METFORMIN IN THE TREATMENT OF ENDOMETROID ENDOMETRIAL CANCER: ESTABLISHING ITS IMPACT AND ASSESSING ITS ROLE

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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SCHOOL OF MEDICAL SCIENCES
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<td>7-AAD</td>
<td>7-actinoaminomycin-D</td>
</tr>
<tr>
<td>4EBP1</td>
<td>4E binding protein 1</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
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<tr>
<td>AEH</td>
<td>Atypical endometrial hyperplasia</td>
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<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
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<td>Akt</td>
<td>Protein kinase B</td>
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<td>AMP</td>
<td>Adenosine monophosphate</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BAA</td>
<td>BODIPY-aminoacetate</td>
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<tr>
<td>BAAA</td>
<td>BODIPY-aminoacetaldehyde</td>
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<td>BMI</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CA-IX</td>
<td>Carbonic anhydrase-9</td>
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<td>DEAB</td>
<td>Diethylaminobenzaldehyde</td>
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<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
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<tr>
<td>(c)DNA</td>
<td>(complementary) Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunoabsorbant assay</td>
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<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
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<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
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<tr>
<td>FIGO</td>
<td>International Federation of Gynecology and Obstetrics</td>
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<tr>
<td>GDF15</td>
<td>Growth differentiation factor 15</td>
</tr>
<tr>
<td>HbA1C</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
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<tr>
<td>HE4</td>
<td>Human epididymis protein 4</td>
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<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor-1 alpha</td>
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<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment-insulin resistance</td>
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<td>IGF-1/2</td>
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<td>Insulin like growth factor binding protein-1</td>
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<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>LN</td>
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<td>LVS1</td>
<td>Lymphovascular space invasion</td>
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<tr>
<td>MAHSC-CTU</td>
<td>Manchester Academic Health Science Centre-Clinical Trials Co-ordination Unit</td>
</tr>
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<td>CRF</td>
<td>Case report form</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
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<td>MFT</td>
<td>Manchester University Hospitals NHS Foundation Trust</td>
</tr>
<tr>
<td>MHRA</td>
<td>Medicine and Healthcare Products Regulatory Authority</td>
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<tr>
<td>MMR</td>
<td>Mismatch repair</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MSI</td>
<td>Microsatellite instability</td>
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<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>NICE</td>
<td>National Institute of Clinical Excellence</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>Polycystic ovary syndrome</td>
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<td>Polymerase chain reaction</td>
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<td>Abbreviation</td>
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<tr>
<td>PGK1</td>
<td>Phosphoglycerate kinase 1</td>
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<td>PI3K</td>
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<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<tr>
<td>RCT</td>
<td>Randomised controlled trial</td>
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<td>R+D</td>
<td>Research and development</td>
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<td>RFS</td>
<td>Recurrence free survival</td>
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<td>RR</td>
<td>Relative risk</td>
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<tr>
<td>RT-qPCR</td>
<td>Real Time quantitative-Reverse Transcription Polymerase Chain Reaction</td>
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<tr>
<td>SEER</td>
<td>Surveillance, Epidemiology and End Results Program</td>
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<td>SEM</td>
<td>Standard error of mean</td>
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<td>SFE</td>
<td>Sphere formation efficiency</td>
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<td>(S)HR</td>
<td>(Sub)hazard ratio</td>
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<td>Sulforhodamine B</td>
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<td>TMA</td>
<td>Tissue microarray</td>
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<td>Trial management group</td>
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<tr>
<td>TSC</td>
<td>Trial Steering Committee</td>
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<td>TSC1/2</td>
<td>Tuberous sclerosis complex 1/2</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>World Health Organisation</td>
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Abstract

**Background:** Endometrial cancer incidence and mortality is increasing due to rising obesity rates, an ageing population and a preponderance of cardiovascular deaths. Novel treatments are required for women who are unfit for or who decline surgery, to reduce the risk of disease recurrence and improve survival. Laboratory work and small uncontrolled window studies suggest that metformin reduces endometrial cancer proliferation through inhibition of the PI3K-Akt-mTOR pathway and selectively targets cancer stem cells responsible for metastasis and disease recurrence in breast and ovarian cancer.

**Methods:** i) The clinical efficacy of metformin was tested in a double-blind, placebo-controlled, randomised trial. Expression of Ki-67 and markers of the PI3K-Akt-mTOR and insulin signalling pathway were compared prior to and following treatment. ii) Cell lines were used to characterise endometrial cancer stem cells and to investigate the effect of metformin on these cells in the context of a model of obesity associated endometrial cancer. iii) The true prevalence of cardiovascular risk factors in women with endometrial cancer was compared with the general population and the impact of universal screening and treatment of modifiable risk factors on 10-year cardiovascular disease risk determined.

**Results:** Standard diabetic doses of metformin for 1-5 weeks prior to surgery did not result in an overall reduction in endometrial cancer proliferation when tested in a methodologically robust randomised controlled trial. BMI significantly influenced response to treatment (p=0.05); non-obese women had a non-significant decrease in Ki-67 expression of 8.3% (95%CI -18.70 to +2.09) following metformin exposure, whilst no effect was seen in obese women (mean difference in post-treatment Ki-67 +5.50%, 95%CI -8.31 to +13.81). This difference was unrelated to serum drug levels. Metformin had no effect on the PI3K-Akt-mTOR pathway and insulin signalling when pre- and post-treatment endometrial biopsies were directly compared. Metformin reduced the number and activity of endometrial cancer stem cells, characterised by high ALDH activity and CD133 positivity, and decreased the expression of cancer stem cell genes at a lower concentration than that required to affect overall cell proliferation in vitro. This effect was, however, abolished in the presence of adipocyte-conditioned media. Women with newly diagnosed endometrial cancer had a 1.4-fold increased 10-year cardiovascular disease risk, as measured by QRISK2 score, compared with the general population due to higher levels of recognised and undertreated risk factors. Screening and treatment of these risk factors could reduce an individual’s absolute risk by 1.82%, requiring 55 women with endometrial cancer to be treated to prevent one cardiovascular event compared with 145 women in the general population.

**Conclusions:** Obese women appear to be resistant to the anti-tumour effects of metformin as a consequence of adipocyte secreted mediators. Metformin has a specific and selective effect on endometrial cancer stem cells in a cell line model; its effect on these cells in vivo should be examined. The increased risk of cardiovascular disease in women with a history of endometrial cancer may be improved by metformin treatment; the effect of long term therapy on overall and cardiovascular specific survival should be examined in a randomised controlled trial.
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Chapter 1  Introduction

1.1  Epidemiology of endometrial cancer

1.1.1  Rising incidence of endometrial cancer

Endometrial cancer is currently the fourth most common female malignancy, with 9324 new diagnoses in 2014 in the UK (Cancer Research UK 2017a). The incidence is rising, with a 48% increase in the number of cases over the last 20 years, and this trajectory is set to continue, with an estimated 3600 new diagnoses expected each year in England and Wales by 2030 (Kitson et al. 2017b). In the USA, endometrial cancer is set to overtake colorectal and lung cancer to become the third most common malignancy in women (Rahib et al. 2014). This disease is not solely a problem of the developed world, however, as the burden of endometrial cancer is anticipated to increase globally, including in low income countries (Arnold et al. 2014).

This increase has been associated with the worldwide rise in the incidence of obesity, with which endometrial cancer has the strongest association amongst the 20 most common tumour types (Renehan et al. 2008). A 5kg/m² increase in body mass index (BMI) has been shown to be associated with a 60% increase in the relative risk of developing endometrial cancer in a meta-analysis of prospective observational studies (Crosbie et al. 2010). This effect is not linear, with a proportionally greater increase in risk per 5kg/m² increase in BMI when the reference BMI is above 27kg/m² compared with a BMI below this threshold. A woman with a BMI of 42kg/m² has a relative risk of developing endometrial cancer that is 9.11 times higher (95% CI 7.26-11.51) than that of a woman with a BMI of 22kg/m² (Crosbie et al. 2010). The strength of association between obesity and endometrial cancer is such that it has been estimated that 41% of cases are directly attributable to obesity (Bhaskaran et al. 2014).

Besides obesity, the rising incidence of endometrial cancer is related to an increase in the prevalence of other risk factors; namely an ageing population (Wartko et al. 2013) and the rising incidence of diabetes (Diabetes UK 2016). There has also been a decrease in risk reducing behaviours, including fewer hysterectomies performed for the treatment of benign disease (Mukhopadhaya and Manyonda 2013), reduced use of combined hormone replacement therapy (Crosbie et al. 2010), older age at first birth and smaller family size (McAlpine et al. 2016;Gentry-Maharaj et al. 2017) and reduced participation in physical activity (Schmid et al. 2015).

Underpinning many of these risk factors is excess oestrogen exposure, either as the result of unopposed oestrogen production by the ovaries in premenopausal women or the aromatisation of androgens to oestrogen by adipose tissue following the menopause. Tamoxifen, a selective oestrogen receptor modulator, used as a long term adjuvant treatment in breast cancer, is associated with a two to three-fold
increase in risk of endometrial cancer, which persists even after discontinuation of the drug (Fisher et al. 1998; Davies et al. 2013). In contrast, ever use of the oral contraceptive pill, which acts to suppress oestrogen induced endometrial proliferation, is associated with a 50% reduction in endometrial cancer risk (Mueck et al. 2010).

Oestrogen excess cannot be the sole mechanism driving endometrial carcinogenesis, however, as endogenous oestrogen levels are not elevated in all women with endometrial cancer. Indeed, total and free oestrogen levels are only a marker of endometrial cancer risk in postmenopausal women and not in those of reproductive age (Kaaks et al. 2002; Potischman et al. 1996). Similarly, whilst polycystic ovary syndrome (PCOS), a triad of anovulation, hyperandrogenism and multiple ovarian follicles, characterised by relative oestrogen excess, is associated with a 2.8-fold increase in the risk of developing endometrial cancer, the majority of women with the condition do not develop uterine malignancies (Barry et al. 2014). Rather, it is those women with both insulin resistance and PCOS (which co-exist in 50-70% of cases) who appear to be at particular risk.

Whilst obesity is a frequent driver of reduced insulin sensitivity and can thus provide an explanation for the majority of extra cases of diabetes diagnosed in the last 20 years, insulin resistance can occur in the absence of excess adipose tissue. Its effect on endometrial cancer risk also appears to be independent of its association with body mass (Nead et al. 2015). Impaired fasting glucose, as a consequence of insulin resistance, has been shown to be associated with a 25% increase in the risk of endometrial cancer, independent of other components of the metabolic syndrome, including obesity, hypertension and hyperlipidaemia (Trabert et al. 2015). Correcting insulin resistance as well as maintaining a healthy body mass are thus both necessary to tackle the rising incidence of endometrial cancer, and could potentially influence disease-specific survival.

1.1.2 Rising mortality rate

Endometrial cancer mortality is increasing, albeit at a slower rate than disease incidence. Given the current rate of increase, by 2030 it is estimated that there will be an additional 850 deaths each year in England and Wales from the disease (Office for National Statistics 2016). This rise is being driven by the growing number of endometrial cancer diagnoses, as 10-year survival has improved from 55% to 78% in the last 40 years (Cancer Research UK 2017b). This is due to a number of factors, including increased awareness of the significance and reporting of postmenopausal bleeding, meaning that over 80% of women will have disease confined to the uterus at presentation (stage I or II) (Cancer Research UK 2016). There has also been an increase in the incidence of the more indolent endometrioid histological sub-type (Evans et al. 2011). Current treatment consists of surgery, in the form of a total hysterectomy and bilateral salpingo-oophorectomy ± lymphadenectomy, in combination with brachytherapy or external beam
radiotherapy for some (Cancer Research UK 2014a). This can thus be considered curative for the majority of women.

For those women with extra-uterine disease at the time of diagnosis, prognosis is notably poorer; five year survival is only 14% for those with stage IV disease (Cancer Research UK 2016). Elderly women (aged 80 years+) are more likely to be diagnosed with extra-pelvic disease than younger individuals, possibly due to delays in seeking medical attention. The higher endometrial cancer mortality rate in older women, in combination with the marked increase in the number of diagnoses in women aged 70-79 years, means that the majority of deaths from endometrial cancer now occur in older women (Cancer Research UK 2016). Treatment options in the case of extra-uterine disease are limited and have not generally been evidence based, with carboplatin and paclitaxel chemotherapy regimens frequently employed due to a similar response rate to that of epithelial ovarian cancer (Sundar et al. 2017). The PORTEC-3 study has provided some much needed evidence in this field, concluding that combined chemoradiotherapy should be considered in preference to external beam radiotherapy alone in women with stage III endometrial cancer to maximise their failure-free survival (HR 0.66, 95%CI 0.45-0.97, adjusted p=0.014) (de Boer et al. 2018). This regime has not been shown to improve overall survival, though, and is associated with significantly more short term side effects. Medical fitness may present a barrier to optimal treatment, as affected women frequently have multiple co-morbid conditions, including obesity.

1.1.3 Obesity and endometrial cancer survival
Whilst obesity can hamper the diagnosis and management of endometrial cancer, it is unclear as to whether it affects endometrial cancer survival per se. A recent systematic review and meta-analysis suggested that there was a dose-response relationship between BMI and all-cause mortality, with the odds ratio for women with a BMI ≥40kg/m² being 1.66 (95%CI 1.10-2.51, p=0.002) compared with those with a BMI <25kg/m² (Secord et al. 2016). The authors acknowledged, however, that there was marked heterogeneity in the included studies, with differences in populations, BMI categories, the survival outcome measures reported and endometrial cancer management. Overall, the quality of studies was considered to be moderate to very low.

Restricted treatment options for obese women are frequently practiced and a notable problem when attempting to determine the impact of body mass on survival. The poorer five-year survival observed in some studies may well reflect sub-optimal disease management rather than a true effect of obesity itself. Randomised controlled trial (RCT) data are superior here as treatment is standardised. In an analysis of data from the MRC ASTEC study, obesity had no independent effect on overall or recurrence-free survival (Crosbie et al. 2012). Similar results were observed in the GOG99 RCT of adjuvant radiotherapy in early stage endometrial cancer; whilst BMI was significantly associated with all-cause mortality, it had
no effect on time to disease recurrence (von Gruenigen et al. 2006). These results would suggest that any increase in mortality observed in obese women with endometrial cancer is due to non-malignant causes.

Whether the treatment of obesity improves survival in women previously diagnosed with endometrial cancer is currently unknown. A recent Cochrane review found a paucity of RCT data in this area (Kitson et al. 2018a). The three included studies were of low and very low methodological quality, randomised only small numbers of women and had too short a follow-up period to accurately determine whether combined lifestyle and behavioural interventions were associated with improvements in overall, cancer-specific and recurrence free survival. A notable problem was that none of the interventions trialled was successful in achieving significant weight loss compared with the placebo arm. The authors concluded that further adequately powered RCTs incorporating pharmacological weight loss strategies, including metformin, and bariatric surgery, and with follow-up of five to 10 years duration were required to address this question.

1.1.4 Diabetes and endometrial cancer mortality

Alongside obesity, diabetes has also been proposed as an adverse prognostic factor. Published studies to date, however, have been heterogeneous in design, have often only considered all-cause mortality, failed to adjust for co-morbidities, including obesity, and are at risk of bias from the use of retrospectively recalled data. As a consequence, the results produced have been unreliable (Currie et al. 2012; Liao et al. 2014; Zhang et al. 2013c). Lindemann et al. (2015) attempted to address some of these issues in their Norwegian study of women recruited into two large health surveys. Self-reported diabetes was associated with a two-fold increase in risk of death from endometrial cancer compared with those without diabetes, after adjustment for competing causes of death, histological subtype and stage of disease (sub-hazard ratio [SHR] 2.62, 95%CI 1.07-6.43). This effect was even more pronounced in women with a BMI <25kg/m², who had a four-fold higher risk of dying from endometrial cancer compared with those with a BMI ≥25kg/m², although this difference was not statistically significant (BMI <25kg/m² SHR 8.27, 95%CI 1.06-64.42, BMI ≥25kg/m² SHR 1.97, 95%CI 0.64-6.14). These results should, however, be viewed with caution, as the reported confidence intervals are wide, suggesting a large degree of uncertainty in the effect size. Diabetes status was also only determined at baseline and not during follow-up, thereby potentially misclassifying some women as non-diabetic. A limitation of this study is that type I and type II diabetes were considered together, despite the two conditions having significantly different pathophysiology. This may explain the association between diabetes, low BMI and increased mortality, though, as weight loss is associated with disease severity in type I diabetes, whilst it is encouraged to reduce the risk of complications in type II diabetes.
The limited and low quality evidence available means that it is difficult to draw firm conclusions as to whether metabolic syndrome increases death from endometrial cancer as opposed to all-causes. Regardless of this, both obesity and diabetes impact on clinical management of endometrial cancer.

1.1.5 Need for novel treatment strategies

Given the high prevalence of obesity among women with endometrial cancer, it is easy to appreciate how challenging treatment of the disease can be. Surgical access may be limited in obese women, preventing extensive dissection, and associated co-morbidities may impact on fitness to undergo the procedure. As a consequence, complete resection of the disease may not be possible and this, in itself, adversely affects survival. Radiotherapy delivery is more technically challenging in women with a raised BMI. There are also an increasing number of women who are being diagnosed with endometrial cancer prior to the menopause, who decline hysterectomy due to a desire to preserve their fertility. These issues point to a need for non-surgical treatment strategies.

1.1.6 Prevention of disease recurrence

Alongside the need for novel treatments for primary disease, additional therapeutic strategies are also required to prevent disease recurrence as, once present, treatment options are limited. Radiotherapy salvage can be achieved amongst those who have not previously received it, and surgery may be appropriate for a highly selective group. Chemotherapy may be offered, although there is little evidence that it improves survival (Sundar et al. 2017). Survival is markedly poorer following disease recurrence than after treatment of primary disease, and is highly dependent upon the number and sites of disease recurrence, treatment options available and the general health of the woman. At present, there are no long term, oral treatments available to prevent recurrent disease, unlike in breast cancer where tamoxifen is widely used (Davies et al. 2013).

Identifying women at high risk of disease recurrence could reduce unnecessary drug exposure and adverse events. Recent molecular classification of tumours by The Cancer Genome Atlas identified four distinct subgroups of prognostic value, marking a shift from the traditional binary description introduced by Bokhman (Cancer Genome Atlas Research et al. 2013;Bokhman 1983). Routine testing of the surrogate markers p53, microsatellite instability and POLE has been shown to be feasible and can be performed using tumour obtained from either a diagnostic pipelle endometrial biopsy or hysterectomy sample (Stelloo et al. 2015;Stelloo et al. 2014). No recurrences were detected in women with POLE mutations, identifying a group of women with a particularly favourable prognosis and who are unlikely to benefit from adjuvant treatment. Prognosis was intermediate in those with microsatellite unstable tumours or those with no specific mutation profile, whereas p53 mutant tumours had the poorest prognosis (Stelloo et al. 2015). The acceptability of this molecular classification system is currently being tested in the PORTEC-
4a study (POTRTEC-4a trial) and is likely to be used to guide decisions regarding adjuvant treatment in the future.

1.2 Window study design

1.2.1 Outline of study design

Use of the ‘window study’ trial design is increasing in the field of cancer research and utilises the time period between initial diagnosis and surgical management of the disease to assess the short term impact of potential new interventions. The limited duration of the ‘window’ means that such studies rely on the use of surrogate markers of long term clinical outcomes but has the advantage of testing tumours in vivo without affecting standard of care. The finite time period also means that results are generated more quickly and at reduced cost compared with traditional adjuvant drug trials. For the researcher, this type of trial design provides access to treatment naive subjects, thereby ensuring a ‘cleaner’ study. The end of study date for each participant, i.e. the date of surgery, can be manipulated to improve recruitment and the attainment of research samples. The benefits for participants in these studies include the rapid screening of drugs, meaning that those not shown to improve outcome can be withdrawn promptly, reducing the number of people exposed to ineffective treatments (Dowsett et al. 2011b). There is also the opportunity for biomarker studies to be simultaneously conducted to identify key components of the patient or their disease which influence treatment response.

The obvious limitation of the finite ‘window period’ in which to test novel therapies is that it may be too short a duration to observe an effect and impossible to establish any dose-response relationship. There is also the question of the validity of the primary endpoints used in ‘window studies’ and their relationship to important clinical outcomes, such as survival.

1.2.2 Reliance on surrogate biological markers of disease response

Biological markers of disease response are required in window studies as surrogates of clinical outcomes. Such biomarkers need to fulfil a number of criteria; be easy to measure, reliable and reproducible with minimal inter- and intra-observer variability, reflect important cancer cell processes and for expression to change promptly in response to effective drug exposure.

Ki-67, a marker of cell proliferation and expressed only during the active phases of the cell cycle, is used widely in breast cancer trials as a primary outcome measure as it has been shown to meet these criteria (Scholzen and Gerdes 2000). Expression is determined by immunohistochemistry, with measurement increasingly standardised through the publication of best practice guidelines based on expert consensus opinion (Dowsett et al. 2011a). Changes in expression of Ki-67 are evident after as little as two weeks of
drug treatment, as demonstrated in the IMPACT window study, which complemented the larger ATAC trial of anastrozole, tamoxifen or combination treatment for post-menopausal women with oestrogen receptor (ER)-positive breast cancer (Dowsett et al. 2011b). The patient-specific decrease in Ki-67 expression was similar after two and 12 weeks of treatment for the majority of women, with no one having a greater response with prolonged treatment, although 15% of women had a rebound rise in Ki-67 by three months, consistent with acquired treatment resistance. Use of such a biomarker means that treatment can be stopped in those patients who fail to show a response after only two weeks. The advantage of using Ki-67 over other pathological variables, such as tumour grade, is that the majority of patients will demonstrate a reduction in cellular proliferation following exposure to an effective cytostatic drug, decreasing the number of participants required for a trial to achieve a statistically significant result.

It is important that biomarkers have both prognostic and predictive value if they are to be meaningful outcome measures in window studies. Expression should be significantly linked to clinical outcomes, such as survival, and be used to identify those patients who will or will not respond to treatment prior to its initiation and to monitor response during therapy (Oldenhuis et al. 2008). Oestrogen and progesterone receptors in endometrial cancer fulfil both of these functions; expression of these steroid receptors are independent prognostic variables and are useful in predicting who will benefit from adjuvant endocrine treatment (Trovik et al. 2013; Singh et al. 2007). Whether they are of value in predicting response to therapy and clinical outcomes in window studies investigating non-hormonal treatments is unknown. Studies into potential serum biomarkers, including Ca-125, HE4 (human epididymis protein 4) and GDF15 (growth differentiation factor 15), have been performed but there is, as yet, insufficient evidence to support their use as outcome measures in window studies (Werner and Salvesen 2014). Indeed the only biomarker that has been used in endometrial cancer window studies is Ki-67, though most of the data supporting its use for this purpose have been derived from other cancer types, most notably breast cancer.

1.2.3 Evidence for the prognostic and predictive value of Ki-67 in other tumour types

Evidence from RCTs and, more latterly, several meta-analyses has established the prognostic value of Ki-67 as a marker of clinical outcome in number of cancers, including gastric (Liu et al. 2017) and renal cell carcinoma (Kim et al. 2017). In prostate cancer, compared with high expression, low Ki-67 expression was associated with a statistically significant improvement in five year disease-free survival with an odds ratio of 0.32 (95%CI 0.23-0.44, p<0.00001) (Berlin et al. 2017). The largest body of research into the prognostic value of Ki-67 has been in breast cancer, where a meta-analysis of over 60,000 women found a pooled hazard ratio for disease-free survival for high versus low Ki-67 expression of 1.50 (95%CI 1.34-1.69, p<0.00001), an effect that appeared independent of traditional pathological prognostic variables,
including tumour size, nodal involvement, oestrogen receptor status, clinical response and grade (Petrelli et al. 2015; Ellis et al. 2008). This effect appears to be consistent across both early and late stage disease and pre- and post-menopausal women, despite lower overall expression in the latter group (Dowsett et al. 2007; DeCensi et al. 2011; Inwald et al. 2013). These results have been replicated in the ‘real life’ unselected population enrolled in the German regional cancer registry, where Ki-67 expression is frequently measured and used to guide decisions regarding adjuvant treatment, despite the lack of national and international recommendations on its use in routine practice (Inwald et al. 2013).

These have not been forthcoming due to problems with identifying cut-off values to denote high and low Ki-67 expression for use in clinical decision making. Petrelli et al. (2015) attempted to address this question in their meta-analysis, observing that a threshold of positive expression in >25% of cells provided the greatest prognostic information. This cut-off should be regarded with some degree of caution, however, as a moderate risk of publication bias was identified and included studies used differing cut-off values dependent upon their own results, with many performed prior to the publication of the International Ki-67 in Breast Cancer Working Group guidelines making the methodology used heterogeneous (Dowsett et al. 2011a). The guidelines proposed by Dowsett et al. (2011a) aimed to standardise the measurement of Ki-67 across laboratories and incorporated recommendations for staining, including sole use of the MIB-1 antibody and heat-induced epitope retrieval, and scoring. They suggested that at least 500 nuclei should be scored across the whole slide, including areas of greatest (hot spot) staining, and that all nuclear staining should be considered positive, regardless of staining intensity. Adherence to these guidelines is associated with improved concordance between laboratories, increasing the reliability of results, and can be enhanced further through the use of automated scoring (Polley et al. 2015; Abubakar et al. 2016). Despite this, the degree of error surrounding quantification of Ki-67 expression between scorers and laboratories is such that if cut-off values should be set this should be done on an individual laboratory basis and for a specific purpose.

These same methodological issues have hampered assessment of the predictive value of Ki-67 in determining the likely benefit to be derived from adjuvant endocrine and chemotherapy (Yerushalmi et al. 2010). Window studies, using the standardised protocol for staining and scoring of Ki-67 expression and the treatment of Ki-67 as a continuous rather than dichotomised variable, have been the most illuminating source in this regard (DeCensi et al. 2011; Ellis et al. 2008). Within the complementary IMPACT and ATAC studies, Ki-67 expression after two weeks of endocrine treatment was predictive of subsequent recurrence-free survival following more prolonged drug therapy, regardless of baseline Ki-67 expression (Dowsett et al. 2011b). These data support the trialling of adjuvant drugs on an individual basis prior to surgery with a view to only continuing those agents associated with a significant reduction in Ki-67 expression. Further work, however, is required to validate this approach to drug screening and the POETIC study will hopefully answer some of these questions (Dowsett et al. 2011b). This study will
randomise 4000 post-menopausal women with ER-positive breast cancer to either anastrazole/letrozole or no treatment for two weeks before and after surgery and has a secondary aim of determining the accuracy of Ki-67 expression following short-term treatment with an aromatase inhibitor in predicting recurrence free survival.

1.2.4 Evidence for prognostic and predictive value of Ki-67 in endometrial cancer

Unlike in breast cancer, change in Ki-67 expression has been incorporated as a primary outcome measure in endometrial cancer window studies of metformin and anastrazole without definitive evidence of its prognostic and predictive value. The published literature contains conflicting reports as to whether increased expression is associated with poorer disease free survival (Liu et al. 2014b; Stefansson et al. 2004; Huvila et al. 2013). Certainly greater expression is seen in higher grade tumours and this, in itself, is known to correlate strongly with outcome (Stefansson et al. 2004; Salvesen et al. 1999). The majority of studies, though, have included fewer than 200 women and have often limited their inclusion criteria to early stage disease, predominately of endometrioid subtype. As a consequence, the number of disease related events occurring during the follow-up period have been insufficient to adequately power the analysis (Huvila et al. 2013; Fanning et al. 2002). Studies have also frequently only reported the association between Ki-67 expression and survival, without stipulating whether this was all-cause or, the more clinically relevant, cancer-specific mortality (Salvesen et al. 1999; Geisler et al. 1999). Similar methodological problems to those seen in breast cancer have been evident as well, with heterogeneous staining and scoring protocols employed across the different studies. Cut-off values of between 10% and 40% Ki-67 expression have been used to denote high expression, determined either on the basis of the distribution of results observed within the same study or through the arbitrary application of cut-offs used in other cancer types, without validating these findings specifically in endometrial cancer (Geisler et al. 1999; Fanning et al. 2002; Liu et al. 2014b). Where multivariate analyses have been performed investigating the prognostic value of Ki-67 on endometrial cancer survival, there have been omissions of other important variables associated with outcome from the analysis, in particular patient age, the presence of lymphovascular space invasion and histological type (Stefansson et al. 2004; Liu et al. 2014b). Altogether these issues have prevented a meaningful meta-analysis from being performed.

Addressing these problems was the aim of a recent study by our own group, in which attention was paid to the staining and scoring of Ki-67 expression in endometrial cancer with the hope of producing standardised, robust guidelines to be used by all groups going forward (Kitson et al. 2017a). Using the International Ki-67 in Breast Cancer Working Group recommendations as a basis, analysis of hysterectomy specimens from 179 patients showed that: (i) whole slide scoring produced more consistent and reliable results than scoring of tissue microarrays (TMAs), (ii) semi-automated scoring was more time efficient and (iii) scoring of areas of greatest Ki-67 expression (hot spots) produced the most consistent results across endometrial biopsies and hysterectomy specimens. Using this optimised methodology, Ki-
67 expression was positively correlated with tumour stage, grade and depth of myometrial invasion and was a significant prognostic indicator of cancer-specific and recurrence-free survival in univariate analyses. A 10% increase in Ki-67 expression was associated with a 31% worsening of cancer specific survival (95% CI 7-60%, p=0.010), providing some context to the published results of endometrial cancer window studies (Sivalingam et al. 2016; Mitsuhashi et al. 2014). The low number of events in the study meant that there was a large degree of uncertainty around the effect size, however, and the relationship was lost in a multi-variate analysis due to the close association between Ki-67 expression and tumour grade. Unlike grade, though, the intra- and inter-observer agreement for Ki-67 expression was ‘almost perfect’, an essential requirement for a primary outcome measure in clinical trials.

Whilst Ki-67 expression has prognostic value in endometrial cancer, no studies have specifically addressed whether it is also of predictive value in determining the benefit for individual patients from adjuvant therapy or whether short-term reductions in cancer cell proliferation translate into improved long-term survival. Confirmation of this would validate the use of Ki-67 expression as a primary outcome measure in window studies.

1.2.5 Effect of hypoxia on expression of phosphorylated and apoptotic markers

The effect of a drug tested in the pre-surgical window period is determined through comparison of biomarker expression prior to and following treatment. In the case of studies conducted in women with endometrial cancer, this has traditionally meant that an endometrial biopsy performed either at the time of recruitment or shortly prior to enrolment in the study is studied alongside the hysterectomy specimen removed at the time of surgery when drug treatment ceases. This does, however, raise a methodological issue. An endometrial biopsy is able to sample the tumour in situ and, if immediately formalin fixed, is an accurate reflection of endometrial cancer cell biology. The hysterectomy specimen, in contrast, is subject to periods of warm ischaemia, once the uterine arteries are clamped during surgery and before the specimen is removed from the body, followed by cold ischaemia until formalin fixation occurs. Many of the biomarkers interrogated as outcome measures in window studies are activated and deactivated through phosphorylation and dephosphorylation, events which have been shown to be transient and particularly susceptible to hypoxia (Baker et al. 2005). Indeed, expression of phospho-Akt (protein kinase B) was noted to be completely absent from surgically resected specimens whilst remaining present in colorectal tumours sampled by biopsy, with loss of expression occurring as quickly as 20 minutes after the colorectal blood supply was interrupted (Baker et al. 2005). Similar results have been seen with pERK1/2 expression in breast cancer samples, with the size of the sample, and hence the time for formalin penetration and fixation to occur, noted to be of additional importance (Pihnel et al. 2010). Expression of oestrogen and progesterone receptors, in contrast, were unaffected by biopsy type or delays in fixation in breast cancer, as these proteins appeared more stable and resistant to the effects of hypoxia (Neumeister et al. 2012). The effect of biopsy type and hypoxia on expression of these markers in
endometrial cancer has not previously been studied, despite the marked difference in tumour sampling techniques employed at the beginning and end of window studies.

These findings call into question the results of earlier window studies of metformin in endometrial cancer (Mitsuhashi et al. 2014; Laskov et al. 2014), where decreased expression of phosphorylated and hypoxia sensitive biomarkers in the surgical specimen may have been incorrectly attributed to short-term drug treatment. When a contemporaneous control group was recruited for comparison, Sivalingam et al. (2016) found decreased expression of pAkt, pACC, pS6 and p4EBP1 (phospho-4E binding protein 1) in the hysterectomy specimen of both the metformin treated and untreated groups, suggesting that this may be related to the method of tumour sampling employed rather than an effect of the drug. Modification of the window study design to include a repeat endometrial biopsy after drug exposure and before commencement of the hysterectomy procedure is a means of avoiding the confounding effects of operative ischaemia and delays in tissue fixation.

1.3 Metformin in the treatment of endometrial cancer

1.3.1 Metformin and reducing cancer mortality—epidemiological evidence

Studies have suggested lower mortality rates for diabetic patients diagnosed with a malignancy if they are taking metformin compared with other hypoglycaemic medication. Similar results have been found in women with endometrial cancer, with several retrospective cohort studies being recently combined in a meta-analysis. Metformin use was associated with a significant reduction in overall mortality compared with non-metformin users (aHR 0.64, 95%CI 0.45–0.89, p=0.009), regardless of the indication for metformin treatment (Perez-Lopez et al. 2017). Whilst there was low to moderate heterogeneity of effect between the included studies, the uncontrolled nature of the observational study design meant that not only could differences in endometrial cancer treatment not be accounted for, there was also the risk of significant differences in the baseline characteristics of patients. As the nature of the variables used in the adjusted analysis was also not consistent between studies, there is considerable uncertainty around the precision of the above hazard ratio.

Whilst exposure to metformin may be associated with a reduction in overall mortality for women with endometrial cancer, the meta-analysis was unable to comment on whether this was due to improvements in cancer specific survival or a reduction in deaths from other causes. Metformin is known to reduce the prevalence of cardiovascular and renal disease in diabetic patients and to lower the mortality rate associated with these conditions, particularly in the obese (UK Prospective Diabetes Study (UKPDS) Group 1998). The reduction in mortality observed may, therefore, be due to improvements in general health rather than because of a direct effect of metformin on the tumour. Indeed, Arima et al. (2017) noted that whilst metformin users had improved survival for causes other than endometrial cancer, particularly
cardiovascular disease, there was no difference between diabetic patients that took metformin or other anti-diabetic medication in terms of cancer specific survival (HR 0.89, 95% CI 0.52-1.54). This also extended to disease recurrence, with no difference in progression free survival between diabetic metformin users and other diabetic patients, when propensity score matching was performed to adjust for potential confounders (Al Hilli et al. 2016).

Epidemiological studies are vulnerable to a number of biases often because of their retrospective nature, reliance on patient recall, incomplete medical records and failure to take into account the progressive nature of diabetes, which results in frequent alterations in drug therapy over time for individuals. Ascribing a difference in survival between groups of diabetic patients to a particular drug treatment can, therefore, be erroneous if this is only checked at baseline and not recorded again during a 10 year follow-up period. Any benefit from exposure to metformin on endometrial cancer may not necessarily lie in the diabetic population; these women are potentially already at a higher risk of death following a cancer diagnosis (Lindemann et al. 2015). Instead, the effect of metformin in non-diabetic women with endometrial cancer requires further investigation.

1.3.2 *In vitro* evidence of the anti-cancer effects of metformin

Preclinical studies have shown that metformin is able to influence cell division and function in a number of cell lines, including those derived from endometrial cancers. It can inhibit cell division through interruption of cell cycle progression and G1 phase arrest and is associated with an increase in expression of the cell cycle blocker p21 (Cantrell et al. 2010; Takahashi et al. 2014). This corresponds to a decrease in deoxyribonucleic acid (DNA) synthesis by the cancer cells (Xie et al. 2011). This effect is potentiated when metformin is used in combination with the chemotherapeutic drugs 5-fluorouracil, carboplatin and paclitaxel in oesophageal, ovarian and endometrial cancer cells, respectively, with an even greater decrease in the proportion of viable cells than when either drug is used alone (Erices et al. 2013; Honjo et al. 2014; Hanna et al. 2012).

In addition, metformin is able to influence hormone receptor expression within endometrial cancer cells, reducing expression of the pro-proliferative oestrogen receptor alpha and increasing expression of the more stabilising progesterone receptor at both the messenger ribonucleic acid (mRNA) and protein level (Zhang et al. 2011; Zhang et al. 2017; Xie et al. 2011). Co-treatment with progesterone in two women with atypical endometrial hyperplasia and PCOS has been shown to overcome previously noted progesterone resistance (Shen et al. 2008). A recent Cochrane review found that there was insufficient evidence, however, to determine whether combined treatment with medroxyprogesterone acetate and metformin was superior to medroxyprogesterone alone in reverting endometrial hyperplasia to normal endometrium (Clement et al. 2017). Only one small RCT randomising 16 women was suitable for inclusion in the review.
and the quality of evidence was judged to be very low due to very serious risk of bias and imprecision associated with a low event rate.

Metformin has also been shown to affect expression of hTERT mRNA, which encodes for a catalytic subunit of the telomerase enzyme necessary for cancer cells to undergo division (Cantrell et al. 2010). Whether this is solely as a consequence of cell cycle arrest or is an independent mechanism through which metformin influences cell proliferation is unknown, as it remains unclear whether hTERT transcription occurs throughout the cell cycle or only at certain points.

Beyond an effect on cell division, metformin is able to induce cell autophagy, a self-destructive process involving the removal of damaged proteins and organelles and inducing cellular senescence at times of nutrient depletion (Takahashi et al. 2014; Glick et al. 2010). It has also been shown to inhibit the invasion and migration capacity of endometrial cancer cells, thereby potentially preventing metastasis and disease recurrence (de Barros Machado et al. 2016; Laskov et al. 2016).

Metformin may also induce apoptosis, though conflicting results have been generated depending upon the different environmental conditions imposed in varying cancer cell types (Takahashi et al. 2014; Ben Sahra et al. 2008). Iglesias et al. (2013) found that the moderately differentiated Hec-1a endometrial cancer cell line was more sensitive to the anti-proliferative effects of metformin and underwent apoptosis in response to drug treatment compared with the well differentiated Ishikawa cell line. The presence of an activating K-ras mutation within the Hec-1a cells appeared to be critical as silencing with siRNA was shown to abolish the effect of metformin. The concentration of glucose within the cell environment also appears to impact on responsiveness to metformin treatment, as demonstrated in the pre-surgical window study in breast cancer undertaken by Cazzaniga et al. (2013). Here, there was a trend towards an increase in apoptosis, as measured by the TUNEL method, with metformin treatment but only in women who were insulin-sensitive. Those with a homeostatic model assessment-insulin resistance (HOMA-IR) >2.8 had a non-significant decrease in apoptosis following active drug treatment. This resistance to metformin under high glucose conditions has been used to provide an explanation for why concentrations of the drug of the order of 300-fold greater than the peak plasma concentration and an even higher magnitude than that found in endometrial tissues are required to observe effects on cancer cell function in vitro (Mitsuhashi et al. 2014). The media commonly used to support cell growth contains 4500mg/dl glucose, which is equivalent to diabetic serum glucose levels, and is thought to lead to a reduction in the responsiveness of cells to metformin treatment. In vivo experiments are, therefore, required to determine whether metformin does have any biological activity on endometrial cancer cell proliferation.
1.3.3 Animal evidence of the anti-cancer effects of metformin

There have been no animal experiments published on the impact of metformin treatment on endometrial cancer growth and progression, although it does appear to affect the endometrium (Erdemoglu et al. 2009). Metformin was found to reverse the effects of tamoxifen and oestrogen induced endometrial hyperplasia in oophorectomized mice, with a decrease in endometrial gland density, lowering of epithelial height and a reduction in mitogenic activity in epithelial, glandular and stroma cells. This effect appeared to be more pronounced in the obese, insulin resistant Zucker rats than their lean counterparts and metformin was able to inhibit the expression of the growth stimulatory genes c-myc and c-fos (Zhang et al. 2013a). These rats have a mutation in the leptin receptor, resulting in an increase in appetite and the development of obesity by 14 weeks of age as well as becoming hyperlipidaemic, hypercholesterolaemic and hyperinsulinaemic (Kava R. 1990). Whilst these animals are, therefore, a useful model to study the effects of the metabolic syndrome on the endometrium, they do not develop endometrial cancer.

Specific animal models of endometrial cancer that have been generated include human tumour xenografts in mice using Hec-1a cells and mice with selective uterine knockout of the DNA repair protein MLH1 and the tumour suppressors liver kinase B1 and PTEN, either singularly or in combination, using Cre-Lox recombination (Zheng et al. 2014;Cheng et al. 2014;Wild et al. 2012). This technology allows genes to be selectively turned off at specific organ sites, thereby circumventing embryonic lethality, which would otherwise occur if expression of these genes was silenced in all cells. By inserting the Cre recombinase into an exon of the progesterone receptor, gene knockout can be restricted to only those cells that express this receptor, such as those found within the endometrium. Tumour penetrance (i.e. the proportion of animals that develop endometrial cancer) can be up to 100%; however, the animals only survive for a short period of time. The PTEN mutation is also so dominant in driving endometrial hyperplasia that the addition of obesity has no effect on the extent of hyperplasia nor on proliferation or mammalian target of rapamycin (mTOR) pathway activity (Iglesias et al. 2017). It is perhaps not surprising, therefore, that metformin treatment similarly had no effect in this context.

More encouraging results have been noted in other cancer types. In breast, bladder and lung cancer animal models, metformin exposure has been associated with a reduction in tumour growth and burden, especially when administered intraperitoneally (Ma et al. 2014;Zhang et al. 2013b;Memmott et al. 2010). Data also support the synergistic action of metformin with endocrine agents, including tamoxifen (Ma et al. 2014). This, again, was associated with a reduction in cell proliferation and cell cycle progression, but not apoptosis, at least in NNK induced lung cancer cells (Memmott et al. 2010).
1.3.4 Window studies of the anti-cancer effects of metformin

The promising *in vitro* work in endometrial cancer and results of animal experiments in other malignancies have prompted investigation of the effect of metformin on endometrial cancer *in vivo*. As metformin is already in widespread clinical use for the treatment of diabetes, researchers have been able to proceed straight to phase II studies without repeating early stage testing of drug pharmacokinetics and safety. All of the studies conducted to date in endometrial cancer have utilised the window study design, allowing the rapid screening of metformin for evidence of efficacy. The six published studies are summarised in table 1-1.

All of these studies have included only small numbers of women and have lacked a placebo treated arm. The majority of studies have focussed solely on patients with endometrioid endometrial cancer, although two studies have included a proportion (<30%) of patients with non-endometrioid histology (Zhao et al. 2017; Laskov et al. 2014). Only the studies conducted by our own group (Sivalingam et al. 2016) and Zhao et al. (2017) included contemporaneous control groups for comparison. Two of the studies used retrospective controls (Mitsuhashi et al. 2014; Laskov et al. 2014), whilst the remaining two studies failed to include any control group at all (Soliman et al. 2016; Schuler et al. 2015). Five of the six studies found a reduction in endometrial cancer cell proliferation, as measured by immunohistochemical expression of Ki-67, with short-term metformin treatment (Mitsuhashi et al. 2014; Laskov et al. 2014; Sivalingam et al. 2016; Schuler et al. 2015; Zhao et al. 2017). Soliman et al. (2016) found no change in proliferation with metformin, but their median duration of treatment was only 10 days, which may be too short a time frame to observe any effect. Investigation of the potential mechanisms of action of metformin have produced more heterogeneous results, with some studies finding a reduction in signalling through the PI3K-Akt-mTor pathway with metformin treatment (Mitsuhashi et al. 2014; Laskov et al. 2014; Schuler et al. 2014; Zhao et al. 2017) and others finding no evidence of an effect (Soliman et al. 2016; Sivalingam et al. 2016). This is potentially related to the aforementioned differences in tumour sampling technique prior to and following intervention, specifically the effects of operative ischaemia and poor fixation of hysterectomy specimens.

Whilst these results are suggestive of a potential benefit from metformin treatment on endometrial cancer proliferation, they have failed to provide definitive proof because of the methodological issues described in box 1 and the lack of meaningful clinical outcome data.
Table 1-1 Window studies of metformin in endometrial cancer

<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of patients</th>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
<th>Placebo arm?</th>
<th>Treatment duration and dose</th>
<th>Primary outcome</th>
<th>Secondary outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitsuhashi et al. (2014)</td>
<td>31</td>
<td>Endometrioid adenocarcinoma</td>
<td>Diabetes, previous metformin use, abnormal blood coagulation, mental or life threatening illness</td>
<td>No. Retrospective control group used for comparison</td>
<td>4 weeks Metformin 750mg od increased by 750mg weekly to max of 2250mg/day</td>
<td>Proportional decrease in Ki-67 expression of 44.2% with metformin treatment (p&lt;0.001) and 36.4% decrease in topoisomerase IIα (p&lt;0.001). No significant change in retrospective control group</td>
<td>Increase in pAMPK, p27 and decrease in pS6, pERK1/2, cyclin D1</td>
</tr>
<tr>
<td>Laskov et al. (2014)</td>
<td>21</td>
<td>Endometrial cancer (all types), no previous treatment</td>
<td>Previous invasive malignancy, diabetes, previous metformin use</td>
<td>No. Retrospective historical control group used</td>
<td>3-6 weeks Metformin 500mg tds until 48 hours prior to surgery</td>
<td>Significant decrease in Ki-67 of 9.7% with metformin treatment (p=0.02). No change in control group</td>
<td>pS6 staining fell by 31% with metformin treatment (p=0.03) though pAMPK did not change</td>
</tr>
<tr>
<td>Schuler et al. (2015)</td>
<td>20</td>
<td>Endometrioid adenocarcinoma, 18-75 years, BMI ≥30, surgery 7-28</td>
<td>Diabetes, on metformin or insulin currently or in last six</td>
<td>No</td>
<td>1-4 weeks Metformin 850mg od until 24 hours before surgery</td>
<td>Significant decrease in Ki-67 expression with metformin treatment of 11.75%</td>
<td>Associated decreases in pAMPK, pAkt, pS6, p4EBP1 and ER expression.</td>
</tr>
<tr>
<td>Study</td>
<td>Duration</td>
<td>Patient Selection</td>
<td>Treatment</td>
<td>Follow-up</td>
<td>Findings</td>
<td></td>
<td></td>
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<tr>
<td>Soliman et al. (2016)</td>
<td>20 days</td>
<td>Days after enrolment months, history of alcoholism or B12 deficiency, pregnancy, hormonal treatment in last four weeks</td>
<td>No</td>
<td>7-24 days</td>
<td>No change in PR expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diabetes, currently taking hypoglycaemic medication, use of metformin or mTOR inhibitor in last 2 years, previous treatment for endometrial cancer, sensitivity to metformin</td>
<td>Metformin 850mg od until day before surgery</td>
<td></td>
<td>No difference in Ki-67 expression with metformin treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diabetes, fasting glucose ≤125mg/dl, creatinine clearance &gt;60cm³/min, serum bilirubin &lt;2.5mg/dl</td>
<td></td>
<td></td>
<td>No difference in pACC, pS6 or caspase-3 expression following metformin treatment. Significant decreases in pAkt and pERK1/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sivalingam et al. (2016)</td>
<td>40 days</td>
<td>Biopsy proven atypical endometrial hyperplasia or endometrioid endometrial</td>
<td>No. Contemporaneous control group recruited who were not</td>
<td>7-30 days</td>
<td>Significant decrease in Ki-67 in metformin treated group of 17.2% after adjustment for baseline Ki-67, age, Decreases in expression of pACC, pAkt, p4EBP1 and pS6 noted in both control and metformin treated</td>
<td></td>
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</tr>
</tbody>
</table>
controls not exposed to study drug)  | cancer, for surgical treatment  | progesterone treatment, window <7 days  | exposed to study drug  | BMI, insulin resistance and change in control group (p=0.002)  | groups resulting in no significant difference between the two

Zhao et al. (2017)  
65 (33 received metformin, 32 declined metformin and were used as contemporaneous controls)  | Histologically confirmed endometrial cancer, any stage, grade and histological type, no prior radio- or chemotherapy, no HRT use, normal renal and liver function tests  | Prior malignancy, diabetes treated with metformin or insulin, unstable angina, use of weight loss drugs, corticosteroids or drugs known to affect gastrointestinal motility, serious comorbid condition within last 3 months  | No. Contemporaneous control group composed of women who declined metformin treatment  | 3-4 weeks Metformin 500mg tds until 48 hours before surgery  | Significant decrease in proportion of patients with positive Ki-67 staining (not defined) with metformin treatment (p=0.03). No effect in untreated control arm  | Significant decreases in proportion of patients with positive PI3K, pAkt, pS6 and p4EBP1 staining with metformin treatment
**Box 1 Methodological issues associated with early window studies of metformin in endometrial cancer**

- Small numbers of recruited patients
- Short duration of treatment with metformin
- Lack of contemporaneous control group for comparison, with the exception of Sivalingam et al. (2016) and Zhao et al. (2017)
- Non-random treatment allocation, with the risk of differences in patient characteristics between groups
- Investigators not blinded to treatment allocation
- Investigators not blinded to biopsy time point
- Different tumour sampling methods prior to and following drug treatment
- Post-treatment sample exposed to long periods of hypoxia and potential delays in fixation resulting in loss of protein expression
- Often inadequate descriptions of immunohistochemical staining and scoring methods

Many of these limitations can only be addressed by conducting an adequately powered, placebo-controlled, double blind randomised trial. This would also provide an opportunity to investigate the effect of metformin in specific subgroups of patients, who, according to the breast cancer paradigm, may respond differentially to drug treatment. For example, metformin reduced breast cancer cell proliferation selectively amongst insulin resistant women and those with higher fasting glucose levels at baseline, with the percentage decrease in fasting glucose corresponding to the degree of change in Ki-67 expression (Bonanni et al., 2012, Niraula et al., 2012). Subgroup analysis of response to metformin in insulin resistant and sensitive women with endometrial cancer would, therefore, be a logical strategy to seek further insight into the pathways through which the drug acts.

### 1.3.5 Mechanism of action of metformin

Despite increasing evidence to support an anti-cancer effect for metformin, its mechanism of action is yet to be firmly established. Several different pathways have been proposed on the basis of pre-clinical work, but their applicability *in vivo* is still debated. These can be broadly divided into two main themes; an indirect effect of the drug mediated through a reduction in circulating insulin levels and a direct effect of metformin on the endometrial cancer cell (Dowling et al. 2012) (figure 1-1).

#### 1.3.5.1 Indirect mechanism of action

Metformin is actively transported into hepatocytes where it acts on mitochondria to inhibit complex I of the respiratory electron transport chain (Pernicova and Korbonits 2014). This leads to a reduction in the generation of ATP (adenosine triphosphate) and a subsequent increase in AMP (adenosine monophosphate) levels. AMP-activated protein kinase (AMPK) is activated through direct binding of AMP to its γ subunit driving a conformational change and phosphorylation of the catalytic α subunit. The end result of this is down regulation of gluconeogenesis (Pollak 2010). As a consequence, there
is a reduction in serum glucose and subsequently insulin levels, itself a potent stimulator of endometrial cancer cell proliferation.

Insulin exerts its effects on endometrial cancer cells through the insulin receptor (IR), a member of the transmembrane tyrosine kinase family (Ottensmeyer et al. 2000). As shown in figure 1-1, activation by ligand binding leads to phosphorylation and activation of insulin-receptor substrates (IRS), which in turn bind SH2-containing proteins downstream. Through these intermediaries, insulin is able to influence key cellular functions, including cell growth through activation of the MAPK/ERK pathway, regulate mTOR and protein synthesis through Akt, influence gene expression by controlling the transcription factor FOXO and regulate apoptosis through BAD (Siddle 2011). The specific pathway activated in the cell is dependent upon the specific insulin receptor to which the ligand binds. Two splice variants of the insulin receptor exist, differing by the presence (IR-B) or absence (IR-A) of 12 amino acid residues at the carboxyl terminus end of the alpha-subunit (Siddle, 2011). Receptor dimerization is required for ligand binding, meaning that three combinations of monomers are possible. IR-B binds only insulin and is thought to affect cellular metabolism, whereas IR-A also binds insulin-like growth factor-2 (IGF-2), a protein with similar function to insulin, and has a greater influence over cellular proliferation.

The importance of insulin in driving endometrial carcinogenesis has been highlighted by the findings of the Women’s Health Initiative Observation Study, where higher insulin levels were associated with an increased risk of developing endometrial cancer (Gunter et al., 2008). This association remained significant even after correction for BMI and oestradiol levels in non-hormone therapy users and was strengthened when analysis was restricted to overweight women or those with endometrioid histological subtype. Whether increased insulin levels are associated with more aggressive disease has yet to be evaluated.
Figure 1-1 Mechanisms of action of metformin

Insulin

IGF-1

IR

IRS

IR

PI3K

PTEN

PDK1,2

Akt

FOXO1

GSK3

Gluconeogenesis

Rheb

AMPK

TSC1,2

mTOR

mRNA

DICER

p53

Endometrial cancer cell

Inhibition of cell proliferation, protein synthesis and possible induction of apoptosis

Growth regulation

ROS production

MITOCHONDRIAL COMPLEX I

Liver

Gluconeogenesis

ATP

Cell survival

Glycogen synthesis

LKB1

SHC

Raf

MEK

MAPK/ERK

ROS production

NF-kB

S6

4EBP1

c-myc

Gene transcription

NF-kB

S6

4EBP1

c-myc

Inhibition of cell proliferation, protein synthesis and possible induction of apoptosis

Metformin
Certainly, endometrial cancers express higher total levels of insulin receptor mRNA than normal endometrium and endometrial cancer cell lines transfected with IR-A cDNA (complementary DNA) have an increased rate of proliferation compared with cells not overexpressing the receptor (Wang et al., 2013a). Increased expression of the phosphorylated, and hence activated, insulin receptor is associated with adverse prognostic features in endometrial cancer, particularly higher grade, more advanced stage disease, deeper myometrial invasion and lymph node metastases (Wang et al., 2012).

1.3.5.2 Direct mechanism of action

Beyond an indirect effect, metformin also appears to directly influence DNA transcription, protein synthesis and cell cycle regulation in cancer cells, with its transporter, Oct1, found to be present in endometrial cancer cell lines and primary endometrial tumours (Iglesias et al. 2013; Staley et al. 2016). The majority of evidence has focussed on the effect of metformin on AMPK, a major regulator of energy homeostasis and controller of protein synthesis and cell proliferation (Brown et al. 2013). As shown in figure 1-1, activation of AMPK by metformin results in phosphorylation of the tumour suppressor tuberous sclerosis complex 1 and 2 (TSC1 and 2) and leads to inactivation of the mTOR complex and a reduction in protein synthesis (Gwinn et al. 2008). AMPK is also able to inhibit signalling through the PI3K-Akt-mTOR pathway by influencing activity at the insulin and IGF-1 receptors (Ning and Clemmons 2010). Phosphorylation of IRS-1 by AMPK prevents activation of phosphoinositide-3-kinase (PI3K) and its target kinase Akt and hence reduces mTOR activity.

The importance of the PI3K-Akt-mTOR pathway in endometrial carcinogenesis has been identified through work performed by The Cancer Genome Atlas Research Network (Cancer Genome Atlas Research et al. 2013). Over 90% of MSI (microsatellite instability) and copy number low tumours had mutations within the PI3K pathway, dropping to 60% for copy number high tumours. These often take the form of activating PIK3CA and PIK3R1 mutations with associated loss of PTEN (phosphatase and tensin homolog) protein expression. This latter tumour suppressor acts to block PI3K signalling through dephosphorylation of phosphatidylinositol 3,4,5-triphosphate and prevents activation of Akt (Courtney et al. 2010). At a protein level, immunohistochemical expression of phosphorylated, activated Akt is increased in endometrial cancer compared with benign, cycling endometrium, with high levels associated with more advanced disease (Wang et al. 2012; Wahl et al. 2010; McCampbell et al. 2006). Inhibition of mTOR using rapamycin has been shown to translate into reduced growth of endometrial cancer cell lines and an increase in apoptotic cell death (Wahl et al. 2010). These results have resulted in increased interest in the use of mTOR inhibitors for the treatment of endometrial cancer, both singularly and in combination with other therapies (Myers 2013). Despite a strong scientific rationale, clinical results have been disappointing and discontinuation rates significant due to treatment-related toxicities (Makker et al. 2016; Del Campo et al. 2016). The disappointing effect of these drugs on progression-free and overall survival may well be a consequence of their action too far downstream to prevent signalling through other PI3K pathways. Interestingly, a subgroup of patients recruited into a study of combination therapy with everolimus (mTORC1 inhibitor) and letrozole for the treatment of recurrent and progressive endometrioid endometrial cancer found that women also
taking metformin had twice the clinical benefit of those not taking the drug (Myers 2013). Whether this was due to an enhancement of the effect of the mTOR inhibitor by metformin or whether it acted on other pathways or use was simply a marker of a group of patients who were more likely to respond to this class of drugs is not yet clear. Metformin has certainly been shown to affect the mTOR pathway in endometrial cancer cell lines, with increased pAMPK and reduced pS6 levels seen in response to treatment in a dose dependent manner in both cell lines and mouse xenograft models (Iglesias et al. 2013; Cantrell et al. 2010). Whether this occurs as a result of a direct effect of metformin on AMPK or is a response to alterations in AMP and ATP levels has not been clearly documented (Brown et al. 2010).

Metformin has been shown to influence other aspects of cell regulation in vitro through AMPK including increased expression of DICER (a RNase involved in mRNA degradation), down-regulation of the regulator genes c-myc and microRNA222 and activation of the tumour suppressor p53 (Blandino et al. 2012; Wang et al. 2013; He et al. 2014) [figure 1-1]. Different mechanisms of action are likely to be important in distinct cell types, however, as metformin selectively inhibited proliferation of a p53 negative colon cancer cell line but was ineffective against p53 negative prostate cancer cells (Buzzai et al. 2007; Ben Sahra et al. 2011). The extent to which any of these mechanisms are important in endometrioid endometrial cancer has yet to be determined, particularly as p53 mutations are seen much less frequently in this histological type (Cancer Genome Atlas Research et al. 2013).

AMPK activation is not the sole means through which metformin is able to influence protein synthesis and cell proliferation. Mouse embryonic fibroblasts in which AMPK has been knocked out continue to exhibit mTOR inhibition in response to metformin treatment, an effect mediated by Rag GTPases (Kalender et al. 2010). The MAPK/ERK transcriptional control pathway has also been shown to be directly inhibited by metformin in hepatocellular, lung and cervical carcinoma cell lines and at much lower concentrations than those required to influence the mTOR pathway (Do et al. 2013). A reduction in immunohistochemical expression of pERK1 and 2 has been similarly found in the endometrial tumours of patients treated with metformin as part of a window study (Mitsuhashi et al. 2014). Activation of the transcription factor nuclear factor kappa B also appears to be inhibited by metformin, resulting in a reduction in endometrial cancer cell invasion, which is unaffected by simultaneous exposure to an AMPK inhibitor (Tan et al. 2011). Additionally, metformin has been able to block endogenous reactive oxygen species production and DNA damage in both AMPK positive and negative mouse embryonic fibroblasts and in a mouse model exposed to paraquat (Algire et al. 2012). This occurred due to an effect on complex I of the respiratory electron transport chain. The importance of these alternative mechanisms of action is unknown.
1.4 Metformin as an adjuvant therapy in endometrial cancer

1.4.1 Concept of cancer stem cells

The cancer stem cell theory holds that tumours are derived from a small number of cells that have acquired capacity for self-renewal and are able to differentiate into multiple different cell types (Pardal et al. 2003). These ‘tumour initiating cells’ can either be normal stem cells within tissues that have acquired mutations resulting in their uncontrolled expansion or, as is more likely to be the case, progenitor cells that have gained the ability to self-renew. Their presence explains why only a limited number of cells are able to form new tumours when transplanted into animal models and accounts for intra-tumoral heterogeneity as one or more cancer stem cells differentiate into mature progeny recapitulating the original cancer. Indeed, these cells appear to not only be responsible for ensuring a pool of cancer stem cells but also for maintaining the tumour bulk, composed of more differentiated cells with a finite life span. The WNT, BMI1, PI3K and Hippo signalling pathways appear critical to the regulation of normal stem cell self-renewal, with dysregulation of these pathways associated with the development of many of the characteristics of cancer stem cells (Reya et al. 2003; Park et al. 2003; Camargo et al. 2007; Zhou et al. 2007). Cells with the characteristics of cancer stem cells have been identified in endometrial cancer cell lines, including Ishikawa and Hec-1a, as well as primary endometrial tumours (Friel et al. 2008; Kato et al. 2010; Rutella et al. 2009). Since being first recognised in the 1960s, there has been increasing focus on methods of identifying and separating cancer stem cells from the bulk tumour population in order to further study their unique characteristics.

These have been broadly divided into two main themes; (i) identification of cells through the expression of cell surface markers and enzyme activity and (ii) the use of functional assays. Whilst cancer stem cells appear to have their own specific profile dependent upon their organ of origin, there are several markers which appear to cross-over and be common to many tumour types. These include CD133 and aldehyde dehydrogenase (ALDH) activity.

CD133, a glycosylated pentaspan membrane protein thought to be involved in organisation of the plasma membrane, has been noted to be differentially expressed in embryonic and adult tissues (Mizrak et al. 2008). Expression of the glycosylated form of the protein is restricted to undifferentiated cells, with loss of expression appearing to promote commitment to differentiation. A population of CD133 expressing cells has been demonstrated to reside within primary endometrial tumours, which have many of the characteristics associated with cancer stem cells; namely the ability to form colonies under adherence free conditions, increased cloning efficiency when plated at a single cell level demonstrating capacity for self-renewal, multipotency forming both CD133+ve and 133-ve daughter cells and increased tumorigenic potential when injected into immunodeficient mice (Rutella et al. 2009; Nakamura et al. 2014; Ding et al. 2017; Friel et al. 2010). There is limited evidence that these cells also appear to express genes associated with embryonic stem cells, including Sox2, Nanog and Oct4, to a greater extent than their CD133-ve counterparts (Ding et al. 2017). These findings were
Based on cells isolated from a single endometrial tumour and should be viewed with this in mind. Based on more robust evidence, CD133+ve cells appear to have increased capacity for invasion and migration (Nakamura et al. 2014), suggesting a role in the metastatic spread of disease, and are resistant to commonly used chemotherapeutic drugs including cisplatin and paclitaxel (Rutella et al. 2009;Ding et al. 2017). Persistence of these cells following adjuvant treatment is thought to explain disease relapse, highlighting a need to target therapies at cancer stem cells. Given the weight of pre-clinical evidence supporting CD133 as a marker of endometrial cancer stem cells, it is perhaps noteworthy that immunohistochemical expression of the protein within endometrial tumours does not correlate with pathological prognostic variables, including grade and histological subtype, or recurrence free survival and that lower expression is seen in patients with more advanced stage disease and lymph node metastases (Rutella et al. 2009;Elbasateeny et al. 2016;Nakamura et al. 2010). This would suggest that whilst CD133 identifies one group of cells with cancer stem properties, it is not the sole marker of this cell type. This is supported by the finding of at least some tumour development and growth in mouse xenograft models injected with CD133-ve cells (Rutella et al. 2009).

ALDH is a detoxifying enzyme responsible for oxidising aldehydes (Ginestier et al. 2007). It is thought to have a role in the early differentiation of stem cells through the oxidisation of retinol to retinoic acid. Increased ALDH activity has been described in normal haematopoietic and neural stem cells as well as cancer stem cell populations, including multiple myeloma, breast and lung cancer (Dave and Chang 2009;Jiang et al. 2009). ALDH activity is determined by measuring the levels of its product within cells (STEMCELL technologies 2017). BODIPY-aminoacetaldehyde (BAAA) is a fluorescent, non-toxic substrate for ALDH which, in the presence of the enzyme, is converted into BODIPY-aminoacetate (BAA). This is retained within the cell and the amount of fluorescent product produced can be measured by flow cytometry. Endometrial cancer cells with high ALDH activity have been shown to have increased colony, organoid and tumour forming capacity, be more invasive and to be resistant to the induction of cell death by cisplatin treatment (Rahadiani et al. 2011;van der Zee et al. 2015). Unlike CD133, immunohistochemical expression of ALDH has been shown to correlate with tumour size, lymph node metastasis and both overall and disease-free survival, with patients in the high (>10%) expressing group having a 3.65 fold (95%CI 1.03-13.0, p=0.045) worse disease-free survival compared with patients with low expressing tumours (Rahadiani et al. 2011). These findings were based on tumours, however, from only 98 women, in whom there were 22 recurrences and 14 disease related deaths and used the cut-off values employed in lung cancer studies to denote high and low ALDH expression. The relationship between CD133+ve and ALDH\textsuperscript{high} endometrial cancer cells and their relative cancer stem activity is currently unknown.

The other broad method of identifying cancer stem cells and determining their activity is through the use of functional assays, including the formation of three-dimensional ‘spheres’ in adherent free conditions, which would normally be associated with cell anoikis (Shaw et al. 2012;Shaker et al. 2017). Passage of these spheres can then be used to determine the effect of treatments on cancer
stem cell self-renewal. Whilst this standardised protocol for the assessment of sphere formation efficiency is used widely in other cancer types, it has not previously been used in endometrial cancer. Instead, groups have used differing methods preventing direct comparison of results. The gold-standard assay of cancer stem cell activity, however, is in vivo transplantation of human cells into immunocompromised mice to directly assess their tumour initiating capacity. Both ALDH$^{\text{high}}$ and CD133+ve cells form tumours in mice at low seeding densities (10-100 cells) in contrast to non-stem cancer cells, where thousands of transplanted cells are required for tumours to form (Rahadiani et al. 2011; Nakamura et al. 2010).

Despite increasing awareness of the importance of cancer stem cells in disease relapse and metastasis (Eyre et al. 2016), there have been relatively few specific therapeutic options examined to target these cells in endometrial cancer. There is some evidence to suggest, though, that metformin may be of value in this regard.

### 1.4.2 Metformin as an adjuvant therapy-epidemiological evidence

Interest in metformin as an adjuvant therapy has stemmed from epidemiological data suggesting that it may act as a chemo- and radiosensitiser, improving the outcomes of patients with breast and colorectal cancer (Jiralerspong et al. 2009; Skinner et al. 2013). Women receiving neoadjuvant chemotherapy for stage I-III breast cancer as part of a clinical trial were found to have significantly improved pathologic complete response rates if they were diabetic and receiving metformin compared with diabetic non-users and non-diabetic patients (Jiralerspong et al. 2009). Similarly, diabetic patients with rectal adenocarcinoma had a 35% pathological complete response rate at the time of surgery if they were exposed to metformin alongside neoadjuvant chemoradiation compared with 16.6% for non-diabetic patients and 7.5% for diabetic patients not receiving metformin ($p=0.03$) (Skinner et al. 2013). This corresponded with significant improvements in disease free and overall survival in metformin users and a trend towards a reduction in distant site recurrence.

A similar effect has been found in endometrial cancer. Diabetic metformin users with stage III-IV and recurrent endometrial cancer who were receiving chemotherapy were found to have a 1.6-fold increase in median overall survival compared with non-diabetic patients, which increased to a 3.6-fold increase over diabetic patients not exposed to metformin ($p=0.006$) (Ezewuiro et al. 2016). After adjustment for stage and age, metformin use was independently associated with a significant improvement in overall survival in diabetic patients. This study does have its limitations, however, in particular its size, including only 58 diabetic patients, assessment of overall rather than cancer-specific survival and its retrospective design. It, therefore, relied upon medical records for diagnoses of diabetes and medication use and did not adjust for changes in diabetes treatment during follow-up.
1.4.3 Pre-clinical evidence of the effect of metformin on cancer stem cells

Evidence of an effect of metformin on cancer stem cells has been described in breast cancer (Hirsch et al. 2009). Here it was found to reduce sphere formation and decrease in vivo tumour formation in a mouse xenograft model. Metformin appeared to act synergistically with doxorubicin reducing tumour growth to a greater extent than when either drug was used alone. The result was prolongation of disease remission, with all mice remaining tumour-free at two months following combination treatment. Similar results have been described in lung, prostate and ovarian xenograft models and with other chemotherapeutic drugs including platinum agents (Iliopoulos et al. 2011; Shank et al. 2012). Metformin appears to have a selective effect on cancer stem cells, reducing ALDH$^{\text{high}}$ ovarian cancer cells and decreasing the proportion of CD133$^+$ pancreatic cells at a concentration (0.05mM) which did not affect proliferation of whole tumour cell lines (Shank et al. 2012; Gou et al. 2013). It also reduced the sphere forming ability of oesophageal cancer cells to a greater extent in those formed from ALDH$^{\text{high}}$ compared with ALDH$^{\text{low}}$ (Honjo et al. 2014). These results have been supported by evidence of a decrease in expression of cancer stem cell related genes, including those involved in determining pluripotency, self-renewal and the Hippo pathway, critical to the regulation of tissue specific stem cells (Honjo et al. 2014; Courtois et al. 2017; Mo et al. 2014). Metformin decreased expression of Sox2, Oct4 and YAP in gastric and oesophageal cancer cell lines, though particularly high concentrations of the drug (5-10mM) were required to observe an effect.

Despite interest in metformin in other aspects of the management of endometrial cancer, there has been no investigation of the effect of the drug on endometrial cancer stem cells in vitro or in women using the window study design. Despite this, there is an ongoing clinical trial in which women with stage III-IV and recurrent endometrial cancer are being randomised to carboplatin and paclitaxel with or without additional metformin (Clinical Trial.Gov 2014). The primary outcome of the trial is progression free and overall survival. Evaluation of the in vivo effect of metformin on the expression of genes and proteins associated with endometrial cancer stem cells in this context would be highly informative.

1.5 Metformin to improve endometrial cancer survival

1.5.1 Causes of death in endometrial cancer survivors

Despite the excellent prognosis for the majority of patients diagnosed with endometrial cancer, such women have a higher mortality rate than the general population (Felix et al. 2017c). Women diagnosed with endometrial cancer have a 15.9-fold (95%CI 15.8-16.0) increased risk of death from all-causes compared with women without a history of the disease according to data obtained from the Surveillance, Epidemiology and End Results Program (SEER) database in the USA. The excess mortality, whilst present in all age groups, is more pronounced in younger women. Rather than dying from their endometrial cancer, however, these women are at markedly increased risk of death from cardiovascular disease, with an almost nine times higher cardiovascular mortality rate than the
general population (Felix et al. 2017b). Overall the risk of death from myocardial infarction and stroke is twice that of the risk of death from endometrial cancer, especially so for those with early stage disease of endometrioid histological subtype (Felix et al. 2017b; Ward et al. 2012). The proportion of cardiovascular related deaths increases also with time from diagnosis; this explains the discrepancy with findings in the MRC ASTEC trial of pelvic lymphadenectomy in early stage endometrial cancer where disease related deaths predominated but median follow-up was only 37 months (The writing committee on behalf of the Astec study group 2009).

That cardiovascular disease is the commonest cause of death in women with a history of endometrial cancer could perhaps have been foreseen given the presence of shared risk factors; namely obesity and diabetes (Stocks et al. 2015). Obesity drives endometrial carcinogenesis by creating an environment of unopposed oestrogen, inflammation, insulin resistance and hyperinsulinaemia (Kitson et al. 2017b; Crosbie et al. 2010; Renehan et al. 2016). Diabetes increases the risk of endometrial cancer two-fold, even after adjustment for BMI, suggesting an independent relationship between the two conditions. Obesity and diabetes are also the two most significant risk factors for cardiovascular disease and contribute to an increased risk of mortality from myocardial infarction and stroke (Bhupathiraju and Hu 2016).

This association between obesity, diabetes and cardiovascular disease may well explain the rising all-cause mortality rate associated with endometrial cancer (Kitson et al. 2017b; Kitson et al. 2018b). Targeting obesity and insulin resistance following a diagnosis of endometrial cancer could, therefore, reduce cardiovascular disease risk and improve overall and cardiovascular-specific survival in this group of women. It may also impact on disease recurrence and endometrial cancer specific survival if results from breast and colorectal cancer studies can be extrapolated to this disease type (Morey et al. 2009; Rock et al. 2013; Stolley et al. 2009).

1.5.2 Strategies to improve overall survival
Promoting weight loss in overweight and obese endometrial cancer survivors would be an obvious approach to try and improve survival. It is, therefore, disappointing that there is rather limited evidence as to its effectiveness as revealed in the recent Cochrane review (Kitson et al. 2018a). Even in breast cancer, where weight loss following treatment has been shown to improve biomarkers associated with disease recurrence, including oestradiol, adiponectin and inflammatory cytokines, there are no published trials of the effect of weight loss interventions on clinical outcomes (Irwin et al. 2015; Rock et al. 2013). Two RCTs of weight loss interventions in breast cancer survivors have completed recruitment, however, and are due to report shortly on the impact of their intervention on five-year disease recurrence and disease-free survival (ClinicalTrials.gov 2018; Villarini et al. 2012). Weight loss is certainly associated with a reduction in overall mortality in obese individuals (Ma et al. 2017). Estimates of the likely benefit from dietary and physical activity modification on cardiovascular mortality are less robust due to the small number of events in the trials eligible for inclusion in the meta-analysis and difficulties in obtaining cause-specific mortality data. Moderate quality evidence
suggested no significant effect of weight loss strategies based on lifestyle modification of at least one year duration on cardiovascular mortality (RR [relative risk] 0.93, 95%CI 0.67 to 1.31, p=0.81). Neither did they appear to impact on the risk of new cardiovascular events (RR 0.93, 95%CI 0.83 to 1.04, p=0.83). The amount of weight loss was relatively small, however, with a mean difference after one year of -3.42kg (95%CI -4.09 to -2.75kg) decreasing to -2.56kg (95%CI -3.50 to -1.62kg) after three or more years. There was also significant heterogeneity between trials due to the wide variation in weight loss regimes used, which were often poorly described. There is evidence demonstrating a reduction in the risk and number of myocardial infarctions and strokes in individuals who have undergone bariatric surgery compared to obese non-surgical controls, though, potentially due to the larger and more sustained weight loss that is usually achieved with surgery (Colquitt et al. 2014; Kwok et al. 2014).

Reducing insulin resistance and improving glycaemic control in women who are already diabetic would also reduce cardiovascular disease event frequency, though, again, whether this improves survival has yet to be definitively proven (Hayward et al. 2015). Individuals randomised to tight glycaemic control had 8.6 fewer major cardiovascular events per 1000 person-years than those receiving standard care. Weight loss is a central component of diabetes prevention but metformin too is effective in not only preventing but also treating hyperglycaemia (Knowler et al. 2002; Duckworth et al. 2009). A combination strategy incorporating both of these treatment options could be a useful approach to reducing the risk of fatal cardiovascular events in women with a history of endometrial cancer. Whether improvements in glycaemic control impact specifically on survival in cancer patients and, in particular, those with a history of endometrial cancer, is as yet unknown.

Whilst obesity and diabetes account for the majority of cardiovascular disease risk, there are additional risk factors whose modification may have a notable impact on cardiovascular disease mortality (Bitzur 2011). The introduction of statins for the treatment of hypercholesterolaemia reduces the incidence of myocardial infarctions and strokes and improves mortality in individuals both with confirmed diabetes and non-diabetic hyperglycaemia. Treatment of hypertension reduces the frequency of major cardiovascular events as well as mortality, with every 10mmHg decrease in systolic blood pressure being associated with 20% (95%CI 17 to 23%) reduction in fatal and non-fatal myocardial infarctions, strokes, heart failure and sudden cardiac death (Ettehad et al. 2016). Smoking cessation similarly improves cardiovascular mortality and is of benefit even in individuals over the age of 60 years (Mons et al. 2015).

A multifaceted approach incorporating weight loss, treatment of hyperglycaemia, hypercholesterolaemia, hypertension and smoking cessation aimed at reducing the risk of cardiovascular disease in women with a history of endometrial cancer would, therefore, be a logical next step. Despite this, such an approach has not been trialled. Indeed, even estimates of the likely clinical and cost benefits from screening and treating risk factors in terms of lowering cardiovascular event frequency have not been performed. This is required before a systematic programme of
cardiovascular risk factor identification and modification is introduced into routine oncological follow-up for women with endometrial cancer.

1.5.3 Cardiovascular risk assessment

The accurate determination of an individual's risk of cardiovascular disease is critical, therefore, in not only identifying those most in need of risk factor modification but also in quantifying the benefit derived from such treatment. In the UK, the QRISK2 score has been universally introduced into primary and secondary care on the advice of The National Institute for Health and Care Excellence (NICE) to be used for those without a prior history of cardiovascular disease (NICE 2016). Risk estimates are calculated online based on variables including age, sex, blood pressure, height, weight and the presence of significant co-morbidities such as diabetes. Whilst this calculator has been specifically validated in the UK population, the variables included are similar to those incorporated in other risk assessment tools, including the Framingham cardiovascular risk calculator. Comparison with results from other countries is, therefore, possible. The specific advantage to using the QRISK2 calculator, however, is that it accounts for interactions between ethnicity, deprivation (as measured by residential postcode), family history and significant cardiovascular disease risk factors (Hippisley-Cox et al. 2008). Such risk assessment tools can be used longitudinally to evaluate the impact of interventions, such as weight loss, which potentially modify multiple individual risk factors simultaneously on overall cardiovascular risk.

1.6 Summary and research question

The increasing incidence of endometrial cancer in response to the global rise in obesity rates has made this condition a significant concern for the UK population and health service. New treatments are required for women unfit for or unwilling to undergo standard surgical management and to improve disease-free and overall survival. Epidemiological evidence has suggested a potential role for metformin in this regard, with use in diabetic patients associated with improved survival. This has been supported by in vitro evidence of both an indirect effect of the drug on cancer cells through improvement in insulin sensitivity and a direct effect on both bulk and cancer stem cells, whereby it is able to influence protein synthesis, cell cycle regulation, migration and possibly apoptosis through multiple pathways. Its effect on the central cellular metabolic regulator AMPK and the mTOR pathway may well be the most important mechanism of action in endometrial cancer cells. Small, uncontrolled window studies have shown a reduction in proliferation in endometrial tumours with short term metformin exposure. Methodologically rigorous testing of the drug for efficacy is required, however, before metformin can be introduced into routine oncological practice for the treatment of endometrial cancer and the prevention of disease recurrence.
1.6.1 Hypotheses

The hypotheses of this study were:

1. Short-term metformin treatment would be associated with a reduction in the proliferation of endometrial cancer cells and that this would be due to inhibition of the PI3K-Akt-mTOR and insulin signalling pathways and an increase in apoptosis

2. Metformin could have a specific and selective effect on endometrial cancer stem cells (and may be of use as maintenance therapy to reduce the risk of disease recurrence)

3. Women with newly diagnosed endometrial cancer are at greater risk of cardiovascular disease than the general population (and metformin could, therefore, be used as part of a multipronged approach to improve overall survival in this group)

1.6.2 Aims and objectives

This study aimed to determine the short-term biological effect of metformin in endometrioid endometrial cancer and atypical hyperplasia and to quantify the potential impact of screening and treating cardiovascular risk factors in women newly diagnosed with endometrial cancer.

The objectives included:

1. Optimising the design of the pre-surgical window study in endometrial cancer to ensure the generation of methodologically rigorous results

2. Conducting a randomised controlled trial of metformin versus placebo using the optimised window study design in women with endometrioid endometrial cancer or atypical endometrial hyperplasia to determine the short term effect of metformin on endometrial cancer cell proliferation

3. Determining the in vivo impact of metformin on the PI3K-Akt-mTOR, insulin signalling and apoptotic pathways in endometrial tumours and the preferential mechanism through which it exerts its effects

4. Investigating response to metformin treatment in specific subgroups, including obese and insulin resistant women

5. Characterising endometrial cancer stem cells using functional assays and marker and gene expression and investigating the effect of metformin on these cells in vitro

6. Determining the true prevalence of cardiovascular risk factors in women with newly diagnosed endometrial cancer compared to the general population and the potential impact of universal screening and treatment of modifiable risk factors on 10-year cardiovascular disease risk
Chapter 2  Optimisation of window study endpoints

2.1 Introduction
The accurate quantification of clinically significant biomarkers prior to and following drug treatment is critical to the success of pre-surgical window studies. Interruption to the blood supply of specimens during the surgical procedure results in hypoxia, with as little as 10 minutes of anoxia being sufficient to induce significant biochemical alterations (Srinivasan et al. 2002). Phosphorylated markers, such as those of the PI3K-Akt-mTOR pathway, are particularly susceptible to hypoxia and expression can be markedly altered within 20 minutes of vascular clamps being applied (Baker et al. 2005). This can be exacerbated by delays in fixation of specimens; chronic ischaemia leads to protein degradation which can be halted by formaldehyde forming stabilising cross-links with proteins (Thavarajah et al. 2012). The size of the sample, and hence time it takes for penetration of formalin into the specimen, is critical to ensuring preservation of protein expression (Pinhel et al. 2010). Reliance on large surgical specimens, therefore, for a readout of tumour biology could be risky (Mann et al. 2005).

This study, therefore, sought to compare an endometrial biopsy taken immediately prior to the start of surgery with tumour from the hysterectomy specimen for the expression of biomarkers commonly used in window studies, including Ki-67, phosphorylated markers of the PI3K-Akt-mTOR and insulin signalling pathway, oestrogen and progesterone receptors and the cancer stem cell markers ALDH and CD133, to determine if there was a notable difference in immunohistochemical expression between the two samples. The degree of hypoxia and the extent to which this was responsible for any differences in protein expression was quantified through expression of the hypoxia markers HIF-1α and CA-IX.

2.2 Materials and methods

2.2.1 Patient and tissue selection
Tumour tissue was sampled from women recruited into a placebo-controlled, randomised trial of metformin prior to hysterectomy for the treatment of atypical endometrial hyperplasia or endometrioid endometrial cancer. Full details of the clinical trial are provided in chapter 3. Samples included matched endometrial biopsies, which were obtained immediately prior to hysterectomy with a vacuum aspiration device as well as representative tumour blocks from the hysterectomy specimen. All women participating in the trial were eligible for inclusion regardless of the pre-surgical treatment they had been randomised to receive.

The endometrial biopsies were immediately formalin fixed in theatre before being embedded in paraffin. The hysterectomy specimens were either immediately placed in formalin or were transferred dry to the pathology department for directed tumour biopsy before being fixed and paraffin embedded. Four micrometre whole sections were cut using a microtome and mounted onto a histological glass
slide before immediately undergoing Ki-67 immunohistochemical staining. This slide preparation technique had been previously identified by Kitson et al. (2017a) (appendix 3) as the most reliable and reproducible method for quantifying tumour proliferation. An experienced gynaecological histopathologist reviewed haematoxylin and eosin stained slides from all endometrial biopsies and hysterectomy specimens and marked representative areas of tumour from which triplicate cores were taken to construct tumour microarrays (TMA). These TMAs were created to conserve tumour and were subsequently used for all immunohistochemical staining, except Ki-67, where whole slides were used.

2.2.2 Immunohistochemistry

Immunohistochemistry was performed using the Leica Bond Max (Leica Biosystems, Wetzlar, Germany) with heat induced epitope retrieval, unless otherwise stated. Primary antibody detection was performed using the Refine Detection Kit (Leica Biosystems), which contains a rabbit anti-mouse IgG secondary antibody and anti-rabbit poly-HRP IgG antibody and utilises 3,3’-diaminobenzidine as a chromogen. Counterstaining of slides was performed using haematoxylin. Negative (isotype) and appropriate positive controls were used for each antibody.

2.2.2.1 Ki-67

Staining of whole slides for Ki-67 expression was performed in accordance with the previously described protocol (Kitson et al. 2017a). In brief, antigen retrieval was performed at pH9 for 20 minutes and a 30 minute casein block was applied to reduce non-specific antibody binding. Slides were incubated with the MIB-1 antibody (X0931 Dako, Carpinteria, CA) at a dilution of 1:100 for one hour at room temperature.

2.2.2.2 Phosphorylated markers (pAkt, p4EBP-1, pIR and pIGF1R)

Expression of phosphorylated markers of the PI3K-Akt-mTOR and insulin/IGF1 signalling pathways was measured using the TMAs. Antigen retrieval was performed at pH6 (pIGF1R) or pH9 (p-Akt, p4EBP-1, pIR) for 20 minutes in the absence (pAkt) or presence (p4EBP-1, pIR, pIGF1R) of a 30 minute casein block. Primary antibody incubation was performed for one hour at room temperature with the following antibodies; pAkt (Ser473, #4060) at dilution 1:50, p4EBP-1 (Thr 37/46, #2855) at dilution 1:800, pIR (Y1361, ab60946) at dilution 1:1000 and pIGF1R (Y1161, ab39398) at dilution 1:50. Antibodies raised against pAkt and p4EBP-1 were obtained from Cell Signalling (Beverley, MA, USA) and against pIR and pIGF1R from Abcam (Cambridge, UK).

2.2.2.3 Markers of cancer stem cell activity (CD133 and ALDH)

Expression of markers of cancer stem cell activity (CD133 and ALDH) was also determined using the TMAs. Antigen retrieval was performed at pH6 (CD133) or pH9 (ALDH) for 20 minutes followed by a 30 minute casein block. Primary antibody incubation was performed for one hour at room temperature with either CD133 (#130-090-422, Miltenyi Biotec, Surrey, UK) at dilution 1:25 or ALDH (611194, BD Biosciences, Oxford, UK) at dilution 1:100.
2.2.2.4 Hormone receptors (Oestrogen receptor and progesterone receptor)

Immunohistochemistry to determine the expression of oestrogen (ER) and progesterone receptors (PR) was performed in the clinical histopathology laboratory at Manchester University NHS Foundation Trust using a standardised protocol and the TMAs. In brief, staining was performed using the automated Ventana BenchMark ULTRA IHC/ISH Staining Module (Ventana, Tucson, AZ, USA), using heat induced epitope retrieval and an EDTA buffer (pH 8.4). Slides were incubated with ultraviolet inhibitor blocking solution before being exposed to the primary antibody (anti-ER [SP1], # 790-4324, anti-PR [1E2] #790-2223, both from Ventana, Tucson, AZ, USA, and pre-diluted to optimal concentration) for 20 minutes at room temperature. Antibody detection was performed using a horseradish peroxidase linked secondary antibody, DAB chromogen and substrate and copper enhancer. Haematoxylin and a blueing reagent were used for counterstaining.

2.2.2.5 Hypoxia markers

The effect of hypoxia on expression of the above markers was quantified through the immunohistochemical detection of the transcriptional regulator, hypoxia inducible factor-1 alpha (HIF-1α) and its target protein, carbonic anhydrase-9 (CA-IX), on the TMAs. Antigen retrieval was performed at pH9 for 40 and 20 minutes, respectively, before slides were incubated with the primary antibody (HIF-1α BD 610959, BD Biosciences, Oxford, UK, dilution 1:50, CA-IX NB100-417, NOVUS Europe/UK, Abingdon, UK, dilution 1:2000) for one hour.

2.2.3 Scoring using Definiens Developer

The digitisation of slides was performed using the Leica SCN400 Slide Scanner (Leica Microsystems, Wetzlar, Germany). Semi-automated scoring was undertaken by applying an optimised computer algorithm to manually selected malignant endometrial cancer glands using Definiens Developer software. Glands were manually compared prior to and following application of the solution to ensure the correct classification of cells as positively or negatively stained by two independent researchers.

2.2.3.1 Whole sections

Quantification of Ki-67 expression was performed using whole sections, with all nuclei considered positive regardless of staining intensity. Scoring was performed by manually selecting the three areas of greatest Ki-67 expression (hot spots) across the slide at x10 magnification (figure 2.1a-d), ensuring at least 2000 nuclei were scored and expressed as the percentage of positively stained nuclei.

2.2.3.2 TMAs

All malignant glands within each of the triplicate cores were scored in their entirety (figure 2.1e-g). With the exception of the cancer stem cell activity markers, CD133 and ALDH, staining was quantified using an H-score, which takes into account staining intensity (0=none, 1=weak, 2=moderate, 3=strong) and the percentage of cells at each staining intensity and has a maximum value of 300. Staining was assessed when present either within the nucleus only (ER, PR, HIF-1α), nucleus and cytoplasm (pAkt, p4EBP1) or at the cell membrane and/or cytoplasm (pIR, pGF1R, CA-IX). CD133
and ALDH expression were scored as the percentage of cells with positive apical membrane or cytoplasmic staining, respectively, regardless of staining intensity.
Figure 2-1 Scoring of whole slides and TMAs using the semi-automated Definiens Developer software (a, b x1 magnification, c, d x20 magnification, e-g x4 magnification). Whole sections stained with MIB-1 antibody (a) were used to determine Ki-67 expression. The three areas of greatest Ki-67 expression, referred to as hot spots, were manually selected (b) before the optimised solution was applied (c and d). TMAs were used to determine expression of all other markers, with a representative core stained with the ALDH antibody shown in e). All malignant glands within the core were selected (f) and the specific optimised solution applied (g). B and f orange-malignant glands, dark blue-stroma, pale blue-white space, d and g yellow-positively stained cells, blue-negatively stained cells.
Figure 2.1 cont.
2.2.4 Data collection
Demographic and pathological data were collected by interview and from paper and electronic medical records.

2.2.5 Statistical analysis
Data were summarised using means and standard deviations. Immunohistochemistry scores of the matched endometrial biopsies and hysterectomy specimens were compared using the Wilcoxon signed-rank test. Normally distributed data were compared using the student T-test with Pearson’s correlation coefficient used to examine the relationship between ordinal variables. A p-value ≤0.05 was considered statistically significant; asterisks were used to denote significant results as *p≤0.05, **p≤0.01, ***p≤0.001 and ****p≤0.0001. The statistical analysis was conducted using SPSS version 23, Stata version 14 and Graph Pad Prism 7.

2.3 Results
In total, matched endometrial biopsies and hysterectomy specimens of sufficient size and quality to be suitable for analysis were available from 75 women.

2.3.1 Effect of tumour sampling technique on commonly used biomarkers in endometrial cancer window studies
Ki-67 expression was significantly lower in the hysterectomy specimen compared with the corresponding endometrial biopsy (p<0.0001, figure 2-2). The mean Ki-67 expression in the endometrial biopsies was 41.8% (SD 18.6%) compared with 33.2% (SD 18.7%) in the hysterectomy specimen.
Figure 2-2 Ki-67 expression in endometrial biopsies and corresponding hysterectomy specimens. There was a significant difference in Ki-67 expression between the endometrial biopsy and its matched hysterectomy specimen, with expression, on average, 8.6% (SD 15.9) lower in the hysterectomy specimen (p<0.0001). Representative images x4 magnification.

A significant reduction in immunohistochemical expression of phosphorylated markers of the PI3K-Akt-mTOR and insulin signalling pathways was also found between endometrial biopsies and hysterectomy specimens (figure 2-3, all p<0.0001). Indeed, expression of these markers in the hysterectomy specimens was almost completely absent (figure 2-4).
Figure 2-3 Comparison of expression of phosphorylated markers of the PI3K-Akt-mTOR and insulin signalling between endometrial biopsies and corresponding hysterectomy specimens a) pAkt b) p4EBP1 c) pIR d) pIGF1R. The mean H score decreased from 42.6 (SD 40.0) in the endometrial biopsy to 3.3 (SD 4.8) in the hysterectomy specimen for pAkt, from 79.3 (SD 40.7) to 6.7 (SD 21.5) for p4EBP1, from 200.8 (SD 38.2) to 55.1 (SD 50.9) for pIR and from 221.8 (SD 40.1) to 126.2 (SD 82.7) for pIGF1R (all p<0.0001).
Figure 2-4 Expression of phosphorylated markers that were widely present in endometrial biopsies was often found to be absent in the corresponding hysterectomy specimen a) pAkt b) p4EBP1 c) pIR d) pIGF1R (x4 magnification).
Similarly, the immunohistochemical expression of hormone receptors, ER and PR, was also significantly lower in the hysterectomy specimen compared with the matched endometrial biopsy (figure 2-5). The mean H-score for ER expression decreased from 271 (SD 48.2) in the endometrial biopsy to 211.6 (SD 69.5) in the hysterectomy specimen (p<0.0001), whilst the mean H-score for PR expression decreased from 206.9 (SD 67.1) to 143.1 (SD 69.7, p<0.0001). Despite lower ER expression in the hysterectomy specimen, there was no discrepancy in overall oestrogen receptor status between the two samples. There was, however, a discrepancy in progesterone receptor status in two of 63 cases (3.17%), where loss of receptor expression was noted in the hysterectomy specimen.

Contrary to the above findings, there was no difference in expression of the cancer stem cell markers ALDH and CD133 between the endometrial biopsy and the matched hysterectomy specimen (figure 2-6). Indeed, there was a non-significant increase in mean ALDH expression in the hysterectomy specimen (endometrial biopsy 58.4%, SD 26.3% vs. hysterectomy 64.8%, SD 24.4%, p=0.09). CD133 expression was static between the two tumour specimens, though overall markedly lower than ALDH expression (mean difference endometrial biopsy 3.3%, SD 4.7% vs. hysterectomy 2.7%, SD 3.7%, p=0.48).

Figure 2-5 Immunohistochemical expression of the hormone receptors ER and PR was significantly lower in the hysterectomy specimen compared with the matched endometrial biopsy a) ER b) PR. Representative images x4 magnification.
There was no loss of expression of the cancer stem cell markers ALDH and CD133 in the hysterectomy specimens a) ALDH b) CD133. Representative images x4 magnification.

The difference in immunohistochemical expression of these commonly studied biomarkers between the endometrial biopsy and hysterectomy specimen was closely correlated with baseline expression in the endometrial biopsy (table 2-1). The greater the immunohistochemical expression in the endometrial biopsy, the greater the loss of expression in the corresponding hysterectomy specimen.

**Table 2-1 Correlation between baseline immunohistochemical expression of biomarkers in endometrial biopsies and the difference in expression between the biopsy and hysterectomy specimen.** For all proteins studied, loss of expression in the hysterectomy specimen was significantly correlated with baseline expression in the endometrial biopsy.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Pearson correlation coefficient</th>
<th>Number of samples compared</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>0.42</td>
<td>69</td>
<td>0.0003***</td>
</tr>
<tr>
<td>pAkt</td>
<td>0.99</td>
<td>70</td>
<td>&lt;0.0001****</td>
</tr>
<tr>
<td>p4EBP1</td>
<td>0.89</td>
<td>73</td>
<td>&lt;0.0001****</td>
</tr>
<tr>
<td>pIIR</td>
<td>0.55</td>
<td>73</td>
<td>&lt;0.0001****</td>
</tr>
<tr>
<td>pIGF1R</td>
<td>0.58</td>
<td>73</td>
<td>&lt;0.0001****</td>
</tr>
<tr>
<td>ER</td>
<td>0.40</td>
<td>64</td>
<td>0.001***</td>
</tr>
<tr>
<td>PR</td>
<td>0.62</td>
<td>63</td>
<td>&lt;0.0001****</td>
</tr>
</tbody>
</table>
The extent to which loss of expression of one biomarker correlated with a reduction in expression of another was examined (figure 2-7). Loss of expression of p4EBP1 in hysterectomy specimens was associated with significant reductions in expression of Ki-67 ($r=0.24$, $p=0.05$, 68 patients) and pIGF1R ($r=0.32$, $p=0.007$, 71 patients). Loss of expression of pIR correlated with a reduction in expression of pAkt ($r=0.23$, $p=0.05$, 70 patients). Loss of expression of pIGF1R was associated with reductions in expression of ER ($r=0.29$, $p=0.02$, 63 patients) and PR ($r=0.36$, $p=0.004$, 61 patients), whilst loss of PR expression also correlated with loss of pIR ($r=0.33$, $p=0.008$, 62 patients), pIGF1R ($r=0.36$, $p=0.004$, 61 patients) and, in particular, ER expression ($r=0.65$, $p<0.0001$, 61 patients).

Figure 2-7 Pairwise correlation matrix of differences in biomarker expression between endometrial biopsies and corresponding hysterectomy specimens

The magnitude of staining loss in the hysterectomy specimen compared with the corresponding endometrial biopsy was correlated with clinico-pathological variables. No association was found between loss of expression of these commonly studied biomarkers and age, BMI, depth of myometrial invasion or need for adjuvant therapy (all $p>0.05$, table 2-2). The difference in immunohistochemical expression of biomarkers between endometrial biopsies and hysterectomy specimens was smaller in tumours of higher grade and stage and those with lymphovascular space invasion (LVSI) and lymph node metastases, but these results did not consistently reach statistical significance.
Table 2-2 Association between loss of immunohistochemical expression of commonly studied biomarkers in hysterectomy specimens and clinico-pathological variables. There was no clear association between loss of expression and patient age, BMI, depth of myometrial invasion and need for adjuvant therapy. There were, however, smaller discrepancies in immunohistochemical staining between the endometrial biopsy and hysterectomy specimen in endometrial cancers of higher grade and stage and those with LVSI and lymph node metastases, although these results did not consistently reach statistical significance.

<table>
<thead>
<tr>
<th></th>
<th>Ki-67 diff</th>
<th>pAkt diff</th>
<th>p4EBP1 diff</th>
<th>pIR diff</th>
<th>pIGF1R diff</th>
<th>ER diff</th>
<th>PR diff</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>9.4 (16.7)</td>
<td>24.2 (24.2)</td>
<td>55.9 (45.0)</td>
<td>138.6 (62.9)</td>
<td>105.3 (102.4)</td>
<td>100.7 (69.3)</td>
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Results presented as mean difference between endometrial biopsy and corresponding hysterectomy specimen (SD)

LVSI lymphovascular space invasion, LN lymph node, mets metastases
Loss of protein expression in the hysterectomy specimen was also correlated with specimen characteristics and handling to determine whether differences in immunohistochemical staining could be explained by delays in achieving adequate formalin fixation of tissues (table 2-3). No clear association was found between the extent of staining loss and tumour size, specimen weight, day of surgery or location of pathology services either on- or off-site (all p>0.05). The difference in expression of biomarkers between the endometrial biopsy and corresponding hysterectomy specimen was lower in cancers removed by laparotomy, a generally faster surgical procedure than total laparoscopic hysterectomy, although the result was not statistically significant. Whilst there was no significant overall correlation between tumour-serosal distance and loss of immunohistochemical staining, this relationship was strengthened when only unopened specimens were considered. This was supported by a trend towards smaller differences in immunohistochemical staining between endometrial biopsies and hysterectomy specimens if the uterus had been bisected prior to placement in formalin, although, with the exception of p4EBP1, this also did not reach statistical significance.
Table 2-3 Association between loss of immunohistochemical expression of commonly studied biomarkers in hysterectomy specimens and specimen characteristics and handling.

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<th>p4EBP1 diff</th>
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<th>pIGF1R diff</th>
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<td><strong>Tumour-serosal distance (unopened specimens, mm)</strong></td>
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Results presented as mean difference in biomarker expression (SD) or Pearson’s correlation coefficient, p value and number of samples compared.
2.3.2 Role of hypoxia in driving loss of protein expression

Paradoxically, expression of the hypoxia inducible transcription factor HIF-1α and its downstream effector CA-IX were found to be higher in the endometrial biopsy than the hysterectomy specimen (figure 2-7, both p<0.0001). This was despite the uterine blood supply being clamped for at least 20 minutes during surgery and obvious discolouration of the uterus occurring even before it had been removed from the body as a consequence of poor perfusion.

Figure 2-8 Immunohistochemical expression of the hypoxia-induced proteins HIF-1α and CA-IX was significantly lower in the hysterectomy specimen compared with the matched endometrial biopsy a) HIF-1α b) CA-IX. The mean H-score decreased from 95.1 (SD 46.9) in the endometrial biopsy to 58.2 (SD 39.9) in the hysterectomy specimen for HIF-1α and from 26.2 (SD 26.9) to 10.1 (SD 15.5) for CA-IX (both p<0.0001). Representative images x4 magnification.
2.4 Discussion

Immunohistochemical expression of Ki-67, phosphorylated markers of the PI3K-Akt-mTOR and insulin signalling pathways and the hormone receptors, ER and PR, was significantly lower in tumour obtained from the hysterectomy specimen than from an endometrial biopsy taken prior to the start of surgery. In contrast, expression of cancer stem cell markers, ALDH and CD133, was unaffected by tumour sampling technique. Despite observational evidence of reduced tissue perfusion during surgery, immunohistochemical expression of hypoxia inducible transcription factors and their downstream effectors was significantly lower in the hysterectomy specimen suggesting that loss of detectable protein expression had occurred. Depletion of immunohistochemical staining in the hysterectomy specimen correlated with baseline tumour expression and was reduced if specimens were bisected before being placed in formalin.

These findings would suggest that delays in achieving adequate fixation of the tumour within the hysterectomy specimen have a significant role to play in the subsequent loss of detectable protein expression. The slow penetration of formalin (1mm per hour) means that it can be several hours before the fixative reaches the tumour located in the endometrial cavity (Srinivasan et al. 2002). A time interval of 16 hours or more between surgical removal of a breast cancer and its fixation has been shown to significantly reduce immunohistochemical expression of Ki-67 (Arima et al. 2016). A means of avoiding this is to immediately dissect and section large specimens before placing them in formalin to improve exposure of the tumour to the fixative (Hewitt et al. 2008).

That the cancer stem cell markers CD133 and ALDH are unaffected by hypoxia and delays in fixation may initially appear surprising, though there have been no previous studies comparing the expression of these proteins in biopsies and surgical excision specimens. Rather than triggering cell death, as occurs in the bulk of tumour cells, hypoxia has been shown to actually enrich for cancer stem cells (Crowder et al. 2014). As a result, expression of CD133 and ALDH is upregulated at both the mRNA and protein level, with an increase in the proportion of cells expressing these markers in response to growth in 1% oxygen in glioblastoma and ovarian cancer cell lines (Seidel et al. 2010;Crowder et al. 2014;Seo et al. 2016). Any loss of protein expression through poor fixation of the tumour in the hysterectomy specimen is likely, therefore, to be counteracted by the increased expression of these cancer stem cell markers by the cells.

In order to avoid loss of protein expression, the use of endometrial biopsies for immunohistochemistry is to be encouraged. The smaller sample size means that formalin fixation occurs more rapidly and it is thus a better reflection of tumour biology. These findings have been replicated in breast cancer, where immunohistochemical expression of pAkt and pERK1/2 was found to be significantly lower in surgical excision specimens compared with core-cut biopsies, with almost absent staining in a proportion of samples (Pinhel et al. 2010). In contrast to this study, ER and PR appeared to be more resistant to the effects of delays in fixation in breast cancer, though this was based on only 29 samples and, although not significant, there was a trend towards lower expression of ER in the
resected specimens (Pinhel et al. 2010). A major concern is that despite the loss of protein expression being relatively modest, inappropriate decisions regarding adjuvant treatment may be made if based on the immunohistochemical staining of excised specimens. A study by Mann et al. (2005) estimated that up to 9% of women with breast cancer could be denied beneficial hormonal therapy if hormone receptor status was determined solely using lumpectomy/mastectomy specimens. For window studies, which rely on detecting small changes in cellular processes to determine drug efficacy, the impact of these inaccuracies could be even more pronounced. As a consequence, the hysterectomy specimen can no longer be generally considered sufficiently reliable to be used in the assessment of primary and secondary outcomes. Future studies should be designed to detect differences in expression of such proteins between an endometrial biopsy performed at diagnosis and one performed prior to the start of the hysterectomy, as was the case with the PREMIUM study.

The strength of my study was the comparison of several different groups of biomarkers commonly evaluated in endometrial cancer window studies in a large number of matched endometrial biopsies and hysterectomy specimens from women enrolled in a randomised controlled trial. One person performed the staining and scoring of all slides and was blinded to sample type. A second, independent, researcher scored a subset (20%) of slides to ensure consistency. The use of semi-automated software to quantify immunohistochemical expression within tumour samples was more time efficient than manual scoring and potentially improved the reliability and reproducibility of the scores obtained (Kitson et al. 2017a).

There was a paucity of data available regarding the handling of the surgical specimen once it had been removed from the body, in particular the time interval before placement in formalin and how long it had remained in the fixative before sectioning. This meant that surrogate measures had to be used in their place to investigate the relationship between delays in or prolongation of fixation and loss of immunohistochemical staining, including size of tumour and specimen and tumour-serosal distance. The extent to which these variables correlate with tumour fixation has not, however, been previously determined. Whilst data were available regarding whether the uterus had been opened prior to placement in formalin in order to obtain a tumour biopsy, records were not kept of those specimens that had been bisected without a biopsy being performed, which happened on several occasions. Inclusion of this information may have strengthened the association between ease of formalin penetration and loss of immunohistochemical staining.

Correlation between the degree of hypoxia within the hysterectomy specimen and the reduction in expression of Ki-67 and phosphorylated markers of the PI3K-Akt-mTOR and insulin signalling pathways was not possible due to loss of HIF-1α and CA-IX protein expression, most likely as a consequence of delays in achieving tissue fixation. Alternative methods of determining intra-tumoral hypoxia without relying on the immunohistochemical expression of proteins, however, are either invasive or expensive, negating their use (Walsh et al. 2014). Regardless of the extent to which loss
of protein expression occurs prior to or following removal of the uterus from the body, the end result is a sample that no longer represents the tumour from which it was obtained.

2.4.1 Conclusion

Immunohistochemical expression of proteins commonly examined in endometrial cancer window studies, including the proliferation marker Ki-67, phosphorylated markers of the PI3K-Akt-mTOR and insulin signalling pathways and the hormone receptors, ER and PR, is significantly lower in hysterectomy specimens than endometrial biopsies performed immediately before surgery is commenced. This is likely to be the result of intra-tumoral hypoxia following devascularisation of the uterus and, in particular, delays in achieving adequate tissue fixation in large surgical specimens. The expression of cancer stem cell markers, however, is unaffected, probably because of enrichment of these cells in hypoxic niches. In order for methodologically robust results to be generated from window studies in endometrial cancer, going forward an endometrial biopsy should be used in preference to the hysterectomy specimen for end of treatment analyses.
Chapter 3  PRE-surgical Metformin In Uterine Malignancy-results of the PREMIUM randomised controlled trial

3.1 Introduction
Epidemiological evidence suggesting improved overall survival in diabetic women with endometrial cancer (Perez-Lopez et al. 2017) and pre-clinical data demonstrating a reduction in endometrial cancer cell proliferation (Cantrell et al. 2010), inhibition of invasion and migration (de Barros Machado et al. 2016) and a potential increase in apoptosis (Takahashi et al. 2014) with metformin treatment has resulted in a plethora of clinical trials in the field. To date, six window studies have been performed, treating women with diabetic doses of metformin for the short time period between diagnosis and surgical treatment of their endometrial cancer (Mitsuhashi et al. 2014; Soliman et al. 2016; Schuler et al. 2015; Sivalingam et al. 2016; Laskov et al. 2014; Zhao et al. 2017). With the exception of one study (Schuler et al. 2015), all found a reduction in endometrial cancer proliferation, as measured by immunohistochemical expression of Ki-67, following exposure to metformin. The effect of metformin on key endometrial carcinogenic pathways, including the PI3K-Akt-mTOR and MAPK/ERK pathways, has produced more heterogeneous results (Mitsuhashi et al. 2014; Soliman et al. 2016), possibly as a consequence of different methods of tumour sampling prior to and following intervention (Sivalingam et al. 2016). The absence of contemporaneous control groups for comparison may also have potentially resulted in normal changes in endometrial tumour biology being erroneously assigned to metformin exposure.

The PREMIUM study was designed as a methodologically rigorous randomised controlled trial primarily to provide definitive evidence as to whether metformin was efficacious in reducing endometrial cancer proliferation. Secondarily, it sought to determine the importance of proposed direct and indirect mechanisms of action of the drug and the tolerability of treatment.

3.2 Materials and methods

3.2.1 Clinical trial design

3.2.1.1 Trial design and participants
A phase III, double-blind, placebo-controlled, randomised trial utilising the pre-surgical window period was conducted in five centres within the North West of England (Manchester University Hospitals NHS Trust, The Christie NHS Foundation Trust, Pennine Acute Hospitals NHS Trust, Wrightington, Wigan and Leigh NHS Foundation Trust and Tameside and Glossop Integrated Care NHS Foundation Trust) between 2015 and 2017. This built upon an earlier phase II study carried out by our
group (Sivalingam et al. 2016) and aimed to demonstrate effectiveness of metformin compared to a placebo and to identify adverse effects of the drug.

Potential eligible women were identified shortly after their diagnosis at one of two multi-disciplinary team meetings held at Manchester University Hospitals NHS Trust (MFT) and The Christie NHS Foundation Trust on a weekly basis. The full inclusion and exclusion criteria for the trial are described in Table 3-1.

Table 3-1: Inclusion and exclusion criteria for PREMIUM randomised controlled trial

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
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<tbody>
<tr>
<td>Biopsy proven atypical endometrial hyperplasia</td>
<td>Current treatment with metformin</td>
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<tr>
<td>or endometrioid endometrial cancer</td>
<td></td>
</tr>
<tr>
<td>Scheduled surgical treatment by hysterectomy in following 5-35 days</td>
<td>Diabetic on hypoglycaemic medication</td>
</tr>
<tr>
<td>Able to provide informed consent</td>
<td>Inability to consent due to lack of capacity or language barriers</td>
</tr>
<tr>
<td>Age 18 years or more</td>
<td>Unable to comply with treatment protocol</td>
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<tr>
<td></td>
<td>Non-endometrioid endometrial cancer on diagnostic biopsy</td>
</tr>
<tr>
<td></td>
<td>Severe renal impairment (serum creatinine &gt;130μmol/L or eGFR &lt; 45ml/min/1.73m²)</td>
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<tr>
<td></td>
<td>Severe hepatic impairment (abnormal liver function tests to be discussed on individual basis with hepatologist)</td>
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<tr>
<td></td>
<td>Current alcohol abuse</td>
</tr>
<tr>
<td></td>
<td>Sensitivity/hypersensitivity to biguanides</td>
</tr>
<tr>
<td></td>
<td>Current treatment with other mTOR inhibitors or chemotherapeutic agents</td>
</tr>
<tr>
<td></td>
<td>Currently pregnant</td>
</tr>
<tr>
<td></td>
<td>Current progesterone treatment</td>
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</table>

Current progesterone treatment (either as a contraceptive or for the management of endometrial cancer related bleeding) was a contraindication as progestins have been previously shown to decrease the mitotic index of endometrial cancer cells and reduce Ki-67 expression (Zaino et al. 2014). Interpretation of the effect of metformin on endometrial cancer proliferation would, therefore, have been hindered in this context.

An outline of the trial protocol is provided in figure 3-1.
At the recruitment visit, a medical history was taken, alongside assessment of serum renal and liver function and a pregnancy test (if pre-menopausal) to confirm eligibility. In addition, the following anthropometric measurements were obtained:

- Height (performed bare footed with a stadiometer)
- Weight (performed clothed using calibrated electronic scales)
- BMI (calculated as weight (kg)/height (m)^2)
• Waist circumference (measured using a tape measure in accordance with recommendations from the WHO, using the midpoint between the lower margin of the last palpable rib and the top of the iliac crest as landmarks (WHO 2008))
• Hip circumference (measured with a tape measure at the widest portion of the buttocks (WHO 2008))

Women were requested to have fasted for at least six hours before undergoing venepuncture to obtain serum for assessment of baseline insulin resistance and markers of adiposity. If possible, a repeat endometrial biopsy was performed using an endometrial sampling device in the outpatient clinic and was immediately formalin fixed. A formalin fixed paraffin embedded (FFPE) block from the diagnostic endometrial biopsy was also requested from the relevant histopathology department.

Following randomisation, women were instructed to take the study drug (metformin or matched placebo) once daily after food for the first three days followed by twice a day thereafter for 1-5 weeks. In the case of metformin, this equated to 850mg for three days followed by 1700mg for the remainder of the study period. Women discontinued treatment on the evening before their scheduled surgery to reduce the risk of lactic acidosis associated with peri-operative metformin administration. The date of surgery was determined by local surgeon availability. The decision to include a stepped dose regime was made following analysis of pilot study data, where a gradual increase in dose was associated with fewer adverse events and improved compliance (Sivalingam et al. 2016). Regular telephone contact (at the point of dose escalation and at least once a week thereafter) was made by me with participants to monitor compliance and adverse events.

On the day of surgery, repeat physical and fasted physiological assessments were performed. An endometrial biopsy was obtained in theatre prior to the start of the hysterectomy and was immediately fixed in formalin. Directed tumour biopsies were taken from the hysterectomy specimen, if possible, by a gynaecological histopathologist. FFPE blocks of the tumour specimen were obtained from the relevant hospital once the diagnostic work had been completed. Participation in the study ended at the time of surgery.

In the event that surgery was delayed beyond five weeks from randomisation, women were asked to return for a second clinic visit at which the same anthropometric measurements were performed, alongside fasted venepuncture and an endometrial biopsy. The latter was used in place of the hysterectomy specimen for the primary and secondary outcome analyses.

All women provided written, informed consent to participate in the study. The trial was sponsored by Manchester University Hospitals NHS Foundation Trust and was approved by the North West Research Ethics Committee (14/NW/1236), Medicine and Healthcare Products Regulatory Authority (MHRA, reference 21387/0232/001-0001) and local Research and Development (R+D) departments.
The trial was prospectively registered on both UK (ISRCTN 88589234) and European (Euradact number 2014-000991-25) clinical trial databases.

3.2.1.2 Changes to trial design
Following commencement of the trial, the inclusion criteria were broadened to incorporate changes in the definition and management of precancerous endometrial lesions introduced by the World Health Organisation (WHO) in 2015 (Emons et al. 2015). The trial eligibility criteria had initially included only women with biopsy proven severe atypical endometrial hyperplasia, however, following the publication of the WHO guidelines, all atypical endometrial hyperplasia, regardless of severity, was regarded as being premalignant necessitating treatment by hysterectomy. The inclusion criteria for the trial were, therefore, amended accordingly.

The original trial protocol stated that an endometrial biopsy would be obtained at the time of recruitment for all women for use in primary and secondary immunohistochemical analyses. Where it was not possible to obtain a repeat sample, the diagnostic biopsy would be requested from the referring hospital and used in its place. Early in recruitment it became apparent that the majority of women would not consent to a repeat endometrial biopsy and the decision was taken to use the diagnostic biopsy in all cases for baseline immunohistochemical assessment to ensure consistency. In the absence of treatment, Ki-67 expression appears static over time and this amendment to the trial design was not, therefore, expected to influence the primary outcome of the study (Kitson et al. 2017a).

During recruitment, it was agreed that the sample size for the trial would be increased to replace women who had not taken any of the study drug, either because they had withdrawn consent immediately after randomisation or because their surgery date had been brought forward to within five days of randomisation, or where a tumour biopsy was not available from the final visit. As the trial was powered to assess the effect of metformin on expression of a histological biomarker, it was not felt that this amendment would impact upon the validity of conducting a modified intention-to-treat analysis.

All substantial amendments to the trial protocol and participant paperwork received approval from the research ethics committee, MHRA and local R+D departments.

3.2.1.3 Randomisation and blinding
Prior to commencement of recruitment, computer generated randomisation lists were produced by an independent clinical trials unit (Manchester Academic Health Science Centre-Clinical Trials Coordination Unit, MAHSC-CTU) using the permuted block method. This involved the creation of four blocks of size 30, with a 1:1 ratio of metformin and placebo. The lists were used by the Pharmacy Manufacturing Unit at Guy’s and St Thomas’ NHS Foundation Trust during packaging of the drug into tamper-proof bottles to ensure that each bottle was labelled with a unique two-letter code. Following recruitment, women were randomised by computer to the next available bottle of drug, using this
unique code, by telephoning the central randomisation line at MAHSC-CTU. The drug was dispensed from the Clinical Trials pharmacy at MFT in bottles containing 70 tablets.

With the exception of the trial statistician, who was involved in the generation of the randomisation lists, participants, investigators and other members of the clinical trial team remained blinded to treatment allocation for the duration of trial recruitment, participation and analysis. This was possible due to the identical appearance, packaging, labelling and dosing regimen for the placebo and active drug (figure 3-2). Should a serious adverse event have occurred, emergency unblinding to reveal treatment allocation was possible by a member of the Clinical Trials pharmacy team, who had access to sealed, opaque envelopes containing details of treatment assignment. This was not required during the trial.
Figure 3-2 Trial medication and bottle a) Tablets containing metformin or placebo were identical in appearance. Study tablet compared in size to 10 pence piece (diameter 23mm). b) The bottle of tablets dispensed was uniform in appearance to ensure blinding of patients and the research team to treatment allocation.

3.2.2 Primary outcome

3.2.2.1 Ki-67 immunohistochemistry

Determination of Ki-67 expression by immunohistochemistry was performed as previously described (chapter 2). In brief, 4µm sections were cut from FFPE blocks of diagnostic endometrial biopsies and corresponding hysterectomy specimens using a microtome and mounted onto histological glass slides. Whole sections were stained using the Leica Bond Max (Leica Biosystems, Wetzlar, Germany), using a heat-induced epitope retrieval protocol at pH9 for 20 minutes and a 30 minute
casein block. Slides were incubated with the MIB-1 antibody (X0931 Dako, Carpinteria, CA, dilution 1:100) for one hour at room temperature. Primary antibody detection was performed using the Refine Detection Kit (Leica Biosystems).

3.2.2.2 Ki-67 scoring
Semi-automated scoring using Definiens Developer software was performed as previously described [chapter 2, (Kitson et al. 2017a)]. In brief, slides were digitised using the Leica SCN400 Slide Scanner (Leica Microsystems, Wetzlar, Germany). Malignant glands were manually selected in the three fields identified at x10 magnification as exhibiting the greatest Ki-67 staining (hot spots), before applying an optimised computer algorithm to detect positively and negatively stained nuclei. At least 2000 nuclei per slide were included. Results were expressed as the percentage of positively stained nuclei, regardless of staining intensity. Scoring was performed by two independent assessors, who were blinded to treatment allocation. Absolute discrepancies of greater than 10% were settled by consensus. Quality control was maintained by manually comparing slides prior to and following application of the algorithm to ensure the correct classification of nuclei.

Images demonstrating the selection of hot spots and the detection of positively and negatively stained nuclei using Definiens Developer software are shown in chapter 2.

3.2.3 Secondary outcomes

3.2.3.1 Tissue microarray construction
Tissue microarrays were constructed from FFPE blocks of endometrial biopsies taken at baseline and following treatment with metformin or placebo. Representative areas of tumour were demarcated by an experienced gynaecological histopathologist on haematoxylin and eosin stained slides, from which triplicate cores were obtained and used to create the TMAs. This enabled staining for multiple immunohistochemical markers to be performed on small amounts of tissue.

3.2.3.2 Immunohistochemistry
Immunohistochemical expression of markers of apoptosis (cleaved caspase-3), the PI3K-Akt-mTOR pathway (pAkt, pACC, p4EBP-1, pS6) and insulin signalling (pIβR, total IGF1R, pIGF1R, IGFBP-1) were determined using the Leica Bond Max (Leica Biosystems, Wetzlar, Germany). Due to cross-reactivity of the pIGF1R antibody with the insulin receptor, total as well as phosphorylated levels of IGF1R were determined. In view of the effect of hypoxia on phosphorylated marker expression (chapter 2), the TMAs constructed from endometrial biopsies taken immediately prior to surgery were used in preference to the hysterectomy specimen and were compared with baseline endometrial biopsy TMAs.

Staining was performed using a heat induced epitope retrieval protocol. Full details of the antibodies and conditions employed are detailed in table 3-2. The Refine Detection Kit (Leica Biosystems) was used for primary antibody detection, with 3,3' diaminobenzidine as a chromogen and haematoxylin
counter-staining. Negative (isotype) and appropriate positive controls were used for each antibody for quality assurance as stipulated.

Table 3-2 Antibodies and experimental conditions used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Catalogue number</th>
<th>Dilution</th>
<th>Host</th>
<th>Antigen retrieval</th>
<th>Casein blocking step</th>
<th>Positive control tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 (MIB-1 clone)</td>
<td>Dako</td>
<td>X0931</td>
<td>1:100</td>
<td>Monoclonal mouse</td>
<td>EDTA pH9</td>
<td>Included</td>
<td>Tonsil</td>
</tr>
<tr>
<td>Cleaved caspase-3</td>
<td>Cell Signaling</td>
<td>#9661</td>
<td>1:200</td>
<td>Mouse monoclonal</td>
<td>Citrate buffer pH6</td>
<td>Not included</td>
<td>Normal colon</td>
</tr>
<tr>
<td>pAkt (Ser 473)</td>
<td>Cell Signaling</td>
<td>#4060</td>
<td>1:50</td>
<td>Rabbit monoclonal</td>
<td>EDTA pH9</td>
<td>Not included</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>pACC (Ser 79)</td>
<td>Cell Signaling</td>
<td>#3661</td>
<td>1:300</td>
<td>Rabbit polyclonal</td>
<td>EDTA pH9</td>
<td>Included</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>pS6 (Ser 235/236)</td>
<td>Cell Signaling</td>
<td>#4858</td>
<td>1:400</td>
<td>Rabbit monoclonal</td>
<td>EDTA pH9</td>
<td>Included</td>
<td>Colon cancer</td>
</tr>
<tr>
<td>p4EBP1 (Thr37/46)</td>
<td>Cell Signaling</td>
<td>#2855</td>
<td>1:800</td>
<td>Rabbit monoclonal</td>
<td>EDTA pH9</td>
<td>Included</td>
<td>Colon cancer</td>
</tr>
<tr>
<td>pIR (Y1361)</td>
<td>Abcam</td>
<td>ab60946</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
<td>EDTA pH9</td>
<td>Included</td>
<td>Placenta</td>
</tr>
<tr>
<td>IGF1R beta</td>
<td>Cell Signaling</td>
<td>#3027S</td>
<td>1:600</td>
<td>Rabbit polyclonal</td>
<td>EDTA pH9</td>
<td>Not included</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>pIGF1R</td>
<td>Abcam</td>
<td>ab39398</td>
<td>1:50</td>
<td>Rabbit polyclonal</td>
<td>Citrate buffer pH6</td>
<td>Included</td>
<td>Placenta</td>
</tr>
<tr>
<td>IGFBP1</td>
<td>Abcam</td>
<td>ab111203</td>
<td>1:100</td>
<td>Rabbit polyclonal</td>
<td>Citrate buffer pH6</td>
<td>Included</td>
<td>Placenta</td>
</tr>
</tbody>
</table>

pAkt phospho-Akt, pACC phospho-ACC, pS6, phospho-S6, p4EBP1 phospho-4EBP1, pIR phospho-IR, pIGF1R phospho-IGF1R

3.2.3.3 Immunohistochemical scoring

Scoring of TMA cores was similarly performed on digitalised versions of stained slides using Definiens Developer software. All malignant glands within each of the triplicate cores were scored in their entirety. The presence or absence of staining was assessed within the nucleus and cytoplasm (cleaved caspase-3, pAkt, pS6, p4EBP-1), cytoplasm alone (pACC), cytoplasm and cell membrane...
(IGFBP1) or at the cell membrane alone (pIR, IGF1R, pIGF1R). With the exception of cleaved caspase 3, the immunohistochemical expression of each of the antibodies was quantified using an H-score; the product of staining intensity (0=no staining, 1=weak, 2=moderate, 3=strong) and the percentage of cells at each stain intensity, with a maximum value of 300. Cleaved caspase-3 expression was calculated as the percentage of positively stained cells, regardless of staining intensity. Images of cores stained with pACC, pS6 and pIR of different intensities are shown in figure 3-3.

Scoring was performed by two independent assessors, blinded to treatment allocation; absolute discrepancies of greater than 10% were settled by consensus.
Figure 3-3 TMA cores stained with a) pACC, b) pS6 and c) pI of different intensities

<table>
<thead>
<tr>
<th>None</th>
<th>Weak</th>
<th>Moderate</th>
<th>Strong</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td>b)</td>
<td>c)</td>
<td></td>
</tr>
</tbody>
</table>

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3.2.3.4 Physiological analyses
At baseline and following treatment with metformin or placebo, fasted serum was collected for measurement of glucose and insulin levels, from which HOMA-IR was calculated using the formula HOMA-IR = (glucose mmol/l x insulin mU/l)/22.5. In addition, glycated haemoglobin levels (HbA1C) were determined as a marker of long term glucose control. These analyses were performed by the clinical biochemistry laboratory at MFT using a routine automated assay for which standard operating procedures were in place.

Insulin-like growth factor-1 (IGF-1), insulin-like growth factor binding protein-1 (IGFBP-1), leptin and adiponectin levels were measured using Quantikine (IGF-1) and DuoSet (IGFBP-1, leptin, adiponectin) sandwich enzyme linked immunoabsorbant assay (ELISA) kits from R+D Systems (Abingdon, UK), according to the manufacturer’s instructions. Sample concentrations were determined by extrapolation from standard curves. Intra-assay coefficients of variation were 4.3%, 3.6%, 5.3% and 3.2%, respectively, for IGF-1, IGFBP-1, leptin and adiponectin. The corresponding inter-assay coefficients of variation were 8.3%, not determined, 7.2% and 9.3%.

3.2.3.5 Treatment compliance and adverse event monitoring
Compliance with the treatment regime was determined through three different means; regular telephone consultation with the women, completion of a study diary where each dose taken was recorded, and counting of returned tablets at the time of surgery. The latter was performed independently by myself and the Clinical Trials Pharmacy team, and was compared with the expected number of returned tablets given the duration of the individual treatment window and the dispensing of uniform bottles containing 70 tablets.

Adverse event monitoring was performed by direct symptom enquiry during weekly telephone consultations with participants, or by self-reporting of symptoms, which could be noted on the study diary or telephoned through if further advice was required. An adverse event was defined as the appearance or worsening of any undesirable symptom, sign or condition occurring after commencement of the study, regardless of whether it was felt to be related to the study drug. A serious adverse event was one resulting in significant or persistent disability/incapacity, a congenital birth defect, inpatient hospitalisation or was regarded as being medically significant (i.e. jeopardised the participant or required medical or surgical intervention to prevent one of the above).

Adverse events were categorised according to their nature, severity, seriousness, relationship to the study drug, duration and whether action was warranted. The severity of adverse events was graded as follows:

Mild-easily tolerated, causing minimal discomfort and not interfering with everyday activities
Moderate-sufficiently discomforting to interfere with every day activities
Severe-preventing every day activities
Dose modifications and temporary or permanent cessation of the study drug were advised by myself depending upon the nature and severity of adverse events. All women received a seven day supply of cyclizine (an anti-emetic) along with the trial drug in case of nausea and vomiting as this is the most common adverse event experienced by those taking metformin.

All serious adverse events required reporting to MAHSC-CTU and the Sponsor within 24 hours of first knowledge, with appropriate onward referral to the research ethics committee and MHRA. Trial investigators were responsible for following up adverse events until their resolution and implementing appropriate urgent safety measures to protect women from any immediate threat to their health and safety.

3.2.4 Molecular and hormonal profiling of endometrial cancers

3.2.4.1 TP53 status
The TP53 mutational status of tumours was determined by immunohistochemical expression of the corresponding protein in the hysterectomy specimen. Staining was performed by the clinical histopathology laboratory at MFT using the TMA slides and the automated Ventana BenchMark ULTRA (Ventana Co., Tucson, AZ, USA) and UltraView DAB Detection System (Ventana Co.) Heat induced epitope retrieval was performed for 36 minutes at pH 8.4 before incubating the slides with an ultraviolet inhibitor blocking solution followed by the primary, anti-p53 (DO-7), antibody (1:50 dilution, M7001, Dako, Carpinteria, CA) for 32 minutes. Antibody detection was undertaken using a horseradish peroxidase conjugated secondary antibody, with DAB as a chromogen and haematoxylin counterstaining.

Expression of the p53 protein was determined manually, in accordance with previously published recommendations (McCluggage et al. 2011; Nout et al. 2012). p53 staining was considered ‘mutant like’ if >50% of tumour cells exhibited strong nuclear staining, discrete geographical areas of tumour had >50% positive nuclear staining or there was a complete absence of tumour staining.

3.2.4.2 Hormone receptor status
Immunohistochemical staining for ER and PR status was performed using TMAs constructed from the hysterectomy specimens as described in chapter 2. Scoring was performed manually by two independent researchers, with tumours demonstrating any degree of nuclear staining classified as receptor positive.

3.2.5 Serum metformin quantification
Plasma taken on the morning of surgery, approximately 12 hours after the last dose of the study drug, was used to quantify trough metformin levels. The concentration of metformin was determined at the Department of Clinical Pharmacology and Pharmacy, University of Southern Denmark using liquid chromatography and tandem mass spectrometry (LC-MS/MS). The LC-MS/MS system consisted of
an Ultimate 3000 UHPLC system connected to a TSQ Quantiva Triple Quadropole Mass Spectrometer with heated electrospray ionization (Thermo Scientific, San Jose, CA). Metformin was quantitated by positive ionisation at the transition from (m/z) 130.4 – 71.1, and with (m/z) 130.4 – 60.1 as a qualifier trace. Analytical separation was performed using hydrophilic interaction chromatography as described by Nielsen et al. (2014). Plasma samples underwent a single protein precipitation step, in which 10 μL of 25 μg/mL metformin-d6 (internal standard), 20 μL of 0.53M ammonium acetate and 390 μL acetonitrile was added to 100 μL of plasma. The sample was vortexed and then centrifuged at 3000g for 15 minutes. A volume of 10μl of supernatant was injected onto the LC-MS/MS system. Calibration curves and quality control samples were run alongside study samples. The limit of quantification was 10ng/ml and limit of detection 1ng/ml.

3.2.6 Data handling
Case report forms (CRFs) were completed for each woman at the recruitment and end of study visits and following every telephone consultation. Original versions were sent to the data manager at MAHSC-CTU at regular intervals and copies were retained in individual folders, held in a secure location at MFT. Data were checked for errors, inconsistencies and missing information by MAHSC-CTU and queries were raised in response and directed to myself for clarification.

Individual participant results from the primary and secondary outcomes were compiled by myself into Excel spreadsheets and were sent to the trial statistician for analysis. Unblinding of the study was not performed until all primary and secondary outcome assessments had been completed.

3.2.7 Trial monitoring
Oversight of each recruiting centre was performed by an independent trial monitor from MAHSC-CTU, who reviewed individual source documents, CRFs and the site files to ensure the rights and wellbeing of women were protected, reporting of trial data was complete, accurate and verifiable and that the study was being conducted in accordance with the study protocol, regulatory requirements, as applicable, and Good Clinical Practice.

Routine monitoring of the trial was undertaken by the Trial Management Group (TMG), who met on a three monthly basis, and was composed of the Chief and Co-Investigators, statistician, trial manager, data manager and sponsor representative. The TMG was responsible for identifying and resolving issues surrounding data collection, protocol deviations, safety data, the preparation of trial reports and ensuring the well-being of women. Overall supervision of the trial was devolved to the Trial Steering Committee (TSC), consisting of the members of the TMG in addition to an independent chair person, two independent clinicians and a patient representative. TSC meetings were held annually to ensure the study was progressing towards meeting its objectives, the interests of the women were being safeguarded, that the safety and efficacy of the study intervention were regularly assessed and to monitor the overall conduct of the trial.
3.2.8 Statistical analysis

The trial sample size was calculated using preliminary results from the earlier pilot study conducted by our group (Sivalingam et al. 2016). No losses to follow-up were observed in this trial and non-compliance with the drug regime was less than 20%. For a two arm, randomised controlled trial, a sample size of 88 would give 90% power at the \( p=0.05 \) level to detect a 10% difference in Ki-67 using an intention to treat analysis, where the standard deviations in the intervention and control arms are 20.6% and 28.6%, respectively, and the correlation between Ki-67 expression at baseline and hysterectomy is 0.82. Ninety-three women were recruited to allow replacement of individuals who withdrew from the study before taking any of the trial medication or who stopped taking it more than 72 hours before their post-treatment samples were obtained, as this was likely to promote a rebound increase in tumour cell proliferation (unpublished results). A modified intention-to-treat analysis was performed including all women who had taken at least one dose of the study drug and for whom a tumour biopsy was available within 72 hours of discontinuing treatment.

Descriptive statistics were summarised using means and standard deviation for normally distributed data and medians and ranges for non-parametric data. The two groups were compared using the Mann-Whitney-U test for continuous data and Chi squared and Fisher’s exact test for categorical data. For the immunohistochemical expression of proteins, which had been scored by two independent observers, the mean score of the two observers for each participant was used in the analysis. The effect of metformin treatment on the primary and secondary outcomes was determined using an analysis of covariance model with baseline expression as a covariate. Pre-specified subgroup analyses were performed to investigate the effect of baseline BMI, insulin resistance (as measured by homeostatic model assessment-insulin resistance [HOMA-IR] or HbA1C), endometrial cancer grade, hormone receptor status and mutational profile of tumours, and total dose of metformin treatment on post-treatment Ki-67 expression. Correlations between variables were assessed using Pearson correlation coefficient for parametric data and Spearman’s rank test for non-parametric data.

Compliance was recorded as the percentage of women missing two or fewer doses of the trial medication. Adverse events were documented as free text and summarised using preferred terms by myself. The safety set included all women who had taken at least one dose of the trial medication and contained one more case than the modified intention-to-treat analysis. The worst grade of any adverse event reported by an individual as well as the frequency of specific adverse events thought to be causally related to metformin exposure were documented.

Statistical analysis was performed using Stata version 13 and GraphPad Prism version 7. A \( p \) value \( \leq 0.05 \) was considered statistically significant, with asterisks used to denote significant results as *\( p \leq 0.05 \), **\( p \leq 0.01 \), ***\( p \leq 0.001 \).
3.3 Results: Participants and adverse events

3.3.1 Recruitment

Between February 2015 and February 2017, 564 women were assessed for eligibility, of which 177 were approached and 96 women were screened (figure 3-4). Three women did not meet the inclusion criteria for randomisation due to poor renal function, leaving 93 women who were randomised. Five women were withdrawn from the study; three at their own request immediately following randomisation and before taking any of the trial medication, one before the drug was dispensed as the date of surgery was brought forward to within five days of randomisation and one because the date of surgery was delayed beyond five weeks from randomisation and it was not possible to obtain a repeat endometrial biopsy due to cervical stenosis. Eighty-eight randomised women completed treatment and were included in the modified intention-to-treat analysis; 43 received placebo and 45 received metformin.

In order to ensure participation in the trial did not compromise clinical care, data were collected on the length of time between diagnostic endometrial biopsy and surgery for women randomised in the trial and 232 women who did not meet the trial inclusion criteria or declined to participate. There was no significant difference in the interval between diagnosis and surgery for women who were or were not randomised in the trial (median biopsy to surgery interval 49 days [24-89 days] for randomised patients vs. 46 days [14-368 days] for non-randomised patients, p=0.084).
564 patients assessed for eligibility

387 excluded
- Type II endometrial cancer n=143
- Window period too short n=67
- Diabetic n=66
- Not for surgical treatment n=55
- On progesterone treatment n=19
- Operation at non-recruiting site n=17
- Unable to give informed consent n=10
- Poor renal function n=4
- On chemotherapy n=3
- Severe liver disease n=1
- Other n=2

177 patients given PIS

81 patients declined to participate

96 patients screened

3 patients not eligible due to poor renal function

93 patients eligible and randomised

Placebo (n=46)

Metformin (n=47)

3 withdrawals

2 withdrawals

Analysed
3.3.2 Demographic and baseline characteristics

The demographic and baseline characteristics of randomised participants are shown in table 3.3. The two groups were balanced with regards these characteristics. Over half (50/88) of women in the trial were obese, with almost one fifth (15/88) being super-obese, defined as a BMI ≥40kg/m². The median waist:hip ratio in the two groups was close to the 0.85 threshold used by the WHO to define abdominal obesity (WHO 2008).

Seven percent (3/88) of women in the metformin and placebo arms had been previously diagnosed with non-diabetic hyperglycaemia or diabetes and were being managed with dietary modification. Eleven percent (10/88) of women, however, had previously undiagnosed non-diabetic hyperglycaemia, a finding replicated in the broader endometrial cancer population (chapter 5). One woman in the metformin arm was diagnosed with type 2 diabetes on the basis of her baseline HbA1C measurement.

Ninety-five percent (84/88) of women had endometrial cancer, with the majority (72/88) having low grade, early stage (I and II) disease, in keeping with the usual stage distribution in the UK. Twenty percent (9/45) of women in the metformin arm and 7% (3/43) in the placebo arm had extra-uterine disease at the time of surgery. The hormonal status and mutational profile of tumours was relatively homogeneous with almost all found to be oestrogen and progesterone receptor positive. Fewer than 10% (6/88) of tumours were classed as p53 mutant.
Table 3-3 Demographic and baseline characteristics. Results are presented as median (range) or n (%). Undiagnosed non-diabetic hyperglycaemia and type 2 diabetes were defined as an HbA1C >42mmol/mol and >48mmol/mol, respectively, in a person previously not known to have the condition.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Metformin arm (n=45)</th>
<th>Placebo arm (n=43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>64.0 (29.6-83.7)</td>
<td>67.1 (39.8-85.2)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25</td>
<td>31.0 (20.2-54.2)</td>
<td>32.0 (17.8-47.6)</td>
</tr>
<tr>
<td>25-29.9</td>
<td>8 (17.8)</td>
<td>9 (20.9)</td>
</tr>
<tr>
<td>30-34.9</td>
<td>11 (24.4)</td>
<td>10 (23.3)</td>
</tr>
<tr>
<td>35-39.9</td>
<td>11 (24.4)</td>
<td>11 (25.6)</td>
</tr>
<tr>
<td>≥40</td>
<td>8 (17.8)</td>
<td>5 (11.6)</td>
</tr>
<tr>
<td>Waist:hip ratio</td>
<td>0.84 (0.74-0.99)</td>
<td>0.83 (0.70-0.96)</td>
</tr>
<tr>
<td>Diagnosed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diagnosed Diabetes</td>
<td>0 (0.0)</td>
<td>2 (4.7)</td>
</tr>
<tr>
<td>Non-diabetic hyperglycaemia</td>
<td>3 (6.7)</td>
<td>1 (2.3)</td>
</tr>
<tr>
<td>Undiagnosed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undiagnosed Diabetes</td>
<td>1 (2.2)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Non-diabetic hyperglycaemia</td>
<td>7 (15.6)</td>
<td>3 (7.0)</td>
</tr>
<tr>
<td>Tumour grade at hysterectomy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEH</td>
<td>2 (4.4)</td>
<td>2 (4.7)</td>
</tr>
<tr>
<td>1</td>
<td>26 (57.8)</td>
<td>23 (53.5)</td>
</tr>
<tr>
<td>2</td>
<td>10 (22.2)</td>
<td>12 (27.9)</td>
</tr>
<tr>
<td>3</td>
<td>6 (13.3)</td>
<td>6 (14.0)</td>
</tr>
<tr>
<td>4</td>
<td>1 (2.2)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>FIGO (2009) stage at hysterectomy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>29 (64.4)</td>
<td>23 (53.5)</td>
</tr>
<tr>
<td>1b</td>
<td>4 (8.9)</td>
<td>11 (25.6)</td>
</tr>
<tr>
<td>2</td>
<td>1 (2.2)</td>
<td>4 (9.3)</td>
</tr>
<tr>
<td>3</td>
<td>9 (20.0)</td>
<td>3 (7.0)</td>
</tr>
<tr>
<td>4</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>N/A</td>
<td>2 (4.4)</td>
<td>2 (4.7)</td>
</tr>
<tr>
<td>ER expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>44 (88.9)</td>
<td>43 (100.0)</td>
</tr>
<tr>
<td>Negative</td>
<td>1 (2.2)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>PR expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>42 (93.3)</td>
<td>42 (97.7)</td>
</tr>
<tr>
<td>Negative</td>
<td>3 (6.7)</td>
<td>1 (2.3)</td>
</tr>
</tbody>
</table>
### 3.3.3 Duration of treatment and compliance

The mean duration of treatment in the metformin arm was $20.5\pm7.3$ days and $21.5\pm6.6$ days in the placebo arm. Compliance was generally good with 80.0% of women in the metformin arm and 86.0% of women in the placebo arm missing two or fewer doses of the trial medication.

### 3.3.4 Tolerability of treatment

Metformin was well tolerated with no women discontinuing treatment due to adverse events. Women in the metformin arm were more likely to report any adverse event (37/45) than those in the placebo arm (24/44) and these were of greater severity ($p=0.001$). In particular, women in the metformin arm were significantly more likely to report nausea and vomiting (37.8% vs. 13.6%, $p=0.015$), diarrhoea (51.1% vs. 13.6%, $p=0.0003$) and loss of appetite (20.0% vs. 0.0%, $p=0.003$, table 3-4).

<table>
<thead>
<tr>
<th>p53 status</th>
<th>Wild type</th>
<th>Mutant</th>
<th>Missing cores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>39 (86.7)</td>
<td>4 (8.9)</td>
<td>2 (4.4)</td>
</tr>
<tr>
<td></td>
<td>40 (93.0)</td>
<td>2 (4.7)</td>
<td>1 (2.3)</td>
</tr>
</tbody>
</table>

AEH atypical endometrial hyperplasia, FIGO International Federation of Gynecology and Obstetrics
Table 3-4 Adverse events. For the purpose of adverse event reporting, all participants who took at least one dose of the trial medication were included (metformin=45, placebo=44).

<table>
<thead>
<tr>
<th>Adverse events</th>
<th>Metformin, n (%)</th>
<th>Placebo, n (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worst grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 0</td>
<td>8 (17.8)</td>
<td>19 (43.2)</td>
<td>0.001***</td>
</tr>
<tr>
<td>Grade 1</td>
<td>26 (57.8)</td>
<td>23 (52.3)</td>
<td></td>
</tr>
<tr>
<td>Grade 2</td>
<td>10 (22.2)</td>
<td>2 (4.7)</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>1 (2.2)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>11 (24.4)</td>
<td>6 (13.6)</td>
<td>0.283</td>
</tr>
<tr>
<td>Nausea and vomiting</td>
<td>17 (37.8)</td>
<td>6 (13.6)</td>
<td>0.015*</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>23 (51.1)</td>
<td>6 (13.6)</td>
<td>0.0003***</td>
</tr>
<tr>
<td>Flatulence/bloating</td>
<td>6 (13.3)</td>
<td>2 (4.5)</td>
<td>0.267</td>
</tr>
<tr>
<td>Anorexia</td>
<td>9 (20.0)</td>
<td>0 (0.0)</td>
<td>0.003**</td>
</tr>
<tr>
<td>Taste disturbance</td>
<td>3 (6.7)</td>
<td>2 (4.5)</td>
<td>1.000</td>
</tr>
<tr>
<td>Rash/itching</td>
<td>6 (13.3)</td>
<td>2 (4.5)</td>
<td>0.267</td>
</tr>
<tr>
<td>Renal dysfunction</td>
<td>1 (2.2)</td>
<td>0 (0.0)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

3.3.5 Effect of metformin on Ki-67 expression

Overall, metformin had no effect on endometrial cancer proliferation (figure 3-5). The mean difference between the metformin and placebo arms in post-treatment Ki-67 expression was -0.57% (95%CI -7.57% to +6.42%, p=0.87), after adjusting for baseline Ki-67 expression.
Figure 3-5 Effect of metformin on Ki-67 expression. There was no significant reduction in post (treatment) Ki-67 expression with metformin exposure compared to placebo, after adjustment for pre (treatment) Ki-67 expression.

The primary outcome was based on analysis of Ki-67 expression in the diagnostic endometrial biopsy (pre-treatment) and hysterectomy specimen (post-treatment). In light of the findings described in chapter 2, in which Ki-67 expression of hysterectomy specimens was found to be significantly lower than that of matched endometrial biopsies, the analysis was repeated comparing pre- and post-treatment endometrial biopsies. The result was essentially unchanged (mean difference -2.04%, 95%CI -10.06% to +5.98%, p=0.61).

Pre-specified subgroup analysis, however, revealed a differential response to metformin treatment according to baseline BMI. For women with a BMI <30kg/m², metformin treatment resulted in a change in post-treatment Ki-67 expression of -8.3% (95%CI -18.70 to +2.09) compared to the placebo group (figure 3-7a). In contrast, for women with a BMI ≥30kg/m², post-treatment Ki-67 expression was +5.50% (95%CI -3.57 to +14.57) higher in the metformin arm compared to the placebo arm (figure 3-7b). The difference in effect of metformin in the two BMI groups was of statistical significance (p=0.05).
Figure 3-6 Differential response to metformin treatment according to baseline BMI. a) BMI <30kg/m² b) BMI ≥30kg/m². Metformin treatment was more effective in reducing endometrial cancer proliferation in leaner women. c) Images of tumours stained for Ki-67 from non-obese and obese women prior to and following metformin treatment (x 4 magnification).
The differential response to metformin in non-obese and obese women was, however, unrelated to plasma drug levels. Whilst there was a trend towards lower trough metformin levels in women with a higher BMI, this was not significant ($r=-0.08$, 95%CI -0.37 to 0.23, $p=0.62$, figure 3-8a). No correlation was found between change in Ki-67 expression (final Ki-67 expression-baseline Ki-67 expression) and trough metformin levels ($r=0.08$, 95%CI -0.22 to 0.38, $p=0.59$, figure 3-8b).
Figure 3-7 Interaction between trough metformin levels, BMI and change in Ki-67 expression with metformin treatment. A non-significant trend was found between trough metformin levels and BMI, with lower drug levels found in those with the greatest body mass index (a). Despite this, no correlation was found between trough drug levels and change in Ki-67 expression (b).
Further subgroup analyses did not reveal any other significant modulating effect on response to metformin treatment. Baseline insulin resistance, as measured by HOMA-IR (p=0.52) or HbA1C (p=0.60), total dose of metformin administered (p=0.71) and grade of endometrial cancer (p=0.16) did not influence change in Ki-67 expression.

The relative homogeneity of the hormone receptor and mutational status of tumours within the trial meant that planned subgroup analyses according to these variables could not be performed.

### 3.3.6 Effect of metformin on the PI3K-AKT-mTOR pathway

Metformin treatment was not associated with an increase in AMPK, as determined by phosphorylated expression of its substrate acetyl-CoA carboxylase (ACC). The mean difference in post-treatment pACC levels between the metformin and placebo arms was +11.18 (95%CI -10.59 to +32.96, p=0.31, figure 3-8a). Similarly, exposure to metformin did not result in a reduction in signalling through the PI3K-AKT-mTOR pathway. Neither pS6 nor p4EBP1 or pAkt levels were significantly lower in the metformin treated group compared with the placebo arm (mean difference pS6 -15.96, 95%CI -37.60 to +5.69, p=0.15, p4EBP1 +2.16, 95%CI -17.19 to +21.51, p=0.82, pAkt -5.08, 95%CI -20.39 to +10.24, p=0.51, figure 3-8b-d).
Figure 3-8 Effect of metformin on the PI3K-AKT-mTOR pathway. Metformin treatment was not associated with an increase in pACC levels and neither did it reduce signalling through the PI3K-Akt-mTOR pathway a) pACC, b) pS6, c) p4EBP1, d) pAkt

a)

b)
3.3.7 Effect of metformin on insulin resistance and signalling

As expected, metformin treatment was associated with a significant reduction in post-treatment glucose levels compared with exposure to placebo (mean difference -0.40mmol/l, 95%CI -0.68 to -0.11, p=0.007). This did not, however, translate into improvements in insulin resistance, as measured by either HbA1C or HOMA-IR, or a reduction in serum insulin, IGF-1 or adiponectin levels (table 3-5). Insulin signalling within the endometrial tumours was also unaffected by metformin treatment (table 3-5).
**Table 3-5 Effect of metformin on insulin resistance and signalling.** The only significant effect of metformin was on serum glucose levels (p=0.007); however, this did not translate into any effect on serum or tissue markers of insulin sensitivity or signalling.

<table>
<thead>
<tr>
<th>Serum and tumour markers</th>
<th>Mean difference (95%CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>-0.40 (-0.68 to -0.11)</td>
<td>0.007**</td>
</tr>
<tr>
<td>Insulin (log pmol/l)</td>
<td>-0.07 (-0.31 to +0.17)</td>
<td>0.57</td>
</tr>
<tr>
<td>HOMA-IR (log)</td>
<td>-0.16 (-0.43 to +0.12)</td>
<td>0.26</td>
</tr>
<tr>
<td>HbA1C (mmol/mol)</td>
<td>-0.53 (-1.31 to +0.25)</td>
<td>0.18</td>
</tr>
<tr>
<td>IGF-1 (ng/ml)</td>
<td>+2.05 (-4.90 to +8.99)</td>
<td>0.56</td>
</tr>
<tr>
<td>IGFBP1 (log ng/ml)</td>
<td>-0.18 (-0.49 to +0.13)</td>
<td>0.25</td>
</tr>
<tr>
<td>Adiponectin (mg/l)</td>
<td>-0.15 (-0.35 to +0.05)</td>
<td>0.13</td>
</tr>
<tr>
<td>Tumour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pIR</td>
<td>-1.66 (-23.03 to +19.71)</td>
<td>0.88</td>
</tr>
<tr>
<td>Total IGF1R (log)</td>
<td>-0.37 (-0.88 to +0.14)</td>
<td>0.15</td>
</tr>
<tr>
<td>pIGF1R</td>
<td>+6.67 (-10.92 to +24.26)</td>
<td>0.45</td>
</tr>
<tr>
<td>IGFBP1</td>
<td>+6.08 (-26.85 to +39.01)</td>
<td>0.71</td>
</tr>
</tbody>
</table>

**3.3.8 Effect of metformin on adiposity**

The mean difference in post-treatment weight between the metformin and placebo arms was -0.76kg (95%CI -1.54 to +0.03), however, this was not statistically significant (p=0.06). Similarly, metformin treatment had no effect on the waist:hip ratio (mean difference -0.004, 95%CI -0.01 to +0.02, p=0.60). There was no difference in post-treatment serum leptin levels between the metformin and placebo treated arms (mean difference -3.54 ng/ml, 95%CI -8.83 to +1.75, p=0.19).

**3.3.9 Effect of metformin on apoptosis**

Exposure to metformin was not associated with an increase in apoptosis of endometrial cancer cells, as measured by cleaved caspase-3 expression (log mean difference -0.14, 95%CI -0.80 to +0.53, p=0.69).

**3.3.10 Correlation between changes in Ki-67 expression and secondary outcome measures**

Given the heterogeneous effect of metformin on endometrial cancer proliferation, in order to try and elucidate the mechanism of action of the drug, change in Ki-67 expression following metformin treatment was correlated with secondary outcome measures. Changes in Ki-67 expression following exposure to metformin did not correlate with changes in expression of markers of the PI3K-Akt-
mTOR pathway, serum or tissue markers of insulin sensitivity and signalling or apoptosis (all p>0.05, figure 3-9).
Figure 3-9 Changes in Ki-67 expression with metformin treatment did not correlate with changes in expression of markers of the PI3K-Akt-mTOR or insulin signalling pathways or apoptosis.
3.4 Discussion

In contrast to previously published work, metformin did not reduce endometrial cancer proliferation in all patients when tested in a methodologically rigorous, double-blind, randomised-controlled trial. Instead, a differential response to treatment according to baseline BMI was apparent, with non-obese women demonstrating a reduction in post-treatment Ki-67 expression whilst obese women failed to respond. Metformin did not reduce activation of the PI3K-Akt-mTOR or insulin signalling pathways and did not increase insulin sensitivity or apoptosis within endometrial tumours. The lack of response in obese women was not due to lower metformin levels, suggesting that they are resistant to the anti-tumour effects of the drug.

The marked difference in findings with regards the effect of metformin on endometrial cancer proliferation between this trial and earlier window studies (Mitsuhashi et al. 2014; Laskov et al. 2014; Soliman et al. 2016; Sivalingam et al. 2016; Zhao et al. 2017) was unexpected. Earlier studies had, however, been small in size, uncontrolled and often lacked a contemporaneous control arm for comparison. The major strength of this trial is its robust design, including the incorporation of a placebo arm, blinding of participants and the research team to treatment group allocation for the duration of the study, adequately powered sample size and the use of a standardised, previously published protocol for the staining and quantification of Ki-67 expression (Kitson et al. 2017a). This recommended the use of hot spot scoring as this was the most reliable and reproducible method and had a close correlation with traditional pathological prognostic variables, including grade, stage and depth of myometrial invasion. The other studies had used their own individual staining procedures and had universally employed a whole slide scoring approach, which was often poorly described. Even allowing for this, however, the results of this trial were unchanged; whole slide scores were found to correlate closely with those obtained through the identification of hot spots and there was no difference in post-treatment whole slide Ki-67 expression between metformin and placebo treated groups (results not shown).

A similar situation has previously arisen in breast cancer where the promising results of small, uncontrolled window studies, which had suggested a reduction in breast cancer proliferation with metformin treatment (Hadad et al. 2011; Niraula et al. 2012), failing to be replicated in a larger, randomised trial with a placebo arm (Bonanni et al. 2012). As here, however, baseline patient characteristics appeared to be predictive biomarkers of response with, in the case of breast cancer, insulin resistance (as measured by HOMA-IR) stratifying patient response. In contrast to our results, however, in breast cancer BMI appears to be positively correlated with change in Ki-67 expression with metformin treatment. Women with a BMI ≤27kg/m² had a non-significant increase in Ki-67 expression of +8.2% (95%CI -3.4 to +21.3%) whilst those with a BMI >27kg/m² had a non-significant reduction in Ki-67 expression of 8.0% (95%CI -23.2 to +10.3%, p=0.14). Direct comparison with the endometrial cancer population is difficult, however, particularly as the proportion of obese breast cancer patients randomised was only 13% compared with 56.8% in our study. The earlier pilot work undertaken by our group in endometrial cancer found a similar negative trend to the one described.
here between BMI and change in Ki-67 expression, with leaner women demonstrating a greater response to metformin treatment (Spearman's correlation coefficient $r=0.37$, 95% CI $-0.02$ to $+0.66$, $p=0.054$) (Sivalingam et al. 2016). Metformin is not bound to plasma proteins and, as such, has a large volume of distribution, which is closely linked to lean body weight (Tucker et al. 1981; Bardin et al. 2012). Despite this, the lack of significant correlation between trough metformin levels and BMI or change in Ki-67 expression suggests that adipocyte mediated factors are responsible for the reduction in sensitivity of endometrial cancer cells to metformin. These findings are supported by laboratory experiments conducted in chapter 4 in which exposure to adipocyte conditioned media made endometrial cancer cell lines resistant to metformin treatment. Elucidating the adipokines responsible for this phenomenon may allow the development of targeted therapies to overcome metformin resistance.

The absence of an effect of metformin on phosphorylated markers of the PI3K-Akt-mTOR and insulin signalling pathways is contrary to the findings of some of the earlier endometrial cancer window studies and is likely to be related to the different sampling techniques employed to obtain tumour for the end of trial analyses (Mitsuhashi et al. 2014; Laskov et al. 2014; Dowling et al. 2015; Zhao et al. 2017). On the basis of the findings discussed in chapter 2, the endometrial biopsy taken immediately prior to the start of surgery was used for all immunohistochemical secondary outcome measurements rather than the hysterectomy specimen. This meant that any effect of hypoxia on the expression of these markers was removed and issues related to delays in fixation of large surgical specimens were avoided (Pinhel et al. 2010).

A limitation in interpreting the trial data is its reliance on surrogate biomarkers as its primary outcome instead of clinical measures. The window study design was chosen to allow rapid screening of drug efficacy and, as previously discussed, means that there is insufficient time for an effect on tumour grade and stage, let alone survival, to be realised. The use of Ki-67 expression as a replacement for these variables has not been formally validated and, whilst it has prognostic value in relation to cancer-specific survival, its predictive capacity remains to be determined (Kitson et al. 2017a). The absence of an effect of metformin on endometrial cancer proliferation does not, therefore, necessarily mean that treatment is without any beneficial effect.

The need for limited duration of treatment in order to avoid compromising standard patient care may be an explanation for why no effect of metformin on endometrial tumours was observed. Longer term treatment, of up to six months duration, may have been more effective and would be consistent with the use of metformin for the treatment of type 2 diabetes, where three to six months of treatment is generally required for HbA1C levels to reach target values (NICE 2017). The median treatment period in PREMIUM was similar to that of other window studies, though, which had previously described a reduction in cell proliferation. The sample size calculation performed to determine recruitment was based upon provisional data from the earlier pilot study conducted by our group, which had found a reduction in Ki-67 expression with metformin treatment. The trial was, therefore, adequately powered.
to detect overall differences in endometrial cancer proliferation, but not to specifically address the differential response to metformin treatment in specific subgroups, such as those stratified by BMI. Further research into the potential benefit of metformin as a primary treatment for endometrial cancer in non-obese women is, however, not required as these women are already highly likely to be cured of their disease with standard surgical management.

The results of the PREMIUM study do not necessarily mean there is no role for metformin in other settings for women with or at risk of endometrial cancer. There are currently 91 clinical trials registered on the ClinicalTrials.gov website as investigating the effect of metformin in cancer care (Clinical Trial.Gov 2017a), with many studying the drug in combination with chemo- and radiotherapy to determine its potential to reduce the risk of and treat disease recurrence and metastasis. Long-term adjuvant metformin may be of value in improving overall survival in women with early stage endometrial cancer as part of a multi-modal approach to reducing cardiovascular disease risk (chapter 5). There is also the potential for metformin to be effective in primary endometrial cancer prevention (Kitson et al. 2017b). The results of an ongoing trial of the drug in overweight and obese premenopausal women in terms of its effect on breast cancer risk are eagerly awaited (Clinical Trial.Gov 2017b).

### 3.4.1 Conclusion

Metformin treatment was not associated with a reduction in overall endometrial cancer proliferation, signalling through the PI3K-AKTmTOR pathways or insulin receptors and neither did it increase apoptosis within the endometrial tumour when tested in a high quality, double-blind, placebo-controlled randomised trial. This was due to a lack of response to the drug in obese women, potentially because of adipocyte mediated resistance to its anti-tumour effects. A role for metformin in the primary treatment of endometrial cancer is, therefore, unlikely unless the adipocyte secreted mediators responsible for this phenomenon can be characterised and targeted agents developed to improve metformin sensitivity in obese women. These results emphasise the need for methodologically robust clinical trials to be performed before drugs are repurposed and used in routine oncological practice.
Chapter 4  Characterisation of endometrial cancer stem cells and the effect of metformin

4.1 Introduction

Despite the identification of populations of cells with stem-like properties in endometrial cancer cell lines and primary endometrial tumours using the surface marker CD133 and activity of the ALDH enzyme, characterisation of these cells has been limited and the extent of overlap between CD133\textsuperscript{+ve} and ALDH\textsuperscript{high} cells is unknown (Rutella et al. 2009; Kato et al. 2010; Rahadiani et al. 2011). This extends to both functional studies of cancer stem cell activity, including the well described 'mammosphere' assay, and expression of genes controlling self-renewal, pluripotency and epithelial-mesenchymal transition (EMT) (Sato et al. 2016; Shaker et al. 2017). The latter is an important driver of tumour invasion; overexpression of the EMT-transcription factors Twist, Snai1 and Zeb1 have been found in cancer stem cells in head and neck carcinoma and confers a stem-like phenotype on cancer cells (Chen et al. 2011; Mani et al. 2008).

There has been a similar lack of investigation into potential therapeutic options to target endometrial cancer stem cells. On the basis of evidence from other cancer types, metformin may be of value in this regard as it has been shown to affect cancer stem cells in breast, ovarian and prostate cancer among others (Hirsch et al. 2009; Iliopoulos et al. 2011; Shank et al. 2012).

Whilst a close association between obesity and endometrial cancer risk is well described, the exact means by which excess adiposity drives endometrial carcinogenesis remains unknown and its effect on disease recurrence is still debated. A potential mechanism through which obesity could initiate tumour formation and promote disease relapse is through modulation of endometrial cancer stem cell activity. Such an effect has been described in ovarian and prostate cancer stem cells, with adipocyte secreted proteins, in particular leptin, found to be responsible for increasing cell invasion and migration, and the promotion of stemness related gene expression and behaviour (Tang et al. 2016; Kato et al. 2015). As the endometrium and adipose tissue are anatomically distant, any communication between the two is also likely to be via adipokine mediators.

This study, therefore, sought to further define CD133\textsuperscript{+ve} and ALDH\textsuperscript{high} cells, comparing their phenotype and expression of stem cell and EMT-transcription factor genes, and to determine the extent of overlap between these two populations. The effect of metformin on endometrial cancer stem cells was examined using both in vitro assays and the expression of markers of cancer stem cell activity in vivo. The impact of adipocyte secreted mediators on both endometrial cancer stem cell activity and the sensitivity of these cells to metformin was investigated using adipocyte conditioned media.
4.2 Material and methods

4.2.1 Endometrial cancer cell lines

Two established endometrial cancer cell lines, Ishikawa and Hec-1a, were used for experiments. Ishikawa cells were purchased from HPA Culture Collection (Salisbury, UK) and are a well differentiated endometrioid endometrial cancer cell line with positive oestrogen and progesterone receptor status and wild type K-Ras (Iglesias et al. 2013). Hec-1a is a moderately differentiated endometrioid endometrial cancer cell line with defective oestrogen receptors, activating K-ras mutations and positive PTEN expression (Iglesias et al. 2013). They were obtained from ATCC (Middlesex, UK). A third cell line, KLE (ATCC, Middlesex, UK), was also initially used as it was considered representative of poorly differentiated endometrial cancer cells with no oestrogen receptor expression or K-ras mutations (Richardson et al. 1984). This cell line, however, did not grow well in culture, frequently changed phenotype after being passaged and produced inconsistent results when used to form spheres. There has also been some debate over whether it is an undifferentiated endometrioid endometrial cancer cell line or is actually derived from a non-endometrioid tumour (Gao et al. 2012). For these reasons its use in experiments was discontinued.

All cell culture was performed in sterile conditions in a laminar flow hood. Cells were cultured in growth media composed of DMEM/F12 media (Gibco, Paisley, UK) with a glucose concentration of 4.5g/l, supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Dorset, UK) and additional glutamine (1%, Gibco, Paisley, UK). Cells were grown in a humidified incubator at 37°C and 5% CO₂.

4.2.1.1 Thawing cells

Cells were removed from liquid nitrogen storage and thawed rapidly in a water bath at 37°C. Growth media was added and the cells were centrifuged at 500g for 4 minutes. The media was then aspirated and the cell pellet resuspended in fresh growth media before being transferred to a vented flask and placed in an incubator.

4.2.1.2 Sub-culture of cells

Routine passage was performed when cells reached 80-90% confluency. The growth media was aspirated and cells were washed with phosphate buffered saline (PBS). Pre-warmed trypsin/ethylenediaminetetracetic acid (EDTA, Sigma-Aldrich, Dorset, UK) was added to the flask and the cells returned to the incubator for five minutes to detach. The trypsin was neutralised with the addition of fresh growth media and cells were reseeded in an appropriately sized vented flask at a density of 1x10^5 cells/ml.

4.2.1.3 Freezing cells

Cells were harvested as described above and cell count performed using a haemocytometer or automated cell count, where available. Viable cells were distinguished from dead cells through the use of trypan blue. Cells were pelleted by centrifugation at 500g for 4 minutes. The growth media was aspirated and the pellet resuspended in an appropriate volume of freezing media, consisting of
DMEM/F12 with 20% fetal bovine serum and 10% dimethyl sulphoxide (DMSO) before being transferred to a cryovial for storage. The final concentration of cells was $1 \times 10^6$ cells/ml. Cryovials were placed in an isopropanol gradient freezing pot in a -80°C freezer overnight and then liquid nitrogen for longer term storage.

4.2.2 Adipocyte culture

In order to investigate the impact of obesity on endometrial cancer biology, endometrial cancer cell lines were exposed to conditioned media from primary pre- and mature adipocytes.

4.2.2.1 Primary pre-adipocyte culture from omental biopsies

Pre-adipocytes were extracted from omental biopsies performed at the time of routine surgery for endometrial cancer or from patients undergoing investigation and treatment of pelvic pain and endometriosis. Ethics approval was obtained from the NRES committees North West-Haydock and South West-Cornwall and Plymouth (15th May 2013) for this purpose. The omental tissue was washed twice in PBS to remove excess blood and diathermised sections of tissue were removed. The tissue was finely dissected with scissors into 1-2mm$^3$ pieces before being transferred into a centrifuge tube. Collagenase/dispase (Roche, Sussex, UK) was dissolved in pre-adipocyte growth media (DMEM/F12 media plus 10% FBS, 1% glutamine and 1% penicillin/streptomycin [Sigma-Aldrich, Dorset, UK]) to give a final concentration of 1mg/ml and was added to the dissected omental tissue. The tissue suspension was placed on a flask shaker for two hours at 37°C to aid digestion before being filtered through a 100µm filter to remove connective tissue and undigested fat. The remaining cell suspension was centrifuged at 400g for five minutes to form a cell pellet, which was resuspended in pre-adipocyte growth media and transferred to a humidified incubator for culture. The growth media was changed every 3-4 days until cells were confluent.

If cells were not required immediately for differentiation or conditioned media experiments they were harvested as per endometrial cancer cell lines and stored in liquid nitrogen in specific pre-adipocyte freezing media. This consisted of FBS with 10% DMSO and 10% pre-adipocyte growth media.

4.2.2.2 Pre-adipocyte differentiation

Confluent pre-adipocytes were treated with adipocyte differentiation media (Sigma-Aldrich, Dorset, UK) for up to 15 days. Attainment of a mature adipocyte phenotype was confirmed by a change in morphology of cells from an elongated to a rounded shape and Oil Red O staining.

4.2.2.3 Oil Red O staining

Oil Red O staining was performed to identify neutral triglycerides and lipids within adipocytes as confirmation of their maturation. A working solution was made fresh for each experiment by diluting three parts of a stock solution (Sigma Aldrich, Dorset, UK) with two parts distilled water before passing it through a 0.2µm syringe filter to remove any debris. The solution was kept in the dark until required. The media was aspirated from wells containing confluent mature adipocytes and 10% formaldehyde in PBS added and left for 10 minutes for fixation to occur. Cells were subsequently
washed twice with PBS before the working solution of Oil Red O stain was applied and left for 15 minutes. Residual extracellular stain was removed by washing with distilled water until the water was clear before cells were visualised under a light microscope.

4.2.2.4 Conditioned media
Pre- and mature adipocyte-conditioned media was used to investigate the relationship between adipocytes and endometrial cancer cell proliferation and stem cell activity. Once pre-adipocytes had reached confluency and full differentiation of mature adipocytes was complete, media was removed and replaced with DMEM/F12 (Gibco, Paisley, UK) supplemented with 1% glutamine and 1% penicillin/streptomycin without the addition of FBS. Conditioned media was subsequently removed at 24 hours and either used immediately or stored at -80°C in cryovials until required.

4.2.3 Drug preparation
Metformin hydrochloride 500mg (Sigma-Aldrich, Dorset, UK) was used to make a working stock solution of 100mM metformin in growth media and was diluted as required.

4.2.4 Sulforhodamine B (SRB) cytotoxicity assay
The SRB cytotoxicity assay was used to study the effects of metformin and conditioned media treatment on endometrial cancer cell viability. Ishikawa and Hec-1a cells were plated in 96 well plates in growth media at a density of 1000 cells/well or 10,000 cells/well, the latter in the case of conditioned media experiments where cell proliferation was markedly reduced due to the absence of FBS in the conditioned media. After 24 hours, the growth media was replaced with either fresh growth media or conditioned media with or without the addition of metformin and maintained in an incubator for four (conditioned media experiments) or five days. Cells were then fixed with 10% trichloracetic acid for one hour at 4-8°C before being washed with PBS and left to dry in a fume cupboard overnight. The following day, 0.4% SRB (Sigma-Aldrich, Dorset, UK) was added to the wells and left for 15 minutes before unbound stain was removed by washing with 1% acetic acid. Bound protein was solubilised with 10mM Tris and absorption measured at 540nM using a microplate photometer.

4.2.5 Sphere formation and passaging
Cancer stem cell activity was investigated by determining the sphere formation efficiency of endometrial cancer cell lines in the presence and absence of metformin. The effect of metformin on cancer stem cell self-renewal was examined by passaging spheres.

4.2.5.1 Sphere formation
A poly-HEMA solution was made by adding 12g of poly (2-hydroxyethylmethacrylate) [Sigma-Aldrich, Dorset, UK] to one litre of 95% ethanol. The solution was placed on a heated shaker for 12 hours until the poly-HEMA had dissolved completely. Two mls of the solution was added to each well of a six well plate, which was placed in an oven at 50°C for five days until the solution had evaporated.
The low-adherent plates were placed in a laminar flow hood for 10 minutes under UV light before experiments were commenced to reduce the risk of contamination. Stem cell media was made using phenol free, high glucose DMEM/F12 (Gibco, Paisley, UK) to which 1% penicillin/streptomycin, 20ng/ml EGF and 10ml B27 (Gibco, Paisley, UK) was added. Endometrial cancer cells were harvested as previously described and the trypsin neutralised with growth media. Cell count was performed and 100,000 cells transferred to a clean tube before being centrifuged at 500g for four minutes. The subsequent cell pellet was resuspended in stem cell media and passed through a 25G needle and syringe to ensure a single cell suspension was formed. Two mls of stem cell media, with or without additional metformin, was pipetted into each well of a six well plate to which 5000 Ishikawa cells or 2000 Hec-1a cells were added. Plates were transferred to a humidified incubator where they were left for five days undisturbed before the number of spheres formed was counted.

Sphere count was performed at x40 magnification by light microscopy using an eye piece graticular and systematically recording the number of spheres >50µm in size. The sphere formation efficiency (SFE) was calculated by dividing the number of spheres formed by the number of single cells initially plated in suspension.

4.2.5.2 Sphere passaging
After performing a sphere count, media was aspirated from all replicate wells and passed through a 1000µl tip to disrupt the sphere structure. Cells were centrifuged at 450g for two minutes before the media was aspirated and 500µl trypsin added to the cell pellet to encourage cell separation. Cells were placed in the incubator for 90 seconds after which the trypsin was neutralised by the addition of an equal volume of growth media. Cells were centrifuged once more at 600g for five minutes, resuspended in stem cell media and replated at their original cell density assuming 100 cells per sphere formed at passage zero (Shaw et al. 2012). A further sphere count was performed five days later.

The self-renewal capacity of the endometrial cancer cell lines was calculated by dividing the total number of secondary spheres formed by the total number of primary spheres.

4.2.6 Flow cytometry
Flow cytometry was used to investigate the expression of markers of cancer stem cell activity and the mitochondrial mass of these cells and was performed using the BD LSR II flow cytometer (BD Biosciences, Oxford, UK) and analysed with FlowJo software. Cell sorting was performed by the Flow Cytometry team at CRUK-MI using the Aria II or III flow cytometer (BD Biosciences, Oxford, UK), according to availability.

4.2.6.1 CD133
Ishikawa and Hec-1a cells were grown in growth media, as previously described, in the presence or absence of metformin for five days. Cells were harvested and a cell count performed and 1 x 10⁶-10⁷
cells transferred to a centrifuge tube and spun at 500g for four minutes. The cell pellet was resuspended in a buffer composed of 0.5% bovine serum albumin (BSA), 2mM EDTA and PBS and was incubated with either CD133-APC or CD133-VioBright FITC antibodies (both monoclonal mouse, Miltenyi Biotec, dilution 1:500) and FcR blocking reagent (Miltenyi Biotec, dilution 1:5) or isotype control antibody (mouse IgG1, Miltenyi Biotec, dilution 1:11) for 10 minutes in the dark at 2-8°C. Following incubation, cells were washed and resuspended in the buffer for flow cytometry.

4.2.6.2 ALDH activity
ALDH activity was measured using the ALDEFLUOR assay (STEMCELL technologies, Cambridge, UK) according to the manufacturer’s protocol. The ALDEFLUOR reagent, BODIPY-aminoacetaldehyde (BAAA) is a fluorescent substrate for the ALDH enzyme and is able to freely diffuse into live, intact cells. In the presence of ALDH, it is converted into BODIPY-aminoacetate (BAA), which is unable to pass out of cells due to an efflux inhibitor contained within the ALDEFLUOR buffer. The amount of fluorescent product formed is measured by flow cytometry and is directly proportional to the activity of the ALDH enzyme. An inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), is used to control for background fluorescence.

Ishikawa and Hec-1a cells were harvested and $1 \times 10^6$-$10^7$ cells were transferred to a 1.5μl eppendorf and centrifuged at 600g for five minutes. The subsequent cell pellet was resuspended in 1ml ALDEFLUOR buffer, which had been pre-warmed to room temperature. Five microlitres of ALDEFLUOR Reagent was added to the ‘test’ tube and mixed thoroughly before 500μl of the mixture was transferred to a separate ‘control’ tube containing ALDEFLUOR DEAB reagent. Both tubes were incubated for 45 minutes at 37°C on a heat block. Cells were centrifuged again at 600g for five minutes, washed and the cell pellet resuspended in 500μl of fresh buffer and transferred to fluorescence activated cell sorting (FACS) tube for analysis. The stain 7-actinomycin-D (7AAD) was used at a concentration of 5μl per million cells to discriminate live and dead cells and was added to the FACS tube shortly prior to performing flow cytometry.

Where dual staining with CD133 was undertaken, the ALDEFLUOR assay was performed up until the second centrifugation step at which point the cell pellet was resuspended in ALDEFLUOR buffer containing CD133-APC antibody (dilution 1:500) and FcR blocking reagent (dilution 1:5) or isotype control (dilution 1:11). Cells were incubated in the dark at 2-8°C for 10 minutes before being centrifuged at 600g for five minutes, washed and resuspended in fresh buffer for analysis.

4.2.6.3 Mitochondrial mass
Cancer stem cells in other cancer types have been shown to be characterised by increased mitochondrial mass (Farnie et al. 2015). If this were similarly the case with endometrial cancer stem cells, it could be hypothesised that they would be more susceptible to inhibitors of mitochondrial function, such as metformin, than bulk tumour cells. The mitochondrial mass of Ishikawa and Hec-1a cells was determined using the MitoTracker Deep Red dye according to the manufacturer’s protocol. In brief, cells were incubated with 25nM MitoTracker dissolved in DMSO for 45 minutes at 37°C either
at the same time as the ALDEFLUOR reagent or prior to the addition of the CD133 antibody. The remaining methodology followed that of the corresponding cancer stem cell marker.

4.2.7 RNA extraction

Total RNA was extracted from single cells sorted on the basis of expression of the cancer stem cell markers CD133 and high ALDH activity and unsorted Ishikawa and Hec-1a cells grown in normal growth media with or without metformin supplementation. This was subsequently used in PCR experiments to study the expression of stem cell and EMT genes in the presence and absence of metformin.

RNA extraction was performed using the RNeasy Micro Kit (Qiagen, Manchester, UK) according to the manufacturer’s protocol. In brief, sorted cells were centrifuged at 300g for five minutes and the cell pellet resuspended in buffer RLT with β-mercaptoethanol to denature any RNases present. The lysate, once mixed, was added directly to a QIAshredder column and centrifuged at high speed (≥8000g) for two minutes before being added to a gDNA eliminator column to remove genomic DNA. Following further high speed centrifugation, 70% ethanol was added to the flow through to precipitate RNA. The sample was then transferred to an RNeasy MinElute spin column and centrifuged at high speed for 15 seconds to purify the RNA. Washes with buffers RW1 and RPE were performed before 80% ethanol was added to bind the RNA to the column. The column was then placed in a clean collection tube and centrifuged again to remove all ethanol before 14µl RNAse free water was added and the resulting flow through following centrifugation was collected. Quantification and quality assurance of the extracted RNA was performed by the Molecular Biology Core Facility at CRUK-MI using the Agilent 2100 Bioanalyzer and Qubit RNA HS Assay kit (Fisher Scientific, Loughborough, UK). Samples were stored at -80°C until required.

4.2.8 Reverse transcription

Extracted RNA was diluted to a final concentration of 1.5ng/ml using RNase-free water to allow comparison across samples. Reverse Transcription Mastermix (PN 100-6297, Fluidigm, San Francisco, USA) containing buffer, dNTPS, primers, a ribonuclease inhibitor and an engineered RNaseH+ MMLV reverse transcriptase was added to the diluted RNA. Samples were subsequently mixed, centrifuged and placed in a thermal cycler (PTC-200 Thermal Cycler, MJ Research, Minnesota, USA). The thermal cycler was programmed for 5 minutes at 25°C for primer annealing, followed by 30 minutes at 42°C for extension and 5 minutes at 85°C to inactivate the reverse transcriptase. The cDNA formed was immediately pre-amplified.

4.2.9 Pre-amplification of cDNA

The list of genes to be amplified was selected on the basis of their increased expression in cancer stem cells and role in determining pluripotency (Sox2, Nanog, Oct4, BMI1, Lin28a, Hey1, Hes1),
controlling EMT (Twist1, Snai1 and 2, Zeb1), Wnt pathway activation (Wnt2, β-catenin) and Hippo signalling (Taz, Yap1). Epithelial (E-cadherin, Occludin, Claudin, Desmplakin, EpCam) and mesenchymal (Vimentin, N-cadherin) markers were also chosen. PGK1 was used as a housekeeper gene, to which expression of all other genes was normalised.

TaqMan® gene expression assays (table 4-1, Fisher Scientific, Loughborough, UK), containing pre-optimised forward and reverse primers and sequence-specific probes, were pooled and diluted to a final concentration of 0.2X in DNA Suspension Buffer (10mM Tris, pH8.0, 0.1mM EDTA, PN T0221, Teknova, Hollister, USA). cDNA and TaqMan PreAmp MasterMix (OPN 4391128, Fisher Scientific, Loughborough, UK) were added to the pooled assay mix and the reactions were briefly mixed and centrifuged. Pre-amplification was performed in a thermal cycler (PTC-200 Thermal Cycler, MJ Research, Minnesota, USA) programmed for 95°C for 10 minutes for initial denaturation, followed by 15 cycles at 95°C for 15 seconds for denaturation and 60°C for 4 minutes for annealing/extension. After cycling, the reaction was diluted 1:5 using DNA Suspension Buffer (Teknova, Hollister, USA) and stored at -20°C for up to 2 weeks.
Table 4-1 TaqMan assays used for gene expression analysis

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene Name</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX2</td>
<td>SOX2</td>
<td>Hs01053049_s1</td>
</tr>
<tr>
<td>NANOG</td>
<td>NANOG</td>
<td>Hs02387400_g1</td>
</tr>
<tr>
<td>OCT4</td>
<td>POU5F1</td>
<td>Hs04260367_gH</td>
</tr>
<tr>
<td>BMI1</td>
<td>BMI1</td>
<td>Hs00995520_g1</td>
</tr>
<tr>
<td>LIN28A</td>
<td>LIN28A</td>
<td>Hs00702808_s1</td>
</tr>
<tr>
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<td>HEY1</td>
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<tr>
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<td>HES1</td>
<td>Hs00172878_m1</td>
</tr>
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<td>WNT2</td>
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<tr>
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<td>B-CATENIN</td>
<td>Hs00355045_m1</td>
</tr>
<tr>
<td>YAP1</td>
<td>YAP1</td>
<td>Hs00902712_g1</td>
</tr>
<tr>
<td>TAZ</td>
<td>TAZ</td>
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</tr>
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<td>Hs01675818_s1</td>
</tr>
<tr>
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<td>SNAI1</td>
<td>Hs00195591_m1</td>
</tr>
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<td>SNAI2</td>
<td>SNAI2</td>
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</tbody>
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4.2.10 Real Time quantitative-Reverse Transcription-Polymerase Chain Reaction

Real Time quantitative-Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) was performed using the Flex Six™ Integrated Fluidic Circuit (IFC, Fluidigm, San Francisco, USA). It is composed of six 12 assays-by-12 samples partitions which can be run either simultaneously or on separate occasions and utilises smaller volumes of reagents and samples than traditional PCR experiments performed on 96-well plates. At the first time of use, the Flex Six Chip was primed by injecting control line fluid into each accumulator and running the Prime Script (153x) in the IFC Controller HX. Assay stocks (x10) were prepared using the TaqMan® gene expression assays of interest and a housekeeper gene, phosphoglycerate kinase 1 (PGK1), diluted in Assay Loading Reagent (PN 100-5359, Fluidigm, San Francisco, USA). Pre-amplified cDNA was added to a sample pre-mix composed of
TaqMan Fast Advanced Master Mix (2X, PN 4444557, Fisher Scientific, Loughborough, UK) and 20x GE Sample Reagent (PN 100-6311, Fluidigm, San Francisco, USA). All assay and sample solutions were vortexed and centrifuged before being pipetted into the IFC inlets. The IFC was returned to the IFC Controller HX and the Load Mix (153x) script run. Once complete, the Flex Six chip was transferred to the BiomarkHD system in which the qPCR was performed. This was programmed for a thermal mix consisting of 25°C for 30 minutes, 70°C for 60 minutes and 50°C for 2 minutes followed by a Hot Start at 95°C for 1 minute and 35 cycles composed of 96°C for 5 seconds for denaturing and 60°C for 20 seconds for annealing. Upon completion, the Flex Six IFC was returned to the IFC Controller HX for the Post Run Process to relax the valves and ensure the 90-day lifetime of the chip.

Data were analysed using the Biomark Real-Time PCR Analysis Software.

The fold change in gene expression was determined using the formula:

\[ \text{fold change} = 2^{\Delta \Delta \text{Ct}} \]

where Ct is the threshold cycle, \( \Delta \text{Ct} = \text{Ct of the specific gene} - \text{Ct of PGK1 (housekeeper gene)} \) and \( \Delta \Delta \text{Ct} = \Delta \text{Ct of ALDH}^{\text{high}}/\text{CD133}^{+} \text{cells} - \Delta \text{Ct of ALDH}^{\text{low}}/\text{CD133}^{-} \text{cells}. \)

### 4.2.11 Immunohistochemistry

Immunohistochemical staining for CD133 and ALDH was performed as described in chapter 2. In brief, TMA was constructed from endometrial cancers sampled by in situ biopsy prior to and following treatment with either metformin or matched placebo as part of the PREMIUM study (chapter 3). Staining of 4µm sections of the TMA was performed using the Leica Bond Max with heat induced epitope retrieval at pH 6 (CD133) and pH 9 (ALDH). Primary antibody incubation was performed for one hour at room temperature with CD133 (#130-090-422, Miltenyi Biotec, Surrey, UK) at dilution 1:25 or ALDH (611194, BD Biosciences, Oxford, UK) at dilution 1:100. The primary antibody was detected using the Refine Detection Kit.

Slides were digitised using the Leica SCN400 slide scanner and scored with the Definiens Developer software, with the scorer blinded to treatment group allocation. The percentage of cells with positive apical membrane (CD133) or cytoplasmic (ALDH) staining was scored, regardless of staining intensity, in all endometrial cancer glands contained within triplicate repeat cores.

### 4.2.12 Statistical analysis

All experiments were performed in triplicate on at least three independent occasions to ensure consistency of results and to reduce the risk of error. Results are presented as the mean ± standard error of the mean (SEM). The student’s T test or analysis of variance was used to compare normally distributed, paired data. The effect of metformin on the immunohistochemical expression of ALDH and CD133 was determined using an analysis of covariance model with baseline expression as a covariate. A p value ≤0.05 was considered statistically significant, with asterisks used to denote
significant results as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$. The statistical analysis was conducted using SPSS version 23.0 and Graph Pad version 7.

4.3 Results

4.3.1 Characterising endometrial cancer stem-like cells

4.3.1.1 Sphere formation and self-renewal
Both Ishikawa and Hec-1a endometrioid endometrial cancer cell lines contained a small proportion of cells with the ability to survive, proliferate and form three-dimensional spherical structures in attachment-free conditions. The characteristic spheres formed are demonstrated in figure 4-1.

Figure 4-1 Spheres formed in low adherent conditions by a) Ishikawa cells b) HEC-1a cells at x40 magnification. ‘Spheres’ formed by the two cell lines had differing appearances, with Ishikawa cells forming truly spherical structures whilst ‘spheres’ formed by Hec-1a cells were less compact and had a more irregular outline.

Hec-1a cells had significantly greater SFE than Ishikawa cells (2.01%±0.13 vs. 0.71%±0.06, $p<0.0001$), reflecting their less well differentiated phenotype and greater cancer stem cell activity.

The self-renewal capacity of Ishikawa and Hec-1a cancer stem cells was demonstrated through the serial passage of spheres. Spheres formed from Ishikawa cells contained viable cells to at least passage 2 and Hec-1a cells continued to form spheres to at least passage 3. The self-renewal capacity of both cell lines increased with each passage, suggesting positive selection for cancer stem cells (results not shown).
4.3.1.2 ALDH activity

A small proportion of Ishikawa and Hec-1a cells demonstrated high ALDH activity. As anticipated, the proportion of ALDH\(^{\text{high}}\) cells was greater in the Hec-1a cell line than the Ishikawa cell line (3.4\%\pm1.1 vs. 0.4\%\pm0.1, \(p=0.07\)). A representative example of the flow cytometry analysis and gating for ALDH\(^{\text{high}}\) cells is shown in figure 4-2.

Figure 4-2 Representative example of flow cytometry analysis and gating for ALDH\(^{\text{high}}\) cells. ALDH\(^{\text{high}}\) cells were discriminated from ALDH\(^{\text{low}}\) cells using the ALDH inhibitor DEAB.

ALDH activity was identified as a marker of sphere-forming activity. ALDH\(^{\text{high}}\) cells in both Ishikawa and Hec-1a cell lines formed more spheres than ALDH\(^{\text{low}}\) cells, though this was only statistically significant for Hec-1a cells (figure 4-3).
Figure 4-3 ALDH is a marker of sphere forming activity in a) Ishikawa and b) Hec-1a cells. Ishikawa cells with high ALDH activity had increased SFE compared with cells with low ALDH activity (SFE 0.55%±0.15 vs. 0.25%±0.02, p=0.15). Similarly, ALDH^{hi} Hec-1a cells had twice the SFE of ALDH^{lo} cells (SFE 0.66%±0.07 vs. 0.32%±0.05, p=0.005).

4.3.1.3 Expression of CD133
Less than one fifth of Ishikawa cells were identified as being CD133+ve and these cells had greater sphere forming capacity than CD133-ve cells, suggesting it measures cancer stem cell activity in this cell line (figure 4-4). In contrast, no CD133+ve cells were identified in the Hec-1a cell line. A representative example of flow cytometry analysis and gating for CD133+ve cells is shown in figure 4-5.
Figure 4-4 CD133 is a marker of sphere-forming activity in Ishikawa cells. CD133+ve cells had 3.5 times the SFE of CD133-ve cells (SFE 0.58%±0.7 vs. 0.16%±0.02, p=0.0006).

Figure 4-5 Representative example of flow cytometry analysis and gating for CD133+ve cells. CD133+ve cells were discriminated from CD133-ve cells using an isotype control antibody.
4.3.1.4 Co-expression of cancer stem-like cell markers

Given that both ALDH activity and CD133 identify populations of Ishikawa cells with cancer stem cell activity, the extent of overlap between these two markers and their relative sphere forming activity was determined. Double positive cells had greater cancer stem cell activity than single marker positive and double marker negative cells, forming the most spheres in low-adherent culture (figure 4-6). Double negative cells, as expected, had the lowest SFE (ALDH<sup>high</sup>CD133<sup>-ve</sup> 1.43%±0.53 vs. ALDH<sup>low</sup>CD133<sup>-ve</sup> 0.18%±0.04, p=0.0002). Single marker positive cells formed an intermediate number of spheres. Dual staining revealed that the two markers selected for almost exclusive cell populations (ALDH<sup>high</sup>CD133<sup>+ve</sup> cells 0.01%, figure 4-7).

Figure 4-6 ALDH<sup>high</sup> CD133<sup>-ve</sup> cells have greatest sphere formation efficiency in the Ishikawa cell lines. ALDH<sup>high</sup>CD133<sup>-ve</sup> cells had the greatest SFE whilst ALDH<sup>low</sup>CD133<sup>-ve</sup> cells had the lowest SFE. ALDH activity appeared to be a stronger marker of cancer stem cell activity than CD133 as ALDH<sup>high</sup> cells had greater SFE than CD133<sup>+ve</sup> cells.

The proportion of and relationship between ALDH<sup>high</sup> and CD133<sup>+ve</sup> cells in Ishikawa and Hec-1a cell lines is summarised in figure 4-7.
Figure 4-7 Relationship between cancer stem and non-stem (bulk) cells in endometrial cancer cell lines. The Ishikawa cell line contained distinct populations of cells with high ALDH activity and CD133 positivity, with minimal overlap between the two. In contrast, the Hec-1a cell line contained only ALDH\textsuperscript{high} cells (images not to scale).

4.3.1.5 Mitochondrial mass
Ishikawa and Hec-1a cancer stem cells, identified by ALDH\textsuperscript{high} activity, had a 1.5-2.3 fold higher mitochondrial mass, as measured by MitoTracker mean fluorescent intensity (MFI), than bulk cells with ALDH\textsuperscript{low} activity (both p<0.05, figure 4-8a and b). Similarly, Ishikawa cancer stem cells positive for CD133 had significantly greater mitochondrial mass than CD133\textsuperscript{ve} cells (p<0.001, figure 4-8c).
Figure 4-8 Cancer stem cells are characterised by higher mitochondrial mass. Cells were sorted according to ALDH activity in a) Ishikawa cells and b) Hec-1a cells and according to CD133 positivity in Ishikawa cells only (c). Cells with high ALDH activity in both the Ishikawa and Hec-1a cell lines had greater mitochondrial mass than cells with low ALDH activity. Ishikawa cells positive for CD133 also had 1.3-fold the mitochondrial mass of CD133 negative cells (p=0.0009).

4.3.1.6 Stem cell and EMT-transcription factor gene expression

RT-qPCR was used to establish whether cells with high ALDH activity and CD133 expression did indeed express key genes associated with pluripotency, self-renewal and a cancer stem cell phenotype. Cells with high ALDH activity had increased expression of SOX2, LIN28A and HEY1 compared to cells with low ALDH activity in both the Ishikawa and Hec-1a cell lines (all p<0.05 except
LIN28A p=0.07, figure 4-9a). SOX2 expression, in particular, was up to 57 times higher in Ishikawa cells with high ALDH activity compared with ALDH[^low] cells (p=0.02). NANOG, BMI and HES1 expression was also significantly increased in ALDH[^high] cells but only in the Ishikawa cell line (all p<0.05).

In contrast, CD133[^+ve] Ishikawa cells were found to have increased expression of only LIN28A, HEY1 and HES1 (all p<0.05, except HEY1 p=0.08, figure 4-9b). The fold change in gene expression in CD133[^+ve] compared with CD133[^-ve] Ishikawa cells was, however, less than that seen in Ishikawa cells sorted according to ALDH activity. Expression of SOX2, NANOG and BMI1 was lower in CD133[^+ve] cells than in their negative counterparts, consistent with earlier findings that CD133 positive cells may not have the same level of cancer stem activity as those identified through high ALDH activity.

The OCT4 gene was not amplified in any of the samples tested.
Figure 4-9 Expression of stem cell genes in ALDH$^{\text{high}}$ and CD133$^{+ve}$ cells. a) ALDH$^{\text{high}}$ cells in Ishikawa and Hec-1a cell lines, b) CD133$^{+ve}$ cells in the Ishikawa cell line. Ishikawa cells with high ALDH activity demonstrated increased expression of genes associated with pluripotency and self-renewal when compared to ALDH$^{\text{low}}$ cells. ALDH$^{\text{high}}$ cells in the Hec-1a cell line had increased expression of SOX2, LIN28A and HEY1 only compared with their ALDH$^{\text{low}}$ counterparts. In contrast, CD133$^{+ve}$ Ishikawa cells exhibited increased expression of LIN28A, HEY1 and HES1, but had lower expression of the major regulators of a stem cell phenotype, SOX2, NANOG and BMI1.

a)

b)
Given that increased activation of the Wnt and Hippo signalling pathways is associated with the development of cancer stem cell traits, the relative expression of markers of these pathways in endometrial cancer cells with high ALDH activity and CD133 positivity was determined. There was evidence of increased activation of the Wnt pathway in Ishikawa cells with high ALDH activity, as determined by expression of the genes β-CATENIN and WNT2 (figure 4-10a). In the Hec-1a cell line, expression of only the β-CATENIN gene was increased. There was evidence of overactivity of Hippo signalling in ALDH\textsuperscript{high} cells in both cell lines studied, with a 57.5±13.7 and 3.5±0.6 fold increase in YAP1 expression in the Ishikawa and Hec-1a cell lines respectively (figure 4-10a). The TAZ gene was not amplified in any of the ALDH\textsuperscript{high} cells used in the replicate experiments and, therefore, its relative expression could not be determined.

In contrast, Wnt pathway activity was significantly lower in CD133\textsuperscript{+}ve cells than CD133\textsuperscript{-}ve cells (fold change \textit{WNT2} 0.07±0.00, \textit{p}≤0.0001, \textit{β-CATENIN} 0.67±0.04, \textit{p}=0.001, figure 4-10b). Expression of the Hippo pathway target gene \textit{TAZ} was also found to be significantly lower in CD133\textsuperscript{−}ve Ishikawa cells compared with CD133\textsuperscript{−}ve cells, whilst YAP1 expression was marginally increased (YAP1 fold change 1.3±0.03, \textit{p}=0.003, figure 4-10b).
Figure 4-10 Relative activity of Wnt and Hippo signalling pathways in ALDH$_{\text{high}}$ and CD133$^{+ve}$ endometrial cancer cells. a) ALDH$_{\text{high}}$ cells in Ishikawa and Hec-1a cell lines b) CD133$^{+ve}$ cells in Ishikawa cell line.

Overactivity of both the Wnt and Hippo signalling pathways was found in ALDH$_{\text{high}}$ cells in both cell lines studied, with the exception of lower WNT2 gene expression in ALDH$_{\text{high}}$ Hec-1a cells. In contrast, there was decreased expression of markers of the Wnt and Hippo signalling pathways in CD133$^{+ve}$ Ishikawa cells, though expression of the Hippo target gene $YAP1$ was found to be slightly increased.
Expression of transcription factors involved in controlling EMT, and hence cancer metastasis, were also studied. Expression of *SNAI1* and *SNAI2* was significantly upregulated in ALDH<sup>high</sup> cells from both Ishikawa and Hec-1a cell lines (all p≤0.001, figure 4-11a). In the Ishikawa cell line, there was a 12.2±2.4 fold increase in *TWIST* gene expression in ALDH<sup>high</sup> cells, whilst in the Hec-1a cell line *ZEB1* was overexpressed (*ZEB1* fold change 191.1±48.4, p=0.02).

CD133 positivity, however, was not associated with increased expression of EMT-transcription factors (figure 4-11b). Expression of the genes *TWIST, SNAI1* and *ZEB1* was the same or lower in CD133<sup>+</sup> cells compared with CD133<sup>−</sup> cells, with *ZEB1* expression 5.9±0.02 fold lower in CD133<sup>−</sup> cells than in CD133<sup>+</sup> cells. *SNAI2* was not amplified in the CD133<sup>+</sup> cells used in any of the replicate experiments and, therefore, its relative expression in this cell population could not be determined.
Figure 4-11 The gene expression of EMT-transcription factors in endometrial cancer stem cells identified by a) high ALDH activity, b) CD133 positivity. ALDH$^{\text{high}}$ cells in both Ishikawa and Hec-1a cell lines had greater gene expression of $SNAI1$ and $SNAI2$ than their ALDH$^{\text{low}}$ counterparts. The expression of $TWIST$ and $ZEB1$ differed according to the cell line studied, with higher gene expression of $TWIST$ seen in Ishikawa ALDH$^{\text{high}}$ cells whilst $ZEB1$ was more highly expressed in Hec-1a ALDH$^{\text{high}}$ cells. In contrast, expression of EMT-transcription factors was the same or lower in CD133$^{\text{+ve}}$ cells compared with CD133$^{\text{-ve}}$ cells.
4.3.1.7 Distinct populations of cancer stem cells

To further define the characteristics of cancer stem cells identified through ALDH activity and CD133 positivity, the expression of markers associated with an epithelial-like and mesenchymal-like state were determined. As shown in table 4-2, ALDH\textsuperscript{high} cells demonstrated increased expression of both epithelial and mesenchymal genes, whilst CD133\textsuperscript{+ve} cells demonstrated a reduction in genes associated with an epithelial-like state, including \textit{E-CADHERIN}, and a corresponding increase in the expression of \textit{VIMENTIN}, which is associated with a more mesenchymal phenotype (both \(p\leq0.001\)).

Table 4-2 Expression of genes associated with epithelial-like and mesenchymal-like states in ALDH\textsuperscript{high} and CD133\textsuperscript{+ve} cells.

<table>
<thead>
<tr>
<th>EMT marker</th>
<th>Ishikawa ALDH\textsuperscript{high}/ALDH\textsuperscript{low} fold change log(2)</th>
<th>Hec-1a ALDH\textsuperscript{high}/ALDH\textsuperscript{low} fold change log(2)</th>
<th>Ishikawa CD133\textsuperscript{+ve}/CD133\textsuperscript{+ve} fold change log(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{E-CADHERIN}</td>
<td>4.14</td>
<td>2.62 (0.18)</td>
<td>-3.00 (0.18)</td>
</tr>
<tr>
<td>\textit{OCCLUDIN}</td>
<td>3.31</td>
<td>5.04 (0.02)</td>
<td>-2.22 (0.07)</td>
</tr>
<tr>
<td>\textit{CLAUDIN}</td>
<td>4.10</td>
<td>2.89 (0.20)</td>
<td>-2.24 (0.06)</td>
</tr>
<tr>
<td>\textit{DESMOPLAKIN}</td>
<td>6.10</td>
<td>2.42 (0.15)</td>
<td>-2.96 (0.04)</td>
</tr>
<tr>
<td>\textit{EPCAM}</td>
<td>3.68</td>
<td>0.10 (0.11)</td>
<td>-1.47 (0.12)</td>
</tr>
<tr>
<td>Mesenchymal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{VIMENTIN}</td>
<td>3.82</td>
<td>2.41 (0.18)</td>
<td>1.13 (0.07)</td>
</tr>
<tr>
<td>\textit{N-CADHERIN}</td>
<td>3.81</td>
<td>2.11 (0.10)</td>
<td>-3.54 (0.15)</td>
</tr>
</tbody>
</table>

Results presented as mean (SEM)

4.3.2 Effect of metformin on cancer stem cell markers and activity

The effect of metformin on cancer stem cell number and activity in the two endometrial cancer cell lines was investigated.

4.3.2.1 Effect of metformin on cancer stem cell markers

Metformin at a concentration of 1mM significantly decreased the proportion of cancer stem cells identified by ALDH\textsuperscript{high} activity in Hec-1a cells (figure 4-12). In contrast, the same dose of metformin had no effect on Ishikawa cells, though the population of ALDH\textsuperscript{high} cells in this cell line was markedly smaller.
**Figure 4-12** Effect of metformin on proportion of ALDH$^{\text{high}}$ cells in two endometrial cancer cell lines. Metformin at a concentration of 1mM resulted in a 1.7-fold reduction in the proportion of ALDH$^{\text{high}}$ cells in the Hec-1a cell line (p≤0.05). The same concentration had no effect on the proportion of ALDH$^{\text{high}}$ cells in the Ishikawa cell line due to low cell number.

When the effect of metformin on Ishikawa cancer stem cells identified through expression of the marker CD133 was investigated, both 0.5mM and 1mM concentration significantly reduced the proportion of cancer stem cells in a dose dependent manner (figure 4-13). Treatment with 1mM metformin almost completely eliminated the CD133$^{\text{+ve}}$ population of cells.
Figure 4-13 Metformin decreases the proportion of CD133\(^{+ve}\) cells in Ishikawa cells in a dose-dependent manner. Treatment with 0.5mM metformin resulted in a 1.4-fold reduction in the proportion of CD133\(^{+ve}\) cells (p=0.003). When the dose of metformin was increased to 1mM, the reduction in the proportion of CD133+ve cells was even greater (10-fold reduction, ps<0.0001).

### 4.3.2.2 Effect of metformin on cancer stem cell activity

Metformin had a dose dependent effect on cancer stem cell activity, as measured by sphere formation efficiency, in both Ishikawa and Hec-1a cell lines (figure 4-14). Treatment with 0.5mM metformin resulted in a 1.3-fold decrease in Ishikawa SFE (p=0.03) and a 1.6-fold decrease in Hec-1a SFE (p=0.0008). At a concentration of 20mM metformin, almost no spheres were formed in either cell line.
Figure 4-14 Metformin has a dose dependent effect on sphere formation efficiency a) Ishikawa cells b) Hec-1a cells. Metformin, from a concentration of 0.5mM, reduced the sphere formation efficiency of both Ishikawa and Hec-1a cells.
Whilst metformin decreased cancer stem cell activity in Ishikawa and Hec-1a cells, it had no effect on the self-renewal capacity of these cells (figure 4-15).

**Figure 4-15** Metformin has no effect on the self-renewal capacity of a) Ishikawa and b) Hec-1a cancer stem cells. Treatment of Ishikawa and Hec-1a cells in non-adherent culture with metformin did not affect the self-renewal capacity of either cell line, as measured by the number of second generation spheres formed.

The reduction in SFE of Ishikawa and Hec-1a cells with metformin treatment was the result of a decrease in cancer stem cell activity rather than due to an effect on cell viability, however, as higher concentrations of the drug were required to reduce cell number (figure 4-16). The relative IC\textsubscript{50} values for metformin to inhibit endometrial cancer cell viability and stem cell activity are shown in table 4-3.
Figure 4-16 Higher concentrations of metformin are required to affect a) Ishikawa and b) Hec-1a cell viability. Metformin reduced the viability of Ishikawa and Hec-1a cells but higher concentrations were required to have the equivalent effect to that seen on cancer stem cell activity.
Table 4-3 Comparison of the IC₅₀ values of metformin to reduce cell viability and cancer stem cell activity in Ishikawa and Hec-1a cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Effect</th>
<th>IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ishikawa</td>
<td>Viability</td>
<td>6.77mM</td>
</tr>
<tr>
<td></td>
<td>Cancer stem cell activity</td>
<td>1.60mM</td>
</tr>
<tr>
<td>Hec-1a</td>
<td>Viability</td>
<td>3.72mM</td>
</tr>
<tr>
<td></td>
<td>Cancer stem cell activity</td>
<td>1.46mM</td>
</tr>
</tbody>
</table>

4.3.2.3 Effect of metformin on stem cell and EMT-transcription factor gene expression

Metformin reduced the expression of genes associated with pluripotency and self-renewal in a dose-dependent manner in Ishikawa cells, though it had a less pronounced effect in Hec-1a cells (figure 4-17a and b).
Figure 4-17 Effect of metformin on stem cell genes in a) Ishikawa and b) Hec-1a cell lines. Metformin significantly reduced the expression of genes associated with the development of a stem cell-like phenotype in a dose-dependent manner in Ishikawa cells. The effect in Hec-1a cells was less pronounced and only resulted in a significant reduction in expression of \( SOX2 \) and \( BMI1 \).

a) Ishikawa

![Ishikawa graph]

b) Hec-1a

![Hec-1a graph]
Metformin treatment of Ishikawa cells also resulted in a significant reduction in Wnt pathway activation and Hippo signalling in a dose-dependent manner (figure 4-18a). In Hec-1a cells, 1mM metformin reduced expression of β-CATENIN and YAP1 but resulted in a 2.5±0.4 fold increase in WNT2 expression (figure 4-18b).

TAZ was not amplified in Hec-1a cells in any of the replicate experiments and, therefore, the effect of metformin on its expression could not be determined.
Figure 4-18 Effect of metformin on Wnt and Hippo signalling in endometrial cancer cells a) Ishikawa, b) Hec-1a. Metformin reduced the expression of markers of the Wnt (WNT2 and β-CATENIN) and Hippo (YAP1 and TAZ) signalling pathways in a dose-dependent manner in Ishikawa cells, but had a more variable effect in Hec-1a cells.

**Ishikawa**

![Graph showing fold change in Wnt and Hippo signalling markers in Ishikawa cells after metformin treatment.]

**Hec-1a**

![Graph showing fold change in Wnt and Hippo signalling markers in Hec-1a cells after metformin treatment.]

WNT2 β-CATENIN YAP1 TAZ

0.0 0.5 1.0 1.5

Fold change (compared to control cells)

Control 0.5mM metformin 1mM metformin

WNT2 β-CATENIN YAP1 TAZ

0 1 2 3 4

Fold change (compared to control cells)

Control 0.5mM metformin 1mM metformin

WNT2 β-CATENIN YAP1
The effect of metformin on EMT-transcription factors was more heterogeneous. Treatment with 1mM metformin resulted in a two-fold reduction in the expression of *TWIST* and *SNAI1* (both p≤0.005) in Ishikawa cells (figure 4-19a) but had no significant effect on their expression in Hec-1a cells (figure 4-19b). In contrast, 1mM metformin increased the expression of *SNAI2* and *ZEB1* in Ishikawa cells by 4.7- and 1.3-fold, respectively, but reduced expression of the latter gene in Hec-1a cells (fold change 0.9±0.03, p=0.05).
Figure 4-19 Effect of metformin on EMT-transcription factors in a) Ishikawa cells and b) Hec-1a cells. Metformin, at a concentration of 1mM, significantly reduced the expression of the EMT-transcription factors TWIST and SNAI1 in Ishikawa cells but had no effect on their expression in Hec-1a cells. The same concentration of metformin, however, decreased the expression of ZEB1 in Hec-1a cells whilst resulting in increased expression of the same transcription factor in Ishikawa cells.
4.3.3 Impact of obesity on cancer stem cell activity and metformin sensitivity

To investigate the impact of obesity on endometrial cancer stem cell activity, endometrial cancer cell lines were exposed to conditioned media from primary pre- and mature adipocytes before being used in a sphere formation assay. In light of the potential role of adiposity in modulating responsiveness to metformin highlighted in chapter 3, a comparison of the effect of metformin on endometrial cancer stem cell activity in the presence and absence of adipocyte conditioned media was performed.

4.3.3.1 Optimising adipocyte differentiation

Optimisation of the duration of treatment with adipocyte differentiation media for pre-adipocytes extracted from primary omental biopsies to achieve full maturation was undertaken. Pre-adipocytes not exposed to adipocyte differentiation media retained their fibroblast-like phenotype; they did not contain lipid droplets and did not demonstrate positive staining with Oil Red O (figure 4-20a). Pre-adipocytes treated for 15 days with differentiation media showed 100% differentiation into mature adipocytes, with a rounded morphology and the presence of lipid droplets, which became more obvious with Oil Red O staining (figure 4-20b). Treatment for between 10 and 15 days resulted in lesser degrees of differentiation in a proportion of cells only.

Figure 4-20 Optimisation of pre-adipocyte differentiation a) cells treated with control (pre-adipocyte) media b) cells treated with adipocyte differentiation media (x100 magnification)
4.3.3.2 Effect of adipocyte-conditioned media on cancer stem cell activity and sensitivity to metformin treatment

Pre- and mature adipocyte-conditioned media were generated using pre-adipocytes derived from omental biopsies obtained from women with endometrial cancer. No micro or macroscopic metastases were present in any of the omental specimens.

A significant increase in the cancer stem cell activity of Ishikawa cells was observed in response to treatment with conditioned media from three of the six pre-adipocyte cultures tested and four of the six corresponding adipocyte cultures (figure 4-21a). Adipocyte-conditioned media had a greater effect on cancer stem cell activity than conditioned media from undifferentiated pre-adipocytes, increasing SFE, on average, 3.76±0.81-fold, compared with cells treated with control (unconditioned growth) media (p=0.02).

The normal inhibitory effect of metformin on endometrial cancer stem cell activity, as determined by sphere formation in non-adherent culture, was lost when cells were pre-treated with adipocyte-conditioned media for 96 hours (figure 4-21b). Rather than SFE decreasing with 1mM metformin, as occurred in the control treated cells, SFE was unchanged in Ishikawa cells exposed to adipocyte-conditioned media prior to metformin treatment.
Figure 4-21 Effect of pre-adipocyte (PACM) and adipocyte-conditioned media (ACM) on sphere formation efficiency of Ishikawa cells and their response to metformin treatment. a) Adipocyte-conditioned media increased the SFE of Ishikawa cells to a greater extent than pre-adipocyte conditioned media; b) the effect of metformin on endometrial cancer stem cell activity was abolished when cells were pre-treated with adipocyte-conditioned media.
The effect of adipocyte-conditioned media on the SFE of Ishikawa cells was not related to an increase in proliferation of the endometrial cancer cell line. The number of viable cells was not significantly different between those that had been treated with control or conditioned media (figure 4-22, p=0.27).

Figure 4-22 Pre-adipocyte and adipocyte-conditioned media had no effect on the viability of endometrial cancer cells. Treatment of Ishikawa cells with pre-adipocyte or adipocyte-conditioned media for 4 days had no effect on cell viability as determined by a SRB assay.

The effect of metformin on the viability of Ishikawa cells grown in monolayer was also unaltered by exposure to adipocyte conditioned media (figure 4-23, all p>0.05). This suggests that under these experimental conditions, adipocyte-conditioned media had a selective effect on cancer stem cell activity and did not affect the sensitivity of bulk tumour cells to low concentrations of metformin.
Figure 4.23 Adipocyte-conditioned media did not alter the sensitivity of Ishikawa cells grown in monolayer to metformin. Cell viability was not significantly different between cells that had been grown in normal growth media and those that had been exposed to adipocyte-conditioned media at any metformin concentration studied.

**4.3.4 In vivo effect of metformin on endometrial cancer stem cells**

The effect of metformin on endometrial cancer stem cells *in vivo* was determined by comparing immunohistochemical expression of ALDH and CD133 in endometrial tumours from patients randomised to receive metformin or matched placebo prior to surgery within the context of the PREMIUM study (chapter 3). Pre- and post-treatment endometrial biopsies were available for 68 patients; 33 patients had received metformin for 1-5 weeks and 35 patients had received placebo. The age, BMI, grade and stage of endometrial cancer of patients included in this study was similar to that of women randomised in the full trial.

Overall, metformin treatment was not associated with a significant reduction in immunohistochemical expression of ALDH (figure 4.25a). The mean difference in post-treatment ALDH expression between the metformin and placebo treated groups was -1.52% (95%CI -12.59 to +9.56, p=0.79), after adjusting for baseline ALDH expression. Subgroup analysis did, though, reveal a differential effect of metformin on ALDH expression according to baseline BMI. For women with a BMI <30kg/m², post-treatment ALDH expression was -7.95% (95%CI -25.74 to +9.84) after exposure to metformin (figure
4-25b). In contrast, women with a BMI ≥30kg/m² had an increase in ALDH expression of +3.08% (95%CI -11.05 to +17.21) following metformin treatment (figure 4-25c). The difference in effect of metformin in each of the BMI groups was not, however, statistically significant (p=0.34).

Figure 4-24 Effect of metformin on immunohistochemical expression of the cancer stem cell marker ALDH. Short-term treatment with metformin was not associated with a significant overall reduction in ALDH expression (a). A differential effect according to baseline BMI was observed, however, with non-obese women having a decrease in post-treatment ALDH expression of 7.95% with metformin treatment (b). Metformin had no effect in obese women, with post-treatment ALDH expression 3.08% higher after exposure to the drug (c).
Representative images of endometrial tumours from obese and non-obese women prior to and following treated with metformin are shown in figure 4-25.
Figure 4-25 Representative images of ALDH expression in endometrial tumours from non-obese and obese women prior to and following short-term metformin treatment (x4 magnification). Exposure to metformin was associated with a reduction in immunohistochemical staining for ALDH in non-obese women, but not in obese women. Despite strong immunohistochemical staining for ALDH in the stroma, the manual selection of endometrial cancer glands using Definiens Developer software ensured that only these areas of the tumour were scored.

Similarly, metformin had no effect on the overall immunohistochemical expression of CD133 in endometrial tumours (figure 4-26a). The mean difference in post-treatment log(CD133+0.01) expression between the metformin and placebo treated groups was -0.16 (95%CI -0.97 to +0.66).
after adjusting for baseline log(CD133+0.01) expression (p=0.71). Subgroup analysis again, however, revealed a differential effect according to baseline BMI, although the differences in post-treatment log(CD133+0.01) expression with exposure to metformin were much smaller (figure 4-26b and c). Non-obese women had a 0.55 (95%CI -3.78 to +2.68) decrease in post-treatment log(CD133+0.01) expression following exposure to metformin whilst obese women had a relative increase of 0.34 (95%CI -2.26 to +2.94) with metformin treatment. This difference in the effect of metformin in non-obese and obese women was also not statistically significant (p=0.67).

**Figure 4-26 Effect of metformin on expression of the cancer stem cell marker CD133.** Overall, metformin had no effect on expression of log(CD133+0.01), with no significant difference in post-treatment expression between the active drug and placebo treated arms. Women with a BMI <30kg/m\(^2\), however, had a decrease in post-treatment log(CD133+0.01) expression of 0.55 following metformin treatment (b), whilst women with a BMI ≥30kg/m\(^2\) had an increase in log(CD133+0.01) expression of 0.34 following exposure to metformin (c).
Representative examples of immunohistochemical expression of CD133 in endometrial tumours from non-obese and obese women prior to and following metformin treatment are shown in figure 4-27.
Figure 4-27 Representative images of endometrial tumours stained by immunohistochemistry for CD133 from non-obese and obese women prior to and following exposure to metformin (x4 magnification). Short-term metformin treatment reduced the immunohistochemical expression of CD133 in endometrial tumours in non-obese women but not in obese women. Immunohistochemical staining for CD133 was restricted to the apical membrane of cells.
4.4 Discussion

High ALDH activity and CD133 positivity identify two almost mutually exclusive populations of cells in the endometrial cancer cell lines Ishikawa and Hec-1a which have increased cancer stem cell activity, as determined by the ability to form spheres in non-adherent culture. ALDH$^{\text{high}}$ cells appear to have the greater cancer stem cell activity and have increased expression of stem cell and EMT-transcription factor genes compared with CD133$^{\text{+ve}}$ cells. Endometrial cancer stem cells have greater mitochondrial mass than bulk tumour cells, making them susceptible to mitochondrial inhibitors, including metformin. Indeed, metformin inhibits endometrial cancer stem cell activity and reduces the number of cancer stem cells present at concentrations lower than that required to affect cell proliferation. It also reduces the expression of genes associated with self-renewal, pluripotency and induction of EMT, but only in Ishikawa cells. These results are supported by evidence of an effect of short-term pre-surgical metformin on the immunohistochemical expression of endometrial cancer stem cell markers in the PREMIUM trial samples, though response to treatment was highly modulated by baseline BMI. Obesity could potentially drive endometrial carcinogenesis through an effect on cancer stem cells; secreted adipokines selectively increase cancer stem cell activity but also reduce the sensitivity of these cells to the effects of metformin.

The proportions of ALDH$^{\text{high}}$ and CD133$^{\text{+ve}}$ cells in the two endometrial cancer cell lines reported here are similar to those seen in other studies (Kiyohara et al. 2017; Zhang et al. 2016; Nakamura et al. 2010). This is the first study, however, in which the two populations of endometrial cancer stem cells have been directly compared at both a functional and genetic level. Ding et al. (2017) investigated the expression of stem cell genes in CD133$^{\text{+ve}}$ and CD133$^{\text{-ve}}$ endometrial cancer cells from a single human tumour, finding a significant increase in expression of SOX2 and NANOG in CD133$^{\text{+ve}}$ cells alongside raised protein levels of Epcam. This is contrary to the results of this study, where RNA expression of all three stem cell and epithelial markers was found to be lower in CD133$^{\text{+ve}}$ cells than CD133$^{\text{-ve}}$ cells. This could be explained by the fact that cell lines were used here rather than primary samples and that the findings reported by Ding et al. (2017) were based on results obtained using tumour samples from one patient only, who had a low grade, early stage endometrial cancer. The only study investigating differences in gene expression between ALDH$^{\text{high}}$ and ALDH$^{\text{low}}$ cells in endometrial cancer cell lines focussed solely on expression of EMT genes, observing that only expression of TWIST1 and 2 differed between the two cell populations (van der Zee et al. 2015).

Whilst in breast cancer, distinct populations of cancer stem cells appear to exist in either an epithelial-like or mesenchymal-like state with little overlap between the two (Liu et al. 2014a), this does not appear to be the case in the two endometrial cancer cell lines studied here. ALDH$^{\text{high}}$ cells in Ishikawa and Hec-1a cell lines overexpress both epithelial and mesenchymal markers compared with ALDH$^{\text{low}}$ cells, whilst the opposite is generally true for CD133$^{\text{+ve}}$ cells, with the exception of VIMENTIN, whose expression is increased two-fold. Whilst a strong link between EMT and gain of cancer stem-like cell properties has been proven, complete EMT in the majority of cancers is unusual, with most expressing both epithelial and mesenchymal markers (Mani et al. 2008; De Craene and Berx 2013).
Indeed the balance between EMT and its reverse process, mesenchymal-epithelial transition, appears to be important in not only driving metastasis but also in the establishment of new tumours at distant sites. If this is the case, cancer stem cells would be expected to express increased levels of both epithelial and mesenchymal markers as well as the transcription factors Snai1, Twist and Zeb1 that control this process. Low dose metformin, at least in endometrial cancer cell lines, appears to target this process by reducing the expression of EMT transcription factors, though this has yet to be confirmed in primary endometrial cancer samples (Laskov et al. 2016).

The effect of metformin on the proportion and activity of endometrial cancer stem cells is consistent with that described in breast, prostate and ovarian cancer, where it has previously been shown to reduce mammosphere formation and decrease tumour growth in xenograft models (Shank et al. 2012;Iliopoulos et al. 2011;Hirsch et al. 2009). Whilst not specifically examined in this study, the targeting of cancer stem cells by metformin resulted in prolongation of disease remission and an increase in disease-free survival in a breast cancer xenograft model (Hirsch et al. 2009).

That the effect of metformin on endometrial cancer stem cells is at least attenuated, if not lost completely, when cells are pre-treated with adipocyte conditioned media is an important original finding given the high prevalence of obesity in the endometrial cancer population and one that was found here to be replicated in vivo. These results would suggest that this is related to increased adipokine secretion from mature adipocytes. Identification and targeting of the mediators responsible for this phenomenon is essential if metformin is to be used effectively in the adjuvant setting for women with endometrial cancer.

The active role that adipocytes appear to play in the biology of endometrial cancer is becoming well established. A recent study by Sahoo et al. (2017) found that adipocyte conditioned media increased the colony forming capacity of Ishikawa cells and reduced their responsiveness to paclitaxel when co-treated for 72 hours. They proposed that vascular endothelial growth factor (VEGF) could be important in mediating this effect, though whether it is also responsible for the resistance to metformin observed in obese individuals has yet to be determined. Certainly, VEGF expression is greater in visceral adipose tissue, which appears to have a more potent effect on endometrial cancer cells than subcutaneous adipose tissue (Klopp et al. 2012;Sahoo et al. 2017). This corresponds with epidemiological evidence supporting an increased risk of cancer in patients with central obesity, defined by an elevated waist:hip ratio (De Pergola and Silvestris 2013;Aune et al. 2015). Given the already published data on the greater biological activity of visceral adipocytes and the close anatomical proximity of the omentum to the uterus, it was decided to focus on the effect of omental-derived adipocyte-conditioned media on endometrial cancer stem cell activity only.

In contrast to the findings of our study, however, Sahoo et al. (2017) described an increase in endometrial cancer cell proliferation following exposure to adipocyte-conditioned media. The authors had used conditioned media generated from a mouse pre-adipocyte cell line that had been induced to
differentiate rather than primary adipocytes and they used a cell viability assay that detected ATP production rather than the number of cells present. This is an important methodological point as adipocyte-conditioned media has been shown to increase the rate of oxidative phosphorylation in ID8 ovarian cancer cells and hence this could explain the increase in ATP production observed rather than a true increase in cell number (Tebbe et al., 2014). The SRB assay used in our study, in contrast, relies on the binding of SRB to proteins to determine cell mass and thus avoids the potential pitfalls associated with assays which detect the metabolic activity of cells (Orellana and Kasinski, 2016).

The strengths of my study include the replication of experiments in two endometrial cancer cell lines, which differed in their differentiation status and mutational profile, thereby increasing the generalisability of the results obtained. Endometrial cancer stem cells were characterised using a multifaceted approach, based on their phenotype, including expression of surface markers, enzyme activity and mitochondrial mass, and genotype. The effect of metformin on both the number and function of cancer stem cells in vitro was investigated and correlated with clinical samples from participants in a pre-surgical window study to determine the applicability of laboratory findings to endometrial cancer patients. This is the first study to specifically investigate the effect of diabetic doses of metformin on cancer stem cell number in humans and allowed the assessment of the impact of adiposity on drug response. The modelling of obesity associated endometrial cancer provided further mechanistic insights into the role of adipocytes in the development of the disease and resistance to metformin treatment.

Despite this, cell lines do have their limitations; including the risk of introducing genotypic and phenotypic variation through serial passaging, taking them away from the cell of origin (Pan et al. 2009) and their limited reflection of intra- and inter-tumoral heterogeneity. Replication of experiments using primary endometrial cancer cells would help negate these problems. This work focussed on endometrial cancer stem cells identified through high ALDH activity and CD133 expression on the basis of previously published, although limited, work in the stem cell field. The fact that cells with a negative phenotype continue to exhibit some cancer stem cell activity, forming spheres in non-adherent culture, suggests that these are not the only endometrial cancer stem cell markers, something which should be investigated further. The ‘gold standard’ method of assessing cancer stem cell activity, in vivo transplantation, was not performed due to time limitations but would be a natural progression of this work to confirm these findings in a more sophisticated endometrial cancer model. The concentration of metformin at which an effect on endometrial cancer stem cell activity was observed was lower than that used in other experiments in endometrial cancer, but was again several-fold higher than plasma levels in humans (chapter 3) or that measured in endometrial cancer tissue (Mitsuhashi et al. 2014; Cantrell et al. 2010). This may be related to experimental conditions, particularly the use of high glucose containing media, which has been shown to alter the responsiveness of cells to metformin treatment (de Barros Machado et al. 2016). Replication of the proliferation assays using low (1g/l) and high (4.5g/l) glucose containing adipocyte-conditioned media
in addition to metformin did not alter the results, however (results not shown). The fact that short-term exposure to diabetic doses of metformin was found to reduce the immunohistochemical expression of CD133 and ALDH in endometrial cancers from non-obese patients is encouraging and suggests that these laboratory based findings may be of clinical relevance.

4.4.1 Conclusion

ALDH activity and CD133 expression identify two distinct populations of endometrial cancer cells with different cancer stem cell activity and expression of stem cell and EMT genes. Metformin, at a concentration lower than that required to affect proliferation, reduces the number and activity of endometrial cancer stem cells in vitro and decreases the immunohistochemical expression of markers of endometrial cancer stem cell activity in patients. This effect, however, is diminished in the presence of obesity; identification of the adipokines responsible for this phenomenon is a priority area if metformin is to be of clinical value in this setting.
Chapter 5 Unrecognised and undertreated cardiovascular risk factors in women newly diagnosed with endometrial cancer

5.1 Introduction

Despite accumulating evidence for higher rates of death from cardiovascular disease among endometrial cancer survivors, there is currently no guidance for screening these women for cardiovascular risk factors in the UK or elsewhere. Before recommendations can be made, however, the magnitude of the problem needs to be established and areas for improvement identified. At present, the true prevalence of hyperglycaemia, hypertension and hypercholesterolaemia in women with a history of endometrial cancer is unknown. Previous studies have focussed solely on established diagnoses, thereby potentially underestimating the actual prevalence of these conditions, which are frequently asymptomatic (Burzawa et al. 2011; Soliman et al. 2006; Trabert et al. 2015).

This study, therefore, had three aims. Firstly, to determine the true prevalence of cardiovascular disease risk factors in women with newly diagnosed endometrial cancer compared with women of the general population. Secondly, to establish whether women with a history of endometrial cancer were indeed at higher risk of cardiovascular disease than those without a history of the disease. Thirdly, whether the implementation of a screening and treatment programme directed at cardiovascular risk factors would be of benefit to women following primary treatment for their endometrial cancer.

5.2 Materials and methods

5.2.1 Study design and data collection

A case-control study was conducted in the UK between 2016 and 2017

5.2.1.1 Selection of cases and data collection

Consecutive patients with a new diagnosis of endometrioid endometrial cancer or its precursor, atypical endometrial hyperplasia, receiving treatment in the North West of England, and who had consented to participate in research were recruited. A detailed medical history was obtained from interview and checked against medical records and included known diagnoses of diabetes, non-diabetic hyperglycaemia, hypercholesterolaemia, hypertension and cardiovascular disease. The latter was defined as a previous myocardial infarction, angina, cerebrovascular accident, transient ischaemic attack or coronary artery bypass graft. A complete drug history was recorded and current medical therapy for the above conditions was considered as evidence of a prior diagnosis. Smoking status was documented as never smoker, ex-smoker or current smoker and included the number of cigarettes smoked per day.
Anthropometric measurements were obtained, including:

- Height, measured bare-footed using a stadiometer
- Weight, measured fully clothed using calibrated electronic scales
- BMI, calculated as weight(kg)/ height(m)^2

Serum was obtained by venepuncture after at least a six hour fast and sent to the Clinical Biochemistry Department at MFT for routine analysis. This included measurement of HbA1C and cholesterol (total and high density lipoprotein [HDL]) levels by high performance liquid chromatography and an enzymatic colorimetric method, respectively. Blood pressure was measured at rest using a validated, automated sphygmomanometer.

5.2.1.2 Selection of controls and data collection

Each endometrial cancer case was matched with up to five female controls for age (±5 years) and ethnicity from the Health Survey for England 2014, the details of which have been published previously (Health and Social Care Education Centre 2015). In brief, surveys are conducted annually of 8000 individuals selected at random by postcode using a standardised questionnaire to gain information about the general health of the nation. Individual level data is freely available through the NHS digital website. Details of the past medical history of controls were obtained, in particular known diagnoses of hypertension, hypercholesterolaemia, diabetes and cardiovascular disease, as well as current medication use for these conditions. Measurements of weight and height as well as fasted serum total and HDL cholesterol and HbA1C were performed as part of the survey using similar methodology to that described for cases. These results were also retrieved from the data repository. As information regarding a previous diagnosis of cancer and, in particular, endometrial cancer, was not recorded individually within the database, cases could not be excluded on the basis of whether they had a history of malignancy.

5.2.2 Outcome definitions

New diagnoses of non-diabetic hyperglycaemia and type 2 diabetes were defined as an HbA1C measurement of 42-47mmol/mol and greater than or equal to 48mmol/mol, respectively, in a person not previously known to have these conditions or fasting plasma glucose of 5.5-6.9 mmol/l and ≥7.1 mmol/l, respectively. These thresholds are in accordance with recommendations from the World Health Organisation (WHO 2006) and National Institute of Clinical Excellence (NICE 2012).

A new diagnosis of hypertension was defined as a systolic blood pressure greater than or equal to 140mmHg in a person who had not previously been diagnosed by a physician as having high blood pressure (NICE 2016). Inadequately treated hypertension was regarded as being present if the systolic blood pressure was 140mmHg or higher in a person already taking antihypertensive medication.
Hypercholesterolaemia was newly diagnosed if the total:HDL cholesterol ratio was greater than 4.5 in a person not currently taking statin therapy (Millan et al. 2009). Inadequately treated hypercholesterolaemia was considered present if the total:HDL cholesterol ratio remained greater than 4.5 despite treatment with statins.

Obesity was defined as a BMI greater than or equal to 30kg/m² and was, by definition, considered to be undertreated.

5.2.3 QRISK 2 score

The 10 year cardiovascular risk score was calculated using the UK-validated, online version of the QRISK2 calculator 2016, available at https://www.qrisk.org/2016/. Data was inputted on age, ethnicity, gender, residential postcode, smoking status, presence of diabetes, atrial fibrillation, chronic kidney disease, antihypertensive treatment, rheumatoid arthritis, systolic blood pressure, total:HDL cholesterol ratio, height and weight. Missing data were imputed by the calculator using age and gender specific average values. For the control group, information relating to co-morbidities, in particular atrial fibrillation, chronic kidney disease, rheumatoid arthritis, and home postcode were not available in the database and, therefore, these data were not entered into the calculator for either group in the final analysis.

In order to estimate the likely benefit to be derived from implementation of a screening and treatment programme for cardiovascular risk factors in women with newly diagnosed endometrial cancer, the effect of optimisation of modifiable variables was calculated. An optimised QRISK2 score was derived based on quitting smoking, reduction of systolic blood pressure to 140mmHg, lowering of total:HDL cholesterol ratio to 4.5 and achieving a BMI of 25kg/m². The absolute change in risk was calculated by subtracting the optimised risk score from the predicted risk prior to risk factor optimisation (Rutter et al. 2016).

5.2.4 Statistical analysis

A power calculation was performed a priori based on the median age of women with endometrial cancer (Cancer Research UK 2014b). Assuming 22.9% of women aged 65 years have a 10 year cardiovascular risk ≥20%, 124 cases and 620 controls were required to detect a two-fold increase in QRISK2 score in women with endometrial cancer compared with the general population, with 90% power, 5% error and five matched controls per case (Hippisley-Cox et al. 2008).

Continuous data were summarised as medians and interquartile ranges due to their non-parametric distribution and were compared using the Mann-U Whitney test. Categorical data were compared using the χ² and Fisher’s exact test. Comparisons of related samples were performed using the Wilcoxon signed rank test.
A p value ≤0.05 was considered statistically significant, with asterisk used to denote significant results as *p≤0.05, **p≤0.01, ***p≤0.001 and ****p≤0.0001. The statistical analyses were conducted using SPSS version 23 and Graph Pad version 7.

5.3 Results

5.3.1 Description of cases and controls

One hundred and fifty women with endometrioid endometrial cancer (referred to hereafter as endometrial cancer, n=144) and its precursor, atypical endometrial hyperplasia (n=6), were recruited. Reflecting the stage distribution in the general endometrial cancer population in the UK, 87% (125/144) of women with endometrial cancer had early stage (Stage I and II) disease at presentation.

Cases were matched for age and ethnicity with 746 female controls from the Health Survey for England (2014). There were insufficient women of non-white ethnic background and aged 75 years and over included in the Health Survey for each case to be matched fully with five controls. No data were missing for cases and missing data for the Health Survey for England controls was limited to 2% (absent cholesterol measurements 1.9%, HbA1C levels 2.1%, blood pressure measurements 1.2%, information on statin use 0.5% of controls).

The demographic details of cases and controls are shown in table 5-1. As expected, women with endometrial cancer had significantly greater BMI than those without the disease (BMI ≥30kg/m² 60.7% cases vs. 32.4% controls, p<0.0001, figure 5-1). Over one fifth (34/150) of endometrial cancer cases were super-obese, defined as a BMI ≥40kg/m², compared with 3.4% (25/746) of controls. Despite this, the proportion of women already diagnosed with cardiovascular disease was over 50% lower in women with a history of endometrial cancer than the general population (6.0% cases vs. 15.7% controls, p=0.002). This was unrelated to smoking status as the proportions of current and ex-smokers were similar in the two groups.
## Table 5-1 Demographic details of cases and controls.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (n=150)</th>
<th>Controls (n=746)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median yrs (IQR)</td>
<td>65 (57-72)</td>
<td>64 (54-71)</td>
<td>0.093</td>
</tr>
<tr>
<td>BMI, median kg/m² (IQR)</td>
<td>32.5 (26.9-38.8)</td>
<td>27.2 (24.0-31.5)</td>
<td>&lt;0.0001****</td>
</tr>
<tr>
<td>&lt;25</td>
<td>25 (16.7)</td>
<td>241 (32.3)</td>
<td></td>
</tr>
<tr>
<td>25-29.9</td>
<td>34 (22.7)</td>
<td>258 (34.6)</td>
<td></td>
</tr>
<tr>
<td>30-34.9</td>
<td>37 (24.7)</td>
<td>152 (20.4)</td>
<td></td>
</tr>
<tr>
<td>35-39.9</td>
<td>20 (13.3)</td>
<td>64 (8.6)</td>
<td></td>
</tr>
<tr>
<td>≥40</td>
<td>34 (22.7)</td>
<td>25 (3.4)</td>
<td></td>
</tr>
<tr>
<td>Missing data</td>
<td>0 (0.0)</td>
<td>6 (0.8)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity, n (%)</td>
<td></td>
<td></td>
<td>0.081</td>
</tr>
<tr>
<td>White</td>
<td>137 (91.3)</td>
<td>680 (91.1)</td>
<td></td>
</tr>
<tr>
<td>Indian</td>
<td>6 (4.0)</td>
<td>29 (3.9)</td>
<td></td>
</tr>
<tr>
<td>Pakistani</td>
<td>4 (2.7)</td>
<td>20 (2.7)</td>
<td></td>
</tr>
<tr>
<td>Black/African/Caribbean</td>
<td>3 (2.0)</td>
<td>17 (2.3)</td>
<td></td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td></td>
<td></td>
<td>0.271</td>
</tr>
<tr>
<td>Never smoked</td>
<td>88 (58.7)</td>
<td>394 (52.8)</td>
<td></td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>43 (28.7)</td>
<td>265 (35.5)</td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>19 (12.7)</td>
<td>87 (11.7)</td>
<td></td>
</tr>
<tr>
<td>Diagnosed cardiovascular disease, n (%)</td>
<td></td>
<td></td>
<td>0.002**</td>
</tr>
<tr>
<td>No</td>
<td>141 (94.0)</td>
<td>629 (84.3)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>9 (6.0)</td>
<td>117 (15.7)</td>
<td></td>
</tr>
</tbody>
</table>

### 5.3.2 Prevalence of known cardiovascular risk factors in cases and controls

The prevalence of previously diagnosed diabetes was similar in both cases and controls (11.3% cases vs. 8.3% controls, p=0.350). Likewise, there was no difference in the proportion of women already taking statin therapy for hypercholesterolaemia in the two groups (28.0% cases vs. 24.3% controls, p=0.354). The only noticeable difference was in the number of women currently taking antihypertensive medication, with almost half of women with endometrial cancer already being treated for hypertension compared with one third of controls (46.7% cases vs. 33.6% controls, p<0.0001). Non-diabetic hyperglycaemia was not recorded as a diagnosis in the Health Survey for England data; therefore, a comparison of the known prevalence of this condition in the two populations was not possible. Four cases (3.2%) had been previously diagnosed with non-diabetic hyperglycaemia.
5.3.3 Prevalence of adequately treated and inadequately treated/screen detected risk factors in cases and controls

The prevalence of screen detected type 2 diabetes was significantly higher in women with a history of endometrial cancer than those without a history of the disease (6.0% cases vs. 1.3% controls, p=0.002, figure 5-1). Similarly, the proportion of cases with undiagnosed non-diabetic hyperglycaemia was significantly greater than that of controls; over four times as many women with endometrial cancer had previously unrecognised non-diabetic hyperglycaemia than the general population. Indeed, over half of women with endometrial cancer included in the study had screen detected non-diabetic hyperglycaemia (51.2% cases vs. 11.3% controls, p<0.0001). Due to the number of missing HbA1C values, it was not possible to reliably determine the proportion of known diabetic patients with adequately and inadequately controlled disease.

Whilst there was no difference in the prevalence of adequately treated hypertension in cases and controls (20.7% cases vs. 22.9% controls, p=0.593), women diagnosed with endometrial cancer were significantly more likely to have inadequately treated (26.0% cases vs. 10.1% controls, p<0.0001) and undiagnosed hypertension (23.3% cases vs. 2.7% controls, p<0.0001). Overall, therefore, a greater proportion of cases were hypertensive than controls (70.0% cases vs. 46.6% controls, p<0.0001, figure 5-1).

These results were replicated when a diagnosis of hypercholesterolaemia was considered. The proportion of women with adequately treated hypercholesterolaemia was similar in both cases and controls (23.3% cases vs. 21.3% controls, p=0.588), whilst women with endometrial cancer were significantly more likely to have inadequately treated (5.3% cases vs. 1.7% controls, p=0.015) and undiagnosed (23.3% cases vs. 12.5% controls, p=0.001) disease. Overall, cases were significantly more likely to have raised cholesterol levels than controls (50.0% cases vs. 35.0% controls, p=0.0006).
Figure 5-1 Prevalence of adequately treated and inadequately treated/screen detected cardiovascular risk factors in cases and controls. The prevalence of adequately treated diabetes, hypertension and hypercholesterolaemia was similar in both cases and controls. In contrast, a significantly greater proportion of women with endometrial cancer had inadequately treated and screen detected non-diabetic hyperglycaemia, diabetes, hypertension, hypercholesterolaemia and obesity than the general population.

When all cardiovascular risk factors were considered together, 90.0% of women with endometrial cancer had at least one risk factor compared with 68.3% of controls (p<0.0001, figure 5-2). Again, this was due to a greater proportion of women with endometrial cancer having previously unrecognised and inadequately treated risk factors than the general population. Over one fifth of cases were at particularly high risk of cardiovascular disease with three or more risk factors in contrast to controls where fewer than one in fourteen women had multiple concurrent cardiovascular risk factors (22.0% cases vs. 6.9% controls, p<0.0001).
Figure 5-2 Prevalence of any one, two and three or more cardiovascular risk factors in cases and controls. Women newly diagnosed with endometrial cancer were significantly more likely to have one, two or three or more concurrent cardiovascular risk factors than the general population due to a higher prevalence of undiagnosed and inadequately treated disease (all p values ≤0.0001).

5.3.4 Predicted 10-year cardiovascular disease risk in cases and controls

In order to determine the predicted 10-year cardiovascular disease risk of cases and controls, individual QRISK2 scores were calculated. As the QRISK2 score is only valid for those without a prior diagnosis of cardiovascular disease, type I diabetes, familial hypercholesterolaemia and aged between 25-85 years, 11 cases and 124 controls were excluded from this analysis. This left 139 cases and 622 controls for whom a QRISK2 score could be calculated. Despite the exclusions, the two groups remained well matched for age and ethnicity and continued to demonstrate significant differences in the prevalence of overall, screen detected and inadequately treated cardiovascular risk factors, despite similar proportions having previously recognised risk factors (figure 5-3).
Figure 5-3 a) Prevalence of diagnosed and screen detected/inadequately treated individual cardiovascular risk factors in QRISK2 analysis. b) Prevalence of one, two or three or more concurrent cardiovascular risk factors in QRISK2 analysis. In accordance with the results observed in the whole population, the prevalence of overall and screen detected/inadequately treated individual cardiovascular risk factors was significantly higher in cases than controls, although the prevalence of known disease was similar in the two groups. The only exception to this was diabetes, with no statistical difference in the proportions of women with and without endometrial cancer being affected (12.9% cases vs. 7.8% controls, p=0.068). Women with endometrial cancer were again more likely to have at least one risk factor for cardiovascular disease, with 17.9% having three or more concurrent risk factors compared with 4.9% of women in the general population (p<0.0001).
QRISK2 scores were calculated for women with newly diagnosed endometrial cancer both including and excluding data on co-morbid conditions and residential postcode as a surrogate marker of deprivation. Inclusion of these additional data did not have a significant effect on the score obtained (median QRISK2 score 13.3% [IQR 6.4-21.1] compared with 12.6% [6.6-21.4%] \( p=0.349 \)), making comparisons with the Health Survey for England population, where these data were not available, valid.

NICE recommends that individuals with a 10-year cardiovascular disease risk of 10% or greater, as predicted by their QRISK2 score, should be offered statin therapy for primary disease prevention. Two thirds of women with endometrial cancer met this threshold compared with under half of women without the disease (63.3% cases vs. 46.6% controls, \( p=0.0005 \), table 5-2). The increase in cardiovascular disease risk between the groups was even more marked when the higher threshold of a 20% QRISK2 score was considered; 29.5% of women with endometrial cancer were deemed at high risk of cardiovascular disease compared with 16.9% of the general population \( p=0.001 \). These differences were reflected in the higher median QRISK2 score in cases compared with controls (12.6% cases vs. 8.8% controls, \( p<0.0001 \)).
Table 5-2 QRISK2 score analysis. Women with newly diagnosed endometrial cancer were significantly more likely to meet the NICE threshold for statin therapy for primary cardiovascular disease prevention (QRISK2 score ≥10%) and be deemed at high risk for cardiovascular disease in the following 10 years (QRISK2 score ≥20%). Screening and treatment of modifiable risk factors was estimated to be over three times more effective in reducing the QRISK2 score of cases than controls.

<table>
<thead>
<tr>
<th>10 year cardiovascular risk</th>
<th>Cases (n=139)</th>
<th>Controls (n=622)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10%, n (%)</td>
<td>51 (36.7)</td>
<td>322 (53.4)</td>
<td>0.0005***</td>
</tr>
<tr>
<td>≥10%, n (%)</td>
<td>88 (63.3)</td>
<td>290 (46.6)</td>
<td></td>
</tr>
<tr>
<td>≥20%, n (%)</td>
<td>41 (29.5)</td>
<td>105 (16.9)</td>
<td>0.001***</td>
</tr>
</tbody>
</table>

Median (IQR) before risk factor optimisation

<table>
<thead>
<tr>
<th>Median (IQR) before risk factor optimisation</th>
<th>12.6% (6.6-21.4%)</th>
<th>8.8% (3.5-17.1%)</th>
<th>&lt;0.0001****</th>
</tr>
</thead>
</table>

Median (IQR) after risk factor optimisation

<table>
<thead>
<tr>
<th>Median (IQR) after risk factor optimisation</th>
<th>11.6% (6-18.9%)</th>
<th>8.4% (3.2-15.9%)</th>
<th>0.0004***</th>
</tr>
</thead>
</table>

Absolute percentage change in cardiovascular risk following optimisation

<table>
<thead>
<tr>
<th>Absolute percentage change in cardiovascular risk following optimisation</th>
<th>-1.82</th>
<th>-0.69</th>
</tr>
</thead>
</table>

Estimated number needed to treat to prevent one cardiovascular event in following 10 years

<table>
<thead>
<tr>
<th>Estimated number needed to treat to prevent one cardiovascular event in following 10 years</th>
<th>55</th>
<th>145</th>
</tr>
</thead>
</table>

5.3.5 Estimated effect of risk factor optimisation

In order to estimate the impact of introducing a screening and treatment programme for cardiovascular risk factor optimisation in endometrial cancer survivors, the likely effect of improving modifiable risk factors was calculated. Individual QRISK2 scores were recalculated assuming the success of a weight reduction plan to achieve a BMI of 25kg/m², smoking cessation, treatment of hypertension to reduce systolic blood pressure to 140mmHg and lipid lowering medication to achieve a total:HDL ratio of 4.5. Risk factor optimisation resulted in an absolute reduction in QRISK2 score of 1.82% for women with endometrial cancer compared with a 0.69% reduction for women without the disease. This equated to needing to treat 55 endometrial cancer survivors to prevent one cardiovascular event (myocardial infarction, transient ischaemic attacked or cerebrovascular accident).
in the following 10 years. In contrast, 145 women in the general population would need to be treated
to observe the same reduction in events.

5.4 Discussion
The prevalence of diabetes, non-diabetic hyperglycaemia, hypertension, hypercholesterolaemia and
obesity was significantly higher in women newly diagnosed with endometrial cancer than the general
population. Indeed, almost all women diagnosed with endometrial cancer in our study had at least one
cardiovascular risk factor and 22% had three or more concurrent risk factors. This ‘true’ prevalence of
cardiovascular risk factors in this population was only apparent, however, when non-selective
screening was undertaken. Whilst similar proportions of women with and without a history of
endometrial cancer had been previously diagnosed with hyperglycaemia, hypertension and
hypercholesterolaemia, the prevalence of unrecognised cardiovascular risk factors was significantly
higher in the former group. Even when diagnosed, women with endometrial cancer are more likely to
have sub-optimally controlled blood pressure and lipid levels and be obese. The knock on effect of
this is an elevated 10 year risk of cardiovascular disease; women with newly diagnosed endometrial
cancer had a 1.4-fold higher QRISK2 score than the general population and were more likely to be
considered at high risk of cardiovascular disease as defined by a QRISK 2 score of ≥10% or 20%.
Treatment of these modifiable risk factors following a diagnosis of endometrial cancer would be an
effective strategy with an estimated reduction in individual QRISK 2 scores of 1.82%. This is predicted
to be more beneficial than the introduction of a similar programme in the general population, which is
already advocated by NICE for people aged 40 years and older (NICE 2016).

These results support the introduction of universal screening for previously unrecognised
cardiovascular risk factors and assessment of glycaemic, blood pressure and lipid control for those
already diagnosed. Thereafter, there are several treatment options available to optimise these risk
factors with the aim of reducing the incidence of subsequent cardiovascular disease. Weight loss has
been shown to improve insulin resistance, reduce blood pressure and normalise cholesterol levels
and has the additional benefits of being low cost and associated with minimal risk of harm (Vidal
2002;Look and Wing 2010). Traditionally, it has been reported to be notoriously difficult to achieve by
diet and exercise alone, even within the context of clinical trials, and especially hard to sustain in the
long term (Laskey et al. 2016;Knowler et al. 2002). The recent DIRECT study conducted in primary
care in Scotland and Tyneside has contested this, finding that significant weight loss of ≥15kg at 12
months is possible in motivated individuals and can lead to remission of type 2 diabetes (Lean et al.
2017). Initiation of antihypertensive medication and statin therapy may be required by some and could
be combined with metformin treatment, which has similarly been shown to improve multiple
cardiovascular risk factors. In particular, exposure to metformin has been found to prevent the
development of diabetes in those at high risk of the disease and, indeed, may be more effective than
weight loss in this regard for individuals with a BMI ≥35kg/m^2 and with high fasting plasma glucose,
both of which are highly prevalent in women diagnosed with endometrial cancer (Knowler et al. 2002).
Metformin itself has also been shown to promote weight loss, with individuals in the Diabetes Prevent Programme study losing, on average, 2.1kg over a 2.8 year follow-up period with a decrease in calorie intake and a reduction in central adiposity. It has a favourable impact on cholesterol levels as well, with a lowering of low-density lipoprotein and an increase in HDL levels. Whether these effects translate into a reduction in the incidence of cardiovascular disease for individuals both with and without diabetes is currently unknown. Despite being used ubiquitously for the treatment of type 2 diabetes, there have been very few randomised controlled trials of metformin conducted with cardiovascular end points and those that have been performed have been at significant risk of bias, including small numbers of events and recruiting young participants with poorly controlled diabetes only (Griffin et al. 2017). As the authors of the meta-analysis note, however, further RCTs examining the impact of metformin on cardiovascular outcomes in diabetic patients are unlikely as the weight of evidence supporting its use in the management of the disease would make withholding treatment unethical. Well-designed cohort studies, such as the proposed continued follow-up of individuals in the Diabetes Prevention Programme for the long term effect of metformin on cardiovascular event frequency, will provide useful alternative sources of evidence.

This is the first study to investigate the risk of cardiovascular events in women newly diagnosed with endometrial cancer compared to the general population. A small number of previous studies have examined the prevalence of individual cardiovascular risk factors in women with endometrial cancer, however, none of these have considered hypercholesterolaemia in their assessment and most have commented on known diagnoses only. When non-selective screening for diabetes was performed by Burzawa et al. (2011), they described a prevalence of known type 2 diabetes of 30.3% in women newly diagnosed with endometrial cancer and made a new finding of insulin resistance in a further 36% of women, similar to the prevalence of 54.4% of non-diabetic hyperglycaemia seen in our own study. The lower rate of overt diabetes reported by ourselves (17.3%) may be a reflection of the different ethnic backgrounds of included women and distinct healthcare systems, with differing access to opportunistic screening.

Within the SEER database, Felix et al. (2017c) found significantly more deaths from cardiovascular disease in women with a history of endometrial cancer than in the general population. In contrast, a retrospective analysis of participants within the Iowa Women’s Health Study noted that endometrial cancer survivors had a 25% lower risk of cardiovascular mortality compared with age and BMI matched controls (Felix et al. 2017a). This latter study, however, was reliant upon information obtained from death certificates to determine disease specific mortality rates and is thus at risk of the inherent inaccuracies associated with use of these types of data. The median BMI of women included in the study at 28kg/m² was not only lower than that observed in our own cohort but BMI matching of cases to controls also eliminated the impact of obesity on other cardiovascular risk factors, all of which are intimately related. Involvement in a longitudinal study of lifestyle factors on cancer incidence may have also influenced participant behaviour, encouraging women to make positive changes to their diet and activity levels. This could explain why there was no difference in the rate of
non-fatal cardiovascular events in women with and without a history of endometrial cancer in the Women’s Health Initiative (Felix et al. 2017d). As with the Iowa Women’s Health Study, participants were healthier, with a lower prevalence of obesity and hypertension than in our study, potentially as a result of the ‘healthy bias’ associated with the selective recruitment of women into clinical trials.

The main strength of my study is the universal screening of all cases and controls for four cardiovascular risk factors, namely diabetes, hypertension, hypercholesterolaemia and obesity. This has provided data on the true prevalence of these conditions in women with endometrial cancer and allowed a reliable estimation of their 10 year cardiovascular disease risk to be calculated. The Health Survey for England participants provide a true reflection of the health of the general population as, in contrast to the control group of many studies, individuals were asked to take part on the basis of their postcode rather than through self-recruitment, thereby avoiding the ‘healthy control’ biases which are otherwise introduced. This ensured that they were a suitable comparison group for our cases with endometrial cancer. The study was adequately powered to detect a difference in cardiovascular disease risk between women newly diagnosed with endometrial cancer and those without the disease, even after exclusion of participants with known cardiovascular disease and those ineligible for QRISK2 assessment.

The lack of information on co-morbid conditions for participants in the Health Survey for England meant that the QRISK2 score had to be calculated based on a limited data set. As demonstrated, however, this does not appear to have had a significant impact on the predicted cardiovascular disease risk due to the low prevalence of these conditions within the populations studied. Similarly, the format of the Health Survey for England data meant that it was not possible to reliably exclude a prior diagnosis of endometrial cancer for individuals included in the control group. This may have resulted in the misclassification of cases as controls, but is again unlikely to have affected the conclusions reached as it would have biased the results towards the null. The prevalence of cardiovascular disease within the HSE population was determined through self-reporting of a physician diagnosis of a heart attack, angina or stroke, but also included heart murmurs and abnormal heart rhythms. This may have resulted in the overestimation of the prevalence of diagnosed cardiovascular disease within the control population, although it does not discount the possibility that a reluctance to seek medical attention, as is often found in obese women, may have also contributed to the disparity in known cardiovascular disease prevalence (Amy et al. 2006). The lack of HbA1C data for individuals already diagnosed with diabetes meant that it was not possible to assess the glycaemic control of cases and controls and, therefore, comment upon whether the findings of undertreatment of other cardiovascular risk factors extended to the management of diabetes. Regardless, glycaemic control would form a central component of any screening and treatment programme for cardiovascular risk factors in endometrial cancer survivors. The QRISK2 score has only been validated for individuals with a BMI between 18-40kg/m² and automatically limits the BMI to the upper or lower limit should the inputted height and weight provide a value outside of this range. This has the effect of overestimating the QRISK2 score for individuals with a very low BMI, but, of more relevance...
to women with a history of endometrial cancer, it is likely to markedly underestimate the 10 year cardiovascular risk for those with a BMI greater than 40kg/m². Given that over one fifth of cases were super-obese, this means that the median QRISK2 score calculated here is likely to be conservative and the difference in risk between women with and without a history of endometrial cancer may be even greater than proposed.

5.4.1 Conclusion
Women newly diagnosed with endometrial cancer have a higher prevalence of individual and multiple concurrent cardiovascular risk factors and are more likely to have undiagnosed and undertreated diabetes, non-diabetic hyperglycaemia, hypertension, hypercholesterolaemia and obesity than the general population. This puts them at greater risk of cardiovascular disease. Following primary treatment of their endometrial cancer, screening for and intervention to mitigate these risks should be performed in order to reduce this risk and potentially lead to improvements in overall survival. Metformin, alongside lifestyle modification, antihypertensive and statin therapy, could be a central component of such a treatment programme. Further evidence from adequately powered, long term trials of metformin for the primary prevention of cardiovascular disease in women is required before it can be routinely recommended for all endometrial cancer survivors.
Chapter 6  Conclusions and future directions

Despite initial promise of a role for metformin in the treatment of endometrial cancer from pre-clinical work and small uncontrolled window studies, standard diabetic doses of the drug for 1-5 weeks prior to surgery did not result in an overall reduction in endometrioid endometrial cancer cell proliferation when tested within a methodologically rigorous randomised controlled trial. Response to the drug was not consistent, however; body mass appeared to be critical in identifying a subgroup of women (BMI < 30 kg/m²) for whom short-term treatment with metformin did reduce Ki-67 expression. Lack of response in obese women could not be correlated with lower drug levels, signifying that obesity may be responsible for the development of resistance to the anti-tumour effects of metformin.

Whilst contrary to the findings of earlier studies of the effect of short-term metformin on endometrial cancer proliferation, these conclusions are based on high quality clinical trial data, using an optimised study design that has taken into account the loss of detectable protein expression that occurs in large surgical specimens. Subgroup analysis of the modulating effect of BMI on response to metformin was determined a priori, although the specific cut-off values used were data driven. This novel finding of resistance to the anti-proliferative effects of metformin in endometrial tumours in obese women was supported by pre-clinical data, where pre-treatment with adipocyte conditioned media abrogated the reduction in cancer stem cell activity seen with low (in vitro) concentrations of the drug. These results suggest that adipocytes may be directly responsible for inducing drug resistance, through the release of adipokines. Investigation of the specific mediators secreted by these cells should be undertaken, and can be evaluated using multiplex adipokine ELISA kits designed to detect leptin, adiponectin, TNF-α, VEGF-α and inflammatory cytokines, among others, in adipocyte conditioned media. The impact of each of these adipokines in turn on endometrial cancer proliferation as well as cancer stem cell number, activity and response to metformin should be interrogated.

Even in non-obese women, in whom a non-significant reduction in cell proliferation was observed, metformin is unlikely to have a role in the primary treatment of endometrial cancer. The majority of these women are already cured of their disease by undergoing standard surgical treatment ± adjuvant radio- and chemotherapy. The only exception to this is younger women that desire fertility preservation, for whom a hysterectomy is an unacceptable option. Logically this group, especially because of the close association with polycystic ovary syndrome and insulin resistance, would be anticipated to benefit the most from treatment with an insulin sensitizer that reduces tumour proliferation. Baseline insulin sensitivity, however, did not affect tumour response to the drug in the PREMIUM study and the mean reduction in Ki-67 expression in women with a BMI ≤ 30 kg/m² was relatively modest at 8.3%, suggesting that metformin is not effective enough to be used alone as a primary treatment in this group. Combination therapy with, for example a levonorgestrel-releasing intra-uterine device, as is currently being trialled in Taiwan, may be more effective in inducing disease remission (Clinical Trials.Gov 2018). Contrary to the secondary objectives of the ongoing Taiwanese trial, however, this combination is unlikely to improve clinical pregnancy rates because of the
contraceptive effect of the intra-uterine device. Persistence of histological response beyond 12 months, despite dual treatment for only half of this time, is also improbable, especially if weight loss is not encouraged. Maintenance treatment with metformin may be hypothesised to be more effective in preventing disease relapse based on the findings presented in this thesis of the effect of the drug on endometrial cancer stem cells.

Metformin had a selective and specific effect on endometrial cancer stem cells, decreasing their number and activity at a concentration lower than that required to affect the proliferation of bulk tumour cells. It also reduced the expression of stem cell and EMT genes. Whilst these findings are encouraging, there is a caveat that the experiments were conducted using endometrioid endometrial cancer cell lines, which have their limitations in reflecting tumour biology in vivo. Replication of the experiments using primary endometrial cancer cultures is necessary as a first step to determine if these results hold true in more representative physiological conditions and studying the effects of the drug in different molecular subgroups of endometrial cancer, which have a higher propensity to metastasise and recur, is also critical (Stelloo et al. 2015). Consistent with other pre-clinical studies of metformin in endometrial cancer, the dose of the drug used in the laboratory experiments conducted in this thesis was 150-fold higher than the average trough metformin level measured in patients involved in the PREMIUM study (129,000ng/ml vs.865ng/ml). Whilst the increased dose of the drug required to observe a biological effect in vitro has been assumed to be due to the culture conditions used to maintain the growth of cell lines, the results of the PREMIUM study have neatly demonstrated that in vivo testing of metformin is critical before drugs are repurposed and used in clinical care. Developing and assessing the effect of the drug on tumour growth and relapse in a mouse xenograft model of endometrial cancer is, therefore, required and is the ‘gold standard’ means of determining an effect of the drug on cancer stem cell activity. It would be hypothesised that metformin treatment would reduce tumour volume and prolong disease remission in this context and, in so doing, would decrease the number and activity of CD133^+ve and ALDH\textsuperscript{high} endometrial cancer cells, as determined through flow cytometry and the sphere formation assay.

The establishment of drug efficacy on endometrial cancer stem cells and the translation of this into improvements in disease-free survival in an animal model is of critical importance before undertaking taking expensive, time-consuming trials in humans, in which large numbers of women could potentially be exposed to an ineffective treatment with not inconsiderable adverse effects. It is noted with some concern, therefore, that 540 women have already been recruited to a phase II/III randomised trial of paclitaxel and carboplatin with or without additional metformin in advanced and recurrent endometrial cancer (ClinicalTrials.gov 2016). The trial aims to determine whether the addition of metformin improves progression free and overall survival. The findings presented in this thesis make a positive result from this trial unlikely; firstly, metformin is only being given during chemotherapy cycles and, therefore, exposure to the drug will be limited and interrupted by periods off treatment and secondly, as obese women are not excluded, any effect is likely to be diluted across the patient population. Attempts to determine the predictive and prognostic significance of key targets
of metformin/mTOR pathway will be hampered by using primary tumour from the hysterectomy specimen, in which decreased expression of these markers are likely to be found due to hypoxia and poor tissue fixation. The lack of inclusion of a biopsy at trial entry also means that translational work investigating the effect of metformin on endometrial cancer stem cells will not be possible.

A more robust approach would be to undertake a pilot window study, to determine the feasibility of recruiting women with a BMI <30kg/m² who are at high risk of local and distant endometrial cancer recurrence, due to high grade and advanced stage disease, non-endometrioid histology, deep myometrial and lymphovascular space invasion. Women would be randomised to receive either metformin or placebo for 1-5 weeks before surgery and endometrial biopsies taken prior to and following treatment would allow the effect of the drug on cancer stem cell number and activity to be determined. It would be hypothesised that metformin treatment would be associated with a decrease in the proportion of CD133⁺ve and ALDH³⁰⁰ cells and a reduction in the sphere forming efficiency of endometrial cancer cells. The use of functional sphere assays and the quantification of cancer stem cell marker expression as outcome measures in endometrial cancer trials have not been previously tested and the clinical utility of these biomarkers does need to be determined. The prognostic value of flow cytometry and immunohistochemically detected expression of CD133 and ALDH, and endometrial cancer sphere formation in determining disease-free and cancer-specific survival needs to be assessed, particularly as immunohistochemistry for ALDH detects only the presence of the enzyme and not its activity (Ablett et al. 2012). Incorporation of all three methods of determining endometrial cancer stem cell number and activity as primary endpoints in a window study of metformin would also allow their capacity to predict tumour response to the drug and longer term clinical outcomes to be assessed. If validated as an approach, changes in endometrial cancer stem cell activity and marker expression with metformin treatment could be used to infer an effect of the drug on clinical outcome and would support larger scale trials using metformin as a maintenance therapy for up to five years to prevent disease relapse and improve survival.

As well as potentially impacting on recurrence and mortality rates, metformin may also improve overall survival in women diagnosed with endometrial cancer, who remain at a higher risk of death than the general population despite advances in the management of their malignancy. This is due to a preponderance of cardiovascular risk factors, which, as shown in this thesis, are more likely to be undiagnosed and undertreated than in women without a history of endometrial cancer, and, as a consequence, women newly diagnosed with endometrial cancer have a greater risk of fatal and non-fatal cardiovascular events in the 10 years following primary treatment. These data, from a large, well-conducted case-control study, support the immediate implementation of a systematic screening programme to identify cardiovascular risk factors in women with newly diagnosed endometrial cancer, including measurement of BMI, HbA1C, blood pressure and serum lipids. These can then be used to calculate an individual woman’s risk of cardiovascular disease, using the QRISK2 score. High risk individuals (QRISK2 score ≥10%) should be encouraged to lose weight and commence appropriate
medical therapy to normalise their blood pressure and glucose levels and should be advised to start statin treatment, regardless of baseline cholesterol measurements.

As discussed in a recent Cochrane review, weight loss in women with a history of endometrial cancer would be hypothesised to improve overall and cardiovascular survival as well as potentially increasing disease-free survival (Kitson et al. 2018a). Despite this, the impact of weight loss in this group is an under-researched area. The small studies that have been conducted have used dietary and behavioural interventions that have been ineffective in promoting weight loss compared to usual care and, hence, have failed to show any benefit on survival. The DiRECT study has shown that weight loss is feasible and sustainable in motivated individuals at a primary care level and can involve as little as eight hours of structured training by a specialist dietitian (Lean et al. 2017). A trial randomising obese women to either a weight loss programme, such as Counterweight-Plus, or usual care following primary treatment of endometrial cancer should be performed, with overall, cardiovascular-specific and disease-free survival as endpoints. The effectiveness of the programme in inducing weight loss and resolution of hyperglycaemia in this population should also be evaluated. Such a trial would require nationwide recruitment, with randomisation at a hospital level, and long term (five-10 year) follow-up.

An alternative, but not mutually exclusive, strategy would be to investigate the effect of long term metformin on survival in obese women with a history of endometrial cancer. By preventing type 2 diabetes in women with non-diabetic hyperglycaemia and favourably impacting on weight loss and cholesterol levels, metformin would be expected to decrease the incidence of cardiovascular disease in this group and thereby improve both cardiovascular-specific and overall survival (Knowler et al. 2002). In order to avoid the long follow-up and large sample size needed to detect a statistically significant reduction in the number of myocardial infarctions, strokes and mortality rate with metformin treatment, change in cardiovascular disease risk, as determined by QRISK2 score, could be used as a primary outcome measure in a randomised controlled trial. This is a valid surrogate marker from which information about the impact of metformin on cardiovascular event frequency could be extrapolated.

In conclusion, the findings presented in this thesis suggest that obese women appear resistant to the anti-proliferative effects of metformin in endometrial cancer and that this is a likely consequence of adipocyte secreted mediators. Metformin has a selective and specific effect on endometrial cancer stem cells, decreasing their number and functional activity in a cell line model. Women with a history of endometrial cancer are at higher risk of cardiovascular disease than the general population and are more likely to have unrecognized and undertreated risk factors. The effect of a longer duration of metformin treatment on overall and cardiovascular-specific survival and cancer stem cells in women with endometrial cancer should be tested in well-designed randomised controlled trials.
References


Appendix 1

Identifying High-Risk Women for Endometrial Cancer Prevention Strategies: Proposal of an Endometrial Cancer Risk Prediction Model

Sarah J. Kitson¹,², D. Gareth Evans³, and Emma J. Crosbie¹,²

Abstract

Already the fourth most common cancer in women in the developed world, the incidence of endometrial cancer is increasing rapidly, in line with the increasing prevalence of obesity. Relatively few studies have been undertaken of risk-reducing interventions aimed at limiting the impact of the disease on both individuals and the health service. Those that have been performed have demonstrated only modest results due to their application in relatively unselected populations. A validated risk prediction model is therefore urgently required to identify individuals at particularly high risk of endometrial cancer who may benefit from targeted primary prevention strategies and to guide trial eligibility. On the basis of a systematic review of the literature, the evidence for inclusion of measures of obesity, reproduction, insulin resistance, and genetic risk in such a model is discussed, and the strength of association between these risk factors and endometrial cancer is used to guide the development of a pragmatic risk prediction scoring system that could be implemented in the general population. Provisional cutoff values are described pending refinement of the model and external validation in large prospective cohorts. Potential risk-reducing interventions are suggested, highlighting the need for future studies in this area if the increasing tide of endometrial cancer is to be stemmed.

Introduction

Endometrial cancer is the fourth most common cancer in women in the United Kingdom, with more than 9,000 new diagnoses made in 2013 (1). The incidence is increasing not only in the developed world, where case numbers have more than doubled in the last 20 years but is also expected to increase in lower income countries as the global burden of obesity worsens (2). Given the current trajectory, it is predicted that by 2030, there will be an additional 3,700 new cases of endometrial cancer diagnosed each year in the United Kingdom (Fig. 1; refs.3, 4). In line with this, mortality rates are also increasing, albeit to a lesser extent, with a further 850 endometrial cancer deaths per year anticipated in England and Wales alone by 2030 (3). While endometrial cancer usually presents early, the morbidity associated with treatment, particularly in an increasingly elderly population, is not insignificant and disease recurrence, despite adjuvant treatment, continues to be a problem. Intervention is urgently required to stem this increasing tide of endometrial cancer at the earliest opportunity, both for individual patients and for the health service, are not to become overwhelming.

Reducing the incidence of endometrial cancer requires the introduction of risk-reducing measures used selectively in those at greatest disease risk and targeted at key mechanisms driving endometrial carcinogenesis. Previously studied interventions have often been found to have only a modest effect on disease risk, mainly due to their application in relatively unselected populations with the result that more pronounced benefits for specific subgroups may be diluted (Table 1). This highlights the importance of developing better risk prediction models to identify specific patient groups in whom these candidate risk-reducing interventions can be trialed to maximize their potential impact.

Here, we propose a pragmatic risk prediction model to stratify the general female population into low-, medium-, and high-risk groups for endometrioid endometrial cancer, the most common histologic subtype (75% of all endometrial cancers; ref. 5) and for which there is the greatest understanding of underlying risk factors and potential carcinogenic mechanisms. Given that the number of cases peaks when women are in their mid to late 60s, such a model would be aimed at women aged 45–55 years with an intact uterus, allowing sufficient time for any benefit from prophylaxis to be realized. Experimental and epidemiologic evidence will be used to argue for the inclusion of measures of obesity (obesity score), unopposed estrogen exposure (reproductive risk score), insulin resistance (insulin resistance risk score), and family history (genetic risk score) to identify individuals at greatest risk and will include protective factors which may negate these risks. The rationale for using specific risk-reducing measures in subgroups based on their predominant endometrial cancer risk factor will also be explored.
There are 2 limitations to this approach, which must be appreciated at the outset. While such a model is likely to have maximal impact on disease burden, it may not significantly reduce endometrial cancer mortality, as non-endometroid tumors are more biologically aggressive and associated with poorer prognosis. The second point is that it may fail to protect women with undiagnosed Lynch syndrome in whom endometrial cancer often presents at an earlier age (<45 years); however, the model is designed to target the general population rather than those at a particularly high genetic risk of the disease (6).

**Obesity Score**

Any risk prediction model for endometrial cancer will be centered on measures of excess adiposity. It is estimated that up to 41% of endometrial cancer cases are directly attributable to women being overweight or obese and endometrial cancer has the strongest link with obesity of the 20 most common tumor types (6, 7). Several underlying mechanisms linking excess adiposity and endometrial cancer have been described; excess estrogen production, insulin resistance, and inflammation (Fig. 2). Each is discussed further in the relevant sections.

Numerous measures of obesity exist, but the most commonly used, cheapest and easiest to apply in a clinic setting is body mass index (BMI), calculated using the formula weight (kg)/height (m)\(^2\).

**BMI**

Meta-analyses of prospective observational studies have shown that a 5 kg/m\(^2\) increase in BMI is associated with a 60% increase in the relative risk of developing endometrial cancer (6, 8). The effect is nonlinear although, with a proportionally greater increase in risk for each 5 kg/m\(^2\) increase in BMI above 27 kg/m\(^2\), such that a woman with a BMI of 42 kg/m\(^2\) has a 9.11 times [95% confidence interval (CI), 7.26–11.51] greater risk of developing endometrial cancer than a woman with a BMI of 22 kg/m\(^2\) (8). This is reflected in the final model, with additional weighting given to the presence of super obesity (Table 2).

Given this association, it would appear reasonable to offer weight loss surgery to reduce the risk of endometrial cancer in those at greatest risk of the disease (BMI ≥ 40 kg/m\(^2\) along with additional risk factors for the disease). It is already known that there is a not insignificant prevalence of asymptomatic endometrial hyperplasia of 8.6% to 10% in the bariatric surgery population (women with BMI ≥ 40 kg/m\(^2\) or BMI ≥ 35 kg/m\(^2\) in the presence of obesity-related co-morbidities, such as diabetes mellitus or obstructive sleep apnea; refs. 9–11). This risk is reduced by weight loss surgery; the prevalence of endometrial cancer has been shown to decrease from 1.4% to 0.4% in obese women following bariatric surgery (12). Even those persistently obese women, benefit from a 50% lowering of endometrial cancer risk following surgery, suggesting that metabolic changes, such as improvements in insulin sensitivity, are also important in this context (12).

Additional health benefits associated with bariatric surgery include a reduction in the incidence of other obesity-related cancers, including postmenopausal breast and colorectal cancer, as well as resolution of diabetes, hypertension, angina, and obstructive sleep apnea (13). These benefits need to be incorporated into cost-effectiveness studies when determining the value of weight loss surgery in cancer prevention.

Focusing solely on women with the highest BMI (≥ 40 kg/m\(^2\)) however, limits the benefits from endometrial cancer prevention to only 3% of the female population (14). Other measures of adiposity, such as central obesity and weight gain over time, can also be used to identify those women with lower BMIs who also have a particularly high risk of developing endometrial cancer.

**Body Fat Distribution**

Body fat distribution is potentially a better predictor of cancer risk for obesity-associated malignancies than BMI, especially in breast cancer (15). Measures which assess the extent of central versus peripheral obesity can, therefore, be useful to further
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<th>Intervention</th>
<th>Target population</th>
<th>Mechanism of action</th>
<th>Current evidence</th>
<th>Side effects</th>
<th>Contraindications</th>
<th>Potential problems</th>
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<tr>
<td>Low-fat diet (&lt;20% of energy from fat)</td>
<td>BMI &gt; 30 kg/m²</td>
<td>Decrease adiposity and weight</td>
<td>- Decrease serum estrone, estradiol, and testosterone levels (67) - Increase sex hormone-binding globulin levels (67) - Improved insulin sensitivity (68)</td>
<td>Low-fat diets per se do not prevent endometrial cancer if they are not associated with significant weight loss (69) - Self-reported prior weight loss of 20 lbs or more in a single episode associated with a nonsignificant 7% reduction in risk of endometrial cancer (70) - Lower insulin and HOMA-IR levels found after 3 mo of an intermittent fasting diet, where only 600-650 cal/d are consumed on 2 d/wk, compared with a continuous low-calorie diet. No difference in amount of weight loss between groups but reduction in fat mass and improved compliance in intermittent fasting group (68). No studies of the effect of intermittent fasting on cancer prevention in humans have yet been published.</td>
<td>Nil</td>
<td>Long-term compliance often low with weight gain noted after discontinuing intervention. - Excessive rebound weight gain may exacerbate endometrial cancer risk</td>
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<td>Physical activity</td>
<td>BMI ≥ 25 kg/m²</td>
<td>Decrease adiposity and weight</td>
<td>- Decrease adiposity and weight</td>
<td>Increase insulin sensitivity and reduce insulin levels (71) - Reduce serum estradiol and increase sex hormone-binding globulin levels (71) - May improve innate and acquired immune responses (71)</td>
<td>One hour daily of moderate intensity activity likely to reduce endometrial cancer risk, with the most active women benefitting from a 20%–30% risk reduction, independent of adiposity (72). Higher intensity, longer duration exercise likely to be bestest, though all activity types lower endometrial cancer risk by a similar amount. Benefit restricted to overweight/obese women (73). - No clinical trials undertaken looking at increasing physical activity as a primary prophylactic intervention against endometrial cancer</td>
<td>Nil</td>
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<th>Interventions</th>
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<td>Decrease adiposity and weight (either through caloric restriction, malabsorption, or decrease in appetite)</td>
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<td>Bariatric surgery associated</td>
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<td>Surgical complications, including anastomotic leak</td>
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<td>Patient not motivated to undergo procedure</td>
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<td>Estimated that 71 bariatric procedures would need to be conducted to prevent 1 incident endometrial cancer, although patients with BMI ≥ 40 kg/m² or ≥ 35 kg/m² in the presence of obesity-related co-morbidities, e.g., diabetes, hypertension, obstructive sleep apnea, and increased risk of breast and cervical cancers (risk returns to normal once use discontinued)</td>
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<td>Improvement in insulin sensitivity and decrease in oxidative stress and inflammation</td>
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<td>Lowering insulin and leptin levels and increase in adiponectin</td>
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<td>Metaformin</td>
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<td>Gastrointestinal upset - nausea, vomiting, diarrhea, rash</td>
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<td>Headache, breast tenderness, breakthrough bleeding, increased risk of venous thromboembolism, increased risk of endometrial cancer (risk returns to normal once use discontinued)</td>
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<td>Metformin associated with resolution of diabetes and improvements in cardiovascular disease (77)</td>
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<td>Increased endometrial progesterone receptor expression (79)</td>
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<td>Treatment with metformin associated with resolution of atypia and reduction in insulin, glucose, and testosterone levels (81–83).</td>
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<tr>
<td>Metformin associated with a 40%–50% reduction in endometrial cancer risk, with benefit continuing even after the procedure (79). In animal studies (78), inhibition of TNF-α signaling, at least in vascular endothelial cells, reduces endometrial proliferation.</td>
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<tr>
<td>Inhibition of TNF-α signaling, at least in vascular endothelial cells</td>
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<tr>
<td>Reduction in endometrial proliferation</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Oligomenorrhea</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lynch syndrome</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Decision analytical model suggested that 5 y of COCP use in obese women was unlikely to be cost-effective for decreasing endometrial cancer incidence, although failed to take into account the reduction in ovarian cancer risk. Selection of subgroups on the basis of pre-existing cardiovascular disease, family history of thrombosis, morbid obesity, diabetes, obesity, age ≤ 35 y, PCOS, and Lynch syndrome.</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additional benefit of reducing ovarian cancer risk by 20% for each 5 years of use</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td></td>
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<td>Additional benefit of reducing ovarian cancer risk by 20% for each 5 years of use</td>
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</tbody>
</table>
### Table 1. Candidate prophylactic interventions trialed in endometrial cancer prevention and their relative merits (Cont'd)

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Target population</th>
<th>Mechanism of action</th>
<th>Current evidence</th>
<th>Side effects</th>
<th>Contraindications</th>
<th>Potential problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levonorgestrel-releasing intrauterine system (Mirena)</td>
<td>Tamoxifen users, Estrogen-only HRT users, Obese women</td>
<td>Downregulation of endometrial estrogen receptors and reduction in cellular proliferation (89)</td>
<td>Use of levonorgestrel-releasing intrauterine system for the treatment of heavy menstrual bleeding associated with a 54% reduction in endometrial cancer compared with premenopausal controls and up to 75% reduction with prolonged use (32). Follow-up limited to age 55, so may have underestimated benefit by excluding age group with highest endometrial cancer incidence. Use associated with protection against endometrial hyperplasia in tamoxifen and estrogen-only HRT users (90). Current ongoing study by our own group investigating the role of the levonorgestrel-releasing intrauterine system in the primary prevention of endometrial cancer in obese women.</td>
<td>Irregular bleeding (usually settles within 6 mo), coil expulsion, failed insertion, uterine perforation during insertion, endometritis, breast tenderness, mood swings</td>
<td>Breast cancer, unexplained vaginal bleeding, cervical cancer, liver disease, stroke, untreated pelvic infection</td>
<td>Benefit in asymptomatic, obese population yet to be determined</td>
</tr>
<tr>
<td>Aspirin</td>
<td>BMI ≥ 30 kg/m²</td>
<td>Anti-inflammatory effect, Reduction in aromatase and estrogen levels (61), Increased apoptosis (62)</td>
<td>Meta-analysis of observational studies found a small, nonsignificant reduction in endometrial cancer risk with long-term aspirin in the general population (91). Obese women may derive greater benefit, although. Similar results seen for women with Lynch syndrome taking aspirin for 4 years for the primary prevention of endometrial cancer (64). In colorectal cancer cell lines, nitric oxide donating aspirin suppressed microsatellite instability in MMR-deficient cells and is thought to lower the threshold for apoptosis in response to DNA damage (92).</td>
<td>Indigestion, gastrointestinal bleeding, peptic ulcer</td>
<td>Bleeding disorders, allergy to nonsteroidal anti-inflammatories, renal disease, caution in asthma</td>
<td>Minimal benefit seen in general population, further studies required to determine whether particular subgroups likely to derive greater benefit from aspirin prophylaxis</td>
</tr>
</tbody>
</table>

(Continued on the following page)
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Table 1. Candidate prophylactic interventions trialed in endometrial cancer prevention and their relative merits (Cont’d)

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Target population</th>
<th>Mechanism of action</th>
<th>Current evidence</th>
<th>Side effects</th>
<th>Contraindications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D</td>
<td>BMI &gt; 30 kg/m²</td>
<td>Increase sex hormone-binding globulin levels (19)</td>
<td>Limited to animal studies using obese Pen (+/−) mice (95)</td>
<td>Insomnia, restlessness, tachycardia, headache, nausea/vomiting (related to caffeine)</td>
<td>None with doses up to 10,000 IU/day</td>
</tr>
<tr>
<td>Coffee consumption</td>
<td>Non/low coffee consumers</td>
<td>Increase Pten expression and MAPK activity (17)</td>
<td>Increased coffee consumption, particularly in reducing endometrial cancer risk (99)</td>
<td>No studies investigating coffee drinking specifically in endometrial cancer prophylaxis</td>
<td>No evidence of benefit in reducing endometrial cancer risk (18)</td>
</tr>
</tbody>
</table>

Effect of Weight Change

While current BMI has a significant influence on endometrial cancer risk, weight change over time is also important and is factored into the risk prediction model. This is based on results from the meta-analysis discussed above, in which an increase in weight between the ages of 18 and 20 years and middle age was associated with a higher endometrial cancer risk, even after adjusting for current BMI (19). For each 5-kg increase in weight over this time period, the risk of endometrial cancer increased by 18% (95% CI, 15%–21%). Importantly, this result has been replicated in a non-Western population, with lower overall levels of obesity, and may be more pronounced in women with a higher starting BMI in their late teens/early twenties (20). The caveat to the use of weight gain in a predictive model of endometrial cancer risk is its reliance on estimates of historical weight and the inaccuracies inherent to such data.

Adipokines

In addition to clinical measurements of body mass and adiposity distribution, adiponectin levels are also included as a serum biomarker of obesity and an adverse metabolic phenotype. Adiponectin is secreted by adipose tissue, although levels are inversely correlated with BMI (21). Biologically, it has an anticancer effect, acting as an anti-inflammatory and improving insulin sensitivity, while inhibiting angiogenesis and downregulating vascular adhesion molecule expression (22). This is achieved through activation of AMPK and inactivation of ERK and MAPK (Fig. 2). It is also able to increase apoptosis by inducing expression of p53 and Bax, thereby acting as a negative regulator of tumor formation (23). Higher serum levels of adiponectin are associated with a reduction in endometrial cancer risk (summary: OR, 0.47; 95% CI, 0.34–0.65) with evidence of a dose–response relationship (24). For each 5 μg/mL increase in adiponectin levels, the risk of endometrial cancer has been found to decrease by 18%, an effect consistent across analyses adjusted for confounding factors, such as menopausal status, BMI, and hormone replacement therapy (HRT) use. This supports the distinction between
metabolically healthy and metabolically unhealthy obese individuals and is incorporated into the risk prediction model as a protective factor (25).

At present, there is insufficient evidence to support the inclusion of the other important adipokine, leptin, in the risk model. It is also secreted by adipocytes and is involved in energy homeostasis, with levels increasing in proportion with body mass (26). It has multiple cellular effects in vitro, any or all of which are associated with an increased risk of tumor formation, including proinflammatory, proangiogenic, mitogenic, and antiapoptotic effects, through activation of MAPK, PI3K, and STAT pathways and increases in aromatase activity (26). While a meta-analysis of observational studies found that women with leptin levels in the upper tertile had a 2-fold increase in their risk of endometrial cancer compared with those with the lowest levels, independent of BMI, the included studies were heterogeneous in design and insufficient data were available to determine whether a dose–response relationship existed. Further work is, therefore, required to quantify the relationship between leptin levels and endometrial cancer risk before it can be included in any prediction model.

Each of the obesity measures discussed is derived from good quality epidemiologic and in vitro evidence demonstrating a dose–response relationship between excess adiposity and endometrial cancer risk. While they are included to measure different aspects of this association, to avoid “double counting” obesity in the risk prediction model, the highest score of any of the clinical obesity measures added to the serum adiponectin score will be combined with the reproductive, insulin, and genetic risk scores to derive the overall score.

Reproductive Risk Score

Established reproductive risk factors for endometrial cancer can be interpreted in light of the “unopposed estrogen theory”. Estrogen induces endometrial proliferation through local production of IGF-1, increasing the risk of accumulation of genetic mutations in proto-oncogenes and tumor suppressor genes (27). It is also responsible for an increase in free radical–mediated DNA damage and inhibition of apoptosis (26, 27). Increased lifetime exposure to estrogen, through early menarche (<12 years) or late menopause (≥55 years), is, not surprisingly, associated with an increased risk of endometrial cancer (28). While estrogen only HRT is a time-honored risk factor for endometrial cancer, it is now so rarely used in women with an intact uterus that it has not been included in the risk prediction model. Conversely, use of the combined oral contraceptive pill (COC) for ≥5 years is associated with a significant reduction in endometrial cancer risk due to suppression of endogenous estrogen levels and increased exposure to progesterone throughout the menstrual cycle (29). For the same reason, increasing parity is a protective factor; a meta-analysis of 46 studies showed that, compared with nulliparous women, women who had had one child had a 27% lower risk of developing endometrial cancer (RR, 0.73; 95% CI, 0.64–0.84) and those with 2 children a 38% reduction in endometrial cancer risk (RR, 0.62; 95% CI, 0.53–0.74; ref. 30). While there was some evidence of a dose–response relationship between parity and endometrial cancer risk, the numbers of included women with 3 or more children were too small to draw meaningful conclusions from.

For postmenopausal women, adipose tissue becomes the dominant source of estrogen, responsible for the conversion of...
Table 2. Proposed endometrial cancer risk prediction model

<table>
<thead>
<tr>
<th>Risk score</th>
<th>Risk factor</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMI</td>
<td>25</td>
<td>30</td>
<td>35</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Waist circumference</td>
<td>90</td>
<td>100</td>
<td>110</td>
<td>120</td>
<td>130</td>
</tr>
<tr>
<td>Obesity</td>
<td>Weight gain</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>between 18-25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>and 45-55 y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin</td>
<td>&gt;5 μg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reproductive</td>
<td>Early menarche</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(&lt;12 y) or late</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>menopause (&gt;55 y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OR</td>
<td>Anovulation</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6 mo of more,</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>unrelated to</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>pregnancy,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>breastfeeding, or</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>contraceptive use)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td>2+</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥5 y</td>
<td></td>
<td>Never or &lt;5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever use of tamoxifen</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free testosterone</td>
<td>≤17 pmol/L</td>
<td></td>
<td>&gt;17 pmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>Type 2 diabetes</td>
<td>Absent</td>
<td>Present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCOS</td>
<td>Absent</td>
<td>Present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-peptide</td>
<td>(non-fasting)</td>
<td>≤0.76 nmol/L</td>
<td>&gt;0.76 nmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic</td>
<td>Family history of</td>
<td>No first- or second-degree relatives affected</td>
<td>First-degree relative diagnosed at ≤50 years of age</td>
<td>Two or more first- or second-degree relatives diagnosed</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>endometrial cancer</td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

NOTE: Points are assigned as described for each individual risk factor. The highest single clinical obesity score is then added to the serum adiponectin score to give the final obesity score. This is combined with the total reproductive, insulin, and genetic scores to give an overall total, which is used to assign patients into risk categories: 0–2 low risk, 3–7 medium risk, ≥8 high risk.
endometrial cancer (by a median of 11.2 years), allowing ade-
quate time for prophylactic intervention to be instituted. Mea-
surement of serum-free androgens also has the advantage that
levels are unaffected by the menstrual cycle, avoiding the com-
plexities of timing blood sampling that is seen with other sex
hormones. It is as yet unclear whether elevated androgen levels are
associated with an increased risk of developing premenopausal
endometrial cancer as the study by Clendenen and colleagues (40)
found no association if a diagnosis was made prior to the age of 55
years, although their analysis was based on only 49 cases and 86
controls. The molecular effect of testosterone on the endometri-
um and endometrial cancer cells is still debated, but it would
appear logical for it to be included in the prediction model, given
the close association between elevated androgen levels, obesity,
and estrogen production in postmenopausal women and PCOS
in younger individuals (40)

Measurement of serum estrogen levels was discounted from the
model on the basis that it was only of value in determining
endometrial cancer risk in postmenopausal women. Several
case–control and prospective cohort studies have found increased
levels of endogenous total and free estrogen in postmenopausal
women with endometrial cancer compared with controls, with
estradiol levels in the upper tertile being associated with a 2- to 4-
fold increase in endometrial cancer risk (27, 41, 42). In premen-
opausal women, however, this relationship is not evident, lim-
iting its applicability in our target population (43). There are no
published studies evaluating progesterone as a marker of endo-
mtrial cancer risk, although as levels vary dramatically through-
out the menstrual cycle, attempting to control for this would be
difficult (27).

Insulin Risk Score

The third component of the risk prediction model, and an area
receiving increasing attention, is the effect of insulin resistance on
the development of endometrial cancer. There is now substantial
in vitro evidence for a direct effect of insulin and IGF-1 on
endometrial cancer cells, with activation of the insulin receptor
resulting in an increase in cell proliferation and inhibition of
apoptosis (44, 45). These effects are mediated through both the
MAPK and PI3K/Akt pathways (Fig. 2). Insulin and IGF-1 also
stimulate β-catenin, a signaling pathway involved in early tumor
formation, and through this the oncogene Ras. By increasing the
breakdown of IGFBP-3, insulin is able to act to increase levels of
free IGF-1 and thus enhance its tumor-promoting capacity.
Beyond these direct effects, hyperinsulinemia is also involved in
increasing ovarian androgen production and peripheral aroma-
tization to estrogen, reducing sex hormone–binding globulin and
adiponectin levels and stimulating leptin secretion, highlighting
the interdependence of these mechanisms (44).

In line with this, a diagnosis of type 2 diabetes mellitus is
included in the model as its presence is associated with a greater
than 2-fold elevation in endometrial cancer risk, even after adjust-
ment for activity levels and BMI (46). Similarly, PCOS, while
featuring in the reproductive risk score because of its link with
hyperandrogenemia, is also included in the insulin risk score; 50% to
70% of patients with PCOS are also insulin-resistant and this
group has a particularly high endometrial cancer risk (47).
Despite the epidemiologic evidence supporting an increased risk
of endometrial cancer for those with elevated insulin levels, large-
scale testing is not possible due to the lack of a standardized
protocol for sample preparation and testing and the absence of
validated cutoff values to stratify patients into high- and low-risk
groups (48–51). For these reasons, surrogate measures of insulin
sensitivity, such as HOMA-IR and QUICKI, which rely on accurate
insulin level measurements, have also not been included. The
gold-standard test of insulin sensitivity is the euglycemic clamp
test, but this is too expensive and time-consuming to be used apart
from on an individual patient basis (52). While measurement of
IGF-1 levels would circumvent many of these problems, no
consistent association between serum IGF-1 and endometrial
cancer risk has been demonstrated, suggesting that local endo-
mtrial IGF-1 production may be more relevant than systemic
levels (51).

On the basis of current evidence and with mind to the practi-
calities of screening a large number of patients, we propose
incorporating the pro-insulin protein, C-peptide, into a risk
prediction model. It is stored intracellularly with insulin and the
2 are released together in equal amounts; higher levels of C-
peptide thus reflect increased endogenous insulin secretion and
insulin resistance. It has the advantage of having a longer half-life
than insulin and more accurately reflects insulin levels if there is
variation in fasting time. An absolute requirement for fasting
samples is also not necessary. Five observational studies have
been conducted examining the relationship between C-peptide
evels and endometrial cancer, the results of which were combined
in a meta-analysis (49). Both fasting and non-fasting levels were
significantly higher in patients who subsequently developed
endometrial cancer than in controls, with evidence of a dose-
response relationship (51, 53). Only one study reported on actual
C-peptide levels rather than study-specific quintiles; a level greater
than 0.76 nmol/L is associated with 1.5- to 2-fold elevation in
endometrial cancer risk and is used in the model (53).

Glycosylated hemoglobin (HbA1C) is now part of both the
World Health Organization (WHO) and National Institute for
Health and Care Excellence (NICE) recommendations for diag-
nosing type 2 diabetes and validated clinical laboratory protocols
are already in place for its measurement. It represents glycemic
control over a preceding 8- to 12-week period and can be mea-
sured at any time of day without the requirement for fasting,
making it easier to measure than fasting glucose levels or perform-
ing an oral glucose tolerance test (OGTT). There is, however,
sufficient evidence to support its inclusion in the risk prediction
model, at present. Only one study has been performed examining
the relationship between HbA1C levels and endometrial cancer
risk and was insufficiently powered to determine cutoff values for
inclusion here (54). It did suggest, although, that even modest
elevations in HbA1C in nondiabetic patients may significantly
increase cancer risk. Further work is clearly warranted in this area.

Genetic Risk Score

The risk of endometrial cancer in women with Lynch syndrome
(mutations in the DNA mismatch repair genes MSH2, MSH6,
MLH1, PMS2, or EPCAM) is significantly elevated, with a cumu-
lative risk of endometrial cancer of 16% to 71% by the age of 70
years, depending upon the specific gene affected (55, 56). Despite
this, the role of screening for endometrial cancer in women with
Lynch syndrome and the value of prophylactic intervention to
reduce this risk have yet to be clearly defined and is the subject of
ongoing research. As this model has been developed for use in the
general population, this topic will not be discussed further here.
Irrespective of the underlying genetic predisposition, a family history of endometrial cancer is associated with a significant increase in endometrial cancer risk, particularly if a first-degree relative was diagnosed before the age of 50 years (HR, 6.68; 95% CI, 4.02–11.1; P < 0.001; ref. 57). This risk is increased further if 2 or more first- or second-degree relatives have previously had endometrial cancer (HR, 8.73; 95% CI, 4.25–17.9; P < 0.001). The risk of endometrial cancer for women with a family history of colorectal cancer is much lower and overall not significantly higher than for women without a family history. While both inherited mutations in genes critical to endometrial carcinogenesis and the presence of shared risk factors (including obesity) for the condition may explain this association, the exact mechanisms have yet to be determined.

Inflammation

While not directly incorporated at present, future work may well see measures of inflammation feature in the risk prediction model. Adipose tissue is increasingly being recognized as playing an active role in many diseases, including cancer, through the release of adipokines, cytokines, and sex hormone metabolism (58). Obesity is, itself, a state characterized by chronic inflammation (59). Cytokines are produced by activated adipocytes and infiltrating macrophages in response to adipose tissue expansion and localized hypoxia. Increasing BMI and waist circumference are associated with elevated levels of cytokines including IFNs, IL6, IL8, IL1 receptor antagonist (IL-1Ra), and C-reactive peptide (CRP; refs. 26, 60, 61).

Endometrial carcinogenesis may be promoted by this inflammatory milieu. Chronic inflammation results in the generation of free radicals, increased concentrations of COX2 and prostaglandin E2, and leads to cell proliferation and DNA damage (62). Activation of the NF-kB pathway by inflammatory cytokines is responsible for inhibition of apoptosis, overcoming cell-cycle arrest and the transcription of genes encoding proinflammatory cytokines, thereby establishing a vicious cycle of inflammation, resulting in tumor formation (Fig. 2). Inflammation also contributes to the development of insulin resistance and IL6 stimulates aromatase activity and the conversion of androgens into estrogen within adipose tissue (61). Nested case–control studies within the EPIC and Women's Health Initiative cohorts found higher levels of inflammatory mediators to precede a diagnosis of endometrial cancer, although the association was largely dependent on the degree of adiposity (61, 63). There is, however, some debate about which cytokines are specifically elevated in endometrial cancer and the optimal laboratory technique for their measurement. In particular, these proteins may be too nonspecific to be used in a risk prediction model; levels are elevated transiently in numerous situations, including subclinical infection. Longitudinal, prospective cohort studies are required to evaluate the role of inflammatory cytokines, such as IL6 and CRP, in endometrial cancer risk stratification and to determine whether repeated measures over time are of greater predictive value than one-off measurements. Should this evidence be forthcoming, it would support the targeted use of aspirin as a prophylactic intervention for those with an increased inflammation risk score. This has already been shown to be the case for women with Lynch syndrome in the CAP2 study, where treatment with aspirin for ≥2 years was associated with a 53% reduction in the incidence of endometrial cancer, although the mechanism underpinning this effect may well be different (64).

Using the Risk Prediction Model to Target Prophylaxis

The 4 individual components of the risk prediction model, genetic (G), insulin (I), reproductive (R), and obesity (O) scores, are combined to give an overall assessment of endometrial cancer risk, stratified into low-, medium-, and high-risk groups (Table 2, Fig. 3). On the basis of an absolute lifetime risk of the disease of 2.4%, this approximates to an absolute risk of endometrial cancer of up to 4.9%, 7.3% to 17.1%, and ≥19.5% for the low-, medium-, and high-risk groups, respectively (65). The

![Figure 3](https://example.com/figure3.png)
predominant risk factor identified can be used to determine the type of prophylactic intervention trialed, for example, metformin when the insulin score is particularly high, the COCP or levonorgestrel-releasing intrauterine device if the reproductive score predominates.

The ‘optimal’ model for risk prediction will include all the clinical and serum biomarkers incorporated into Table 2, to identify undiagnosed risk factors, particularly the presence of insulin resistance, within an asymptomatic population. Where blood draw is not possible, a model based on the clinical risk factors alone can be employed, although this is likely to understate disease risk in some women. For those deemed low risk, diet and exercise advice alone is required; this can be as simple as encouragement to maintain a normal BMI for those with a negative risk score to more intensive dietetic input and exercise advice for those with a BMI ≥ 25 kg/m². Lifestyle education such as this is vital not only to limit endometrial cancer risk but also to prevent an increase in risk of other malignancies and cardiovascular disease. Whether women given an individualized risk assessment are more likely to heed advice about lifestyle modification to induce weight loss is currently unknown; the concept of a ‘teachable moment’ to positively influence behavior is a hotly debated topic.

Women within the medium-risk group could receive the diet and exercise advice along with aspirin and metformin or a levonorgestrel-releasing intrauterine system (Mirena, Table 1), depending upon whether their highest score is in the reproductive or insulin risk categories. For those patients already taking metformin, a review of the dose and compliance with treatment is warranted, with the addition of further hypoglycemic medication indicated if glycemic control cannot be optimized further.

Those within the high-risk category require multimodal intervention to reduce their endometrial cancer risk, including diet and exercise advice, aspirin, metformin, and a Mirena coil. For women with a BMI ≥ 40 and other endometrial cancer risk factors (particularly diabetes), bariatric surgery should also be offered; such a procedure would not only provide endometrial protection but also be associated with significant reductions in weight and improvements in insulin resistance.

Reassessment of endometrial cancer risk using the prediction model is likely to be required every 5 years. This allows the Mirena coil to be replaced, if necessary, to ensure continuing efficacy and change or introduce other prophylactic treatments depending upon an individual’s risk score. Such assessments will continue until age 70, at which point the number of cases of the disease naturally declines and evidence for the validity of the components of the risk prediction model and prophylactic treatments discussed becomes more circumspect.

Conclusion

Mechanistic and epidemiologic studies have provided useful information on which to guide the development of a prediction model for endometrial cancer risk. We propose that such a model should include measures of obesity, reproductive hormones, insulin resistance, and family history, reflecting the interconnection of these mechanisms in driving endometrial cancer development. As it stands, this model is purely theoretical and requires formal testing in a large prospective cohort of asymptomatic women for whom long-term outcome data are available. This will allow the model to be refined, using random decision forests and unconditional logistic regression, to optimize the weighting of included variables and ensure its accuracy in identifying individuals at high and low risk of the disease. Once calibrated, we propose to validate the model in a second, independent cohort, thereby verifying its applicability to the general population. The UK Biobank, with its recruitment of more than 250,000 women and inclusion of anthropometric, biochemical, and clinical follow-up data, will provide the ideal resource in which to conduct this work (66). With periodic release of information, the Biobank is a not-for-profit organization established to assist researchers in understanding disease-specific risk factors and the development of such prediction models. This information would not only allow the identification of individuals with a particularly high risk of developing endometrial cancer but also potentially guide the development of prophylactic treatment aimed at specific disease-causing targets, such as insulin resistance and inflammation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The views expressed in this publication are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

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References

Identifying High-Risk Women for Endometrial Cancer Prevention Strategies: Proposal of an Endometrial Cancer Risk Prediction Model

Sarah J. Kitson, D. Gareth Evans and Emma J. Crosbie


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Appendix 2

Interventions for weight reduction in obesity to improve survival in women with endometrial cancer (Review)

Kitson S, Ryan N, MacKintosh ML, Edmondson R, Duffy JMN, Crosbie EJ

Kitson S, Ryan N, MacKintosh ML, Edmondson R, Duffy JMN, Crosbie EJ.
Interventions for weight reduction in obesity to improve survival in women with endometrial cancer.
DOI: 10.1002/14651858.CD012513.pub2.

www.cochranelibrary.com
# Interventions for weight reduction in obesity to improve survival in women with endometrial cancer (Review)

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A B S T R A C T

Background
Diagnoses of endometrial cancer are increasing secondary to the rising prevalence of obesity. Obesity plays an important role in promoting the development of endometrial cancer, by inducing a state of unopposed oestrogen excess, insulin resistance and inflammation. It also affects treatment, increasing the risk of surgical complications and the complexity of radiotherapy planning, and may additionally impact on subsequent survival. Weight-loss interventions have been associated with improvements in breast and colorectal cancer-specific survival as well as a reduction in the risk of cardiovascular disease, a frequent cause of death in endometrial cancer survivors.

Objectives
To determine the impact of weight-loss interventions, in addition to standard management of endometrial cancer, on overall survival and the frequency of adverse events.

Secondary objectives include an assessment of weight-loss interventions on endometrial cancer-specific survival, weight loss achieved, cardiovascular event frequency and quality of life both overall and stratified according to patient body mass index (BMI), where possible.

Search methods
This review searched Cochrane Central Register of Controlled Trials, MEDLINE, Embase and reference lists of articles, trial registries, and international gynaecological oncology conference abstracts from inception to January 2018.

Selection criteria
Randomised controlled trials (RCTs) of interventions to facilitate weight loss in overweight or obese women undergoing treatment for, or previously treated for, endometrial cancer were selected.

Data collection and analysis
Two review authors independently selected studies, assessed trial quality, and extracted data with disagreements resolved by a third review author. Study authors were contacted to obtain missing data, including details of any adverse events.
Main results

We included three RCTs in the review, randomising a total of 161 overweight and obese women with endometrial cancer. All studies compared combined behavioural and lifestyle interventions to facilitate weight loss through dietary modification and increased physical activity. The included RCTs were of low or very low quality, due to high risk of bias by failing to blind participants, personnel and outcome assessors, and significant loss to follow-up (attrition rate up to 29%).

Combined behaviour and lifestyle interventions were not associated with improved overall survival (risk ratio (RR mortality), 0.23 95% confidence interval (CI) 0.01 to 4.55, P = 0.34, one RCT, 37 participants; very low-certainty evidence) compared with usual care at 24 months. There was no evidence that such interventions were associated with improvements in cancer-specific survival or cardiovascular event frequency as no cancer-related deaths, myocardial infarctions or strokes were reported in the included studies. None of the included RCTs reported data for the outcome of recurrence-free survival. Combined behaviour and lifestyle interventions were not associated with significant weight loss at either six months (mean difference (MD) -1.88 kg, 95% CI -5.98 to 2.21 kg, P = 0.37, three RCTs, 131 participants, I²= 0%; low-certainty evidence) or 12 months (MD -8.98 kg, 95% CI -19.88 to 1.92 kg, P = 0.11, two RCTs, 91 participants, I²= 0%; very low-certainty evidence) when compared with usual care. Combined behaviour and lifestyle interventions were not associated with increased quality of life, when measured using either the SF-12 Physical Health questionnaire or FACT-G at six months (FACT-G MD 2.51, 95% CI -5.61 to 10.64, P = 0.54, two RCTs, 95 participants, I²= 83%; very low-certainty evidence), or by FACT-G alone at 12 months (MD 2.77, 95% CI -0.65 to 6.20, P = 0.11, two RCTs, 89 participants, I²= 0%; very low-certainty evidence) when compared with usual care. No serious adverse events, for example hospitalisation or deaths, were reported in included trials. Lifestyle and behavioural interventions were associated with a higher risk of musculoskeletal symptoms (RR 19.03, 95% CI 1.17, 310.52, P = 0.04, two RCTs, 91 participants; low-certainty evidence).

Authors’ conclusions

There is currently insufficient high-quality evidence to determine the effect of combined lifestyle and behavioural interventions on survival, quality of life, or significant weight loss in women with a history of endometrial cancer compared to those receiving usual care. The limited evidence suggests that there is little or no serious or life-threatening adverse effects due to these interventions, although musculoskeletal problems were increased, presumably due to increased activity levels. Our conclusion is based on low- and very low-quality evidence from a small number of trials and very few patients. We therefore have very little confidence in the evidence: the true effect of weight-loss interventions in obese women with endometrial cancer is currently not known.

Further methodologically-rigorous, adequately-powered RCTs are required with follow-up of 5 to 10 years duration. These should focus on the effects of varying dietary modification regimens, pharmacological treatments associated with weight loss and bariatric surgery on survival, quality of life, weight loss and adverse events.

Plain Language Summary

Weight-loss interventions in endometrial cancer survivors

Background

Endometrial or womb cancer is a common cancer in women and the number of cases is rising. This is due, in part, to increasing levels of obesity, which is a major risk factor for the disease. Whilst survival following endometrial cancer is generally excellent if diagnosed early, affected women are more likely to die early due to an increased risk of heart attacks and strokes and to have poorer quality of life. This review assessed the evidence for weight-loss interventions in overweight and obese endometrial cancer survivors to determine whether they were of benefit compared with usual care.

Study characteristics

We included three randomised controlled trials in which women were allocated at random to receive one of several interventions (treatments) and which involved 161 obese participants. The trials were conducted in the USA and the UK. All compared lifestyle advice (diet and exercise) plus self-help techniques (to encourage adherence to the advice) with usual care. The evidence is current to January 2018.

Key results

We found no benefit for endometrial cancer survivors from receiving lifestyle advice in terms of survival, cardiovascular events or quality of life, though such interventions were not associated with significant or serious harms to participants. They did, however, report
higher rates of musculoskeletal symptoms, presumably due to increases in physical activity. Whilst some women lost weight with these interventions, others did not, meaning that overall there was little or no benefit.

**Quality of the evidence**

The quality of included studies was, however, low or very low and all were small in terms of the number of participants and not designed to specifically look at the effect of their intervention on survival. Additional high-quality studies are required in this field and currently there are five ongoing trials.
### Summary of Findings for the Main Comparison

Lifestyle intervention versus usual care compared to placebo for weight reduction in obesity to improve survival in women with endometrial cancer

**Patient or population:** weight reduction in obesity to improve survival in women with endometrial cancer  
**Setting:** university hospitals in the USA  
**Intervention:** Lifestyle intervention versus usual care  
**Comparison:** placebo

<table>
<thead>
<tr>
<th>Outcomes</th>
<th>Anticipated absolute effects* (95% CI)</th>
<th>Relative effect (95% CI)</th>
<th>n of participants (studies)</th>
<th>Certainty of the evidence (GRADE)</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>Overall survival (24 months) (Number of deaths from any cause)</td>
<td>100 per 1000 (1 to 455)</td>
<td>RR 0.23 (0.01 to 4.55)</td>
<td>37 (1 RCT)</td>
<td>⊕⊕⊕⊕ VERY LOW</td>
<td>Risk ratio for mortality calculated</td>
</tr>
<tr>
<td>Adverse events-musculoskeletal (Number of musculoskeletal adverse events reported)</td>
<td>0 per 1000 (0 to 0)</td>
<td>RR 19.03 (1.17 to 310.52)</td>
<td>91 (2 RCTs)</td>
<td>⊕⊕⊕ LOW</td>
<td>Unable to calculate assumed and corresponding risk as no events in control groups</td>
</tr>
<tr>
<td>Recurrence-free survival (24 months) (Number of disease recurrence or death)</td>
<td>See comment</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No RCTs reported this outcome</td>
</tr>
<tr>
<td>Cancer-specific survival (24 months) (Number of cancer-related deaths)</td>
<td>See comment</td>
<td>not estimable</td>
<td>37 (1 RCT)</td>
<td>⊕⊕⊕⊕ VERY LOW</td>
<td>Unable to calculate risk ratio for mortality as no cancer related deaths reported in either arm of the study</td>
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<tr>
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<tr>
<td>Weight loss (12 months)</td>
<td>Change in weight from baseline in kg; positive values = weight gain, negative values = weight lost</td>
<td>The mean weight loss (12 months) was + 1.5 kg, MD 8.98 lower to 1.92 higher</td>
<td>-</td>
<td>91</td>
<td>VERY LOW 12910</td>
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<td>Cardiovascular and metabolic event frequency (12 months) (Number of strokes, myocardial infarctions and hospitalisations for heart failure)</td>
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<td>See comment</td>
<td>-</td>
<td>93</td>
<td>VERY LOW 1289</td>
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<tr>
<td>Quality of life FACT-G (12 months) (Change in QOL on FACT-G questionnaire from baseline; positive values = improved QOL, negative values = worsening QOL)</td>
<td>The mean quality of life FACT-G (12 months) ranged from 0 to + 2 units, MD 2.77 units higher</td>
<td>-</td>
<td>-</td>
<td>89</td>
<td>VERY LOW 2911</td>
</tr>
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</table>

* The risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).

CI: Confidence interval; MD: mean difference; RR: Risk ratio;

**GRADE Working Group grades of evidence**

**High certainty:** We are very confident that the true effect lies close to that of the estimate of the effect

**Moderate certainty:** We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different

**Low certainty:** Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect

**Very low certainty:** We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect
Although participants, personnel and outcome assessors were not blinded to treatment group allocation this is unlikely to affect this specific outcome measure.

Downgraded by one point as included study at high risk of attrition bias due to incomplete outcome reporting.

Downgraded by one point due to indirect results (included study contained two patients who, in addition to receiving the intervention, underwent gastric bypass during follow-up and were included in the final analysis).

Downgraded by one point due to imprecision as low event number in included study and wide confidence intervals.

Downgraded by one point as two of the included studies were at high risk of attrition bias due to incomplete outcome reporting.

Downgraded by one point due to imprecision as no events in control arms of included studies and wide confidence intervals.

One of the included studies contained two patients who, in addition to receiving the intervention, had undergone gastric bypass during follow-up. This was not felt to impact on the number of adverse musculoskeletal events experienced and, therefore, the study was not downgraded for this reason.

Downgraded by one point due to imprecision as no events in any study.

Downgraded by one point due to indirect results (one of the included studies contained one patient, who by this time, had undergone a gastric bypass in addition to receiving the specific study intervention and was included in the final analysis).

Downgraded by one point due to imprecision as wide confidence intervals in all included studies, which cross the line of unity.

Downgraded by one point due to high risk of performance and detection bias in all included studies. Participants, personnel and outcome assessors were unblinded to treatment group allocation, which may have affected the subjective results.

The assumed (control) risk is the median weight change from baseline among the control groups in the included studies.

The assumed (control) risk is the range of scores for change in QOL from baseline at 12 months in the control groups from the included studies, presented in preference to the median change score due to significant variation.
BACKGROUND

Description of the condition

Endometrial cancer is a cancer of the lining of the womb and is the fourth most common cancer in women in the developed world (Cancer Research UK 2014a). Each year, 9,000 new cases of endometrial cancer are diagnosed in the UK, and 60,000 in the USA (Cancer Research UK 2014a; NCI 2016). The incidence of the disease has doubled in the last 20 years, and this trajectory is expected to continue. Endometrial cancer has a generally good prognosis if diagnosed early, with eight out of 10 women still alive at five years after diagnosis (Cancer Research UK 2014b). With more women than ever surviving initial treatment for endometrial cancer, interventions aimed at reducing the risk of disease recurrence and optimising general health in the long term (at least 5 to 10 years following diagnosis) are required.

Endometrial cancer has a strong link with obesity and it is this relationship that is thought to underpin the rising number of cases (Renahan 2008). As the percentage of the female population who are obese has increased, so has the number of diagnoses of endometrial cancer. Three biological mechanisms, or themes, have been proposed to explain this association: unopposed oestrogen, insulin resistance, and the presence of an inflammatory milieu (tumour environment).

Oestrogen is a potent stimulator of endometrial cell proliferation or turnover, an effect that is normally counteracted by progesterone during the menstrual cycle. Unopposed oestrogen occurs in two different scenarios; if progesterone levels are low because of absent ovulation (anovulation), such as in polycystic ovary syndrome, or if oestrogen levels exceed progesterone levels. This occurs in obese postmenopausal women, when the ovaries no longer produce progesterone, but testosterone, secreted by the ovaries and adrenal glands, is converted into oestrogen by excess fat (adipose) tissue. Unopposed oestrogen is associated with an increased risk of endometrial cancer. It increases the rate of turnover of endometrial cells and thus the chance of acquiring alterations (mutations) within key genes associated with cancer development. Epidemiological studies have confirmed an increased risk of endometrial cancer in women with high oestrogen levels (Dossus 2013).

Insulin is also able to stimulate endometrial cell proliferation, activating many of the pathways shown to be critical to endometrial cancer development. Obese women have higher insulin levels than their normal-weight counterparts; excess fat tissue reduces the responsiveness of the body to the effects of insulin, so levels increase to compensate. Elevated serum insulin levels have been shown to be present in women with endometrial cancer, compared with those without the disease (Dossus 2013).

Thirdly, fat tissue produces inflammatory and carcinogenic (cancer promoting) proteins, hence obese women have elevated levels compared with normal-weight women. Any, or all of these proteins, may be responsible for the increase in endometrial cancer rates seen in this population (Dossus 2013).

Obesity plays an important role in promoting the development of endometrial cancer, and potentially affects treatment and subsequent survival. The mainstay of treatment for endometrial cancer is surgery to remove the uterus (womb), cervix, fallopian tubes and ovaries. This may be followed by radiotherapy, chemotherapy or both in some women. Obese women often have other health problems, including diabetes and sleep apnoea, which can adversely affect their medical fitness to undergo an operation, and increase the risk of complications associated with surgery and radiotherapy. This may lead to compromises in treatment (Papadia 2006). There is debate in the literature as to whether being overweight or obese has a negative impact on survival. Results from two large cohort studies, in which groups of women with endometrial cancer were followed up, have suggested that obese women, with a body mass index (BMI) of 30 or more, are twice as likely to die during this period as women of a healthy weight. This increases to a six-fold elevation in risk if their BMI is over 40 (Calle 2003; Reeves 2007). However, these studies did not take into account differences in the cancer grade (how abnormal the cells appeared), stage (how far the disease had spread), or the type of treatment received.

When women with endometrial cancer received standardised treatment in the context of a randomised controlled trial (RCT), researchers were able to demonstrate that BMI had no impact on the risk of recurrence or overall survival. This was despite a high proportion of obese women having poorer general health (Crosbie 2012). The extra deaths observed in obese women with endometrial cancer may well be unrelated to their cancer. Women with early stage disease are twice as likely to die from cardiovascular disease, for example heart attacks and strokes, as they are to die from their endometrial cancer (Ward 2012). Excessive weight gain following diagnosis, and indeed, significant weight loss, may be more important than body mass per se. Data from observational studies demonstrate that large weight gains have a detrimental effect on survival, even after adjustment for other factors that influence prognosis, such as cancer grade and stage (El-Safadi 2012; Matsuo 2016). Therefore, measures taken to reduce body weight following treatment for endometrial cancer may be beneficial in improving survival, either by reducing the risk of death from endometrial cancer, or by lowering the chance of dying from other causes, in particular cardiovascular disease.

Description of the intervention

This review focused on interventions designed to promote weight loss as their primary goal, and includes non-pharmacological, pharmacological, and surgical interventions. These may be used alone, or in combination. Non-pharmacological or ‘lifestyle’ interventions are those aimed at reducing nutrient intake and increasing physical activity, through diet and exercise, and may be used alongside psychological interventions such as stress management,
stimulus control, and problem solving (addressing barriers to adhering to diet and exercise regimens) to induce permanent changes in behaviour. Pharmacological interventions include drugs that act to either reduce fat absorption, the most widely used of which is orlistat, or suppress appetite. Bariatric surgery encompasses procedures designed to limit food intake (e.g. gastric banding), cause malabsorption (e.g. intestinal bypass), or both (e.g. gastric bypass; Figuls 2013).

How the intervention might work

Weight-loss interventions may improve survival by influencing any, or all of the pathways described above that link obesity and endometrial cancer, and have already been shown to be beneficial for survivors of other obesity-related cancers, including breast and colorectal cancer (Morey 2009; Rock 2015; Stolley 2009). Like endometrial cancer, breast cancer also appears to be hormonally driven, and weight-loss interventions that have been associated with a loss of 5% or more body weight have been shown to reduce total and free oestradiol (a type of oestrogen) levels in women following treatment for this cancer type, which may reduce the risk of disease recurrence (Rock 2013). Similarly, weight-loss interventions have already been shown to lower levels of both insulin and adiponectin (a marker of insulin resistance), and improve insulin sensitivity in women following treatment for breast cancer (Rock 2013; Swisher 2015). They have also been associated with a reduction in the expression of inflammatory and cancer-promoting proteins, and this may explain why they reduce the risk of disease recurrence (Irwin 2015).

In addition to potential improvements in cancer-specific outcomes, weight-loss interventions may also improve overall survival by reducing the risk of cardiovascular disease. This shares many of the same risk factors with endometrial cancer, including obesity and high blood pressure, both of which were improved when individuals with breast and colorectal cancer underwent intentional weight loss following treatment (Rock 2015). A previous Cochrane review concluded that physical activity may have a positive effect on quality of life in multiple different cancers, with reductions in anxiety, fatigue, sleep disturbance, and improved emotional wellbeing. These results should be interpreted cautiously, as included studies were at risk of considerable bias (Mishra 2012). In particular, there was a high risk of performance bias (significant differences between groups beyond simply which intervention they received), as due to the nature of the intervention (i.e. exercise), it was not possible to conceal the treatment allocation from the participants and researcher. A proportion of the included studies were also assessed to be at high risk of selectively reporting only some of the outcomes (reporting bias), failing to be transparent in their allocation of participants to treatment groups (allocation bias), and not managing incomplete outcome data appropriately (attrition bias). The differences in exercise regimens tested meant it was difficult to combine the results to give an overall conclusion.

Why it is important to do this review

The impact of obesity on women’s health has recently been highlighted in a number of high-profile publications, including the UK Chief Medical Officer’s report in December 2015 (Department of Health 2015), and the publication of the British Journal of Obstetrics and Gynaecology’s themed issue, Obesity and Reproductive Health, in January 2016 (Crosbie 2016). The impact of lifestyle changes, including weight loss, on outcomes following treatment for endometrial cancer was also identified as one of the top 10 research priorities in endometrial cancer in the recent James Lind and Womb Cancer Alliance Priority Setting Partnership (Wan 2016). Therefore, this review is timely in its aim to establish the availability of evidence about the effects of weight-loss interventions on survival and quality of life following treatment for endometrial cancer. There have been no previous Cochrane reviews of this topic, and such information will set the scene for high-quality research to assess the feasibility, effectiveness, and cost-effectiveness of such interventions.

OBJECTIVES

To determine the impact of weight-loss interventions, in addition to standard management of endometrial cancer, on overall survival and the frequency of adverse events.

Secondary objectives include an assessment of weight-loss interventions on endometrial cancer-specific survival, weight loss achieved, cardiovascular event frequency and quality of life, both overall and stratified according to body mass index (BMI) and tumour characteristics, where possible.

METHODS

Criteria for considering studies for this review

Types of studies

We included randomised controlled trials (RCTs), which are considered the highest level of evidence in clinical trials, to maximise the quality of included studies. We included studies reported as full text, those published as abstract only, and unpublished data, to ensure all relevant trials were incorporated.

Types of participants

We included trials that enrolled women of all ages, who were either overweight (BMI more than or equal to 25 kg/m²) or obese (BMI more than or equal to 30 kg/m²), and who were currently undergoing, or had been previously treated for endometrial cancer, of...
any grade, stage, or histological subtype. Trials were included regardless of primary treatment modality, i.e. surgery, radiotherapy, hormonal treatment, or a combination. When studies of participants with mixed BMI were identified but subgroup data were not provided, we contacted the study authors to request the subgroup data for overweight and obese participants only. If authors were unable or unwilling to provide these data, the study was not included in the meta-analysis.

Types of interventions

We included studies reporting on interventions designed to promote weight loss as one of their primary stated goals, in any healthcare setting, including community-based studies. These could include:

- lifestyle interventions, including dietary and physical activity regimens;
- behavioural strategies to improve adherence to treatment, which may include self-monitoring of eating habits and physical activity, stress management, or stimulus control (eliminating environmental cues associated with undesired eating);
- pharmacological interventions (such as, but not limited to, appetite suppressants, drugs that cause fat malabsorption or serotonin receptor antagonists (drugs that affect appetite) of any dose, route of delivery, or duration);
- surgical interventions (including gastric band, sleeve (surgical removal of part of the stomach), or bypass procedure).

Any of these interventions were compared with any other intervention, usual care, or placebo.

Types of outcome measures

Primary and secondary outcome measures were described in terms of the effect of the weight-loss intervention on survival, weight loss, cardiovascular events or quality of life, important measures that help determine whether these interventions should be included in routine clinical practice. Inclusion of these outcomes in the study design were not determinants of the eligibility of the trial for this review.

Primary outcomes

- Overall survival; determined as the time from randomisation until death from any cause
- Frequency of adverse events, of any nature

Secondary outcomes

- Recurrence-free survival; length of time from randomisation to recurrence of the disease or death
- Cancer-specific survival; length of time from randomisation to death from endometrial cancer
- Weight loss; amount of weight lost between randomisation and end of study
- Cardiovascular and metabolic event frequency; specifically the number of strokes, myocardial infarctions, and hospitalisations for heart failure
- Quality of Life as measured on any validated scale

Search methods for identification of studies

We imposed no language restrictions on our searches. Where necessary, we translated the reports.

Electronic searches

We searched the following electronic databases from inception to January 2018:

- Cochrane Central Register of Controlled Trials (CENTRAL, the Cochrane Library, 2017, Issue 12, Appendix 1);
- MEDLINE Ovid SP (1946 to January week 2 2018, Appendix 2);
- Embase Ovid SP (1980 to 2018 week 4, Appendix 3).

Searching other resources

We handsearched the citation lists of included studies and previous systematic reviews and contacted experts in the field to identify further reports of trials. Where additional information was required, we contacted the principal investigator of the trial.

Unpublished and grey literature

We searched the following for ongoing clinical trials.

- International Standard Randomised Controlled Trial Number (ISRCTN) - metaRegister of Controlled Trials (www.isrctn.com/)
- www.controlled-trials.com/rct
- www.clinicaltrials.gov
- PsycINFO

Handsearching

We also handsearched the reports of conferences in the following sources.

- Gynecologic Oncology (Annual Meeting of the American Society of Gynecologic Oncologist)
- International Journal of Gynecological Cancer (Annual Meeting of the International Gynecologic Cancer Society)
- British Journal of Cancer
- NCRI Cancer Conference
- Annual Meeting of European Society of Medical Oncology (ESMO)
• Annual Meeting of the American Society of Clinical Oncology (ASCO)

We searched for other conference abstracts and proceedings using ZETOC and WorldCat Dissertations.

**Data collection and analysis**

**Selection of studies**

We downloaded all titles and abstracts retrieved by electronic searching to a reference management database (EndNote) and removed duplicates. Two review authors (SK and NR) independently examined the remaining references. We excluded studies that clearly did not meet the inclusion criteria, and obtained full-text copies of potentially relevant references. Two review authors (SK and NR) independently assessed the eligibility of the retrieved reports and publications. We resolved any disagreement through discussion, or if required, we consulted a third person (MM). We identified and collated multiple reports of the same study so that each study, rather than each report, was the unit of interest in the review. We recorded the selection process in sufficient detail to complete a PRISMA flow diagram and Characteristics of included studies table (Liberati 2009).

**Data extraction and management**

Two review authors (SK and NR) independently extracted study characteristics and outcome data from included studies onto a pre-piloted data collection form. We noted in the Characteristics of included studies table if outcome data were not reported in a usable format. We resolved any disagreement through discussion, or if required, we consulted a third person (MM). One review author (SK) transferred data into the Review Manager file (RevMan 2014). We double-checked that data were entered correctly, by comparing the data in the RevMan file with the study reports. A second review author (MM) spot-checked study characteristics for accuracy against the trial report. In the case where an included study had more than one report, we collated the available data to ensure maximal information yield and gave priority to the publication with the longest follow-up associated with our review's primary and secondary outcomes. We extracted the following data.

- Author, year of publication, and journal citation (including language)
- Country
- Setting
- Inclusion and exclusion criteria
- Study design, methodology
- Study population (total number enrolled; baseline patient characteristics: age, co-morbidities (e.g. diabetes, cardiovascular disease); European Cooperative Oncology Group (ECOG) performance status; BMI; type of endometrial cancer; grade and stage of disease; timing of intervention in relation to treatment of endometrial cancer (i.e. before or after definitive treatment, nature of primary endometrial cancer treatment (e.g. surgery, radiotherapy, hormonal)).
- Intervention details (type of intervention; dose, route of administration; duration of treatment; additional information as appropriate)
- Comparison (nature of intervention; dose, route of administration; duration of treatment; additional information as appropriate)
- Risk of bias in study (see below)
- Duration of follow-up
- Outcomes: For each outcome, we extracted the outcome definition and unit of measurement (if relevant). For adjusted estimates, we recorded variables adjusted for in the analyses.
- Results: We extracted the number of participants allocated to each intervention group, the total number analysed for each outcome, and the missing participants.
- Notes: Funding for trial, and notable conflicts of interest of trial authors.

We extracted the results as follows.

- For time-to-event data (survival and disease progression), we extracted the log of the hazard ratio [log (HR)] and its standard error from trial reports. If these were not reported, we attempted to estimate the log (HR) and its standard error using the methods of Parmar 1998. If this were not possible for survival data, they were treated as dichotomous outcomes and the risk ratio was estimated.
- For dichotomous outcomes (e.g. adverse events, cardiovascular events or deaths), if it were not possible to calculate a hazard ratio, we estimated a risk ratio; we extracted the number of patients in each treatment arm who experienced the outcome of interest and the number of patients assessed at endpoint.
- For continuous outcomes (e.g. quality of life measures, weight loss), we extracted the mean and standard deviation of the outcome of interest and the number of patients assessed in each treatment arm at specific time points and used this to estimate the mean difference and its standard deviation.

If reported, we extracted both unadjusted and adjusted statistics. Where possible, we extracted data relevant to an intention-to-treat analysis, in which case participants were analysed in the groups to which they were assigned. We noted the time points at which outcomes were collected and reported.

**Assessment of risk of bias in included studies**

We assessed and reported on the methodological risk of bias of included studies in accordance with the Cochrane Handbook of Systematic Reviews of Interventions (Higgins 2011a), which recom-
mends the explicit reporting of the following individual elements for RCTs.

- Selection bias: random sequence generation and allocation concealment
- Performance bias: blinding of participants and personnel (patients and treatment providers)
- Detection bias: blinding of outcome assessment
- Attrition bias: incomplete outcome data
- Reporting bias: selective reporting of outcomes

Two review authors (SK and NR) independently applied the ‘Risk of bias’ criteria; we resolved differences by discussion, or by appealing to a third review author (MM). We checked clinical trial registries for a priori primary and secondary outcome measures to assess the risk of selective reporting. We judged each item as being at high, low, or unclear risk of bias, as set out in the criteria provided by Higgins 2011b and Higgins 2011a. We provided a quote from the study report and a statement to justify the judgement for each criteria. We summarised results in both a graph and a narrative summary. When interpreting treatment effects and meta-analyses, we took into account the risk of bias for the studies that contributed to that outcome. Where information on risk of bias related to unpublished data or correspondence with a trialist, we noted this in the ‘Risk of bias’ table.

Measures of treatment effect

We used the following measures of the effect of treatment.

- For time-to-event data, we used the hazard ratio (HR), if possible. Where this was not the case, the data were treated as a dichotomous outcome and the risk ratio (RR) was estimated using the Mantel-Haenszel method.
- For dichotomous outcomes, we analysed data based on the number of events and the number of people assessed in the intervention and comparison groups. We used these to calculate the RR and 95% confidence interval (CI) using the Mantel-Haenszel method.
- For continuous outcomes, we analysed data based on the mean, standard deviation (SD), and number of people assessed for both the intervention and comparison groups, to calculate mean difference (MD) between treatment arms with a 95% CI. If the MD was reported without individual group data, we used this to report the study results. If more than one study measured the same outcome using different tools, we planned to calculate the standardised mean difference (SMD) and 95% CI using the inverse variance method in RevMan 2014.

We undertook meta-analyses only where this was meaningful, i.e. if the treatments, participants, and the underlying clinical question were similar enough for pooling to be appropriate. We described skewed data reported as medians and interquartile ranges. Where multiple trial arms were reported in a single trial, we included only the relevant arms and divided the ‘shared’ comparison group equally between the number of treatment groups, to avoid ‘double-counting’.

Unit of analysis issues

The unit of analysis was the participant. If any trials had multiple treatment groups, we combined similar intervention arms and control arms together in order to create single pair-wise comparisons.

Dealing with missing data

We attempted to contact study authors to obtain missing data (participant, outcome, or summary data). Where possible, we conducted analysis of participant data on an intention-to-treat basis; otherwise, we analysed data as reported. We reported on the levels of loss to follow-up, and assessed this as a source of potential bias. We did not impute missing outcome data.

Assessment of heterogeneity

Where we considered studies similar enough (based on participants, intervention, comparison, settings and outcome measures) to pool the data using meta-analysis, we assessed the degree of heterogeneity by visually inspecting forest plots, by estimating the percentage of heterogeneity (I² statistic) between trials that cannot be ascribed to sampling variation (Higgins 2003), by formally testing the significance of the heterogeneity (Chi² statistic; Deeks 2001), and if possible, by conducting subgroup analyses. We used these I² statistic levels as a rough guide to assess heterogeneity as:

- 0% to 40%; might not be important;
- 30% to 60%; may represent moderate heterogeneity;
- 50% to 90%; may represent substantial heterogeneity;
- > 75%; considerable heterogeneity.

We evaluated the value of the I² statistic alongside the magnitude and direction of effects, and the P value for the Chi² test (Higgins 2011). If there was evidence of substantial clinical, methodological, or statistical heterogeneity across included studies, we did not report pooled results from the meta-analysis, but instead used a narrative approach to data synthesis. In this event, we investigated and reported the possible clinical or methodological reasons for this.

Assessment of reporting biases

We aimed to minimise reporting bias by systematically searching for all eligible studies, including unpublished data and ongoing clinical trials, and by not including any language restrictions. Updates of this review will deal with any time lag bias. Had we included 10 or more studies that investigated a particular outcome, we planned to examine funnel plots that correspond to the meta-analysis of the outcome to assess the potential for small-study effects, such as publication bias. We planned to visually
assess funnel plot asymmetry; if asymmetry was suggested by a visual assessment, we planned to perform exploratory analyses to investigate it.

Data synthesis
If sufficient, clinically similar studies (in terms of participants, intervention, comparison, settings and outcome measures) were available to ensure meaningful conclusions, we pooled their results in meta-analyses using the random-effects model in RevMan. Given the number of possible interventions that could have been included in the incorporated studies, we only planned to perform the following meaningful comparisons.

- Lifestyle interventions in addition to usual care versus usual care
- Behavioural interventions in addition to usual care versus usual care
- Pharmacological interventions in addition to usual care versus usual care
- Surgical interventions in addition to usual care versus usual care
- Lifestyle interventions versus behavioural interventions
- Lifestyle interventions versus pharmacological interventions
- Lifestyle interventions versus surgical interventions
- Behavioural interventions versus pharmacological interventions
- Behavioural interventions versus surgical interventions
- Pharmacological intervention versus surgical interventions.

The specific method for pooling data depended upon the nature of the outcome measure. If we were unable to pool the data statistically using meta-analysis, we conducted a narrative synthesis of results. We presented the major outcomes and results, organised by intervention categories, according to the major types or aims of the identified interventions.

'Summary of findings' table
We assessed and reported the quality of the evidence for each outcome, using the GRADE approach and these domains: study limitations (suggesting a high likelihood of bias), inconsistency (unexplained heterogeneity), imprecision (wide confidence intervals), indirectness of evidence, and publication bias. We created a 'Summary of findings' table, using GRADEpro GDT software (GRADEpro GDT), and two review authors (SK and NR) independently assessed the quality of the evidence, using Chapter 12.2 of the Cochrane Handbook of Systematic Reviews of Interventions as a guide (Schünemann 2011). We used a checklist to maximise consistent GRADE decisions, and the GRADE Working Group quality of evidence definitions (Meader 2014). We downgraded the evidence from high quality by one level for serious limitations (or by two for very serious limitations) for each outcome, and outlined our rationale in the footnotes.

- High quality: We are very confident that the true effect lies close to that of the estimate of the effect.
- Moderate quality: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
- Low quality: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect.
- Very low quality: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect.

We included the following outcomes in the 'Summary of findings' table.

- Overall survival
- Adverse events
- Recurrence-free survival
- Cancer-specific survival
- Weight loss
- Cardiovascular and metabolic event frequency
- Quality of life

If meta-analyses had not been possible, we planned to present results in a narrative 'Summary of findings' table format, such as that used in the Cochrane review Chan 2011.

Subgroup analysis and investigation of heterogeneity
We performed subgroup analyses for the following factors, where possible.

- BMI
- Histological type, stage, and grade of endometrial cancer

Sensitivity analysis
If adequate data were available, we planned to perform a sensitivity analysis comparing studies with high and unclear risk of bias and low risk of bias for attrition and outcome reporting, and allocation concealment (the latter is relevant only to pharmacological interventions).

 RESULTS

Description of studies

Results of the search
The electronic search retrieved 873 records. Thirty references were potentially eligible and were retrieved as full-text articles. Three
studies (five references) met the inclusion criteria and five studies were ongoing. Please see study tables: Characteristics of included studies; Characteristics of excluded studies and Characteristics of ongoing studies and the PRISMA flow chart (Figure 1).
Figure 1. Study flow diagram.

871 records identified through database searching

2 additional records identified through other sources

21 duplicate records removed

852 records screened

822 of records excluded

25 full-text articles excluded:
10 duplicates
6 wrong study design (systematic reviews)
3 wrong indication
1 wrong patient population
5 ongoing studies

30 full-text articles assessed for eligibility

3 studies (5 refs) included in qualitative synthesis

3 studies (5 refs) included in quantitative synthesis (meta-analysis)
Included studies

Study design and setting
Three randomised controlled trials (RCTs) were included in the review. Two RCTs were conducted in a single centre (McCarroll 2014; von Gruenigen 2009) and one was a multi-centre trial (Allison 2016). All trials were conducted in university hospitals in the USA (Allison 2016; McCarroll 2014; von Gruenigen 2009).

Participants
Included trials randomised 161 overweight and obese female participants previously treated for endometrial cancer and with good performance status (0-2, a way of quantifying the general wellbeing and physical activity levels of cancer patients) (Allison 2016; McCarroll 2014; von Gruenigen 2009). The mean age of participants ranged from 54 years (von Gruenigen 2009) to 62 years (Allison 2016). Two RCTs included only patients with stage I or II disease (McCarroll 2014; von Gruenigen 2009). The other RCT did not provide details of the stage of disease of participants (Allison 2016). All patients underwent surgery as the primary treatment of their endometrial cancer (Allison 2016; McCarroll 2014; von Gruenigen 2009). In addition, one RCT included participants who had also received adjuvant brachytherapy, radiotherapy, or chemotherapy (McCarroll 2014). One RCT specifically excluded patients who had received, or were due to receive adjuvant treatment (Allison 2016), whilst the other trial did not provide details of radio- and chemotherapy exposure (von Gruenigen 2009).

Interventions
All studies compared combined behavioural and lifestyle interventions to facilitate weight loss through dietary modification and increased physical activity, with usual care. Two RCTs utilised a two-arm design, comparing one intervention with usual care (McCarroll 2014; von Gruenigen 2009). One RCT had a three-arm design, comparing two types of lifestyle interventions with usual care (Allison 2016). Counselling was provided either on an individual basis by telephone or text (Allison 2016) or a combination of face-to-face group and individual sessions (McCarroll 2014; von Gruenigen 2009).

Primary outcome

Overall survival

• 3/3 RCTs reported overall survival, defined as the number of deaths occurring during follow-up (Allison 2016; McCarroll 2014; von Gruenigen 2009).

Adverse events

• 2/3 RCTs reported adverse events, defined as any undesirable symptom or sign occurring after the study had commenced, even if not thought to be directly related to the intervention (McCarroll 2014; von Gruenigen 2009). These were reported as two separate categories; mild to moderate adverse reactions and life-threatening adverse reactions.

Secondary outcome

Recurrence-free survival

• No trials reported recurrence-free survival

Cancer-specific survival

• 3/3 RCTs reported cancer-specific survival, defined as the number of deaths secondary to endometrial cancer occurring during follow-up (Allison 2016; McCarroll 2014; von Gruenigen 2009).

Weight loss

• 3/3 RCTs reported change in weight from baseline, measured in kilograms (Allison 2016; McCarroll 2014; von Gruenigen 2009).

Cardiovascular and metabolic event frequency

• 3/3 RCTs reported cardiovascular events, defined as the number of myocardial infarctions, strokes, and hospitalisations for heart failure occurring during follow-up (Allison 2016; McCarroll 2014; von Gruenigen 2009).

Quality of life

• 3/3 RCTs reported change in quality of life score from baseline (Allison 2016; McCarroll 2014; von Gruenigen 2009). Quality of life was measured by four different instruments.
  • 1/3 RCTs used SF-12 Physical Health questionnaire (Allison 2016).
  • 2/3 RCTs used Functional Assessment of Cancer Therapy-General (FACT-G) (McCarroll 2014; von Gruenigen 2009).
We contacted the principal investigator of each of the included RCTs for unpublished data where it was felt to be important to the results of the review. Full and detailed responses were obtained from the study authors (Table 1).

**Excluded studies**

Ten full-text articles were excluded from the review for the following reasons.

- 6/10 full-text articles were systematic reviews (Babatunde 2016; Fasching 2009; Gil 2007; Koutoukidis 2015; Lin 2016; Smits 2015).
- 1/10 RCTs included a different patient population, enrolling patients with breast and colon cancer and only one patient with endometrial cancer (Beck 2015).
- 3/10 RCTs were for the wrong indication. One incorporated a physical activity-based intervention for the treatment of cancer-related fatigue rather than weight loss (Donnelly 2011), the primary aim of another was to study the effect of a diet and physical activity intervention on quality of life (Koutoukidis 2017) and another assessed the feasibility and effectiveness of physical activity and changes in self-efficacy, outcome expectation and self-regulation (Rossi 2016).

**Risk of bias in included studies**

Please refer to Characteristics of included studies; Figure 2; Figure 3

![Risk of bias graph](image-url)
Figure 3. ‘Risk of bias’ summary: review authors’ judgements about each risk of bias item for each included study.
Allocation
All RCTs were at low risk of selection bias related to random sequence generation (Allison 2016; McCarroll 2014; von Gruenigen 2009). One RCT used computer-generated randomisation (Allison 2016). The other two RCTs used block randomisation methods, stratifying patients according to baseline BMI (McCarroll 2014; von Gruenigen 2009).

One RCT was at low risk of selection bias related to allocation concealment as they used appropriate methods of sequentially numbered envelopes (Allison 2016). Two RCTs were at unclear risk of bias for allocation concealment as they did not describe the methods used (McCarroll 2014; von Gruenigen 2009).

Blinding
All RCTs were at high risk of performance bias related to blinding of participants and personnel (Allison 2016; McCarroll 2014; von Gruenigen 2009). Due to the nature of the intervention (either group or individual counselling sessions regarding weight loss and physical activity or usual care involving no additional counselling or generic health advice only), it was not possible to blind participants and the research team to group allocation.

It would, however, be possible to blind outcome assessors for all primary and secondary outcomes, thereby reducing the risk of detection bias. All RCTs were at high risk of detection bias as they used unblinded members of the research team to measure all outcomes (Allison 2016; McCarroll 2014; von Gruenigen 2009). We considered that blinding was unlikely to affect the findings for the primary outcomes of overall survival and adverse events, nor the secondary outcomes of recurrence-free and cancer-specific survival, weight loss and cardiovascular event frequency, but that it may affect quality of life assessments.

Incomplete outcome data
One RCT was considered at low risk for attrition bias as they had no withdrawals from the study and no missing data (Allison 2016). The other two RCTs were considered to be at high risk for attrition bias as they had a participant withdrawal and missing data rate more than 10% (McCarroll 2014; von Gruenigen 2009). McCarroll 2014 had a withdrawal rate of 16/75 (21.3%) and von Gruenigen 2009 had a withdrawal rate of 7/45 (15.6%) and missing data for an additional 2/22 (9.1%) of participants in the control arm.

Selective reporting
None of the three RCTs published their protocols prospectively but all were registered prior to commencement of recruitment on clinicaltrials.gov and reported all of their prespecified outcomes (Allison 2016; McCarroll 2014; von Gruenigen 2009). These were, therefore, deemed at low risk of reporting bias.

Other potential sources of bias
No studies reported significant differences in baseline characteristics between their intervention and control groups. Only 30/41 (73.2%) of participants in one RCT had completed their outcome assessments at the time of correspondence with the study authors for this review (Allison 2016). Additional data will be available for future updates of the review. An additional source of bias was identified in one RCT where two participants in the intervention arm underwent gastric bypass during follow-up and continued to be included in the final analysis (von Gruenigen 2009). There were insufficient studies investigating each outcome to construct a funnel plot to assess for publication bias.

Effects of interventions
See: Summary of findings for the main comparison

1. Lifestyle intervention compared with usual care
   All three RCTs compared combined lifestyle and behavioural interventions with usual care (Allison 2016; McCarroll 2014; von Gruenigen 2009).

Primary outcomes

1. Overall survival (six, 12 and 24 months)
   Insufficient data were available to calculate the effect of combined lifestyle and behavioural interventions on overall survival using the hazard ratio. Instead, mortality was treated as a dichotomous outcome and the risk ratio (RR) determined.
   There was no evidence that a combined lifestyle and behavioural intervention, incorporating dietary and physical activity advice with self-monitoring and stimulus control techniques, was associated with an improvement in overall survival at six months as no deaths were observed in the intervention or usual care groups of the two studies that reported this outcome (Analysis 1.1) (Allison 2016; McCarroll 2014). A risk ratio could not, therefore, be calculated and a meta-analysis could not be performed. Neither sensitivity nor subgroup analyses were possible.
   There was no evidence that lifestyle and behavioural interventions were associated with an improvement in overall survival at 12 months as no deaths were observed in either the intervention or...
usual care groups of the one study that reported this outcome (Analysis 1.2) (McCarroll 2014). A risk ratio could not, therefore, be calculated. Sensitivity and subgroup analyses were not possible. Lifestyle and behavioural interventions were not associated with an improvement in overall survival at 24 months (RR (mortality) 0.23, 95% confidence interval (CI) 0.01 to 4.55, P = 0.34, one RCT, 37 participants, very low-certainty evidence) (Analysis 1.3; Figure 4) (von Gruenigen 2009). Two deaths occurred in the control arm. Sensitivity and subgroup analyses were not possible.

Figure 4. Forest plot of comparison: 1 Lifestyle intervention versus. usual care, outcome: 1.3 Overall survival (24 months).

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Experimental Events</th>
<th>Control Events</th>
<th>Weight</th>
<th>Risk Ratio M-H, Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Gruenigen 2008</td>
<td>0</td>
<td>17</td>
<td>2</td>
<td>0.23 [0.01, 4.55]</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>17</td>
<td>20</td>
<td>100.0%</td>
<td>0.23 [0.04, 4.55]</td>
</tr>
<tr>
<td>Total events</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Not applicable</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect Z = 0.96 (P = 0.34)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Adverse events

Mild to moderate adverse events

One RCT reported no mild to moderate adverse events related to the study intervention (von Gruenigen 2009).

One RCT (McCarroll 2014) reported 13 musculoskeletal events in 10 participants in the intervention group, including knee and leg pain and muscle weakness, which were felt to be possibly related to the study intervention. Participants receiving combined lifestyle and behavioural interventions had a higher risk of musculoskeletal events than those receiving usual care (RR 19.03, 95% CI 1.17 to 310.52, P = 0.04, two RCTs, 91 participants, low-certainty evidence) (Analysis 1.4; Figure 5) (McCarroll 2014; von Gruenigen 2009).
Two participants in the study by McCarroll 2014 also reported episodes of diarrhoea, which were felt to be possibly related to the study intervention. Lifestyle and behavioural interventions were not associated with an increased risk of diarrhoea (RR 4.53, 95% CI 0.23 to 90.51, P = 0.32, two RCTs, 91 participants, low-certainty evidence) (Analysis 1.14) (McCarroll 2014; von Gruenigen 2009).

Life-threatening adverse events

No life-threatening adverse events related to the study intervention were reported in any of the RCTs.

Secondary outcomes

1. Recurrence-free survival

No RCTs reported this outcome.

2. Cancer-specific survival (six, 12 and 24 months)

There was no evidence that combined lifestyle and behavioural interventions were associated with an improvement in cancer-specific survival at 6 months as no cancer-specific deaths were reported (Analysis 1.5) (Allison 2016; McCarroll 2014). A risk ratio could not, therefore, be calculated and a meta-analysis could not be performed. No sensitivity or subgroup analyses were possible.

There was no evidence that combined lifestyle and behavioural interventions were associated with an improvement in cancer-specific survival at 12 months as no deaths were reported in either group in the one study reporting this outcome (Analysis 1.6) (McCarroll 2014). A risk ratio could not, therefore, be calculated. No sensitivity or subgroup analyses were possible.

There was no evidence that combined lifestyle and behavioural interventions were associated with an improvement in cancer-specific survival at 24 months as no cancer-specific deaths were reported (Analysis 1.7) (von Gruenigen 2009). A risk ratio could not, therefore, be calculated. No sensitivity or subgroup analyses were possible.

3. Weight loss (six, 12 and 24 months)

Combined lifestyle and behavioural intervention was not associated with weight loss at six months compared to usual care (mean difference (MD) -1.88 kg, 95% CI -5.98 to 2.21, P = 0.37, three RCTs, 131 participants, I² = 0%, very low-certainty evidence) (Analysis 1.8) (Allison 2016; McCarroll 2014; von Gruenigen 2009). Subgroup analysis according to baseline BMI was performed and did not affect the result (Analysis 1.9) (McCarroll 2014; von Gruenigen 2009). Insufficient data were available to perform a subgroup analyses according to histological type, stage and grade of endometrial cancer. No sensitivity analyses were possible.

Lifestyle and behavioural intervention was not associated with weight loss at 12 months compared to usual care (MD -8.98 kg, 95% CI -19.88 to 1.92, P = 0.11, two RCTs, 91 participants, I² = 0%, very low-certainty evidence) (Analysis 1.10). Although some individuals lost a lot of weight, most of the participants lost none or very little, which is why this result was not statistically significant. Subgroup analysis demonstrated no effect of baseline BMI on weight loss following the intervention (Analysis 1.11) (McCarroll 2014; von Gruenigen 2009). No sensitivity analysis was possible.

Overall, a lifestyle and behavioural intervention was not associated with weight loss at 24 months compared with usual care (MD -18.26 kg, 95% CI -38.73 to 2.21, P = 0.08, one RCT, 25 participants, very low-certainty evidence) (Analysis 1.12) (von Gruenigen 2009). There was no evidence that combined lifestyle and behavioural interventions were associated with an improvement in cancer-specific survival at 24 months as no cancer-specific deaths were reported (Analysis 1.7) (von Gruenigen 2009). A risk ratio could not, therefore, be calculated. No sensitivity or subgroup analyses were possible.
Subgroup analysis demonstrated significant differences in amount of weight lost according to baseline BMI (Chi^2 = 10.10, df = 1, P = 0.001). Participants with a BMI < 40 kg/m^2 did not achieve greater weight loss following the intervention compared with those receiving usual care at 24 months (MD 2.12 kg, 95% CI -20.82 to 25.06, P = 0.86, one RCT, 13 participants, very low-certainty evidence) (von Gruenigen 2009). Participants with a BMI greater than or equal to 40 kg/m^2 who received the intervention, however, did achieve greater weight loss at 24 months than those receiving usual care (MD -54.58 kg, 95% CI -80.97 to -28.19, P < 0.0001, one RCT, 12 participants, very low-certainty evidence) (von Gruenigen 2009).

Participants with a BMI greater than or equal to 40 kg/m^2 who received the intervention, however, did achieve greater weight loss at 24 months than those receiving usual care (MD -54.58 kg, 95% CI -80.97 to -28.19, P < 0.0001, one RCT, 12 participants, very low-certainty evidence) (von Gruenigen 2009).

Participants with a BMI greater than or equal to 40 kg/m^2 who received the intervention, however, did achieve greater weight loss at 24 months than those receiving usual care (MD -54.58 kg, 95% CI -80.97 to -28.19, P < 0.0001, one RCT, 12 participants, very low-certainty evidence) (von Gruenigen 2009). Participants with a BMI greater than or equal to 40 kg/m^2 who underwent bariatric surgery during follow-up and lost a large amount of weight as a consequence. No sensitivity analysis was possible.

4. Cardiovascular and metabolic event frequency (six and 12 months)

No cardiovascular or metabolic events were reported at six and 12 months. (Analysis 1.15; Analysis 1.16)

5. Quality of life (six and 12 months)

Six months

**SF-12 Physical Health questionnaire**

Combined lifestyle and behavioural intervention was not associated with improvement in quality of life at six months compared with usual care when measured using the SF-12 Physical Health questionnaire (MD -2.29, 95% CI -7.34 to 2.76, P = 0.37, one RCT, 30 participants, moderate-certainty evidence) (Allison 2016).

Twelve months

The effect of lifestyle and behavioural intervention on quality of life was measured at 12 months by two RCTs, both of which used the FACT-G questionnaire (McCarroll 2014; von Gruenigen 2009). Lifestyle and behavioural intervention was not associated with improvement in quality of life at 12 months (MD -2.77, 95% CI -0.65 to 6.20, P = 0.11, 89 participants, I^2=0%, very low-certainty evidence) (Analysis 1.20) (McCarroll 2014; von Gruenigen 2009). The QoL response to the intervention did not differ according to baseline BMI in a subgroup analysis (Analysis 1.21). A sensitivity analysis was not possible.
DISCUSSION

Summary of main results

The limited evidence suggests that combined lifestyle and behavioural interventions had no effect on overall survival. There was no evidence that combined lifestyle and behavioural interventions affected cancer-specific or recurrence-free survival or reduced the number of cardiovascular and metabolic events in endometrial cancer survivors over a 12 month follow-up period as either no events were recorded in the studies or the outcome was not reported. Dietary and physical activity advice, in combination with behavioural strategies to improve compliance, are not associated with significant weight loss or improvement in quality of life for women with a history of endometrial cancer over a similar follow-up period, when compared with those receiving usual care. Body mass index (BMI) at baseline did not affect these results. These results should be viewed with caution, however, as only three randomised controlled trials (RCTs) met the eligibility criteria for inclusion in this review, all of which were small in size and meant that no events were recorded for many of these outcomes. At 24 months, super-obese participants (BMI greater than or equal to 40 kg/m²) in one RCT (von Gruenigen 2009) lost significantly more weight than those receiving usual care. However, there were biases in the design of this study, namely the inclusion of participants who underwent gastric bypass surgery during follow-up. Despite a lack of benefit with regards to the outcomes included in this review, lifestyle and behavioural interventions to induce weight loss in endometrial cancer survivors were associated with a significant risk of musculoskeletal side effects, though the low event numbers make relative risk estimates unreliable and none of the adverse events recorded were considered serious or life-threatening.

The 'Summary of Findings' table summarises the main outcomes (Summary of findings for the main comparison).

Overall completeness and applicability of evidence

The evidence for each of the outcomes was limited as only three studies met the inclusion criteria and each had enrolled small numbers of participants. Two of the included studies were undertaken by the same study authors recruiting from the same hospital and pool of endometrial cancer survivors and were carried out as a pilot study (von Gruenigen 2009), followed by a definitive RCT using similar methodology (McCarroll 2014). This is likely to impact on the applicability of their findings to other populations.

All of the included studies were at high risk for performance bias, as due to the nature of the interventions, they were unable to blind participants and personnel to treatment group allocation. The RCTs were also at high risk for detection bias due to the use of unblinded outcome assessors. Whilst this is unlikely to have affected objective outcomes, such as weight loss and survival, it may have impacted on more subjective outcomes, such as quality of life. The use of independent, blind outcome assessors in future studies would remove this potential source of bias.

Two different questionnaires were used to measure quality of life in the three studies included in this review. The results presented in the 'Summary of findings' table are based on use of the FACT-G (Functional Assessment of Cancer Therapy-General) questionnaire as this was used by two studies and, hence, pools the individuals results from the greatest number of participants. These findings were considered, however, to be based on very low-certainty evidence due to the risk of bias in the included studies. The study using the SF-12 Physical Health Component questionnaire, whilst providing evidence of greater certainty, was based on a small number of participants and considered different aspects of quality of life, preventing pooling in the meta-analysis. The overall findings of all three studies were, however, similar, with no significant improvement in quality of life found at six months following weight-loss interventions. In order to improve the quality of evidence and to allow future meta-analyses of the effect of weight-loss interventions on quality of life to be conducted, it would be advisable for all studies going forward to use a common quality of life assessment tool.

While the study authors were able to provide additional data on the outcome measures included in this review, overall and cancer-specific survival and cardiovascular and metabolic event frequency were not specific outcomes of these studies. This explains the paucity of data provided, which were insufficient to allow the calculation of hazard ratios for these outcomes. The short duration of the intervention (six months) and limited follow-up time of the included RCTs, which was between six and 24 months, explains why so few deaths and cardiovascular and metabolic events were recorded by the study authors. Any conclusions with regards the effect of lifestyle and behavioural interventions on survival should, therefore, be made with caution. For weight-loss interventions to be shown to impact on survival for women with a history of endometrial cancer, the duration of both the intervention and follow-up period will need to be considerably longer (five to 10 years).

The only studies that met the inclusion criteria for this review had focused solely on lifestyle and behavioural strategies. There were no studies of pharmacological or surgical interventions, which are likely to be more effective than diet and physical activity advice in achieving significant sustained weight loss and hence impacting on the outcomes measured in this review (Bray 2016). Randomised controlled trials comparing these interventions with placebo/usual care are, therefore, required.

There were limited data available about the baseline characteristics of participants in the included studies, in particular with regards to their baseline BMI and histological type, stage and grade of endometrial cancer, which restricted the number of subgroup analyses that could be conducted. This information is vital to investigate whether all endometrial cancer survivors derive a similar
benefit from weight-loss interventions or whether efforts should be targeted at specific subpopulations, such as those with the greatest BMI. Adequately powered studies including participants with both early and late stage, endometrioid and non-endometrioid endometrial cancer are required to explore these issues further.

Quality of the evidence
There were only three RCTs that met the inclusion criteria for the review, meaning that a meta-analysis could rarely be performed. The small number of studies also meant that assessment of the heterogeneity between studies is unlikely to be reliable, particularly with regard to dichotomous outcomes. Ideally, the calculation of confidence intervals for I² and sensitivity analyses would have been performed, but neither were possible in RevMan.

Using the GRADE method of assessment, the certainty of the evidence for all outcomes was either low or very low, meaning that our confidence in the effect estimate was limited or very limited and that the true effect may, or is likely to, be substantially different from the estimate of effect. The reasons for downgrading certainty of the evidence included serious and very serious risk of bias in the primary studies (for example, unblinded participants, study personnel and outcome assessors, significant, unexplained, loss of participants to follow-up), imprecision due to small-study sizes and the risk of introducing an indirect comparison. The latter applied particularly to the study with the longest follow-up period of 24 months (von Gruenigen 2009), which was the only one to show an effect of lifestyle and behavioural interventions on weight loss. The fact that this was only observed at 24 months and not at six or 12 months, despite the intervention being limited to six months duration, is noteworthy, especially as the study was not originally planned to follow participants beyond 12 months and that, by this point, of the 25 participants remaining, two had undergone gastric bypass and continued to be included in the final analysis.

Potential biases in the review process
The search strategy was overseen by the Cochrane Gynaecological, Neuro-oncology and Orphan Cancer group to reduce the risk of introducing bias into the review process. No limitations with regards to language or date of publication were applied and deliberate efforts were made to search for ongoing clinical trials. Additional unpublished data were gained through correspondence with study authors and were included in the review. Decisions regarding the eligibility of studies for inclusion, ‘Risk of bias’ assessment, data collection and grading of evidence were performed by two review authors independently, with disagreements settled by a third review author. The main bias relates to the small number of included studies, all of which had only limited participant numbers and were of low or very low methodological quality, which meant that it was frequently not possible to conduct a meta-analysis and prevented the drawing of firm conclusions regarding the clinical effectiveness of the intervention. It also meant that it was not possible to assess for publication bias. No conflicts of interest were identified for any of the study authors.

Agreements and disagreements with other studies or reviews
Despite increasing awareness of the need to improve survival and quality of life in women with a history of endometrial cancer, there is little published literature evaluating weight-loss interventions in this regard. Of the four systematic reviews previously conducted, three have included at least some of the data from three of the studies incorporated here (Allison 2016; McCarroll 2014; von Gruenigen 2009), though do not appear to have had the same access to unpublished data as this review’s authors. Chlebowski 2016 described the results of the SUCCEED trial (McCarroll 2014) and preliminary findings from the study by Allison 2016 on weight loss and quality of life, but did not attempt a meta-analysis. Where a meta-analysis has been performed, the results have been similar to those reported here. Lin 2016 focused on the effect of interventions to increase physical activity, but noted that only one study used an exercise intervention alone without combining it with some form of lifestyle/dietary modification. They found no benefit of these interventions on health-related quality of life (standardised mean difference (SMD) 0.05, 95% CI -0.28 to 0.37, P = 0.78), though there were significant improvements in BMI compared with those receiving usual care. The authors included studies conducted in survivors of all gynaecological malignancies and did not attempt to evaluate the effects of physical activity in specific cancer subtypes. There was also substantial methodological heterogeneity between RCTs, which had widely differing physical activity regimens, ranging from residential rehabilitation courses comprising physical activity education to pelvic floor exercises, which was not investigated further in their analysis. When the eligibility criteria for included studies was extended to non randomised trials the results were again similar, with no improvement seen in quality of life at three and six months (Smits 2015). A fourth systematic review included only epidemiological studies, two single-arm intervention studies and five cross-sectional studies of physical activity, and concluded that increased exercise could contribute to better quality of life in endometrial cancer survivors (Babarunde 2016). They did not, however, conduct a meta-analysis and had undertaken only a limited search of the literature. No other individual RCT or review to date has evaluated the role of weight-loss interventions in improving survival for women with endometrial cancer. The only evidence available showed a trend towards increased mortality with greater levels of television viewing, as a surrogate marker of inactivity, in women recruited into the NIH-AARP Diet and Health Study and who had developed endometrial cancer during long-term follow-up, though this result

Interventions for weight reduction in obesity to improve survival in women with endometrial cancer (Review)
was not significant (Arem 2016). There was no association between self-reported activity levels following diagnosis and overall survival and unfortunately the study was underpowered to look specifically at cardiovascular and cancer-related deaths. An adequately powered RCT incorporating survival outcomes is, therefore, required to address this question.

**Authors’ Conclusions**

**Implications for practice**

There is limited evidence available regarding the efficacy of weight-loss interventions in improving survival and reducing cardiovascular and metabolic event frequency in endometrial cancer survivors. There is very low-certainty evidence that combined lifestyle and behaviour interventions are not associated with significant weight loss at 12 months and that there is no improvement in quality of life compared to those receiving usual care. The small number and size of the included randomised controlled trials (RCTs) in this review mean that any effect size estimates should be viewed with caution, however. Whilst demonstration of a significant benefit from receiving diet and physical activity education has not been possible, the low-certainty evidence available suggests that it may not be associated with significant or serious adverse events, apart from an increase in musculoskeletal symptoms, and could easily be incorporated into routine follow-up reviews at low cost.

**Implications for research**

Further trials are required to specifically address the effects of weight-loss interventions on overall, cancer-specific and recurrence-free survival and to compare different dietary modification regimens, including intermittent fasting versus continuous low-calorie diets, pharmacological treatments associated with weight loss, such as orlistat and metformin, and bariatric surgery, all of which may be more effective in achieving and sustaining significant weight loss and hence impacting upon these outcomes. Bariatric surgery, in particular, has already been shown to result in greater weight loss than non-surgical weight management, which is maintained in the longer term, and leads to the resolution of diabetes, reducing overall and cardiovascular-caused mortality as well as improving some aspects of quality of life in non-cancer patients (Arterburn 2015; Colquitt 2014). It would be anticipated that women treated for endometrial cancer would derive similar benefits from undergoing weight-loss surgery, though whether they would also notice improvements in cancer-caused mortality is currently unknown. Any future trials in this area should be of high methodological quality, adequately powered and with at least five years of follow-up to allow time for the impact of these interventions on survival to be determined. Larger trials would also allow the relative benefit of weight-loss interventions on specific subgroups of endometrial cancer survivors, such as the super-obese and those diagnosed with early and late stage disease, to be evaluated.

Of the five ongoing RCTs that could not be included in this version of the review, four will not address any of these issues as they involve randomisation to different lifestyle and/or behavioural interventions or usual care and do not include survival in their outcome measures (Bantum 2015; Basen-Engquist 2016; Nock 2011; Yeh 2015). The exception to this is the RCT Hawkes 2014, in which participants with early stage endometrioid endometrial cancer are being randomised to a levonorgesterol-intrauterine device alone or in combination with either metformin or a subscription to a weight-loss programme at a ratio of 3:3:5. Whilst the follow-up period is only of six months duration, the primary outcome of the study is absence of endometrial cancer or atypical endometrial hyperplasia at this time point. This will allow the effect of metformin on short-term recurrence-free survival to be evaluated.

**Acknowledgements**

We would like to thank Jo Morrison (Co-ordinating Editor) for clinical and editorial advice, Jo Platt (Information Specialist) for designing the search strategy and Gail Quinn, Clare Jess, and Tracey Harrison (Managing and Assistant Managing Editors), for their contribution to the editorial process.

Dr Emma Crosbie was awarded funding via the Cochrane Review Support Programme to expedite the completion of this review which is a priority topic area.

This project was supported by the National Institute for Health Research (NIHR), via Cochrane Infrastructure funding to the Cochrane Gynaecological, Neuro-oncology and Orphan Cancer Group. The views and opinions expressed therein are those of the authors and do not necessarily reflect those of the Systematic Reviews Programme, NIHR, National Health Service (NHS) or the Department of Health.

We would like to thank the referees for many helpful suggestions and comments, some of these include Rebecca Beeken, Evangelos Kontopantelis and Katharine Tylko-Hill.
References to studies included in this review

Allison 2016 [published and unpublished data]

McCarroll 2014 [published and unpublished data]

von Gruenigen 2009 [published and unpublished data]

von Gruenigen 2009 [published and unpublished data]

References to studies excluded from this review

Babatunde 2016 [published data only]

Beck 2015 [published data only]

Donnelly 2011 [published data only]

Fasching 2009 [published data only]

Gil 2007 [published data only]

Koutoukidis 2015 [published and unpublished data]

Koutoukidis 2017 [published and unpublished data]

Lin 2016 [published data only]

Rossi 2016 [published data only]

Smits 2015 [published data only]

References to ongoing studies

Bantum 2015 [published data only (unpublished sought but not used)]

Basen-Engquist 2016 [published data only (unpublished sought but not used)]

Hawkes 2014 [published data only (unpublished sought but not used)]
Hawkes AL, Quinn M, Gelski V, Armes J, Brennan D, Janda M, et al. Improving treatment for obese women with early stage cancer of the uterus: rationale and design of the

Nock 2011 [published data only (unpublished sought but not used)]

Yeh 2015 [published data only (unpublished sought but not used)]
Yeh J. Survivorship promotion In reducing IGF-1 Trial (SPIRIT). Clinical Trials.gov 2015.

Additional references

Arem 2016

Arterburn 2015

Bray 2016

Calle 2003

Cancer Research UK 2014a

Cancer Research UK 2014b

Chan 2011

Chlebowski 2016

Colquitt 2014

Crosbie 2012

Crosbie 2016

Deeks 2001

Department of Health 2015

Dossus 2013

El-Safadi 2012

Figuls 2013

GRADEpro GDT [Computer program]

Higgins 2003

Higgins 2011a
Interventions for weight reduction in obesity to improve survival in women with endometrial cancer (Review)

Higgins 2011b

Irwin 2015

Liberati 2009

Matsuo 2016

Meader 2014

Mishra 2012

Morey 2009

NCI 2016

Papadia 2006

Parmar 1998

Reeves 2007

Renehan 2008

RevMan 2014 [Computer program]

Rock 2013

Rock 2015

Schünemann 2011

Stolley 2009

Swisher 2015

Wan 2016

Ward 2012
References to other published versions of this review

Kitson 2017

* Indicates the major publication for the study
### Characteristics of included studies  
**[ordered by study ID]**

**Allison 2016**

| Methods | \begin{tabular}{p{4cm}p{30cm}}
Design & Comment: Parallel design, 3-arm, open-label randomised controlled trial  
Quote: "...randomised, controlled study..."  
Setting & Comment: Multicentre study in USA.  
Quote: "...multi-site, pilot feasibility study..."  
Follow-up & Duration: 6 months  
Quote: "...6 month follow-up..."
\end{tabular} |
| --- | --- |

| Participants | \begin{tabular}{p{4cm}p{30cm}}
Number of participants enrolled & 41 women were randomised; 13 into Arm A, 13 into Arm B and 15 into Arm C. Six-month follow-up data were only available for 30 women at the time of undertaking this review (11 Arm A, 10 Arm B, 9 Arm C)  
Inclusion criteria & Women aged 18 years or older  
Biopsy proven endometrial cancer of any histological type  
BMI greater than or equal to 30 kg/m²  
ECOG PS 0-1  
No concurrent or planned chemo-radiation  
Access to wireless Internet and/or smartphone  
Life expectancy > 1 year  
Exclusion criteria & Significant medical condition that would affect compliance with protocol or ability to participate, e.g. uncontrolled hypertension, symptomatic cardiac disease  
Current participation in another weight-loss programme or taking weight-loss medication  
Another invasive malignancy in last five years (excluding non-melanoma skin cancer)  
Autoimmune or immunosuppressive condition  
Currently taking immunosuppressant medication  
Currently pregnant  
Baseline participant characteristics & The mean age of participants was 62.2 years (SD 8.7 years), with a mean BMI of 39.1 kg/m² (range 30 kg/m² to 67 kg/m²). Details of co-morbid conditions not collected by study authors. Participants had both type I and type II endometrial cancer, though the grade and stage of their malignancy was not provided. All had ECOG PS 0-1 and had undergone surgical treatment of their endometrial cancer. Baseline characteristics of participants according to group allocation were not provided.
\end{tabular} |
| --- | --- |

| Interventions | \begin{tabular}{p{4cm}p{30cm}}
Arm A & Comment: Telemedicine arm. Telephone-based weight-loss counselling undertaken by trained interventionists with guided digital measurements of weight, lean and fat mass.  
Counselling and weight-loss measurements occurred at least weekly for the six months duration of the intervention
\end{tabular} |
| --- | --- |
Quote: “The telemedicine arm included a Wifi scale that recorded at least weekly weights of participants. The scale automatically graphs the weights on a password protected website which permitted counsellors to have immediate feedback during weekly 15-20 minute counselling sessions teach standard weight-loss skills, including self-monitoring, problem-solving, enlisting social support, and overcoming negative thoughts according to a standard curriculum.”

Arm B

Comment: Text4Diet Group. Participants received 3-5 Short Message Service (SMS) text messages each day for the six-month intervention period. The text message provided tips and reminders to encourage healthy eating and weight loss. Participants also received a digital scale to track and report weight and were prompted to do so once a week by text message.

Quote: “The texting arm receives personalized text messages daily, following different monthly themes, e.g. Do not go to a party hungry. Eat a healthy snack before or bring a healthy dish with you to share. You will be more likely to stick to your goals! Since you have been meal planning do you find that you eat out less often? Y or N-remember the restaurant website is a great way to help you plan a healthy meal to order. Different styles included encouraging statements, yes/no questions or multiple choice questions.”

All participants in Arms A and B recorded dietary intake and restricted calories to 1200 kcal/day to 1500 kcal/day. They were given an exercise goal of 50 minutes/week to 175 minutes/week of moderate, aerobic physical activity, e.g. brisk walking.

Arm C

Comment: Enhanced Usual Care Group. Participants provided with handouts based on American Cancer Society guidelines on healthy eating and exercise and did not receive any additional input from the research team.

Quote: “...printed information from American Cancer Society guidelines on healthy eating and exercise...encourage weight loss through dietary monitoring and a walking program...these efforts were not reinforced or monitored by study staff...”

Outcomes

Primary outcomes
Overall survival: No deaths were reported in any arm for the duration of the study
Adverse events: Not reported
Second outcomes
Recurrence-free survival: Not reported
Cancer-specific survival: No cancer-specific deaths were reported in any arm for the duration of the study
Weight loss: Change in weight from baseline at 6 months reported
Cardiovascular and metabolic event frequency: No events reported in any arm
QoL: Change in QoL from baseline at 6 months reported using SF-12 Physical Health component change score

Quote: “Change in quality of life from baseline... SF-12 Physical Health component change score”

Power

Comment: No power calculation performed. Aim of study was to provide estimates of the effect size of the intervention in order to power a full-scale trial.

Quote: “The purpose will be to provide estimates for the size of an intervention effect achievable by the experimental intervention in order to power and justify a grant application for a full-scale trial of a weight loss program in women with endometrial cancer. With a sample size of 30 participants per group, the true difference in mean weight loss...”
between the groups can be estimated with a 95% confidence interval size of ±0.50\( \sigma \), where \( \sigma \) is the population standard deviation of weight loss, assumed in this calculation to be the same in each of the two intervention groups and the control group. We will assess the comparability of variance across the groups and do exploratory analyses of possibly variance-stabilizing transformations. Because this is a pilot study to derive parameters to design an appropriately-powered study, hypothesis testing is not a primary goal of the statistical analysis of the data, although P-values will be calculated.”

Notes
Study not yet published. Information obtained through correspondence with research team

<table>
<thead>
<tr>
<th>Bias</th>
<th>Authors’ judgement</th>
<th>Support for judgement</th>
</tr>
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<tbody>
<tr>
<td>Random sequence generation (selection bias)</td>
<td>Low risk</td>
<td>Comment: Computer-generated algorithm used at co-ordinating centre to produce randomisation envelopes for each site</td>
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<tr>
<td></td>
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<td>Quote: “The coordinating center used a computer generated algorithm to produce the randomization envelopes for each clinical site, with the general parameters of randomizing 1:1:1 across the three conditions.”</td>
</tr>
<tr>
<td>Allocation concealment (selection bias)</td>
<td>Low risk</td>
<td>Comment: Next envelope chosen for each enrolled participant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quote: “The envelopes are then chosen sequentially as each participant was enrolled.”</td>
</tr>
<tr>
<td>Blinding of participants and personnel (performance bias) All outcomes</td>
<td>High risk</td>
<td>Comment: Participants and personnel were unblinded</td>
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<tr>
<td></td>
<td></td>
<td>Quote: “There was no blinding.”</td>
</tr>
<tr>
<td>Blinding of outcome assessment (detection bias) All outcomes</td>
<td>High risk</td>
<td>Comment: Outcome assessments performed by unblinded study co-ordinators</td>
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<tr>
<td></td>
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<td>Quote: “The outcome assessments were conducted by study coordinators and trained medical personnel (for blood draws, DEXA). The coordinators knew which condition the participants were in, but other medical personnel were not informed.”</td>
</tr>
<tr>
<td>Incomplete outcome data (attrition bias) All outcomes</td>
<td>Low risk</td>
<td>Follow-up Entered the study: 13 into Arm A, 13 into Arm B and 15 into Arm C</td>
</tr>
</tbody>
</table>
Allison 2016 *(Continued)*

| Source of funding                                                                 | Comment: Source of funding described  
|----------------------------------------------------------------------------------| Quote: “Cross-TREC study funded by NCI U54-CA155850 - University of Pennsylvania; U54 CA155626 - Harvard University; U54 CA155496CC - Washington University; U01 CA116850 - Fred Hutchinson Cancer Research Center.”  
|                                                                                   | Ethical approval:  
|                                                                                   | Comment: Ethical approval obtained  
|                                                                                   | Conflicts of interest:  
|                                                                                   | Comment: No conflicts of interest reported  
| Other sources                                                                     | Other sources:  
|                                                                                   | The study failed to enrol 30 participants into each group within their allotted time.  
|                                                                                   | The reasons for this were not provided.  
|                                                                                   | Four centres open to recruitment although only the Universities of Washington and Pennsylvania enrolled patients into the study  
|                                                                                   | Only 30 participants had completed 6 months of follow-up at the time of correspondence with the study's chief investigator. Further results will be available for inclusion when the review is updated  
| Withdrawn from study: 0 in Arm A, 0 in Arm B, 0 in Arm C  
| Completed the study (at the time of correspondence): 11 in Arm A, 10 in Arm B, 9 in Arm C  
| No missing data reported.  
| Intention-to-treat analysis:  
| Comment: Not performed  
| Quote: “Given we only had pre-post assessment data and our main analyses used paired t-tests and correlations, we were unable to do intention-to treat analyses.” | Selective reporting (reporting bias):  
| Low risk                                                                         | Comment: Protocol not published but trial registered prospectively on clinicaltrials.gov and all prespecified outcomes reported  
<p>| Other bias                                                                       | Unclear risk |</p>
<table>
<thead>
<tr>
<th>Methods</th>
<th>Design</th>
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<tbody>
<tr>
<td>Comment: Parallel design, two-arm, randomised controlled trial</td>
<td></td>
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<tr>
<td>Quote: &quot;...two-group randomised trial...Patients were randomised to either: 1) a lifestyle intervention (SUCCEED) group that received nutrition, exercise, and behavioral modification counselling and 2) a usual care (UC) group...&quot;</td>
<td></td>
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<tr>
<td>Setting</td>
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<tr>
<td>Comment: Single-centre study in Ohio, USA</td>
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<td>Quote: &quot;The Case Comprehensive Cancer Center (affiliates University Hospitals Case Medical Center and Cleveland Clinic) tumor registry was used to identify potential subjects. A letter was sent to potential patients describing the study and women were invited to attend an informational session.&quot;</td>
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<tr>
<td>Follow-up</td>
<td></td>
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<tr>
<td>Comment: 12 months</td>
<td></td>
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<tr>
<td>Quote: &quot;Outcome measures were assessed in both groups at baseline, 3, 6, and 12 months. &quot;</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Participants</th>
<th>Number of participants enrolled</th>
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<tbody>
<tr>
<td>75 participants enrolled; 41 in the intervention arm and 34 in the control arm</td>
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<tr>
<td>Inclusion criteria</td>
<td></td>
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<tr>
<td>Histologically-confirmed endometrial cancer diagnosed within last three years</td>
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<tr>
<td>Stage I or II</td>
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<tr>
<td>Undergone surgical treatment of endometrial cancer in the form of total abdominal hysterectomy and bilateral salpingo-oophorectomy +/- lymphadenectomy</td>
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<tr>
<td>No evidence of disease at time of enrolment</td>
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<tr>
<td>Performance status 0-2</td>
<td></td>
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<tr>
<td>BMI greater than or equal to 25 kg/m²</td>
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<tr>
<td>Medical clearance from primary care physician and approval to contact patient by treating gynae-oncologist</td>
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<tr>
<td>Exclusion criteria</td>
<td></td>
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<tr>
<td>Unable to read consent form</td>
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<tr>
<td>Severe depression, dementia or cognitive deficit</td>
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<tr>
<td>Unavailable for longitudinal follow-up assessment</td>
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<tr>
<td>Pre-existing medical conditions that prevent participation in unsupervised walking</td>
<td></td>
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<tr>
<td>Participation in weight-loss or exercise programme in preceding six months</td>
<td></td>
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<tr>
<td>Baseline participant characteristics</td>
<td></td>
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<tr>
<td>There were no significant differences in the baseline characteristics of participants between the two groups</td>
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<tr>
<td>The mean age of participants in the intervention arm was 57 years (SD 8.6 years) compared with 58.9 years (SD 10.9 years) in the control arm</td>
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<tr>
<td>Overall, the mean BMI was 36.5 kg/m²; 36.4 kg/m² (SD 5.5) in the intervention arm and 36.5 kg/m² (SD 9.6) in the control arm.</td>
<td></td>
</tr>
<tr>
<td>The co-morbidities hypertension and diabetes were present in 31.7% and 17.1% of participants in the intervention arm compared with 35.3% and 26.5% of participants in the control arm, respectively. All participants had a performance status of 0-2</td>
<td></td>
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<tr>
<td>All participants underwent surgical treatment of their endometrial cancer, on average, 20.7 months earlier. In addition, 39.0% of participants in the intervention arm and 35.3% of participants in the control arm had undergone adjuvant radiotherapy. Details of grade, stage and histological type of endometrial cancer were not provided</td>
<td></td>
</tr>
</tbody>
</table>
### Interventions

**Comment:** Sixteen group sessions focusing on diet and physical activity over six months and an additional three face-to-face counselling visits at 3, 6 and 12 months. Feedback and support were provided by a registered dietitian after the end of the group sessions by phone, email and newsletters.

*Quote:* “Sixteen group sessions were conducted (10 weekly followed by 6 bi-weekly) in the SUCCEED group. Physician face-to-face counselling visits occurred at 3, 6 and 12 months. Group topics included PA, nutrition and improving diet quality and behavior modification designed to increase women’s self-efficacy. Sessions were 60 min in length with 8-10 women per group. The RD weighed participants in private at the beginning of each session and weekly food/activity records were reviewed. After 6 months when the group sessions ended, additional feedback and support was provided by the RD via newsletters, telephone and email. Newsletter topics included holiday recipes, reinforcement of goals for increasing calcium, decreasing sodium, and ways to increase PA. The intervention followed a stepwise, phased approach using strategies outlined by social cognitive theory, indicating that the optimal intervention for a major behavior change should focus on establishing short-term goals, enabling the person to build self-efficacy.”

**Control arm**

Comment: Received information brochure only. Participants also attended physician counselling sessions at 3, 6 and 12 months, but these visits did not include any lifestyle advice related to weight loss, physical activity or nutrition.

*Quote:* “Patients randomized to the (control) group received an informational brochure (‘Healthy Eating & Physical Activity Across Your Lifespan, Better Health and You’)

### Outcomes

**Primary outcomes**

Overall survival: No deaths reported for the duration of the study period (12 months)

Adverse events: Reported adverse events in both intervention and control arms

*Quote:* "(Adverse events were reported) as required by the IRB...The true adverse events were all in the intervention group”

Second outcomes

Recurrence-free survival: Not reported

Cancer-specific survival: No deaths reported for the duration of the study period (12 months)

Weight loss: Weight change from baseline at 3, 6 and 12 months reported.

Cardiovascular and metabolic event frequency: No events reported

QoL: Change in QoL from baseline measured at 3, 6 and 12 months using FACT-G questionnaire.

*Quote:* "Quality of life (QoL) was measured by the Functional Assessment of Cancer Therapy-General (FACT-G)...

FACT-G was measured at baseline, 3, 6, and 12 months post-baseline.”

**Power**

Comment: A power calculation was performed and sufficient detail was provided to allow it to be replicated.

*Quote:* “Approximately 37 patients per group were needed to provide 80% power to detect a difference between groups in mean weight change from baseline to 12 months of 4.0 kg or greater (alpha= 0.05, two-sided, SD= 6.0; effect size= 0.67) and to assess changes in PA with a similar effect size. Effect sizes of 0.5 are considered medium and 0.8 or greater large.”
### Risk of bias

<table>
<thead>
<tr>
<th>Bias</th>
<th>Authors’ judgement</th>
<th>Support for judgement</th>
</tr>
</thead>
</table>
| Random sequence generation (selection bias)                         | Low risk           | Comment: Block randomisation performed according to baseline BMI  
Quote: "Randomization was stratified using block sizes of 6 or 8 by baseline BMI (25.0-39.9 versus > 40)"                                                                                     |
| Allocation concealment (selection bias)                            | Unclear risk       | Comment: No details of allocation concealment provided by study authors                                                                                                                                                    |
| Blinding of participants and personnel (performance bias)           | High risk          | Comment: Blinding of participants and personnel not possible due to nature of intervention. Principle investigator involved in delivery of intervention so aware of randomisation  
Quote: "Due to the interventions performed by the study team (dietitian, Physical therapist, psychologist, etc.), they were able to know who was in each group." |
| Blinding of outcome assessment (detection bias)                     | High risk          | Comment: Principle investigator performed outcome assessments and was unblinded to treatment group allocation. This is unlikely to affect weight measurements but may impact upon quality of life assessments |
| Incomplete outcome data (attrition bias)                           | High risk          | Follow-up  
Entered into the study: 41 in intervention arm and 34 in control arm  
Withdrawn from study: 6 in intervention arm and 10 in control arm  
Completed study: 35 in intervention arm and 24 in control arm  
Reasons for withdrawal from study not provided by authors. The study was underpowered at 12 months to detect a weight loss of 4.0 kg or greater in the intervention arm  
Quote: "Attrition in the trial overall was 21.3%. Six (14.6%) patients in the LI group versus 10 (29.4%) in UC did not complete..." |

Notes: This is the definitive RCT following on from the pilot study also included in this review (von Gruenigen 2009)
the twelve-month assessments, \( P = 0.159 \). Thirty-one (75.6\%) participants in the (intervention arm) attended 14 or more of the 16 sessions; mean adherence was 84.1\% Intention-to-treat analysis Comment: Analyses were conducted according to an intention-to-treat protocol, however, only 85.4\% of participants in the intervention arm and 70.6\% of participants in the control arm attended for the 12 month assessments. Missing data were imputed by multiple imputation Quote: "Analyses were done according to intention-to-treat principles. Missing data were examined and imputed by multiple imputation"

<table>
<thead>
<tr>
<th>Selective reporting (reporting bias)</th>
<th>Low risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comment: Protocol not published but trial registered prospectively on clinicaltrials.gov and all prespecified outcomes reported</td>
<td></td>
</tr>
</tbody>
</table>

Other bias Low risk

Source of funding Comment: Source of funding described Quote: "This research was supported by the American Cancer Society.”
Ethical approval Comment: Ethical approval was obtained Quote: "Institutional review board approval was granted…”
Conflicts of interest Comment: No significant conflicts of interest noted

von Gruenigen 2009

<table>
<thead>
<tr>
<th>Methods</th>
<th>Design</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comment: Parallel design, two-arm, randomised controlled trial Quote: &quot;...prospective, two-group randomized controlled trial...“</td>
<td></td>
</tr>
<tr>
<td>Setting</td>
<td></td>
</tr>
<tr>
<td>Comment: Single-centre study in Ohio, USA Quote: &quot;...women included in the cancer registry at the Ireland Cancer Center diagnosed from 2001-2004…”</td>
<td></td>
</tr>
<tr>
<td>Follow-up</td>
<td></td>
</tr>
<tr>
<td>Comment: 24 months</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Participants</th>
<th>Number of participants enrolled</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 participants enrolled</td>
<td>23 into the intervention arm and 22 into the control arm</td>
</tr>
<tr>
<td>Inclusion criteria</td>
<td></td>
</tr>
<tr>
<td>Histologically-confirmed endometrial cancer</td>
<td></td>
</tr>
</tbody>
</table>
Stage I or II  
Undergone surgical treatment of endometrial cancer in the form of total abdominal hysterectomy and bilateral salpingo-oophorectomy +/- lymphadenectomy  
No evidence of disease at time of enrolment  
Performance status 0-2  
BMI > 25 kg/m²  

Exclusion criteria  
Clear cell or papillary serous histology  

Baseline participant characteristics  
There were no significant differences in the baseline characteristics of participants between the two groups  
The mean age of participants in the intervention arm was 54 years (SEM 2.0 years) compared with 55.5 years (SEM 1.6 years) in the control arm  
Overall, the mean BMI was 42.3 kg/m²; 43.5 kg/m² (SEM 2.1) in the intervention arm and 41.1 kg/m² (SEM 2.2) in the control arm.  
The co-morbidities hypertension, diabetes and metabolic syndrome were present in 65.2%, 17.4% and 26.1% of participants in the intervention arm compared with 36.4%, 27.3% and 27.3% of participants in the control arm, respectively. All participants had a performance status of 0-2  
All participants underwent surgical treatment of their endometrial cancer, on average, 2 years earlier. Details of adjuvant treatment and grade, stage and histological type of endometrial cancer were not provided  

Interventions  
**Intervention arm**  
Comment: Consisted of group sessions based on other nutrition and exercise goals and delivered by a registered dietitian, principle investigator and psychologist for 6 months. Participants were encouraged to gradually increase walking or other aerobic activity to 5 days per week for 45 minutes or more per session. Reinforcement of the content of group sessions was provided on an individual basis by the principle investigator at 3, 6 and 12 months  
Quote: "...Group session topics included: weight loss readiness and goal-setting, physical activity, portion sizes and food intake per mypyramid.gov, emotional eating/negative thinking, behavior modification, grocery shopping and reading food labels, relapse prevention, eating out and in social situations, and stress management...(The groups) met weekly for 6 weeks, bi-weekly for 1 month, and monthly for 3 months. Participants were contacted by the RD (MBK) by phone or newsletter every week that the group did not meet. Phone calls were structured in content and included reinforcement and discussion regarding the previous week's topic. Participants were also given feedback on individual progress towards physical activity and nutrition goals...Pedometers were provided to and used by the LI group for patient feedback...Study participants in both groups saw the PI at 3, 6 and 12 months. Both groups received counselling regarding overall health concerns and LI participants received specific reinforcement of group session topics. “  

**Control arm**  
Comment: Received usual care and were provided with a generic booklet on improving health. Individual meetings were held with the principle investigator at 3, 6 and 12 months, however, these consisted of counselling regarding overall health concerns rather than a discussion about weight loss and physical activity  
Quote: "the (control arm) received only an informational brochure after randomization ("Better Health and You,” Weight Control Information Network, June 2004)...(control..."
arm) participants did not receive any advice related to weight loss, physical activity or nutrition at these visits...

<table>
<thead>
<tr>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary outcomes</strong>&lt;br&gt;Overall survival: Deaths reported for the duration of the study period (24 months) but insufficient data available to determine hazard ratio&lt;br&gt;Quote: “Within 24-months, 2 patients deceased: n=1 to brain aneurysm and n=1 to kidney cancer.”&lt;br&gt;Adverse events: No reported adverse events in either intervention and control arms&lt;br&gt;Quote: “(Adverse events reported) as required by the IRB. No adverse events due to study procedures occurred.”&lt;br&gt;Second outcomes&lt;br&gt;Recurrence-free survival: Not reported&lt;br&gt;Cancer-specific survival: Deaths reported for the duration of the study period (24 months) but insufficient data available to determine hazard ratio&lt;br&gt;Quote: “During the study period...2 patients deceased: n=1 to brain aneurysm and n=1 to kidney cancer. Both deaths were in the control arm...”&lt;br&gt;Weight loss: Weight change from baseline at 3, 6, 9, 12 and 24 months reported&lt;br&gt;Cardiovascular and metabolic event frequency: No events reported up to 24 months follow-up&lt;br&gt;QoL: Change in QoL from baseline at 3, 6, 9 and months reported using FACT-G questionnaire&lt;br&gt;Quote: “QoL and self-efficacy were assessed at baseline and at 3, 6, and 12 months... QoL was measured by the Functional Assessment of Cancer Therapy-General (FACT-G), a valid and reliable questionnaire evaluating physical, functional, family-social, and emotional well-being domains. A fatigue subscale (-F) and an endometrial symptom subscale (-En) were also used...”&lt;br&gt;<strong>Power</strong>&lt;br&gt;Comment: A power calculation was performed and sufficient detail was provided to allow it to be replicated&lt;br&gt;Quote: “Approximately 25 patients per group were needed to provide 80% power to detect a difference between groups in mean weight change from baseline to 12 months of 5 kg (11 lb) or greater, representing approximately 5% for an obese female (alpha = 0.05, two-sided, SD = 5.0). Five percent weight change is considered clinically relevant and a recommended goal for weight loss over 6 months.”</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Notes</th>
</tr>
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<tbody>
<tr>
<td>The follow-up was described as being of 12 months duration in the publication, however, when contacted the authors were able to provide data for weight change up to 24 months&lt;br&gt;This was the pilot study preceding the definitive trial, which is also included in this review (McCarroll 2014)</td>
</tr>
</tbody>
</table>

### Risk of bias

<table>
<thead>
<tr>
<th>Bias</th>
<th>Authors’ judgement</th>
<th>Support for judgement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random sequence generation (selection bias)</td>
<td>Low risk</td>
<td>Comment: stratified block randomisation based on BMI employed&lt;br&gt;Quote: “After enrolment, participants were randomly assigned (to intervention or con-</td>
</tr>
</tbody>
</table>
Randomization was stratified according to patient BMI (25-39.9 versus ≥40 kg/m²) using a stratified blocked randomization scheme.

<table>
<thead>
<tr>
<th>Bias</th>
<th>Risk</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allocation concealment (selection bias)</td>
<td>Unclear risk</td>
<td>No details of allocation concealment provided by study authors</td>
</tr>
<tr>
<td>Blinding of participants and personnel (performance bias)</td>
<td>High risk</td>
<td>Blinding of participants and personnel not possible due to nature of intervention. Principle investigator involved in delivery of intervention so aware of randomisation. Principle investigator performed outcome assessments and was unblinded to treatment group allocation. This is unlikely to affect weight measurements but may impact upon quality of life assessments.</td>
</tr>
<tr>
<td>Blinding of outcome assessment (detection bias)</td>
<td>High risk</td>
<td>Principle investigator performed outcome assessments and was unblinded to treatment group allocation. This is unlikely to affect weight measurements but may impact upon quality of life assessments.</td>
</tr>
<tr>
<td>Incomplete outcome data (attrition bias)</td>
<td>High risk</td>
<td>Follow-up entered into the study: 23 in intervention arm, 22 in control arm. Withdraw from study: 5 in intervention arm, 2 in control arm. Completed study: 17 in intervention arm, 20 in control arm (though data from assessment at 12 months missing for 2 participants in the control arm). Two withdrawals in the intervention arm were due to issues with work, the reason for the other three withdrawals in this group were not stipulated. The two withdrawals from the control arm occurred prior to the first assessment at 3 months and the reasons were not stipulated. Attribute in the trial overall was 16% [2 patients (10%) in the UC group versus 5 (22%) in the LI group; P = 0.242], therefore 84% completed follow-up assessments. Specifically, 78% of patients [LI: 17/23 (74%), UC: (18/22) (82%)] completed the 12-month assessment time point and there was no difference between groups.</td>
</tr>
</tbody>
</table>
Intention-to-treat analysis

Comment: Analyses were conducted according to an intention-to-treat protocol. There was, however, significant missing data; 19% of weight values and 15% to 19% of QoL data were missing. Missing data were imputed using three different techniques; last and next average (average of last and next known values), previous row mean method and last observation carried forward. All produced similar findings and so only the results obtained using the first approach were included in the journal publication.

Quote: “Imputation was done for 19% (35/180) of weight values, 10 patients (LI: 6 and UC: 4) had weight values imputed for the final weight. These patients opted to not complete the assessment and values were imputed based on the most recent physician visit, if they had one or were imputed... Imputation was done on between 15-19% of values for the various QoL and eating behavior measures.”

<table>
<thead>
<tr>
<th>Selective reporting (reporting bias)</th>
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<th>Comment: Protocol not published but trial registered prospectively on clinicaltrials.gov and all prespecified outcomes reported</th>
</tr>
</thead>
</table>
| Other bias                          | High risk| Source of funding  
Comment: Source of funding described  
Quote: “This research was supported by a grant from the Lance Armstrong Foundation”  
Ethical approval  
Comment: Ethical approval was obtained  
Quote: “Institutional review board approval was obtained...”  
Conflicts of interest  
Comment: No significant conflicts of interest noted  
Other sources  
Study failed to recruit sufficient numbers to meet a priori total in time frame. One patient in the intervention arm underwent gastric bypass at 9 months after the start of the intervention and another between 12 and 24 months. Both were in-
von Gruenigen 2009  (Continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Reason for exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Babatunde 2016</td>
<td>Comment: Systematic review rather than randomised controlled trial</td>
</tr>
</tbody>
</table>
| Beck 2015          | Comment: Wrong patient population  
Quote: “Obese (Mean BMI = 35.8) female patients (Mean age 58.41) with breast (n = 15), colon (n = 1), and endometrial cancers (n = 1) were recruited and randomly assigned to receive exercise and nutrition intervention without (POWER, n = 10) or with an additional mindfulness component (MORE POWER, n = 7)” |
| Donnelly 2011      | Comment: Wrong indication  
Quote: “To determine the feasibility and effectiveness of a physical activity (PA) behavioural change intervention in managing cancer-related fatigue (CRF) among gynaecological cancer survivors during and post anti-cancer treatments” |
| Fasching 2009      | Comment: Systematic review rather than randomised controlled trial                    |
| Gil 2007           | Comment: Systematic review rather than randomised controlled trial                    |
| Koutoukidis 2015   | Comment: Systematic review rather than randomised controlled trial                    |
| Koutoukidis 2017   | Comment: Wrong indication  
Quote: “Aim....(to determine if) Shape-up following cancer treatment programme is more effective than usual care in improving the health-related quality of life of endometrial cancer survivors” |
| Lin 2016           | Comment: Systematic review rather than randomised controlled trial                    |
| Rossi 2016         | Comment: Wrong indication  
Quote: “...aims of this study were to 1) assess the feasibility of a 12-week physical activity intervention for obese socioculturally diverse endometrial cancer survivors in Bronx, NY; 2) determine the probable effectiveness of the intervention on physical activity, waist circumference, physical function and quality of life; and 3) evaluate changes in self-efficacy, outcome expectations, social support, and self-regulation during the 12-week physical activity intervention.” |
| Smits 2015         | Comment: Systematic review rather than randomised controlled trial                    |

BMI: body mass index; ECOG: European Cooperative Oncology Group; FACT-G: Functional Assessment of Cancer Therapy-General; QoL: quality of life; RCT: randomised controlled trial; SD: standard deviation; SEM: standard error of the mean

Characteristics of excluded studies  [ordered by study ID]

<table>
<thead>
<tr>
<th>Study</th>
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Quote: “To determine the feasibility and effectiveness of a physical activity (PA) behavioural change intervention in managing cancer-related fatigue (CRF) among gynaecological cancer survivors during and post anti-cancer treatments” |
| Fasching 2009      | Comment: Systematic review rather than randomised controlled trial                    |
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| Lin 2016           | Comment: Systematic review rather than randomised controlled trial                    |
| Rossi 2016         | Comment: Wrong indication  
Quote: “...aims of this study were to 1) assess the feasibility of a 12-week physical activity intervention for obese socioculturally diverse endometrial cancer survivors in Bronx, NY; 2) determine the probable effectiveness of the intervention on physical activity, waist circumference, physical function and quality of life; and 3) evaluate changes in self-efficacy, outcome expectations, social support, and self-regulation during the 12-week physical activity intervention.” |
| Smits 2015         | Comment: Systematic review rather than randomised controlled trial                    |

BMI: body mass index
### Characteristics of ongoing studies  [ordered by study ID]

#### Bantum 2015

<table>
<thead>
<tr>
<th>Trial name or title</th>
<th>Hula-based exercise program in increasing physical activity in breast, cervical, endometrial, or ovarian cancer survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methods</td>
<td>Parallel-design, open-label, randomised trial</td>
</tr>
<tr>
<td>Participants</td>
<td>Women aged 21 and older, living in Oahu, Hawaii, diagnosed with primary breast, cervical, endometrial or ovarian cancer (stage I-III), completed initial regional and systemic breast cancer treatment at least 2 months earlier, physically capable of doing hula-based physical activity, not currently undergoing chemo- or radiation therapy</td>
</tr>
</tbody>
</table>
| Interventions       | Arm I: Hula-based exercise programme consisting of warm-up, conditioning and cool-down over 60 minutes, twice a week and a home-based hula practice for 10-15 minutes, three times per week for six months  
                      | Arm II: The same hula-based exercise programme beginning six months after study enrolment                        |
| Outcomes            | Primary outcome measure: feasibility of programme (compliance, satisfaction)  
                      | Secondary outcome measures: biomarkers (sex hormones, cytokines, inflammatory markers e.g. CRP, leptin, IGF-1, IGFBP-3), DNA methylation patterns, self-reported physical activity, quality of life, depression, affective states, social constrains and cognitive functioning |
| Starting date       | September 2013                                                                                                    |
| Contact information | Erin Bantum University of Hawaii Cancer Center, USA                                                                |
| Notes               | Clinical trials.gov identifier: NCT02351479                                                                        |

#### Basen-Engquist 2016

<table>
<thead>
<tr>
<th>Trial name or title</th>
<th>Feasibility of the NEXT steps weight loss intervention +/- resistance training for endometrial cancer survivors: Effect on lean mass &amp; biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methods</td>
<td>Parallel-design, unblinded, three-arm, randomised controlled trial</td>
</tr>
<tr>
<td>Participants</td>
<td>Women aged 18 and over diagnosed with stage I and II endometrial cancer in the preceding three years and at least six months following treatment, with a BMI between 30 kg/m² to 45 kg/m², with access to a computer or smartphone and Wifi for syncing Fitbit devices and willing to travel to MD Andersen, Texas, USA</td>
</tr>
</tbody>
</table>
| Interventions       | NEXT Steps-Aerobic Exercise and Resistance Training (NS-ART). Participants entered into exercise plan focused on physical activity and resistance training of six months duration. Physical activity guidelines workbook distributed along with activity monitor. Participants receive phone calls and text messages for support in reaching diet and exercise goals. Participants also given resistance bands and exercise handouts  
                      | NEXT Steps-Aerobic Exercise (NS-AE). Partipants placed into an exercise plan of six months duration focused on physical activity only. Participants receive phone calls and text messages for support in reaching diet and exercise goals  
                      | Standard Care Control Group (CG). Participants receive standard care consisting of phone calls asking about their health and self-help materials for six months |
### Basen-Engquist 2016 (Continued)

<table>
<thead>
<tr>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary outcome measure: feasibility of interventions (consent, retention, adherence and satisfaction rates)</td>
</tr>
<tr>
<td>Second outcome measure: change in lean body mass (weight and measured by dual-energy x-ray absorptiometry)</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Starting date</th>
</tr>
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<tbody>
<tr>
<td>October 2016</td>
</tr>
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<table>
<thead>
<tr>
<th>Contact information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karen Basen-Engquist, MD Andersen Cancer Center, USA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Notes</th>
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</thead>
<tbody>
<tr>
<td>Clinical Trials.gov identifier: NCT02774759</td>
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</tbody>
</table>

### Hawkes 2014

<table>
<thead>
<tr>
<th>Trial name or title</th>
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</thead>
<tbody>
<tr>
<td>Improving treatment for obese women with early stage cancer of the uterus: rationale and design of the levonorgestrel intrauterine device +/- metformin +/- weight loss in endometrial cancer (feMME) trial</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parallel design, open-label, three-arm, randomised trial with patients randomised in a 3:3:5 ratio to the interventions</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Participants</th>
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</thead>
<tbody>
<tr>
<td>Grade 1 endometrioid, endometrial cancer, apparent stage I disease on CT and MRI scan, no lymphovascular space invasion on endometrial curettings, no or minimal myometrial invasion on MRI scan and a normal (less than or equal to 30 U/mL) CA-125 level</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Interventions</th>
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</thead>
<tbody>
<tr>
<td>Levonorgesterol-IntraUterine Device only. Standard, Australian Therapeutic Goods Administration approved device to be inserted into the uterine cavity and left for six months</td>
</tr>
<tr>
<td>Levonorgesterol-IntraUterine Device plus Metformin at a dose of 1000 mg daily, given orally with meals for six months</td>
</tr>
<tr>
<td>Levonorgesterol-IntraUterine Device plus weight-loss intervention. Participants will be provided with a voucher for a comprehensive subscription to a weight-loss program (Weight Watchers®) and are encouraged to attend the face-to-face group meetings and to use the online tools and social networking opportunities for six months</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary outcome measure: Absence of invasive endometrial cancer or atypical endometrial hyperplasia at six months on dilatation and curettage</td>
</tr>
<tr>
<td>Secondary outcome measures: change in weight and physical activity, quality of life, anxiety and depression symptomatology, health service usage, pelvic floor distress, diet, serum and tissue predictive biomarkers</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Starting date</th>
</tr>
</thead>
<tbody>
<tr>
<td>October 2012</td>
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</table>

<table>
<thead>
<tr>
<th>Contact information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andreas Obermair Queensland Centre for Gynaecological Cancer, Australia</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Notes</th>
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<tbody>
<tr>
<td>Clinical trials.gov identifier: NCT01686126</td>
</tr>
</tbody>
</table>
### Nock 2011

<table>
<thead>
<tr>
<th>Trial name or title</th>
<th>Assisted exercise in obese endometrial cancer patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methods</td>
<td>Parallel-design, open-label, randomised trial</td>
</tr>
<tr>
<td>Participants</td>
<td>Adult women with histologically-confirmed grade 1-2, stage I endometrial cancer diagnosed in last four years, have not received adjuvant chemotherapy and completed treatment at least three months earlier, successfully completed a cardiopulmonary stress test, medical clearance by treating team to participate in exercise programme, BMI greater than or equal to 30 kg/m²</td>
</tr>
<tr>
<td>Interventions</td>
<td>'Assisted Rate' Exercise Intervention: cycling on stationary, recumbent exercise bike with motor assistance to maintain pedaling rate 35% greater than their voluntary rate. Participants will complete 45 to 60 minute sessions three times per week for eight weeks 'Voluntary Rate' Exercise Intervention: cycling on stationary, recumbent exercise bike at preferred pedaling rate for 45 to 60 minutes, three times per week for eight weeks</td>
</tr>
<tr>
<td>Outcomes</td>
<td>Primary outcome measures: changes in body weight, fitness, bi-manual dexterity, exercise motivation and self-reported eating behaviour Secondary outcome measures: changes in food behaviour in response to high- and low-calorie visual stimuli under fed and starved conditions, genetic (e.g. dopamine receptor and transporter) and serum biomarkers (e.g. leptin)</td>
</tr>
<tr>
<td>Starting date</td>
<td>September 2011</td>
</tr>
<tr>
<td>Contact information</td>
<td>Nora Nock Case Comprehensive Cancer Center, Cleveland, USA</td>
</tr>
<tr>
<td>Notes</td>
<td>Clinical Trials.gov identifier: NCT01870947</td>
</tr>
</tbody>
</table>

### Yeh 2015

<table>
<thead>
<tr>
<th>Trial name or title</th>
<th>Survivorship Promotion In Reducing IGF-1 Trial (SPIRIT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methods</td>
<td>Parallel-design, single-blinded, three-arm, randomised controlled trial</td>
</tr>
<tr>
<td>Participants</td>
<td>Women and men aged 18 years and older, with a prior diagnosis of a solid malignancy (including endometrial cancer), who have completed surgical, chemo- or radiation therapy at least three months previously and with an anticipated treatment-free lifespan of more than 12 months, BMI greater than or equal to 25 kg/m² and less than 400 lbs, with internet and phone access and willingness to change diet, physical activity and weight</td>
</tr>
<tr>
<td>Interventions</td>
<td>Active Comparator: Self-Directed. Meeting with trial team at beginning of study and provision of written information about weight management Experimental: Coach-Directed Behavioral Weight Loss.Remote Lifestyle Coaching Intervention-behaviour based telephonic coaching with web-based support to promote healthy lifestyle and weight loss. The goal of the intervention is to achieve at least 5% weight loss in the first six months and to maintain these improvements through month 12 by meeting dietary and exercise goals Experimental: Metformin up to 2000 mg per day. Dosing can be flexible, depending on tolerance, and given 2-3 times per day orally with meals for 12 months</td>
</tr>
</tbody>
</table>
Outcomes

- Primary outcome measures: IGF-1 levels, IGF-1:IGFBP3 ratio at 6 months
- Secondary outcome measures: IGF-1 levels, IGF-1:IGFBP3 ratio at 12 months
- Other outcome measures: change in weight, BMI, dietary intake, physical activity, glucose, insulin, HbA1C, IL-8, CRP levels and side effects in experimental arms

Starting date
- May 2015

Contact information
- Jessica Yeh, John Hopkins, Maryland, USA

Notes
- Clinical Trials.gov identifier: NCT02431676

BMI: body mass index; CA-125: cancer antigen 125; CRP: C-reactive protein; CT: computed tomography; iGF-1: insulin growth factor; IGFBP-3: insulin-like growth factor binding protein-3; IL-8: interleukin-8; MRI: magnetic resonance imaging
## DATA AND ANALYSES

Comparison 1. Lifestyle intervention versus usual care

<table>
<thead>
<tr>
<th>Outcome or subgroup title</th>
<th>No. of studies</th>
<th>No. of participants</th>
<th>Statistical method</th>
<th>Effect size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Overall survival (6 months)</td>
<td>2</td>
<td>99</td>
<td>Risk Ratio (M-H, Random, 95% CI)</td>
<td>0.0 [0.0, 0.0]</td>
</tr>
<tr>
<td>2 Overall survival (12 months)</td>
<td>1</td>
<td>59</td>
<td>Risk Ratio (M-H, Random, 95% CI)</td>
<td>0.0 [0.0, 0.0]</td>
</tr>
<tr>
<td>3 Overall survival (24 months)</td>
<td>1</td>
<td>37</td>
<td>Risk Ratio (M-H, Random, 95% CI)</td>
<td>0.23 [0.01, 4.55]</td>
</tr>
<tr>
<td>4 Adverse events-musculoskeletal</td>
<td>2</td>
<td>91</td>
<td>Risk Ratio (M-H, Random, 95% CI)</td>
<td>19.03 [1.17, 310.52]</td>
</tr>
<tr>
<td>5 Cancer-specific survival (6 months)</td>
<td>2</td>
<td>99</td>
<td>Risk Ratio (M-H, Random, 95% CI)</td>
<td>0.0 [0.0, 0.0]</td>
</tr>
<tr>
<td>6 Cancer-specific survival (12 months)</td>
<td>1</td>
<td>59</td>
<td>Risk Ratio (M-H, Random, 95% CI)</td>
<td>0.0 [0.0, 0.0]</td>
</tr>
<tr>
<td>7 Cancer-specific survival (24 months)</td>
<td>1</td>
<td>37</td>
<td>Risk Ratio (M-H, Random, 95% CI)</td>
<td>0.0 [0.0, 0.0]</td>
</tr>
<tr>
<td>8 Weight loss (6 months)</td>
<td>3</td>
<td>131</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-1.88 [-5.98, 2.21]</td>
</tr>
<tr>
<td>9 Weight loss stratified by BMI (6 months)</td>
<td>2</td>
<td>101</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-3.11 [-9.32, 3.10]</td>
</tr>
<tr>
<td>9.1 BMI &lt;40 kg/m$^2$</td>
<td>2</td>
<td>63</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-3.18 [-10.29, 3.93]</td>
</tr>
<tr>
<td>9.2 BMI &gt;/40 kg/m$^2$</td>
<td>2</td>
<td>38</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-2.89 [-15.65, 9.88]</td>
</tr>
<tr>
<td>10 Weight loss (12 months)</td>
<td>2</td>
<td>91</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-8.98 [-19.88, 1.92]</td>
</tr>
<tr>
<td>11 Weight loss stratified by BMI (12 months)</td>
<td>2</td>
<td>90</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-5.23 [-11.59, 1.12]</td>
</tr>
<tr>
<td>11.1 BMI &lt;40 kg/m$^2$</td>
<td>2</td>
<td>55</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-4.08 [-11.20, 3.04]</td>
</tr>
<tr>
<td>11.2 BMI &gt;/40 kg/m$^2$</td>
<td>2</td>
<td>35</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-9.76 [-23.84, 4.32]</td>
</tr>
<tr>
<td>12 Weight loss (24 months)</td>
<td>1</td>
<td>25</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-18.26 [-38.73, 2.21]</td>
</tr>
<tr>
<td>13 Weight loss stratified by BMI (24 months)</td>
<td>1</td>
<td>25</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-25.84 [-81.40, 29.72]</td>
</tr>
<tr>
<td>13.1 BMI &lt;40 kg/m$^2$</td>
<td>1</td>
<td>13</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>2.12 [-20.82, 25.06]</td>
</tr>
<tr>
<td>13.2 BMI &gt;/40 kg/m$^2$</td>
<td>1</td>
<td>12</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-54.58 [-80.97, -28.19]</td>
</tr>
<tr>
<td>14 Adverse events-diarrhoea</td>
<td>2</td>
<td>91</td>
<td>Risk Ratio (M-H, Random, 95% CI)</td>
<td>4.53 [0.23, 90.51]</td>
</tr>
<tr>
<td>15 Cardiovascular and metabolic event frequency (6 months)</td>
<td>3</td>
<td>131</td>
<td>Risk Ratio (M-H, Random, 95% CI)</td>
<td>0.0 [0.0, 0.0]</td>
</tr>
<tr>
<td>16 Cardiovascular and metabolic event frequency (12 months)</td>
<td>2</td>
<td>93</td>
<td>Risk Ratio (M-H, Random, 95% CI)</td>
<td>0.0 [0.0, 0.0]</td>
</tr>
<tr>
<td>17 Quality of life-SF12 Physical Health component (6 months)</td>
<td>1</td>
<td>30</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>-2.29 [-7.34, 2.76]</td>
</tr>
<tr>
<td>18 Quality of life FACT-G (6 months)</td>
<td>2</td>
<td>95</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>2.51 [-5.61, 10.64]</td>
</tr>
<tr>
<td>19 Quality of life stratified by BMI (6 months FACT-G)</td>
<td>2</td>
<td>95</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>4.69 [1.39, 7.99]</td>
</tr>
<tr>
<td>19.1 BMI &lt;40 kg/m$^2$</td>
<td>2</td>
<td>60</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>4.01 [-5.48, 13.51]</td>
</tr>
<tr>
<td>19.2 BMI &gt;/40 kg/m$^2$</td>
<td>2</td>
<td>35</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>4.18 [-0.13, 8.49]</td>
</tr>
<tr>
<td>20 Quality of life FACT-G (12 months)</td>
<td>2</td>
<td>89</td>
<td>Mean Difference (IV, Random, 95% CI)</td>
<td>2.77 [-0.65, 6.20]</td>
</tr>
<tr>
<td>Study or subgroup</td>
<td>Experimental</td>
<td>Control</td>
<td>Risk Ratio M-H, Random, 95% CI</td>
<td>Weight</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------</td>
<td>---------</td>
<td>---------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>Allison 2016</td>
<td>0/21</td>
<td>0/9</td>
<td>Not estimable</td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>0/41</td>
<td>0/28</td>
<td>Not estimable</td>
<td></td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>62</strong></td>
<td><strong>37</strong></td>
<td><strong>Not estimable</strong></td>
<td></td>
</tr>
</tbody>
</table>

Total events: 0 (Experimental), 0 (Control)
Heterogeneity: not applicable
Test for overall effect: not applicable
Test for subgroup differences: Not applicable
### Analysis 1.2. Comparison 1 Lifestyle intervention versus. usual care, Outcome 2 Overall survival (12 months).

**Review:** Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

**Comparison:** 1 Lifestyle intervention versus. usual care

**Outcome:** 2 Overall survival (12 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Risk Ratio M-H Random 95% CI</th>
<th>Weight</th>
<th>Risk Ratio M-H Random 95% CI</th>
</tr>
</thead>
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<td>n/N</td>
<td>n/N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>0/35</td>
<td>0/24</td>
<td>Not estimable</td>
<td></td>
<td>Not estimable</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>35</strong></td>
<td><strong>24</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total events: 0 (Experimental), 0 (Control)

Heterogeneity: not applicable

Test for overall effect: not applicable

Test for subgroup differences: Not applicable

### Analysis 1.3. Comparison 1 Lifestyle intervention versus. usual care, Outcome 3 Overall survival (24 months).

**Review:** Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

**Comparison:** 1 Lifestyle intervention versus. usual care

**Outcome:** 3 Overall survival (24 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Risk Ratio M-H Random 95% CI</th>
<th>Weight</th>
<th>Risk Ratio M-H Random 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n/N</td>
<td>n/N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>0/17</td>
<td>2/20</td>
<td>100.0 %</td>
<td>0.23 [ 0.01, 4.55 ]</td>
<td></td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>17</strong></td>
<td><strong>20</strong></td>
<td></td>
<td>100.0 %</td>
<td>0.23 [ 0.01, 4.55 ]</td>
</tr>
</tbody>
</table>

Total events: 0 (Experimental), 2 (Control)

Heterogeneity: not applicable

Test for overall effect: Z = 0.96 (P = 0.34)

Test for subgroup differences: Not applicable

---

*Interventions for weight reduction in obesity to improve survival in women with endometrial cancer (Review)*

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Analysis 1.4. Comparison 1 Lifestyle intervention versus usual care, Outcome 4 Adverse events-musculoskeletal.

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1 Lifestyle intervention versus usual care

Outcome: 4 Adverse events-musculoskeletal

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental n/N</th>
<th>Control n/N</th>
<th>Risk Ratio M-H, Random, 95% CI</th>
<th>Weight</th>
<th>Risk Ratio M-H, Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>McCarroll 2014</td>
<td>10/31</td>
<td>0/28</td>
<td></td>
<td>100.0%</td>
<td>19.03 [1.17, 310.52]</td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>0/16</td>
<td>0/16</td>
<td></td>
<td></td>
<td>Not estimable</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>47</td>
<td>44</td>
<td></td>
<td>100.0%</td>
<td>19.03 [1.17, 310.52]</td>
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</table>

Total events: 10 (Experimental), 0 (Control)

Heterogeneity: not applicable

Test for overall effect: Z = 2.07 (P = 0.039)

Test for subgroup differences: Not applicable
**Analysis 1.5. Comparison 1 Lifestyle intervention versus. usual care, Outcome 5 Cancer-specific survival (6 months).**

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1 Lifestyle intervention versus. usual care

Outcome: 5 Cancer-specific survival (6 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Risk Ratio</th>
<th>Weight</th>
<th>Risk Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>n/N</td>
<td>M-H, Random, 95% CI</td>
<td>M-H, Random, 95% CI</td>
<td></td>
</tr>
<tr>
<td>Allison 2016</td>
<td>0/21</td>
<td>0/9</td>
<td>Not estimable</td>
<td>Not estimable</td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>0/41</td>
<td>0/28</td>
<td>Not estimable</td>
<td>Not estimable</td>
<td></td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>62</strong></td>
<td><strong>37</strong></td>
<td>Not estimable</td>
<td>Not estimable</td>
<td></td>
</tr>
</tbody>
</table>

Total events: 0 (Experimental), 0 (Control)

Heterogeneity: not applicable

Test for overall effect: not applicable

Test for subgroup differences: Not applicable

**Analysis 1.6. Comparison 1 Lifestyle intervention versus. usual care, Outcome 6 Cancer-specific survival (12 months).**

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1 Lifestyle intervention versus. usual care

Outcome: 6 Cancer-specific survival (12 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Risk Ratio</th>
<th>Weight</th>
<th>Risk Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>n/N</td>
<td>M-H, Random, 95% CI</td>
<td>M-H, Random, 95% CI</td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>0/35</td>
<td>0/24</td>
<td>Not estimable</td>
<td>Not estimable</td>
<td></td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>35</strong></td>
<td><strong>24</strong></td>
<td>Not estimable</td>
<td>Not estimable</td>
<td></td>
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</tbody>
</table>

Total events: 0 (Experimental), 0 (Control)

Heterogeneity: not applicable

Test for overall effect: not applicable

Test for subgroup differences: Not applicable
Analysis 1.7. Comparison 1 Lifestyle intervention versus. usual care, Outcome 7 Cancer-specific survival (24 months).

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1 Lifestyle intervention versus. usual care

Outcome: 7 Cancer-specific survival (24 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Risk Ratio M-H Random 95% CI</th>
<th>Weight</th>
<th>Risk Ratio M-H Random 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Gruenigen 2009</td>
<td>0/17</td>
<td>0/20</td>
<td>Not estimable</td>
<td></td>
<td>Not estimable</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td>17</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total events: 0 (Experimental), 0 (Control)

Heterogeneity: not applicable

Test for overall effect: not applicable

Test for subgroup differences: Not applicable

0.01 0.1 1 10 100

Favours usual care Favours intervention
### Analysis 1.8. Comparison 1 Lifestyle intervention versus usual care, Outcome 8 Weight loss (6 months).

**Review:** Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

**Comparison:** 1 Lifestyle intervention versus usual care

**Outcome:** 8 Weight loss (6 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Weight</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean(SD)[kg]</td>
<td>N</td>
<td>Mean(SD)[kg]</td>
<td>IV,Random,95% CI</td>
</tr>
<tr>
<td>Allison 2016</td>
<td>21</td>
<td>-4.78 (7.09)</td>
<td>9</td>
<td>-3.5 (5.1)</td>
<td>82.7 %</td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>41</td>
<td>-3.9 (19.348)</td>
<td>28</td>
<td>0.6 (25.787)</td>
<td>13.3 %</td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>16</td>
<td>-6.25 (29.499)</td>
<td>16</td>
<td>-0.59 (29.636)</td>
<td>4.0 %</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>78</strong></td>
<td><strong>53</strong></td>
<td><strong>100.0 %</strong></td>
<td><strong>-1.88 [-5.98, 2.21]</strong></td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Tau² = 0.0; Chi² = 2 (P = 0.82); I² =0.0%
Test for overall effect: Z = 0.90 (P = 0.37)
Test for subgroup differences: Not applicable
### Analysis 1.9. Comparison 1 Lifestyle intervention versus. usual care, Outcome 9 Weight loss stratified by BMI (6 months).

**Review:** Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

**Comparison:** 1 Lifestyle intervention versus. usual care

**Outcome:** 9 Weight loss stratified by BMI (6 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Weight</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean(SD)[kg]</td>
<td>N</td>
<td>Mean(SD)[kg]</td>
<td>IV,Random,95% CI</td>
</tr>
<tr>
<td>BMI &lt;40 kg/m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>29</td>
<td>-3.82 (14.78244)</td>
<td>18</td>
<td>-0.04 (13.05601)</td>
<td>59.0 %</td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>8</td>
<td>-3 (18.33574)</td>
<td>8</td>
<td>-1.9 (11.32327)</td>
<td>17.3 %</td>
</tr>
<tr>
<td><strong>Subtotal (95% CI)</strong></td>
<td><strong>37</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>76.3 %</td>
<td>-3.18 [-10.29, 3.93]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI &gt;40 kg/m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>12</td>
<td>-4.23 (15.9546)</td>
<td>10</td>
<td>-3.18 (20.4041)</td>
<td>16.0 %</td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>8</td>
<td>-3.62 (19.84407)</td>
<td>8</td>
<td>3.07 (25.46078)</td>
<td>7.7 %</td>
</tr>
<tr>
<td><strong>Subtotal (95% CI)</strong></td>
<td><strong>20</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.7 %</td>
<td>-2.89 [-15.65, 9.88]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100.0 %</td>
<td>-3.11 [-9.32, 3.10]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Tau² = 0.0; Chi² = 1, df = 1 (P = 0.76); I² =0.0%

Test for overall effect: Z = 0.88 (P = 0.38)

Test for subgroup differences: Chi² = 0.00, df = 1 (P = 0.97), I² =0.0%
**Analysis 1.10. Comparison 1 Lifestyle intervention versus usual care, Outcome 10 Weight loss (12 months).**

**Review:** Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

**Comparison:** 1 Lifestyle intervention versus usual care

**Outcome:** 10 Weight loss (12 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Weight</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean(SD)[kg]</td>
<td>N</td>
<td>Mean(SD)[kg]</td>
<td>IV Random, 95% CI</td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>14</td>
<td>-7.51 (28.248)</td>
<td>18</td>
<td>1.63 (28.679)</td>
<td>30.1 %</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>49</strong></td>
<td><strong>42</strong></td>
<td><strong>100.0 %</strong></td>
<td><strong>-8.98 [-19.88, 1.92]</strong></td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: $\tau^2 = 0.0; \chi^2 = 0.00, df = 1 (P = 0.98); I^2 = 0.0$

Test for overall effect: $Z = 1.61 (P = 0.11)$

Test for subgroup differences: Not applicable
Analysis 1.11. Comparison 1 Lifestyle intervention versus usual care, Outcome 11 Weight loss stratified by BMI (12 months).

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1 Lifestyle intervention versus usual care

Outcome: 11 Weight loss stratified by BMI (12 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental Mean (SD) [kg]</th>
<th>Control Mean (SD) [kg]</th>
<th>Weight Mean Difference</th>
<th>Weight Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI &lt;40 kg/m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>26 -6.24 (12.79597)</td>
<td>15 -3.02 (13.0282)</td>
<td>59.7 % -3.22 [ -11.45, 5.01 ]</td>
<td></td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>6 -7.29 (14.65478)</td>
<td>8 -0.65 (11.57599)</td>
<td>20.0 % -6.64 [ -20.85, 7.57 ]</td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal (95% CI)</strong></td>
<td><strong>32</strong> 100.0 % -4.08 [ -11.20, 3.04 ]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Tau² = 0.0; Chi² = 0.17, df = 1 (P = 0.68); I² =0.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect: Z = 1.12 (P = 0.26)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 BMI &gt;40 kg/m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>9 -3.31 (19.0656)</td>
<td>9 4.24 (23.18116)</td>
<td>10.5 % -7.55 [ -27.16, 12.06 ]</td>
<td></td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>8 -8.2 (19.18842)</td>
<td>9 3.91 (23.34786)</td>
<td>9.9 % -12.11 [ -32.35, 8.13 ]</td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal (95% CI)</strong></td>
<td><strong>17</strong> 20.4 % -9.76 [ -23.84, 4.32 ]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Tau² = 0.0; Chi² = 0.10, df = 1 (P = 0.75); I² =0.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect: Z = 1.36 (P = 0.17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>49</strong> 100.0 % -5.23 [ -11.59, 1.12 ]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Tau² = 0.0; Chi² = 0.77, df = 3 (P = 0.86); I² =0.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect: Z = 1.61 (P = 0.11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for subgroup differences: Chi² = 0.50, df = 1 (P = 0.48), I² =0.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Analysis 1.12. Comparison 1 Lifestyle intervention versus usual care, Outcome 12 Weight loss (24 months).

**Review:** Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

**Comparison:** 1 Lifestyle intervention versus usual care

**Outcome:** 12 Weight loss (24 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Weight</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Gruenigen 2009</td>
<td>11 -16.48 (24.405)</td>
<td>14 1.78 (27.73)</td>
<td>100.0%</td>
<td>-18.26 [-38.73, 2.21]</td>
<td></td>
</tr>
</tbody>
</table>

**Total (95% CI)** 11 14 100.0% -18.26 [-38.73, 2.21]

*Heterogeneity: not applicable*

*Test for overall effect: Z = 1.75 (P = 0.080)*

*Test for subgroup differences: Not applicable*

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1. Lifestyle intervention versus usual care

Outcome: 13. Weight loss stratified by BMI (24 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Weight</th>
<th>Mean Difference</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 BMI &lt;40 kg/m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>von Gruenigen 2009</td>
<td>6 2.92 (25.19)</td>
<td>7 0.8 (14.78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Subtotal (95% CI)</td>
<td>6 50.7%</td>
<td>7 2.12 [ -20.82, 25.06 ]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 BMI &gt;/40 kg/m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>von Gruenigen 2009</td>
<td>5 -28.77 (23.91)</td>
<td>7 25.81 (21.64)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Subtotal (95% CI)</td>
<td>5 49.3%</td>
<td>7 -54.58 [ -80.97, -28.19 ]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>11</td>
<td>14</td>
<td>100.0%</td>
<td>-25.84 [ -81.40, 29.72 ]</td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Tau² = 1438.26; Chi² = 14.91, df = 1 (P = 0.001); I² = 90%

Test for overall effect: Z = 0.91 (P = 0.36)

Test for subgroup differences: Chi² = 10.10, df = 1 (P = 0.001), I² = 90%
### Analysis 1.14. Comparison 1 Lifestyle intervention versus usual care, Outcome 14 Adverse events-diarrhoea.

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1 Lifestyle intervention versus usual care

Outcome: 14 Adverse events-diarrhoea

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Risk Ratio M-H Random 95% CI</th>
<th>Weight</th>
<th>Risk Ratio M-H Random 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>n/N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>2/31</td>
<td>0/28</td>
<td></td>
<td>100.0%</td>
<td>4.53 [0.23, 90.51]</td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>0/16</td>
<td>0/16</td>
<td>Not estimable</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>47</strong></td>
<td><strong>44</strong></td>
<td></td>
<td>100.0%</td>
<td>4.53 [0.23, 90.51]</td>
</tr>
</tbody>
</table>

Total events: 2 (Experimental), 0 (Control)
Heterogeneity: not applicable
Test for overall effect: Z = 0.99 (P = 0.32)
Test for subgroup differences: Not applicable

### Analysis 1.15. Comparison 1 Lifestyle intervention versus usual care, Outcome 15 Cardiovascular and metabolic event frequency (6 months).

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1 Lifestyle intervention versus usual care

Outcome: 15 Cardiovascular and metabolic event frequency (6 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Risk Ratio M-H Random 95% CI</th>
<th>Weight</th>
<th>Risk Ratio M-H Random 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>n/N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allison 2016</td>
<td>0/21</td>
<td>0/9</td>
<td>Not estimable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>0/41</td>
<td>0/28</td>
<td>Not estimable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>0/16</td>
<td>0/16</td>
<td>Not estimable</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>78</strong></td>
<td><strong>53</strong></td>
<td>Not estimable</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total events: 0 (Experimental), 0 (Control)
Heterogeneity: not applicable
Test for overall effect: not applicable
Test for subgroup differences: Not applicable
### Analysis 1.16. Comparison 1 Lifestyle intervention versus usual care, Outcome 16 Cardiovascular and metabolic event frequency (12 months).

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1 Lifestyle intervention versus usual care

Outcome: 16 Cardiovascular and metabolic event frequency (12 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Risk Ratio M-H, Random 95% CI</th>
<th>Weight</th>
<th>Risk Ratio M-H, Random 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>n/N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>0/34</td>
<td>0/24</td>
<td>Not estimable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>0/17</td>
<td>0/18</td>
<td>Not estimable</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>51</strong></td>
<td><strong>42</strong></td>
<td><strong>Not estimable</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total events: 0 (Experimental), 0 (Control)

Heterogeneity: not applicable

Test for overall effect: not applicable

Test for subgroup differences: Not applicable

![Favours intervention vs Favours usual care](0.01 0.1 1 10 100)
Analysis 1.17. Comparison 1 Lifestyle intervention versus usual care, Outcome 17 Quality of life-SF12 Physical Health component (6 months).

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1 Lifestyle intervention versus usual care

Outcome: 17 Quality of life-SF12 Physical Health component (6 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Mean(SD)</td>
<td>N Mean(SD)</td>
<td>IV,Random,95% CI</td>
<td>IV,Random,95% CI</td>
</tr>
<tr>
<td>Allison 2016</td>
<td>21 4.61 (6.14)</td>
<td>9 6.9 (6.6)</td>
<td>-2.29 [ -7.34, 2.76 ]</td>
<td>100.0 %</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>21</strong></td>
<td><strong>9</strong></td>
<td><strong>100.0 %</strong></td>
<td><strong>-2.29 [-7.34, 2.76]</strong></td>
</tr>
</tbody>
</table>

Heterogeneity: not applicable

Test for overall effect: Z = 0.89 (P = 0.37)

Test for subgroup differences: Not applicable

Analysis 1.18. Comparison 1 Lifestyle intervention versus usual care, Outcome 18 Quality of life FACT-G (6 months).

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1 Lifestyle intervention versus usual care

Outcome: 18 Quality of life FACT-G (6 months)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Mean(SD)</td>
<td>N Mean(SD)</td>
<td>IV,Random,95% CI</td>
<td>IV,Random,95% CI</td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>38 6.8 (9.320933)</td>
<td>27 0.13 (9.944003)</td>
<td>6.67 [ 1.89, 11.45 ]</td>
<td>49.9 %</td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>14 0.985 (7.972825)</td>
<td>16 2.61 (4.512483)</td>
<td>-1.63 [ -6.35, 3.10 ]</td>
<td>50.1 %</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>52</strong></td>
<td><strong>43</strong></td>
<td><strong>100.0 %</strong></td>
<td><strong>2.51 [-5.61, 10.64]</strong></td>
</tr>
</tbody>
</table>

Heterogeneity: Tau² = 28.50; Chi² = 5.85, df = 1 (P = 0.02); I² =83%

Test for overall effect: Z = 0.61 (P = 0.54)

Test for subgroup differences: Not applicable

Interventions for weight reduction in obesity to improve survival in women with endometrial cancer (Review)
### Analysis 1.19. Comparison 1 Lifestyle intervention versus usual care, Outcome 19 Quality of life stratified by BMI (6 months FACT-G).

**Review:** Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

**Comparison:** 1 Lifestyle intervention versus usual care

**Outcome:** 19 Quality of life stratified by BMI (6 months FACT-G)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Weight</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N   Mean(SD)</td>
<td>N   Mean(SD)</td>
<td>IV,Random,95% CI</td>
<td></td>
<td>IV,Random,95% CI</td>
</tr>
<tr>
<td><strong>BMI &lt;40 kg/m²</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>28 6.25 (8.143228)</td>
<td>18 -1.9 (11.34486)</td>
<td>29.8 %</td>
<td>8.15 [ 2.10, 14.20 ]</td>
<td></td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>6 0.266 (11.32549)</td>
<td>8 1.93 (5.026714)</td>
<td>11.6 %</td>
<td>-1.66 [-11.37, 8.05 ]</td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal (95% CI)</strong></td>
<td>34</td>
<td>26</td>
<td>41.3 %</td>
<td>4.01 [-5.48, 13.51 ]</td>
<td></td>
</tr>
<tr>
<td><strong>BMI &gt;=40 kg/m²</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>10 8.11 (12.57908)</td>
<td>9 3.83 (6.918173)</td>
<td>13.4 %</td>
<td>4.28 [-7.73, 13.29 ]</td>
<td></td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>6 5.28 (5.282051)</td>
<td>10 1.13 (4.012481)</td>
<td>45.3 %</td>
<td>4.15 [-0.75, 9.05 ]</td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal (95% CI)</strong></td>
<td>16</td>
<td>19</td>
<td>58.7 %</td>
<td>4.18 [-0.13, 8.49 ]</td>
<td></td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td>50</td>
<td>45</td>
<td>100.0 %</td>
<td>4.69 [1.39, 7.99 ]</td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Tau² = 31.08; Chi² = 2.83, df = 1 (P = 0.09); I² = 65%
Test for overall effect: Z = 0.83 (P = 0.41)

Heterogeneity: Tau² = 0.0; Chi² = 0.00, df = 1 (P = 0.98); I² = 0%
Test for overall effect: Z = 1.90 (P = 0.057)

Heterogeneity: Tau² = 0.0; Chi² = 2.96, df = 3 (P = 0.40); I² = 0%
Test for overall effect: Z = 2.78 (P = 0.0054)
Test for subgroup differences: Chi² = 0.00, df = 1 (P = 0.97), I² = 0%

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer.

Comparison: 1. Lifestyle intervention versus usual care.

Outcome: 20. Quality of life FACT-G (12 months).

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Weight</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean(SD)</td>
<td>N</td>
<td>Mean(SD)</td>
<td>IV(Random,95% CI)</td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>34</td>
<td>5.35 (10.86217)</td>
<td>25</td>
<td>2.09 (9.617692)</td>
<td>42.6 %</td>
</tr>
<tr>
<td>von Grunigen 2009</td>
<td>14</td>
<td>2.77 (5.598273)</td>
<td>16</td>
<td>0.36 (7.032958)</td>
<td>57.4 %</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>48</strong></td>
<td></td>
<td><strong>41</strong></td>
<td></td>
<td><strong>100.0 %</strong></td>
</tr>
</tbody>
</table>

Heterogeneity: Tau^2 = 0.0; Chi^2 = 0.6, df = 1 (P = 0.81); I^2 =0.0%

Test for overall effect: Z = 1.59 (P = 0.11)

Test for subgroup differences: Not applicable
Analysis 1.21. Comparison 1 Lifestyle intervention versus usual care, Outcome 21 Quality of life stratified by BMI (12 months FACT-G).

Review: Interventions for weight reduction in obesity to improve survival in women with endometrial cancer

Comparison: 1 Lifestyle intervention versus usual care

Outcome: 21 Quality of life stratified by BMI (12 months FACT-G)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Experimental Mean (SD)</th>
<th>Control Mean (SD)</th>
<th>N</th>
<th>Mean Difference</th>
<th>Weight</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 BMI &lt;40 kg/m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>3.99 (11.20)</td>
<td>1.85 (6.35)</td>
<td>26</td>
<td>25.4 %</td>
<td>2.14</td>
<td>[ -3.17, 7.45 ]</td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>5.4 (2.48)</td>
<td>2.02 (5.51)</td>
<td>5</td>
<td>40.4 %</td>
<td>3.38</td>
<td>[ -0.83, 7.59 ]</td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td></td>
<td></td>
<td>31</td>
<td>65.8 %</td>
<td>2.90</td>
<td>[ -0.40, 6.20 ]</td>
</tr>
<tr>
<td>Heterogeneity: Tau² = 0.0; Chi² = 0.13, df = 1 (P = 0.72); I² = 0.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect: Z = 1.72 (P = 0.085)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 BMI &gt;/40 kg/m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCarroll 2014</td>
<td>9.29 (9.86)</td>
<td>2.61 (13.59)</td>
<td>8</td>
<td>5.7 %</td>
<td>6.68</td>
<td>[ -0.83, 7.59 ]</td>
</tr>
<tr>
<td>von Gruenigen 2009</td>
<td>1.61 (1.61)</td>
<td>-0.27 (7.82)</td>
<td>6</td>
<td>28.5 %</td>
<td>1.88</td>
<td>[ -3.14, 6.90 ]</td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td></td>
<td></td>
<td>14</td>
<td>34.2 %</td>
<td>2.68</td>
<td>[ -1.90, 7.26 ]</td>
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<td>Heterogeneity: Tau² = 0.0; Chi² = 0.59, df = 1 (P = 0.44); I² = 0.0%</td>
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<td>Test for overall effect: Z = 1.15 (P = 0.23)</td>
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**ADDITIONAL TABLES**

Table 1. Authors’ responses to additional information request

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<tr>
<th>Study</th>
<th>Principle Investigator contacted</th>
<th>Additional information requested</th>
<th>Answers provided</th>
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<tr>
<td>Allison 2016</td>
<td>Kelly Allison</td>
<td>Randomisation process, Blinding process, How was the study analysed, Exclusion criteria, How was missing data dealt with, Baseline characteristics, Duration of study intervention, Was a power calculation performed?</td>
<td>“The coordinating center used a computer generated algorithm to produce the randomization envelopes for each clinical site, with the general parameters of randomizing 1:1:1 across the three conditions. The envelopes are then chosen sequentially as each participant...”</td>
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</table>
Table 1. Authors’ responses to additional information request (Continued)

| Results-overall survival, adverse events, recurrence-free survival, cancer-specific survival, weight loss from baseline, cardiovascular and metabolic event frequency, change in quality of life from baseline | Funding source was enrolled. |
| Conflicts of interest | “There was no blinding. The outcome assessments were conducted by study coordinators and trained medical personnel (for blood draws, DEXA). The coordinators knew which condition the participants were in, but other medical personnel were not informed.” |
| | “Given we only had pre-post assessment data and our main analyses used paired t-tests and correlations, we were unable to do intention-to-treat analyses.” |
| | “Exclusion criteria included: age less than 18, current or recent participation in a weight loss program or use of weight loss medications; uncontrolled serious medical or psychiatric condition(s) that would affect the patient’s ability to participate in the interventional study; invasive malignancy other than EC or non-melanoma skin cancer which required active treatment currently or within the last 5 years, or current pregnancy.” |
| | “Given the pre-post assessment design, were excluded participants for variables that were not completed.” |
| | See Characteristics of included studies. Data on co-morbidities, performance status and type of endometrial cancer were not provided” |
| | “No - From the grant: The purpose will be to provide estimates for the size of an intervention effect achievable by the experimental intervention in order to power and justify a grant application for a full-scale trial of a weight loss program in women with endometrial cancer. With a sample size of 30 participants per group, the true difference in mean weight loss between the groups can be estimated with a 95% confidence interval” |
Table 1. Authors’ responses to additional information request (Continued)

<table>
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<td>Single- or multi-centre study?</td>
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<td>Reasons for non-attendance at follow-up visits</td>
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<tr>
<td></td>
<td>Methods of group allocation concealment</td>
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<tr>
<td></td>
<td>Prospectively published protocol?</td>
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<td></td>
<td>Results—overall survival, adverse events, recurrence-free survival, cancer-specific survival, weight loss from baseline, cardiovascular and metabolic event frequency, change in quality of life from baseline</td>
</tr>
</tbody>
</table>

confidence interval size of ±0.50σ, where σ is the population standard deviation of weight loss, assumed in this calculation to be the same in each of the two intervention groups and the control group. We will assess the comparability of variance across the groups and do exploratory analyses of possibly variance-stabilizing transformations. Because this is a pilot study to derive parameters to design an appropriately-powered study, hypothesis testing is not a primary goal of the statistical analysis of the data, although p-values will be calculated.

See Data and analyses. No data provided on adverse events, recurrence-free and cancer-specific survival.

"Cross-TREC study funded by NCI U54-CA155850 - University of Pennsylvania; U54 CA155626 - Harvard University; U54 CA155496CC - Washington University; U01 CA116850 - Fred Hutchinson Cancer Research Center."

None declared.
Table 1. Authors’ responses to additional information request  (Continued)

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<td>Michele McCarrroll</td>
<td>Single- or multi-centre study?</td>
<td>Reasons for non-attendance at follow-up visits</td>
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<td>Prospectively published protocol?</td>
<td>Results—overall survival, adverse events, recurrence-free survival, cancer-specific survival, weight loss from baseline, cardiovascular and metabolic event frequency, change in quality of life from baseline</td>
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</tbody>
</table>

No

See Data and analyses

APPENDICES

Appendix 1. CENTRAL search strategy

#1 MeSH descriptor: [Uterine Neoplasms] explode all trees
#2 ((uterus or uterine or endometri* or womb or corpus uteri) near5 (cancer* or tumor* or tumour* or neoplas* or carcinoma* or adenocarcinoma* or malignan*))
#3 #1 or #2
#4 MeSH descriptor: [Body Mass Index] this term only
#5 BMI
#6 MeSH descriptor: [Obesity] explode all trees
#7 MeSH descriptor: [Body Weight] explode all trees
#8 MeSH descriptor: [Adiposity] this term only
#9 obese or obesity or overweight or weight or adiposity or excess body fat

Interventions for weight reduction in obesity to improve survival in women with endometrial cancer (Review)

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Appendix 2. MEDLINE Ovid search strategy

1. exp Uterine Neoplasms/
2. ((uterus or uterine or endometri* or womb or corpus uteri) adj5 (cancer* or tumor* or tumour* or neoplas* or carcinoma* or adenocarcinoma* or malignan*)).mp.
3. 1 or 2
4. body mass index/
5. BMI.mp.
6. exp obesity/
7. exp body weight/
8. Adiposity/
9. (obese or obesity or overweight or weight or adiposity or excess body fat).mp.
10. 4 or 5 or 6 or 7 or 8 or 9
11. randomized controlled trial.pt.
12. controlled clinical trial.pt.
13. randomized.ab.
14. placebo.ab.
15. clinical trials as topic.sh.
16. randomly.ab.
17. trial.ti.
18. 11 or 12 or 13 or 14 or 15 or 16 or 17
19. 3 and 10 and 18

Appendix 3. Embase search strategy

1. exp uterus cancer/
2. ((uterus or uterine or endometri* or womb or corpus uteri) adj5 (cancer* or tumor* or tumour* or neoplas* or carcinoma* or adenocarcinoma* or malignan*)).mp.
3. 1 or 2
4. body mass/
5. BMI.mp.
6. exp obesity/
7. exp body weight/
8. (obese or obesity or overweight or weight or adiposity or excess body fat).mp.
9. 4 or 5 or 6 or 7 or 8
10. crossover procedure/
11. double-blind procedure/
12. randomized controlled trial/
13. single-blind procedure/
14. random*.mp.
15. factorial*.mp.
16. (crossover* or cross over* or cross-over*).mp.
17. placebo*.mp.
18. (double* adj blind*).mp.
19. (singl* adj blind*).mp.
20. assign*.mp.
21. allocat*.mp.
22. volunteer*.mp.
23. 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22
CONTRIBUTIONS OF AUTHORS
All review authors contributed to the study conception and design.
Aquisition of data was undertaken by Sarah Kitson, Neil Ryan and Michelle MacKintosh
Analysis and interpretation were undertaken by Sarah Kitson, Neil Ryan, James Duffy, Richard Edmondson and Emma Crosbie.
Drafting of the manuscript was performed by Sarah Kitson, James Duffy, and Emma Crosbie and was reviewed by all authors.
The review update will be undertaken by Emma Crosbie.

DECLARATIONS OF INTEREST
Sarah Kitson: None known.
Neil Ryan: None known.
Michelle MacKintosh: None known.
Richard Edmondson: None known.
James Duffy: None known.
Emma Crosbie: None known.

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Dr Emma Crosbie has been awarded funding via the Cochrane Review Support Programme to expedite the completion of this review which is a priority topic area.

External sources
• National Institute for Health Research Clinician Scientist Fellowship, UK.
Dr Emma Crosbie and Dr Sarah Kitson are funded through an National Institute for Health Research (NIHR) Clinician Scientist award (NIHR-CS-012-009). The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.
• MRC, UK.
Dr Neil Ryan is an Medical Research Council Doctoral Research Fellow (MR/M018431/1)
DIFFERENCES BETWEEN PROTOCOL AND REVIEW

For the outcomes of overall and cancer-specific survival insufficient data were available from published reports or correspondence with study authors to allow the calculation of hazard ratios. Instead, survival was treated as a dichotomous outcome and the risk ratio for survival was calculated in its place. Depending upon the assembled research, the study authors had planned to organise the data by population and, within the data categories, to explore the main comparisons of the review. Due to the small number of studies and participants included in the review this was not possible.
Appendix 3

Ki-67 in endometrial cancer: scoring optimization and prognostic relevance for window studies

Sarah Kitson1,2,9, Vanitha N Sivalingam1,2,9, James Bolton3, Rhona McVey3, Mashid Nickkho-Amiry1,2, Melanie E Powell4, Alexandra Leary5, Hans W Nijman6, Remi A Nout7, Tjalling Bosse8, Andrew G Renehan1, Henry C Kitchener1, Richard J Edmondson1,2 and Emma J Crosbie1,2

1Division of Molecular and Clinical Cancer Sciences, Faculty of Biology, Medicine and Health, University of Manchester, St Mary’s Hospital, Manchester, UK; 2Department of Obstetrics and Gynaecology, Central Manchester University Hospitals NHS Foundation Trust, Manchester, Academic Health Science Centre, Manchester, UK; 3Department of Histopathology, Central Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, UK; 4Department of Clinical Oncology, Barts Health NHS Trust, London, UK; 5INSERM U981 and Department of Medicine, Gynecology Unit, Gustave Roussy, Villejuif, France; 6Department of Gynecology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; 7Department of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands and 8Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands

Ki-67, a marker of cellular proliferation, is increasingly being used in pre-surgical window studies in endometrial cancer as a primary outcome measure. Unlike in breast cancer, however, there are no guidelines standardizing its measurement and its clinical relevance as a response biomarker is undetermined. It is, therefore, imperative that Ki-67 scoring protocols are optimized and its association with patient survival rigorously evaluated, in order to be able to clinically interpret the results of these studies. Using the International Ki-67 in Breast Cancer Working Group guidelines as a basis, whole slide, hot spot and invasive edge scoring protocols were evaluated using endometrial biopsies and hysterectomy specimens from 179 women. Whole sections and tissue microarrays, manual and semi-automated scoring using Definiens Developer software were additionally compared. Ki-67 scores were related to clinicopathological variables and cancer-specific survival in uni- and multivariate analysis. Against criteria of time efficiency, intra- and inter-observer variability and consistency, semi-automated hot spot scoring was the preferred method. Ki-67 scores positively correlated with grade, stage and depth of myometrial invasion (P-values all < 0.03). By univariate analysis, higher Ki-67 scores were associated with a significant reduction in cancer-specific survival (P ≤ 0.05); however, this effect was substantially attenuated in the multivariate model. In conclusion, hot spot scoring of whole sections using Definiens is an optimal method to quantify Ki-67 in endometrial cancer window study specimens. Measured this way, it is a clinically relevant marker, though further work is required to determine whether reductions in Ki-67 in neoadjuvant intervention studies translate into improved patient outcome.

Modern Pathology (2017) 30, 459–468; doi:10.1038/modpathol.2016.203; published online 2 December 2016

Despite the rising incidence and corresponding increase in deaths from endometrial cancer, there is a noticeable lack of research into new prevention and treatment strategies.1,2 There is a dearth of high quality clinical trial evidence to inform the management of women with advanced or recurrent endometrial cancer. Similarly, there is a lack of robust evidence to guide the clinical care of women who are unfit for surgery or who desire fertility-sparing treatment.

Clinical trials require large numbers of participants over long follow-up periods to demonstrate the superiority of one treatment over another on
clinically important outcomes, such as overall and cancer-free survival. In cancer types that are amenable to diagnostic sampling, novel interventions can be screened for efficiently using the pre-surgical window study design, whereby tissue endpoints are compared before (at diagnosis) and after treatment (at definitive surgery) using biomarkers as surrogates for clinical endpoints. Ideally such biomarkers should have prognostic utility and be able to predict response to adjuvant treatment and longer term outcome. Their use allows the rapid screening of new interventions so that time, effort, and financial resources can be directed at treatments that hold the most promise.

Window studies in endometrial cancer are hampered by the lack of validated biomarkers meeting these criteria. In breast cancer, the nuclear protein Ki-67 is an established prognostic and predictive biomarker. Expressed only during the active G1, S, and G2 phases of the cell cycle, its expression is a marker of cellular proliferation and is readily detected by immunohistochemistry. The International Ki-67 in Breast Cancer Working Group set standards for the staining, scoring and analysis of Ki-67 in breast cancer to ensure the reproducibility, reliability and accuracy of studies using Ki-67 as their primary outcome measure. In brief, these included:

- Sole use of the MIB-1 antibody with heat-induced epitope retrieval
- Inclusion of positive and negative controls in all batches
- Scoring at least three high-power fields (x40 magnification) across whole sections, incorporating the invasive edge of the tumor and hot spots
- Assessment of nuclear staining only (intensity of staining not relevant)
- Counting at least 500 (and preferably 1000) malignant cells
- Expressing the Ki-67 score as the percentage of positively stained cells among the total number of malignant cells assessed

Despite ambiguity in the literature about the value of Ki-67 as a biomarker in endometrial cancer, pre-surgical window studies using a change in Ki-67 as their primary endpoint have begun in earnest. Although Ki-67 expression has been shown to positively correlate with tumor grade, there is a lack of consensus as to whether it has prognostic value. Heterogeneity of staining, scoring, and analysis protocols, including the use of study-specific cut-off values, have also hampered the validation of findings in other cohorts and, by extension, hindered the clinical interpretation of results from the aforementioned window studies. Furthermore, most previous studies were published over 10 years ago, using the now superseded FIGO 1988 staging criteria, limiting their applicability to modern clinical research.

The aims of this study were three-fold: to identify the most reliable, reproducible and time efficient method of Ki-67 scoring using the recommendations of the International Ki-67 in Breast Cancer Working Group as a guide; to determine the correlation between Ki-67 and known pathological prognostic variables; and to investigate whether higher Ki-67 expression is associated with a shorter cancerspecific survival and, therefore, has clinical value as a biomarker in endometrial cancer trials.

Materials and methods

Patient and Tissue Selection

The study was designed, analyzed, and reported in accordance with the REMARK guidelines for tumor marker prognostic studies. Tumor tissues from 179 patients undergoing hysterectomy for endometrial cancer were retrospectively selected. This included 128 consecutive patients who had donated tissue for research to the Manchester BRC Biobank from 2009 to 2014. Due to a preponderance of low grade and stage disease in this cohort, an additional 51 high-risk patients, for whom tissue and clinical follow-up data were available, were included from partner institutions of the TransPORTEC consortium (Leiden University Medical Center, The Netherlands; University Medical Center Groningen, The Netherlands; University College London, United Kingdom; and Gustave Roussy Paris, France) to ensure a representative population. This latter group included patients who had undergone primary surgery between 1991 and 2010. All grades, stages, and histological subtypes of endometrial cancer were included. All patients underwent surgery. Patients with intermediate or high-risk disease were given adjuvant treatment according to local protocols.

Tumor from hysterectomy specimens and, for a subset of tumors, corresponding endometrial biopsies taken immediately before the start of surgery, were formalin fixed and paraffin embedded, and stored at room temperature for up to 24 years. Four-μm thick sections were cut from representative paraffin blocks using a cryostat and mounted onto a histological glass slide. Slides were either stained immediately or stored at +4 °C pending immunohistochemistry. Whole hematoxylin and eosin-stained slides were reviewed by experienced gynaecological histopathologists (JB, RM, and TB) to confirm FIGO (2009) stage, histological subtype, grade, depth of myometrial invasion and the presence or absence of lymphovascular space invasion. Tissue microarrays were created by the study histopathologists from hysterectomy specimens for a subset of the Manchester patients and the transPORTEC cohort using triplicate tumor cores. This allowed the effect of slide preparation technique to be determined by comparing Ki-67 scores from whole sections and tissue microarrays obtained from the same tumor.
**Immunohistochemistry**

Immunohistochemistry was performed using the Leica Bond Max (Leica Biosystems, Wetzlar, Germany) with heat-induced epitope retrieval. This fully automated system is routinely used in many hospitals and ensures consistent staining across runs. Staining was performed using the optimized protocol recommended by the International Ki-67 in Breast Cancer Working Group. Antigen retrieval was undertaken at pH 9 for 20 min. A casein block of 30 min duration was carried out to reduce nonspecific antibody binding. Slides were incubated at room temperature for 1 h with the MIB-1 antibody (monoclonal mouse, anti-human Ki-67 antibody; Dako, Carpinteria, CA), at a dilution of 1:100. Primary antibody detection was undertaken using the Refine Detection Kit (Leica Biosystems), which contains a rabbit anti-mouse IgG secondary antibody and anti-rabbit poly-HRP IgG antibody and utilizes 3,3'-diaminobenzidine as a chromogen. Slides were counterstained with hematoxylin. Negative (isotype control) and positive (tonsil) controls were used for quality assurance.

**Ki-67 Scoring**

Slides were digitized using the Leica SCN400 Slide Scanner (Leica Microsystems, Wetzlar, Germany). A semi-automated score was obtained by applying a computerized algorithm (Definiens Developer) to the malignant glands (Figure 1a and b). Manual selection of malignant glands guaranteed that scoring was limited to these areas and that stromal and inflammatory cells were excluded. In the case of carcinosarcomas, only malignant glands (the carcinoma component) were selected for scoring. Manual selection of malignant glands was repeated prior to each application of the algorithm. Malignant glands were visually compared prior to and following application of the Definiens Developer solution to ensure the correct classification of nuclei as positively and negatively stained and that debris and artifact were reliably excluded. All stained nuclei were counted as positive, irrespective of staining intensity. Different algorithms were tried and their accuracy checked for whole section and tissue microarray analyses, although similar rules and thresholds applied. For each algorithm, the accuracy of nuclei detection was confirmed using a subset of 12 randomly selected slides. For whole slides, the Ki-67 proliferation index, referred to hereafter as the Ki-67 score, was the percentage of positively stained nuclei scored according to three methods: whole slide, hot spot, and invasive edge scoring. For manual scoring, the percentage of positively stained nuclei within three high-powered fields (×40 magnification) randomly selected across the tumor was calculated, ensuring at least 1000 nuclei were counted. Using the semi-automated system, all nuclei within three (hot spot and invasive edge) or five (whole slide) representative high-powered fields (×20) were scored (at least 2000 nuclei in total). The areas to be scored were selected randomly across the section to take into account the heterogeneous proliferation seen in endometrial tumors (whole slide scoring), from areas of maximal Ki-67 staining (hot spot scoring) or from the endometrial/myometrial interface (invasive edge scoring) by two independent scorers (SK and VS), who were blinded to patient outcome (Figure 1c–h). For the tissue microarrays, all malignant glands of each tumor core were scored in their entirety.

Individual tumor cores and full sections were scored three times (twice by SK, once by VS) and the final Ki-67 score was calculated as the mean value of the three repeats. For the tissue microarrays, the final Ki-67 score for each tumor was the average of nine measurements; three cores from each tumor scored on three separate occasions. Discordant results of >10% (between SK and VS) were settled by consensus. The time to score individual slides was measured using a stopwatch.

**Follow-up Data Collection**

Demographic, pathology, and follow-up data were obtained from electronic and hard copy patient records. In Manchester, patients were reviewed in specialist clinics every 4 months for the first two years and six monthly thereafter for a total of five years. The detection of recurrent disease was by way of symptom enquiry and clinical examination, with imaging as required. Cause of death was determined from primary care and mortuary records. For the transPORTEC patients, clinical follow-up data were provided by individual clinicians and stored in a secure database. All cases without events were censored at the last follow-up visit.

**Statistical Analysis**

Tumor availability and consent for follow-up data collection limited the sample size to 179 patients; similar numbers to previous studies of Ki-67 in endometrial cancer. Importantly, this cohort included 26 endometrial cancer-related deaths and 41 recurrences, ensuring that the study was adequately powered to investigate the effect of Ki-67 on endometrial cancer recurrence and survival. Ki-67 was measured as a continuous score using the hot spot method and data conformed to a negatively skewed distribution (Figure 2). Intraclass correlation coefficient. Bland–Altman plots were constructed to compare scores from endometrial biopsies and corresponding hysterectomy specimens and different slide preparation techniques, with 95% limits of agreement interpreted clinically. The association between Ki-67 and other pathological and clinical variables was tested using the...
Mann–Whitney U-test for non-parametric data and Spearman rank correlation for continuous and ordinal variables. Kaplan–Meier curves were constructed to estimate cancer-specific survival according to Ki-67 score and the log-rank test for trend used to compare curves. Cancer-specific survival was defined as the time between date of surgery and death from endometrial cancer. Recurrence-free survival was the interval between date of surgery and first documentation of recurrent disease. A Cox proportional hazard regression model was used in uni- and multivariate analyses of cancer-specific and recurrence-free survival, after confirming that the data complied with the proportional hazards assumption using log–log curves. These analyses examined Ki-67 as a continuous variable, using 10% increments to derive hazard ratios. The univariate analysis included previously documented important co-variates; age, body mass index (<30 kg/m² vs ≥30 kg/m²), grade (1, 2, and 3), stage (1, 2, 3, and 4), histological type (endometrioid vs non-endometrioid), lymphovascular space invasion (presence vs absence), depth of myometrial invasion (<50% vs ≥50%), and adjuvant therapy use (yes vs no). The multivariate analysis utilized the significant

Figure 1 Ki-67 immunohistochemistry and scoring using Definiens Developer on whole sections and tissue microarrays. The accuracy of the solution to correctly identify individual positively and negatively stained nuclei was manually checked by comparing individual endometrial cancer glands with and without the solution applied. (a) Photomicrograph of endometrial cancer gland with Ki-67 immunohistochemistry applied (b) digital scoring output (>20 magnification). Positively stained nuclei are yellow, negatively stained nuclei are blue. (c) Representative tissue microarray core following Ki-67 immunohistochemistry. (d) Same tissue microarray core following the application of Definiens Developer solution, with endometrial cancer glands shown in orange and the surrounding stroma in dark blue. Areas of the slide without the presence of tissue are colored pale blue. (e) Whole section of tumor following Ki-67 immunohistochemistry. Compared with the tissue microarray, a significantly greater tumor area is present on a whole section and is a better representation of the heterogeneity in proliferation seen across endometrial cancers. (f) The same section of tumor with the five areas selected at random using Definiens Developer software highlighted in orange to determine the whole slide score. (g) Three areas of greatest proliferation identified to provide a hot spot score. (h) Three areas along the endometrial/myometrial interface to quantify the invasive edge score.

Figure 2 Frequency distribution of Ki-67 scores, as measured by the hot spot scoring method in 179 patients. The median Ki-67 score was 40%, with an interquartile range of 24–52%.
prognostic variables identified in the univariate analysis. The model was developed using forward stepwise regression and confirmed using backward stepwise regression. Both methods produced identical results. A $P$-value of $\leq 0.05$ was regarded as being of statistical significance. The statistical analysis was carried out using SPSS version 22 and GraphPad Instat.

**Results**

**Optimization of Ki-67 Scoring**

The semi-automated platform, combined with whole slide and hot spot scoring methods, demonstrated excellent intra- and inter-observer agreement, comparable to that seen with manual scoring (Table 1). The intra-class correlation coefficient values of 0.906–0.962 correspond to ‘almost perfect’ agreement between repeated measurements by the same and different observers. Invasive edge scoring, in contrast, had lower reproducibility (intra-class correlation coefficient 0.750–0.868) and could only be performed on the 50% of available slides in which the endometrial/myometrial interface was sampled, limiting the value of this scoring method. Semi-automated scoring was considerably more time efficient than manual scoring, saving over 4 min per slide (2.2–3.1 min vs 7.7 min).

Whole slides and tissue microarrays from the same tumor were available for a subset of the Manchester patients ($n=17$) and 50 of the 51 TransPORTEC patients. In general, there was poor agreement between whole slide and tissue microarray scores for individual patients (Figure 3a and Table 2), particularly when slides had been cut at the same time but stained several months apart. Delayed staining (of 3 months or more) resulted in much lower Ki-67 scores (data not shown). Within tissue microarrays, there was substantial variation in scores between individual cores from the same tumor and between observers (inter-observer intra-class correlation coefficient 0.701).

As the window study design necessitates analysis of tumor tissue prior to and following pre-surgical intervention, the consistency of Ki-67 scores across different tumor sampling techniques is important. Scores determined using the whole slide scoring method and Definiens software varied significantly between endometrial biopsies taken immediately prior to surgery and the corresponding hysterectomy specimen (Figure 3b, 95% limits of agreement −18 to +38%). Hot spot scoring (Figure 3c) was more consistent, with the exception of a single outlier, which, when removed, reduced the 95% limits of agreement to −7 to 13%. On the basis of these findings, hot spot scoring was deemed the optimal scoring method and was applied in survival analyses to determine the clinical relevance of Ki-67.

<table>
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<th>Semi-automated scoring n=179</th>
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<td>Intra-observer intra-class correlation coefficient</td>
<td>0.91</td>
<td>0.96</td>
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<tr>
<td>Inter-observer intra-class correlation coefficient</td>
<td>0.92</td>
<td>0.96</td>
</tr>
<tr>
<td>Percentage of slides possible to score in cohort</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Time per slide (min)</td>
<td>7.7</td>
<td>2.3 (+2 min to run solution)</td>
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Table 1: Comparison of manual and semi-automated scoring of Ki-67 expression

Invasive edge scoring had lower intra- and inter-observer reproducibility than manual whole slide or hot spot scoring, and could only be performed on the 50% of available slides in which the endometrial/myometrial interface was sampled. Manual scoring had ‘almost perfect’ intra- and inter-observer reproducibility but took considerably longer to perform than whole slide and hot spot scoring using the Definiens Developer algorithm.
Clinical Relevance of Ki-67

The cohort included 116 endometrioid and 63 non-endometrioid type (including serous, clear cell, carcinosarcoma, mixed, and undifferentiated) cancers, of which 108 were FIGO stage 1 (60%), 22 were stage 2 (12%), 42 were stage 3 (24%), and 6 were stage 4 (3%). The estimated median follow-up time, using the reverse Kaplan–Meier method, was 39.5 months, during which time 41 (23%) patients had local (22, 12%) and/or distant recurrences (35, 20%). There were 47 deaths (26%), of which 26 (15%) were from endometrial cancer. For grade 1/2 endometrioid, grade 3 endometrioid and non-endometrioid type cancers, 5-year cancer-specific survival rates were 93%, 88%, and 43% ($P < 0.001$), respectively.

The median Ki-67 score in the overall cohort was 40%, with an interquartile range of 24–52%. The relationship between Ki-67 and patient clinicopathological characteristics was investigated (Table 3). As expected, Ki-67 score was closely associated with tumor grade ($P \leq 0.001$). In addition, it was also positively correlated with patient age, stage, depth of myometrial invasion, and adjuvant therapy use ($P$-values all $\leq 0.04$). Scores were higher in those tumors with lymphovascular space invasion present and non-endometrioid histology, though these results did not reach statistical significance.

Ki-67 scores were divided into two equal groups using the median score of 40% to denote low and high expression, to explore the relationship between Ki-67 and cancer-specific survival. The Kaplan–Meier curves suggested that greater tumor proliferation was associated with a significant reduction in survival; 5-year cancer-specific survival rates were 58% for those tumors with high Ki-67 expression, compared with 88% for those with tumors with low Ki-67 expression (Figure 4, $P = 0.05$).

In a univariate analysis, Ki-67 score, as a continuous variable, as well as age, grade, stage, and histological type of endometrial cancer, presence or absence of lymphovascular space invasion and depth of myometrial invasion, was a prognostic indicator of cancer-specific survival (Table 4). A 10% increase in Ki-67 was associated with a 31% (95% CI 7–60%) worsening of cancer-specific survival. After adjustment for important clinicopathological variables and Ki-67 score, only age,
stage and histological type of endometrial cancer remained independent prognostic variables for cancer-specific survival (Table 3). Ki-67 failed to reach statistical significance in the multivariate analysis.

Table 3 Relation between patient characteristics and Ki-67 score

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients (%)</th>
<th>Median Ki-67 score % (interquartile range)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>179</td>
<td>40 (24–52)</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (years)</td>
<td>68</td>
<td></td>
<td>0.022*</td>
</tr>
<tr>
<td>Interquartile range (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td></td>
<td></td>
<td>0.663</td>
</tr>
<tr>
<td>&lt; 25</td>
<td>22 (12)</td>
<td>44 (26–58)</td>
<td></td>
</tr>
<tr>
<td>25–29.9</td>
<td>35 (20)</td>
<td>28 (17–47)</td>
<td></td>
</tr>
<tr>
<td>≥ 30</td>
<td>64 (36)</td>
<td>41 (26–51)</td>
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<tr>
<td>Missing data</td>
<td>58 (32)</td>
<td>43 (28–55)</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td>&lt;0.0001****</td>
</tr>
<tr>
<td>1</td>
<td>39 (22)</td>
<td>23 (10–39)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>36 (20)</td>
<td>41 (26–50)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>104 (58)</td>
<td>44 (30–55)</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td>0.014**</td>
</tr>
<tr>
<td>1</td>
<td>108 (60)</td>
<td>36 (22–50)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>22 (12)</td>
<td>37 (24–55)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>42 (24)</td>
<td>44 (29–56)</td>
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<tr>
<td>4</td>
<td>6 (3)</td>
<td>54 (45–67)</td>
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<tr>
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<td>1 (1)</td>
<td>17 (17–17)</td>
<td></td>
</tr>
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<td>Histological type</td>
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<td></td>
<td>0.246</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>116 (65)</td>
<td>37 (23–51)</td>
<td></td>
</tr>
<tr>
<td>Non-endometrioid</td>
<td>63 (35)</td>
<td>44 (28–54)</td>
<td></td>
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<tr>
<td>Lymphovascular space invasion</td>
<td></td>
<td></td>
<td>0.138</td>
</tr>
<tr>
<td>Absent</td>
<td>93 (52)</td>
<td>37 (22–50)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>70 (39)</td>
<td>42 (25–55)</td>
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<tr>
<td>Missing data</td>
<td>16 (9)</td>
<td>45 (31–53)</td>
<td></td>
</tr>
<tr>
<td>Depth of myometrial invasion</td>
<td></td>
<td></td>
<td>0.030*</td>
</tr>
<tr>
<td>&lt; 50%</td>
<td>83 (46)</td>
<td>32 (20–50)</td>
<td></td>
</tr>
<tr>
<td>≥ 50%</td>
<td>92 (51)</td>
<td>43 (28–53)</td>
<td></td>
</tr>
<tr>
<td>Missing data</td>
<td>4 (2)</td>
<td>42 (33–51)</td>
<td></td>
</tr>
<tr>
<td>Any adjuvant therapy</td>
<td></td>
<td></td>
<td>0.031*</td>
</tr>
<tr>
<td>No</td>
<td>61 (34)</td>
<td>32 (22–49)</td>
<td></td>
</tr>
<tr>
<td>Yesb</td>
<td>102 (57)</td>
<td>44 (27–55)</td>
<td></td>
</tr>
<tr>
<td>Missing data</td>
<td>16 (9)</td>
<td>35 (26–45)</td>
<td></td>
</tr>
</tbody>
</table>

Ki-67 expression positively correlated with age at the time of surgery, grade, and stage of endometrial cancer. Higher scores were seen in tumors with >50% myoinvasion compared with more superficially invasive cancers and women receiving adjuvant therapy. There was no association between Ki-67 and body mass index, histological type of endometrial cancer, and lymphovascular space invasion (P > 0.05).

bGrade 3 endometrioid tumors are classified within the endometrioid subtype. Non-endometrioid tumors include serous, clear cell, carcinosarcomas, mixed, and undifferentiated cancers.

bAdjuvant treatment included external beam radiotherapy (44, 25%), vaginal brachytherapy (21, 12%) or both (25, 14%), and/or chemotherapy (39, 22%), which was single-agent carboplatin (2, 1%) or carboplatin/paclitaxel-based (29, 16%). Data on chemotherapy regime absent in 8 (5%) of cases. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Analyses were repeated using recurrence-free survival as the outcome of interest and produced similar results.

Discussion

This is the first study to compare semi-automated scoring using Definiens Developer software with manual Ki-67 scoring in endometrial cancer. Although unable to differentiate between malignant glands and stromal tissue, when the areas to be scored were manually selected by observers blinded to outcome, the accuracy and reproducibility of scoring by Definiens was extremely high. Automated scoring was superior to manual scoring in terms of speed and it was reliable across time and between scorers.

Compared with whole slide and invasive edge scoring, hot spot scoring was the most reproducible scoring method for Ki-67, with excellent intra- and inter-observer agreement, and the most consistent across different endometrial tumor-sampling techniques. This is of particular importance for window studies using Ki-67 as a primary outcome measure, where an endometrial biopsy taken prior to intervention is frequently compared with the hysterectomy specimen at the end of treatment to determine response.

The scoring of whole slides was found to be superior to that of tissue microarrays in terms of both reproducibility and consistency. There are no published comparisons of Ki-67 assessment by tissue microarray and whole slide scoring in the breast cancer literature for guidance, but the International Ki-67 in Breast Cancer Working Group do note anecdotal evidence for lower scoring on tissue microarrays and advise avoiding their use when establishing quantitative relationships with clinical
outcomes. A study in ovarian cancer similarly showed that Ki-67 staining of tissue microarray cores may not be representative of the results obtained from whole section immunohistochemistry. Appreciation of the heterogeneity of staining seen within whole sections of endometrial tumors is lost when only a small area is sampled in a core, reflected in the poor correlation of scores. This becomes even more evident if there is a time interval between slides being cut and stained; a delay in staining of more than 6 weeks resulted in lower Ki-67 scores. This has previously been described for sections stored under varying conditions; even at 4 °C the resulting hydrolysis negatively impacts on antigenicity. The authors, therefore, recommend undertaking staining on freshly cut sections to avoid this problem and limiting assessment to whole sections only. If freshly cut sections are not available, it is important that all slides are cut at the same time and later stained together for accurate comparison within a study, with the caveat that this limits comparability between studies.

Using the optimized methodology of semi-automated hot spot scoring, Ki-67 score was strongly associated with known pathological prognostic variables, including grade, stage, and depth of myometrial invasion. Although not independent of other prognostic factors, high Ki-67 was associated with poor cancer outcomes. These data are consistent with those of Salvesen et al, Stefansson et al, Geisler et al, and Liu et al, who described Ki-67 as a prognostic biomarker in endometrial cancer, although significance was generally lost after adjusting for important pathological variables like grade of disease and histological subtype. These studies were considerably larger than those of Fanning et al and Huvila et al, who published conflicting results; the latter studies had fewer disease events, shorter follow-up periods and were fundamentally underpowered to detect a significant effect of Ki-67 on cancer-specific outcomes.

Table 4 Univariate and multivariate analysis of associations between Ki-67 score and standard variables and cancer-specific survival in 179 women with endometrial cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th></th>
<th></th>
<th>Multivariate analysis</th>
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<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>P-value</td>
<td>HR</td>
<td>95% CI</td>
<td>P-value</td>
</tr>
<tr>
<td>Age (1 year)</td>
<td>1.10</td>
<td>1.05–1.15</td>
<td>&lt; 0.0001****</td>
<td>1.06</td>
<td>1.001–1.13</td>
<td>0.028*</td>
</tr>
<tr>
<td>Body mass index</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 30 kg/m²</td>
<td>1.00</td>
<td>—</td>
<td>0.52</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>≥ 30 kg/m²</td>
<td>1.51</td>
<td>0.43–5.25</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>—</td>
<td>0.014**</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>0.584</td>
<td>0.05–6.44</td>
<td>—</td>
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<tr>
<td>3</td>
<td>4.975</td>
<td>1.17–21.12</td>
<td>—</td>
<td>—</td>
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<td>—</td>
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<td>Stage</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>—</td>
<td>&lt; 0.0001****</td>
<td>1.00</td>
<td>—</td>
<td>0.004**</td>
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<tr>
<td>2</td>
<td>0.96</td>
<td>0.12–7.99</td>
<td>—</td>
<td>3.27</td>
<td>0.34–31.78</td>
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<tr>
<td>3</td>
<td>7.8</td>
<td>3.03–20.11</td>
<td>—</td>
<td>6.44</td>
<td>2.07–20.06</td>
<td>—</td>
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<tr>
<td>4</td>
<td>23.36</td>
<td>6.49–84.05</td>
<td>—</td>
<td>16.09</td>
<td>2.83–91.36</td>
<td>—</td>
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<td>Histological type</td>
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<td></td>
</tr>
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<td>Endometrioid</td>
<td>1.00</td>
<td>—</td>
<td>0.0001****</td>
<td>1.00</td>
<td>—</td>
<td>0.002**</td>
</tr>
<tr>
<td>Non-endometrioid</td>
<td>8.16</td>
<td>3.27–20.39</td>
<td>—</td>
<td>5.72</td>
<td>1.93–16.95</td>
<td>—</td>
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<tr>
<td>Lymphovascular space invasion</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Present</td>
<td>4.54</td>
<td>2.21–9.33</td>
<td>&lt; 0.0001****</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Depth of myometrial invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 50%</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>≥ 50%</td>
<td>2.46</td>
<td>1.03–5.90</td>
<td>0.043*</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Any adjuvant therapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.00</td>
<td>—</td>
<td>0.202</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Yes</td>
<td>2.05</td>
<td>0.68–6.19</td>
<td>—</td>
<td>—</td>
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<td>—</td>
</tr>
<tr>
<td>Ki-67 score (10% increase)</td>
<td>1.31</td>
<td>1.07–1.60</td>
<td>0.010**</td>
<td>1.14</td>
<td>0.91–1.41</td>
<td>0.257</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; HR, hazard ratio.
Ki-67 was no longer statistically significantly associated with cancer-specific survival when included in the multivariate analysis. Variables found to be statistically significantly associated with cancer-specific survival in the univariate analysis (P ≤ 0.05) were included in the multivariate analysis; ‘—’ represents not statistically significant in the multivariate analysis. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
Detailed clinical follow-up and expert pathology review are strengths of this study. The included population was sufficiently large to ensure that the study was adequately powered; 10–25 events are required per prognostic variable under investigation.19 Metcful documentation of date of recurrence and cause of death allowed cancer-specific and recurrence-free survival to be calculated, arguably more clinically relevant endpoints than the overall survival used in other studies.16,18

The median Ki-67 score was similar to that of other studies (40% vs 33–40%),14,15,18 who used median Ki-67 to dichotomize tumors into low and high Ki-67 expression. This approach is crude and prevents extrapolation across study populations. Ours is the first study to consider Ki-67 as a continuous variable, equating a 10% increase in Ki-67 expression with a cancer-specific survival hazard ratio of 1.31. This information is important for clinical trials using Ki-67 as a primary endpoint as it provides some degree of clinical context in which to interpret the results. The magnitude of effect seen in this study is similar to that shown in breast cancer studies, where Ki-67 expression is routinely log transformed to normalize the data. In breast cancer, the hazard ratio per 2.7-fold increase in Ki-67 expression was 1.95 for recurrence-free survival.23 Applying the same methodology to our findings for ease of comparison, the hazard ratio for recurrence-free survival in endometrial cancer was 1.94 (95% CI 1.10–3.43).

These findings are unsurprising, given that cancer is a disorder of unregulated cell proliferation.24 When measured by different methodologies, including S-phase fraction by flow cytometry, immunohistochemical staining of proliferative cell nuclear antigen, Ki-67 or manual counting of mitotic figures, cell proliferation increases across the spectrum of endometrial cancer development, from normal endometrium through to hyperplasia and cancer, with the highest rates seen in grade 3, serous, and clear cell cancers.25,26 It is also closely associated with tumor grade and stage, known important prognostic variables in endometrial cancer.15,27 It is logical to hypothesize, therefore, that those cancers with the greatest cell proliferation will have the poorest clinical outcome and that the fastest dividing areas of the tumors (the hot spots) will be closely associated with disease metastasis and recurrence.

A limitation of this study was that too few tumors were available to adequately power the assessment of Ki-67 in a multivariate analysis, controlling for all known prognostic clinicopathological variables. The aim of this study, however, was not to ascertain whether Ki-67 could replace pathological prognostic variables, but rather to determine its value as a primary tissue endpoint for use in clinical trials, where the window is of treatment is too short to observe changes in grade and stage of disease. In breast cancer, a drop in Ki-67 following short-term treatment with neoadjuvant chemotherapy predicts long-term response to that drug in the adjuvant setting.6 Our data suggest that Ki-67 could be used to stratify patients for entry into endometrial cancer adjuvant drug trials, excluding those whose prognosis is so good that they are unlikely to derive benefit from further therapeutic intervention beyond surgery. This is tentative speculation that requires formal testing. Ideally, this should include testing the same novel therapy before and after surgery and assess changes in pre-surgical Ki-67 score alongside longer-term cancer-specific and recurrence-free survival as outcome measures. Response to treatment could then be stratified according to baseline Ki-67 score. Such data are clearly required for drugs like metformin, which is increasingly being investigated in endometrial cancer window studies, if there is to be sufficient evidence of clinical efficacy for them to be used in routine practice.

In conclusion, these data provide evidence that semi-automated scoring of Ki-67 using Definiens Developer software is reliable, reproducible, and more time efficient than manual scoring and that hot spot methodology should be employed in future clinical trials as it is the most consistent across endometrial biopsies and hysterectomy specimens. When measured using standardized protocols of immunohistochemical staining, Ki-67 is associated with endometrial cancer survival and is, therefore, a clinically relevant endpoint, though further work is required to determine whether it fulfills all of the criteria to be used as a biomarker of treatment response.

Acknowledgments

We would like to thank Professor Mitch Dowsett for his expert guidance and advice regarding study design. Assistance with slide preparation and optimization of the immunohistochemistry staining protocol was kindly provided by the Histology Department at the Cancer Research UK Manchester Institute. Slide scanning and training in the use of Definiens Developer software was provided by the Advanced Imaging Facility at the CRUK Manchester Institute. VS is funded through a Wellcome Trust/Wellbeing of Women Research Training Fellowship. EC and SK are funded through a National Institute for Health Research (NIHR) Clinician Scientist Fellowship (award reference NIHR-CS-012-009). This article presents independent research funded by the NIHR and facilitated by the Greater Manchester Local Clinical Research Network. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Disclosure/conflict of interest

The authors declare no conflict of interest.
References


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Appendix 4

Measuring the biological effect of presurgical metformin treatment in endometrial cancer

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Background: Preclinical studies in endometrial cancer (EC) show that metformin reduces cellular proliferation by PI3K-AKT-mTOR inhibition. We tested the hypothesis that short-term presurgical metformin reduces cellular proliferation in atypical endometrial hyperplasia (AEH) and endometrioid EC, and assessed the feasibility of using phosphorylated PI3K-AKT-mTOR proteins as tissue end points.

Methods: Women with AEH or EC received metformin 850 mg twice a day or no drug in the presurgical window between diagnosis and hysterectomy. Before and after the window, tissue samples were obtained; serum markers of insulin resistance (e.g. homeostasis model of assessment of insulin resistance index) were determined; and anthropometrics measured (e.g. BMI). Cell proliferation (Ki-67) and PI3K-AKT-mTOR phosphostatus were assessed by immunohistochemistry and scored blinded to treatment.

Results: Twenty-eight metformin-treated and 12 untreated patients, well matched for age and BMI, completed the study. Metformin treatment (median 20 days, range 7–34) was associated with a 17.2% reduction in tumour Ki-67 (95% CI 27.4, 7.0, P = 0.002), in a dose-dependent manner. Tumour PI3K-AKT-mTOR protein phosphostatus varied but the effects were not significant after adjusting for changes in controls.

Conclusions: Short-term metformin was associated with reduced Ki-67 expression in EC. Changes in tumour PI3K-AKT-mTOR protein phosphostatus were seen in both groups. Future studies should address the variability attributed to different sampling techniques including devascularisation of the uterus at hysterectomy.

The incidence of endometrial cancer (EC) is rising (Cancer Research UK, 2014). A major contributor to this rise is the obesity epidemic. Worldwide, the proportion of women with a BMI of 25 kg m⁻² or greater has increased from 30% to 38% over a 30-year period (Ng et al, 2014), and as many as 34% of all ECs are directly attributable to patients being overweight or obese (Arnold et al, 2015). Endometrial cancer ranks highest among all cancers in its association with obesity, with every 5 kg m⁻² increase in BMI conferring a 1.6-fold increased risk of the disease (Renehan et al, 2008; Crosbie et al, 2010). Women with type 2 diabetes mellitus (T2DM) have a two-fold increased risk of EC compared with non-diabetic women (Friberg et al, 2007), and a prospective study found up to 36% of patients with EC have undiagnosed insulin resistance (Burzawa et al, 2011). The mechanisms underpinning...
this link between obesity, insulin resistance and endometrial carcinogenesis is incompletely understood.

Metformin is first-line medical therapy for T2DM. Epidemiological data have suggested that diabetic patients taking metformin have a lower incidence of cancer compared with those taking other hypoglycaemic agents (Evans et al, 2005; Libby et al, 2009). Preclinical studies have demonstrated a growth static effect of metformin on breast, prostate, ovarian and EC cell lines, effected both through alterations in glucose metabolism and inhibition of the PI3K-AKT-mTOR signalling pathway (Zakikhani et al, 2006, 2010; Cantrell et al, 2010; Sarfstein et al, 2013). Metformin accumulates in the tumour tissue and activates AMPK, an inhibitor of the mTOR pathway (Zakikhani et al, 2006). The impact of metformin on tumour growth has been assessed in vivo using presurgical window studies, where expression of the proliferation marker Ki-67 is measured before and after treatment with metformin in patients awaiting breast (Hadad et al, 2011; Bonanni et al, 2012; Niraula et al, 2012), prostate (Joshua et al, 2014) and EC surgery (Laskov et al, 2014; Mitsuhashi et al, 2014; Schuler et al, 2015). Three previous studies (Laskov et al, 2014; Mitsuhashi et al, 2014; Schuler et al, 2015) report that metformin administration reduced Ki-67 expression in endometrial tumours when given for 2–4 weeks before hysterectomy, but all three lacked a contemporaneous control group, and thus one cannot conclusively attribute these changes to metformin.

As a long-term strategy, we wish to develop large trials using metformin in the pre- or postoperative setting in women with EC. Measurement of tumour Ki-67 expression is a useful and readily performed surrogate biomarker assay, but it is nonspecific. The putative cancer-relevant cellular mechanism for metformin offers an opportunity to include tumour biomarkers, such as phospho-4EBP-1 expression, as surrogates of response, but interpretation in human studies is not trivial, and is confounded by many factors, including sample timing in relation to drug administration and tissue handling. Our long-term aim is to test the hypothesis that metformin has a growth inhibitory effect in EC. Given the potential pitfalls listed above, in the present study, our aim was first to establish that metformin is well tolerated in this oncological setting, and then test the hypothesis that short-term metformin use reduces cellular proliferation in women with atypical endometrial hyperplasia (AEH) and endometrioid EC, and additionally assess the feasibility of using related phosphorylated PI3K-AKT-mTOR proteins as tumour end points.

**MATERIALS AND METHODS**

Clinical trial study design. This was a non-randomised trial of metformin or no drug taken during the presurgical window period between diagnosis and hysterectomy. Women with biopsy-proven AEH or endometrioid EC scheduled for hysterectomy were eligible to take part. Women with diabetes on hypoglycaemic medication, those with non-endometrioid histology and those on concomitant progestosterone therapy were excluded from the study. Women in the metformin group received metformin 850 mg twice daily for 7 to 30 days until the evening before hysterectomy. Women who declined metformin, whose window period was too short (<7 days) or whose renal function was impaired (eGFR < 45 ml min\(^{-1}\) per 1.732) were recruited to the control group, and received no drug (Figure 1).

Women were recruited from St Mary’s Hospital, Manchester and Tameside General Hospital between October 2012 and February 2014. All participants gave written, informed consent. Approvals were received from the North West Centre for Research Ethics Committee and the Medicines and Healthcare Products Regulatory Agency (MHRA). The study was prospectively registered on the European (EudraCT 2011-001382-40) and UK (ISRCTN 81570194) clinical trial databases.

Women taking metformin were monitored for toxicity by telephone call. Adverse events (AEs) were graded using Common Terminology Criteria for Adverse Events (CTCAE) v.3.0 (National Institute of Health, 2010). Where gastrointestinal side effects were intolerable, women withheld metformin until they subsided and recommended at 850 mg daily, followed by 850 mg twice daily when tolerated. A final pill count established cumulative exposure.

![CONSORT diagram for the study indicating patient screening and accrual for the course of the study](image)

**Figure 1.** Flow chart showing study enrolment, withdrawals and exclusions. Concerns about starting a new drug with potential gastrointestinal side effects, inability to adhere to strict follow-up procedures and psychological distress at their recent cancer diagnosis were the most common reasons given for declining participation in the study.

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and treatment compliance. The cumulative dose was divided by days on treatment to calculate the average daily dose.

To assess the known effects of metformin on weight and markers of insulin resistance, at baseline and hysterectomy, height, weight and BMI; waist and hip circumference; and fasting blood glucose, insulin, C-peptide, adiponectin, leptin and high-sensitivity C-reactive protein (hsCRP) were measured. The homoeostasis model of assessment of insulin resistance index (HOMA-IR) is the product of fasting glucose and insulin by 22.5 (Matthews et al., 1985). Tumour samples were taken at recruitment and at hysterectomy for histopathology and immunohistochemistry (IHC) analyses. A blind biopsy was taken at recruitment using a Pipelle endometrial sampling device; the final tumour sample was taken from the hysterectomy specimen, sampled and processed for clinical decision-making according to standard protocols. The diagnostic Pipelle was used as the baseline biopsy when hysterectomy was scheduled for <7 days’ time or the recruitment biopsy was not obtained or insufficient for analysis. Consultant gynaecological histopathologists assessed all histopathology samples. Histological subtype, grade, stage, depth of myometrial invasion and the presence of lymphovascular space invasion were assessed using the FIGO 2009 Endometrial Cancer Staging System.

Immunohistochemical analysis. The primary end point was change in Ki-67 proliferation index. This was the percentage of tumour nuclei positively stained for Ki-67 at hysterectomy compared with baseline. Automated IHC staining was performed on 4-μm formalin-fixed paraffin-embedded sections using the Leica Bond Max (Leica Biosystems, Wetzlar, Germany) with heat-induced epitope retrieval. The primary antibody, Ki-67 MIB-1 clone (Dako, Carpinteria, CA, USA), was incubated for 60 min at a 1:100 dilution. Primary antibody detection was performed using the Refine Detection Kit (Leica Biosystems). The slides were counterstained using haematoxylin and a bluing agent. Negative (isotype control) and positive (tensil) controls were used for quality assurance.

Full slides were digitised using the Leica SCN400 Slide Scanner (Leica Microsystems, Wetzlar, Germany). To reduce bias and heterogeneity, RM selected equivalent areas to be scored on the pre- and postintervention sections using the haematoxylin and eosin slides. She was blinded to treatment group and intensity of staining for Ki-67 proliferation index was determined from staining for Ki-67 in the areas she selected. The Ki-67 proliferation index was determined from >2000 nuclei scored in >3 high powered fields (×20). A semiautomated score was obtained by applying a computerised algorithm (Definiens Developer) to the malignant glands, which had been selected manually (Supplementary Figure S1). The Pipelle baseline sample was a scrape from the tumour surface while the hysterectomy specimen provided full tumour thickness. To reduce the bias inherent to comparing tumour from two different sampling methods, we restricted Ki-67 scoring to the luminal (surface) aspect of the tumour in the hysterectomy specimen. All scoring was performed by two independent scorers (VS, SK) who were blinded to time point and treatment group. The interobserver intra-assay correlation coefficient (ICC) was 0.97 (95% CI 0.96, 0.98) and any discrepancies were reviewed together and resolved by consensus agreement.

Secondary end points included phosphorylated proteins from the PI3K-AKT-mTOR pathway and apoptotic markers. Tissue microarrays (TMAs) were created from tric太平 cores of equivalent areas in pre- and postintervention biopsies selected by the study histopathologist (RM), who was blinded to treatment group. Automated IHC was performed using the Leica Bond Max (Leica Biosystems) with heat-induced epitope retrieval. The primary antibodies were: (1) phospho-AKT (p-AKT, Ser 473) at 1:50 dilution; (2) phospho-S6 (p-S6, Ser 235/236) at 1:400 dilution; (3) phospho-acetyl-CoA carboxylase (p-ACC, Ser 79) at 1:300 dilution; (4) phospho-4EBP1 (p-4EBP1, Thr 37/46) at 1:800 dilution; (5) PTEN Clone 6H2.1 (Dako) at 1:600 dilution; and (6) cleaved caspase-3 at 1:200 dilution. All antibodies were from Cell Signalling (Beverley, MA, USA), unless otherwise stated.

p53, oestrogen receptor (ER) and progesterone receptor (PR) status were analysed in the clinical histopathology laboratory according to standard protocols using the automated Ventana BenchMark XT (Ventana, Tucson, AZ, USA). The primary antibodies used were: (1) p53 Clone D07 (Leica Biosystems) at 1:50 dilution; (2) ER Clone SP1 (Roche, Basel, Switzerland); and PR Clone 1E2 (Roche). The same horseradish peroxidase-linked secondary antibody (Ventana) was used for all analyses; the chromagens were sequential DAB and copper. The slides were counterstained using haematoxylin and a bluing agent.

p-AKT, p-ACC, p-4EBP1, ER and PR staining was assessed by modified H-score, the product of area score (proportion of positively stained core, scored 0–6) and intensity of staining score (0 = none, 1 = mild, 2 = moderate, 3 = strong). p-AKT, p-ACC and p-S6 staining was assessed in the cytoplasm and nucleus, whereas p-4EBP1, ER and PR were nuclear. PTEN was positive (strong staining of entire section) or negative (<10% staining of malignant glands despite strong staining of adjacent stroma) (Garg et al., 2012). p53 was ‘mutant-like’ if >50% of the tumour nuclei showed strong staining, when discrete geographical patterns showed strong staining, or when no p53 was found in the entire tumour (McClugge et al., 2011; Nout et al., 2012). Cleaved caspase-3 (cc3) staining was scored by Definiens computerised algorithm that detected positive and negative nuclei and generated a cc3-positive index (percentage of cells positive for cc3). These were checked manually in view of the low proportion of cc3-positive cells. All TMA scoring was completed by two independent scorers (VS, SK) who were blinded to time point and treatment group. The interobserver ICCs were all >0.94 and any discrepancies were reviewed together and resolved by consensus agreement.

Enzyme-linked immunosorbsent assay. Fasting serum glucose, insulin and C-peptide were measured by automated assay according to routine clinical care standard operating procedures. Adiponectin and leptin were measured using a DuoSet ELISA Development Kit (R&D Systems, Abingdon, UK). High-sensitivity CRP was measured by an in-house antibody sandwich ELISA technique with anti-human CRP primary antibodies, calibrators and controls from Abcam (Cambridge, UK). Intra-assay coefficients of variability (CV) were 3%, 5% and 5% for adiponectin, leptin and hsCRP, respectively. Interassay CVs were 9%, 7% and 6%, respectively.

Statistical analysis. The study was powered to observe a 20% reduction in Ki-67 following treatment. Assuming a median baseline Ki-67 proliferation index of 50%, a standard deviation of 20% (in house unpublished data) and a correlation of 70% between pre- and postintervention measurements, a sample size of 29 would have 80% power to detect a 20% change in Ki-67 at the P = 0.05 significance level. We aimed to recruit 30 women to receive metformin, with opportunistic recruitment of as many contemporaneous controls as possible.

Treatment effect was analysed using an analysis of covariance linear regression model, with post-treatment score as the response variable, and baseline score, age, BMI, insulin resistance (HOMA-IR) and treatment group as covariates. The effect of treatment on serum markers of insulin resistance used the same analysis of covariance, but excluded HOMA-IR as a covariate. Correlations were calculated using Spearman’s rank-sum correlation coefficients. Descriptive statistics, including mean and s.d. for normally distributed data, and median and interquartile range (IQR), for nonparametric data, were used to compare the two groups of patients.
Study population and baseline parameters. In total, 28 women received metformin and 12 received no drug in the presurgical window period between diagnosis and hysterectomy (Figure 1). Baseline demographics are shown in Table 1. The two groups were evenly matched in age (mean 64 vs 68 years) and BMI (mean 35 vs 32 kg m\(^{-2}\)) in the treated and untreated groups, respectively. Eighty percent of all women were overweight or obese. Four had undiagnosed diabetes (fasting serum glucose > 7.0 mmol l\(^{-1}\)) and 60% were insulin resistant (fasting glucose 6.0–6.9 mmol l\(^{-1}\) or HOMA-IR > 2.8). Most women had low-grade, early-stage tumours (22 out of 28 of metformin-treated and 9 out of 12 untreated women, respectively).

Duration and tolerability of metformin treatment. Women received metformin for a median of 20 days (IQR 17, 24). Seventy-five percent of women experienced AEs but 96% of these were scored as grade 1 AEs (Table 2). Four patients withdrew from the study completely due to unacceptable gastrointestinal side effects. Thirteen others omitted one or more dose to reduce side effects. The median daily dose received was 1573 mg (IQR 1475, 1659).

Effects of metformin on Ki-67 proliferation index. Baseline Ki-67 levels were similar in the two groups (mean 50.9% (s.d. 17.1%) in the metformin-treated vs 55.6% (s.d. 25.1%) in the untreated women) (Table 3). Baseline Ki-67 was significantly associated with tumour grade (Spearman’s correlation coefficient 0.37, 95% CI 0.06, 0.62, \(P = 0.018\); Supplementary Figure S2). There was also a significant negative correlation between baseline Ki-67 expression and insulin resistance status (HOMA-IR) (Spearman’s correlation coefficient -0.43, 95% CI -0.66, -0.13, \(P = 0.006\)), but no relationship with BMI, age, stage or treatment group.

Ki-67 proliferation index was 17.2% lower following metformin treatment (adjusted mean difference –17.2% (95% CI –27.4%, –7.0%), \(P = 0.002\)) after adjustment for baseline Ki-67, age, BMI, insulin resistance (HOMA-IR) and change in the untreated women. Each line in Figure 2A represents the postintervention change in Ki-67 for an individual woman. A lower Ki-67 was shown to occur in the metformin-treated and -untreated women (median 40 days (IQR 37, 43)). The remaining five (18%) showed static or increased Ki-67 levels were similar in the two groups (mean 50.9% (s.d. 17.1%) in the untreated group (Table 3). Baseline Ki-67 was significantly associated with tumour grade (Spearman’s correlation coefficient 0.37, 95% CI 0.06, 0.62, \(P = 0.018\); Supplementary Figure S2). There was also a significant negative correlation between baseline Ki-67 expression and insulin resistance status (HOMA-IR) (Spearman’s correlation coefficient -0.43, 95% CI -0.66, -0.13, \(P = 0.006\)), but no relationship with BMI, age, stage or treatment group.

DISCUSSION

This is the largest study of presurgical metformin treatment in EC conducted to date. A particular strength of the study is the untreated control group, as the variability of serum and tissue biomarkers between diagnosis and hysterectomy has not been studied before. Although not randomised, the two groups were evenly matched in terms of age, BMI, insulin resistance status, tumour grade and stage. We found that Ki-67 expression was stable on sequential biopsies taken before hysterectomy (data not shown) and a significant reduction in Ki-67 expression was only observed at the time of hysterectomy in the metformin-treatment group. By contrast, a reduction in the expression of phosphorylated PI3K-AKT-mTOR pathway proteins was observed at hysterectomy in both the metformin-treated and -untreated women. Hysterectomy specimens were bisected and immersed in formalin within 30 min of resection. This fixation protocol is standard for routine clinical care and achieves adequate preservation of tissue architecture and the expression of stable proteins like Ki-67, but unstable phosphorylation events may be lost. Future studies should consider taking a blinded biopsy at hysterectomy before devascularisation of the uterus; this would allow preservation of unstable phosphorylation events and facilitate the comparison of tumour biomarkers pre/postintervention on sequential biopsies achieved using the same sampling method.

Most of our patients were overweight or obese and the prevalence of undiagnosed T2DM and insulin resistance was striking. These observations are consistent with previous work (Burzawa et al, 2011; Crosbie et al, 2012). Cancer clinicians should have heightened awareness that diabetes (known and undiagnosed) is common amongst women with EC. We observed changes in biomarkers of insulin resistance and adiposity between baseline and hysterectomy in both groups. Whilst weight loss and its associated impact on insulin resistance is a recognised consequence of advanced stage cancer (Fearon et al, 2011), the majority of our patients had good prognosis tumours diagnosed at an early stage. The mediator of these alterations may therefore be anxiety-induced change in fasting glucose –0.3 mmol l\(^{-1}\); insulin –7.0 mU l\(^{-1}\); HOMA-IR –2.7; and leptin –2.3 ng ml\(^{-1}\)), but these were not statistically significant after adjusting for changes in the untreated group (Table 3).
Table 1. Baseline patient and tumour characteristics at recruitment.

<table>
<thead>
<tr>
<th>Baseline parameters</th>
<th>Metformin (Mean, Median)</th>
<th>28 S.d.</th>
<th>Q1</th>
<th>Q3</th>
<th>Control (Mean, Median)</th>
<th>12 S.d.</th>
<th>Q1</th>
<th>Q3</th>
<th>P-value*</th>
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<td>70.0</td>
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<td>7.0, 70.5</td>
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<td>&gt; 80</td>
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<td>Body mass index (kg m⁻²)</td>
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<td>5</td>
<td>41.7%</td>
<td>9</td>
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<td>Waist/hip girth ratio</td>
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<td>0.84</td>
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<td>Tumour grade at hysterectomy</td>
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<td>FIGO stage at hysterectomyb</td>
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<td>Positive</td>
<td>28</td>
<td>100.0%</td>
<td>11</td>
<td>91.7%</td>
<td>0</td>
<td>0.0%</td>
<td>1</td>
<td>8.3%</td>
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<tr>
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<td>0.0%</td>
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<tr>
<td>PR expression</td>
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<td></td>
</tr>
<tr>
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<td>100.0%</td>
<td>12</td>
<td>100.0%</td>
<td>0</td>
<td>0.0%</td>
<td></td>
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<tr>
<td>Negative</td>
<td>0</td>
<td>0.0%</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<td>PTEN expression</td>
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<tr>
<td>Wild type</td>
<td>19</td>
<td>67.9%</td>
<td>9</td>
<td>75.0%</td>
<td>9</td>
<td>32.1%</td>
<td>3</td>
<td>25.0%</td>
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</tr>
<tr>
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<td>9</td>
<td>32.1%</td>
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<td></td>
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<tr>
<td>Wild type</td>
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<td>96.4%</td>
<td>11</td>
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<td>1</td>
<td>3.6%</td>
<td>1</td>
<td>8.3%</td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
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<td>3.6%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AEH = atypical endometrial hyperplasia; EC = endometrial cancer; ER = oestrogen receptor; FIGO = International Federation of Gynecology and Obstetrics; PR = progesterone receptor.

aWilcoxon's rank-sum test used to compare baseline characteristics in metformin-treated and controls.
bTwo control patients were excluded as the final histology was atypical endometrial hyperplasia.
cTwo controls did not have cancer in the final hysterectomy specimen and were discharged from clinical follow-up postsurgery.
dTwo metformin-treated patients received adjuvant chemotherapy alone for concurrent primary ovarian tumours, but would only have received clinical follow-up stage 1A endometrial tumours. Only one patient received chemotherapy alone for EC.
behavioural change or intentional weight loss in preparation for surgery. Previous window studies in EC (Laskov et al, 2014) and breast cancer (Niraula et al, 2012) reported significant changes in biomarkers of adiposity and insulin resistance after short-term metformin treatment, but the lack of a control group hinders interpretation of these data. A large randomised window study in breast cancer that adjusted for changes in untreated controls found no effect of metformin on BMI or insulin resistance after four weeks of treatment (DeCensi et al, 2014). The latter study had a lower prevalence of overweight/obesity (40%) and insulin resistance (27%) compared with that we report here. Other studies have demonstrated a beneficial impact of metformin on BMI and markers of insulin resistance after a full six months’ treatment in breast cancer patients (Goodwin et al, 2015) as well as euglycaemic obese healthy women (Worsley et al, 2014), suggesting that the lack of demonstrable effect of metformin on biomarkers of adiposity and insulin resistance reflects the short duration of treatment in this study.

Metformin was generally well tolerated, although 4 out of 36 patients withdrew from the study due to gastrointestinal side effects. When treating T2DM, it is standard to commence metformin at a low dose and build up gradually to limit gastrointestinal toxicity. In this study, metformin was commenced at full dose to maximise the total amount of metformin received before hysterectomy. It is not known whether standard diabetic doses of metformin are sufficient for anticancer activity in vivo. In preclinical laboratory studies, supradiabetic concentrations of metformin are required to achieve a growth static effect using cancer cell lines (Cantrell et al, 2010; Sarfstein et al, 2013; Lengyel et al, 2015). Mitsuhashi et al (2014) found metformin at concentrations of 1.2–5.1 mmol kg⁻¹ in EC, equivalent to ~20% of circulating serum levels. The effective concentration of metformin in EC is therefore 1/400 lower compared with concentrations required to suppress proliferation in vitro. Optimal anticancer doses of metformin to be used in clinical studies have yet to be established. No studies have performed a dose-escalation protocol and previous window studies have given typical diabetic doses of metformin.

### Table 2. AEs experienced by all patients who participated in the metformin-treatment group

<table>
<thead>
<tr>
<th>Summary of AEs experienced by all patients who received metformin treatment</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients who received at least one dose of metformin</td>
<td>35 (100)</td>
</tr>
<tr>
<td>Patients who developed any AEs</td>
<td>27 (77)</td>
</tr>
<tr>
<td>Number of AEs</td>
<td></td>
</tr>
<tr>
<td>Grade 1 AE</td>
<td>98 (100)</td>
</tr>
<tr>
<td>Grade 2 AE</td>
<td>94 (96)</td>
</tr>
<tr>
<td>Grade 3 AE</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Grade 4 AE</td>
<td>1 (1)</td>
</tr>
</tbody>
</table>

### No. of patients experiencing an AE

| Loss of appetite | 4 (11) |
| Nausea/vomiting | 27 (77) |
| Diarrhoea | 24 (69) |
| Abdominal pain | 12 (34) |
| Skin changes | 3 (9) |
| Headache | 3 (9) |
| Fatigue | 2 (6) |
| Bloating | 2 (6) |
| Abnormal baseline bloods | 10 (29) |
| Others | 11 (31) |

Mean patient tolerability scores (0 = not tolerable, 10 = very tolerable): 29 (6.1 (s.d. 2.5))

### Table 3. Change from baseline following intervention

<table>
<thead>
<tr>
<th>Tumour and metabolic parameters</th>
<th>Pretreatment</th>
<th>Post-treatment</th>
<th>Pretreatment</th>
<th>Post-treatment</th>
<th>Adjusted mean difference</th>
<th>Upper</th>
<th>Lower</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 proliferation index</td>
<td>%</td>
<td>50.9 (11.1)</td>
<td>37.4 (60.9)</td>
<td>55.6 (25.1)</td>
<td>58.1 (65.2)</td>
<td>−17.2</td>
<td>−21.4</td>
<td>−7.0</td>
</tr>
<tr>
<td>Body mass index</td>
<td>kg m⁻²</td>
<td>35.5 (11.3)</td>
<td>34.1 (26.2)</td>
<td>32.0 (5.9)</td>
<td>31.9 (6.0)</td>
<td>−0.1</td>
<td>−0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Waist/hip girth ratio</td>
<td>0.9 (0.1)</td>
<td>0.9 (0.1)</td>
<td>0.9 (0.1)</td>
<td>0.9 (0.1)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.97</td>
</tr>
<tr>
<td>Glucose</td>
<td>mmol l⁻¹</td>
<td>6.0 (1.5)</td>
<td>5.3 (4.9)</td>
<td>5.7 (0.7)</td>
<td>5.3 (0.6)</td>
<td>0.2</td>
<td>−0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Insulin</td>
<td>mU l⁻¹</td>
<td>16.0 (9.4)</td>
<td>14.5 (9.4)</td>
<td>12.3 (8.0)</td>
<td>9.6 (4.5)</td>
<td>12.0</td>
<td>−5.9</td>
<td>2.3</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td></td>
<td>4.4 (2.8)</td>
<td>4.6 (3.8)</td>
<td>3.2 (2.3)</td>
<td>2.3 (1.1)</td>
<td>−0.3</td>
<td>−1.5</td>
<td>0.8</td>
</tr>
<tr>
<td>C-peptide</td>
<td>pmol l⁻¹</td>
<td>1076.1 (482.3)</td>
<td>1055.0 (560.0)</td>
<td>896.4 (341.0)</td>
<td>781.0 (461.2)</td>
<td>44.7</td>
<td>−206.2</td>
<td>295.5</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>mg l⁻¹</td>
<td>3.3 (1.5)</td>
<td>3.2 (1.5)</td>
<td>3.4 (1.3)</td>
<td>3.1 (1.2)</td>
<td>−0.3</td>
<td>−0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Leptin</td>
<td>ng ml⁻¹</td>
<td>54.1 (42.6)</td>
<td>55.0 (42.7)</td>
<td>42.9 (23.0)</td>
<td>45.5 (23.5)</td>
<td>−2.1</td>
<td>−13.2</td>
<td>9.0</td>
</tr>
<tr>
<td>Ln (hsCRP)</td>
<td>mg l⁻¹</td>
<td>1.3 (1.3)</td>
<td>1.3 (1.3)</td>
<td>0.9 (1.1)</td>
<td>0.6 (1.2)</td>
<td>0.0</td>
<td>−0.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**Abbreviations:** ANCOVA = analysis of covariance; BMI, body mass index; CI, confidence interval; HOMA-IR = homeostasis model of insulin resistance; hsCRP = high-sensitivity C-reactive protein. The treatment effect (adjusted mean difference) was analysed using an ANCOVA with post-treatment measurement as the response variable and baseline measurement, age, BMI, and insulin resistance (HOMA-IR) and treatment arm as covariates. As some data were not normally distributed, median and quartiles are also presented. The italic entries show that certain figures are the median and IQR, whereas the other figures are the mean and s.d.
doses of 500–2250 mg metformin per day (Laskov et al, 2014; Mitsuhashi et al, 2014; Schuler et al, 2015). In this study, we observed a Ki-67 drop associated with metformin treatment and this was positively correlated with the average daily dose of metformin received. It is interesting to speculate whether higher doses would have had even greater impact. Metformin is not bound to plasma proteins (Tucker et al, 1981) and has a very high volume of distribution. The effective circulating dose of metformin may therefore vary with BMI. We found some evidence of this, with greater reductions in post-metformin Ki-67 observed in leaner patients. Based on these data, we hypothesise that higher doses of metformin may achieve superior anticancer effects, particularly in obese and morbidly obese women. There is considerable inter-individual variation in glycaemic response to metformin in T2DM, partly explained by genetic differences in organic cation transporter-1 (OCT-1) expression levels in hepatic and skeletal tissue (Graham et al, 2011; Berstein et al, 2013). No studies have measured OCT-1 expression levels in EC, but differences in levels may explain why some patients responded to metformin but others did not. Metformin accumulates in endometrial tissue but has a half-life of 6 h; it is not known whether the timing of the last dose of metformin before serum and endometrial sampling affected our results.

The baseline level of apoptosis was very low in this study and there was no correlation with tumour grade. Apoptosis is poorly documented in EC; however, a similar window study investigating the effects of medroxyprogesterone acetate reported comparable low baseline values (Zaino et al, 2014). We also found no evidence for a proapoptotic effect of metformin in EC. In preclinical studies using EC cell lines, apoptosis is only induced at much higher concentrations of metformin compared with those required to inhibit cell growth (Cantrell et al, 2010).

Ki-67 is an established prognostic and predictive biomarker in breast cancer (Dowsett et al, 2005, 2006, 2007), but there is little evidence for its use as a surrogate marker in EC. We and others have shown that high-grade tumours have higher Ki-67 levels; tumour grade is an established independent prognostic biomarker in EC. Several studies have found an association between high Ki-67, other biomarkers of poor prognosis in EC and EC-specific mortality (Salvesen et al, 1998, 1999; Stefansson et al, 2004; Liu et al, 2014), but there is little consensus regarding optimal staining and scoring protocols to generate robust and reproducible data. We have adapted the International guidelines for Ki-67 staining and scoring in breast cancer established by Dowsett et al (2011) for this study. We developed a protocol for semiautomated scoring that is both reproducible and demonstrates excellent agreement with manual scoring. In breast cancer, a significant Ki-67 drop following short-term treatment with neoadjuvant chemotherapy is

Figure 2. (A) Line graph showing the adjusted mean difference in Ki-67 proliferation index in paired pre- and postintervention endometrial tumours from metformin-treated and control patients. (B and C) Endometrial tumour stained for Ki-67 before (B) and after (C) treatment with metformin at ×20 magnification.

Figure 3. Phosphorylation changes in (A) AKT, (B) ACC, (C) S6 and (D) 4EBP1 using box and whisker plots representing the median modified H-score (middle line) and the first and third quartile from paired pre- and postintervention endometrial biopsies for metformin-treated and control patients. The whiskers represent the maximum and minimum values.
predictive of tumour responsiveness to that drug (Dowsett et al., 2005, 2006, 2007). Thus, presurgical window studies have been an efficient way of screening novel therapeutic strategies for breast cancer. This trial design also has great potential in EC, as a trial powered to assess the impact of a new drug in the adjuvant setting using recurrence or EC-specific survival as the end point would be extremely expensive to conduct, requiring thousands of participants over many years of follow-up. Furthermore, like the breast, the endometrium lends itself to sampling in the outpatient setting, facilitating the comparison of matched biopsies taken before and after intervention in the presurgical window period.

Our data add to the growing body of evidence supporting biological activity of metformin in EC that may have therapeutic potential. This is an exciting area of research that is likely to produce further evidence over the next few years. feMMe, a phase II randomised clinical trial, is assessing the additional benefit of metformin or weight loss in combination with the levonorgestrel-releasing intrauterine device in non-surgical patients with AEH and early EC (Hawkes et al., 2014). Another study is assessing the impact of metformin with paclitaxel and carboplatin for advanced stage or recurrent EC. In addition to its therapeutic role, it is interesting to speculate whether metformin could be used for primary prevention of EC in high-risk groups. Reducing insulin resistance, promoting modest weight loss or preventing further weight gain would seem plausible strategies for EC risk reduction in morbidly obese women. A study assessing the impact of short-term treatment with metformin or placebo with or without a lifestyle intervention program designed to achieve weight loss and increase activity levels is underway, using endometrial Ki-67 as the primary end point. The data from these and similar studies are eagerly awaited.

**CONCLUSION**

Short-term presurgical metformin treatment is associated with a significant drop in Ki-67 expression in EC. Changes in phosphorylated mTOR proteins and serum markers of insulin resistance are observed to some extent in both groups, emphasising the need for a control group to adjust for the variability of biomarkers over time. Indeed, the phosphorylation status of mTOR proteins in EC at hysterectomy may be more indicative of devascularisation of the uterus than study interventions. Future studies based on tissue end points should compare pre- and postintervention endometrial biopsies taken using the same sampling method and before devascularisation of the uterus.

**DISCLAIMER**

This article presents independent research funded by the National Institute for Health Research (NIHR). The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


**CONFLICT OF INTEREST**

The authors declare no conflict of interest.


Appendix 5

The unrecognized burden of cardiovascular risk factors in women newly diagnosed with endometrial cancer: A prospective case control study

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HIGHLIGHTS
• CVD risk was measured in a prospective study of 150 EC patients and 746 controls.
• EC patients had significantly more CVD risk factors than other women.
• Most CVD risk factors were either unrecognized or inadequately treated.
• EC patients had a significantly higher 10-year risk of CVD (QRISK2) than controls.
• Identifying and treating CVD risk factors could improve outcomes for EC survivors.

Abstract
Background. Cardiovascular disease is a major cause of death in endometrial cancer survivors. The aim of this study was to determine whether women newly diagnosed with endometrial cancer have a higher prevalence of cardiovascular risk factors than the general population.

Methods. The prevalence of adequately treated and unrecognized/inadequately treated cardiovascular risk factors and the corresponding 10-year cardiovascular risk by QRISK2 score was measured in 150 consecutive women undergoing primary treatment for endometrioid endometrial cancer in the North West of England, and 746 age and ethnicity-matched control women from the Health Survey for England 2014.

Results. Women with endometrial cancer had higher proportions of obesity (BMI ≥30 60.7% vs. 32.4%, p < 0.0001) and a preponderance of unrecognized and inadequately treated cardiovascular risk factors. Compared with controls, endometrial cancer cases had a higher prevalence of incident hyperglycemia (57.2% vs. 11.5%, p < 0.0001), total: HDL cholesterol ratio > 4.5 (26.7% vs. 13.7%, p < 0.0001), and were more likely to have three or more cardiovascular risk factors (22% vs. 6%, p < 0.0001). This equates to a higher 10-year cardiovascular risk (median QRISK2 score 12.6% vs. 8.8%, p < 0.0001). Optimization of risk factors would have a greater impact on absolute cardiovascular disease risk for cases than controls (QRISK2 score reduction 1.8% vs. 0.7%).

Conclusions. Women undergoing primary treatment for endometrial cancer have a higher prevalence of cardiovascular risk factors than women without the disease. Early identification and treatment of these risk factors could improve outcomes for endometrial cancer survivors.

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Keywords:
Endometrial cancer
Obesity
Hyperglycemia
Hypertension
Hypercholesterolemia
Cardiovascular disease
QRISK2
Survival

1. Introduction

Endometrial cancer, the fourth most common female malignancy, affects over 9000 women each year in the UK and its incidence is rising, such that by 2030 it is estimated that there will be an additional 3600 new cases diagnosed every year in England and Wales alone [1–3]. Similar trends have been reported in other countries, for example in the
angioplasty, coronary artery bypass graft, stroke or transient ischemic attack.

test abnormalities, pre-diabetic hyperglycemia, hypertension, hypercholesterolemia

during open surgery [6]. More women than ever are thus surviving a diagnosis of endometrial cancer, with 79% and 77.5% of women expected to live at least five and 10 years respectively following diagnosis [7].

Despite this, women with a history of endometrial cancer have a higher mortality rate than the general population, particularly if diagnosed at a young age [8]. Rather than dying of their endometrial cancer, however, cardiovascular deaths predominate in those with early stage disease and this becomes more pronounced the longer women survive their cancer diagnosis. Overall, the risk of death from myocardial infarction and stroke is estimated to be two-fold higher than the risk of death from endometrial cancer and nearly nine-fold higher than that of women from the general population [8,9].

This is not surprising given that endometrial cancer and cardiovascular disease share the common risk factors of obesity and diabetes [10]. Obesity is the strongest risk factor for endometrial cancer, driving carcinogenesis through unopposed estrogen excess, hyperinsulinemia and insulin resistance [2,11,12]. Women with diabetes have a two-fold higher risk of endometrial cancer compared with non-diabetic women, even after adjustment for body mass index (BMI), suggesting an independent relationship between the two conditions.

Yet screening for, and optimization of, cardiovascular risk factors is not routinely undertaken in endometrial cancer survivors. Indeed, the true prevalence of hypercholesterolemia, hypertension and hyperglycemia in women with endometrial cancer is unknown. Previous estimates have been based on established diagnoses of cardiovascular risk factors, which may underestimate the true prevalence of conditions that are often asymptomatic [13–15]. This makes it difficult to estimate the benefit to be gained from introducing a program of routine testing and treatment of cardiovascular risk factors in endometrial cancer survivors.

In the current study, we asked whether women newly diagnosed with endometrial cancer have a higher prevalence of known and unrecognized cardiovascular risk factors than the general female population. Using the QRISK2 score, a widely-used UK-based validated cardiovascular risk calculator [16], we estimated the 10-year risk of cardiovascular disease in the two groups and calculated the likely benefit to be derived from optimization of modifiable risk factors.

2. Methods

2.1. Study design

This was a prospective case-control study performed between 2016 and 2017 in the North West of England.

2.2. Research ethics

The study was approved by the West of Scotland Research Ethics Committee (reference 16/WS/0040) and was prospectively registered on the NIHR Clinical Research Network Portfolio.

2.3. Selection of cases and data collection

We recruited consecutive patients with newly diagnosed endometrioid endometrial cancer referred for primary treatment by hysterectomy, who provided written, informed consent to participate in the study. A detailed medical history was obtained through interview and checked against medical records regarding known diagnoses of diabetes, pre-diabetic hyperglycemia, hypertension, hypercholesterolemia and cardiovascular disease, defined as a previous myocardial infarction, angina, coronary artery bypass graft, stroke or transient ischemic attack. Current medications used for the aforementioned conditions were also considered evidence of a prior diagnosis. Smoking status categorised women as never smokers, ex-smokers or current smokers and the number of cigarettes smoked per day was recorded.

Anthropometric measurements were recorded in a standard fashion; height was determined using a stadiometer and performed barefooted and weight measured using electronic scales after removal of bulky clothing. BMI was calculated using the formula weight (kg)/height (m)². Venepuncture was performed after an overnight fast of at least six hours duration and blood sent to the Clinical Biochemistry Department of the Manchester University NHS Foundation Trust for routine analysis. Determination of glycosylated hemoglobin (HbA1C) was undertaken using high performance liquid chromatography whilst total and high density lipoprotein (HDL) cholesterol levels were measured using an enzymatic colorimetric method, all according to standard operating procedures. Measurement of blood pressure was performed at rest in a seated position using a calibrated, automated sphygmomanometer.

2.4. Selection of controls and data collection

Each endometrial cancer case was matched for age (± 5 years), female sex and ethnicity to five participants in the Health Survey for England (HSE) 2014, the details of which have been previously published [17]. In brief, the survey is performed annually and collects information on the general health of 8000 adults randomly selected by postcode from across England using standardised questionnaires. Participants are representative of the general population, with each region proportionally sampled in a similar age distribution to the wider UK population [17]. In particular, the prevalence of overweight and obese individuals are equivalent to whole population estimates (HSE 58% vs. National Statistics 58%) [17,18]. Individual level data is made freely available through the NHS Digital website. Information on previous medical history, in particular a prior diagnosis of cardiovascular disease, hypertension and diabetes, and drug history is available. Weight and height measurements and fasted serum levels of total and HDL cholesterol and HbA1C are measured in a comparable way to that of cases and recorded on line. Limited information was available in the Health Survey for England on cancer status, in particular a prior history of endometrial cancer, and participants were therefore not excluded on this basis.

2.5. Outcome definitions

New diagnoses of non-diabetic hyperglycemia and type 2 diabetes were defined as an HbA1C between 42 and 48 mmol/mol and >48 mmol/mol, respectively, in a woman not previously diagnosed with these conditions. The HbA1C values used are in accordance with recommendations from the World Health Organisation [WHO, [19]].

A new diagnosis of hypercholesterolemia was defined as a total:HDL cholesterol ratio >4.5 in a woman not previously prescribed treatment with statins [20]. Inadequately treated hypercholesterolemia was defined as a total:HDL cholesterol ratio >4.5 in a woman already taking statin therapy.

Newly diagnosed hypertension was defined as a systolic blood pressure of >140 mm Hg in a person not previously known to have a physician-obtained diagnosis of hypertension, or taking antihypertensive therapy [16]. Inadequately treated hypertension was the persistence of a systolic blood pressure greater than this threshold in someone already taking antihypertensive medication.

2.6. QRISK2 score

The QRISK2 score was calculated using the validated 2016 version of the online calculator available at https://www.qrisk.org/2016/. QRISK2 derives cardiovascular disease risk estimates based on prospective...
data from the UK primary care population. It is therefore infrequently used in other countries because risk estimates may not be appropriately calibrated outside of the UK. Data were input on ethnicity, gender, smoking status, diabetic status, antihypertensive treatment, total:HDL cholesterol, systolic blood pressure, height and weight. Missing data were imputed by the calculator by substituting gender and aged-based average values. Data on comorbidities, including atrial fibrillation, renal disease and rheumatoid arthritis, family history of cardiovascular disease and postcode were unavailable for the Health Survey for England cohort and so were not included in the final analysis.

The predicted 10-year cardiovascular disease risk for patients without pre-existing cardiovascular disease was compared with an optimized risk for that individual, based on the treatment of underlying modifiable risk factors (i.e. quitting smoking, systolic blood pressure reduced to 140 mm Hg, total: HDL ratio decreased to 4.5, BMI reduced to 25 kg/m²). The absolute change in predicted risk through the optimization of risk factors was calculated by subtracting the optimized risk from the predicted risk prior to risk factor optimization [21].

2.7. Statistical analysis

A power calculation was performed based on the median age of women with endometrial cancer in our study. We assumed that 22.9% of women aged 65 years would have a QRISK2 score of >20% [6,22]. To detect a two-fold difference in QRISK2 score in women with endometrial cancer compared to those without the disease with 90% power, 5% error and five matched controls per case, we calculated that 124 cases and 620 controls would be required.

Data are reported as median and interquartile ranges due to their non-parametric distribution. Groups were compared using the Mann-U Whitney test for continuous data and χ² and Fisher’s exact tests for categorical data.

A p value of ≤0.05 was considered statistically significant. All statistical analysis was conducted using SPSS version 23 and Graph Pad Prism 7.

3. Results

3.1. Description of cases and controls

One hundred and fifty women with endometrioid endometrial cancer (hereafter referred to as endometrial cancer, n = 144) or its precursor lesion, atypical endometrial hyperplasia (n = 6), were recruited. Eight-nine percent of women with endometrial cancer had early stage disease (stages I and II) at presentation, reflecting the stage distribution seen in the general endometrial cancer population in the UK.

Cases were matched with 746 controls from the Health Survey for England (2014) for age and ethnic background. There were insufficient female participants aged 75 years and over and of non-white ethnic backgrounds included in the survey for all cases to be matched with five controls. There were no missing data for cases and 2% missing data for Health Survey for England controls. Missing data was restricted to absent total and HDL cholesterol levels (1.9% of controls), HbA1C levels (2.1% of controls), blood pressure measurements (1.2% of controls) and information on statin use (0.5% of controls).

The demographic details of cases and controls are shown in Table 1. Whilst there was no difference in the proportion of women who were current or ex-smokers in the two groups, women with endometrial cancer had significantly higher BMIs than those without the disease, reflecting the strong association between endometrial cancer and obesity (BMI ≥ 30 60.7% cases vs. 32.4% controls, p < 0.0001). In contrast, the proportion of cases already diagnosed with cardiovascular disease was significantly lower than that seen in the control group (6.0% cases vs. 15.7% controls, p = 0.002).

3.2. Prevalence of known and screen-detected cardiovascular risk factors in cases and controls

The prevalence of physician-diagnosed cardiovascular risk factors, specifically diabetes and hypercholesterolemia, was similar in the case and control groups (Fig. 1). Significantly more women with endometrial cancer, however, were likely to be taking antihypertensives than those in the general population (46.7% cases vs. 29.8% controls, p = 0.0001). Non-diabetic hyperglycemia had been previously diagnosed in 3.2% of cases. It was not recorded as a diagnosis in the Health Survey for England data, thereby preventing comparison of its prevalence between the two groups.

In contrast to the equivalent proportions of known cardiovascular risk factors in the two populations, the prevalence of screen detected and undertreated risk factors was significantly higher in women with endometrial cancer than those without the disease (Fig. 2). A new diagnosis of diabetes and non-diabetic hyperglycemia was made according to elevated HbA1C values in 6.0% and 51.2% of cases compared with 1.3% and 10.2% of controls, respectively (p < 0.0001). Similarly, over a quarter of cases had an elevated total:LDL cholesterol ratio > 4.5, either in the absence of or despite statin therapy, compared with less than one in seven women in the control group (26.7% cases vs. 13.7% controls, p = 0.0002). Despite the higher proportion of women with endometrial cancer already receiving treatment for hypertension, more than twice as many cases had a systolic blood pressure > 140 mm Hg than controls (49.3% cases vs. 23.7% controls, p < 0.0001).

Overall, a similar proportion of cases and controls were found to have at least one cardiovascular risk factor that had been previously diagnosed (42.7% cases vs. 34.9% controls, p = 0.08, Fig. 3). The marked difference, however, was in the true underlying prevalence of these risk factors in the two populations. Significantly more women with endometrial cancer were found to have screen-detected or undertreated cardiovascular risk factors (88.7% cases vs 54.3% controls, p = 0.0001) and approximately a fifth were found to have at least three of these risk factors, which were not being adequately treated (19.3% cases vs. 3.5% controls, p < 0.0001).

| Table 1: Demographic data for cases and controls. |
|-----------------|-----------|-----------|----------|
| Characteristic   | Cases     | Controls  | p value  |
| Age, median yrs. [IQR] | 65 (57–72) | 64 (54–71) | 0.093    |
| BMI, median kg/m² [IQR] | 32.5 | 27.2 | <0.0001*** |
| <25              | 25 (16.7) | 241 (32.3) |
| 25–29.9         | 34 (22.7) | 258 (34.6) |
| 30–34.9         | 37 (24.7) | 152 (20.4) |
| 35–39.9         | 20 (13.3) | 64 (8.6)   |
| ≥40             | 34 (22.7) | 25 (3.4)   |
| Missing data    | 0 (0.0)   | 6 (0.8)    |
| Ethnicity, n (%) | 137 (91.3) | 680 (91.1) | 0.081    |
| White           | 137 (91.3) | 680 (91.1) |
| Indian          | 6 (4.0)   | 29 (3.9)   |
| Pakistani       | 4 (2.7)   | 20 (2.7)   |
| Black/African/Caribbean | 3 (2.0) | 17 (2.3) | 0.271 |
| Smoking status, n (%) | 88 (58.7) | 394 (52.8) | 0.002** |
| Never smoked    | 88 (58.7) | 394 (52.8) |
| Ex-smoker       | 43 (28.7) | 265 (35.5) |
| Current smoker  | 19 (12.7) | 87 (11.7)  |
| Diagnosed cardiovascular disease, n (%) | 141 (94.0) | 629 (84.3) | 0.002** |
| No              | 141 (94.0) | 629 (84.3) |
| Yes             | 9 (6.0)   | 117 (15.7) |

** p < 0.01.
*** p < 0.001.
**** p < 0.0001.
3.3. Predicted 10-year cardiovascular disease risk in cases and controls

We calculated the QRISK2 score for cases and controls to determine the proportion of women at high risk who would benefit from primary cardiovascular disease prevention. As the QRISK2 score is only valid for patients without a history of cardiovascular disease, type I diabetes, familial hyperlipidemia and aged between 25 and 85 years, 11 cases and 124 controls were excluded from the analysis, leaving 139 cases and 622 controls for whom a QRISK2 score could be calculated. The resulting groups remained well matched for age and ethnic background and continued to demonstrate significant differences in the prevalence of overall, screen detected and undertreated cardiovascular risk factors whilst having similar levels of known risk factors (Supplementary Fig. 1a and b).

Two thirds of women with endometrial cancer compared with under half of controls met the National Institute of Clinical Excellence (NICE) threshold of a 10-year cardiovascular disease risk of 10% or greater for the introduction of statin therapy (63.3% cases vs. 46.6% controls, p = 0.0005, Table 2). Almost a third of cases had a QRISK2 score of 20% or greater, identifying them as being at high risk of cardiovascular disease in the next 10 years (29.5% cases vs. 16.9% controls, p = 0.001). The higher predicted cardiovascular disease risk in women with endometrial cancer compared with the general population was...
reflected in their higher median QRISK2 scores (12.6% in cases vs. 8.8% in controls, p < 0.0001).

3.4. Estimated effect of risk factor optimisation

Finally, we estimated the likely benefit to arise from a screening programme aimed at diagnosing and optimizing treatment of cardiovascular risk factors in endometrial cancer survivors. Interventions to promote weight loss, aiming for a BMI of 25 kg/m², smoking cessation and optimization of hypertension and hypercholesterolemia treatment were shown to result in an absolute percentage reduction in cardiovascular risk of 1.8% for women with endometrial cancer compared with a reduction of 0.7% if undertaken in the control population. This equates to the treatment of 55 women with endometrial cancer to prevent one cardiovascular event (heart attack, transient ischemic attack or cerebrovascular accident) in the next 10 years. In contrast, 145 women in the general population would need to receive treatment to observe the same effect.

4. Discussion

In this study, the prevalence of obesity, diabetes, non-diabetic hyperglycemia, hypertension and hypercholesterolemia was significantly higher in women diagnosed with endometrial cancer than the general population. Almost all of the women with endometrial cancer had more than one risk factor for cardiovascular disease, with 22% having three or more concurrent risk factors. The true prevalence of these conditions, however, only becomes obvious when non-selective screening is performed, as endometrial cancer patients were much more likely to have cardiovascular risk factors that had not been detected and treated in primary care. Even when recognized, lipid and blood pressure control was frequently suboptimal. As a result, the women with endometrial cancer in this study had a 1.5-fold higher 10-year risk of cardiovascular disease, as measured using the QRISK2 score, compared with the general population. Many of these risk factors are modifiable and with optimization this absolute risk could be reduced by up to 1.8%, although it is likely to remain, on average, higher than for women without endometrial cancer. This is related to the fact that many endometrial cancer patients have multiple cardiovascular risk factors. Introduction of screening and treatment of cardiovascular risk factors in women following primary treatment for endometrial cancer would be predicted to be more effective than a similar program aimed at the general population, which is already advocated by NICE for people aged over 40 years [16].

Being diagnosed with cancer is a highly emotive experience that inevitably leads to questions about etiology, risk factors and prevention strategies. Some studies have shown that this ‘teachable moment’ is

<table>
<thead>
<tr>
<th>10 year cardiovascular risk</th>
<th>Cases (n = 139)</th>
<th>Controls (n = 622)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10%, n (%)</td>
<td>51 (36.7)</td>
<td>332 (53.4)</td>
<td>0.0005***</td>
</tr>
<tr>
<td>≥10%, n (%)</td>
<td>88 (63.3)</td>
<td>290 (46.6)</td>
<td>0.001***</td>
</tr>
<tr>
<td>≥20%, n (%)</td>
<td>41 (29.5)</td>
<td>105 (16.9)</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Median (IQR) before risk factor optimization</td>
<td>12.6% (6.6–21.4%)</td>
<td>8.8% (3.5–17.1%)</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Median (IQR) after risk factor optimization</td>
<td>11.6% (6–18.9%)</td>
<td>8.4% (3.2–15.9%)</td>
<td>0.0004***</td>
</tr>
<tr>
<td>Absolute percentage change in cardiovascular risk following optimization</td>
<td>−1.82</td>
<td>−0.69</td>
<td></td>
</tr>
<tr>
<td>Estimated number needed to treat to prevent one cardiovascular event over 10 years</td>
<td>55</td>
<td>145</td>
<td></td>
</tr>
</tbody>
</table>

*** p < 0.001.

**** p < 0.0001.

Fig. 3. Proportion of cases and controls with one or more adequately treated or inadequately treated/screen detected cardiovascular risk factors. There was no significant difference in the prevalence of adequately treated risk factors between women with endometrial cancer and those without. The difference in the proportion of women with one, two or three or more cardiovascular risk factors between the two groups was due to the higher prevalence of previously undiagnosed and inadequately treated risk factors in women undergoing primary treatment for endometrial cancer.
an opportunity for the successful introduction of lifestyle changes that improve overall survival [23]. Cardiovascular risk factor optimization could form part of that discussion; indeed, this is arguably more important than the efforts made in routine follow up to identify recurrent disease in women who have generally been cured of their endometrial cancer [24]. Weight loss, with its favorable impact on insulin resistance, blood pressure and cholesterol profiles, particularly given its low cost, low risk of harm, and added benefits for quality of life is the obvious answer [25,26], but one that is very difficult to achieve and sustain by dietary restriction and lifestyle change [27]. A strategy of identifying and correcting hitherto unrecognized or undertreated cardiovascular risk factors with appropriate drug therapy may therefore offer a reasonable alternative for improving outcomes for endometrial cancer survivors. Bariatric surgery may also be appropriate for some women [28,29].

4.1. Comparison with other studies

This is the first study to investigate the risk of non-fatal cardiovascular events in women with endometrial cancer. Few studies have previously measured the prevalence of individual risk factors for cardiovascular disease in this population, and they have often relied on self-reported co-morbidities or health records for known diagnoses only, and neither have they considered hypercholesterolemia in their assessment [15,30]. The high prevalence of obesity [31] and diabetes [32] is well documented and a few studies have reported the burden of unrecognized insulin resistance and non-diabetic hyperglycemia in endometrial cancer patients [13,33]. When 99 women with newly diagnosed endometrial cancer underwent screening with fasting serum glucose, 30.3% were found to have physician diagnosed diabetes and a further 36% were noted to have previously unrecognized insulin resistance [13]. These results are similar to our own, where 54.4% of women with a history of endometrial cancer were found to have non-diabetic hyperglycemia, although only 17.3% of women in our study had overt diabetes. This difference may be explained by differences in ethnicity and patients being part of distinct healthcare systems with differing rates of opportunistic screening.

Felix, Bower [8] found that deaths from cardiovascular disease were significantly more prevalent in women with a history of endometrial cancer in the Surveillance, Epidemiology and End Results Program (SEER) than in the general population. These results were not replicated in the Iowa Women's Health Study, though, where endometrial cancer survivors were noted to have a 25% lower risk of cardiovascular disease mortality compared with age and BMI matched women without a history of the disease [32]. The latter study was reliant on information recorded on death certificates to determine disease specific mortality rates and thus vulnerable to the inherent inaccuracies of these type of data. Cases were not only leaner than those in our study, with a median BMI of 28 kg/m², but they were also BMI-matched to the controls. This eliminates the impact of obesity on other cardiovascular risk factors, all of which are strongly correlated. As part of a longitudinal study of lifestyle factors on cancer incidence, it is possible that participation in the study led to positive behavior change in women who developed endometrial cancer, reducing their subsequent risk of cardiovascular disease. This may also explain why there was no difference in the rates of non-fatal cardiovascular disease events in women with and without a history of endometrial cancer enrolled in the Women's Health Initiative [34]. As with the Iowa Women's Health Study, participants were healthier, with a lower prevalence of obesity and hypertension than in our study, potentially as a result of the ‘healthy bias’ associated with selective recruitment of women into clinical trials.

4.2. Strengths and limitations

The present study recorded known cardiovascular risk factors but additionally screened for asymptomatic, previously unidentified and inadequately treated hyperglycemia, hypertension and hypercholesterolemia to provide accurate prevalence data and a reliable estimation of 10-year cardiovascular disease risk in women undergoing primary treatment for endometrial cancer. The women enrolled in the Health Survey for England are highly representative of the general population, being selected at random on the basis of postcode rather than relying on self-recruitment into a study, which is known to introduce ‘healthy control’ bias. This makes them a reliable control group for comparison with the endometrial cancer cases. The study was also adequately powered to detect any differences in cardiovascular disease risk between the two populations, even after exclusion of individuals with a known history of cardiovascular disease and those not suitable for assessment using the QRISK2 score.

There were insufficient data contained within the Health Survey for England database to accurately determine whether individuals had a history of malignancy and of endometrial cancer in particular. This may have resulted in the misclassification of cases as controls, but would not have impacted upon the conclusions reached as indeed it would have biased results toward the null. This is even more likely given the limits imposed by the QRISK2 calculator with regards to extremes of body mass. As the model has only been validated for use in individuals with a BMI between 20 and 40 kg/m², values outside of these are automatically replaced with the limit figure. Given the high prevalence of extreme obesity in the endometrial cancer group, this is likely to result in an underestimate of their cardiovascular disease risk and hence the benefit that may be derived from introducing screening and treatment for such risk factors. In addition, there was a paucity of individual level data in the Survey on the presence of renal disease, atrial fibrillation and rheumatoid arthritis, meaning that they could not be included as variables in the QRISK2 score for either group. However, this potential limitation is unlikely to have a significant impact on the final scores because the prevalence of these conditions is low in the general population. Whilst the QRISK2 score has only been validated in the UK population, the variables used to derive risk estimates are the same as those used in other risk calculators, including the Framingham cardiovascular risk calculator. Similar results would be expected if other risk calculators had been used.

4.3. Future work

Our data support the routine screening of women newly diagnosed with endometrial cancer for cardiovascular risk factors. We advocate measuring BMI, blood pressure, HbA1C and serum lipids with a view to calculating an individual woman's risk of cardiovascular disease using a validated risk prediction model, like QRISK2. Cardiovascular risk calculators could be added to the SGO Obesity Toolkit [35] to remind physicians to consider long term health issues for obese endometrial cancer patients, with prompts embedded in electronic patient records. Women at high risk of cardiovascular disease should be supported to reduce their risk through healthy lifestyle change to achieve weight loss and appropriate drug treatment to normalize their blood pressure, blood sugar and lipid levels. All women with a QRISK2 score ≥ 10% should be commenced on a statin, regardless of serum cholesterol [16]. Future research questions should focus on determining the impact of systematic screening and optimization of cardiovascular risk factors on cardiovascular event frequency as well as the optimal management strategy. Of particular interest is whether drug therapy for individual risk factors is superior to weight loss, achieved through dietary modification or bariatric surgery, for improving outcomes in endometrial cancer survivors [36].

5. Conclusions

Women undergoing primary treatment for endometrial cancer have a high prevalence of unrecognized and undertreated cardiovascular risk factors. Screening for and optimization of these conditions could favorably impact on future cardiovascular event frequency and improve overall survival in this population.
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2017.11.019.

Trial registration
NIHR Clinical Research Network Portfolio Study Identifier 30,602-Metabolic syndrome prevalence in endometrial cancer. The study was prospectively registered before data acquisition. The protocol originally stated that a diagnosis of metabolic syndrome would be used as a surrogate marker of cardiovascular risk. After taking advice from experts in the field, however, the QRISK2 score was substituted as it was deemed a more accurate and reliable measure of cardiovascular risk. This amendment was performed prior to data analysis.

Details of contributors
SK, MR and EC designed the study. SK performed data collection and analyses and drafted the manuscript. JL, VS, ML and NR contributed to data collection. RE, ML, MR and EC contributed to data analysis. MR and EC contributed to the draft of the manuscript. All authors contributed to the final manuscript. SK and EC had access to the data and can take responsibility for the integrity of the data and the accuracy of the data analysis.

Competing interests
All authors declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

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