.9 kg/m^2	Class I obesity	Obese
BMI 35-39.9 kg/m^2	Class 2 obesity	Severe obesity
BMI ≥40 kg/m^2	Class 3 obesity	Morbid obesity
BMI ≥45 kg/m^2		Supra morbid obesity / Super obesity

A population-based study assessed the estimated global "population attributable fractions" (PAF) of cancer incidence after taking into consideration a presumed 10-year time lag between onset of obesity and cancer development (101). They also examined the role of smoking and HRT use, common confounders in research of this nature. It was estimated that globally, in 2012, 481,000 (3.6%) new cancers were attributable to high BMI ten years prior. When known obesity related cancers were considered separately, 12.8% of all new cancers were related to high BMI in 2002. Significant

variation in the contribution of obesity was seen, geographically and by cancer site: for uterine cancer the PAF was 41% (95% CI 37.9-43) (101), and was higher still in HRT non-users. It was concluded that one quarter of all obesity related cancers diagnosed in 2012 could be attributed to the rise in the incidence of obesity that was seen between 1982 and 2002.

In the MRC ASTEC trial which included more than 1400 women from across Europe with early endometrial cancer, 80% of women with type 1 cancer were overweight (BMI > 25kg/m^2) and 50% obese (BMI > 30kg/m^2)(8). The association between obesity and cancer is seen most strongly in endometrial cancer, with a large 2008 meta-analysis demonstrating a relative risk of 1.59 per 5kg/m^2 increase in BMI (156). The association becomes stronger once BMI exceeds 27kg/m^2, and in women who have never used HRT (Table 9) (69). A woman with a BMI of 42kg/m^2 has almost a ten-fold increase in the risk of endometrial cancer compared to a woman whose weight is within normal limits. The mediatory effect of HRT on endometrial cancer risk is presumed to be a reflection of the use of continuous combined HRT providing exogenous progesterone antagonism for endogenous oestrogenic stimulation.

TABLE 9: EFFECTS OF OBESITY AND THE USE OF HRT ON ENDOMETRIAL CANCER RISK (69,156)

Patient Characteristics	RR	95% CI
BMI 27kg/m^2	1.22	1.19-1.24
BMI 32kg/m^2	2.09	1.94-2.26
BMI 37kg/m^2	4.36	3.75-5.10
BMI 42kg/m^2	9.11	7.26-11.51
BMI 42kg/m^2, never used HRT	20.70	8.28-51.84
Per 5kg/m^2 increase, never used HRT	1.90	1.57-2.31
Per 5kg/m^2 increase, used HRT	1.18	1.06-1.31
Per 5kg/m^2 increase, all	1.59	1.50-1.68

Features have been identified which are common to both obesity and to endometrial cancer and many studies have queried whether the two share a genetic cause. Data was pooled from 5 population based case control studies and included 3,376 cases of endometrial cancer and 3,867 controls. A BMI genotype risk score was found to be significantly associated with endometrial cancer risk but after adjusting for BMI this association disappeared, meaning that possessing a large number of BMI risk alleles does not increase endometrial cancer risk over that which is conferred by obesity alone (158).

The EPIC biomarker study recruited 370,000 women in whom there were 233 incident cases of endometrial cancer (50 premenopausal and 183 postmenopausal, average age at diagnosis 60 years). These were matched to 446 controls, matched for menopausal status, age, fasting status and phase of menstrual cycle. Cases of endometrial cancer had higher BMI and waist circumference, an older age at menopause and less OCP/more HRT use than matched controls. Postmenopausal women with endometrial cancer were found to have significantly higher levels of oestradiol than controls, and higher levels of glucose, C peptide, testosterone and oestrone were seen in all cases as compared to controls. Levels of adiponectin, SHBG and insulin like growth factor binding proteins (IGFBP) were lower in endometrial cancer than in controls (159).

1.4.2 Perception of risk

Public and physician appreciation of the magnitude of the association between bodyweight and endometrial cancer is limited (160,161). Henretta et al assessed obese women presenting for weight loss surgery's perception of their risk (n=93, mean age 45, mean BMI 49kg/m^2) (162). Of this cohort 38% had diabetes and 57% were hypertensive; 32% had had a previous hysterectomy, in a population where 6/1000 (0.6%) is the norm, 50% reported heavy menstrual bleeding. Over 45% perceived it as "not likely or not possible" that they would get uterine cancer. Of the women who self-categorised themselves as overweight, as opposed to obese, mean BMI was 44 kg/m^2. Women who perceived their bodyweight to be "normal" had a mean BMI of 35 kg/m^2, which in this selected population of women seeking weight loss surgery is likely to reflect the normalisation of obesity in society as it becomes more prevalent.

Within this cohort, compliance with available screening regimes was good (96.8% up to date with cervical screening), however this cannot be extrapolated to other cohorts of women presenting for weight loss surgery, as healthcare provision, socioeconomic status and level of education may differ significantly. Even in its own geographical and healthcare setting the cohort described is only representative of obese women who were motivated to seek bariatric surgery, rather than obese women in general.

A strong inverse relationship between BMI and the likelihood of undergoing cervical screening has been demonstrated in a systematic review of 11 studies that included 170,689 women (163). Various barriers to care have been described by obese women, such as their perception that they are treated with less respect because of their weight, feeling embarrassed when being weighed or having experienced equipment in healthcare settings being unable to accommodate them, being counselled to lose weight or perceiving the physician to have a negative attitude towards them because of their obesity (162).

1.4.3 Effect of obesity on survivorship

Whilst in other obesity related cancers such as breast, colon and rectal cancers, obesity is seen to negatively affect outcome in terms of relapse and survival, endometrial cancer has not yet been shown to follow suit in this regard. Other cancer types such as cervical, thyroid and renal cell cancers, have also demonstrated that obesity does not worsen prognosis. Hypotheses to explain perceived worse outcomes in obese endometrial cancer patients have included the practice of capping chemotherapy doses at body surface area of $2m^2$ or radiation treatment being compromised due to logistic or dosing difficulties. Alternatively, as obesity is a risk factor for well-differentiated, early stage endometrial cancer, this may explain why obesity does not necessarily translate to a poorer outcome. Obesity related endometrial cancer may be associated with features of favourable prognosis, such as PTEN mutations (164).

The Million Women Study demonstrated an increase in mortality from endometrial cancer with increasing BMI, with women who had a BMI $\geq 30kg/m^2$ having a 2.28 relative risk of death (165). Calle et al also reported increased mortality in obese women with endometrial cancer (495,477 women, 694 deaths from uterine cancer in

16 years follow up, BMI ≥ 40kg/m^2 RR mortality 6.25, 95% CI 3.75-10.42) (166) and Arem et al reported an association between higher pre-diagnosis BMI and an increase in overall and disease specific mortality from endometrial cancer (167).

These findings were not substantiated by the results of the MRC ASTEC trial, which did not detect a difference in mortality in obese women with endometrial cancer. ASTEC was restricted to women with early stage disease, with 70% of participants having low risk disease (8). The ASTEC secondary analysis (8) examined post-diagnosis BMI and mortality risk and concluded that across BMI categories there was no significant difference in disease related mortality or relapse free survival. Reeves et al reporting on the Women's Health Initiative Study in 2011 commented that whilst elevated BMI and waist to hip ratio (WHR) both increased the risk of endometrial cancer, they did not appear to influence the prognosis (n=86,937) (168).

The point at which BMI is measured may partly explain these differences. Pre and post diagnosis BMI are not interchangeable, there is evidence of weight loss after cancer diagnosis, which correlates with pre-diagnosis BMI. Variable study conclusions may be an example of the Will Rogers phenomenon, where patients with poor prognosis disease lose what may be a clinically unappreciable amount of weight between their pre and post diagnosis BMI being recorded and attenuate the effect of post-diagnosis BMI on outcomes (169).

Higher BMI is associated with increased all-cause mortality, and increased BMI is associated with shorter overall survival, which may be explained as death related to obesity rather than cancer progression. A recent meta-analysis of the association between BMI and all-cause mortality in women with endometrial cancer included 665,694 women diagnosed with endometrial cancer between 1974 and 2009, with follow up data of between 1.6 and 16 years. An increased risk of all-cause mortality was seen in obese endometrial cancer patients (164).

Von Gruenigen et al conducted a Gynecologic Oncology Group (GOG) study wherein they retrospectively reviewed data from 380 patients participating in a RCT of adjuvant radiotherapy for endometrial cancer. Amongst those with a BMI <40kg/m^2, 45-48%

died of endometrial cancer, whereas above this BMI threshold 16.7% died of endometrial cancer and 66.7% died of other (non-cancer) causes (170). SEER data would seem to concur with this and shows that by five years post diagnosis endometrial cancer survivors were more likely to die of cardiovascular disease than endometrial cancer (171). Comparable increases in all-cause mortality are seen in obese individuals without cancer. Pooled data from 19 prospective studies (n=1.46 million) demonstrated HR of all-cause mortality of 1.99 (95% CI 1.9-2.09) in adults with a BMI 40-49.4kg/m^2, compared with those with BMI 22.5-24.9kg/m^2. This increase was most pronounced in women who had never smoked (Table 10) (172).

TABLE 10: OBESITY IS ASSOCIATED WITH INCREASED RISK OF ALL CAUSE MORTALITY (164)

BMI	OR	95% CI	P
25-29.9 kg/m^2	1.01	0.77 – 1.32	0.9
30-34.9 kg/m^2	1.17	0.85 – 1.61	0.3
35-39.9 kg/m^2	1.26	0.78 – 2.03	0.3
≥40 kg/m^2	1.66	1.10 – 2.51	0.02*
Per 10% increase	1.09	1.03 – 1.16	0.007*

Morbidity of treatment is also thought to be higher in obese patients. Surgery is often more complex, and may require concurrent apronectomy to facilitate surgical access, with accompanying increases in wound infection rates, operating time and duration of inpatient stay. The postoperative course is more likely to be complicated by infective morbidity or thromboembolic events. The super morbidly obese patient is more likely to receive primary radiotherapy rather than standard treatment if surgery is deemed to be too high risk. The delivery of adjuvant radiotherapy is more likely to be suboptimal and increased BMI has been associated with increased cutaneous toxicity of radiotherapy (170).

A recent meta-analysis of 4 studies (n=1362 endometrial cancer survivors) also demonstrated that obese endometrial cancer survivors are significantly more likely

than non-obese endometrial cancer survivors to report lower quality of life scores, in the domains of physical, social and role functioning (173).

1.4.4 Cost of care

Brooks et al have reported significantly higher costs of caring for endometrial cancer patients who are morbidly obese due to longer in patient stay and additional mechanical ventilation and intensive care requirements. Other studies have also demonstrated increased costs associated with comorbidities and treatment related complications (174).

1.5 Weight gain & weight loss

Weight gain and expansion of fat mass is due to adipogenesis (an increase in the number of fat cells) and increasing adipocyte size due to increased lipid storage. The physiological determinants of adiposity are thought to involve the central nervous system, sympathetic nervous system and various endocrine regulators such as insulin, growth hormone, prolactin, leptin and ghrelin.

It is generally accepted that 5% or more weight loss will produce clinically measurable improvements (175). Lifestyle modification can result in 4-6% weight reduction over two to four years, and anti-obesity medication can result in 7-10% weight reduction although both methods have high levels of weight regain (50-60%) (176). Intensive medical interventions (with not insignificant cost and difficulty to administer) report up to 9.7% weight loss at 2 years, with 31% demonstrating 5% weight loss at 2 years. Poor retention of participants is a recognised problem in weight loss studies, with attrition rates of up to 52% commonly seen. Intermittent Energy Restriction programmes have been shown to be superior to Continuous Energy Restriction programmes in terms of short-term improvements in insulin resistance and reduction of body fat, and potentially in adherence and weight loss maintenance also (175,177). Dietary treatment of obesity may be suboptimal due to the tendency to regain weight lost once the diet is completed (178).

Weight loss surgery is the only treatment shown to effect more than 15% weight loss over a 15-year period. It also has positive durable effects on cardiovascular risk factors, improvement of glucose metabolism and overall mortality. NICE guidelines produced in 2006 recommend bariatric surgery for people with a BMI ≥40kg/m^2 as this is when it is most cost effective (179). Comparability of bariatric surgery cohorts from around the world can be limited by key differences in baseline characteristics, due to access to surgery and prevalence of comorbidities.

1.5.1 Weight loss surgery

Bariatric surgery describes a group of procedures performed to facilitate weight loss, and the three types of surgery performed most often in the UK are laparoscopic adjustable gastric banding, laparoscopic or open Roux-en-Y gastric bypass, and laparoscopic or open sleeve gastrectomy (Figure 10). Around three times as many

women as men undergo bariatric surgery and they tend to be in the age range of 40-54 years. Complications of weight loss surgery include bleeding, infection, bowel obstruction and anastamotic leaks as well as medical complications such as venous thromboembolism or respiratory or urinary tract infection (180). There are variable degrees of morbidity and mortality between centres and depending upon the type of surgery performed. Success of surgery is measured by excess weight loss achieved. An excess weight loss of >50% is deemed a treatment success, and <25% a treatment failure (181).

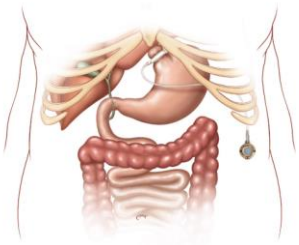
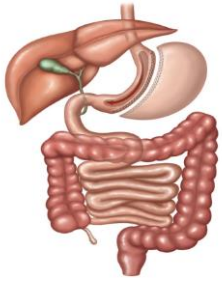
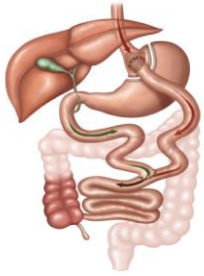
$$\%\ Excess\ Weight\ Loss = \left(\frac{[Preop\ weight - Postop\ weight]}{[Preop\ weight - Ideal\ weight]}\right) x100$$

Laparoscopic adjustable gastric banding has the lowest complication rates but is associated with less weight loss than the other, more invasive, surgical techniques. It involves the placement of an adjustable silicone band around the upper stomach, which restricts the amount of food that can be eaten. Patients can expect to lose between 40-50% of their excess body weight in the 2 years after surgery (181).

Sleeve gastrectomy divides the stomach vertically and reduces the volume of the stomach by 75%. As well as restricting consumption it also reduces appetite due to the effect of partial resection of the stomach on enteric hormone levels. Patients can expect to lose an average of 60-70% of their excess weight over 2 years (181).

The current gold standard is the Roux-en-Y gastric bypass (RYGB), which works by a combination of restriction, malabsorption and appetite suppression. The so-called BRAVE effects describe the physiological changes seen: 1) Bile flow alteration, 2) Reduction of gastric size, 3) Anatomical gut rearrangement and altered flow of nutrients, 4) Vagal manipulation and 5) Enteric gut hormone modulation. On average patients who undergo a gastric bypass lose 65-75% of their excess weight over 2 years (181) and are more likely to show resolution of type 2 diabetes than with other surgical procedures. The vast majority of bariatric surgery operations are performed laparoscopically, and the level of postoperative adverse outcomes is relatively low.

FIGURE 10: TYPES OF BARIATRIC SURGERY (182)

Gastric band	Sleeve gastrectomy	Gastric bypass
		
40-50% excess body weight lost within 2 years 50% resolution type 2 diabetes 1:5 regain weight	60-70% excess body weight lost within 2 years 60-65% resolution type 2 diabetes Less risk than bypass	65-75% excess body weight lost within 12-18 months 80-85% resolution type 2 diabetes 1:10 regain weight - 10 yrs

1.5.2 Mechanisms of action of bariatric surgery

The entero-insular axis, the connection between gastrointestinal, endocrine and pancreatic secretions controlled by the incretin hormones Glucagon-like Peptide 1 (GLP-1), Glucose Dependent Insulinotropic Peptide (GIP), ghrelin and Peptide Tyrosine Tyrosine (PYY), was first described in 1969 (183). Incretins are produced by the gastrointestinal tract in response to nutrient entry (glucose), and lead to insulin secretion. The two main incretins are GIP and GLP-1, and they are responsible for between 50 and 70% of the insulin response to oral glucose in healthy individuals.

GIP is secreted by the K cells of the intestine and stimulates insulin secretion and β cell production. GLP-1 is secreted by the L cells of the ileum and colon and stimulates insulin secretion, improves β cell function and reduces gut motility and gastric emptying.

Subsequently, studies have demonstrated that when caloric intake is reduced, glucose metabolism improves independently of weight loss as incretin production and insulin secretion are reduced. Pories hypothesised that the effects of weight loss must occur in two phases (184):

1. Short Term: changes in glucose metabolism and insulin resistance due to caloric restriction, modulated by the entero-insular axis and involving foregut hormones

2. Long Term: reduction in fat mass, mediated by changes in adipokines

Alterations in ghrelin levels are thought to be key to the success of attempted weight loss. Ghrelin is an endogenous ligand for GH secretagogue receptor produced primarily by A cells in the fundal oxyntic glands of the stomach which is thought to function as a hunger signal, as it peaks before food intake and decreases after eating. It is also thought to modulate gastric acid secretion, gastric motility and leptin secretion. In addition it protects lipids stored in ghrelin sensitive depots from utilisation, promoting the expansion of abdominal fat and, ultimately, the development of metabolic syndrome (185).

Weight loss due to caloric restriction or vertical banded gastroplasty (VBG), the predecessor to laparoscopic adjustable gastric banding, leads to increasing levels of ghrelin in correlation with the reduction in BMI. Gastric bypass surgery conversely leads to a reduction in ghrelin levels, despite more significant weight loss/reduction in BMI. It is hypothesised that this reflects the exclusion of the gastric fundus from the food transit process in gastric bypass surgery, as compared to VBG where the anatomy of the gastrointestinal tract is not altered in the same way (133). It would appear that weight loss programmes need to interfere with ghrelin secretion to be most effective.

1.5.3 Systemic effects of bariatric surgery

The Swedish Obese Subjects (SOS) study was a prospective, matched, surgical intervention study that enrolled 2010 obese patients who underwent bariatric surgery and 2037 matched controls between 1987 and 2001 (180). Surgical patients underwent gastric bypass (13%), gastric banding (19%) or vertical banded gastroplasty (VBG) (68%). Matched controls received standard non-surgical obesity management from their primary care provider. In the surgical cohort mean weight loss was maximal at 1-2 years (mean -23%, bypass > VBG > banding), and thereafter weight increases were seen in all surgical groups but remained significantly lower than at baseline.

The primary endpoint was mortality. Even taking into consideration the high rate of open surgery in this cohort (89%), and the poorer morbidity and mortality figures in the time period the study was initiated, surgery was associated with a 30% risk reduction in overall mortality after multivariate adjustment for baseline conditions (HR 0.71, 95% CI 0.54-0.92). The beneficial effect of bariatric surgery on mortality only became statistically significant after 13 years. The study was underpowered to show differences in specific cause mortality but cancer was the most common cause of death (n=47 control, n=29 surgery).

Diabetes prevention and remission were secondary endpoints. Two years post bariatric surgery 72% of participants with type 2 diabetes at the time of surgery were in remission. By ten years post-surgery 50% of these had relapsed but no cases of relapse were seen in the gastric bypass group. Bariatric surgery was found to reduce the incidence of new type 2 diabetes by 96%, 84% and 78% by 2, 10 and 15 years post bariatric surgery. Furthermore, bariatric surgery was associated with a reduced incidence of myocardial infarction (adjusted HR=0.71; p=0.02) and stroke (adjusted HR=0.66; p=0.008). Whilst high baseline glucose or insulin levels were predictive of beneficial treatment effects, high baseline BMI was not. Data from this study have also shown that over a period of 15 years post bariatric surgery, health care costs for patients with diabetes were equivalent to the costs of those who did not undergo bariatric surgery, but higher for patients who were euglycaemic or had impaired glucose tolerance (186).

More recently the Longitudinal Assessment of Bariatric Surgery (LABS) Consortium have published three year follow up data of their multicentre observational cohort study carried out across 10 institutions across the USA (187). The importance of this study in addition to the SOS study is that surgical techniques have improved since the SOS study was carried out, with greater use of laparoscopic techniques and the virtual replacement in clinical practice of the VBG technique with laparoscopic adjustable gastric banding (LAGB). Surgery was performed between 2006 and 2009 (RYGB n=1738, LAGB n=610), and 79% were female, median baseline BMI 46kg/m^2, median age 46 years.

Greatest weight loss was seen in the first 12 months post bariatric surgery but at three years was still considerable (31.5% reduction total body weight from baseline RYGB, 15.9% LAGB). Three years post bariatric surgery 67.5% RYGB and 28.6% LAGB participants had at least partial diabetes remission, and incident diabetes (new-onset) in each group was 0.9% and 3.2% respectively. Remission of hypertension was seen in 38.2% of RYGB and 17.4% LAGB participants at three-year follow up.

1.5.4 Bariatric surgery and cancer risk

There is now a growing body of evidence to show a reduction in cancer risk following bariatric surgery, an effect that favours women and is most pronounced in the case of postmenopausal breast and endometrial cancers. In a retrospective study Adams et al have shown that 12.5 years after bariatric surgery there is a 46% reduction in cancer mortality and a 38% reduction in obesity related cancers, apparently due to reduced incidence rather than improved survival. The incidence of uterine cancer in particular was reduced by bariatric surgery. A seven-fold reduction in incident cancer risk was seen for endometrial cancer (14 cases per 6596 surgical patients, compared to 98 cases per 9442 controls, HR 0.22, $p<0.0001$) (46).

A more recent retrospective cohort study that included 103,797 female bariatric surgery patients from multiple centres across the USA estimated that bariatric surgery was associated with a 77-81% reduction in risk of uterine malignancy. In a total study population of 7,431,858 hospital admissions 44,345 new diagnoses of uterine malignancy were made. The relative risk of uterine malignancy in women who had had bariatric surgery compared with obese women with no history of bariatric surgery was 0.29 (95% CI 0.26-0.32). This risk was lower in women who reached and maintained a normal weight post bariatric surgery than women who remained overweight or obese post-surgery (RR 0.19, 95% CI 0.17-0.22 cf. RR 0.48, 95% CI 0.43-0.55) (188).

Whilst the SOS study reported a decrease in cancer mortality of 42.5%, it did not show the reduction in cancer incidence to be proportional to the amount of weight loss achieved (189) and this effect was limited to women. Women who underwent bariatric surgery had less new cancer diagnoses during follow up than those in the control arm, n=79 and n=130 respectively (HR=0.58, 95% CI 0.44-0.77, p=0.0001). The effect of

bariatric surgery on cancer incidence was not significantly related to baseline BMI (p=0.9). If the reduction in cancer risk is not proportional to degree of weight loss declining cancer risk may be a function of metabolic alterations or changing central obesity, rather than of absolute weight changes. An alternative explanation is that BMI is not the best measure by which to assess adiposity related risk.

Such evidence is the strongest indicator yet that weight reduction in obese individuals reduces the risk of cancer. Several potential mechanisms have been suggested; reduced caloric intake and increased physical activity, mediation of pro-inflammatory cytokines, alterations in insulin sensitivity and SHBG and sex steroid levels and modulation of adipokine levels have all been discussed.

Neff et al surveyed members of the Society of Gynecologic Oncologists (SGO) (n=327, response rate 30%) and found that only 11% of respondents had had any formal training in obesity management with 63% believing they could adequately counsel patients about weight loss. Significantly fewer offered patients weight loss counselling than would offer smoking cessation counselling. Less than half of respondents agreed that bariatric surgery was more effective at inducing weight loss than a non-surgical intervention. There remains a knowledge gap between physician's awareness of the health detriments of obesity and their ability to offer weight loss solutions (191).

1.5.5 The effect of weight loss on cancer related biomarkers

In view of the dose-response association of BMI and endometrial cancer risk, and the differing profiles of biomarker expression in lean and obese subjects both with and without endometrial cancer a longitudinal assessment of cancer associated biomarkers pre and post non-surgical weight loss was carried out (192). The participants were selected from those who had been recruited to the RENEW study (193) and undergone an intensive 12 month diet and lifestyle intervention (n=89, female, BMI ≥ 35kg/m^2 plus 43 stable weight controls). Average weight loss in the intervention arm was 12.8kg over 12 months, and statistically significant reductions in E-selectin, VEGF, IL-6 and statistically significant increases in GH and adiponectin were observed at 12 months. Soluble E-selectin is an adhesion protein involved in endothelial function that is increased in the presence of inflammatory milieu such as obesity, and has been

implicated in endometrial carcinogenesis. No significant change in TNFα or resistin was observed following non-surgical weight loss, and data from a bariatric surgery cohort concurs with this (194).

Obesity has a negative impact on quality of life and also, in addition to this, associations have been drawn between circulating adipokine levels and quality of life. Studies have suggested BMI independent links between leptin, adiponectin and resistin with low mood and depressive illness. Using biomarker and quality of life data from women achieving clinically significant weight loss (≥ 5% body weight) following non-surgical weight loss interventions (n=52), Linkov et al explored associations between changes in adipokine levels (leptin, adiponectin, resistin) and SF-36 scores (195). They found that only change in leptin level was significantly related to improvement in the physical summary score of the SF-36 questionnaire, once BMI was adjusted for in a linear regression. BMI was also significantly related to improvement in the physical summary score.

As with many measurable parameters or biomarkers it is difficult to know what is clinically significant rather than, or in addition to, being statistically significant. Varady et al designed a small prospective study to assess what degree of weight loss was required to improve adipose tissue physiology (adipokine levels and fat cell size). Women were recruited from a bariatric surgery clinic, who were to be placed on a 40% energy restriction diet prior to gastric banding surgery to reduce the size of the liver and as a consequence potential intra-operative complications (n=13, mean BMI 50 kg/m^2). Of these women, six lost < 5% total body weight over a three-week period and no significant change in insulin, glucose, adiponectin, leptin or resistin was seen, although it may be that the study was underpowered to show this. The other seven lost between 5-10% total body weight and had statistically significant improvements in insulin, glucose, adiponectin, leptin and resistin levels. They also found that adipocyte size was reduced further with 5-10% weight loss than with < 5% weight loss (196).

A prospective cohort study by Geloneze et al (n=43) assessed the effects of bariatric surgery on serum/plasma markers including leptin, glucose and insulin (197). At 12

months post bariatric surgery BMI had dropped from a median of 54.1kg/m^2 to a median of 34.6kg/m^2 and a significant linear reduction in serum leptin was seen, which was closely correlated to change in BMI. The conclusions of the multiple regression analysis were that 56-66% of the variance in leptin levels could be explained by the change in BMI (depending upon glucose tolerance) but that change in leptin levels was not related to insulin levels.

A prospective study of pre and postmenopausal women who underwent bariatric surgery demonstrated significant reductions in DHEAS and 4-androstene metabolites but did not show significant changes in progestagen or oestrogen metabolites. Significant changes in inflammatory and insulin resistance markers were also seen (67).

1.5.6 The effect of weight loss on the endometrium

Argenta et al performed a prospective cohort study assessing the prevalence of endometrial abnormality before and after bariatric surgery (66). At baseline they reported three cases of simple endometrial hyperplasia and one case of complex endometrial hyperplasia, without atypia. Of these, 12 months following bariatric surgery, two cases had resolved and two resolved after 18 months or treatment with oral contraceptive. The authors suggested this could demonstrate that weight loss had the potential to ameliorate endometrial abnormality by removing the stimulation of adiposity, insulin resistance and possibly hyperoestrogenaemia. It may also represent a normal finding in the natural course of the disease. There is evidence to show that <5% of non-atypical endometrial hyperplasia progresses over a 30 year period, and therefore it is likely that this represents an abnormality with the capacity to spontaneously regress.

Further analysis of these 46 paired samples assessed tissue microarrays (TMA) made from the formalin fixed paraffin embedded (FFPE) endometrial biopsies for the Proliferation Index (Ki-67) and expression of ER, PR and androgen receptors (198). Hyperplastic endometrium was found to have a differing profile of hormone receptor expression than normal endometrium, having higher median ER and PR expression (median ER in hyperplasia 261/300 cf. 171/300 in normal endometrium, p=0.001) and

ER expression was seen to increase in one case where normal endometrium became hyperplastic at 12 months (non-atypical).

Normal endometrium did not show any significant difference in ER, PR, androgen receptor or Ki-67 expression following 12 months of bariatric surgery induced weight loss. The reduction in ER and PR expression in hyperplastic endometrium was significant (n=4) and said to move towards the population mean. Proliferation Index (PI) was 10% pre surgery and 6% post-surgery, which represented a non-significant change. No mention was made of efforts to control for menstrual cycle timing, and 30% of the cohort reported progestagen use, which inhibits proliferation. This may account for the low mean PI across the cohort.

The same samples have also been assessed for the effect of weight loss on immune markers CD3 and CD20 and PTEN and no significant changes were seen (199). Approximately 50% of TMA's had insufficient tissue for analysis, and samples were PTEN null in 1 and 2 cases at baseline and 12 months respectively, making them unlikely to demonstrate a difference in PTEN before and after weight loss surgery.

1.5.7 Weight loss in endometrial cancer survivors

A single centre retrospective survey of endometrial cancer survivors who were treated for their disease between 2011 and 2012 received responses from 108 patients (46% response rate). Median BMI was 29.8kg/m^2 and 85% correctly self-identified their BMI category; 29% reported being told of the association between endometrial cancer and obesity and 52% reported attempting to lose weight following diagnosis. Being counselled to lose weight was significantly associated with trying to lose weight ($p<0.001$) (200).

In their cohort study of women who had been treated for atypical endometrial hyperplasia or stage 1 or 2 endometrial cancer Jernigan et al reported less uptake of weight loss opportunities (201). Of 106 women recruited (median BMI 40.9kg/m^2) 6 were already under the care of a bariatric specialist. The other 100 women were offered referral to a bariatric physician and 66 were offered referral to a bariatric surgeon as they met the criteria for bariatric surgery in the United States. Of these, 57

women declined referral to a specialist (31 due to fear of surgery, 18 due to location and distance of travel, 13 for financial reasons), 8 women accepted referral to a bariatric surgeon (1 of whom attended an appointment with the surgeon), and 35 accepted referral to a physician (16 of whom attended the appointment). Independent attempts at weight loss were made by 42 women; 41 women made no weight loss attempt. The study found that women were more likely to accept a bariatric referral if they had a higher BMI (p=0.035) or diabetes (p=0.033) and if they were offered referral within the first year after cancer diagnosis (47% vs. 35% at more than one year, p=0.059).

Many of the factors that affect weight loss seeking behaviour are non-modifiable, but taking the opportunity to offer a referral to a weight loss specialist and counselling patients about the importance of weight loss are both modifiable, physician dependent factors. A RCT showed that weight loss can be achieved with intensive behavioural and nutritional support but in reality not all endometrial cancer survivors are highly motivated trial participants and it is not clear what amount of weight loss would constitute clinically significant weight loss in the context of a cancer survivor (202). AHA guidelines propose 5% weight loss to be a significant reduction, although biomarker studies would suggest 5-10% is more meaningful (196).

Different mechanisms of administering a weight management programme have been studied. Haggerty et al conducted a RCT comparing a 6-month weight loss lifestyle intervention by either text messaging or telephone contact. 60% of women with BMI ≥30kg/m^2 who had endometrial hyperplasia or endometrial cancer (median BMI 34.5kg/m^2, median age 60) expressed an interest in the weight loss programme. More weight loss was seen in the telephone group than the text message group (7.6% vs. 4.1%). Weight loss was maintained at 12 months by 20% of the telephone group and 30% of the text message group.

1.6 Identifying the Research Question

1.6.1 Hypothesis

Despite the strong epidemiological link between obesity and endometrial cancer we have little knowledge of the effects of morbid obesity and weight loss on the endometrium.

With this in mind I hypothesised that morbidly obese women have a high-risk endometrium, which, as a response to major weight loss will exhibit changes that will offer insight into biomarkers of endometrial neoplasia that could be used to identify women who may benefit from targeted prevention interventions. To address this hypothesis, I conducted a prospective cohort study of morbidly obese women undergoing surgical and non-surgical weight management. Endometrial biopsies and serum samples were taken before and after weight loss to assess the impact of obesity and weight loss on endometrial cancer risk.

I hypothesised that bariatric surgery would result in reductions in circulating oestrogen, free androgen index, SHBG and leptin, in conjunction with increases in adiponectin levels, and that these changes would take longer to manifest than improvements in insulin resistance, which would be observed through a reduction in insulin levels and fasting glucose fairly rapidly after surgery. Such alterations in glucose homeostasis were expected to occur before significant weight loss as a result of the metabolic alteration and caloric restriction associated with bariatric surgery. It was proposed that the degree of these changes would be greater than those seen in the non-surgical weight loss group who would lose less weight more slowly.

I further hypothesised that the effects of obesity would be observed at a molecular level in histologically normal endometrium, and that higher levels of baseline pAKT, pERK and Ki-67 would be seen with increasing degrees of obesity, that weight loss would reduce the expression of these molecules in proliferative endometrium, and that their relationship with BMI would not be a linear one, as the reduction in cancer risk with BMI is not linear.

Based upon previously reported prevalence data it was expected that endometrial hyperplasia would be detected in approximately 0 to 12% of subjects, and endometrial

cancer in 0 to 3% of subjects, and that the prevalence would vary depending upon menopausal status.

1.6.2 Aims

Whilst endometrial cancer is often cured by hysterectomy and bilateral salpingo-oophorectomy it remains a significant clinical problem, particularly in morbidly obese women in whom the surgical risk is increased by obesity. Current gold standard treatment is laparoscopic hysterectomy, which in the face of severe obesity is not always possible. An alternative approach is apronectomy and abdominal hysterectomy; however this is associated with greater operative morbidity and prolonged recovery time. Not infrequently younger women are presenting with endometrial cancer and wish to preserve their fertility, which precludes standard operative management.

Now, more than ever, it is important to know if there is an opportunity to prevent endometrial cancer through screening of morbidly obese women and if there is a place for weight loss surgery or other targeted preventative measures (LNG-IUS, COCP, metformin), to reduce cancer risk or to treat pre-malignant endometrial abnormalities.

The overall aim of this project was to provide insight into the relationship between obesity and weight loss and the impact of these on the endometrium. By clarifying such associations it may be possible to identify targets for future work into the prevention and treatment of endometrial cancer. It may also further the discourse on the role of screening for high-risk groups, as well as the acceptability and feasibility of Pipelle endometrial biopsy as a screening tool in obese women.

The specific aims were to:

1. Determine the prevalence of endometrial abnormalities in pre and post-menopausal women undergoing surgical or non-surgical weight loss management for morbid obesity, and assess the impact of weight loss upon the endometrial histology

2. Determine the expression of biomarkers of proliferation and signal transduction molecules which have been implicated in endometrial carcinogenesis, before and after weight loss

3. Determine the effect of weight loss on menstrual function, gonadotrophin levels and circulating oestrogen, CRP, leptin and adiponectin levels

1.6.3 Objectives

The following specific research objectives were as follows:

1. Recruit 100-200 women into a clinical study to take blood and endometrial samples before and after weight loss
2. Assess the histopathology of the baseline samples to determine the rate of endometrial atypia and cancer in asymptomatic morbidly obese women
3. Use immunohistochemical techniques to examine changes in Ki-67 +/- AKT and pAKT, ERK 1,2 and pERK 1,2, PTEN, ER and PR which occur with weight loss
4. Measure changes in FSH, LH, glucose, insulin, oestradiol, progesterone, testosterone, CRP, FAI and SHBG and markers of inflammation and correlate with weight loss and alterations in menstrual function

2. METHODS

2.1 CLINICAL STUDY

2.1.1 STUDY DESIGN

A prospective observational cohort study entitled The Impact of Weight Loss on the Malignant Potential of Endometrium (ISRCTN17241389) was conducted (Figure 11), sponsored by Central Manchester Foundation Trust (Study ID R01331) and approved by the North West Ethics Committee on the 23/01/2012 (12/NW/0050) (Appendix 10.1). The study was co-adopted onto the NIHR portfolio by metabolic-endocrine and cancer (UKCRN ID 16828).

2.1.2 RECRUITMENT

Pre and post-menopausal female participants who were undergoing either surgical or non-surgical weight management were recruited from the regional weight management service at Salford Royal NHS Foundation Trust (SRFT).

The aims were to recruit 100 women undergoing bariatric surgery, and a comparison group of women undergoing non-surgical weight management, to document the prevalence of endometrial pathology in morbidly obese women and to observe the endometrial effects of weight loss.

"Bariatric surgery" is an umbrella term used to describe procedures such as sleeve gastrectomy, gastric bypass, gastric banding or balloon insertion. To be eligible for NHS funded bariatric surgery according to current UK guidelines (203) patients must have a BMI > 50kg/m^2 or have a BMI > 40kg/m^2 with a significant obesity-related comorbidity such as diabetes mellitus, which could be expected to improve with weight loss, and have primary care trust (PCT) funding for surgery (which requires a GP referral, fulfillment of one of the above two criteria and the completion of a minimum of 6 months non-surgical weight management clinic attendance). Weight loss surgery performed was laparoscopic Roux-en-Y gastric bypass, laparoscopic sleeve gastrectomy or laparoscopic adjustable gastric banding.

Participants who were managed non-surgically received lifestyle advice, dietetic input and regular outpatient follow up, were advised regarding hypo-caloric diets and

occasional use of medication such as metformin, Orlistat or liraglutide to aid weight loss.

Eligible patients were seen individually by the Clinical Research Fellow (Dr. Michelle MacKintosh) or by the Principal Investigator (Dr. Emma Crosbie) to discuss the study. They were provided with a Patient Information Sheet (Appendix 10.3) and informed written consent was obtained (Appendix 10.4) there and then or at a later date if the participant elected to take more time to consider their involvement. All recruits were made aware that participation was entirely voluntary and consent could be withdrawn at any time. The participant's General Practitioner and the weight management team were informed of their recruitment into the study.

2.1.3 STUDY ENTRY

Participants were deemed eligible for recruitment if all inclusion criteria were met and none of the exclusion criteria applied (Table 10).

TABLE 10: INCLUSION AND EXCLUSION CRITERIA

Inclusion Criteria	Exclusion Criteria
Female	Previous hysterectomy
Written informed consent	Pregnancy
Aged 18 years or over	IUD or LNG-IUS in situ
Undergoing bariatric surgery or non-surgical weight management	Previous endometrial ablation
	Current tamoxifen use

2.1.4 BASELINE ASSESSMENTS

At baseline a full medical history was taken and height, weight, waist and hip circumference recorded. All information was recorded on standardised case report forms (CRF). Height was measured with a wall-mounted stadiometer and weight was measured using standardised digital weighing scales specifically designed for bariatric patients. The Body Mass Index (BMI) was calculated as weight in kg divided by height in metres squared. Waist and hip circumference was recorded in centimetres using a

non-tension tape measure. The waist circumference was measured at the midpoint between the iliac crest and the most inferior palpable rib. If the bony landmarks were not palpable the patient was asked to place the tape measure around their narrowest circumference. The hip circumference was taken as the widest circumference around the buttocks.

Fasting venous bloods (approximately 30 mls) and an endometrial sample were taken using either a Pipelle© (Carefusion, United Kingdom) or MedGyn Endosampler© (MedGyn, IL, USA) on the day of bariatric surgery or at the participant's first visit to the research clinic at St Mary's Hospital if they were not undergoing bariatric surgery.

2.1.5 Post intervention assessments

Follow up assessments were performed at 2 months and 12 months following baseline sampling, during which an abbreviated medical and gynaecological history was taken. Further blood and endometrial samples were taken and height, weight, hip and waist circumference were again recorded. The timing of the follow up assessments were chosen to assess the effects of weight loss induced metabolic improvement, which manifest as early as one week post bariatric surgery (2 months) and the effects of maximal weight change (bariatric surgery related weight loss plateaus at 12 months).

2.1.6 Sample handling & storage

2.1.6.1 Blood

Blood samples were tested for serum FSH, LH, oestradiol, progesterone, free androgen index, testosterone, sex hormone binding globulin, CRP, HbA1c, insulin and glucose. Serum insulin was transported on ice to the Central Manchester Foundation Trust (CMFT) central laboratory and processed within one hour according to standard operating procedures (SOP).

Blood samples were centrifuged for 10mins at 3000rpm before being aliquoted and assayed using automated systems (Modular 8000, Roche Cobas). Additional samples were centrifuged, separated into serum, plasma and buffy coat in 0.5 - 1.0ml aliquots, and stored at -80°C in a Human Tissue Authority licensed biobank facility.

Once participants had completed follow up these were subjected to biomarker analyses for high sensitivity CRP (hsCRP), IL-6, adiponectin and leptin, using commercially available and validated enzyme-linked immunosorbent assay (ELISA) kits Individual patients' samples were analysed concurrently to minimise inter-assay variation in the results. Mr. P Pemberton, Senior Clinical Scientist in the Specialist Assay Laboratory, CMFT, performed these assays.

2.1.6.2 ENDOMETRIUM

Endometrial specimens were processed in the CMFT pathology department where they were formalin-fixed, embedded in paraffin, sectioned, and stained using a standard haematoxylin and eosin preparation. Two independent consultant gynaecological histopathologists assessed morphology. The remainder of the wax block was stored for immunohistochemical analyses that were carried out once the participant had completed follow up.

All clinical samples have been stored in the Trust Biobank at CMFT. The Biobank is licensed by the Human Tissue Authority and has approval from the National Research Ethics Service to operate as a Research Tissue Bank. It is housed in a dedicated, secure facility. Samples are stored anonymously and anonymised clinical data is logged according to the Data Protection Act 1998.

2.1.7 STUDY PROCEDURES

In premenopausal participants the endometrial biopsies were taken during the late proliferative phase of the endometrial cycle, where possible. When endometrial sampling was not possible, for example due to a previous cone biopsy rendering the cervix impassable, the participant was excluded from the remainder of the study. When the endometrial sample was inadequate for diagnosis, the patient was informed. In the absence of abnormal bleeding or other symptoms of concern they were either resampled as per the study protocol or not resampled if they chose to withdraw from the study or if the inadequate biopsy was their final sample.

2.1.8 Management of endometrial abnormalities

A protocol for the management of incidentally detected abnormality was established a priori (Appendix 10.2 Study Protocol). In the event of an abnormal blood or endometrial biopsy result the participant was contacted to inform them of this and further arrangements for investigation or management made as required. The General Practitioner (GP) was informed of abnormalities detected. Abnormal cervical cytology was managed via the cervical screening programme and if colposcopy was indicated this was performed by the participant's local colposcopy clinic. Abnormal blood results were notified to the participants GP for follow up if clinically relevant.

If atypical hyperplasia or cancer was identified on histopathological assessment of the endometrium the participant was referred to a consultant gynaecological oncologist who assessed and counselled them in the gynaecology outpatient department and appropriate management was instigated (conservative, medical or surgical). All cases of neoplasia were discussed at the central gynaecological oncology multidisciplinary team (MDT) meeting.

All cases of endometrial cancer were managed surgically, as per standard care, if the patient consented and was fit to undergo surgery. A trial of medical management was considered, with serial biopsies to ensure no progression of disease, if surgery did not take place. If surgery did not take place, CT or MRI imaging was used to confirm that there was no evidence of more than stage 1a disease.

Symptomatic AEH was managed medically with a LNG-IUS for 6 months with serial endometrial biopsies every 3 months; surgery was offered up front if this would have been the standard treatment (symptomatic disease with no requirement for fertility preservation, patient consents to and fit for hysterectomy). Surgery was also offered if disease progressed on medical treatment or had not resolved after 12 months of medical management.

Asymptomatic AEH was managed either conservatively or medically, as described above, depending upon patient preference and willingness to undergo serial endometrial biopsies. A trial of LNG-IUS was offered in the case of persistent or

progressive disease.

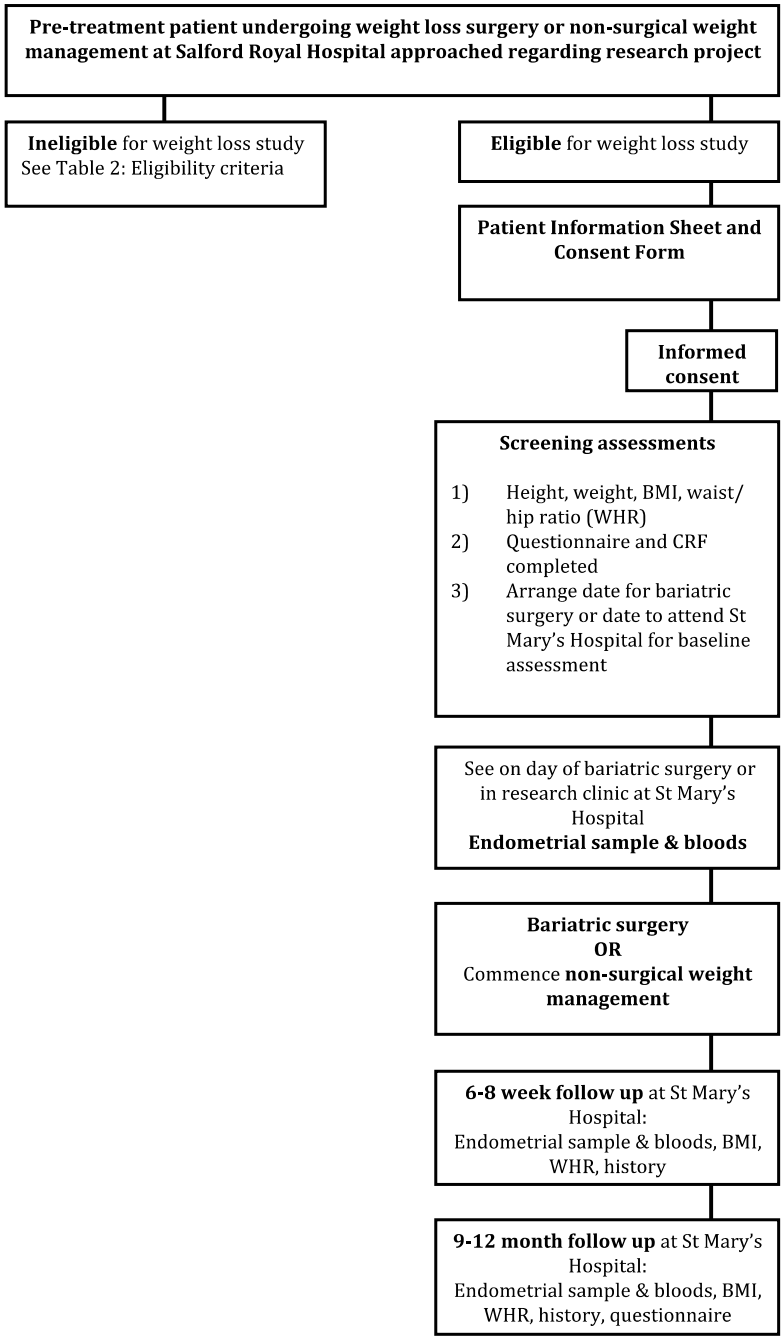
2.1.9 Procedural complications

Endometrial biopsy and hysteroscopy have the potential for harm. Participants were fully informed of the risks of the procedures before their consent was obtained. There is a small risk of uterine perforation (0.002-1.7%) or infection (204). If such complications were suspected the participant was asked to present themselves to the on call gynaecology team at CMFT, or to their local hospital, for further assessment. Participants were made aware of the signs and symptoms of possible complications and given contact details should they need to contact a member of the research team following a biopsy.

2.1.10 Adverse incident reporting

The appearance or worsening of any undesirable sign, symptom or medical condition occurring after the study commenced was considered an adverse event, regardless of whether it was thought to be related to study procedures performed. Abnormal laboratory values or test results constituted an adverse event if they induced clinical signs or symptoms or were considered clinically significant and warranted treatment. The detection of malignant or premalignant abnormalities of the endometrium on histopathology was exempt from Serious Adverse Event (SAE) reporting, as the identification of such abnormalities were included in the aims and objectives of the study. The participant is likely to benefit from earlier detection of these occult abnormalities. All adverse incidents were recorded and managed appropriately.

FIGURE 11: STUDY SCHEMA



2.1.11 Outcome measures

Primary	Secondary
Change in Ki-67 at 2 months	Baseline prevalence of AEH and endometrial cancer
	Change in Ki-67 at 12 months
	Change in pAKT at 2 & 12 months
	Change in pERK 1, 2 at 2 & 12 months
	Change in ER & PR at 2 & 12 months
	Change in PTEN at 2 & 12 months
	Change in glucose metabolism at 2 & 12 months (fasting glucose & insulin, HOMA-IR, HbA1c)
	Change in inflammatory markers at 2 & 12 months (hsCRP, IL-6)
	Change in adipokines at 2 & 12 months (leptin, adiponectin)
	Change in reproductive hormones at 2 & 12 months (oestradiol, progesterone, FSH, LH, testosterone, FAI, SHBG)
	Change in menstrual cycle/bleeding pattern at 2 & 12 months

2.2 PHYSIOLOGICAL ANALYSES

Blood samples were contemporaneously tested for serum FSH, LH, oestradiol, progesterone, FAI, testosterone, SHBG, HbA1c, insulin and glucose in the Clinical Biochemistry Department, CMFT, according to local SOP using standard automated ELISA techniques in current clinical use. All assays underwent preset standardised maintenance and startup QC, and if a result was more than two standard deviations from the mean the system was recalibrated. If a result was more than three standard deviations from the mean the system was recalibrated and the sample retested.

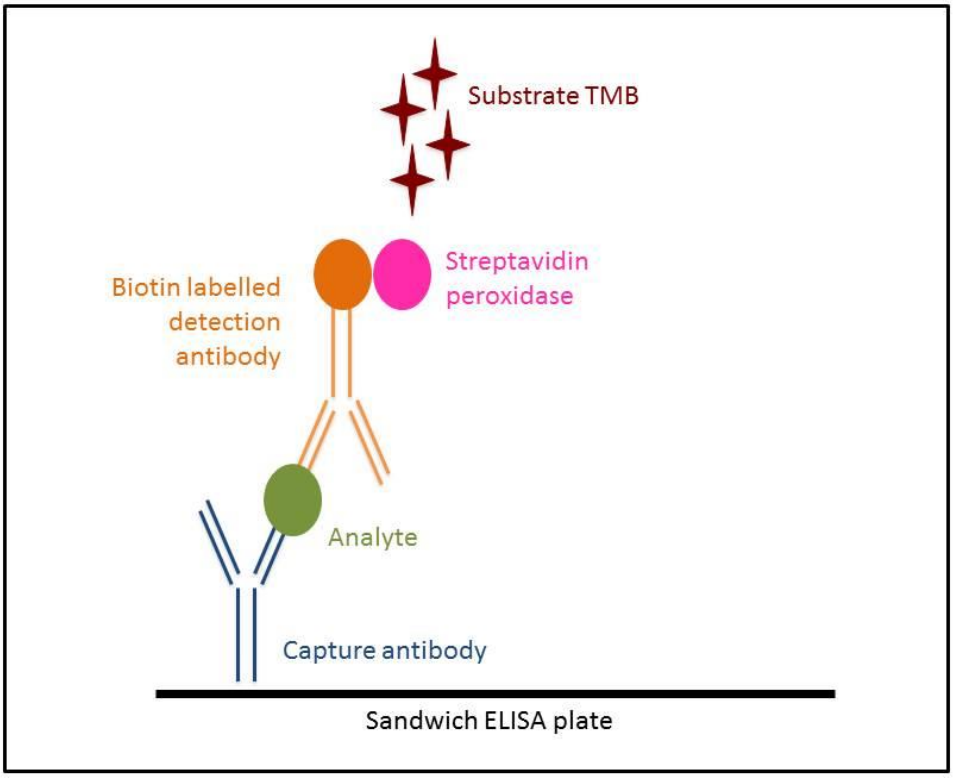
Glucose was measured by a photometric assay and all other biochemical tests were measured using electrochemiluminescence immunoassays. Full details of the assays and their performance are described in Appendix 10.6.

Additional biomarkers were measured in frozen samples of serum, once participants had completed follow up. Materials and methods are described fully in appendix 10.7.

High sensitivity C-reactive protein (hsCRP) was measured in serum by an in-house antibody sandwich ELISA technique. Rabbit anti-human CRP antibodies (unlabelled and horseradish peroxidase-labelled), calibrators, and controls were obtained from Abcam (Cambridge, United Kingdom), *o*-phenylenediamine (Sigma-Aldrich, Poole, Dorset, UK) being used to detect the amount of bound analyte. Colour intensity was measured at **λ** = 490nm on a plate reader (Dynex Technologies, Worthing, UK) and a standard curve generated using Fig P software (Hamilton, ON, Canada) to calculate hsCRP concentration.

Interleukin-6, adiponectin and leptin were all measured with DuoSet ELISA development kits (R&D Systems, Abingdon, United Kingdom) which utilise a sandwich ELISA principle of biotin labelled detection antibody, streptavidin peroxidase and substrate tetramethylbenzidine solution (Figure 12). Colour intensity was measured at **λ** = 450nm on a plate reader (Dynex Technologies, Worthing, UK) and a standard curve generated using Fig P software (Hamilton, ON, Canada) to calculate biomarker concentration.

FIGURE 12: THE SANDWICH ELISA PRINCIPLE OF BIOTIN LABELLED DETECTION AS USED FOR BIOMARKER ANALYSES



2.3 TISSUE ANALYSES

Immunohistochemistry was performed on prepared slides to enable the detection of proteins of interest, Ki-67, ER, PR, pAKT, pERK and PTEN. This process utilises antibodies to detect and visualise antigens, the proteins of interest, in tissues. The expression of these proteins was then quantified / semi-quantified.

2.3.1 NON-AUTOMATED IMMUNOHISTOCHEMISTRY

An initial sample of 20 prepared slides, plus one control (tonsil), were hand stained for Ki-67 using the Novolink polymer detection system (Novocastra). Sections were cut, mounted onto slides (Superfrost Plus, Thermoscientific), baked and deparaffinised in xylene and industrialised methylated spirits (IMS) before being rehydrated under running water. Antigen retrieval was performed using heat for 25 minutes in high pH unmasking solution (1:100 high pH buffer in distilled water). Sections were loaded onto the Shandon Sequenza and incubated with a hydrogen peroxide block for 10 minutes to block endogenous peroxidases, followed by two five-minute washes in Tris Buffered

Saline with Tween, diluted 1:10 with distilled water (TBS). Ki-67 primary antibody (Leica Novocastra) was added (1:50, diluted with TRIS buffered saline with Tween) and incubated for 30 minutes. Horse serum (Vector labs, S-2000) was added to the diluted antibody at a concentration of 1:50. This was followed by two five-minute washes with TBS, incubation with the post-primary block for 30 minutes, two further five-minute washes, 30 minutes incubation with Novolink polymer and two further five-minute washes with TBS. Colour staining was achieved with the addition of 3,3'-diaminobenzidine (DAB) to each slide for five minutes before being placed under running water and then counterstained in haematoxylin, 0.1% acid alcohol and Scott's tap water. Slides were then dehydrated in an increasing IMS and xylene series and coverslipped.

To minimise the quantity of antibody used and maximise output the decision was taken to proceed with automated immunohistochemistry once the process had been learned and understood.

2.3.2 Automated immunohistochemistry

2.3.2.1 Optimisation

Each of the proteins of interest was optimised on the Ventana BenchMark XT IHC Staining Module (Ventana Co., Tucson, AZ, USA) and the outcomes of the optimisation process can be seen in Appendix 10.5. This work was done jointly with Ms. C Keeling, Biomedical Scientist, CMFT. Control tissues were optimised alongside endometrial biopsy samples and are listed in Table 11.

Despite repeated iterations attempting to optimise PTEN on Pipelle samples we were unsuccessful. Although stromal cells stained positively to act as an internal control, the majority of the study participants exhibited universally PTEN negative glands, which would obviously be unexpected in samples that were predominantly of normal endometrium. There was a paucity of data on immunohistochemistry protocols to stain endometrial samples; most data was on solid endometrial tumours – which using our protocol we successfully stained during the optimisation process. We took from this that unlike the other antibodies optimised in this study; staining protocols for PTEN are

substantially different for histologically normal suction curette derived endometrial samples than they are for solid tumour.

TABLE 11: CONTROL TISSUES USED FOR OPTIMISATION

Ki-67	Endometrium Endometrial adenocarcinoma Tonsil
ER	Endometrium
PR	Endometrium
pAKT	Endometrium Endometrial adenocarcinoma Multiblock
pERK	Endometrium Endometrial adenocarcinoma Prostate Kidney
PTEN	Endometrium Endometrial adenocarcinoma Tonsil Colonic adenocarcinoma

Since completion of the laboratory work for this study one paper has been found to contain an alternative staining protocol for PTEN in histologically normal biopsies. Notably the incubation period used in each study was overnight, with a lower antibody concentration than we used (1+300, cf. 1+100 for 60 minutes). The published images show stronger, less specific stromal positivity than we demonstrated in our samples, as a result of these key differences in staining technique. Restaining of all slides for PTEN will be repeated as part of my further work, on the basis of this publication (149).

TABLE 12: IMMUNOHISTOCHEMISTRY PROTOCOLS

Antibody	Clone	Company	Monoclonal Antibody Species	Antigen Retrieval (heat time in CC1 buffer)	Antibody dilution	Antibody Incubation Time	Additional Steps
pAKT (Ser473)	D9E	New England Bioscience	Rabbit	60 min	1+100	60 min	Ultra wash
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	D13.14.4E	New England Bioscience	Rabbit	8 min	1+2500	32 min	Ultra wash
PTEN	6H2.1	Dako	Mouse	60 min	1+100	60 min	None
ERα	SP1	Roche	Rabbit	30 min	RTU	32 min	Ultra wash
PR	1E2	Roche	Rabbit	30 min	RTU	30 min	Ultra wash
Ki-67	MIB-1	Dako	Mouse	30 min	1+100	32 min	Ultra wash

Key: RTU = ready to use

2.3.2.2 STAINING PROTOCOLS

Tissue sections were cut at 4 μm and baked for 20 minutes at 70°C. The automated Ventana BenchMark XT IHC Staining Module (Ventana Co., Tucson, AZ, USA) was used together with the XT Ultraview 3, 3' diaminobenzidine (DAB) v3 detection system (Ventana Co.). Tissue sections were deparaffinised and incubated in EZPrep Volume Adjust (Ventana Co.). At intervals between steps the slides were washed with a TRIS-based reaction buffer, pH 7.6. A heat-induced antigen retrieval protocol was carried out using a TRIS–ethylenediamine tetracetic acid (EDTA)–boric acid pH 8 buffer, Cell Conditioner 1 (CC1) for between 8 and 60 minutes depending upon individual optimisation. The sections were incubated with ultraviolet inhibitor blocking solution for 4 minutes, then with an optimised concentration of antibody. This was followed by incubation with horseradish peroxidase-linked secondary antibody for 8 minutes, DAB chromogen for 8 minutes and copper for 4 minutes. Counterstain (Haematoxylin II) was applied for 4 minutes before a 4-minute incubation with bluing reagent. Slides were then removed from the staining platform and dehydrated through 3 steps of 99% IMS and 3 changes of Xylene. Sections were cover slipped using ClearVue Mount XYL (Thermo Scientific). Table 12 lists the individually optimised final protocols used for each protein of interest.

2.3.3 SCORING OF IMMUNOHISTOCHEMISTRY

Whole slides were independently assessed by two blinded scorers and an average was taken. Slides with more than 10% inter-observer disagreement were rescored and ratified. Glandular and stromal positivity were scored separately if both expressed the protein of interest. Figure 13 demonstrates representative images of the immunostains of study participants.

2.3.3.1 KI-67

Ki-67 is a nuclear protein expressed in proliferating cells, present in G1, S, G2 and M phases of the cell cycle (absent in G0). Endometrial stroma is usually negative and glandular nuclei are positive, to varying degrees (Figure 12). The recommendations of Dowsett et al (205) were used as the basis for the scoring methods developed, from their work on the assessment of Ki-67 in breast tumours which has become the most

widely used method for comparing proliferation between tumour samples in efforts to predict prognosis or response to treatment. Due to wide variation in the use of Ki-67 in research studies a working party was convened and a consensus document produced to try and improve the comparability in study methodology going forward.

In breast cancer there is convincing evidence that Ki-67 is a prognostic indicator in early disease, and predicts response to certain systemic anti-cancer treatment in ER positive breast cancers. Residual Ki-67 at the end of neoadjuvant endocrine treatment correlates with risk of recurrence; it has been used as a primary outcome measure in window of opportunity studies as changes in its expression manifest quickly (2 weeks) and it can be extrapolated as a surrogate marker of other endpoints such as progression free survival (205). Based on our understanding of the effect of endocrine therapy on Ki-67 in breast cancer, a reduction of Ki-67 of 5% is thought to be clinically relevant, as this was the difference seen in responders to tamoxifen treatment for breast cancer (206). It must however be noted that baseline levels of Ki-67 in breast and endometrium, both benign and malignant, are very different

There is a paucity of data on Ki-67 in endometrium and in endometrial cancer in comparison. Villavicencio et al reported a 5% higher median Ki-67 expression in "obese" endometrium compared with "overweight" endometrium. They do not however present their raw data or median Ki-67 scores and the figure of 5% was extrapolated from the histogram. This formed the basis of the sample size calculation, in terms of the change that might be expected with weight loss (102).

Slides were assessed at x10 and x20 magnification to establish representative high-powered fields (HPF). Three representative high-powered fields (x20) were taken and the number of positive nuclei from at least 1000 cells per view were counted and converted to a percentage to produce a proliferation index. Where samples were scanty all glands were scored. Slides where abnormality was present (AEH or endometrial cancer) were first assessed alongside the H&E and the area of abnormality marked by a consultant pathologist, before including this area in the regions of interest selected for scoring. Two independent assessors, blinded to weight, arm of study and

whether samples were baseline or follow up samples, scored the slides and any with significant intra-observer variation were rescored jointly using a double-headed microscope.

Whilst a similar study to ours used tissue microarrays (TMA) to assess the effect of weight loss on Ki-67, the decision was made to score whole slides rather than TMA's in this study to maximize the number of samples available for analysis. This was due to Pipelle endometrial biopsies having a tendency to be disrupted and when endometrial biopsies are taken from asymptomatic women there is a high rate of scanty or insufficient biopsies. Both of these factors may result in the area of endometrium in the wax block being missed when taking a core to prepare a TMA. There is a documented loss of samples for analysis in TMA's due to technical issues that can affect the preparation of the TMA (198). Unpublished data from our group has shown scoring of Ki-67 using TMA's to be less robust compared with whole slides with regards to reproducibility and Interobserver Intraclass Correlation.

2.3.3.2 Oestrogen and progesterone receptors

ERα immunostaining is nuclear, and present in both normal and neoplastic endometrium. Proliferating normal endometrium is generally strongly positive for ERα, and this was our experience in the study samples. Both stroma and glands stain positive but for the purposes of assessing positivity glandular nuclei only were scored. ERα rather than ERβ was assessed as the two have contrasting functions and localisation within the endometrium, and ERα is thought to be pro-proliferative whereas ERβ is thought to be anti-proliferative (207,208).

The PR monoclonal antibody used was directed against an epitope present in the nucleus of cells positive for progesterone receptors. Both PR-A and PR-B isoforms are recognised. Again, normal proliferating endometrium is expected to be strongly positive for PR, in nuclei of both of the glands and the stroma (Figure 12).

The staining of hormone receptors was homogeneous across whole slides and therefore a modified H score was used to quantify ERα and PR. Each slide was divided into six areas and each area given a score between 0-3 for intensity of staining (0 =

negative, 1 = weak, 2 = moderate, 3 = strong) and these were added together to give a cumulative score between 0 and 18.

2.3.3.3 pAKT and pERK

Phosphorylated AKT stains in both normal and abnormal endometrium. Increased levels of pAKT have been reported in endometrial cancer with levels correlating to grade of tumour, and to Ki-67 expression. Comparison of endometrial AKT and pAKT levels in normal, overweight and obese women showed increased expression of pAKT with overweight and obesity but no change in total AKT (102). For this reason we chose to assess pAKT alone. AKT activation occurs at the plasma membrane before translocating through the cytoplasm to the nuclei or mitochondria. This is why membranous, cytoplasmic and nuclear expression is seen (209).

Phosphorylated ERK 1,2 is also expressed in both the nucleus and the cytoplasm, and levels are similar in normal, hyperplastic and malignant endometrium (Figure 12). Both pAKT and pERK 1,2 have been shown to be elevated in obese women on Western Blot analysis (102), hence the decision to further investigate this association by immunohistochemistry.

As these immunostains tend to appear heterogeneous an H score was used to quantify the distribution and intensity of positive staining, and produce a score between 0 and 300 for both pERK 1,2 and pAKT. Some studies have classified these scores in to negative (0-9), low (10-100), medium (101-200) and high (201-300).

H = (3 x % strong staining) + (2 x % moderate staining) + (% weak staining)

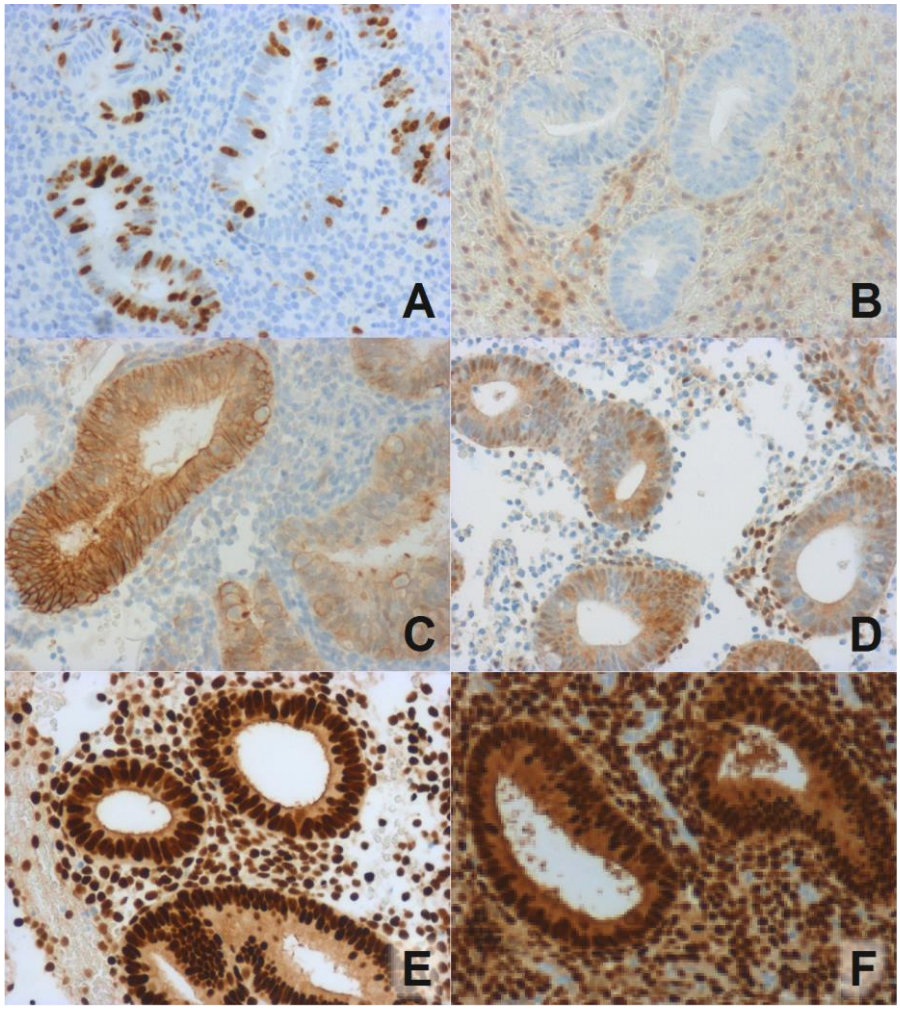
2.3.3.4 PTEN

PTEN immunohistochemistry is thought to be the most accurate reflection of functional PTEN status, as mutation analysis can be difficult due to varied mutations that can be implicated, and because mutation analysis fails to detect PTEN loss due to promoter methylation or epigenetic silencing (210). In a validation study of commercially available PTEN antibodies in endometrial carcinoma, only the 6H2.1 clone was demonstrated to show an inverse correlation with pAKT staining and some correlation with PTEN mutational analyses (211) and therefore this is the clone used in this study.

A variety of scoring methods have been described in the literature, with use of H scores and of discrete positive/negative classification (212). While some samples stain heterogeneously, with negative glands alongside glands where PTEN function is preserved, often the presence of one or more negative gland in a specimen is considered to render that sample PTEN null(149). Difficulty in assessing glandular positivity is recognised where there is pale glandular staining amongst strongly positive stroma. These are variably scored as positive and negative in the literature. The presence of positive stromal cells acts as an internal control (212) (Figure 13).

For the purposes of this study the intended approach was to score samples with one or more negative gland as PTEN null, otherwise it was assumed PTEN function was intact. The stroma was scored using the H score as described for pERK and pAKT, as stromal PTEN expression has been reported to vary throughout the menstrual cycle under the influence of hormones. Menstrual endometrium does not express PTEN protein in the epithelium but it is present in the stroma. During the proliferative phase PTEN expression is ubiquitous across stroma and epithelium. In the secretory phase stromal expression is maintained but epithelial expression reduces, possibly in response to circulating progesterone levels. In the late secretory phase stromal nuclear expression is strong but stromal cytoplasmic expression reduces. PTEN protein in the endometrium basalis, which is less subject to the hormonal variation of the functional layer, demonstrates absent or stable low levels of PTEN (147).

FIGURE 13: IMAGES OF NORMAL ENDOMETRIUM IMMUNOSTAINED FOR KI-67, PTEN, PAKT, PERK 1,2, ER AND PR



Key: images of endometrial biopsy specimens from histologically normal endometrium at x 20 magnification, stained for: A) Ki-67 (nuclei of glands positive and stroma negative), B) PTEN (PTEN null, stroma positive acts as an internal control but glands are negative), C) phosphorylated AKT (note membranous glandular positivity), D) phosphorylated ERK 1,2, (cytoplasmic and nuclear staining), E) oestrogen receptors (ER**α**) and F) progesterone receptors (PR A & B) (both glands and stroma strongly positive as would expect in normal pre-menopausal endometrium).

2.4 Data handling & statistical analyses

2.4.1 Power calculation

Data to inform a sample size calculation was sparse but estimated for the primary outcome measure of change in proliferation (% Ki-67 positivity). Extraction of data from Figure 1 of Villavicencio et al suggests the standard deviation of Ki-67 is approximately 12% with a difference between overweight and obese women of approximately 5% (102). Assuming within-subject correlation between proliferation index at baseline and after weight loss of 0.5, it was estimated that the study would require 51 paired samples to show a difference in Ki-67 of 5% with 80% power. If the correlation between measurements at baseline and after weight loss is greater than 0.5 the sample size needed would reduce, and vice versa.

The difference of 5% was selected as this was the difference in Ki-67 expression seen when Villavicencio et al compared endometrial biopsies of overweight women with endometrial biopsies of obese women (102). In breast cancer studies of adjuvant endocrine therapy this reduction has been shown to be clinically significant with reduced risk of recurrent disease (206), however there are no comparable studies in endometrium to suggest what change one might expect with weight loss or what might constitute a clinically significant change.

2.4.2 Statistical analyses

Statistical analyses were performed using Graphpad Prism version 5.0b for Mac (GraphPad Software, California, USA) and SPSSStatistics version 23.0.0.0 for Mac (IBM, Armonk, NY, USA). Normality was assessed using D'Agostino Pearson omnibus normality test and non-parametric data has been expressed as medians with interquartile range and parametric data expressed as means and standard deviation. Statistical tests performed included Wilcoxon signed rank tests for non-parametric and t-tests for parametric continuous data respectively (198,213). A P value of < 0.05 was considered to be statistically significant. Confirmatory Kruskal Wallis testing has been performed for the primary outcome measure (104). Correlations were assessed using Spearman's Rank Correlation test.

3. Results I

Recruitment to the clinical study, characteristics of the cohort and the prevalence of occult endometrial pathology

3.1 Participant accrual and retention

Between March 2012 and December 2014 248 patients were screened for eligibility and 118 women were enrolled onto the study. Eighty of these underwent baseline assessment, 47 and 34 of whom were followed up at 2 and 12 months respectively. Figure 14 shows the accrual, retention and attrition of study recruits.

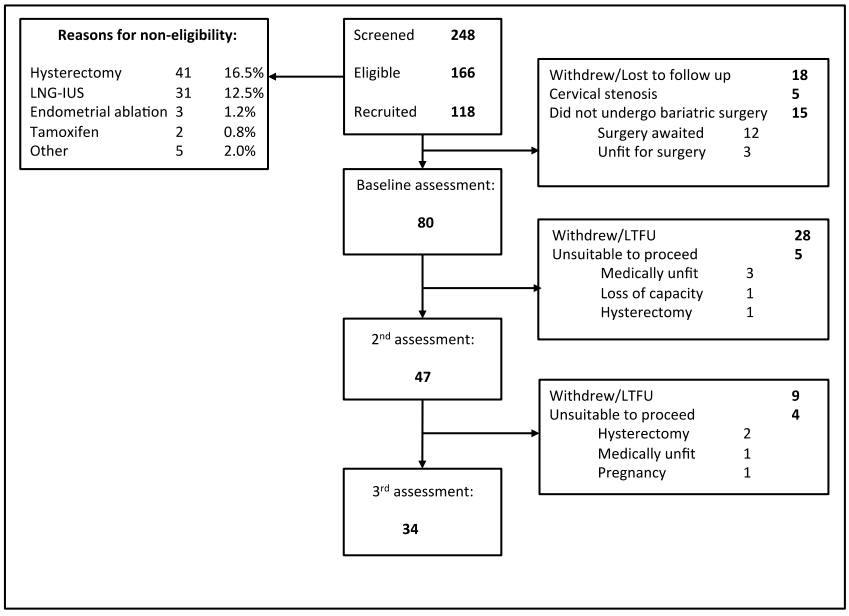
Approximately 70% of eligible women initially consented to participate in the study. The most common reasons for non-eligibility were previous hysterectomy or LNG-IUS (50% and 38% of non-eligible women respectively). Of all 248 women screened, 16.5% had previously had a hysterectomy and 12.5% had a LNG-IUS in situ; menstrual dysfunction was cited as the main indication for both.

There were particular challenges to the recruitment and retention of non-surgical patients. The non-surgical weight management clinics had a higher rate of patients failing to attend appointments, which limited access to them as potential recruits. The non-surgical patients expressed more reluctance to participate on the basis of pre-existing comorbidity. Furthermore, because the baseline biopsy could not be taken opportunistically whilst the patient was anaesthetised for bariatric surgery, women were more reluctant to participate.

Recruitment of non-surgical patients stopped in August 2013, due to these logistical issues and to significant baseline differences between the surgical and non-surgical cohorts. At this point 14 non-surgical patients had been accrued and 8 had undergone baseline assessment, the other 6 having withdrawn from the study prior to baseline assessment. Two more non-surgical patients withdrew following baseline assessment and did not undergo either of the follow up assessments.

When data collection for the purposes of this thesis ended, in December 2014, 12 of the 104 (11.5%) surgical patients enrolled were still awaiting bariatric surgery, and therefore had not undergone baseline assessment, 3 (2.9%) were unfit to undergo bariatric surgery on pre-operative assessment, and 12 (11.5%) had withdrawn their consent to participate. In five patients (4.8%), baseline sampling was not possible due to cervical stenosis, either due to previous cone biopsy or postmenopausal change, and they were withdrawn from the study.

FIGURE 14: ACCRUAL AND RETENTION OF PARTICIPANTS



Following baseline assessment, 28 patients (35%) withdrew or were lost to follow up and a further 5 (6.3%) became unfit or unsuitable to proceed with the study because they lost capacity to consent (1), had a hysterectomy for endometrial cancer (1) or were no longer medically fit to participate in the study (3).

Following assessment at 2 months a further 9 patients were lost to follow up or withdrew from the study (11.3%) and 4 were unsuitable to proceed because they had

had a hysterectomy for endometrial cancer (2), were pregnant (1) or were no longer medically fit to participate in the study (1).

The overall loss to follow up rate was 41% at 2 months and 57.5% at 12 months, which was higher than expected, and more than the sample size calculation was based upon. In summary prevalence data are available for a cohort of 80 women who underwent baseline sampling, 47 paired samples from baseline to 2 months (41 surgical) and 34 triplet samples available at baseline, 2 and 12 months (28 surgical).

3.2 Characteristics of the cohort

3.2.1 Baseline Characteristics

Eighty women underwent baseline sampling (Table 13). Most of the women were premenopausal (62/80, 78%), with an average age of 44 years (range 24-65), median parity of 2 and median BMI of 52.1kg/m^2 (IQR 47-57 kg/m^2). Ninety percent were of white British ethnic origin, which reflects the population attending the weight management service. The most common reason for ineligibility to participate was a history of hysterectomy, which may contribute to the higher prevalence of premenopausal women in this cohort, if the incidence of hysterectomy increases with age. Age was normally distributed according to the D'Agostino & Pearson omnibus normality test, as was parity and weight. BMI was not normally distributed.

Weight loss was instigated by surgical (72/80) and non-surgical (8/80) management. The mean age was 43 vs. 46 years and 55/72 vs. 7/8 were premenopausal in the surgical and non-surgical cohorts, respectively. Median BMI was 52.2kg/m^2 vs. 44.3kg/m^2 ($p=0.0046$) and median weight was 135kg vs. 111kg in the surgical and non-surgical cohorts, respectively. There were also significant differences between the surgical and non-surgical cohorts in fasting glucose ($p<0.0001$), free androgen index ($p=0.002$) and HOMA-IR ($p=0.017$), with these being higher in the surgical cohort where women had a higher BMI.

Obesity related comorbidities were common, in particular type 2 diabetes mellitus, which was seen in 33% (26/80). Twenty-two women (28%) were prescribed metformin

for this, 7 (9%) used insulin and 7 (9%) used other hypoglycaemic medications such as liraglutide, glibenclamide or gliclazide.

The presence of menstrual or reproductive dysfunction was also common. A history of subfertility was reported by 7 women (9%). Polycystic Ovary Syndrome (PCOS) was reported by 14 of the cohort (18%), 2 of whom were prescribed metformin for this indication. Of the premenopausal participants, 12 (15%) were amenorrhoeic and 25 (31%) reported oligomenorrhoea. Abnormal vaginal bleeding (heavy menstrual bleeding, intermenstrual bleeding, postmenopausal bleeding or post-coital bleeding) was reported by 28 women (35%). Only 25 women (31%) reported regular menstrual cycles. Forty four percent of women self-reported being overdue cervical screening or never having undergone cervical screening. Ten women (13%) were using oral or injectable progestagen only contraceptives and 3% (2/80) were using hormone replacement therapy.

TABLE 13: CHARACTERISTICS OF THE COHORT

			Surgical (n=72)	Non-Surgical (n=8)	Total Cohort (n=80)
Demographics					
Age, years		Mean (SD)	43 (9.3)	46 (10.3)	44 (9.35)
Ethnicity	White British Asian Black Other	n (%)	66 (91.7) 3 (4.2) 2 (2.8) 1 (1.4)	6 (75.0) 1 (12.5) 0 (0.0) 1 (12.5)	72 (90.0) 4 (5.0) 2 (2.5) 2 (2.5)
BMI, kg/m^2		Median (IQR)	52.2 (47.0-57.0)	44.3 (41.9-50.3)	52.1 (46.6-56.9)
Weight, kg		Median (IQR)	134.8 (117.0-152.6)	111.0 (107.8-121.6)	136.2 (121.7-153.7)
Reproductive factors					
Parity, number		Median (IQR)	2 (0-2)	2 (1-3)	2 (1-3)
Menopausal status	Pre- Post-	n (%)	55 (76.4) 17 (23.6)	7 (87.5) 1 (12.5)	62 (77.5) 18 (22.5)
Menstrual cycle	Amenorrhoeic Regular Irregular	n (%)	29 (40.3) 23 (31.9) 20 (27.8)	1 (12.5) 2 (25.0) 5 (62.5)	30 (37.5) 25 (31.3) 25 (31.3)
Abnormal bleeding*	HMB IMB PMB PCB	n (%)	17 (23.6) 3 (4.2) 2 (2.8) 3 (4.2)	2 (25.0) 0 (0.0) 0 (0.0) 1 (12.5)	19 (23.8) 3 (3.8) 2 (2.5) 4 (5.0)
Exogenous hormones	HRT Progestagens[§]	n (%)	2 (2.8) 9 (12.5)	0 (0.0) 1 (12.5)	2 (2.5) 10 (12.5)
PCOS		n (%)	13 (18.1)	1 (12.5)	14 (17.5)
Insulin resistance					
Type 2 diabetes		n (%)	23 (31.9)	3 (37.5)	26 (32.5)
Hypoglycaemic medications	Metformin Insulin Other[#]	n (%)	19 (26.4) 7 (9.7) 7 (9.7)	3 (37.5) 0 (0.0) 0 (0.0)	22 (27.5) 7 (8.8) 7 (8.8)

Key: IQR interquartile range; n number, SD standard deviation * HMB Heavy Menstrual Bleeding, IMB Intermenstrual Bleeding, PMB Postmenopausal Bleeding; [§]Progestagen contraceptive (progesterone-only pill, injectable); [#]Other medications (liraglutide, glibenclamide, gliclazide)

3.2.2 Failed or inadequate biopsies

Baseline endometrial sampling was not technically possible in 5 women (4.8%) due to cervical stenosis, due to either previous cone biopsy or post-menopausal status. No baseline biopsies failed due to other technical difficulties. At 2 months 47/49 attempted endometrial biopsies were successful. One endometrial biopsy was not technically possible due to the participant's body habitus (baseline biopsy had been successfully achieved under general anaesthetic) and one attempt failed because the participant could not tolerate it, both withdrew from the study at this point. At 12 months, all 34 attempted endometrial biopsies were successfully completed. Endometrial biopsy was inadequate for histopathological assessment in 2/80 cases at baseline (2.5%), in 1/47 (2.1%) at 2 months and 1/34 (2.9%) at 12 months.

3.2.3 Timing of biopsies

At baseline, 37 (46%) women who underwent biopsy had last menstrual period (LMP) and current day of menstrual cycle documented, the remainder being amenorrhoeic (n=19, 24%), postmenopausal (n=12, 15%) or unsure of the date of their LMP (n=12, 15%). The phase of cycle reported by the histopathologists was also recorded and used to categorise biopsies into proliferative, secretory, menstrual or inactive. Where possible, biopsies were timed to take place as close to day 12 of the cycle as possible. This was limited somewhat by the opportunistic nature of taking endometrial biopsies from surgical patients whilst they were anaesthetised for bariatric surgery.

Most of the 80 baseline biopsies were classified as proliferative endometrium (57%) and 15%, 15% and 13% were secretory, inactive and postmenopausal endometrium respectively; 37 were from women who were premenopausal and menstruating. Follow up biopsies were, as far as possible, taken at a similar time of the menstrual cycle as the individual's preceding biopsies. There was sometimes discrepancy between the phase of the menstrual cycle the patient reported and the histopathological assessment of menstrual cycle phase. This is likely to be in part because of the high prevalence of oligomenorrhoea in this cohort.

3.2.4 Weight management

Eight participants (10%) underwent an established non-surgical weight management programme including regular dietician and physician follow up. Mean weight was 114.8kg, 113.8kg and 104.2kg at baseline, 2 and 12 months respectively. Mean difference in weight was -1.0kg (95% CI -5.4 to 3.4, p=0.81) and -7.2kg (95% CI -13.9 to -0.5, p=0.06) at 2 and 12 months. Mean % total weight change was -0.9% at 2 months (SD 3.4) and -5% at 12 months (SD 4.7), at 2 months only 2/6 had lost any weight, but by 12 months 5/6 had lost weight and 3/6 had lost more than 5% of total body weight.

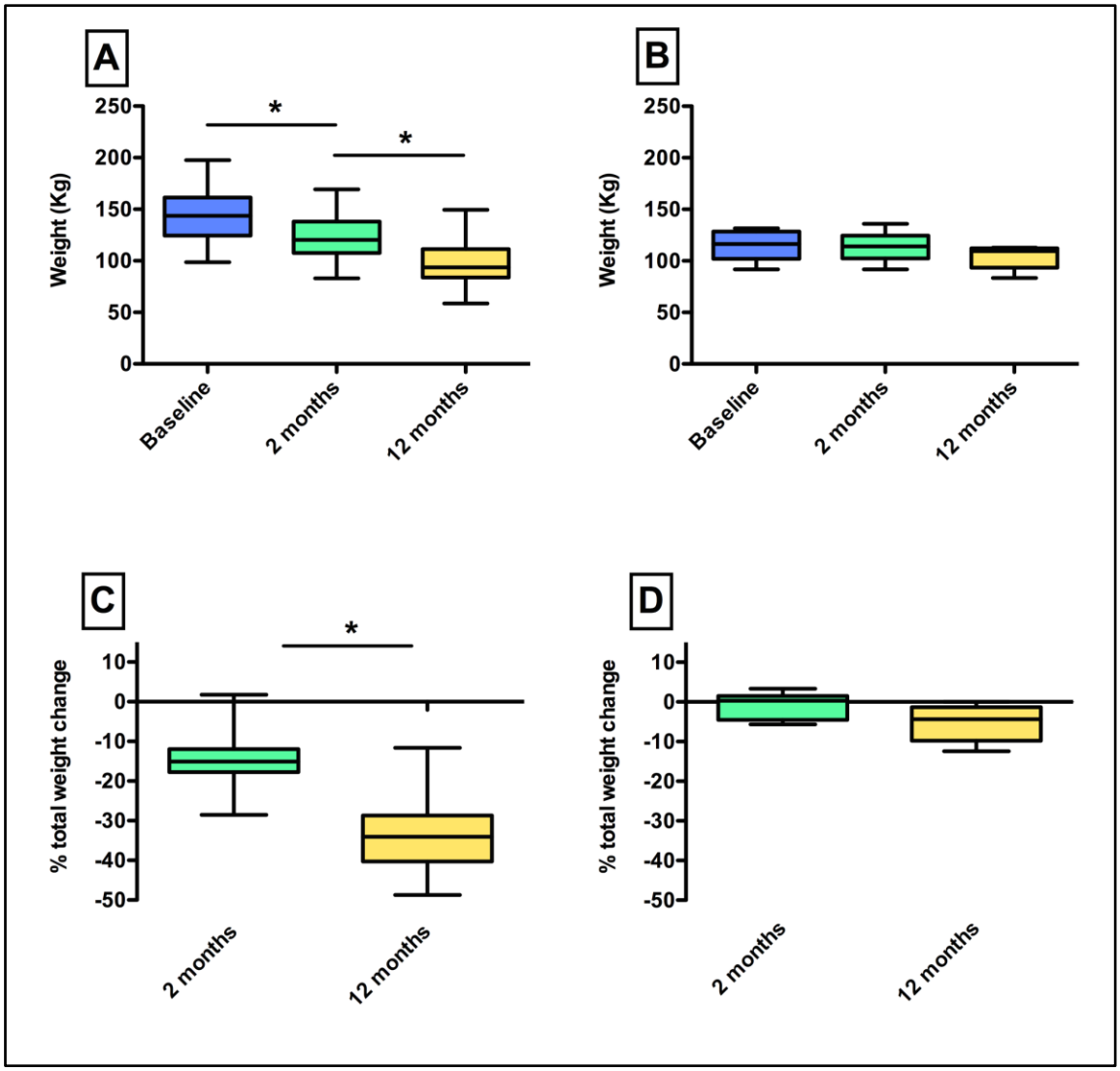
BMI decreased from 46.6kg/m^2 at baseline to 46.2kg/m^2 and 42.6kg/m^2 at 2 and 12 months respectively (mean difference -0.38 (95% CI -2.1 to 1.4), p=0.6 and -2.9 (95% CI -5.7 to 0.2), p=0.04* at 2 and 12 months post non-surgical weight management) (Figure 15, Table 14)

Seventy-two participants underwent weight loss surgery, most commonly laparoscopic gastric bypass surgery (52/72, 72%) or laparoscopic sleeve gastrectomy (17/72, 24%), but also laparoscopic adjustable gastric band (2/72, 3%) and in one case an endoscopic intragastric balloon (1%). There were no cases that necessitated conversion to open surgery. Mean weight was 144.4kg, 122.8kg and 97.7kg at baseline, 2 and 12 months respectively. Mean % total weight change was -15% and -33% at 2 and 12 months (Figure 15, Table 14).

BMI decreased from 53.6kg/m^2 at baseline to 45.6kg/m^2 and 36.1kg/m^2 at 2 and 12 months post bariatric surgery. Mean difference at each time point was -8.21kg/m^2 at 2 months (95% CI -9.3 to - 7.1, p<0.0001*) and -18.81kg/m^2 at 12 months (95% CI -20.8 to -16.7), p<0.0001*.

The surgical cohort lost significantly more weight than the non-surgical cohort (p<0.0001*).

FIGURE 15: WEIGHT LOSS OF SURGICAL AND NON-SURGICAL COHORTS



Key: A) Mean weight at baseline, 2 and 12 months in the surgical cohort, B) mean weight at baseline, 2 and 12 months in the non-surgical cohort, C) % total weight change at 2 and 12 months in the surgical cohort, D) % total weight change at 2 and 12 months in the non-surgical cohort, *p<0.05

TABLE 14: WEIGHT CHANGE FOLLOWING SURGICAL AND NON-SURGICAL WEIGHT MANAGEMENT

		Surgical (n=72) Mean (SD)	**Non-Surgical** (n=8) Mean (SD)	**Total Cohort** (n=80) Mean (SD)	**Significance of differences between time-points in each group**
Weight (kg)	Baseline (T0)	144.4 (24.4)	114.8 (14.75)	139.3 (23.2)	**Mean difference T1 vs. T0 (95% CI)** Surgical: -21.8 (-24.8 to -18.7), p <0.0001* Non-surgical: -1.0 (-5.4 to 3.4), p = 0.81 **Mean difference T2 vs. T0 (95% CI)** Surgical: -47.2 (-53.3 to -41.1), p <0.0001* Non-surgical: -7.2 (-13.9 to -0.5), p = 0.06
	2 months (T1)	122.8 (23.3)	113.8 (15.2)	121.5 (22.4)	
	12 months (T2)	97.7 (22.6)	104.2 (12.1)	98.7 (21.3)	
BMI (kg/m^2)	Baseline (T0)	53.6 (7.7)	46.62 (5.6)	52.6 (7.6)	**Mean difference T1 vs. T0 (95% CI)** Surgical: -8.21 (-9.3 to -7.1), p<0.0001* Non-surgical: -0.38 (-2.1 to 1.4), p=0.60 **Mean difference T2 vs. T0 (95% CI)** Surgical: -18.76 (-20.8 to -16.7), p<0.0001* Non-surgical: -2.9 (-5.7 to 0.2), p=0.04*
	2 months (T1)	45.6 (7.3)	46.23 (5.8)	45.72 (7.2)	
	12 months (T2)	36.1 (7.2)	42.58 (4.9)	37.26 (7.2)	
Change in total body weight (%)	2 months (T1-T0)	-15.16 (5.9)	-0.85 (3.4)	-13.6 (7.5)	**Surgical vs. non-surgical** P< 0.0001*
	12 months (T2-T0)	-32.66 (9.9)	-5.3 (4.7)	-28.68 (13.3)	
Change in BMI (kg/m^2)	2 months (T1-T0)	-8.12 (3.4)	-0.38 (1.7)	-7.04 (4.2)	**Surgical vs. non-surgical** P < 0.0001*
	12 months (T2-T0)	-18.75 (4.6)	-2.96 (2.2)	-15.83 (7.6)	

3.3 Prevalence of occult pathology

3.3.1 Endometrial pathology

Four cases of endometrial cancer (5%) and six cases of atypical endometrial hyperplasia (7.5%) were identified in women who did not have symptoms that would require referral on a suspected cancer pathway. The baseline prevalence of occult malignant and premalignant disease in this cohort was 12.5% (95% CI 6.16 – 21.79). The details of these participants are discussed in Table 15.

In addition, three baseline biopsies were reported as equivocal as they could not be confidently classified as normal or abnormal endometrium, all were normal at the 12-month follow up.

Two endometrial biopsies showed disordered proliferation; both women had normal biopsies at 2 months, one remained normal at 12 months and the other was lost to follow up as she became pregnant 9 months following bariatric surgery. Disordered proliferation is an exaggerated proliferative endometrium without an increase in the ratio of glands to stroma, which forms part of the spectrum between normal proliferative endometrium and non-atypical hyperplasia (214).

In one case, squamous metaplasia was seen on the baseline biopsy, and further investigation in the form of outpatient hysteroscopy and endometrial biopsy at 2 months post bariatric surgery was normal, however, this patient went on to develop atypical endometrial hyperplasia with suspected invasive disease at the 12-month follow up biopsy. Following hysterectomy this was confirmed to be stage 1a grade 1 endometrioid adenocarcinoma of the endometrium. Squamous metaplasia is a benign endometrial lesion which can coexist with hyperplasia or carcinoma, and the presence of these must be excluded as it can be considered a marker of a high risk endometrium (214).

One further case of focal atypical hyperplasia was detected at 12-month follow up in a patient with a previously normal endometrial biopsy.

3.3.2 Management of endometrial pathology

All cases of endometrial cancer diagnosed on baseline biopsy were treated with laparoscopic hysterectomy and bilateral salpingo-oophorectomy following histological diagnosis and discussion at the Gynaecological Oncology MDT. One woman underwent delayed hysterectomy following a trial of medical management as she was 24 years old and nulliparous. After having a LNG-IUS in situ for 12 months, her endometrial biopsies remained concerning for persistent endometrial cancer and so a hysterectomy was performed 15 months after initial diagnosis.

The patient who had squamous metaplasia at baseline, followed by suspected endometrial cancer on her 12-month biopsy also underwent laparoscopic hysterectomy and a cancer diagnosis was confirmed.

Four women had stage 1a grade 1 endometrioid adenocarcinoma of the endometrium, and one had stage 3a grade 2 endometrioid adenocarcinoma of the endometrium on final histopathology and was referred for adjuvant chemotherapy and radiotherapy. This patient had had an MRI scan at diagnosis which had suggested stage 1a disease, however the hysterectomy specimen showed the presence of ovarian micro metastases.

Women with atypical endometrial hyperplasia underwent repeat endometrial sampling at 6-8 weeks, according to the study protocol. Those with persistent abnormalities and abnormal bleeding at second biopsy (n=2) were treated with the LNG-IUS and underwent repeat sampling after 6 months. Both cases showed regression of their endometrial abnormality by 6 months and the LNG-IUS was removed.

Four other cases of AEH were managed expectantly with weight loss, and three of these demonstrated normalisation of the endometrium without treatment. All women with atypical endometrial hyperplasia detected during the study remain under long-term surveillance (endometrial sampling every 6-12 months) to detect recurrent disease.

One patient was found to have a borderline serous ovarian tumour during the course of her expectant follow up for atypical hyperplasia (developed new pelvic pain and

underwent ultrasound scan) and therefore despite improving pathological appearance of her atypical hyperplasia (diffuse to focal) underwent hysterectomy, bilateral salpingo-oophorectomy and omental biopsy for treatment of her ovarian disease. Two years later, she was investigated for right iliac fossa pain and was found to have widespread peritoneal disease consistent with recurrent borderline tumour. A subsequent laparoscopic assessment and biopsy showed operable low-grade serous cancer and the patient therefore underwent laparotomy, tumour debulking, anterior resection with end colostomy and right nephrectomy.

3.3.3 Baseline variation in women with endometrial abnormality

Of the 10 women diagnosed with endometrial pathology at baseline, 8 (80%) were premenopausal, 5 (50%) were known to be diabetic (metformin used in 2), 7 (70%) had an HbA1c greater than the diagnostic threshold for diabetes and 3 (30%) had PCOS. Mean age was 43 years and mean BMI was 53kg/m^2 (cf. 43 years (p=0.91) and 52kg/m^2 (p=0.74) in women with normal endometrium respectively). T tests for equality of means, assuming equal variances, were performed to elicit any key differences in the baseline characteristics of these two groups (Table 16).

Women with abnormal endometrial biopsies had significantly higher baseline HbA1c than women with normal endometrium (66 vs. 43 mmol/mol, mean difference 95% CI -35.9, -10.8, p=0.001). There was no significant difference in age or weight. There was a trend towards higher levels of glucose, insulin, HOMA-IR, hsCRP and lower levels of adiponectin. Adiponectin is inversely and independently associated with endometrial cancer risk, and is lower in obese women than normal weight women. The mean adiponectin level was 1.86 mg/L in women with normal endometrium and 1.2 mg/L in women with abnormal endometrium (p=0.3).

Phosphorylation of AKT in endometrial glands was also higher in abnormal endometrium (mean 42.84 cf. 73; mean difference 95% CI -60.4, 0.09; p=0.05). There was no significant difference in Ki-67 between the two groups (34.3 vs. 25.6%; mean difference 95% CI -23.1, 5.8; p=0.23).

TABLE 15: CHARACTERISTICS OF WOMEN IN WHOM ENDOMETRIAL HYPERPLASIA OR CANCER WAS DETECTED DURING THE STUDY (N=12)

	Baseline characteristics (T0)				Weight loss			Endometrial findings						Management of endometrial pathology (Final diagnosis)	Follow up (time from hysterectomy or date completed study T2)
	Age (yrs)	BMI (Kg/m^2)	Diabetes or insulin resistant (Y/N) *Undiagnosed	Bleeding pattern at baseline	Intervention (surgical/ non-surgical)	T1-T0 (% total body weight)	T2-T0 (% total body weight)	T0 Baseline		T1 6-8 weeks		T2 12 months			
								Morphology	Ki-67 (%)	Morphology	Ki-67 (%)	Morphology	Ki-67 (%)		
1	42	46.1	Y	HMB Regular	Bypass	-15.6	-24.3	Grade 1 EEC	5.2	Grade 2 EEC	54.8	N/A		Hysterectomy (3a G2 EEC)	Recurrence (omental nodules) at 18 months
2	52	51.9	Y	None	Bypass	-12.0	N/A	Grade 1 EEC	10.7	N/A		N/A		Hysterectomy (1a G1 EEC)	No recurrence at 36 months
3	55	54.6	N	None	Bypass	-7.9	N/A	Grade 1 EEC	45.5	N/A		N/A		Hysterectomy (1a G1 EEC)	No recurrence at 14 months
4	24	59.7	N	HMB Oligo PCOS	Non-surg then bypass	+1.7	-13.8	Grade 1 EEC	24.1	Grade 1 EEC	4.2	Grade 1 EEC	2.7	LNG-IUS Hysterectomy (1a G1 EEC)	No recurrence at 14 months
5	44	57.1	Y*	Ameno PCOS	Sleeve	-13.8	-33.0	Squamous metaplasia	22.5	Normal	20.6	Grade 1 EEC	34.8	Hysterectomy (1a G1 EEC)	No recurrence at 24 months

6	35	69.9	N	Normal	Bypass	-17.4	-34.1	AEH, possible invasion	50.3	AEH	49.4	Normal	9.8	LNG-IUS 6/12	Normal endometrium at 28 months
7	43	47.5	Y	HMB Oligo	Sleeve	-16.3	-30.1	AEH	46.6	AEH	49.4	AEH BOT	20.6	Hysterectomy (small focus AEH)	Recurrent low grade serous adenocarcinoma
8	39	56.9	N	HMB Oligo	Sleeve	-19.6	-39.6	AEH	60.8	Normal	0.5	Normal	21.8	No intervention	Normal endometrium at 14 months
9	45	57.0	N	IMB	Bypass	-13.2	-42.5	AEH	65.4	AEH	46.2	Normal	0.4	LNG-IUS 6/12	Normal endometrium at 30 months
10	40	41.6	Y	Oligo	Bypass	-15.0	-15.8	AEH	27.8	Normal	0.1	Normal	23.3	No intervention	Normal endometrium at 24 months
11	51	46.8	Y	HMB Oligo	Bypass	-9.3	-21.5	AEH	37.5	Normal	32.6	Normal	15.0	No intervention	Normal endometrium at 24 months
12	49	54.9	Y*	Normal HMB at T2	Sleeve	-14.0	-34.0	Normal	5.0	Normal	35.5	AEH	27.5	No intervention	Normal endometrium at 12 months

Key: Oligo = oligomenorrhoeic, ameno = amenorrhoeic, HMB = heavy menstrual bleeding, IMB = intermenstrual bleeding, T0 = baseline, T1 = 2 months, T2 = 12 months, BOT = borderline ovarian tumour

TABLE 16: CHARACTERISTICS OF WOMEN WITH ABNORMAL ENDOMETRIAL BIOPSIES COMPARED WITH WOMEN WITH NORMAL ENDOMETRIAL BIOPSIES

Baseline characteristic	Mean		p	95% CI of difference	
	Normal	Abnormal		Lower	Upper
Age (years)	42.92	42.56	0.91	-5.9	6.63
Weight (Kg)	140.0	140.6	0.95	-18.6	17.5
BMI (Kg/m^2)	52.28	53.19	0.74	-6.45	4.62
Glucose (mmol/L)	6.72	8.74	0.051	-4.06	0.0098
Insulin (mU/L)	16.07	20.27	0.36	-13.30	4.90
HOMA-IR	5.31	9.21	0.12	-8.93	1.12
HbA1c (mmol/mol)	42.52	65.89	0.001*	-35.94	-10.81
FSH (IU/L)	11.38	6.46	0.29	-4.31	14.16
LH (IU/L)	9.98	6.77	0.29	-2.90	9.33
LH:FSH	1.17	1.17	0.99	-0.51	0.51
FAI	4.10	3.14	0.48	-1.77	3.68
SHBG (nmol/L)	34.00	30.33	0.68	-14.32	21.66
Testosterone (nmol/L)	0.95	0.94	0.95	-0.37	0.40
Oestradiol (pmol/L)	271.6	280.9	0.91	-173.1	154.5
hsCRP (mg/L)	7.30	10.33	0.28	-8.64	2.59
Adiponectin (mg/L)	1.86	1.20	0.30	-0.61	1.93
Leptin (ng/ml)	57.87	54.43	0.74	-17.74	24.62
Ki-67 (%)	25.64	34.31	0.23	-23.13	5.79
p-AKT glands (0-300)	42.84	73.00	0.05	-60.42	0.09
p-ERK glands (0-300)	52.00	78.00	0.19	-65.73	13.73
p-ERK stroma (0-300)	20.43	35.00	0.33	-44.64	15.50
ER (0-18)	14.00	14.60	0.61	-2.95	1.75
PR (0-18)	12.25	13.10	0.54	-3.59	1.89

Note: variable *n*. Rows from age to oestradiol, n=70 normal and n=10 abnormal. Adiponectin, leptin, hsCRP and endometrial markers n=37 normal and n=10 abnormal. Endometrium and biomarkers only assessed in women who had a repeat sample in follow up, not in those lost to follow up after baseline sampling.

3.3.2 Abnormal cervical cytology

Cervical screening was overdue in 36 (45%) women, and was performed opportunistically. Most had negative cytology (31, 86%), 2 (6%) had high risk HPV negative borderline cytology and 2 (6%) were inadequate; repeat cytology was normal for both. One woman had severe dyskaryosis with possible invasion and following colposcopy and biopsy underwent radical trachelectomy and laparoscopic bilateral pelvic lymphadenectomy for a stage 1b1 squamous cell carcinoma of the cervix.

3.3.3 Occult insulin resistance

Undiagnosed metabolic disease was also detected in addition to the 26 patients (32.5%) with known diabetes at baseline. Three (5.6%) patients who did not have a diagnosis of diabetes were found to have baseline HbA1c levels > 48 mmol/mol which is the World Health Organisation threshold for diagnosing diabetes (215); two of these also had occult endometrial hyperplasia or cancer. Testing HbA1c levels was a Substantial Amendment to the protocol and therefore there is 36% missing data in this regard. No treatment was initiated for these patients as they all underwent bariatric surgery, which could be expected to reverse the majority of cases of type 2 diabetes (216).

The true burden of undiagnosed insulin resistance in morbidly obese women is likely to be greater than this as raised HOMA-IR levels (>2.7) were found in 22 non-diabetic patients (40.7%), although this is not the gold standard test for diagnosing insulin resistance it is a useful surrogate marker for clinical studies.

In some cases the HOMA-IR may have been affected by the blood samples being taken during the bariatric surgery operation; surgery in itself creates an insulin resistant environment, and dexamethasone is commonly used in non-diabetics for its anti-emetic properties. To test for the effect of sampling time I analysed median HOMA-IR levels from patients in the first half of the cohort, where bloods were taken at varying times during surgery, frequently during or at the end of the operation, and compared them with those from the second half of the cohort, where they were taken prior to induction of anaesthesia. No statistically significant difference was seen (median HOMA-IR 4.8 pre-protocol change vs. 3.0 post-protocol change, IQR 1.7-10 vs. 1.7-5.2, p=0.16).

4. Results II

The clinical effects of weight loss

4.1 The effect of weight loss on menstrual function and reproductive hormones

4.1.1 Menstrual function

In women with abnormal bleeding at baseline (intermenstrual, postmenopausal, postcoital or heavy menstrual bleeding), 3/15 (20%) and 2/15 (13%) reported this to have normalized by 2 and 12 months respectively, a 33% improvement overall. Of these, three had AEH and one had disordered proliferation at baseline. In all of these four cases regression of abnormal endometrium was seen alongside normalisation of menstrual bleeding.

Amongst women with abnormal cycles at baseline (oligomenorrhoea or amenorrhoea) 3/23 (13%) and 3/23 (13%) reported restoration of normal menstruation at two and twelve months respectively. One woman achieved a pregnancy 9 months following bariatric surgery; she was amenorrhoeic with disordered endometrial proliferation at baseline. Three women became menopausal (>12 months amenorrhoea) during the course of the study.

Conversely, one participant had normal menstrual bleeding at baseline, which was then reported as heavy at 12 months. Initial endometrial biopsy was normal but at 12 months focal atypical endometrial hyperplasia was noted.

4.1.2 Reproductive hormones

The effects of bariatric surgery on serum reproductive hormones is shown in Figure 16 and compared with the non-surgical cohort, who lost significantly less weight, in Table 18. In some cases the result of serum analyses was categorical rather than continuous, i.e. oestradiol <50pmol/l when it was too low to be accurately measured. In this situation unit imputation was employed, with the value entered being the midpoint of the category assigned to the result, i.e. 25pmol/l, to avoid having to exclude the data point from repeated measures analyses.

Significant improvements towards population norms in serum SHBG, FSH, LH and FAI, were seen at both 2 and 12 months post bariatric surgery (n=41 and n=28 at each time point respectively). A statistically significant change in serum testosterone was seen at 12 months post bariatric surgery. No statistically significant change in serum oestradiol levels was seen. An initial significant change in serum progesterone at 2 months was not borne out at 12 months post-bariatric surgery. No statistically significant changes in reproductive hormones were noted in women in the non-surgical cohort, although this may in part be due to the small sample size (n=6).

4.1.2.1 Sex Hormone Binding Globulin

Mean SHBG levels at baseline, 2 and 12 months were 33, 57 and 70 nmol/L respectively, overall. At 2 months post bariatric surgery the mean difference was an increase of 26.7nmol/L (95% CI 17.5 to 35.9, p<0.0001*) as compared to the non-surgical group where the mean difference was -1.5nmol/L (95% CI -52.7 to 49.7, p=0.88). At 12 months post bariatric surgery the mean difference was 44nmol/L (95% CI 30.5 to 57.5, p<0.0001*) compared with the non-surgical cohort where the mean difference was -7.8nmol/L (95% CI -47.7 to 32.2, p=0.88). This increase after bariatric surgery is likely to be the result of decreased insulin levels in the circulation, as insulin inhibits hepatic synthesis of SHBG.

4.1.2.2 Follicle Stimulating Hormone

Mean serum FSH levels were 10.3, 16.3 and 19.4 IU/L at baseline, 2 and 12 months respectively, overall. At 2 months post bariatric surgery the mean difference was an increase of 5.6IU/L (95% CI 1.4 to 9.8, p=0.0008*) as compared to the non-surgical group where the mean difference was 8.2IU/L (95% CI -7.6 to 24.1, p=0.31). At 12 months post bariatric surgery the mean difference was 8.8IU/L (95% CI 2.9 to 14.8, p=0.0005*) compared with the non-surgical cohort where the mean difference was 10.9IU/L (95% CI –6.7 to 28.7, p=0.19).

4.1.2.3 Luteinising Hormone

Mean serum LH levels were 9.3, 15.1 and 16 IU/L at baseline, 2 and 12 months respectively, overall. At 2 months post bariatric surgery the mean difference was an increase of 6.4IU/L (95% CI 2.1 to 10.8, p=0.015*) as compared to the non-surgical group where the mean difference was 4.3IU/L (95% CI -8 to 16.7, p=0.44). At 12 months

post bariatric surgery the mean difference was 5.2IU/L (95% CI 1.4 to 9, p=0.0051*) compared with the non-surgical cohort where the mean difference was 10.3IU/L (95% CI –5.8 to 26.4, p=0.19).

Increasing FSH and LH levels may reflect the passage of time in a predominantly peri-menopausal cohort, or it may be that decreases in sex steroid levels reduce the suppression of FSH and LH that obesity can cause. However, the samples were not timed to be taken in the early part of the menstrual cycle and therefore are difficult to interpret meaningfully.

4.1.2.4 Free Androgen Index

Mean serum FAI was 3.9, 2.3 and 1.5 at baseline, 2 and 12 months respectively, overall. At 2 months post bariatric surgery the mean difference was -1.9 (95% CI -2.9 to -0.9, p=0.0002*) as compared to the non-surgical group where the mean difference was -0.5 (95% CI -4 to 2.9, p=0.5). At 12 months post bariatric surgery the mean difference was -2.1 (95% CI -3.2 to -1.1, p=0.001*) compared with the non-surgical cohort where the mean difference was 0.3 (95% CI -0.7 to 1.3, p=0.5).

4.1.2.5 Testosterone

Mean serum testosterone was 0.9, 1.0 and 0.8 nmol/L at baseline, 2 and 12 months respectively, overall. At 2 months post bariatric surgery the mean difference was 0.1nmol/L (95% CI -0.4 to 0, p=0.06) as compared to the non-surgical group where the mean difference was 0.1nmol/L (95% CI -0.4 to 0.6, p=0.81). At 12 months post bariatric surgery the mean difference was -0.3nmol/L (95% CI -0.8 to 0.4, p=0.003*) compared with the non-surgical cohort where the mean difference was 0.22nmol/L (95% CI -0.1 to 0.5, p=0.25).

Decreasing FAI reflects increasing SHBG and decreasing testosterone, as it is a function of these levels. Testosterone has been shown to be a product of androstenedione metabolism in both subcutaneous and omental fat, and reduction in adiposity is likely to reduce testosterone levels in this way. This may be why levels were only significantly altered at 12 months post bariatric surgery, as prior to that changes are more related to altered glucose metabolism than loss of fat mass. Testosterone is also SHBG bound so it was expected that levels would fall as SHBG concentrations rose.

4.1.2.6 Progesterone

Mean serum progesterone was 5.8, 4.7 and 6.1 ng/ml at baseline, 2 and 12 months respectively, overall. At 2 months post bariatric surgery the mean difference was -1.5ng/ml (95% CI -5.5 to 2.6, p=0.011*) as compared to the non-surgical group where the mean difference was 6.7ng/ml (95% CI -20.7 to 34, p=0.63). At 12 months post bariatric surgery the mean difference was 1.5ng/ml (95% CI -3.8 to 6.9, p=0.53) compared with the non-surgical cohort where the mean difference was -4.7ng/ml (95% CI -17.2 to 7.8, p=0.63).

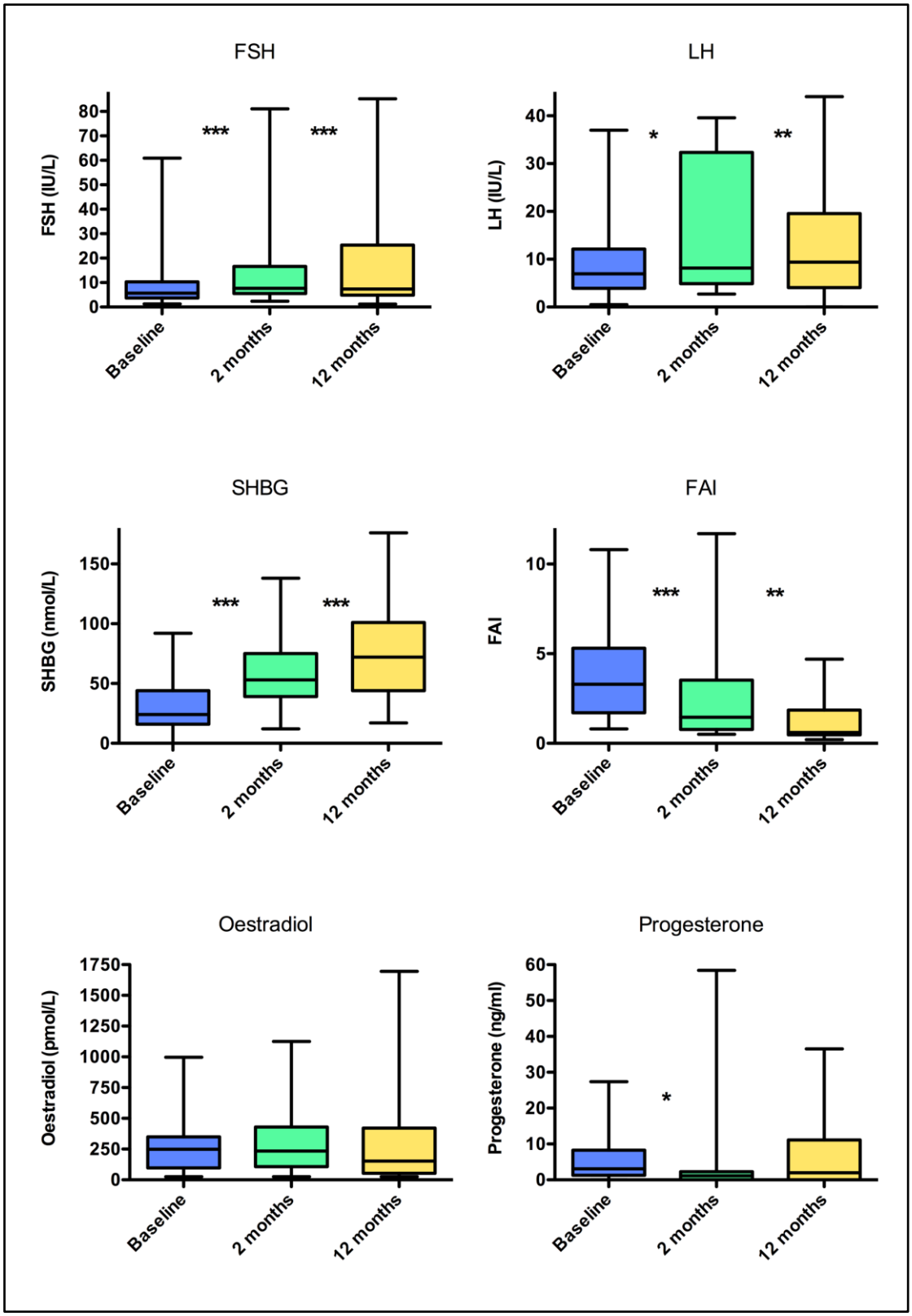
4.1.2.7 Oestradiol

Mean serum oestradiol was 274, 333 and 315 pmol/L at baseline, 2 and 12 months respectively. At 2 months post bariatric surgery the mean difference was an increase of 76.8pmol/L (95% CI -15 to 168, p=0.15) as compared to the non-surgical group where the mean difference was -43.6pmol/L (95% CI -258 to 171, p=0.63). At 12 months post bariatric surgery the mean difference was 9.7pmol/L (95% CI -141 to 161, p=0.55) compared with the non-surgical cohort where the mean difference was 103.4pmol/L (95% CI -503 to 710, p=1).

It was expected that increasing ovulatory cycles with weight loss would translate to an increase in progesterone levels after bariatric surgery in this cohort, and that decreasing fat mass would result in a reduction in oestradiol levels, however we have not successfully demonstrated this. This is likely to be because the blood samples were not taken at the correct times of the menstrual cycle, and so the inherent variation negates any effect we may otherwise have observed. Some studies have only analysed oestrogen change with weight loss in postmenopausal women as the inherent variation in cycling women masks any effect.

The majority of participants were premenopausal but when the oestradiol levels of the postmenopausal participants were analysed in isolation a trend towards falling median oestradiol levels with weight loss was seen, although this did not reach statistical significance (median oestradiol (IQR) at baseline, 2 and 12 months was 79 (45.5-128.5), 51 (25-116.5) and 25 (25-62) pmol/L respectively). These were very small groups however (n=5, 5 and 3 at each time point).

FIGURE 16: THE EFFECT OF BARIATRIC SURGERY ON REPRODUCTIVE HORMONES



Key: * p<0.05, ** p<0.01, *** p<0.001

4.2 The effect of weight loss on circulating markers of insulin resistance and adiposity

4.2.1 Measuring insulin resistance

Insulin resistance was assessed by measuring fasting plasma glucose and serum insulin to calculate HOMA-IR, a homeostatic model assessment that is a surrogate rather than a direct measure of insulin sensitivity (217). An individual with normal insulin sensitivity would derive a HOMA-IR index of 1, but there is significant variation in published reference ranges, and between populations. The upper limit of normal was taken as 2.7 as reported in previous work (217). While there are limitations to this method of assessing insulin resistance, it is more acceptable to patients and less complicated to administer than the gold standard test, the hyperinsulinaemic euglycaemic clamp, and is well established in the literature. The HOMA-IR has been reported to have a reasonable correlation with glucose clamp results, but performs less well in diabetics and in obese subjects (219).

It was not used in isolation to assess insulin resistance within this study; serum adiponectin was also measured, as was HbA1c. HbA1c is formed in a non-enzyme dependent glycation pathway and is used as a marker for average plasma glucose concentration over the preceding three months (as this is the lifespan of red blood cells). As such, it is less susceptible to the inherent variation one expects to see with isolated fasting measurements of glucose or insulin, which will be affected by duration of fast, drugs, acute stress/illness amongst other factors.

4.2.2 The effect of weight loss on insulin resistance

In the surgical cohort statistically significant improvements in fasting plasma glucose, fasting serum insulin, HOMA-IR and HbA1c were seen at both two and twelve months following bariatric surgery. No statistically significant improvements were seen in the non-surgical weight management cohort (Figure 17, Table 18).

4.2.2.1 Fasting glucose and insulin levels and the HOMA-IR

Mean plasma glucose levels were 7.4, 5.1 and 4.9 mmol/L at baseline, 2 and 12 months respectively in the surgical cohort, and 5, 6.1 and 5.4 mmol/L at baseline, 2 and 12 months respectively in the non-surgical cohort. At 2 months post bariatric surgery the mean difference was -2.4mmol/L (95% CI -3.6 to -1.3, p=0.0003*) as compared to the

non-surgical group where the mean difference was 0.83mmol/L (95% CI -0.5 to 2.2, p=0.13). At 12 months post bariatric surgery the mean difference was -3.3mmol/L (95% CI -4.2 to -2.2, p<0.0001*) compared with the non-surgical cohort where the mean difference was -0.02mmol/L (95% CI -0.5 to 0.5, p=0.75).

Mean serum insulin levels were 17.2, 10.9 and 8.5 mU/L at baseline, 2 and 12 months respectively, in the surgical cohort and 16, 17 and 17.2 mU/L at baseline, 2 and 12 months respectively, in the non-surgical cohort. At 2 months post bariatric surgery the mean difference was -6.7mU/L (95% CI -11.8 to -1.6, p=0.02*) as compared to the non-surgical group where the mean difference was 1.8mU/L (95% CI -4.1 to 7.8, p=0.5). At 12 months post bariatric surgery the mean difference was -12.4mU/L (95% CI -18.6 to -6.1, p<0.0001*) compared with the non-surgical cohort where the mean difference was 2.8mU/L (95% CI -3.3 to 8.8, p=0.5).

Median HOMA-IR levels were 6.6, 2.6 and 1.8 at baseline, 2 and 12 months respectively, in the surgical cohort and 3.7, 4.4 and 4.2 at baseline, 2 and 12 months respectively, in the non-surgical cohort. At 2 months post bariatric surgery the mean difference was -4.5 (95% CI -7.4 to-1.5, p=0.0032*) as compared to the non-surgical group where the mean difference was 0.72 (95% CI -0.9 to 2.3, p=0.3). At 12 months post bariatric surgery the mean difference was -6.8 (95% CI -10 to -3.6, p<0.0001*) compared with the non-surgical cohort where the mean difference was 0.51 (95% CI -1.3 to 2.3, p=0.63).

4.2.2.2 HbA1c

HbA1c measurements were not available for all patients as it was a later addition to the study protocol, with 35% missing data. Mean HbA1c levels were 47.7, 35 and 34 mmol/mol at baseline, 2 and 12 months respectively, in the surgical cohort and 46, 78 and 59 mmol/mol at baseline, 2 and 12 months respectively, in the non-surgical cohort.

At 2 months post bariatric surgery the mean difference was -10.5mmol/mol (95% CI -16 to -4.9, n=26, p<0.0001*). There was insufficient data to calculate this for the non-surgical cohort. At 12 months post bariatric surgery the mean difference was -11.3mmol/mol (95% CI -17.9 to -4.7, n=15, p<0.0001*) compared with the non-surgical cohort where the mean difference was 7mmol/mol (95% CI -56.5 to 70.5, n=2, p=0.5).

4.2.3 Measuring adipokines

Adiponectin is predominantly secreted by adipocytes and serum levels are inversely correlated with central obesity and BMI. It is thought to *improve* insulin resistance in peripheral tissues. Low levels have been associated with hyperinsulinaemia, type 2 diabetes and metabolic syndrome, and it has been postulated that low adiponectin levels mediate the effect of obesity on cancer risk, including endometrial cancer and particularly in premenopausal women. It is unclear whether adiponectin acts purely through effects on insulin resistance or if it has direct cellular effects

Leptin is produced predominantly in adipose tissue and *reduces* tissue sensitivity to insulin leading to hyperinsulinaemia. Levels are increased in obesity, and in endometrial cancer as compared to normal subjects.

4.2.4 The effect of weight loss on adipokines

Statistically significant improvements were seen in serum adiponectin and leptin in the bariatric surgery cohort at both two and twelve months post bariatric surgery. No significant change in adiponectin or leptin was noted in the non-surgical weight management cohort (Figure 17, Table 18).

4.2.4.1 Adiponectin

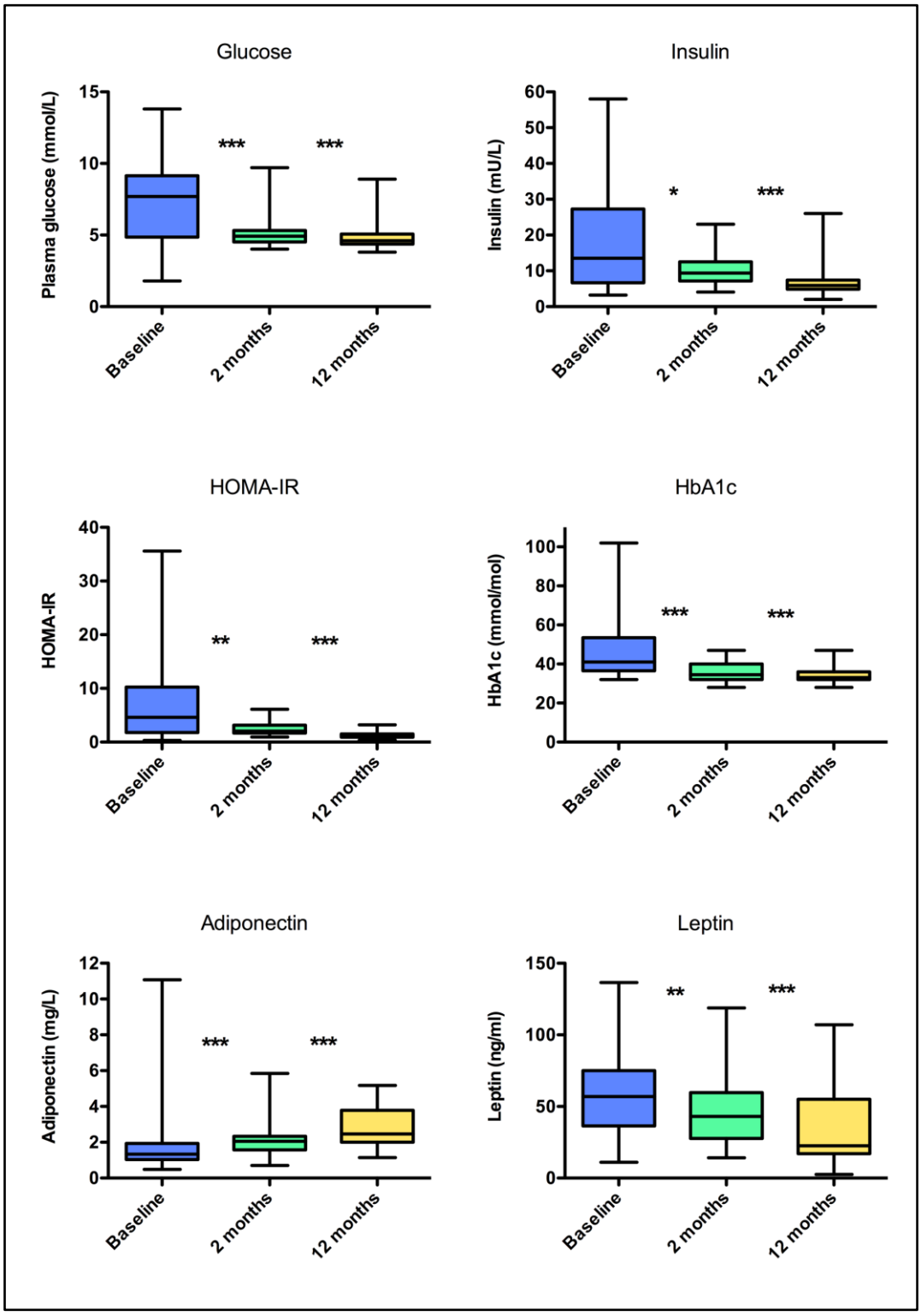
Mean adiponectin levels were 1.71, 2.21 and 2.82 mg/ml at baseline, 2 and 12 months respectively, in the surgical cohort and 1.95, 1.93 and 1.94 mg/ml at baseline, 2 and 12 months respectively, in the non-surgical cohort. At 2 months post bariatric surgery the mean difference was 0.47mg/ml (95% CI 0.17 to 1.1, p<0.0001*) as compared to the non-surgical group where the mean difference was -0.01mg/ml (95% CI -0.44 to 0.42, p=0.81). At 12 months post bariatric surgery the mean difference was 1.3mg/ml (95% CI 0.97 to 1.7, p<0.0001*) compared with the non-surgical cohort where the mean difference was -0.006mg/ml (95% CI -0.57 to 0.56, p=0.81).

4.2.4.2 Leptin

Mean leptin levels were 56, 45 and 33 ng/ml at baseline, 2 and 12 months respectively, in the surgical cohort and 64, 70 and 66 ng/ml at baseline, 2 and 12 months respectively, in the non-surgical cohort. At 2 months post bariatric surgery the mean difference was -8.1ng/ml (95% CI -13.2 to -2.9, p=0.001*) as compared to the non-surgical group where the mean difference was 6.1ng/ml (95% CI -6.6 to 18.8, p=0.44).

At 12 months post bariatric surgery the mean difference was -25.3ng/ml (95% CI -34 to -16.6, p<0.0001*) compared with the non-surgical cohort where the mean difference was 2.2ng/ml (95% CI -14.8 to 19.2, p=1).

FIGURE 17: THE EFFECT OF BARIATRIC SURGERY ON MARKERS OF INSULIN RESISTANCE AND ADIPOSITY



Key: * p<0.05, ** p<0.01, *** p<0.001

4.3 The effect of weight loss on systemic inflammation

4.3.1 Measuring systemic inflammation

Endometrial cancer risk has been shown to be elevated amongst women with increased levels of CRP and pro-inflammatory cytokines, and CRP and IL-6 are strongly correlated with BMI and waist circumference. Serum levels of high sensitivity CRP and IL-6 were assayed to assess the effects of weight loss upon them.

4.3.2 The effect of weight loss on systemic inflammation

Statistically significant reduction in IL-6 was seen at two and twelve months post bariatric surgery, alongside a significant reduction in high sensitivity CRP at twelve months post bariatric surgery. The lack of significant change in hs-CRP at two months and the greater reduction in IL-6 seen at 12 months compared with that at two months may in part reflect the acute inflammation associated with a surgical procedure still in effect at two months post-op. No significant changes in either marker were seen in the non-surgical cohort although a downward trend was noted in both high-sensitivity CRP and IL-6 at both time points (Table 18).

4.3.2.1 High sensitivity CRP

Mean hsCRP levels were 7.4, 6.5 and 3.7 mg/ml at baseline, 2 and 12 months respectively, in the surgical cohort and 11.3, 10.4 and 10.7 mg/ml at baseline, 2 and 12 months respectively, in the non-surgical cohort. At 2 months post bariatric surgery the mean difference was -0.05mg/ml (95% CI -1.7 to 1.6, p=0.35) as compared to the non-surgical group where the mean difference was -0.9mg/ml (95% CI -4.9 to 1.1, p=0.81). At 12 months post bariatric surgery the mean difference was -4.3mg/ml (95% CI -6.3 to -2.3, p<0.0001*) compared with the non-surgical cohort where the mean difference was -0.6mg/ml (95% CI -4.1 to 2.8, p=0.63).

The trend towards higher baseline levels of hsCRP in the non-surgical group may represent a greater degree of pre-existing comorbidity, although the sample size is too small to draw any firm conclusions about the true mean hsCRP in this cohort.

4.3.2.2 IL-6

Mean IL-6 levels were 6, 4.5 and 1.4 pg/ml at baseline, 2 and 12 months respectively, in the surgical cohort and 4, 2.4 and 2.6 pg/ml at baseline, 2 and 12 months respectively,

in the non-surgical cohort. At 2 months post bariatric surgery the mean difference was -1.5pg/ml (95% CI -6.8 to -3.8, p<0.0001*) as compared to the non-surgical group where the mean difference was -1.6pg/ml (95% CI -5.8 to 2.7, p=0.63). At 12 months post bariatric surgery the mean difference was -5.7pg/ml (95% CI -8.9 to -2.6, p<0.0001*) compared with the non-surgical cohort where the mean difference was -1.4pg/ml (95% CI -1.4 to 1.2, p=0.19).

4.4 THE RELATIONSHIP BETWEEN WEIGHT LOSS AND CHANGES IN CIRCULATING MARKERS

Surgical weight loss is associated with significant improvements in markers of insulin resistance, inflammation, adipokines and reproductive hormones at both 2 and 12 months following bariatric surgery. Rapid improvements in glucose metabolism following bariatric surgery are well documented and an expected finding. The early significant changes in reproductive hormones (SHBG, FAI, LH and FSH) which are already apparent by two months post bariatric surgery may suggest that these are more closely related to the improvement in glucose metabolism than to the absolute change in weight or adiposity. Univariate regression analysis failed to identify any significant correlations with weight loss but the study is likely to be underpowered to provide these data.

When all measurements were grouped together BMI was seen to correlate with many of the serum markers, and with phosphorylated AKT, but not with Ki-67 when r is Spearman's correlation coefficient calculated for non-parametric data (Table 17).

TABLE 17: CORRELATION OF SERUM MARKERS, KI-67 AND P-AKT WITH BMI

Y axis	Number of pairs	r	95% CI Lower limit	95% CI Upper limit	p
Ki-67	116	0.12	-0.07	0.3	0.093
pAKT	116	0.19	0.007	0.37	0.019*
SHBG	101	-0.36	-0.52	-0.17	0.0001*
FAI	80	0.40	0.19	0.57	0.0001*
Oestradiol	105	0.17	-0.03	0.35	0.044*
HOMA-IR	98	0.39	0.21	0.55	<0.0001*
HbA1c	84	0.16	-0.063	0.37	0.074
hsCRP	100	0.33	0.14	0.50	0.0003*
Adiponectin	100	-0.35	-0.51	-0.16	0.0002*
Leptin	100	0.61	0.47	0.72	<0.0001*

				Surgical								Non-surgical							
				T1 vs. T0 (n=41)				T2 vs. T0 (n=27)				T1 vs. T0 (n=6)				T2 vs. T0 (n=6)			
				Mean difference	(95% CI)		p value	Mean difference	(95% CI)		p value	Mean difference	(95% CI)		p value	Mean difference	(95% CI)		p value
Circulating markers	Insulin resistance	Glucose	(mmol/L)	-2.4	-3.6	-1.3	0.0003*	-3.3	-4.2	-2.2	<0.0001*	0.83	-0.5	2.2	0.13	-0.02	-0.5	0.5	0.75
		Insulin	(mU/L)	-6.7	-11.8	-1.6	0.02*	-12.4	-18.6	-6.1	<0.0001*	1.8	-4.1	7.8	0.50	2.8	-3.3	8.8	0.50
		HOMA-IR		-4.5	-7.4	-1.5	0.0032*	-6.8	-10.0	-3.6	<0.0001*	0.72	-0.9	2.3	0.30	0.51	-1.3	2.3	0.63
		HbA1c	(mmol/mol)	-10.5	-16.0	-4.9	<0.0001*	-11.3	-17.9	-4.7	<0.0001*	Insufficient data to analyse				7	-56.5	70.5	0.50
		Adiponectin	(mg/L)	0.47	0.17	1.1	<0.0001*	1.3	0.97	1.7	<0.0001*	-0.01	-0.44	0.42	0.81	-0.0056	-0.57	0.56	0.81
	Adiposity	Leptin	(ng/ml)	-8.1	-13.2	-2.9	0.001*	-25.3	-34.0	-16.6	<0.0001*	6.1	-6.6	18.8	0.44	2.2	-14.8	19.2	1
	Inflammation	hsCRP	(mg/L)	-0.05	-1.7	1.6	0.35	-4.3	-6.3	-2.3	<0.0001*	-0.9	-4.9	1.1	0.81	-0.6	-4.1	2.8	0.63
		IL-6	(pg/ml)	-1.5	-6.8	-3.8	<0.0001*	-5.7	-8.9	-2.6	<0.0001*	-1.6	-5.8	2.7	0.63	-1.4	-1.4	1.2	0.19
	Reproductive function	Oestradiol	(pmol/L)	76.8	-15	168	0.15	9.7	-141	161	0.55	-43.6	-258	171	0.63	103.4	-503	710	1
		Progesterone	(ng/ml)	-1.5	-5.5	2.6	0.011*	1.5	-3.8	6.9	0.53	6.7	-20.7	34.0	0.63	-4.7	-17.2	7.8	0.63
		Testosterone	(nmol/L)	-0.2	-0.4	0.0	0.06	-0.3	-0.8	0.4	0.003*	0.1	-0.43	0.63	0.81	0.22	-0.1	0.5	0.25
		SHBG	(nmol/L)	26.7	17.5	35.9	<0.0001*	44.0	30.5	57.5	<0.0001*	-1.5	-52.7	49.7	0.88	-7.8	-47.7	32.2	0.88
		FAI		-1.9	-2.9	-0.9	0.0002*	-2.1	-3.2	-1.1	0.0010*	-0.5	-4.0	2.9	0.50	0.3	-0.7	1.3	0.50
		LH	(IU/L)	6.4	2.1	10.8	0.015*	5.2	1.4	9.0	0.0051*	4.3	-8.0	16.7	0.44	10.3	-5.8	26.4	0.19
		FSH	(IU/L)	5.6	1.4	9.8	0.0008*	8.8	2.9	14.8	0.0005*	8.2	-7.6	24.1	0.31	10.9	-6.7	28.7	0.19

TABLE 18: WEIGHT LOSS INDUCED CHANGES IN MARKERS OF INSULIN RESISTANCE, ADIPOSITY, INFLAMMATION AND REPRODUCTIVE FUNCTION

5. RESULTS III

THE ENDOMETRIAL EFFECTS OF WEIGHT LOSS

5.1 ENDOMETRIAL PATHOLOGY

Six cases of atypical endometrial hyperplasia were diagnosed at baseline, along with three biopsies that were reported as equivocal, and two endometrial biopsies that featured disordered proliferation. All had repeat sampling at 6-8 weeks and women with persistent abnormality and/or symptoms of endometrial abnormality were offered treatment with LNG-IUS. The remainder were managed conservatively and underwent serial endometrial biopsies.

The three biopsies reported as equivocal were normal at two months following weight loss surgery. At twelve months one of these women was found to have at least severe atypical hyperplasia with suspected invasive disease on endometrial biopsy and following hysterectomy final histopathological diagnosis was stage 1a grade 1 endometrioid adenocarcinoma of the endometrium. Of the two cases of disordered proliferation, both were normal at two months and one of these women conceived nine months post bariatric surgery after prolonged amenorrhoea prior to weight loss surgery.

All six women with atypical endometrial hyperplasia had repeat sampling 6-8 weeks later. Two had a LNG-IUS inserted at 6-8 weeks, one due to intermenstrual bleeding and one because of possible invasive disease, at the time of repeat biopsy which showed persistent abnormality in both cases. Four women with atypical hyperplasia did not have symptoms of concern like intermenstrual bleeding, although all of them had oligomenorrhoea and three reported heavy menstrual bleeding. Three had normal endometrial biopsies at 6-8 weeks following weight loss surgery (Figure 21 depicts the improving morphology of one of these cases, alongside the changes in her Ki-67 and pAKT with weight loss). The fourth had persistent endometrial abnormality but declined LNG-IUS and opted for conservative management.

Six months post weight loss surgery this patient was found to have a borderline serous ovarian tumour on ultrasound scan and therefore underwent total abdominal

hysterectomy, bilateral salpingo-oophorectomy and omental biopsy for this indication. The endometrium was reported as having a small residual focus of atypical endometrial hyperplasia, having initially been reported as diffuse, widespread atypical endometrial hyperplasia at baseline.

Two women with symptomatic hyperplasia and abnormal bleeding were managed with LNG-IUS for six months. One had an endometrial biopsy that showed atypical endometrial hyperplasia with possible invasive disease at baseline but due to the participant's age (35 years) and desire for fertility preservation opted to be managed medically. Both had persistent atypical endometrial hyperplasia at two months post bariatric surgery but normal endometrial biopsies twelve months post bariatric surgery. Both remain disease free two to three years post bariatric surgery; previous studies report recurrence of atypical endometrial hyperplasia following medical management to be at least 40% (92).

5.2 Endometrial proliferation

Ki-67 is a nuclear antigen that indicates proliferative activity of the endometrium. It has been shown to increase with worsening grade, stage and histological subtype of endometrial cancer (103,104). It is also present and variable within normal endometrium, being affected by phase of menstrual cycle, exogenous hormones and menopausal status, and has been positively correlated with BMI, serum oestrogen, leptin and insulin (102).

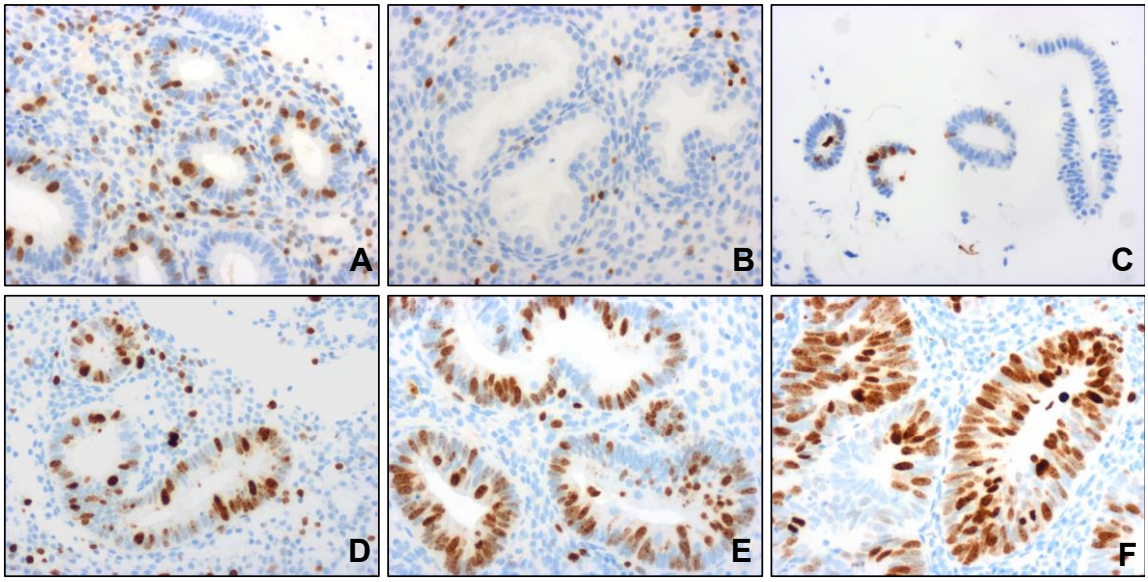
Endometrial immunohistochemistry was scored by two independent scorers (MM and MNA) blinded to treatment arm and time-point. For assessment of Ki-67 positivity three representative high-powered fields at x20 magnification were scored as described in Chapter 2 and an average taken. Biopsies with > 10% discrepancy between scorers were reviewed and ratified. Interobserver Intraclass Correlation (ICC) for Ki-67 scoring (Cronbach's alpha) was 0.976 (0.944-0.990). Ki-67 positivity is reported as a percentage, which may also be referred to as the Proliferation Index.

5.2.1 Determinants of baseline proliferation index

The highest Ki-67 levels were seen in endometrial cancer and AEH (median Ki-67 36%, IQR 21-48% vs. 23%, IQR 5-43% in normal endometrium; p=0.17). In normal

endometrium, Ki-67 was higher in the proliferative phase (median 33%, IQR 15-43%) than the secretory phase (median 20%, IQR 4-52%). Ki-67 levels were lowest in normal postmenopausal endometrium (median 5%, IQR 1-13% vs. median 30% in normal pre-menopausal, IQR 9-44%) (Figure 18).

FIGURE 18: IMAGES OF KI-67 STAINING IN BENIGN, HYPERPLASTIC AND MALIGNANT ENDOMETRIUM



Key: A) benign proliferative endometrium, B) benign secretory endometrium, C) scanty disrupted sample from postmenopausal patient, D) atypical endometrial hyperplasia, E) grade 1 endometrioid endometrial cancer, F) grade 2 endometrioid endometrial cancer.

In women with histologically normal endometrium at baseline there was no significant linear correlation observed between Ki-67 levels and BMI (premenopausal R^2=0.0013, p=0.84; postmenopausal R^2=0.59, p=0.23), HbA1c (premenopausal R^2 0.002, p=0.81; postmenopausal R^2=0.56, p=0.24) or HOMA-IR (premenopausal R^2=0.0008, p=0.89; postmenopausal R^2=0.32, p=0.44). This may reflect the small number of observations included in the analysis or the multiple other variables at play. In an effort to control for the effects of these variables, the same analysis was performed selecting women with histologically normal endometrium who were premenopausal in whom menstrual cycle timing was matched between biopsies. Again no significant correlation was demonstrated between baseline Ki-67 and BMI (R^2=0.18, p=0.07), HbA1c (R^2=0.07, p=0.31) or HOMA-IR (R^2=0.08, p=0.33).

5.2.2 The effect of weight loss on endometrial proliferation

The effect of weight loss on proliferation index has been individually assessed in the surgical and non-surgical cohorts, due to fundamental differences in the mechanism and the effects on glucose metabolism of bariatric surgery as compared with non-surgical weight loss. Due to the inherent variation of proliferation at different phases of the endometrial cycle a subgroup analysis of the surgical weight loss cohort has also been performed, restricted to women not taking exogenous hormones whose biopsies were taken at matched times in the endometrial cycle as reported by histopathological assessment and who did not develop a new endometrial abnormality in follow up (Figure 19 E, F).

Ki-67 was normally distributed at each time point in this subgroup, and also at baseline and 12 months in the surgical cohort, according to the D'Agostino and Pearson omnibus normality test. The non-surgical cohort was too small to test normality (n=6). For consistency mean difference has been reported in Table 20, but medians have been described here.

Median Ki-67 score in the surgical cohort was 28.6%, 13.5% and 12.8% at baseline, two and twelve months respectively and weight loss surgery was associated with statistically significant reductions in proliferation at both 2 and 12 months. The median difference was -15.1% (95% CI -17.1 to -2.01%, p=0.009, n=40) at two months and -15.8% (95% CI -21.8 to -1.1%, p=0.034, n=27) at twelve months post surgery. Kruskal Wallis testing found the medians to vary significantly (p=0.03*) across the 3 groups.

In the non-surgical cohort no significant change in Ki-67 was seen following weight loss intervention, and median score was 20.1%, 20.6% and 41.1% at baseline, two and twelve months respectively. Median difference was 0.5% (95% CI -29.0 to 32.6%, p=0.84, n=6) and 21% (95% CI -11.5 to 42.5%, p=0.31, n=6) at two and twelve months respectively.

Subgroup analysis of surgical patients whose biopsies were taken at matched points in the endometrial cycle, were not using exogenous hormones and did not develop a new endometrial abnormality in the course of follow up, was performed. The intention of this subgroup analysis was to remove the treatment effect of abnormalities being

treated with progesterone as well as other potential confounders, to ascertain the effect on proliferation of weight loss alone.

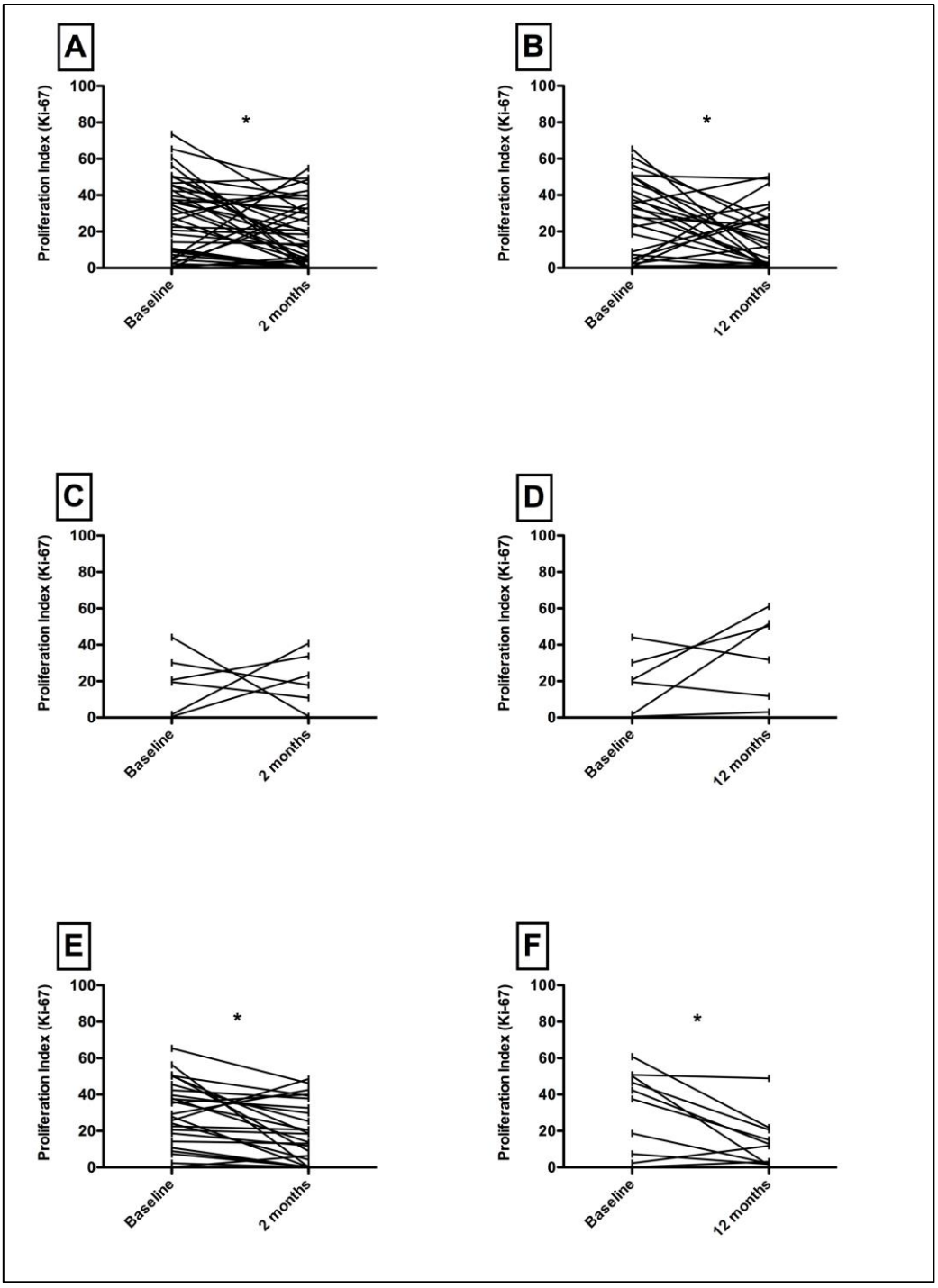
In this group weight loss was associated with statistically significant reductions in Ki-67 at both 2 and 12 months post weight loss surgery. Median Ki-67 score was 29.3%, 18.4% and 12.3% at baseline, 2 and 12 months. Mean difference was -10.9% (95% CI -18.2 to -4.3, p=0.0022, n=25) and -17% (95% CI -31.3 to -4.2, p=0.016, n=10) at 2 and 12 months post weight loss surgery.

Each line in Figure 19 represents the change from baseline in Ki-67 for an individual woman at two months and twelve months. A lower Ki-67 score in follow up was observed in 29/47 (62%) women overall; the remaining 18 (38%) showed static or higher Ki-67 levels. In bariatric surgery patients specifically Ki-67 decreased in 29/41 (71%) at 2 months (range -1.3% to -60.3%) and Ki-67 increased in 11/41 (29%). Graphs E and F in Figure 19 depict the Ki-67 change in the subgroup of surgical patients described above (did not receive exogenous progestagen treatment, did not develop a new endometrial abnormality, endometrial samples taken at matched phases of the menstrual cycle).

Of the women whose Ki-67 score increased during follow up (n=15) some may be explained by development or worsening of an abnormal endometrium (n=3), undergoing sampling during a different phase of the menstrual cycle (n=2), non-surgical (non-significant) weight loss (n=4) or weight loss leading to the establishment of regular menstruation after months or years of amenorrhoea (n=2). In 4 cases there was no obvious explanation for the increase in proliferation during follow up.

Ki-67 score increased in two women who developed endometrial abnormality at 12 month follow up (normal to atypical hyperplasia 5% at baseline to 36%, squamous metaplasia to grade 1 endometrial cancer 23% at baseline to 35%). This may suggest a role for Ki-67 assessment in the monitoring of women being conservatively or medically managed for low-grade endometrial cancer or atypical hyperplasia, as a potential marker or worsening or progressive disease.

FIGURE 19: THE EFFECT OF WEIGHT LOSS ON KI-67 EXPRESSION IN THE ENDOMETRIUM



A Ki-67 scores in the surgical cohort at baseline and 2 months, **B** Ki-67 scores in the surgical cohort at baseline and 12 months, **C** Ki-67 scores in the non-surgical cohort at baseline and 2 months, **D** Ki-67 scores in the non-surgical cohort at baseline and 12 months, **E** Ki-67 scores in the surgical subgroup at baseline and 2 months, **F** Ki-67 scores in surgical subgroup at baseline and 12 months (Subgroup = did not receive exogenous progestagen treatment, did not develop a new endometrial abnormality, endometrial samples taken at matched phases of the menstrual cycle).

5.2.3 The relationship between weight loss and change in Ki-67

No significant linear relationship between 12-month weight change and Ki-67 change was demonstrated (R^2 0.026, p = 0.30). When weight change was categorized into <20% total weight loss (or weight gain), 20-40% total weight loss and > 40% total weight loss no significant difference in Ki-67 change was seen between the groups (p=0.36) either but the trend suggested a relationship between greater weight loss and a bigger drop in Ki-67. Where participants lost <20% of their total body weight by 12 months the mean difference in Ki-67 was 2.96% (n=9), with 20-40% total body weight loss at 12 months mean difference in Ki-67 was -9.34% (n=17) and when there was more than 40% total body weight loss by 12 months mean difference in Ki-67 was -17.5% (n=6).

5.2.4 The relationship between other factors and Ki-67

No significant correlation was seen between the change in Ki-67 expression and the change in HbA1c (R^2 0.0029, p=0.79), HOMA-IR (R^2 0.042, p=0.26) or sex hormone binding globulin (R^2 0.011, p=0.55).

Similarities were seen between Ki-67 change at 2 months in both pre- and post-menopausal women, and whilst they only reached statistical significance in pre-menopausal women this is likely to reflect the greater numbers of pre-menopausal women in the study and the lower Ki-67 at baseline in post-menopausal women. Mean difference in Ki-67 at 2 months in pre-menopausal women was -8.3% (95% CI -16.6 to 0, p=0.04*, n=40) and in post-menopausal women was -6.5% (95% CI -15.1 to 2, p=0.16, n=6). At 12 months post weight loss intervention the trend towards reduction in Ki-67 with weight loss persisted in pre-menopausal women but did not reach statistical significance (mean difference -7.4%, 95% CI -18.2 to 3.5, p=0.17, n=30 vs. post-menopausal mean difference 1.4%, 95% CI -17.6 to 20.3, p=0.75, n=3).

When all measurements at all time points were pooled Ki-67 was found to be weakly correlated with pAKT expression, as well as with serum levels of oestradiol, FAI, HOMA-IR, adiponectin and leptin. Oestradiol was the serum marker that had the strongest correlation with Ki-67. No significant correlation with BMI was seen (Table 19).

TABLE 19: CORRELATION OF KI-67 WITH BMI, P-AKT AND SERUM MARKERS

Y axis	Number of pairs	r	95% CI Lower limit	95% CI Upper limit	p
BMI	116	0.12	-0.07	0.3	0.093
pAKT	124	0.37	0.21	0.52	<0.0001*
SHBG	109	-0.14	-0.33	0.05	0.067
FAI	90	0.25	0.04	0.44	0.0078*
Oestradiol	113	0.38	0.21	0.53	<0.0001*
HOMA-IR	106	0.28	0.09	0.45	0.0018*
HbA1c	88	0.082	-0.14	0.29	0.22
hsCRP	109	0.053	-0.14	0.24	0.29
Adiponectin	109	-0.17	-0.35	0.021	0.036*
Leptin	109	0.19	-0.003	0.37	<0.023*

5.3 Effects on pro-proliferative signal transduction pathways

5.3.1 pAKT

Expression of pAKT was assessed because abnormal signalling of the PI3K/AKT signal transduction pathway is implicated in endometrial carcinogenesis, and activation of this pathway (measured by phosphorylated AKT) is increased in obese and overweight women and may be increased in hyperplastic and neoplastic endometrium. It has also been associated with ER expression.

Whilst baseline pAKT expression was normally distributed, at T1 and T2 pAKT expression was non-parametric. Wilcoxon signed rank test was used to assess the changes in pAKT expression after weight loss intervention at each time point (Table 20).

Median pAKT expression in the surgical cohort was 45, 0 and 10/300 at baseline, two and twelve months respectively and weight loss surgery was associated with statistically significant reductions in pAKT expression at both 2 and 12 months (median difference -45, p=0.027, n=40 and median difference -35, p=0.0015, n=27 respectively).

In the non-surgical cohort no significant change in pAKT expression was seen following weight loss intervention, and median proliferation index was 35, 12.5 and 15/300 at baseline, two and twelve months respectively. Median difference was -22.5, p=0.63, n=6 and -20, p=0.63, n=6 at 2 and 12 months post weight loss intervention.

Subgroup analysis of surgical patients whose biopsies were taken at matched points in the endometrial cycle, were not using exogenous hormones and did not develop a new endometrial abnormality in the course of follow up, was performed (n=25 and n=10 at 2 and 12 months, graphs E and F in Figure 20). In this group weight loss was not associated with statistically significant reductions in pAKT expression at either 2 or 12 months post weight loss surgery. Median pAKT expression was 40, 10 and 0 at baseline, 2 and 12 months respectively, p=0.085 and p=0.058 2 and 12 months post bariatric surgery. The absence of a significant effect in this selected cohort may reflect the smaller sample size associated with the subgroup analysis, but other possible reasons cannot be excluded such as the possibility that different variables affect pAKT than affect Ki-67 and therefore the selection for the subgroup analysis was inappropriate.

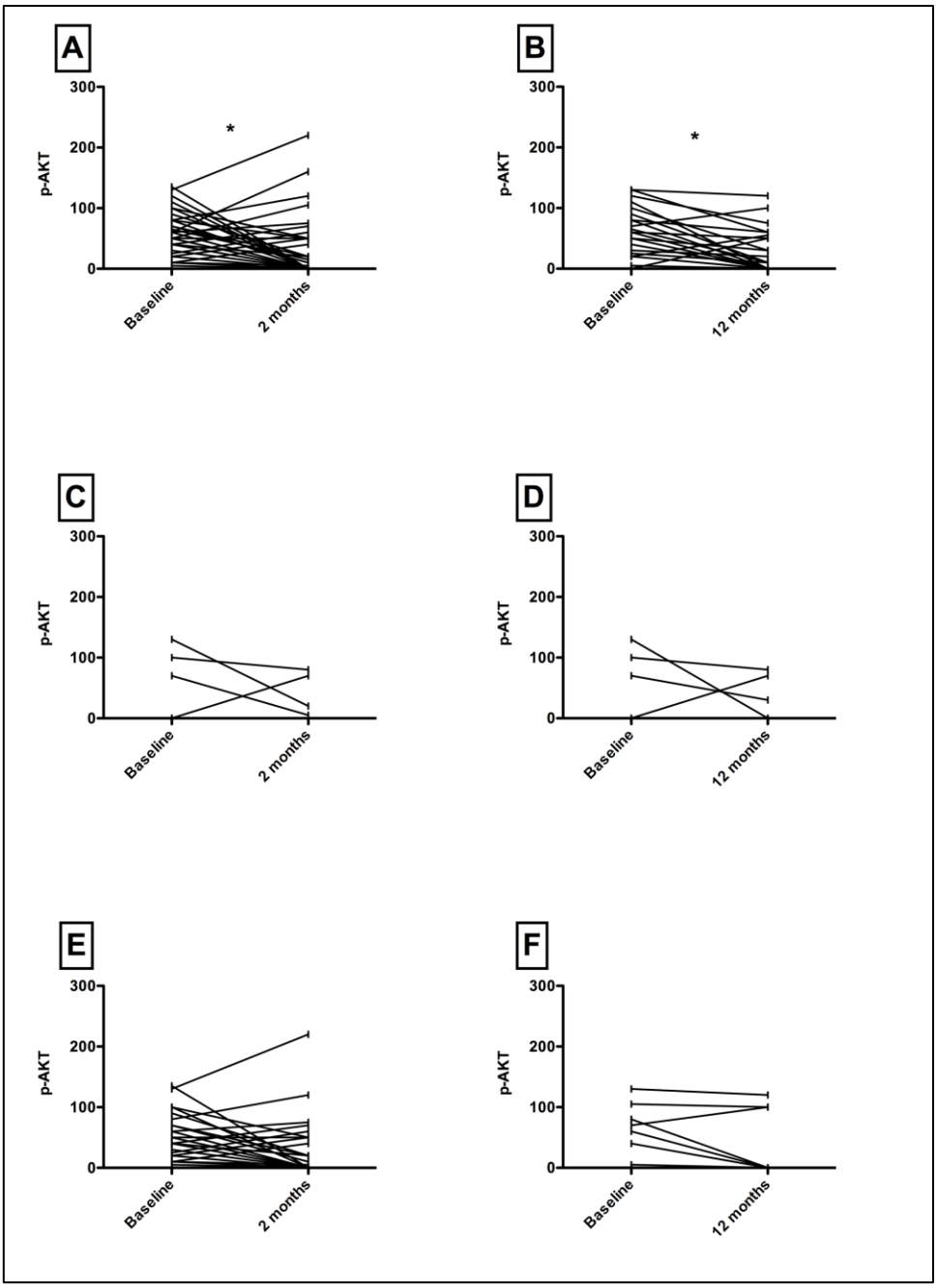
There is no evidence that pAKT is subject to the same variation across the menstrual cycle as Ki-67 is known to be.

5.3.2 pERK 1,2

Enhanced activation of the Ras/ERK 1,2 pathway (measured by proportion of phosphorylated ERK 1,2) is seen in overweight and obese premenopausal women, and also in the presence of endometrial cancer.

The expression of pERK 1,2 in both endometrial stroma and glands was assessed and no statistically significant effect of weight loss was observed in either the surgical or non-surgical cohorts (Table 20). Mean difference in glandular pERK 1,2 was 6.0 (95% CI -15 to 27, p=0.8) at 2 months and -9.6 (95% CI -41 to 22, p=0.45) at 12 months post bariatric surgery and was 11.7 (95% CI -27 to 50, p=0.63) and -13.3 (95% CI -60 to 33, p=1) 2 and 12 months post non-surgical weight loss intervention. Mean difference in stromal pERK 1,2 was 2.0 (95% CI -22 to 26, p=0.92) at 2 months and -18 (95% CI -40 to 4, p=0.2) at 12 months post bariatric surgery and was 8.3 (95% CI -42 to 58, p=1) and 5 (95% CI -22 to 32, p=0.75) 2 and 12 months post non-surgical weight loss intervention.

FIGURE 20: THE EFFECT OF WEIGHT LOSS ON ENDOMETRIAL EXPRESSION OF P-AKT



A pAKT scores in the surgical cohort at baseline and 2 months, **B** pAKT scores in the surgical cohort at baseline and 12 months, **C** pAKT scores in the non-surgical cohort at baseline and 2 months, **D** pAKT scores in the non-surgical cohort at baseline and 12 months, **E** pAKT scores in surgical subgroup at baseline and 2 months, **F** pAKT scores in same surgical subgroup at baseline and 12 months (Subgroup = did not receive exogenous progestagen treatment, did not develop a new endometrial abnormality, endometrial samples taken at matched phases of the menstrual cycle).

5.4 Effects on endometrial hormone receptor expression

Endometrium expresses both oestrogen and progesterone receptors, and their expression varies throughout the menstrual cycle and reproductive lifetime. Higher levels of PR expression have been documented in obese premenopausal women. Loss of ER and PR expression is associated with advanced, poor prognosis endometrial tumours. Table 20 reports the change in ER and PR expression observed with weight loss.

5.4.1 Oestrogen receptors

Weight loss surgery was associated with statistically significant reductions in oestrogen receptor expression in the endometrium at both 2 and 12 months post bariatric surgery (mean difference -1.5, 95% CI -3 to 0.1, p=0.025* and -2.3, 95% CI -4 to -0.3, p=0.04* respectively). No significant change in ER expression was noted in the non-surgical cohort at either time point (mean difference -1.2, 95% CI -6.4 to 4.1, p=0.72 and -0.2, 95% CI -4.4 to 4.1, p=0.88 at 2 and 12 months respectively).

5.4.2 Progesterone receptors

Weight loss surgery was associated with statistically significant reductions in progesterone receptor expression in the endometrium at both 2 and 12 months post bariatric surgery (mean difference -1.4, 95% CI -3 to 0.2, p=0.031* and -3.5, 95% CI -5.8 to -1.1, p=0.004* respectively). No significant change in PR expression was noted in the non-surgical cohort at either time point (mean difference 3.2, 95% CI 0.6 to 5.8, p=0.13 and 3.7, 95% CI -0.2 to 7.6, p=0.13 at 2 and 12 months respectively).

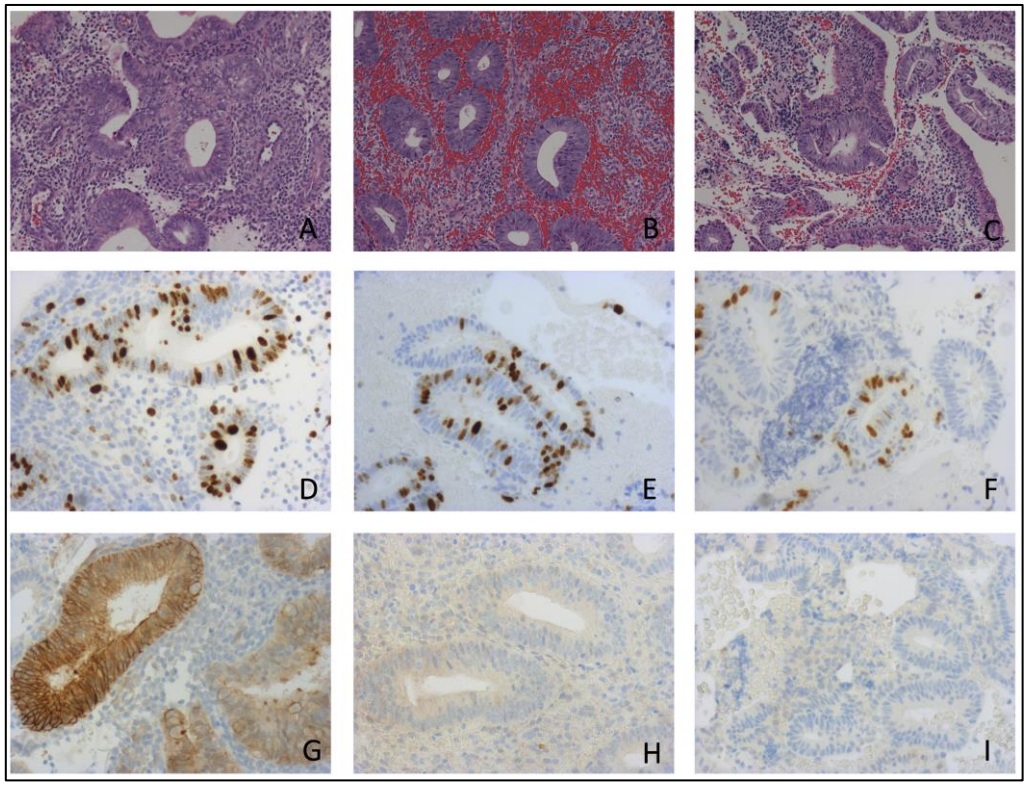
5.4 Effects on PTEN

It was not possible to assess the effects of weight loss on PTEN expression in the endometrium due to not being able to optimise the antibody for PTEN on Pipelle samples, despite multiple iterations. The antibody was successfully optimized on control tissues including endometrial adenocarcinoma sections, and stromal positivity was seen in Pipelle samples but in all histologically normal endometrial samples the glands were PTEN negative, which is obviously not what would be expected. Due to time and cost constraints this will not be not pursued further within the context of this MD, but will form part of the further work subject to funding.

TABLE 20: CHANGES IN THE ENDOMETRIUM OBSERVED WITH WEIGHT LOSS

		Surgical								Non-surgical							
		T1 vs. T0 (n=41)				T2 vs. T0 (n=27)				T1 vs. T0 (n=6)				T2 vs. T0 (n=6)			
		Mean difference	(95% CI)		p value	Mean difference	(95% CI)		p value	Mean difference	(95% CI)		p value	Mean difference	(95% CI)		p value
Endometrial markers	**Ki-67** (%)	-9.6	-17.1	-2	0.01*	-11.5	-21.8	-1.1	0.03*	1.81	-29	32.6	0.84	15.5	-11.5	42.5	0.31
	pAKT g (0-300)	-18.6	-35.2	-2.1	0.023*	-30.6	-45.9	-15.2	0.0002*	-20.8	-85.6	43.9	0.63	-20.0	-88.7	48.7	0.63
	pERK g (0-300)	6.0	-15.2	27.2	0.80	-9.6	-40.8	21.6	0.45	11.7	-26.7	50.0	0.63	-13.3	-60.1	33.4	1
	pERK s (0-300)	2.0	-22.3	26.4	0.92	-18.0	-39.6	3.6	0.20	8.3	-41.5	58.2	1	5.0	-22.2	32.2	0.75
	ER (0-18)	-1.5	-3.0	0.1	0.025*	-2.3	-4.0	-0.3	0.04*	-1.2	-6.4	4.1	0.72	-0.2	-4.4	4.1	0.88
	PR (0-18)	-1.4	-3.0	0.2	0.031*	-3.5	-5.8	-1.1	0.004*	3.2	0.56	5.8	0.13	3.7	-0.2	7.6	0.13

FIGURE 21: CASE STUDY OF PARTICIPANT WL47 WHO DEMONSTRATED NORMALISATION OF ATYPICAL HYPERPLASIA ALONGSIDE REDUCTIONS IN KI-67 AND P-AKT AFTER BARIATRIC SURGERY, WITHOUT TREATMENT



Key: A-C) H&E sections of patient WL47 at baseline, 2 and 12 months showing AEH at baseline which was managed conservatively, followed by 2 normal biopsies 2 and 12 months post bariatric surgery, D-F) Ki-67 stained sections from WL47 showing reduction in proliferation from 37.5% at baseline to 32.6% and 15% at 2 and 12 months post bariatric surgery, G-I) decreasing pAKT expression in the same patient at the same time points.

6. DISCUSSION

6.1 FEATURES OF COHORT

6.1.1 AIMS AND OBJECTIVES

The incidence and mortality of endometrial cancer are increasing, despite improving survival rates. The number of young women being diagnosed with endometrial cancer is also increasing, and a desire for fertility sparing treatment of endometrial cancer and pre-cancer is becoming more prominent. There is an urgent need to know if we have an opportunity to prevent endometrial cancer through screening of morbidly obese women and treatment of precursor lesions, and if there is a place for weight loss surgery or other targeted preventative measures (LNG-IUS, COC, metformin) to reduce cancer risk or to treat pre-malignant endometrial abnormalities. Not enough is known about the effect of obesity on endometrial carcinogenesis, or the effect of treating obesity on risk of disease.

The overall aim of this project was to provide insight into the relationship between obesity and weight loss and the impact they have on endometrium, to identify targets for future work into the prevention and treatment of endometrial cancer, and to enhance the discourse on the role of screening in high-risk women.

The specific aims were to:

1. Determine the prevalence of endometrial abnormalities in pre and post-menopausal women undergoing surgical or non-surgical weight loss management for morbid obesity, and assess the impact of weight loss upon the endometrial histology

2. Determine the expression of biomarkers of proliferation and signal transduction molecules which have been implicated in endometrial carcinogenesis, before and after weight loss

3. Determine the effect of weight loss on menstrual function, gonadotrophin levels and circulating oestrogen, CRP, leptin and adiponectin levels

6.1.2 Baseline characteristics of the cohort

Over a 32 month period 248 women attending weight management clinics were approached and screened for eligibility, 118 women were enrolled onto the clinical study and 80 women underwent baseline assessment. Approximately 70% of eligible women consented to participate, more than ¾ of those not eligible to participate had previously had a hysterectomy or had a Mirena LNG-IUS in situ, and the reason most commonly given for this was menstrual dysfunction. 16.5% of all women approached had had a previous hysterectomy, which would be in keeping with expectations. Whilst the incidence of hysterectomy for benign indications in age-matched women varies greatly internationally, in the UK it has been reported to be around 20% by age 60 (220). Other comparable studies of bariatric surgery cohorts in the US have reported prior hysterectomy rates of 30-33% (67,162).

More than three quarters of women recruited were pre-menopausal, and the average age was 44 years. The median BMI was 52kg/m^2; 72 women (90%) underwent bariatric surgery and 8 (10%) commenced a non-surgical weight management programme. Median BMI was significantly higher in the surgical cohort than the non-surgical cohort. One third of women were diabetic, most commonly treated with metformin. Some women were using insulin or multiple medications for their diabetes. The baseline characteristics were in keeping with cohorts enrolled in other bariatric surgery studies although frequently bariatric surgery cohorts in the US have a lower median BMI than observed in this cohort, due to differing patterns of bariatric surgery use in different healthcare economies (187).

A substantial proportion of women reported a history of menstrual or reproductive dysfunction; 9% gave a history of subfertility, 18% had a previous diagnosis of PCOS, 46% of premenopausal women had amenorrhoea or oligomenorrhoea and 35% reported abnormal vaginal bleeding. None of the women recruited were using combined oral contraceptives, 13% were using progestagen only methods of contraception. Cervical screening was overdue in 44% of women, which is reinforced by the strong inverse relationship that has been demonstrated between BMI and cervical screening uptake in a systematic review of 170,000 women (163).

Data from the LABS-2 study of 1,538 women undergoing bariatric surgery in the US reported a previous diagnosis of PCOS in 13%, particularly in women with onset of obesity prior to the age of 18. Infertility was present in 8%. Oligomenorrhoea was more common in obese premenopausal women than in normal weight women in a study of over 6000 women (26% cf. 14%). Both body composition and menstrual irregularity have been associated with high testosterone and FAI, raised insulin and low SHBG. We found all of these to be true.

HPO axis dysfunction due to PCOS is the most common cause of ovulatory disorders. In reality the diagnosis of PCOS is difficult and lacks specificity and sensitivity. It is likely to exist on a spectrum with significant overlap seen with women who are obese but do not have PCOS. Being overweight mimics the clinical picture of PCOS and many of the same features can be seen. There is a great deal of heterogeneity within PCOS, and probable under and over diagnosis, especially in obese women.

Significant differences in the degree of weight loss were seen between the 2 cohorts. In the non-surgical group mean % total weight change was -0.9% at 2 months and -5% at 12 months, although it is noteworthy that the standard deviation at 12 months was 4.7. The surgical cohort lost an average of 15% and 33% of total body weight at 2 and 12 months respectively. These findings are in keeping with what is known about weight loss achieved by surgical and non-surgical means.

Of note is the fact that three patients had undiagnosed diabetes on the basis of their HbA1c level, and that raised HOMA-IR levels were found in 22 non-diabetic women. This means that 46% of the total cohort had *undiagnosed* diabetes or insulin resistance using HOMA-IR > 2.5 as a surrogate marker for insulin resistance, and that 79% of the total cohort were diabetic or insulin resistant. A prospective cohort study from the MD Anderson found that 2/3 of their patients with newly diagnosed endometrial cancer were insulin resistant, and on multivariate analysis this was significantly associated with BMI and nulliparity.

6.2 Prevalence of endometrial abnormality

6.2.1 The prevalence of disease and how it compares to existing evidence

The baseline prevalence of endometrial cancer and pre-cancer in this morbidly obese cohort was 12.5%. Endometrial cancer was found in four women (5%) and atypical hyperplasia in six (7.5%). In addition to the baseline endometrial pathology detected, cervical cytology performed opportunistically led to the diagnosis of a stage 1b1 cervical cancer. This degree of pathology is far in excess of what was anticipated, and what has been described in the literature to date (66–68).

Existing data from the UKCTOCS nested case control cohort suggests a prevalence in the general population of 0.34% for endometrial cancer and 0.03% for AEH, 82% of whom were asymptomatic at diagnosis (54). Whilst in obese populations this has been shown to be higher, such studies are small. Viola et al detected a prevalence of undiagnosed AEH 2.2% and endometrial cancer of 1-3% in overweight and obese pre and postmenopausal women. The mean BMI in this study was only 35-37kg/m^2, which may explain their lower prevalence (56). Bariatric surgery studies have reported varying results of up to 6.8% non atypical hyperplasia, up to 3.3% atypical hyperplasia, and a 0% undiagnosed endometrial cancer prevalence in cohorts of 30-59 women (66–68).

Clearly the baseline prevalence we have identified exceeds previously published data, and several factors may account for this. This is the largest prospective cross-sectional analysis of endometrial cancer and pre-cancer in a morbidly obese cohort to date, and the only one from the UK. The next largest study by Argenta et al biopsied 59 women prior to bariatric surgery (66). The median age and BMI of their participants was lower than in our study (39 years vs. 43 years and 46kg/m^2 vs. 52kg/m^2), and we know that AEH and endometrial cancer are correlated with BMI and likely to be more common in older women. Crucially, 28% of their participants used oral contraceptives or LNG-IUS, both of which are protective against endometrial cancer – no women within our cohort used combined oral contraception or LNG-IUS, although some used oral progestagens.

Their participants were also more highly selected than ours. Any abnormal bleeding in the preceding 6 months was an exclusion criterion, and abnormal bleeding is common

in this age group, particularly in women who are obese. In our cohort this may have excluded up to 35% of women, and up to 60% of the women diagnosed with endometrial cancer or pre-cancer, depending upon how the authors defined abnormal bleeding. 8.5% of biopsies were insufficient for diagnosis in this study (vs. 2.5% in ours), and the authors do not report the prevalence of diabetes or insulin resistance within their cohort so it is difficult to draw parallels in this regard.

Other studies had smaller numbers of participants (n=30 and n=47), and inexplicably high rates of inadequate samples being obtained (28-38%) (67,68). The reasons for this are unclear but in the absence of a reassuring ultrasound scan it cannot be assumed that lack of an adequate endometrial sample indicates lack of disease, and undeniably obesity can cause technical difficulty achieving endometrial sampling. Whilst Modesitt et al report 40% prevalence of diabetes in their cohort, they do not assess undiagnosed diabetes or insulin resistance, and Kaiyrlykyzy et al do not report the prevalence of diabetes or insulin resistance in their cohort.

6.2.2 Features of women with abnormal endometrium

Eight of the ten women diagnosed with endometrial cancer or atypical hyperplasia at baseline were premenopausal, five were known to be diabetic, two had undiagnosed diabetes, and three had a previous diagnosis of PCOS. Women with endometrial abnormality had significantly higher HbA1c at baseline than those with normal endometrium.

From these findings we can conclude that insulin resistance and diabetes are significant risk factors for harbouring occult endometrial cancers and pre-cancers in these frequently young women. We have demonstrated in this study that undiagnosed insulin resistance and in some cases undiagnosed diabetes are a problem in morbidly obese women. Of the three women found to have undiagnosed diabetes, two of them had an endometrial abnormality, so the importance of detecting and treating diabetes in women who are already at risk of endometrial cancer by virtue of their obesity is clear.

Abnormal endometrium also demonstrated greater activation of the PI3K/AKT/mTOR pathway. Activation of the PI3K pathway is a feature of hyperinsulinaemia, which may explain this observation of increased pAKT in women with endometrial abnormality, although many other factors also interplay with this pathway. No significant difference in age or BMI was seen between the two groups.

Only one of the eight pre-menopausal women found to have endometrial cancer or pre cancer described their menstrual cycle as normal. Four had heavy menstrual bleeding and oligomenorrhoea, one had intermenstrual bleeding, one had oligomenorrhoea and one had heavy regular menstrual bleeding. So, while for the purposes of this study they may be described as asymptomatic, what is actually meant by that is that they had symptoms frequently dismissed or considered normal in women who are obese and approaching the menopause who would not meet the criteria for referral on a suspected cancer pathway (74). In contrast, one in four women in the total cohort had abnormal bleeding or abnormal cycles. This highlights a key point, which is, in women with such a significant increase in their risk of endometrial cancer any menstrual abnormality should be treated as a red flag symptom until proven otherwise.

6.2.3 Is there evidence for a high-risk endometrium?

Key to the study was the assumption based on epidemiological data that morbidly obese women have a high-risk endometrium and that major weight loss drives towards a lower risk endometrium, and the hypothesis was that the results of the study would lend insight to two important lines of enquiry:

1. What are the key detectable features of a high-risk endometrium (i.e. those that altered during the process of weight loss)?
2. How could these features be used to design prevention studies by identifying truly high-risk women and measuring the up or down regulation of these outcome measures in response to treatment?

This study has demonstrated higher median Ki-67 levels than a similar study wherein 30% of women were using LNG-IUS or oral contraceptives (198), along with rising Ki-67 levels in endometrial biopsies that went from normal to abnormal. Although we did not

demonstrate a significant difference in Ki-67 scores between abnormal and normal endometrium, there was a trend towards higher Ki-67 in abnormal endometrium. Ki-67 expression was found to correlate weakly with oestradiol, pAKT, FAI, HOMA-IR, adiponectin and leptin.

Weight loss brought about both short and long term statistically significant reductions in Ki-67 expression, which remained significant on subgroup analysis once variation in menstrual cycle timing and treatment effect in women on exogenous hormones were accounted for. Statistically significant reductions in the phosphorylation of the PI3K/AKT signal transduction pathway were also seen, which could explain the reduction in proliferation and could have resulted from the significant changes seen in insulin, leptin or adiponectin levels or from more esoteric effects of weight loss that have not been elicited herein. In 50% of women with AEH and endometrial cancer a reduction in Ki-67 (that was not the result of cycle timing mismatch) was seen prior to any treatment taking place (underwent 2 month biopsy prior to hysterectomy or LNG-IUS), which may reflect inherent variation but is likely to be the effect of weight loss on endometrial proliferation.

There was no significant difference in BMI between the women with normal endometrium and those with AEH or endometrial cancer; there was however a significant difference in their baseline HbA1c levels. In the ten women with endometrial cancer or AEH at baseline five were known to be diabetic (four were on metformin, but still had HbA1c levels between 60 and 102 mmol/mol) and two had undiagnosed diabetes. It is clear that diabetes and obesity in concert make for a very high risk state in the potential development of endometrial cancer and AEH and that these women would be suitable for targeted screening if this was to be implemented, along with screening of obese women for diabetes as there is significant under diagnosis of insulin resistance.

In pre-menopausal women diagnosed with endometrial abnormality menstrual bleeding abnormality was common and oligomenorrhoea/amenorrhoea should be recognised as a risk factor for the accumulation of endometrial abnormality, and heavy

menstrual bleeding in high-risk women should be regarded as a red flag symptom. In women found to have AEH who choose to undergo non-surgical management the monitoring of Ki-67 may be an indicator of worsening endometrial change.

6.3 Effects of weight loss on circulating markers of insulin resistance, reproductive dysfunction, inflammation and adiposity

6.3.1 Insulin resistance

As expected, insulin resistance improved dramatically across all measured parameters (glucose, insulin, HbA1c, HOMA-IR) both 2 and 12 months following bariatric surgery. No statistically significant changes were seen in the non-surgical group, with an *increase* in insulin levels being evident at 12 months in this group. This was despite a mean weight loss of 5%. Other studies have reported improvements in these parameters with weight loss of 5-10% (196) so the explanations for not seeing an effect are either that this group of women did not lose enough weight to demonstrate a clinically or statistically significant effect on insulin resistance or the numbers studied were too small and the intra-patient variation too great to demonstrate this effect (n=6, standard deviation 4.7) or a combination of the two.

The measured effect of weight loss on HbA1c in this study is limited by 35% missing data as it was a Substantial Amendment to the protocol after the study was underway. There were 26 and 15 results available at 2 and 12 months post bariatric surgery so meaningful conclusions can still be drawn, however in the smaller non-surgical group this information was only available for 1/6 at 2 months and 2/6 at 12 months.

Insulin resistance is linked with obesity related cancers and it can be difficult to separate the two risk factors out. Hyperinsulinaemia may reduce the production of IGFBP's, increasing the levels of bioavailable IGF1, and also inhibits apoptosis, encourages proliferation and tumour genesis, through interactions with both PI3K and MAPK pathways.

It is thought that after bariatric surgery glucose metabolism improves independently of weight loss, as incretin production (GLP-1, GIP, PYY) and therefore insulin secretion are reduced. Short-term caloric restriction alters glucose metabolism and insulin resistance modulated by the entero insular axis and involving foregut hormones. Long-term improvements are the result of reduction in fat mass and mediated by changes in adipokines.

6.3.2 Reproductive hormones

At 2 months post bariatric surgery a significant increase in SHBG was seen alongside a significant decrease in FAI. These findings persisted at 12 months post bariatric surgery, at which point a significant decrease in testosterone was also seen. The early changes in SHBG and FAI may reflect the dramatic reduction in insulin levels seen after bariatric surgery, as insulin inhibits hepatic synthesis of SHBG. These findings are consistent with other studies that have measured the effect of weight loss on androgens and SHBG.

No significant changes in oestradiol or progesterone were seen 12 months post-bariatric surgery. It was hypothesised that weight loss would lead to a reduction in oestradiol levels, because it has been shown to correlate with BMI in postmenopausal women, and because it is recognised that obesity is a hyper estrogenic state as androgens are converted to oestrone and oestradiol in peripheral adipose tissue. Low SHBG levels in obesity are also thought to contribute to increased fractions of unbound oestrogen.

It is difficult to control for the normal variations seen in pre-menopausal circulating oestrogen levels; some studies have only measured oestrogen levels in post-menopausal participants because of this (159). In our study the assessments took place either at a time dictated by the surgical team carrying out the bariatric surgery or in the proliferative phase, as per the study protocol. This was a pragmatic decision to try to control for variables affecting the primary outcome measure – endometrial Ki-67, without increasing the burden of the study on participants and increasing the loss to follow up rates.

Due to this, the recorded levels of oestradiol, and indeed FSH and LH, are not trough levels and as such cannot really be interpreted in a meaningful way. Modesitt et al also failed to demonstrate any effect of bariatric surgery on oestrogen and progesterone metabolites, although their study is likely to be subject to these same limitations due to similarities in study design. Interestingly Villavicencio et al demonstrated increasing oestradiol levels with increasing BMI with fairly low SEM values; levels being statistically significantly higher in obese women than in normal weight women – despite apparently having similar limitations as other studies where no effect has been

seen. They had three small groups of entirely pre-menopausal women (n=10, 9 and 12), do not state when in the menstrual cycle the bloods were taken but do specify that endometrial sampling took place in the proliferative phase, yet have succeeded in demonstrating not only a statistically significant difference across BMI categories, but a strong correlation between Ki-67 and oestradiol (102).

Statistically significant increases in FSH and LH were seen at both 2 and 12 months post bariatric surgery; non-surgical weight management was not associated with significant changes in these levels. As mentioned, these are not trough levels and as such must be interpreted with caution. Over a 12-month period it is not unexpected for FSH and LH levels to rise in women of this age group, due to the passage of time. If oestradiol levels had dropped with weight loss it may suggest that women whose FSH and LH were previously suppressed by high circulating oestrogen levels were no longer suppressed, although there is no evidence this is the case here.

No significant changes were observed in progesterone levels although these too were likely to be subject to a large degree of error due to the timing of sampling, as they vary hugely throughout the menstrual cycle. It might have been expected that the median progesterone would rise after weight loss because women were transitioning from being anovulatory to ovulatory, but this could have been counteracted by other women in the study becoming peri-menopausal over the course of the study.

6.3.3 Inflammatory markers

In this study significant alterations in inflammatory markers were seen. At 2 and 12 months post bariatric surgery there was a statistically significant reduction in IL-6, and at 12 months a significant reduction in high sensitivity CRP was noted. A greater effect on IL-6 was evident at 12 than 2 months. No significant effect was seen in non-surgical patients although a downward trend was noted for both markers. The relatively greater effect at 12 months may reflect the acute inflammation associated with a recent surgical procedure skewing the findings at 2 months.

Inflammation has been linked to the promotion and progression of cancer, through direct and indirect mechanisms of action. CRP and pro-inflammatory cytokines such as

IL-6 and TNFα have consistently been shown to be higher in obesity and endometrial cancer, although are vulnerable to reporting bias as are most biomarker and cancer risk studies.

6.3.4 Adipokines

Weight loss of between 5 and 10% has been demonstrated to increase adiponectin and reduce leptin levels, in a linear manner (196,197). At both 2 and 12 months post bariatric surgery statistically significant improvements in both adiponectin and leptin were evident, in keeping with previous studies and the study hypothesis. No significant differences were noted in the non-surgical group, the potential reasons for this having been discussed previously.

Adiponectin is produced by adipocytes and leptin predominantly from adipose tissue. Leptin reduces tissue sensitivity to insulin leading to hyperinsulinaemia, and is found in high levels in obesity and endometrial cancer. Adiponectin is inversely correlated with obesity and endometrial cancer, and improves insulin sensitivity in peripheral tissues. Both interact with PI3K and MAPK signalling pathways via actions on AMPK; adiponectin activates it by phosphorylation and leptin inactivates it. Previous studies have shown both bariatric surgery and non-surgical weight loss to be effective at improving adipokine levels in the circulation.

6.4 Effects of weight loss on the endometrium

6.4.1 Menstrual function

Menstrual dysfunction was common in this cohort, as is often described in obese women. In women who reported amenorrhoea or oligomenorrhoea at baseline 26% saw restoration of regular menstrual bleeding during the 12 month follow up period. 33% of women with abnormal bleeding (i.e. intermenstrual, postmenopausal, postcoital or heavy menstrual bleeding) reported resolution of this by 12 months after weight loss. In absolute terms this was five women, four of whom had atypical hyperplasia or disordered proliferation on their baseline biopsies, which normalised alongside the improvement in their bleeding. One woman who reported normal menstrual cycles at baseline developed heavy menstrual bleeding at 12 months and was found to have atypical hyperplasia at that point.

These findings highlight the importance of routine enquiry into menstrual function in morbidly obese women when they visit relevant healthcare professionals, and the relevance of heavy menstrual bleeding in this high-risk group as a potential symptom of endometrial abnormality. Whilst weight loss may lead to improvements in menstrual function, this may predominantly be in the context of existing endometrial abnormality as is evident in the five women who experienced resolution of abnormal bleeding after weight loss.

6.4.2 Endometrial pathology

In addition to the 12.5% prevalence at baseline, one patient developed endometrial cancer and one patient developed atypical hyperplasia by their 12-month biopsy. Two baseline biopsies were reported as disordered proliferation, both had normalised without intervention 2 months post weight loss. Four of the six women with atypical hyperplasia were asymptomatic and managed conservatively; three normalised by 2 months after bariatric surgery, one showed a partial response at 12 months. Two were symptomatic of their hyperplasia and were treated with LNG-IUS for 6 months. Both had a complete response at 6 months and remained disease free at 3 years.

Whilst similar studies suggest regression of hyperplasia with bariatric surgery induced weight loss (67), ours is the first to describe multiple examples of women who have had

atypical hyperplasia that spontaneously resolved after bariatric surgery (n=4). Despite studies that have reported on outcomes of treatment of atypical hyperplasia with LNG-IUS reporting high rates of relapse, with a median time to relapse of 6 months, and often more than 6 months treatment required to achieve complete response in obese women, the two women with AEH treated with LNG-IUS demonstrated complete response at 6 months and no recurrence at 3 years.

All women with AEH underwent bariatric surgery and it may be that through this, the removal of the stimulus for the AEH prevents or minimises recurrence after treatment discontinuation due to the significant changes in insulin resistance, inflammation, SHBG, FAI and adipokines we have demonstrated, and increases the likelihood of spontaneous resolution of disease. Admittedly our understanding of the natural history of AEH is not advanced enough to enable us to conclude that AEH would not resolve *spontaneously* in the context of persisting obesity. No additional cases of endometrial abnormality were detected at the 2 month biopsy, only at the 12 month biopsy, which provides reassurance that a) abnormalities are unlikely to have been missed on baseline assessment and b) a screening interval of 12 months may be appropriate were this to ever be considered.

6.4.3 Endometrial proliferation

As previously recognised Ki-67 was higher in premenopausal women than post-menopausal, higher in the proliferative phase of the menstrual cycle than the secretory phase, and higher in endometrial cancer and AEH than in normal endometrium. The data did not substantiate previous data describing a correlation between BMI and Ki-67, although the study may be underpowered to detect this – however this effect has been demonstrated strikingly in smaller cohorts than ours (102). What was observed however, was a trend between increasing amounts of weight loss and more pronounced reductions in Ki-67. On the other hand, Ki-67 appeared to increase in women whose endometrium was becoming more abnormal (n=3) which is to be expected based upon our understanding of Ki-67, and may support the use of Ki-67 as a marker of treatment effect in women undergoing conservative management of AEH. Across all time points Ki-67 levels were shown to correlate weakly with pAKT, FAI,

oestradiol, HOMA-IR, adiponectin and leptin levels. Stronger associations with oestradiol were seen in the study by Villavicencio et al, possible explanations for which are unclear (102).

A decrease from baseline in the median Ki-67 score of 15.1% and 15.8% were seen 2 and 12 months after bariatric surgery ($p=0.009$ and $p=0.034$). In a subgroup of biopsies matched for menstrual cycle timing the median changes were 10.9% and 17% at 2 and 12 months post bariatric surgery which became more statistically significant in spite of smaller numbers ($p=0.002$ and $p=0.027$). The significant change in proliferation by just 2 months would make it likely that this is primarily a response to reduced insulin levels, and therefore reduced activation of pro-proliferative signalling pathways, as the alteration in oestrogen or inflammatory markers were less convincing at this early time point. Increasing adiponectin and decreasing leptin levels may also have served to down regulate the PI3K/AKT and Ras/MAPK pathways that promote proliferation.

No significant changes were seen in women undertaking non-surgical weight loss. Previous studies have suggested 5-10% weight loss as being clinically significant in terms of seeing an effect on mediators of cancer risk but mean weight loss at 12 months in this group was 5% and no significant effect on endometrial proliferation or on bloods was seen. This may mean that in terms of endometrial cancer risk clinically significant weight loss is at a higher level than 5%, but also a great deal of variation was seen within the small non-surgical group (standard deviation 4.7, n=6) so in larger numbers the effects may become significant. It may be that a greater degree of weight loss is required to see an endometrial effect than is required to see an effect on circulating markers. Of the 6 non-surgical women who remained under follow up, at 2 months 3 had lost weight and at 12 months 5 had lost weight (-3 to -12.4%). No sustained reduction in Ki-67 was seen in any but one patient who lost 12.4% of total body weight over 12 months, in whom Ki-67 was 19.5%, 10.9% and 11.8% at baseline, 2 and 12 months respectively, however this patient went from having proliferative endometrium at baseline to postmenopausal endometrium at 12 months.

Immunohistochemistry studies on the Argenta cohort demonstrated no significant change in proliferation 12 months after bariatric surgery (198). There are several key differences between their study and ours that may explain this. Firstly, their median Ki-67 scores were lower to begin with (10% at baseline and 6% 12 months post-surgery vs. 29% and 13%). This may reflect their higher proportion of participants using COC or LNG-IUS treatment, making for a blander, more suppressed endometrium. The lower level of endometrial abnormality may also account for this variation, and indeed it may mean that Ki-67 is an indicator of risk of future endometrial cancer or AEH. If LNG-IUS and COC reduce risk of endometrial cancer and are associated with endometrium with lower median Ki-67 scores than a cohort where use of these treatment modalities was not a feature, perhaps higher Ki-67 scores reflect a high-risk endometrium.

Of course, there are other possible explanations for variation in Ki-67 scores in two groups where samples were processed and assessed differently. Rather than whole slides the Ki-67 was assessed on tissue microarrays by Argenta et al (198), which have issues with loss of samples in the preparation process or tissue being missed when cores are taken from the wax blocks. From experience the Pipelle biopsies of asymptomatic women are often scanty, and by virtue of how Pipelle samples are obtained tend to be disrupted. This may make them less suitable samples for TMA than solid biopsies. Members of our group have found TMA's to be inferior to whole slides in as yet unpublished work comparing different methods of processing and scoring endometrial tumours for Ki-67, as they lack reproducibility and have poorer inter-observer correlation.

6.4.4 Signal transduction pathways

Endometrial cancer has been shown to exhibit more alterations of the PI3K/AKT/mTOR pathway than other tumour groups, and its alteration is thought to happen early in the carcinogenesis process. AKT inhibits apoptosis and mTOR coordinates protein translation, cell growth and persistence. Activation of this pathway is also increased in overweight and obese women with normal endometrium, and as a consequence so is proliferation.

Bariatric surgery was associated with reduced phosphorylation of AKT, with a median difference of -45 at 2 months and -35 at 12 months (p=0.027 and p=0.0015 respectively). When the subgroup of cycle matched biopsies were analysed, this association was no longer statistically significant, however there is no evidence to suggest that pAKT is subject to the same variation by phase of menstrual cycle as Ki-67, and this subgroup selection may be false, but one would expect that activation of pro-proliferative signal transduction pathways would increase in proliferative phase endometrium. No significant change was seen in the non-surgical group.

These findings can be explained by a variety of effects demonstrated in this weight loss study. PI3K is activated by overexpression of ERα, and we demonstrate a reduction of ERα with weight loss. PI3K is inactivated by AMPK, and AMPK is frequently inactivated in obesity and in insulin resistance in a process that is thought to involve the increased leptin levels both situations display. AMPK is activated by adiponectin, which is found in lower levels in obesity and in endometrial cancer. A reduction in leptin levels and increase in adiponectin level were demonstrated after bariatric surgery. The PI3K pathway is stimulated by insulin and vast reductions in insulin levels and resistance were seen soon after bariatric surgery. The PI3K pathway is also stimulated by oestrogen and bariatric surgery was shown to increase SHBG levels, which may have reduced unbound oestrogen fractions, although no significant change was demonstrated in oestrogen levels after bariatric surgery. In postmenopausal women an indirect action of insulin is to increase bioavailable oestrogen through decreasing hepatic SHBG synthesis. In premenopausal women insulin increases endometrial cancer risk by excess stimulation of ovarian androgen synthesis, leading to chronic anovulation, which in the presence of normal oestradiol levels is a well established risk factor for endometrial cancer (159)

No statistically significant effect of weight loss on pERK 1,2 expression in either the stroma or the glands was seen in either group. It has been suggested that the PI3K pathway negatively regulates the Ras/MAPK/ERK 1,2 pathway (221) which may be why phosphorylation of ERK 1,2 does not mirror pAKT with weight loss. Many of the same factors that drive the PI3K pathway drive the ERK 1,2 pathway, and it was expected

that the expression of pERK 1,2 would be down-regulated with weight loss as a result of this, so it is not entirely clear what explains this finding. The evidence to date of differential activation of this pathway depending upon body habitus is from a small study by Villavicencio et al, where on Western Blot pERK was progressively up-regulated in endometrium from overweight and obese women (102).

6.4.5 Hormone receptor expression

Bariatric surgery was associated with statistically significant serial reductions in ERα and PR expression at both 2 and 12 months follow up. No significant changes were noted in the non-surgical group.

PR is induced by ERα therefore ER expression correlates with PR expression, and both are increased in obesity compared with normal weight women. Data on the effect of weight loss on oestrogen and progesterone receptor expression are very limited. A previous study did not demonstrate a significant difference in oestrogen or progesterone expression after bariatric surgery. This may be because this was a highly selected cohort with low detection of endometrial abnormalities, or it may be that 30% use of exogenous hormones resulted in suppressed endometrium. This study did demonstrate higher ERα and PR expression in non-atypical hyperplasia than normal endometrium, and significant reductions in receptor expression with weight loss in non-atypical hyperplasia (198).

6.5 STRENGTHS OF THE STUDY

This study includes the largest cohort of endometrial samples from asymptomatic morbidly obese women to date, to our knowledge, and provides important prevalence data in this high-risk group. It is the only study of this nature so far reported in the UK, and the only study where insulin resistance and coexistent diabetes have been explored in this detail. It also seems to be the only study of this nature where efforts have been made to control the menstrual cycle timing of follow up biopsies to aid interpretation of results. Similar studies have not performed multiple follow up samples, Argenta et al re-biopsied once at 12 months post weight loss surgery.

The advantage of having sampled at 2 and 12 months is a greater opportunity to assess effects seen early in the weight loss process when rapid changes in glucose metabolism have occurred and weight is actively being lost and at a stage when maximal or near maximal weight loss has been achieved and weight loss has slowed or plateaued. Having a repeat biopsy at 2 months also helps to assess whether any endometrial abnormalities were "missed" at baseline biopsy, when evaluating Pipelle as a potential screening tool and the intervals that could be adopted. In this context we saw the development of two new endometrial abnormalities at 12 months but no additional cases at 2 months, which would suggest that yearly screening intervals could be adequate if screening of high risk women was to be implemented.

The study by Argenta et al had stricter inclusion and exclusion criteria than ours and as a result had a more highly selected group, and 29% of their participants were using therapies known to protect against endometrial cancer. This is likely to explain the low level of abnormality they detected. Their reason for this was to enable them to obtain ethical approval to analyse the baseline biopsies after the 12 month biopsy rather than upfront. In not excluding women with abnormal bleeding and excluding women using the LNG-IUS we had greater sensitivity for detecting endometrial abnormality, and abnormalities could be treated or monitored as required, with less progestagen effect skewing the results.

No other study has assessed bloods alongside endometrial samples in this way. Modesitt et al analysed changes in 'global metabolomics' in blood samples from 20 of their cohort, but the follow up samples were not taken at a specified time. They also were not reported to be fasted samples in follow up, which will affect the interpretation of the effect of bariatric surgery.

A further strength of this study was the involvement of strong local pathology input. Histopathological analysis of endometrial biopsies was performed by consultant histopathologists specialising in gynaecological pathology in a tertiary cancer centre teaching hospital, and abnormal results were verified by a second consultant gynaecological histopathologist. Immunohistochemistry was performed on whole slides rather than TMAs, which minimised the loss of samples in the preparation period and also enabled more thorough scoring of the immunostains than a TMA would permit. Scoring methods were decided up front and slides were scored by two independent assessors, blinded to mode of weight loss and time point of sample. Scores that varied by > 10% were rescored and ratified jointly.

6.6 Limitations of the study

6.6.1 Critical review of methodology

This study was designed as a prospective cohort study as it was first and foremost an exploratory study to provide pilot data into the effects of obesity and weight loss on the endometrium and on circulating markers that have been implicated in the process of endometrial carcinogenesis. It would not have been possible to randomise to surgical weight loss or non-surgical weight loss, or to blind the patients or researchers as to which had taken place.

6.6.2 Sample size

Initial sample size calculation estimated that 51 paired endometrial biopsies would be required to show a 5% change in Ki-67 with weight loss with 80% power. This number was not achieved due to greater than expected loss to follow up between baseline assessment and 2-month assessment. 47 pairs were available for analysis at 2 months and 34 pairs at 12 months.

There was a lack of comparable data on which to power the study, and the stated 5% difference in Ki-67 was the difference that was seen in a study comparing the endometrium of overweight women with that of obese women, rather than a weight loss related change (102). The obese cohort in the Villavicencio et al study also had markedly lower BMI's than the obese women in our study. In fact, our results suggest the change in Ki-67 with weight loss could be more than 10%, so by post-hoc analysis the study would be powered to detect this difference.

The lack of significant change in the non-surgical weight loss group may reflect the lesser degree of weight loss they demonstrated, however the group was small due to unanticipated difficulties in their recruitment. The results from the non-surgical group may have been more informative had the group been larger, however it does not invalidate the results from the surgical group. It is clear from the extent of variation seen in hormones and endometrium, and from the effectiveness of pairing between samples from the same participant, that the surgical participants were their own controls and it would have been very difficult to construct a study where they had matched controls that did not undergo surgical weight loss. What is more, surgical

weight loss and non-surgical weight loss are not just different points on a linear scale, the mechanisms are frequently very different and therefore the effects on end organs and circulating markers very different too.

The non-surgical group was very small and has added little to the conclusions that can be drawn from the study. Further studies intending to recruit cohorts of similar women should aim to recruit more widely, from multiple clinics, and have the capacity to devote more time and manpower to this recruitment.

6.6.3 Scoring and measures

As with all measures there was an expected degree of interassay variation. In an attempt to minimise the effect of this all ELISA's were run in pairs or triplets in the same batch and all assays performed in the Trust laboratory were subject to rigorous daily QC protocols. Similar variation was inevitable in the staining and scoring of immunohistochemistry. Staining was performed in large batches using the Ventana automated system, as previously described, as it was felt this would minimise the variation that would be expected with manual staining.

A variety of assays were used to assess glucose metabolism and insulin resistance, including fasting glucose, fasting insulin, HbA1c and HOMA-IR. HbA1c was a substantial amendment to the protocol to give a more long term look at insulin resistance as it was recognised that short term measures of insulin resistance were more likely to be affected by external factors such as intraoperative dexamethasone and duration of fast which it became evident was difficult to control (because post op bariatric surgery patients are advised to eat and drink at regular intervals, and pre-op they had been fasted for more than 9 hours).

HOMA-IR, although not the gold standard test to assess insulin resistance, seemed to be the most appropriate option. The euglycaemic clamp test is described as the gold standard but it was felt inappropriately invasive to use for the purposes of this study. A 70% concordance has been reported between HOMA-IR and the euglycaemic clamp, although it may be less than this in obese patients.

Blood was taken for oestradiol, FSH and LH as part of the baseline and follow up assessments, but true trough levels of these hormones should be taken early in the menstrual cycle. Otherwise in premenopausal women there is too much cyclical variation for the results to be meaningfully interpreted in the context of this study. The numbers of postmenopausal women recruited were too small to perform meaningful subgroup analyses on changes in oestradiol and endometrium with weight loss in postmenopausal women alone, but with greater numbers this may have been feasible.

As the primary outcome measure was endometrial the timing of the follow up assessments was based on standardizing the endometrial biopsies, which was in the proliferative phase of the menstrual cycle. In terms of the timing of endometrial biopsies, more than half were confirmed to be proliferative phase endometrium.

6.6.4 Ascertainment bias

The estimated baseline prevalence detected in this study may be an overestimate of the prevalence in the general obese population, and endometrial abnormality may be over represented in bariatric surgery cohorts. There are two main reasons why this may be the case.

Diabetes is likely to be over represented in bariatric surgery clinics as it frequently forms part of the basis for referral for bariatric surgery, in an effort to improve glucose control or reduce diabetes related morbidity. The prevalence of diabetes in age and BMI matched populations who are not referred for bariatric surgery may well be lower. As a result patients attending bariatric surgery clinics are likely to be at higher risk of endometrial cancer than the morbidly obese population as a whole.

Furthermore, the majority of patients referred to bariatric surgery clinics are peri-menopausal women who may be more likely to have an undiagnosed endometrial abnormality than postmenopausal women who present with postmenopausal bleeding and are investigated, or younger women who have not yet developed endometrial hyperplasia.

6.7 Wider implications of the research project

6.7.1 The size of the problem

Setiawan et al (9) have reported that the aetiology of type 2 tumours might not be entirely oestrogen independent as was previously assumed, and may be associated with risk factors classically associated with type 1 cancers such as COCP use, diabetes, parity and age at menopause. Whilst we already know that 80% of endometrial cancers are endometrioid tumours and that up to 80% of type 1 endometrial cancer may be obesity associated (123), on this basis the obesity problem may be worse than previously thought if it also contributes to the development of non-endometrioid histological subtypes. Molecular profiling studies have suggested that there may be more effective mechanisms of stratifying risk and targeting adjuvant treatment than current methods (10).

6.7.2 Prevention and undiagnosed insulin resistance

In view of our prevalence data these are clearly a high-risk cohort. They are largely unable to access the primary chemo preventative agent in endometrial cancer, combined oral contraception, because BMI > 35kg/m^2 is a UKMEC 3 recommendation (where the risks are likely to outweigh the benefits). Alternative methods of prevention must be considered and investigated in high-risk obese women, such as the LNG-IUS and weight loss, which have been proven to reduce the risk of endometrial cancer. In the absence of significant weight loss improving insulin resistance may reduce the risk of endometrial cancer and hypoglycaemic agents such as metformin may prove effective in this regard. Metformin has also been shown to reduce Ki-67 in endometrial cancers in short term pre-surgical window studies, which may suggest a role for modifying insulin resistance in the adjuvant treatment or prevention of endometrial cancer (123). 33% of women diagnosed with AEH or endometrial cancer in the study were taking metformin, although notably all had HbA1c > 60mmol/mol at diagnosis. Additionally the diagnosis of an obesity related pre-cancer should prompt discussion with patients about the importance of weight loss and offering of a bariatric referral, and should be included in guidelines for referral criteria for bariatric surgery.

Studies have demonstrated a reduction in endometrial cancer risk (77-81% reduction in risk of uterine malignancy at 12.5 years) and risk of death from cancer (HR 0.58, 95 CI% 0.44-0.77) after bariatric surgery but it remains to be seen whether non-surgical weight loss has similar impact. Weight loss surgery is not something that is economically feasible on a population scale, but increased use together with better targeting and awareness of its benefits should be explored and encouraged. Women attending bariatric surgery consultations should be routinely questioned about any history of menstrual dysfunction or abnormal bleeding and any deviation from the norm should prompt further gynaecological assessment.

Given what we know about the effect of insulin resistance on endometrial cancer risk, together with the high levels of undiagnosed diabetes and insulin resistance and the fact that 7 out of 10 women diagnosed with endometrial cancer or AEH in the study were diabetic, screening of morbidly obese women for diabetes and insulin resistance should be encouraged within primary care. A diagnosis of insulin resistance should be a red flag of future cancer risk in morbidly obese individuals, which warrants patient education, and clinicians should have a lower threshold for suspecting cancer in this scenario. Diagnosis of an obesity related cancer in a non-diabetic patient should prompt testing for diabetes.

6.7.3 Diagnosis of endometrial cancer and pre-cancer in high risk groups

In our cohort of 80 women we detected 10 cases of endometrial cancer and pre-cancer, a prevalence of 12.5%, far in excess of the risk in the general population. Such a high prevalence predictably raises the question of screening of high risk groups for occult endometrial lesions, especially when we consider that 80% of screen detected endometrial cancers found in the nested cohort study of UKCTOCS were asymptomatic (54). While we do not have evidence of a survival benefit associated with screening for endometrial cancer, it is generally perceived to be beneficial to diagnose cancer at an early stage and there is a growing body of evidence to suggest that atypical hyperplasia, and possibly well-differentiated endometrial cancers, could be managed non-surgically with the Mirena LNG-IUS. Earlier detection of AEH could facilitate the

avoidance of hysterectomy in a group of women in whom surgery is associated with an increased level of risk due to their obesity and comorbidity.

If high-risk women underwent endometrial screening, inevitably more cases of AEH and endometrial cancer would be detected and many of these women will be young and may wish to retain their fertility. In our study we detected endometrial cancer in a 24 year old patient who failed to respond to 12 months of LNG-IUS and had a hysterectomy, and a 35 year old who had AEH with possible invasive disease who had a complete response to 6 months of LNG-IUS and no relapse at 2 years. Standard surgical treatment of AEH and endometrial cancer may no longer be acceptable to patients such as these if a convincing alternative can be found. For this reason we need to know if weight loss or other strategies have the ability to reverse the changes associated with AEH and well differentiated endometrial cancer, and it is hoped that the Australian led fEMMe trial will go some way to answering these questions.

There is also a need to develop mechanisms of identifying those patients with AEH who are most likely to have concurrent endometrial cancer and those with AEH and endometrial cancer who are unlikely to respond to progesterone treatment. Stathmin has been suggested as having potential to discriminate those women at high risk of harbouring occult endometrial cancer alongside their AEH, and will be pursued as part of further work on study samples going forward.

Increasingly endometrial cancer is being diagnosed in younger women yet the NICE suspected cancer referral guideline does not reflect this. It only advises a suspected cancer referral for women over 55 years with concerning symptoms for endometrial cancer (74). Clearly our experience would suggest that approach to be inadequate in women with this level of risk, as all of the 4 women diagnosed with endometrial cancer were aged 55 or under.

6.7.4 Is there an argument for screening or targeted prevention of high-risk women?

In the general population there is a low prevalence of occult endometrial abnormality and no evidence that screening reduces mortality from endometrial cancer. In selected high-risk populations the number need to screen may however be in the ballpark of

existing screening programmes like faecal occult blood testing or cervical screening (NNS 400-1500). TVUS in asymptomatic premenopausal women is fraught with difficulty in the interpretation of endometrial thickness, and endometrial biopsy can be uncomfortable. It is unknown if endometrial biopsy is perceived to be an acceptable test by women and there is a need for further research into less invasive and more reliable screening techniques for endometrial cancer in premenopausal women.

Whilst recruitment in this study was good (70% of eligible patients) there was a significant loss to follow up rate and there may be a variety of reasons for this. Data collection surrounding the acceptability of endometrial sampling was outwith the remit of this study, and beyond the level of data collection that could have been realistically achieved. It is worthy of comment that perhaps the best indicator of the loss to follow up due to the test being deemed unacceptable to patients is the number of patients who refused a further endometrial biopsy after undergoing a biopsy whilst awake (not anaesthetised at baseline). This was the case in 13 of the 47 women who underwent a 2-month biopsy, but declined a 12-month biopsy (27%). Future studies must account for this loss to follow up rate when designing their methodology.

Concerns have also been voiced as to the feasibility of endometrial sampling as a screening technique in obese women or in asymptomatic women, and indeed studies have reported rates of unsuccessful biopsies in morbidly obese women approaching 40% with some suggesting that the absence of pathology is partly responsible for this (68). This study should provide reassurance that with 4.8% of baseline biopsies unsuccessful due to cervical stenosis and 2.5% of baseline biopsies being inadequate for histopathological assessment, in what were predominantly asymptomatic morbidly obese women, the technique is feasible in similar cohorts of women.

Women with BMI ≥42kg/m^2 have a relative risk of endometrial cancer of 9.11 compared with women of normal BMI. Diabetes has been shown to increase the risk of endometrial cancer by a relative risk of 2.1. Cancer risk is also increased in insulin resistance. Women with PCOS are estimated to have a lifetime risk of endometrial cancer of approximately 9% (independent of BMI), which is similar to that seen in

morbidly obese women. Women who are obese and have PCOS and insulin resistance have a hugely elevated risk of endometrial cancer. Rosato et al (25) estimated the odds ratio for developing endometrial cancer to be as high as 8.4 in such women. Atypical hyperplasia in postmenopausal women has been shown to increase the relative risk of endometrial cancer by a factor of 14, and up to 50% of women with AEH may have coexistent endometrial cancer. As such, AEH is a convincing precursor lesion to target in screening tests.

6.7.5 Weight loss to treat endometrial abnormality or as adjuvant treatment

Resolution of four cases of AEH without treatment and the favourable outcomes and long-term control of two patients with AEH treated with LNG-IUS raise the possibility that risk factor modification promotes normalisation of the endometrium, possibly via the reduction in proliferation and PI3K activation demonstrated in our study. Weight loss must be further investigated as a mechanism to treat endometrial abnormality or as adjuvant treatment to reduce recurrence risk and all-cause mortality after an endometrial cancer diagnosis. A randomised controlled trial would be advantageous at this juncture.

Weight loss surgery is the only weight loss treatment proven to bring about more than 15% weight loss over a 15-year period, and to achieve high levels of weight loss without surgery requires an intensive long-term programme. This needs significant investment as well as motivated and compliant patients, although to an extent so does weight loss surgery. Weight regain is greater with non-surgical weight loss than after bariatric surgery. No significant effects on the endometrium or on cancer related biomarkers in the blood were seen in our study, where non-surgical patients lost 5% total body weight at 12 months, but this does not preclude the potential of non-surgical weight loss in this scenario as other studies have shown improvements in cancer related biomarkers after non-surgical weight loss. In prostate cancer a pre-surgical window study of intensive non-surgical weight loss versus no intervention has shown this to be a feasible approach, and have demonstrated excellent recruitment and protocol adherence in their participants. They intend to assess the effects of pre-surgical weight loss on tumour biology (222).

6.7.6 Obesity as a barrier to care

An alarming 44% of women recruited had never had cervical screening or were overdue cervical screening. One opportunistically performed cervical cytology test culminated in the diagnosis of a stage Ib1 cervical cancer which was treated with fertility sparing surgery and lymph node assessment. As a specialty there is a need to address the issues of perceived barriers to care which obese patients report stop them from attending for cervical screening, such as feeling as though they are treated with less respect due to their weight, or having experience of equipment in healthcare settings being unable to accommodate them.

If such barriers are an issue in cervical screening they will be an issue in other scenarios as well, and are likely to make obese women less likely to seek help for problems such as menstrual irregularity or abnormal bleeding, which may be an opportunity to detect endometrial abnormalities at an early stage where they may be able to be managed non-surgically rather than when cancer has developed. To overcome these barriers a multifaceted approach is required encompassing investment in equipment and education of healthcare professionals, acknowledgement that morbidly obese patients are not demonstrating the same health seeking behaviours as the general population, and mechanisms of reaching high risk groups and educating them about abnormal symptoms and accessing care.

6.8 FUTURE WORK

The following section represents my plans for future work following on from this study, and will involve the ongoing follow up samples, biobanked tissue and blood and residual wax blocks of FFPE endometrium.

My priority is to optimise the PTEN immunohistochemistry protocol and assess PTEN before and after weight loss in the endometrial samples. The consensus is that immunohistochemistry is a better way of assessing functional loss of PTEN, as opposed to mutation analyses which may not detect PTEN loss due to promoter methylation or epigenetic changes (212). A paper by Lacey et al reports their experience of PTEN in Pipelle endometrial samples and describes incubating in primary antibody overnight at 4°C at 1:300, which is how I intend to proceed in attempts to optimise this antibody on my samples (149). Recently a study has reported LNG-IUS reversing PTEN null glands in atypical hyperplasia and it will be fascinating to see if similar findings occur with weight loss (146). One would not expect weight loss to alter the mutation status, so if this occurred it would be assumed that weight loss reversed epigenetic changes or other variations that cause loss of PTEN function in endometrium.

While Ki-67 is present in all replicating phases of the cell cycle it is less specific than pH3 which is a mitosis specific marker, only expressed during mitosis (M phase) (223). Ki-67 was selected as the primary outcome measure as there is a great deal of experience of it in other studies. Immunohistochemistry for pH3 will now be performed on the endometrial samples to validate the findings of the Ki-67 analyses, and add support to conclusions that can be drawn on the effects of weight loss on endometrial proliferation.

Stathmin is an oncoprotein that is a surrogate marker for PI3K and PTEN dysregulation in breast and endometrial cancer, which has also been said to be a predictor of coexistent malignancy in AEH. There is a lack of data on the effects of obesity on Stathmin expression, although obesity has well documented effects upon PTEN and the PI3K pathway. It is also unclear how Stathmin is expressed in normal endometrium. Recent work has described interesting discrete differences between obese and non-

obese women with type 1 endometrial cancer, where gene expression tests demonstrated overexpression of Stathmin in non-obese endometrial cancer patients and overexpressed K-ras and pro-inflammatory genesets in obese endometrial cancer patients. Mutant K-ras facilitates inflammatory pathways in endometrial cancer, and does not interact with PI3K/ERK 1,2 pathways, but KRAS mutations in type 1 endometrial cancer are less common than PI3K and PTEN dysregulation, and PI3K is upregulated in obesity.

Plasma samples pre and post weight loss have been sent to a collaborator in the US for metabolomic and proteomic analyses, and these data will be analysed once this has been completed.

7. CONCLUSION

Despite advances in cancer diagnosis and treatment, the incidence and number of deaths from endometrial cancer continue to rise. More than 2000 British women die of endometrial cancer each year, and while a variety of factors have been attributed to this, by far the biggest culprit seems to be obesity. Obesity has a non-linear dose dependent effect upon endometrial cancer risk and lifetime risk in morbidly obese women may be as high as 20% (69), a stark contrast to the 2-3% seen in the general population.

We find ourselves in the midst of an obesity epidemic. Worldwide the prevalence of obesity has doubled in the last three decades, and one in four UK adults are obese. While this trend continues it is likely that the incidence of endometrial cancer, and in all probability its overall mortality, will continue to rise also.

7.1 KEY FINDINGS OF THE STUDY

The study has demonstrated a baseline prevalence of endometrial cancer and atypical endometrial hyperplasia of 12.5% in morbidly obese women seeking bariatric surgery or non-surgical weight management, none of whom would have been detected using current referral guidelines for suspected cancers. In doing so we also demonstrated that endometrial sampling is technically possible in morbidly obese women with a failure rate due to cervical stenosis or inadequate biopsy of 7.3%. Poor uptake of cervical screening was observed in the cohort (56%), which would be relevant if considering implementing targeted endometrial cancer screening in high-risk groups as it may translate to poor uptake of endometrial screening also, or justify combining the two screening procedures at one visit.

Almost 80% of the women participating in the study were diabetic or insulin resistant, with 5% of women having undiagnosed diabetes and 41% potentially having undiagnosed insulin resistance based upon the HOMA-IR as a model assessment of insulin resistance. The importance of this is cemented by the observation that of the women diagnosed with occult endometrial cancer or AEH at baseline, 50% were diabetic and a further 20% had undiagnosed diabetes. Of the 3 women found to have undiagnosed diabetes, 2 of them had endometrial cancer or AEH. On comparing the

women with normal endometrium to women with endometrial cancer or AEH, baseline HbA1c was significantly higher in women with an abnormality than in women with normal endometrium. There was no significant difference seen in age or BMI. Overall BMI was shown to correlate with leptin, SHBG, FAI, HOMA-IR, adiponectin, hsCRP, oestradiol and pAKT levels.

Bariatric surgery induced weight loss was associated with statistically significant improvements in insulin resistance, inflammatory markers, adipokines, SHBG and FAI. Most improvements were evident by 2 months post weight loss surgery, and persisted at 12 months. No statistically significant change was observed in oestradiol or progesterone, although the timing of blood sampling and the high proportion of pre-menopausal women in the study is likely to have confounded this observation. Statistically significant increases in FSH and LH were observed, but again these were not baseline day 2-5 samples and so are subject to a great deal of variation.

Statistically significant reductions in Ki-67 were observed at both 2 and 12 months post bariatric surgery, and on subgroup analysis when excluding endometrial samples that were not matched for menstrual cycle timing these reductions held true. The more weight women lost the greater reduction in Ki-67 was seen. Significant decreases were also seen in expression of pAKT at both time points and in ERα and PR expression. Improvements in menstrual function were seen after bariatric surgery, particularly in women who had AEH at baseline. Of six women diagnosed with AEH three had complete resolution of disease by 2 months post bariatric surgery, and two were treated with LNG-IUS for 6 months and had good outcomes in terms of complete response and no relapse at 2 years. Ki-67 correlated weakly with oestradiol, pAKT expression, HOMA-IR, FAI adiponectin and leptin levels.

7.2 Possible mechanisms

Obesity and insulin resistance are a toxic combination which increase the promotion of pro-proliferative signal transduction pathways in the endometrium, as well as favouring amenorrhoea or oligomenorrhoea which facilitate the accumulation of tumourigenic mutations.

Bariatric surgery has well documented positive effects on glucose metabolism and is a highly effective mechanism of weight loss. It seems probable that downregulation of the pAKT pathway leads to reduced endometrial proliferation, and this is likely to be induced by lower insulin levels and supported by increasing adiponectin and decreasing leptin levels, as these changes were all evident by 2 months after bariatric surgery.

Increasing SHBG levels after bariatric surgery are due to reductions in insulin levels, which inhibits hepatic synthesis of SHBG. It is to be expected that increasing SHBG will decrease the unbound oestrogen and androgens in the circulation, although we have only demonstrated reduction in androgens in this study. Greater numbers of postmenopausal participants and sampling of oestradiol levels early in the menstrual cycle of pre-menopausal participants may provide a different result.

7.3 Implications of the study

The baseline prevalence of undiagnosed endometrial cancer and AEH in morbidly obese women is likely to be far higher than previously appreciated. Further work is needed on a larger scale to confirm this, but would support the argument for targeted screening of high-risk women. Undiagnosed insulin resistance in obese women would also appear to be common, and may serve to refine the selection of groups for screening. Bariatric surgery has pronounced effects on endometrial proliferation and on the PI3K pathway, which is a key aspect of type 1 endometrial carcinogenesis, as well as on hormone receptor expression. It also brings about significant changes in insulin resistance, inflammation, adipokines and reproductive hormones providing convincing explanations for the alterations in endometrial micro-environment that have been demonstrated.

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10. APPENDICES

10.1 RESEARCH ETHICS COMMITTEE APPROVAL

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Health Research Authority

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23 January 2012

Dr Emma Crosbie
NIHR Clinical Lecturer in Gynaecological Oncology
University of Manchester
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M13 9WL

Dear Dr Crosbie

Study title:	**The impact of weight loss on the malignant potential of endometrium**
REC reference:	**12/NW/0050**
Protocol number:	**Bariatric01**

Thank you for your letter of 22 January 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair. .

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
GP/Consultant Information Sheets	1.0	01 December 2011
Investigator CV	Dr E Crosbie	01 December 2011
Participant Consent Form	1.0	12 December 2011
Participant Consent Form	1- Revised	12 December 2011
Participant Information Sheet: Patient Information Sheet	1.0	12 December 2011
Participant Information Sheet	2	22 January 2012
Protocol	1.0	17 December 2011
REC application	3.4	30 December 2011
Referees or other scientific critique report		17 November 2011
Response to Request for Further Information		22 January 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/NW/0050	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Dr Lisa Booth
Chair

Email: carol.ebenezer@northwest.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Dr Lynne Webster*

The impact of weight loss on the malignant potential of endometrium

Study Protocol

Version: 3.0

Date: 4th July 2013

Sponsor: Central Manchester University Hospitals NHS Foundation Trust (CMFT)

Authorised by:			
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Signature		Date	
Name	Prof Henry Kitchener	Role	Co-investigator
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Mission Statement

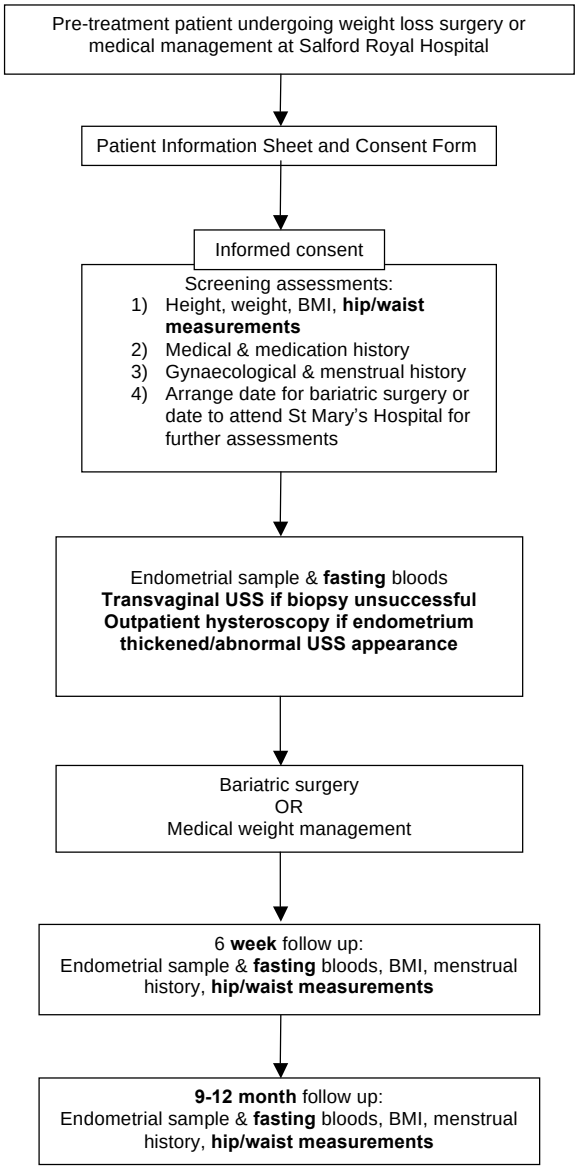
This study will adhere to the conditions and principles which apply to all clinical studies as outlined in the EU Directive 2001/20/EC and Good Clinical Practice. It will be conducted in concordance with the protocol, the Data Protection Act 1998, sponsors' Standard Operating Procedures and other regulatory requirements as appropriate.

Table of Contents

Abbreviations and Glossary

AE	Adverse Event
AKT	A serine / threonine protein kinase that plays a key role in cell proliferation & apoptosis
AMPK	5' AMP-activated protein kinase, an enzyme involved in cellular energy homeostasis
ASR	Annual Safety Report
BMI	Body Mass Index
Caspase 3	Mediator of apoptosis
CMFT	Central Manchester Foundation Trust
C Peptide	A by-product of insulin production
CRF	Case Report Form
CTCAE	Common Terminology Criteria for Adverse Events
DNA	Deoxyribonucleic Acid
ERK 1, 2	Extra-cellular signal regulated kinases 1, 2; signal transduction molecules
FIGO	International Federation of Gynaecology and Obstetrics
FSH	Follicle Stimulating Hormone
GCP	Good Clinical Practice
GP	General Practitioner
H3	Important role in gene expression, marker of proliferation
HOMA	Homeostatic Model Assessment: formula to quantify insulin resistance
HRT	Hormone Replacement Therapy
ICH GCP	International Conference on Harmonisation Good Clinical Practice
IGF	Insulin-like Growth Factor
IGF1R	Insulin-like Growth Factor-1 Receptor
IGFBP	Insulin-like Growth Factor Binding Protein
IHC	Immunohistochemistry
IUD	Intrauterine device
Ki-67	Marker of proliferation
LH	Luteinising Hormone
LNG IUS	Levonorgestrel releasing intrauterine system
MAPK	A serine / threonine specific protein kinase that regulates gene expression, mitosis, differentiation, proliferation and apoptosis
NHS	National Health Service
P	Phosphate
P13K	Protein kinase which plays an important role in apoptosis
PARP	Poly ADP Ribose Polymerase, a DNA repair enzyme
PIS	Patient Information Sheet
PTEN	Phosphatase and tensin homologue, a tumour suppressor gene
R&D	Research and Development
REC	Research Ethics Committee
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
SAS	Supra-regional Assay Service
SHBG	Sex Hormone Binding Globulin
SUSAR	Suspected Unexpected Serious Adverse Reaction
TV	Transvaginal
UK	United Kingdom
USS	Ultrasound Scan

Study Schema



Study Synopsis

Study title
The impact of weight loss on the malignant potential of endometrium

Objectives
- To determine the prevalence of endometrial abnormalities in women undergoing surgical or medical weight loss management for morbid obesity
- To evaluate the histological impact of weight loss on the endometrium
- To determine the expression of biomarkers of proliferation, apoptosis, tumour suppressor / oncoproteins and signal transduction molecules implicated in endometrial carcinogenesis before and after weight loss
- To determine the effect of weight loss on menstrual function, gonadotrophin levels and circulating oestrogen levels
- To explore candidate mechanisms for endometrial neoplasia in obese women and to generate hypotheses regarding obesity related endometrial carcinogenesis

Number of participants
200

Diagnosis and main inclusion criteria
Patients meeting the following criteria can be included in the study:
- Women undergoing surgical or medical weight loss management at Salford Royal Hospital Joint Bariatric Clinic
- Informed consent
- Aged 18 or over

Main exclusion criteria
- Previous hysterectomy
- LNG-IUS or IUD in situ
- Pregnancy
- Previous endometrial ablation
- Treatment with tamoxifen

Primary Outcome Measure
- Ki-67

Secondary Outcome Measures
- Endometrial markers
  - Histopathology: hyperplasia, neoplasia
  - Phospho-H3 (proliferation)
  - Cleaved Caspase 3, cleaved PARP (apoptosis)
  - ERK1,2, phosphorylated ERK 1,2
  - AKT, phosphorylated AKT
  - PTEN, Strathmin
- Physiological markers
  - Fasting serum glucose & insulin, IGFBP-1 and C-peptide levels
  - SHBG, oestrogen, progesterone, free androgen index, FSH, LH, testosterone, CRP
  - Leptin, adiponectin
- Changes in menstrual function

1.0 Summary

1.1 Lay Summary

Endometrial cancer, or cancer of the lining of the womb, has become the most common cancer of the reproductive tract in British women. Obese women are at increased risk of the disease and are also more likely to die from it. The recent obesity epidemic means that more women than ever before are developing the disease. Endometrial cancer can usually be cured by surgery but for obese or elderly women, surgery may be dangerous. It also renders a woman infertile. There is an urgent need to develop preventative strategies for an increasingly obese female population. Understanding more about the mechanisms linking obesity and endometrial cancer will help the development of these.

Bariatric surgery (reduction of stomach capacity by e.g. gastric banding) results in rapid weight loss: 10-15% excess body weight will be lost in six weeks with resolution of body mass index (BMI) to within the normal/overweight range (BMI 25-30) by 12 months. Non-surgical weight loss management can be effective but produces much slower results. Studying the molecular changes in the endometrium following weight loss may provide insights into the mechanisms that drive endometrial cancer development and facilitate the detection of targets for preventative strategies. .

1.2 Abstract and summary of study design

We will study a prospective cohort of around 100 morbidly obese (BMI>40) women who have been offered bariatric surgery and a similar sized cohort on a non surgical weight loss protocol.

The study will assess the effects of weight loss on apoptotic, proliferative and tumour signal transduction molecules present in the endometrium of obese women, in addition to its' effects on serum markers of insulin resistance and hormonal status. Molecular changes resulting from rapid weight loss are likely to be associated with a reduced risk of endometrial cancer.

We will take a detailed gynaecological history, including a history of menstrual abnormalities, hormonal and contraceptive use. Serial endometrial biopsies and **fasting** blood samples of women at baseline and at **6 weeks and 9-12 months** following bariatric surgery, or alternative weight loss management will be obtained.

Endometrial tissue will be subjected to the following: a) histopathology; b) phosphorylation status of AKT and ERK1, 2 as quantified by the NanoPro1000 Firefly platform; c) immunohistochemistry (IHC) for proliferation (Ki-67, p-H3) and apoptotic (cleaved caspase & cleaved PARP) markers as well as the presence of oncoprotein (strathmin) and tumour suppressor proteins (PTEN) thought to play a role in endometrial carcinogenesis. Serum assays for hormonal status (oestrogen, progesterone, free androgen index, LH, FSH, testosterone and SHBG), insulin/glucose metabolism (glucose, insulin, IGFBP-1 and C-peptide) and CRP, adiponectin and leptin levels will be obtained, all of which have been associated with an altered risk of endometrial carcinogenesis in epidemiological studies.

2.0 Introduction and rationale

2.1 Background

The incidence of endometrial cancer has increased substantially over the past two decades. It is now the commonest gynaecological malignancy in the UK (7,045 cases diagnosed in 2006) (1). Obesity and diabetes are established risk factors for endometrial cancer (2). In a recent standardised meta-analysis of 20 cancer types, the association of BMI with cancer risk ranked highest for endometrial cancer, with a relative risk of 1.59 per 5kg/m^2 incremental increase (3). With the current global obesity epidemic, the attribution of excess weight to endometrial cancer risk across populations may be considerable: in Europe, it has been estimated that excess weight may account for 60% of new endometrial cancer cases each year (4). Furthermore, obese women with endometrial cancer have poorer outcomes since obesity is associated with an increased risk of cancer-related mortality (5).

Surgery (by hysterectomy and removal of both ovaries) is the mainstay of treatment for endometrial cancer, often followed by adjuvant radiotherapy. The increasing incidence of endometrial cancer in morbidly obese women (BMI>40) presents very significant challenges for both surgery and radiation therapy. While 5-year overall survival is approximately 70%, 5-year survival rates drop below 50% in advanced tumours and tumours with high-grade histological characteristics (6). Thus, there is a continuing need to prevent this malignancy and improve treatment strategies.

Most established risk factors (early menarche, late menopause) for endometrial cancer probably act through pathways that reflect greater lifetime exposure to oestrogens (7). Oestrogens bind directly to DNA to increase transcription and interact with several growth factor signalling pathways to favour tumorigenesis (8). Obesity is a hyper-oestrogenic state due to the increased aromatisation of androgenic precursors to oestradiol in adipose tissue (9). The mechanism by which obesity contributes to endometrial carcinogenesis is incompletely understood however, and obesity associated hyperinsulinaemia, high circulating leptin and low circulating adiponectin levels may be important (10-12).

The potential role of obesity associated chronic hyperinsulinaemia is supported by observations that high insulin levels are associated with increased incident endometrial cancer risk (13). Insulin may promote endometrial cancer development through direct mitogenic and anti-apoptotic pathways, decreased synthesis of IGFBP-1 and 2 leading to increased bio-availability of IGF-1, and by increasing oestrogen bio-availability through lowered SHBG levels. Adiponectin, a hormone secreted exclusively by adipocytes, and found at higher levels in lean individuals compared to obese individuals, is known to be inversely related to endometrial cancer risk (11). Leptin is known to promote proliferation in endometrial cancer cell lines via AKT and ERK 1, 2 activation (12-14). At a molecular level, abnormal signalling through the P13K/AKT and MAPK/ERK 1, 2 pathways may contribute to endometrial carcinogenesis (15).

The prevalence of endometrial hyperplasia and cancer may be as high as 12% and 3% respectively in asymptomatic obese postmenopausal women (16). Recent data suggest that when compared with endometrial samples taken from normal weight women, endometrial samples from obese women show increased cellular proliferation (Ki-67, p-H3) and increased phosphorylation of AKT and ERK 1, 2, despite normal histology. Proliferation indices are positively correlated with BMI and serum oestrogen, leptin and insulin levels (17). Thus it appears that the effects of obesity could be measured in the endometrium at a molecular level – even in the absence of endometrial pathology.

Data from cohorts of women who have undergone bariatric surgery (a reduction of stomach capacity by gastric banding) suggest that weight loss can reduce the risk of endometrial carcinogenesis (18, 19). There have been no reports to date of the endometrial response to major weight loss. Studying the effects of obesity and surgically-induced weight loss on the endometrium may provide an insight into the pathways associated with endometrial carcinogenesis and clues as to how key events may be blocked, thereby providing a rational basis for disease prevention.

2.2 Rationale and objectives

The aim of this programme of research is to investigate the biological effects of both obesity and weight loss on the endometrium. To do this, we propose to study the proliferative capacity of the endometrium as well as the endometrial expression of various signal transduction molecules in morbidly obese women at several stages of weight loss treatment.

Our specific objectives are as follows:

- To determine the prevalence of endometrial abnormalities in women undergoing surgical or medical weight loss management for morbid obesity
- To evaluate the histological impact of weight loss on the endometrium
- To determine the expression of biomarkers of proliferation, apoptosis and signal transduction molecules implicated in endometrial carcinogenesis before and after weight loss
- To determine the effect of weight loss on menstrual function, gonadotrophin levels and circulating oestrogen levels
- To explore candidate mechanisms for endometrial neoplasia in obese women and to generate hypotheses regarding obesity related endometrial carcinogenesis

3.0 Recruitment into the Study

Patients will be recruited from the Obesity Clinic at Salford Royal Hospital, Manchester. All **new** female patients will be approached regarding the study. Consultants involved in the clinical care of patients undergoing weight management have been informed about the study and have agreed to facilitate our access to appropriate patients. Research Ethics Committee (REC) approval and Trust R&D approval in both sites has been obtained.

Eligible patients will be seen individually at the Obesity Clinic by Dr Michelle MacKintosh, Clinical Research Fellow, Dr Emma Crosbie, Chief Investigator or another appropriately supervised Clinical Research Fellow to discuss the study. They will be provided with the Patient Information Sheet and informed written consent will be obtained from those who choose to participate. This will be done at a later date if they wish to take time to consider their involvement further.

Patients undergoing surgical management of obesity are listed for either gastric bypass, banding or sleeve gastrectomy. They usually wait 2 to 3 months for their surgery. Other patients in the clinic receive medical management of weight loss under the care of the endocrine team, involving hypocaloric diets with or without medication such as Orlistat.

All patients will be offered endometrial sampling and blood tests, with transvaginal ultrasound scan to measure endometrial thickness if biopsy is unsuccessful and

outpatient hysteroscopy with directed biopsy if scan findings are abnormal. This will be performed in a designated research clinic at St Mary's Hospital, Manchester and reasonable travel expenses will be reimbursed, or in theatre at Salford Royal Hospital. The first visit will be before medical weight loss has commenced and follow up visits will be at 6 weeks and 9-12 months. In the surgical group the baseline samples will be taken on the day of bariatric surgery. Fasting bloods will be taken with routine pre-operative blood tests and the endometrial biopsy will be taken under anaesthetic. Where possible endometrial samples will be taken during the proliferative phase, at or close to day 12 of the menstrual cycle, in pre-menopausal patients. It will be clearly recorded what stage of the menstrual cycle the women are in, to ensure consistency with follow up samples. Where possible all blood tests will be taken in conjunction with routine blood tests to avoid unnecessary additional venepuncture. Follow up samples will be arranged at a time of day which would ensure an equivalent fast to that undergone prior to baseline sampling, i.e. overnight in surgical group or more than 4 hours in the medical group.

4.0 Study entry

All eligible patients will be given a Patient Information Sheet and asked to provide written, informed consent prior to recruitment into the study. Their general practitioner will be informed if they agree to take part.

Patients are eligible if all of the following inclusion criteria are met and none of the exclusion criteria apply:

4.1 Inclusion criteria

- Female aged 18 years or more
- Undergoing bariatric surgery or commencing medical weight management therapy
- Written informed consent to participate in the study

4.2 Exclusion criteria

- Previous hysterectomy
- IUD or LNG-IUS in situ
- Previous endometrial ablation
- Treatment with tamoxifen
- Pregnancy

5.0 Assessments

5.1 Baseline screening assessments and samples

Following written informed consent the following assessments will be conducted and samples taken:

- Medical history (to identify comorbidities or medication use that may need to be accounted for in the analysis, e.g. HRT, diabetes, metformin)
- Height and weight (to calculate BMI)
- Hip and waist measurements (to assess distribution of fat)
- Gynaecological history (to assess menstrual function, stage of cycle)
- Blood for physiological analyses (see below)
- Endometrial biopsy (see below)
- TV USS to measure endometrial thickness (in cases where no endometrial sample obtained)

- Outpatient hysteroscopy (to exclude abnormality if no endometrial sample obtained and endometrium abnormal on ultrasound scan, and facilitate directed biopsy)

In surgical patients whose initial sample was taken under anaesthesia only endometrial sampling and fasting serum analyses will be performed.

5.2 Post treatment assessments and samples
At 6 weeks and 9-12 months post-surgery or post-initiation of medical treatment these will all be repeated. This will take place at a designated research clinic at St Mary's Hospital, Manchester, or if patients are unable to travel to St Mary's at the outpatients clinic at Salford Royal Hospital when attending weight management follow up appointments.

5.3 Sample handling and storage
The endometrial biopsies will be taken with an endometrial sampler, or hysteroscopically. The samples will be separated. Half will be snap-frozen liquid nitrogen and transported on dry ice to St Mary's Hospital Gynaecological Oncology Research Laboratories where it will be stored pending analysis. Half will be fixed in formalin, embedded in paraffin and sent to the consultant histopathologist (Dr Rhona McVey) for histopathological analysis. Blood will be collected for the fasting serum analyses. The serum/plasma will be separated from the clotted blood and stored at -80C until required for analysis.

All clinical samples will be stored in the Gynaecological Oncology Laboratory on the 5th floor of St Mary's Hospital, under the supervision of Dr Lynne Hampson, or in the Trust Biobank at St Mary's hospital or at Salford Royal Hospital. All laboratories have a Human Tissue Authority License. Samples will be stored anonymously, with no patient-identifying information on any of the sample tubes or vials. All data created from the analyses of these samples will be handled and stored according to the Data Protection Act 1998.

5.4 Physiological analyses
At baseline and post weight loss, the following will be determined:
- Fasting plasma glucose and serum insulin
  - to calculate HOMA, as an approximation of insulin resistance
- Serum SHBG, oestrogen and progesterone, free androgen index
- Serum gonadotrophins: FSH and LH
- Leptin, adiponectin

The serum gonadotrophins, oestrogen, progesterone, free androgen index, glucose and insulin analyses will be completed in the Clinical Biochemistry Department at CMFT. These analyses are routine clinical analyses for which there are detailed Standard Operating Procedures (SOP) in place. The leptin and adiponectin analyses will be measured using commercially available kits.

5.5 Tissue analyses
In tissue samples taken at baseline and post weight loss (endometrial biopsy), haematoxylin and eosin staining and routine histopathological assessment will be performed. In addition, the following will be determined by immunohistochemistry, Western Blot analysis and NanoPro1000 Firefly analysis:

- Ki-67 and phospho-H3 for cell proliferation;
- Cleaved caspase-3 and cleaved PARP for apoptosis;

- AKT and phosphorylated AKT
- ERK1, 2 and phosphorylated ERK 1,2
- PTEN and strathmin expression

These analyses will be carried out in the Gynaecological Oncology Laboratory at St Mary's Hospital under the supervision of Dr Lynne Hampson or at the Wolfson Molecular Imaging Centre under the supervision of Prof Tony Whetton. These analyses are routine experimental analyses for which there are detailed Standard Operating Procedures (SOP) in place.

6.0 Treatment of participants

The study has the potential for adverse effects on patients, but all procedures are routinely carried out in outpatient clinics across the UK.

6.1 Unexpected findings

If on hysteroscopy or ultrasound scan an unexpected finding is detected, the usual management will apply. The patient and GP will be informed and the patient will be referred to the relevant gynaecology outpatient or rapid access clinic as required. If the biopsy detects endometrial hyperplasia or carcinoma, again the patient and GP will be informed and the patient will be referred to a consultant gynaecologist for further advice/ongoing management. The cases will be discussed between members of the research team on an individual basis to determine whether or not it is appropriate or feasible for the patient to remain in the study, and whether treatment or surveillance are required.

6.2 Complication of procedure

Hysteroscopy and biopsy have the potential for harm to the patients. Before giving their consent to participate they will be fully informed of the risks involved. There is a small risk of uterine perforation and/or infection. In cases where this is suspected the participant will be referred urgently to the on call gynaecology team at St Mary's Hospital, or Salford Royal Hospital, for further investigation and management. The participants will also be made fully aware of the signs and symptoms of possible complications and given contact details and information should they develop any of these.

7.0 Safety reporting

7.1 Adverse events

An adverse event is the appearance or worsening of any undesirable sign, symptom, or medical condition occurring after the study has commenced, even if not considered to be related to the procedures involved. Medical conditions/diseases present before starting the study will only be considered as adverse events if they worsen after the start of the study. Abnormal laboratory values or test results constitute adverse events only if they induce clinical signs or symptoms, are considered clinically significant, or require therapy.

The occurrence of adverse events will be sought by non-directive questioning of the patient during the study. Adverse events also may be detected when they are volunteered by the patient or through physical examination, laboratory test, or other assessment. As far as possible each adverse event will be evaluated to determine:

1. The severity (mild, moderate, severe)
2. Its relationship to the procedure performed
3. Its duration
4. Action taken (no action taken; medication taken; non-drug therapy given; hospitalisation required, surgery required)
5. Whether it is serious, where a serious adverse event (SAE) is defined as one which:

- Is fatal or life-threatening
- Results in persistent or significant disability/incapacity
- Constitutes a congenital anomaly/birth defect
- Requires prolonged hospitalisation (except where it is for routine treatment/monitoring, elective or pre-planned treatment not related to study, for social or respite reasons)
- Is medically significant i.e. defined as an event that jeopardises the patient or may require medical or surgical intervention to prevent one of the above

Unlike routine safety assessments, SAEs are monitored continuously and have special reporting requirements (see below).

All adverse events will be recorded in detail and treated appropriately. Such treatment may include changes in study protocol including possible interruption or discontinuation, changes in the frequency or nature of assessments, hospitalisation, or any other medically required intervention. Once an adverse event is detected it will be followed until its resolution, and assessments will be made at each visit (or more frequently if necessary) of any changes in severity, the suspected relationship to the investigational procedure, the interventions required to treat it, and the outcome.

Evaluation of AEs and SAEs
Seriousness, causality, severity and expectedness will be evaluated for each AE. Cases that are considered serious, possibly, probably or definitely related to study interventions (i.e. serious adverse reactions, SARs) and unexpected (i.e. SUSARs) should be reported as described below.

Assessment of Seriousness
The Investigator should make an assessment of seriousness as defined above (see definitions).

Assessment of Causality
The Investigator must make an assessment of whether the AE/SAE is likely to be related to procedures according to the following definitions. All AEs/SAEs judged as having a reasonable suspected causal relationship (e.g. possibly, probably, definitely) to the study investigation will be considered as ARs/SARs.

Unrelated: an event is not considered to be related to the study procedure
Possibly: although a relationship to the study procedure cannot be completely ruled out, the nature of the event, the underlying disease, concomitant medication or temporal relationship makes other explanations possible.
Probably: the temporal relationship and absence of a more likely explanation suggest the event could be related to the study procedure.
Definitely: The known effects of the study procedure suggest that it is the most likely cause.

Alternative causes such as natural history of the underlying disease, other risk factors and the temporal relationship of the event to the treatment should be considered and investigated.

Assessment of Severity
The Investigator will make an assessment of severity for each AE/SAE and record this on the Adverse Event (AE) Form according to one of the following categories:
Mild: an event that is easily tolerated by the participant, causing minimal discomfort and not interfering with every day activities.
Moderate: an event that is sufficiently discomforting to interfere with normal everyday activities.
Severe: an event that prevents normal everyday activities.

Assessment of expectedness
If an event is judged to be an AR/SAR, the evaluation of expectedness will be made based on knowledge of the intervention and its known complications.

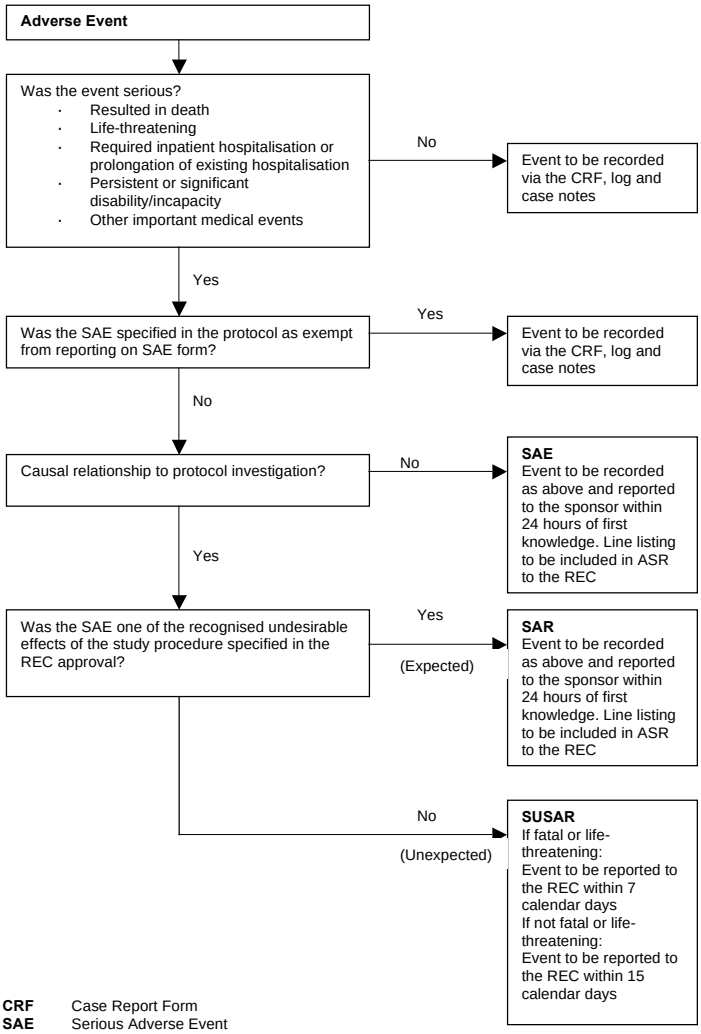
Serious Adverse Event (SAE) reporting
Any SAE will be reported by the Principal Investigator (including a completed SAE form) within 24 hours of first knowledge to the Sponsor. The Principal Investigator will ensure that the patient is appropriately treated. They will also determine whether the SAE is a SUSAR (Suspected Unexpected Serious Adverse Reaction). If it is deemed to be a SUSAR it will be reported immediately to the Sponsor. The Research Ethics Committee will be informed in accordance with Study regulations. An annual safety report will be sent by the Principal Investigator to the Ethics Committee and Sponsor. Completed initial and follow-up Serious Adverse Event forms should be faxed to the sponsor on 0161 276 5766 and addressed 'For the attention of the Quality Manager'. Alternatively, scanned forms can be emailed to adverse.events@cmft.nhs.uk.

Regulatory Reporting Requirements
The sponsor, or their delegate, has a legal responsibility to notify the Research Ethics Committee that approved the study. Fatal or life threatening SUSARs will be reported no later than 7 calendar days, with a further 8 days for follow up information. All other SUSARs will be reported no later than 15 calendar days after the Sponsor is first aware of the reaction.

Follow up procedures
After initially recording an AE or recording and reporting an SAE, the Principal Investigator is required to follow each participant until resolution. Follow up information on an SAE should be reported to the Sponsor. AEs still present in participants at the last study visit should be monitored until resolution of the event or until no longer medically indicated.

Criteria for premature termination of study
These criteria include new safety data, or concerns from safety data (number and nature of SUSARs); or evidence from other studies.

7.2 Flowchart for SAE assessments



CRF Case Report Form
SAE Serious Adverse Event
SAR Serious Adverse Reaction
SUSAR Suspected Unexpected Serious Adverse Reaction
REC Research Ethics Committee

8.0 Study Conduct and Monitoring

8.1 Data Handling

All data will be handled and stored according to the Data Protection Act 1998. CRFs and a study log will be kept in a locked filing cabinet in a locked research office on the 5th floor of St Mary's. Only members of the research team have access to this room. CRFs will be labelled with study specific IDs and no patient-identifying information will be stored in these files. Data will also be stored on a University networked PC in the same office. The data will be stored in a password-restricted database that is only accessible to members of the research team.

8.2 Loss to follow up

If a participant is lost to follow up, the GP **or weight management team** may be contacted to obtain information on the participant's status.

8.3 Participant withdrawal

In consenting to the study, participants are consenting to study procedures, follow-up and data collection. They may withdraw from the study whenever they wish. The GP will be informed. Withdrawal from the study may also be necessary if the responsible physician deems it to be in the best interest of the patient. If a participant explicitly withdraws consent to have any data recorded, their decision must be respected and recorded on the withdrawal form. Details of the withdrawal form should be noted in the participant's records.

8.4 Study end point

The study will end when the final patient undergoes their 9-12 month follow up assessment. An end of study notification will be submitted to the REC and a final report submitted within 12 months. Data analysis will take up to 12 months to complete following the end of the study.

9.0 Informed consent, ethical and regulatory considerations

The protocol will have the favourable opinion of a Research Ethics Committee (REC) as part of the Clinical Study Authorisation. All participants will be informed of the aims of the study, the known possible adverse events, the procedures and possible hazards to which they may be exposed. They will be informed of the strict confidentiality of their data, but that their medical records may be reviewed for study purposes by authorised individuals other than their treating physician.

The participant's consent to take part in the study will be obtained after a full explanation has been given of the procedures involved and the implications of these. The participant's consent will also be sought to notify their GP of their involvement in the study. Participants will be given sufficient time after being given the study Participant Information Sheets (PIS), to consider and discuss participation in the study with friends and family. A contact number will be given to the participant should they wish to discuss any aspect of the study. Following this, the recruiting investigator will determine that the participant is fully informed of the study and their participation is in accordance with UK regulations. Participants will always be asked to sign and date a consent form. One copy will be given to the participant but the original copy will be kept in the study site file and a further copy will be kept with participant's hospital notes.

The right of the participant to refuse to take part in the study without giving reasons must be respected. After the participant has entered the study, the investigator must remain free to give alternative treatment to that specified in the protocol, at any

stage, if he/she feels it to be in the best interest of the participant. However, the reason for doing so will be recorded and the participant will remain within the study for the purpose of follow up and data analysis. Similarly, the participant must remain free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing her further treatment.

The study team will:

- Have in place arrangements to adhere to the principles of Good Clinical Practice (GCP)
- Keep a copy of all essential documents (as defined by GCP) and ensure appropriate archiving and destruction once the study has ended
- Take appropriate urgent safety measures
- Observe reporting requirements to the Ethics Committee as required.

10.0 Sponsorship and indemnity

The sponsor of the study is Central Manchester University Hospitals NHS Foundation Trust. CMFT will be responsible for ensuring that the clinical study is performed in accordance with the following:

- Declaration of Helsinki (South Africa 1996)
- Good Clinical Practice
- Research Governance Framework for Health and Social Care 2001 and subsequent amendments

The Sponsor will ensure the following:

1. That appropriate ethics committee opinion has been sought
2. R&D approval at all sites has been obtained prior to the start of recruitment
3. Amendments have been discussed and reviewed prior to submission for approval
4. That the REC is informed when the study has ended
5. That Annual Safety Reports are submitted to REC within specified timeframes
6. Annual Progress Reports are submitted to the REC within specified timeframes
7. That urgent safety measures are taken as appropriate
8. A report is submitted within 12 months of the end of study notification to the REC

10.1 Negligent harm

CMFT continues to have a duty of care to its patients, whether or not the patient is involved in a clinical study. The Sponsor shall indemnify the Site against claims arising from the negligent acts and/or omissions of the Sponsor or its employees in connection with the Clinical Study (including the design of the Protocol to the extent that the Protocol was designed solely by the Sponsor and the Site has adhered to the approved version of the Protocol) save to the extent that any such claim is the result of negligence on the part of the Site or its employees.

10.2 Data protection

We will act to preserve participant confidentiality and will not disclose or reproduce any information by which participants could be identified. Data will be stored in a secure manner and our studies are registered in accordance with the Data Protection Act 1998.

10.3 Publication policy
Data will be analysed and published as soon as possible. All named investigators will be included in any such publication and the draft manuscript will be approved by the sponsor and all authors prior to submission.

10.4 Finance
The project is funded by CMFT (research fellow salary) and NIHR (project costs).

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Central Manchester University Hospitals NHS
NHS Foundation Trust

Salford Royal NHS
NHS Foundation Trust

Patient Information Sheet

The impact of weight loss on the malignant potential of the endometrium

INVITATION

We would like to invite you to take part in our research study. Before you decide we would like you to under why the research is being done and what it would involve for you. **One of our team will go through information sheet with you and answer any questions you have.** This should take about 15 minutes. T others about the study if you wish. Ask us if there is anything that is not clear.

WHAT IS THE PURPOSE OF THE STUDY?

Endometrial cancer (or cancer of the lining of the womb) is the most common cancer of the reproductive tr British women. If it is caught early, endometrial cancer is curable by surgery. Women who are overweight a increased risk of endometrial cancer. Sometimes endometrial cancer and pre-cancer occur without ca symptoms. This study will screen for abnormalities of the lining of the womb. If we find an abnormality, w arrange appropriate treatment for you. If the lining of the womb is healthy, we would like to test it again afte have lost weight. We want to look at the changes that occur very early on in the cancer pathway and see wh these are affected by weight loss.

WHY HAVE I BEEN CHOSEN?

You have been chosen because you are about to undergo surgery or lifestyle changes to help you lose weight.

DO I HAVE TO TAKE PART?

It is completely up to you to decide if you wish to join the study. If you do not want to take part, this will not affe quality of the medical care you receive.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

The study will be explained by a member of the research team. We will go through this information sheet wit and answer your questions. If you would like to take part we will ask for your written consent. If you would like time to consider taking part in the study we can arrange this. If you consent to take part in the study you will th asked questions regarding your current and past health, medications and lifestyle; this should take no more th minutes.

We will take a blood sample and a sample of the lining of the womb (biopsy). The blood tests will be taken alon routine blood tests where possible to avoid two 'jabs'. If you have surgery we will take the biopsy while yo asleep for your operation. If you do not have surgery, it will be taken at a specialised clinic at St Mary's Ho The biopsy is taken by placing a speculum inside the vagina to allow the doctor to see the neck of the womb cl You may have had this done before if you have had a cervical smear test in the past. A fine straw-like device is placed inside the womb and a sample of the lining of the womb is taken. We may also perform an outp hysteroscopy (camera test to look inside the womb) and internal ultrasound scan if we are unable to take a biop the standard way in order to reassure you and ourselves that everything is normal.

We would like you to come to St Mary's Hospital for repeat blood and biopsy samples 6 and 12 months afte start to lose weight. Reasonable travel expenses to St Mary's Hospital will be reimbursed and where possible will be combined with your routine visits to the weight management clinic.

We want to avoid performing these investigations on women who may be pregnant and ask that contraceptior place in premenopausal participants. The weight management team will have also advised the avoidan pregnancy in the acute weight loss phase. The research team will be asking you at each visit if contracept needed or being used, and what the date of the last menstrual period was. If you feel that there is the possibilit may be pregnant we ask that you let us know. If you or the researchers believe there is a potential for pregna urine pregnancy test will be performed to exclude this before biopsies are performed.

WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART?

You will need to put aside 15 minutes or so to answer questions about your medical health. You will have bloo endometrial biopsies taken before and after weight loss. Blood tests can cause discomfort or bruising. Endom biopsies can be uncomfortable and some patients find intimate examinations embarrassing. Rarely, takir endometrial biopsy or performing an outpatient hysteroscopy can damage or perforate the womb.

WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

If the lining of the womb is healthy, you may find this reassuring. In the unlikely event that we find an abnorı that needs to be treated, we will arrange for you to be seen by a specialist as soon as possible. It is gen accepted that finding cancer and precancerous changes early improves the chances of treatment being succe Most patients taking part in this study will not benefit personally but will contribute to an improved understand how endometrial cancer develops and how we might prevent this from happening in the first place.

WHAT HAPPENS WHEN THE RESEARCH STUDY STOPS?

When the study stops no further samples will be required from you. Your medical treatment and follow u continue as usual. You can, if you wish, be kept informed of the findings of the study.

WHAT WILL HAPPEN IF I DON'T WANT TO CARRY ON IN THE STUDY?

You are free to withdraw from this study at any time without your medical care being affected. If you withdraw the study, we would like to keep all of your samples and to use the data collected up to the point of your withdı However, if you wish, any stored blood or tissue samples already collected will be destroyed.

WHAT IF THERE IS A PROBLEM?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will dc best to answer your questions (0161 701 6912). If you remain unhappy and wish to complain formally, you c this by contacting the patient advice and liaison service (PALS) at the Central Manchester University Hospitals Foundation Trust on 0161 276 8686 or by emailing pals@cmft.nhs.uk. In the event that something does go v and you are harmed during the research and this is due to someone's negligence then you may have grounds legal action for compensation against Central Manchester University Hospitals NHS Foundation Trust but you have to pay your legal costs. The normal National Health Service complaints mechanisms will still be availa you.

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

Your participation in this study is confidential and a record of your participation will be kept with your hc records. Only the study team at the St Mary's Hospital and other healthcare professionals who require acce your medical records will know of your participation. We will also inform your GP of your participation in the stu you are happy for us to do so. Healthcare professionals and scientists with whom we may collaborate outsi Mary's Hospital will not be able to identify you from the data we share.

WILL ANY GENETIC RESEARCH BE DONE?

To try to improve our understanding of the biology of endometrial cancer we may in the future want to look at DNA or RNA (the genetic code that is stored inside your tissues otherwise known as your genes) for muta sequence variation and gene expression differences.

WHAT WILL HAPPEN TO ANY SAMPLES I GIFT?

During your participation in the study, we ask that you gift to us the blood and tissue samples. This means tha blood or tissue left over after the study has been completed may be used in future research without us need ask you again for permission to do so.
All research samples will be collected, processed and stored in the Gynaecological Oncology Research Labo at St Mary's Hospital or the Trust Biobank (Custodian: Dr Emma Crosbie) and will be linked to data from clinical records. Only the study team at St Mary's Hospital will have knowledge of your identity linked to your c record and laboratory samples/data. The study will use donated samples to improve our understanding o biology of endometrial cancer. The results of these investigations are unlikely to have any implications fo personally.

In the future, we would like to keep your samples in a tissue bank linked to your clinical data, for possible u future studies. Where we intend to share samples with researchers at other institutes, including researchers wo for commercial companies, for future studies that cannot yet be specified, the data will be anonymised. The u data/tissue samples in future studies will be subject to additional Research Ethics Committee approval w appropriate.

WHAT WILL HAPPEN TO THE RESULTS OF THIS STUDY?

We aim to widely publish the results of this study at international meetings and in medical journals. We wil provide you with a summary of the results if you wish to receive one.

WHO IS ORGANISING AND FUNDING THIS STUDY?

Dr Emma Crosbie, NIHR Clinical Lecturer in Gynaecological Oncology is the chief investigator. Co-investig include: Professor Henry Kitchener, Professor of Gynaecological Oncology at St Mary's Hospital, Dr Mi MacKintosh, Clinical Research Fellow at St Mary's Hospital, Dr Lynne Hampson, Lecturer in Gynaecol Oncology at St Mary's Hospital and Professor Tony Whetton, Professor of Cancer Cell Biology at the W Molecular Imaging Centre in Manchester.

The University of Manchester funds the study. None of the research team or supervisors will receive payme their involvement. The study is being sponsored by Central Manchester University Hospitals NHS Foundation If you have concerns about the conduct of the study you may wish to contact the Trust Research Office (016 3565) or the Patient Advice & Liaison Service (0161 276 8686).

WHO HAS REVIEWED THE STUDY?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committe protect your interests. This study has been reviewed and given favorable opinion by XXXX.

CONTACT FOR FURTHER INFORMATION?
Dr Michelle MacKintosh (michelle.mackintosh@cmft.nhs.uk) or Dr Emma Crosbie (emma.crosbie@manchester.ac.uk) on 0161 701 6912.

Many thanks for considering taking part in this study.
Yours sincerely,

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10.4 CONSENT FORM

MANCHESTER 1824

The University of Manchester

Central Manchester University Hospitals NHS
NHS Foundation Trust

Salford Royal NHS
NHS Foundation Trust

(

CONSENT'FORM'

Title: The impact of weight loss on the malignant potential of endometrium !

Patient ID for this study:

Principal Investigator: **Tel: 0161 701 6942**

Dr Emma Crosbie
NIHR Clinical Lecturer in Gynaecological Oncology
Academic Unit of Obstetrics & Gynaecology
St Mary's Hospital
(
Thank you for reading the information leaflet about our research project:

1. Patient Information Sheet – version 7.0 11/02/15

If you would like to take part, please read and sign this form ***and initial in the boxes***

1. I have read the attached information sheets on this project (listed above), and have been given a copy to keep. I have been able to ask questions about the project and have had these answered to my satisfaction. ☐
2. I agree to give samples of blood and endometrial tissue for research in this project. I understand how the samples will be collected, that giving samples for this research project is voluntary and that I am free to withdraw my approval for use of these samples at any time without giving a reason and without my medical treatment or legal rights being affected. ☐
3. I give permission for someone from the research team to look at my medical records to get information on my clinical history, investigations and outcome, where relevant to my participation in this study. I also agree to complete a questionnaire and a pictorial menstrual blood loss chart and give permission for the research team to send the same questionnaire and blood loss chart by post for completion at a later date. I understand that the information will be kept confidential. ☐
4. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS trust or regulatory authorities, where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my records, and for them to contact me by telephone/post. ☐
5. I understand that I will not benefit financially if this research leads to the development of a new medical test. ☐

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6. I give permission for the endometrial biopsy specimen taken at St Mary's Hospital, or Salford Royal Hospital to be transferred to the Gynaecological Oncology Research Laboratory at St Mary's Hospital and used for research. ☐

7. I give permission for my General Practitioner to be contacted and informed about my involvement in this trial. ☐

8. **Consent for storage and use in possible future research projects**
I agree that the samples I have given and the information gathered about me can be stored in the Gynaecological Oncology Research Laboratory at St Mary's Hospital for possible use in future projects. I understand that some of these projects may be carried out by researchers other than those who ran the first project. I understand that I will not be identifiable from any of the stored samples or from the clinical information collected about me, which will be fully anonymised. ☐

9. **Consent for research**
I consent to take part in this study. ☐

Name of Patient	**Date**	**Signature**
Name of Person Taking Consent (if different to researcher)	**Date**	**Signature**
Name of Researcher	**Date**	**Signature**

Would you like to be sent information about the progress of the study? Yes ☐ No ☐

Thank you for agreeing to participate in this research

When completed: 1 for patient; 1 for researcher file; 1 (original) to be kept in medical notes

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10.5 AUTOMATED BIOCHEMICAL ANALYSES: MATERIALS AND METHODS

Test	Oestradiol E2
Principle	Competition
First Incubation	Sample + estradiol specific biotinylated monoclonal antibody forms immunocomplex.
Second Incubation	Addition of streptavidin coated microparticles + an estradiol derivative labeled with ruthenium complex fill vacant sites of biotinylated antibodies and form antibody-hapten complex. Interaction of biotin and streptavidin binds complex to solid phase.
Measurement	Microparticles magnetically captured onto surface of electrode. Unbound substances removed. Application of voltage to electrode induces chemiluminescent emission, which is measured by a multiplier.
Stability	2 days at 2-8°C
Running CV	6 - 2

Test	Progesterone
Principle	Competition
First Incubation	Sample + biotinylated monoclonal progesterone specific antibody + progesterone derivative labeled with ruthenium complex, incubated with Danazol. Progesterone competes with labeled progesterone derivative for the antibody binding site.
Second Incubation	Addition of streptavidin coated microparticles, complex becomes bound to solid phase via interaction of biotin + streptavidin. Amount of labelled progesterone bound to solid phase inversely proportional to progesterone content of sample.
Measurement	Microparticles magnetically captured onto surface of electrode. Unbound substances removed. Application of voltage to electrode induces chemiluminescent emission which is measured by a multiplier.
Stability	5 days at 2-8°C
Running CV	9.4 - 3

Test	SHBG
Principle	Sandwich
First Incubation	Sample + biotinylated monoclonal SHBG specific antibody + monoclonal SHBG specific antibody labeled with a ruthenium complex form a sandwich complex
Second Incubation	Addition of streptavidin coated microparticles, complex becomes bound to solid phase via interaction of biotin + streptavidin.
Measurement	Microparticles magnetically captured onto surface of electrode. Unbound substances removed. Application of voltage to electrode induces chemiluminescent emission, which is measured by a multiplier.
Stability	> 12 weeks at 2-8°C
Running CV	2.2 – 1.5

Test	Testosterone
Principle	Competition
First Incubation	Sample + biotinylated monoclonal testosterone specific antibody. Binding sites of labeled antibody become occupied by sample analyte.
Second Incubation	Addition of streptavidin coated microparticles + a testosterone derivative labeled with a ruthenium complex. Interaction of biotin and streptavidin binds complex to solid phase.
Measurement	Microparticles magnetically captured onto surface of electrode. Unbound substances removed. Application of voltage to electrode induces chemiluminescent emission, which is measured by a multiplier.
Stability	> 12 weeks at 2-8°C
Running CV	4.15 – 2.42

Test	LH
Principle	Sandwich
First Incubation	Sample + biotinylated monoclonal LH specific antibody + monoclonal LH specific antibody labeled with a ruthenium complex form a sandwich complex
Second Incubation	Addition of streptavidin coated microparticles, complex becomes bound to solid phase via interaction of biotin + streptavidin.
Measurement	Microparticles magnetically captured onto surface of electrode. Unbound substances removed. Application of voltage to electrode induces chemiluminescent emission, which is measured by a multiplier.
Stability	> 12 weeks at 2-8°C
Running CV	2.09 – 1.1

Test	FSH
Principle	Sandwich
First Incubation	Sample + biotinylated monoclonal FSH specific antibody + monoclonal FSH specific antibody labeled with a ruthenium complex form a sandwich complex
Second Incubation	Addition of streptavidin coated microparticles, complex becomes bound to solid phase via interaction of biotin + streptavidin.
Measurement	Microparticles magnetically captured onto surface of electrode. Unbound substances removed. Application of voltage to electrode induces chemiluminescent emission, which is measured by a multiplier.
Stability	> 12 weeks at 2-8°C
Running CV	2.75 – 2.1

Test	Glucose GLUC3
Principle	UV test. Enzymatic reaction with hexokinase, catalyses phosphorylation of glucose to glucose-6-phosphate by ATP.
Reaction	$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G6P} + \text{ADP}$
Second reaction	$\text{G6P} + \text{NADP}^{+} \xrightarrow{\text{G6PDH}} \text{gluconate6P} + \text{NADPH} + \text{H}^{+}$
Measurement	Rate of NADPH formation is directly proportional to glucose concentration. Measured photometrically.
Stability	3 days at 2-8°C Affected by storage temperature, bacterial contamination, glycolysis. Serum glucose will decrease by approx. 7% an hour due to glycolysis if left to clot and not centrifuged promptly. Can be mitigated by collecting in fluoride tubes.
Running CV	0.95 – 0.7

10.6 BIOMARKER ANALYSES: MATERIALS AND METHODS

Biomarker	**High Sensitivity CRP**
Materials	Rabbit anti-human CRP antibodies (unlabeled and horseradish peroxidase labeled) (Abcam, Cambridge, UK) Phosphate buffered saline, 10 x concentrate (Sigma-Aldrich, Dorset, UK) Immulon 4HBX microtitre plates (Thermo-Scientific, Rochester, NY, USA) Reagent diluent, 1% (w/v) BSA in PBS (x1) Wash buffer, 0.1% Tween-20 in PBS (x1) Sample diluent, 1% (w/v) BSA / 0.1% (v/v) Tween-20 in PBS Substrate solution, o-phenylenediamine (OPD) tablets (Sigma-Aldrich, Dorset, UK) Stop solution, 1.5M sulphuric acid
Methods	1. Capture antibody in PBS was added to microtitre plate 2. Incubated overnight at room temperature to create a solid phase 3. Plate blocked by 1hr incubation with reagent diluent 4. Wash x 3 5. Standards, control and serum (x1000 in reagent diluent) incubated for 2 hrs with solid phase antibody 6. Wash x 3 7. Horseradish peroxidase labeled detection antibody was added and incubated for 1hr 8. Wash x 3 9. Substrate OPD solution was added and colour allowed to develop in proportion to the amount of bound horseradish peroxidase (HRP) 10. Colour development was arrested by the addition of stop solution 11. Colour intensity was measured at **λ** = 490nm on a plate reader (Dynex Technologies, Worthing, UK) 12. Standard curve was generated using Fig P software (Hamilton, ON, Canada) and the concentration of serum hs-CRP calculated
Analytical sensitivity	Minimum detection limit calculated from mean + 2SD of 8 replicate analyses of reagent blank and was found to be 0.1ug/L
Assay dynamic range	Up to 15ug/L
Intra-assay variation	n=14, CV = 4.7%
Inter-assay variation	n=19, CV = 6.2%

Biomarker	**IL-6**
Materials	DuoSet ELISA development kit for IL-6 DY206 (R&D Systems, Abingdon, UK) containing coating antibody, biotin-labelled detection antibody, streptavidin-peroxidase and a recombinant standard Phosphate buffered saline, 10 x concentrate (Sigma-Aldrich, Dorset, UK) Immulon 4HBX microtitre plates (Thermo-Scientific, Rochester, NY, USA) Reagent diluent, 1% (w/v) BSA in PBS (x1) Wash buffer, 0.05% Tween-20 in PBS (x1) Substrate solution, tetramethylbenzidine (TMB) (Sigma-Aldrich, Dorset, UK) Stop solution, 1M sulphuric acid
Methods	1. Capture antibody in PBS was added to microtitre plate 2. Incubated overnight at room temperature to create a solid phase 3. Plate blocked by 1hr incubation with reagent diluent 4. Wash x 3 5. Standards and serum (x1) were incubated for 2 hrs with solid phase antibody 6. Wash x 3 7. Biotin labeled detection antibody was added and incubated for 1hr 8. Wash x 3 9. Streptavidin peroxidase was added and incubated for 30 mins 10. Wash x 3 11. Substrate TMB solution was added and colour allowed to develop in proportion to amount bound HRP 12. Colour development was arrested by the addition of stop solution 13. Colour intensity was measured at **λ** = 450nm on a plate reader (Dynex Technologies, Worthing, UK) 14. Standard curve was generated using Fig P software (Hamilton, ON, Canada) and the concentration of serum IL-6 was calculated
Analytical sensitivity	Minimum detection limit calculated from mean + 2SD of 9 replicate analyses of reagent blank and was found to be 0.5pg/mL
Assay dynamic range	Up to 600pg/mL
Intra-assay variation	n=28, CV = 17.18%
Inter-assay variation	Not determined

Biomarker	**TNFα**
Materials	DuoSet ELISA development kit for TNFα DY210 (R&D Systems, Abingdon, UK) containing coating antibody, biotin-labelled detection antibody, streptavidin-peroxidase and a recombinant standard Phosphate buffered saline, 10 x concentrate (Sigma-Aldrich, Dorset, UK) Immulon 4HBX microtitre plates (Thermo-Scientific, Rochester, NY, USA) Reagent diluent, 1% (w/v) BSA in PBS (x1) Wash buffer, 0.05% Tween-20 in PBS (x1) Substrate solution, tetramethylbenzidine (Sigma-Aldrich, Dorset, UK) Stop solution, 1M sulphuric acid
Methods	1. Capture antibody in PBS was added to microtitre plate 2. Incubated overnight at room temperature to create a solid phase 3. Plate blocked by 1hr incubation with reagent diluent 4. Wash x 3 5. Standards and serum (x1) were incubated for 2 hrs with solid phase antibody 6. Wash x 3 7. Biotin labeled detection antibody was added and incubated for 1hr 8. Wash x 3 9. Streptavidin peroxidase was added and incubated for 30 mins 10. Wash x 3 11. Substrate TMB solution was added and colour allowed to develop in proportion to amount bound HRP 12. Colour development was arrested by the addition of stop solution 13. Colour intensity was measured at **λ** = 450nm on a plate reader (Dynex Technologies, Worthing, UK) 14. Standard curve was generated using Fig P software (Hamilton, ON, Canada) and the concentration of serum TNFα was calculated
Analytical sensitivity	Minimum detection limit calculated from mean + 2SD of 11 replicate analyses of reagent blank and was found to be 2pg/mL
Assay dynamic range	Up to 1ng/mL
Intra-assay variation	n=8, CV = 5.9%
Inter-assay variation	n=8, CV = 13.1%

Biomarker	**Adiponectin**
Materials	DuoSet ELISA development kit for adiponectin DY1065 (R&D Systems, Abingdon, UK) containing coating antibody, biotin-labelled detection antibody, streptavidin-peroxidase and a recombinant standard Phosphate buffered saline, 10 x concentrate (Sigma-Aldrich, Dorset, UK) Immulon 4HBX microtitre plates (Thermo-Scientific, Rochester, NY, USA) Reagent diluent, 1% (w/v) BSA in PBS (x1) Wash buffer, 0.05% Tween-20 in PBS (x1) Substrate solution, tetramethylbenzidine (Sigma-Aldrich, Dorset, UK) Stop solution, 1M sulphuric acid
Methods	1. Capture antibody in PBS was added to microtitre plate 2. Incubated overnight at room temperature to create a solid phase 3. Plate blocked by 1hr incubation with reagent diluent 4. Wash x 3 5. Standards and serum (x1) were incubated for 2 hrs with solid phase antibody 6. Wash x 3 7. Biotin labeled detection antibody added, incubated for 1hr 8. Wash x 3 9. Streptavidin peroxidase was added and incubated for 30 mins 10. Wash x 3 11. Substrate TMB solution was added and colour allowed to develop in proportion to amount bound HRP 12. Colour development was arrested by the addition of stop solution 13. Colour intensity was measured at **λ** = 450nm on a plate reader (Dynex Technologies, Worthing, UK) 14. Standard curve was generated using Fig P software (Hamilton, ON, Canada) and the concentration of serum adiponectin was calculated
Analytical sensitivity	Minimum detection limit calculated from mean + 2SD of 16 replicate analyses of reagent blank and was found to be 0.02ng/mL
Assay dynamic range	Up to 3ng/mL
Intra-assay variation	n=40, CV = 3.2%
Inter-assay variation	n=20, CV = 9.3%

Biomarker	**Leptin**
Materials	DuoSet ELISA development kit for leptin DY398 (R&D Systems, Abingdon, UK) containing coating antibody, biotin-labelled detection antibody, streptavidin-peroxidase and a recombinant standard Phosphate buffered saline, 10 x concentrate (Sigma-Aldrich, Dorset, UK) Immulon 4HBX microtitre plates (Thermo-Scientific, Rochester, NY, USA) Reagent diluent, 1% (w/v) BSA in PBS (x1) Wash buffer, 0.05% Tween-20 in PBS (x1) Substrate solution, tetramethylbenzidine (Sigma-Aldrich, Dorset, UK) Stop solution, 1M sulphuric acid
Methods	1. Capture antibody in PBS was added to microtitre plate 2. Incubated overnight at room temperature to create a solid phase 3. Plate blocked by 1hr incubation with reagent diluent 4. Wash x 3 5. Standards and serum (x1) were incubated for 2 hrs with solid phase antibody 6. Wash x 3 7. Biotin labeled detection antibody added, incubated for 1hr 8. Wash x 3 9. Streptavidin peroxidase was added and incubated for 30 mins 10. Wash x 3 11. Substrate TMB solution was added and colour allowed to develop in proportion to amount bound HRP 12. Colour development was arrested by the addition of stop solution 13. Colour intensity was measured at **λ** = 450nm on a plate reader (Dynex Technologies, Worthing, UK) 14. Standard curve was generated using Fig P software (Hamilton, ON, Canada) and the concentration of serum leptin was calculated
Analytical sensitivity	Minimum detection limit calculated from mean + 2SD of 13 replicate analyses of reagent blank and was found to be 0.04ng/mL
Assay dynamic range	Up to 2ng/mL
Intra-assay variation	n=14, CV = 5.3%
Inter-assay variation	n=11, CV = 7.2%

10.7 Publication: Obesity driven endometrial cancer: is weight loss the answer?

A commentary written for the BJOG in response to the paper by Argenta et al (66). Obesity-driven endometrial cancer: is weight loss the answer? MacKintosh ML, Crosbie EJ. BJOG June 2013; 120(7): 791-794 (112)

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Commentary

Obesity-driven endometrial cancer: is weight loss the answer?

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Endometrial cancer is the fourth most common cancer affecting British women, behind breast, lung and colon cancer. Over the past 20 years, the incidence of endometrial cancer has risen by 40%, and deaths by 20%, despite improved overall survival rates. Currently more than 1900 British women die from endometrial cancer each year, compared with fewer than 1500 at the turn of the century.[1] The aging population, uterine-sparing treatments for menstrual dysfunction and tamoxifen treatment for breast cancer have all contributed to this rise, but escalating obesity rates appear to be the major culprit.

Endometrial cancer ranks highest amongst all cancers in its association with obesity. Every 5 kg/m^2 increase in body mass index (BMI) confers a 1.6-fold increased risk of endometrial cancer. At a BMI of 42, a woman has an almost ten-fold increased risk of endometrial cancer than women of normal weight.[2]Rates of obesity in England and Wales have trebled over the last two decades, and they are still on the increase. Currently, two-thirds of British women are overweight and nearly one-quarter obese. In Europe, excess weight has been estimated to account for 60% of all new endometrial cancer cases per year.[3] Indeed, in the ASTEC trial, a European study of more than 1400 women with early-stage endometrial cancer, 80% of women with type-1 endometrial cancer were overweight (BMI > 25) and 50% were obese (BMI > 30).[4] Thus, although an average woman has a 3% lifetime risk of endometrial cancer, an obese woman has a risk of 9–10%, and for morbidly obese women the risk may be even higher.

How could obesity cause endometrial cancer?

There are two types of endometrial cancer: endometrioid (type-1) cancer and non-endometrioid (type-2) cancer. Obesity is thought to play a major aetiological role in the development of endometrioid tumours but not non-endometrioid tumours, which include several high-risk histotypes that develop via different pathways. Type-1 disease accounts for over 80% of cases, and even though it has a lower death rate than type-2 disease, it still accounts for the majority of deaths.[4] Obesity is a toxic state of hyperestrogenaemia, insulin resistance and inflammation (Figure 1). Obesity causes estrogenic excess because adrenal androgens are aromatised to estrogen by adipose tissue.[5] In addition women who are obese have increased bioavailability of free estrogens because of low levels of sex hormone binding globulin (SHBG), a consequence of hyperinsulinaemia. Estrogens bind directly to endometrial cell DNA to increase transcription and interact with several growth factor signalling pathways, including the PI3K-Akt-mTOR and MAPK/ERK1,2 pathways, to promote proliferation.[6] Excessive or 'unopposed' estrogenic stimulation of the endometrium, for example in postmenopausal or chronic anovulatory states (e.g. polycystic ovary syndrome, PCOS), drives tumourigenesis.[5] Progestogens, naturally released during the second half of the menstrual cycle in ovulating women, or delivered exogenously, 'protect' the endometrium from the tumourigenic effects of estrogen.[7]

Obesity may also increase endometrial cancer risk through hyperinsulinaemia and insulin resistance.[8] Increased circulating levels of insulin lead to reduced blood levels of insulin-like growth factor (IGF) binding proteins, contributing to increased bioavailability of IGF-1. IGF-1 is a growth factor that drives cellular proliferation through the activation of the PI3K-Akt-mTOR pathway. Inhibitors of this pathway include adiponectin, an adipokine found in the serum at concentrations inversely related to insulin resistance. Low

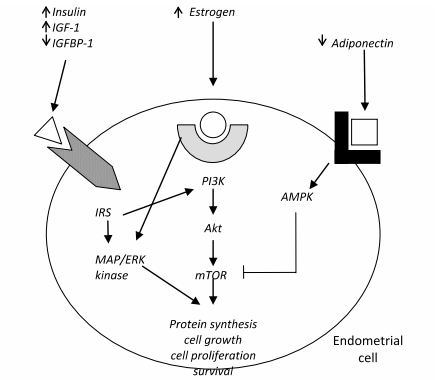

Figure 1. Mechanisms underpinning obesity-related endometrial carcinogenesis include the effects of hyperestrogenism, insulin resistance, altered serum adipokine concentrations and proinflammatory responses (not shown). Key: AR, adiponectin receptors; ER, estrogen receptor; IGF-1R, IGF-1 receptor; IR, insulin receptor.

adiponectin levels are a surrogate marker for insulin resistance, and correlate strongly with endometrial cancer risk, independent of BMI. Adiponectin inhibits the mammalian target of rapamycin (mTOR) pathway through the activation of AMP-activated protein kinase (AMPK). Obesity is a chronic inflammatory state associated with elevated systemic proinflammatory cytokines, promoters and hormones, including tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6), C–reactive protein (CRP) and leptin.[8] Proinflammatory markers have been implicated in tumourigenesis via their direct and indirect effects on cells of the innate and adaptive immune systems, as well as through disturbed tissue homeostasis and increased oxidative stress.

The endometrial effects of obesity

Despite the striking correlations observed between obesity and risk of endometrial cancer, few studies have investigated the effects of obesity on the endometrium. Hysterectomy specimens from women of normal weight (BMI 20–25), and from women who are overweight and obese, show that endometrial proliferation and the activation of key pro-proliferative signalling proteins (pAkt, pERK1,2) are correlated with BMI in premenopausal women. Compared with women of normal weight, women who are obese show increased endometrial proliferation, as evidenced by increased ki-67 expression, as well as increased phosphorylation of Akt (pAkt, 1.6-fold), ERK1,2 (pERK1,2 8.7-fold), despite having normal endometrial histology.[9] Thus the effects of obesity can be observed at a molecular level in apparently normal premenopausal endometrium. Endometrial cancer and its precursor lesion, atypical endometrial hyperplasia, usually present early following the onset of abnormal vaginal (typically postmenopausal) bleeding. However, clinically overt endometrial hyperplasia and neoplasia may be the tip of the iceberg. In a study of asymptomatic obese women, the prevalence of occult endometrial hyperplasia and cancer was 12 and 3% in postmenopausal women, and 5.8 and 1% in premenopausal women, respectively (n = 193).[10] The natural history of occult endometrial pathology is unknown.

Can weight loss reduce endometrial cancer risk?

Emerging evidence suggests that massive, sustained weight loss following bariatric surgery (gastric bypass or banding to reduce stomach capacity) reduces the risk of endometrial cancer. In a prospective Swedish study of more than 4000 matched patients who were morbidly obese undergoing bariatric surgery or medical weight loss management, a sustained weight loss of 20 kg for 10 years or more in the bariatric surgery group was associated with a 38% reduction in cancer incidence amongst women, but not men.[11] A retrospective study of 4977 women who were morbidly obese from Virginia, USA, found a 38% decrease in cancer incidence in the bariatric surgery group compared with controls who were obese, with pronounced reductions in the incidence of endometrial and postmenopausal breast cancer.[12] In another retrospective study, a 24% reduction in incident cancers and 46% reduction in cancer mortality were seen amongst 6596 women undergoing bariatric surgery, compared with 9442 controls who were obese, over a 12.5-year follow-up period in Utah, USA.[13] The lack of an association seen for men may reflect the small numbers of men undergoing bariatric surgery (just 14% of the surgical population in the latter study) and the increased effect of weight loss on the incidence of sex steroid-related tumours, such as breast and endometrial cancer, which predominantly and exclusively affect women, respectively. Interestingly, the most impressive reduction in cancer-specific incidence in the latter study was seen for endometrial cancer, where bariatric surgery resulted in a seven-fold reduction in incident cancer risk (14 cases in 6596 bariatric surgery patients versus 98 cases in 9442 controls who were obese, HR 0.22, $P < 0.0001$).[13] Given that amongst all obesity-related malignancies the link with elevated BMI is strongest for endometrial tumours, this finding is perhaps unsurprising.

Taken together, these observations are exciting and provide proof of principle that endometrial cancer is preventable. Preventing endometrial cancer by weight loss is an attractive strategy as weight loss would also improve cardiovascular fitness, reduce/treat type-2 diabetes and

reduce the risk of other obesity-related cancers, e.g. breast and colon cancer.[14] Bariatric surgery is an effective means of achieving weight loss, but it is not feasible at a population level for reducing cancer risk. Alternative weight loss strategies have not demonstrated convincing efficacy or sustainability. Tackling obesity through diet and exercise is extremely challenging, and is likely to involve complex societal and political initiatives. Meanwhile, the obesity epidemic continues unabated throughout the UK, as well as much of Europe and North America.

The mechanism by which weight loss reduces endometrial cancer risk is complex. Bariatric surgery results in an almost instantaneous improvement in insulin resistance, with measurable effects on metabolism, serum glucose and insulin levels observed far in advance of any appreciable weight loss. Up to 30% of type-2 diabetic patients are discharged home 2–3 days after bariatric surgery with no antiglycaemic medication and normal blood glucose levels.[14] Patients may expect to lose 10–15% of their excess body weight by 6 weeks after surgery, with continued weight loss, albeit at a slower rate, up to about 1 year post-surgery. In general, patients settle at a BMI of 25–30; resolution to a normal weight BMI may never be achieved. The loss of adiposity reduces the aromatisation of adrenal androgens to estrogen, leading to a drop in serum estrogen levels. This is accompanied by higher plasma SHBG concentrations, leading to less bioavailable estrogen in the blood, although the nadir may not be reached until 12 months or longer after surgery. The resolution of chronic inflammatory change may also be slow given that proinflammatory markers are released primarily from white adipose tissue. The effects of surgically induced weight loss on metabolism, plasma sex steroid levels and inflammatory responses are clearly complicated, but in combination they provide a biologically plausible mechanism for the reduction in cancer incidence observed, particularly for estrogen-sensitive tumours.[14]

Can weight loss 'treat' endometrial hyperplasia and neoplasia?

Given the central role of obesity in its pathogenesis, and with compelling evidence that surgically induced weight loss 'prevents' endometrial cancer, an interesting question arises: can weight loss also 'treat' established endometrial hyperplasia and cancer? The study by Argenta et al., published in the current issue of *BJOG*, set out to answer this question.[15] From 45 paired endometrial samples taken before surgery and at 1 year following surgically induced weight loss in asymptomatic women who were morbidly obese, the authors identified a baseline prevalence of endometrial pathology of 6.8% in this largely premenopausal group. There were no cases of occult cancer or atypical endometrial hyperplasia, but three cases of simple hyperplasia and one of complex hyperplasia without atypia. Although not strictly premalignant, hyperplastic change of the endometrium may set the stage for genetic events that pose a steadily increasing risk of progression to cancer.[16] All four cases of endometrial hyperplasia regressed during follow-up: two by 12 months (one simple and one complex hyperplasia), and the other two (both simple hyperplasia) by 18 months, following surgery. In two cases, regression may have been aided by 'treatment' with oral contraceptives. A further case, negative for pathology at surgery, developed simple hyperplasia at 12 months. With such small numbers, a largely premenopausal cohort and the exclusion of women with recent abnormal vaginal bleeding, this study is clearly insufficiently powered to provide conclusive or convincing evidence for the role of bariatric surgery in the treatment of endometrial hyperplasia. However, it does introduce the idea that some hyperplastic abnormalities of the endometrium may be reversible through weight loss. It is conceivable that hyperplasia without atypia could regress following the withdrawal of stimulating factors (e.g. hyperestrogenaemia and hyperinsulinaemia), but it is more difficult to envisage the reversal of atypia or frankly malignant change through weight loss alone. Nonetheless, the bariatric surgery model provides the perfect opportunity to study the molecular changes that underpin endometrial cancer risk reduction. An improved understanding of these will facilitate the development of novel prevention strategies for endometrial cancer.

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