THE ROLE OF METFORMIN IN OBESITY-DRIVEN ENDOMETRIAL CANCER

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

2016

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SCHOOL OF MEDICINE
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<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>4EBP1</td>
<td>4E-binding protein 1</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl CoA Carboxylase</td>
</tr>
<tr>
<td>AE</td>
<td>Adverse event</td>
</tr>
<tr>
<td>AEH</td>
<td>Atypical endometrial hyperplasia</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated kinase</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of covariance</td>
</tr>
<tr>
<td>BD</td>
<td>From b.i.d bis in die or twice daily</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CA-9</td>
<td>Carbonic anhydrase 9</td>
</tr>
<tr>
<td>cc3</td>
<td>Cleaved caspase-3</td>
</tr>
<tr>
<td>CI</td>
<td>Combination index</td>
</tr>
<tr>
<td>COSMIC</td>
<td>Catalogue of Somatic Mutations in Cancer</td>
</tr>
<tr>
<td>CONSORT</td>
<td>Consolidated Standards of Reporting Trials</td>
</tr>
<tr>
<td>CRF</td>
<td>Clinical record form</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EC</td>
<td>Endometrial cancer</td>
</tr>
<tr>
<td>EEF</td>
<td>Endometrioid endometrial adenocarcinoma</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunobead assay</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen Receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynaecology and Obstetrics</td>
</tr>
<tr>
<td>HEC</td>
<td>Hyperinsulinaemic-euglycaemic clamp</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor-1 alpha</td>
</tr>
<tr>
<td>HIER</td>
<td>HIER: Heat-induced epitope retrieval</td>
</tr>
<tr>
<td>Hi-FBS</td>
<td>Hi-FBS: Heat-inactivated fetal bovine serum</td>
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<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment of insulin resistance</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>HS</td>
<td>Hot-spot</td>
</tr>
<tr>
<td>hsCRP</td>
<td>hsCRP: High-sensitivity CRP</td>
</tr>
<tr>
<td>ICC</td>
<td>Intraclass correlation coefficient</td>
</tr>
<tr>
<td>IE</td>
<td>Invasive edge</td>
</tr>
<tr>
<td>IGFBP</td>
<td>IGFBP: Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IR</td>
<td>Insulin receptor : IR</td>
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<td>IGF-1</td>
<td>Insulin-like growth factor 1 : IGF1</td>
</tr>
<tr>
<td>IGF1R</td>
<td>Insulin-like growth factor 1 receptor : IGF1R</td>
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<td>LVSI</td>
<td>Lymphovascular space invasion</td>
</tr>
<tr>
<td>mAB</td>
<td>mAB: Monoclonal antibody</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionization-Time of Flight (Mass spectrometry)</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>OCT-1</td>
<td>Organic cation transporter 1</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
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<tr>
<td>OR</td>
<td>Odds ration</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative Phosphorylation</td>
</tr>
<tr>
<td>pAB</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS++</td>
<td>Phosphate buffered saline with CaCl₂ and MgCl₂</td>
</tr>
<tr>
<td>PCOS</td>
<td>Polycystic ovary syndrome</td>
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<tr>
<td>PFS</td>
<td>Progression-free survival</td>
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<tr>
<td>PIK3CA</td>
<td>Phosphoinositide-3-kinase-catalytic-alpha</td>
</tr>
<tr>
<td>PIS</td>
<td>Patient Information Sheet</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homology</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RFS</td>
<td>Recurrence-free survival</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RS</td>
<td>Recurrence-free survival</td>
</tr>
<tr>
<td>RP</td>
<td>Ribosomal protein</td>
</tr>
<tr>
<td>S6</td>
<td>Ribosomal protein S6</td>
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<tr>
<td>SAE</td>
<td>Serious adverse event</td>
</tr>
<tr>
<td>SAR</td>
<td>Serious adverse reaction</td>
</tr>
<tr>
<td>SUSAR</td>
<td>Suspected unexpected serious adverse reaction</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue Microarray</td>
</tr>
<tr>
<td>TOMM-20</td>
<td>Translocase of Outer Mitochondrial Membrane 20</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>WS</td>
<td>Whole section</td>
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Abstract

Thesis title: The role of metformin in obesity-driven endometrial cancer.
A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences on 28th of March 2016.

Candidate: Vanitha N Sivalingam

Endometrial cancer (EC) is the most common gynaecological cancer affecting women in developed countries. Improving outcomes for women who are unfit for primary surgery or have advanced disease remains a challenge. Metformin use is associated with reduced cancer risk in several observational studies of patients with type 2 diabetes. Pre-clinical studies in EC show that metformin reduces cellular proliferation. The work described in this thesis tests the hypothesis that metformin reduces cellular proliferation in vitro and in vivo in type I EC through actions on the PI3K/AKT/mTOR pro-proliferative pathway. First, an in vitro model of EC using cell lines was established to determine the effect of metformin on cellular proliferation. Metformin was found to be cytostatic in a dose-dependent manner; these effects were potentiated in combination with carboplatin and paclitaxel. Metformin was shown to modulate mTOR phosphorylation proteins by immunoblot. Flow cytometric and metabolic assays found metformin to increase mitochondrial mass, but conversely, reduce mitochondrial function. These in vitro findings varied according to glucose concentration and were attenuated in hypoxia.

Next, staining and scoring protocols for Ki-67, a marker of cellular proliferation, were established using semi-automated scoring on archived EC tumours. Ki-67 correlated with age, tumour grade and myometrial invasion; high Ki-67 expression was associated with an increased risk of disease recurrence, and was thus a prognostic marker. Finally, a presurgical window study of metformin versus no drug in women with EC demonstrated a 17% reduction in tumour Ki-67 with short-term metformin. Ki-67 response varied positively with increased average daily dose of metformin and negatively with increased BMI. High grade tumours were more hypoxic, according to baseline HIF-1α and the Ki-67 response to metformin was lower in hypoxic tumours. The effect on tumour mTOR phosphorylation events varied, but was not significant after adjusting for changes in controls.

In conclusion, these results demonstrated that short-term oral metformin was associated with reduced cellular proliferation in women with EC. The findings from this study require corroboration with a placebo-controlled trial prior to the introduction of metformin as treatment for EC, both as a sole agent and in combination with existing adjuvant therapy. The response to metformin was heterogeneous; tumour hypoxia and metabolic adaptations of cancer cells may lead to metformin-resistance. Future studies should take these modulating effects into account to help identify patients likely to derive clinical benefit from metformin.
Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university of institute of learning.
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1. Introduction

Endometrial cancer (EC) is the most common gynaecological malignancy in developing countries. Endometrial cancer incidence rates have increased overall in the United Kingdom (UK) and almost doubled since 1990 (Figure 1) (CRUK, 2014). Additional data from United States projects the incidence of EC to double again by 2030 (Rahib et al., 2014) with a similar trend expected in the UK. A major contributor to this rise is the obesity epidemic. Worldwide, the proportion of women with a body mass index (BMI) of 25kg/m^2 or greater has increased from 30 to 38% over 30 years, and as many as 34% of all ECs are directly attributable to overweight or obesity (Arnold et al., 2015).

![Figure 1: Uterine cancer, European Age-Standardised Rates per 100,000 Population, Females, Great Britain, 1979-2012. Obtained from Cancer Research UK (CRUK, 2014).](image)

EC is strongly associated with obesity, with every 5kg/m^2 increase in BMI conferring a 1.6-fold increase in risk of developing the disease (Renehan et al., 2008, Crosbie et al., 2010). Type 2 diabetes mellitus (T2DM) is also associated with increased cancer risk (Friberg et al., 2007). Women with T2DM have a two-fold increased risk of EC compared with non-diabetic women (Friberg et al., 2007) and up to 36% of women with EC have undiagnosed insulin resistance (Burzawa et al., 2011).

Most women who present with early stage disease are cured following surgical excision of the uterus, both tubes and ovaries (Kitchener, 2007). Surgery, however can be technically challenging in obese women with prolonged operating times, excessive blood loss, increased post-operative infections and suboptimal debulking (Foley and Lee, 1990, Everett...
et al., 2003, Pavelka et al., 2004, Bouwman et al., 2015). There is an increasing population of unfit women who are too frail to undergo extensive surgery and there are limited medical management options available.

Women with locally advanced recurrent or metastatic EC have a poor prognosis. Hormonal agents, used most commonly for endometrioid EC have modest efficacy with median survival reported at 7-12 months (Thigpen et al., 1999, Bellone et al., 2008, Ma et al., 2004). Combination chemotherapy (commonly carboplatin + paclitaxel) produce objective response rates of approximately 50-60%, but are often poorly tolerated by the older population and again, overall survival is short (Deppe et al., 1994, Fleming et al., 2004). There is a need to identify improved medical therapy, both for primary and adjuvant use with better tolerability and efficacy.

An improved understanding of the molecular drivers linking obesity, insulin resistance and endometrial carcinogenesis has led to the identification of novel targeted therapies. Targeting the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway is of increasing interest as a therapeutic strategy in many tumours (Oza et al., 2011). Metformin is a safe oral therapy used extensively as the first-line medical therapy for T2DM. Numerous data have suggested that metformin may act as an mTOR inhibitor and cause a cytostatic effect on multiple tumours, including EC. A number of novel mechanisms of metformin in cancer have been discovered using laboratory models. Over the last ten years, interest in repurposing metformin as an anti-cancer agent has grown exponentially.
1.1 The endometrium and endometrial cancer

1.1.1 The reproductive and postmenopausal endometrium
The endometrium is composed of three different layers, the basal, intermediate and superficial layers. These layers are composed of surface epithelial glandular cells, connective tissue stroma and blood vessels (Ludwig and Spornitz, 1991). The superficial and intermediate layers are functional layers which surround the lumen of the uterine cavity and undergo cyclic regeneration under hormonal influence.

The endometrium undergoes monthly cyclical changes secondary to stimulation by oestrogen and progesterone during the reproductive years. Oestrogen, in the form of oestradiol drives endometrial proliferation, while progesterone halts proliferation and preserves the breakdown of the endometrium. The ratio and duration of each hormone determines the histologic appearance of the endometrium from proliferative to secretory endometrium and finally withdrawal and breakdown (Mutter et al., 2007). Any alteration in the normal balance of these hormones may result in abnormal histology.

There is a progressive failure of ovarian function and decline in ovarian oestrogen production towards the end of reproductive life. The main source of oestrogen following the menopause is from androgenic adrenal steroids converted by aromatase enzymes to oestrone in the peripheral fat (Gusberg, 1994). In postmenopausal women, oestrogen-induced proliferation is no longer moderated by progesterone. An imbalance of oestrogen and progesterone occurs in obese postmenopausal women and in premenopausal women with obesity-induced chronic anovulation and/or polycystic ovary syndrome (PCOS). This can lead to hyperplasia and in some cases EC.

1.1.2 Endometrial hyperplasia and cancer
Endometrial hyperplasia is defined as an overgrowth of both endometrial stroma and glands and is characterised by a proliferative glandular pattern with or without a degree of atypia (Mutter et al., 2000). Prolonged oestrogenic stimulation causes the endometrial glands to continue proliferation, becoming larger and more complex. An overall increase in endometrial gland density is seen in hyperplasia.

There are currently two systems of endometrial pre-cancer nomenclature in common usage: 1) The WHO94 schema and 2) the endometrial intraepithelial neoplasia diagnostic schema developed by the International Endometrial Collaborative Group (Mutter, 2000). The World Health Organisation (WHO) criteria categorises endometrial hyperplasia based on the presence or absence of nuclear atypia and the degree of architectural crowding.
The four categories are simple hyperplasia, complex hyperplasia, simple hyperplasia with atypia and complex hyperplasia with atypia. The presence of atypia correlates with progression to malignancy; patients with both simple and complex atypical hyperplasia were significantly more likely to develop carcinoma (8% vs 1%, 29% vs 3%, with and without atypia) (Kurman et al., 1985). More recently, Lacey et al. demonstrated through a case-control study that the cumulative risk of progression to EC increased from 8.2% through 4 years to 27.5% through 19 years after atypical hyperplasia (Lacey et al., 2011). The risk of concurrent endometrial cancer following a diagnosis of hyperplasia is increased with age (>60), higher BMI (>35kg/m²) and the presence of T2DM (Matsuo et al., 2015). The WHO94 schema is the one most commonly used by pathologists at present, but there are moves to transition to the endometrial intraepithelial neoplasia nomenclature. In this schema, the three disease categories will include 1) benign (benign endometrial hyperplasia), 2) premalignant [endometrial intraepithelial neoplasia (EIN)], and 3) malignant (endometrial adenocarcinoma, endometrioid type). The diagnosis of EIN must meet five criteria in a single fragment including architectural crowding, altered cytology, minimum size of 1mm, exclusion of carcinoma and exclusion of mimics. This can be summarised as a focus of clustered endometrial glands exceeding a gland to stroma ratio of 1:1, with altered cytology from a background of endometrium and which comprise a sufficient volume of 1mM (Baak et al., 2005).

1.1.3 The dualistic classification of EC

There are two broad subtypes of EC, type I and type II. Bokhman proposed a dualistic model of endometrial tumourigenesis in 1983 based on correlations between clinical, pathological and epidemiological findings (Bokhman, 1983). The dualistic model of EC postulates that the clinical and histopathological differences in the two types of EC are mirrored in their molecular and genetic variations. Approximately 80% of EC are histologically type 1 or endometrioid endometrial adenocarcinomas (EECs). Morphologically, EECs resemble normal endometrium and are often preceded by endometrial hyperplasia. The term “endometrioid” refers to endometrial-type glands of varying differentiation easily recognisable on microscopy (Clement and Young, 2002)

Bokhman observed that type I EECs arose from a background of obesity, hyperlipidaemia and hyperoestrogenism. These patients often had co-existing T2DM, and the tumours were highly and moderately differentiated with a good prognosis. Type II ECs were described as non-endometrioid; high grade tumours that are associated with a higher risk of metastasis and poor prognosis (Bokhman, 1983). The dichotomous classification was also supported
by molecular data. Type I tumours are preferentially associated with mutations in PTEN, KRAS, CTNNB1 and PIK3CA, whereas serous carcinomas show HER2 amplification and recurrent TP53 mutations (Dedes et al., 2011, Matias-Guiu and Prat, 2013). These divisions are not rigid and it is recognised that many ECs have overlapping clinical, morphological and molecular features of both tumour types. Some EECs show high grade cytological and architectural features. It is conceivable that these carcinomas progress from lower grade tumours (Lax et al., 2000). Some tumours also display mixed histology, which may represent two separate original pathologies or progression of foci of low-grade disease.

This dualistic classification holds true for a majority of cases and is incorporated into clinical decision algorithms. The prognostic value, however, remains limited as up to 20% of endometrioid (type I) ECs relapse, while 50% of type II tumours do not (Bokhman, 1983, Murali et al., 2014). Type I and II tumours also share multiple risk factors including obesity (Renehan et al., 2016) and T2DM, while increasing parity, use of oral contraceptives and smoking are associated with reduced risk (Setiawan et al., 2013, Brinton et al., 2013).

1.1.4 Genetic alterations and molecular classifications of type I and type II tumours
An understanding of the genetic alterations observed in EC is imperative in the development of targeted therapies. The common genetic alterations using the dualistic model are presented in Table 1. EECs generally have a high mutational load, particularly in the PI3K/AKT/mTOR signalling pathway and Wnt/β-catenin signalling pathway. These pathways regulate cell growth and survival (Funaki et al., 2000) and gene transcription. The phosphate and tensin homolog (PTEN) is a negative regulator of the PI3K/AKT/mTOR pathway. Loss of function is usually caused by deletional and mutational events. PTEN mutations have been reported in up to 80% of EECs and in about 55% of patients with endometrial hyperplasia (Mutter et al., 2000). PTEN heterozygous mice develop endometrial hyperplastic lesions and up to 20% develop EC, suggesting that PTEN inactivation is an early event in the pathogenesis of EC (Risinger et al., 1993, Terakawa et al., 2003).

The PIK3CA pathway is the most frequently altered in type I tumours with mutations reported in more than 90% of lesions (Kandoth et al., 2013). There is a high co-existence with PTEN mutations and double mutations of both PIK3CA and PTEN have an additive effect on PI3K activation (Oda et al., 2005). KRAS is mutated in about 43% of EC and frequently co-exists with PIK3CA mutations in endometrioid tumours. PIK3CA mutations are also seen in 46% of serous ECs while PTEN and KRAS mutations are less frequently
observed (Lax et al., 2000, Urick et al., 2011). The PI3K/AKT/mTOR pathway may also be activated by HER2 gene amplifications in serous cancers (Dedes et al., 2011).

β-catenin is a component of the E-cadherin-catenin unit, essential for cell differentiation and maintenance of normal tissue architecture. Increased levels of β-catenin may play a role in uncontrolled cellular proliferation and carcinogenesis, independent of PTEN (Fukuchi et al., 1998). Mutations in the Wnt/β-catenin signalling pathway are seen in up to 50% of endometrioid tumours and up to 80% of serous carcinomas (Moreno-Bueno et al., 2003, Dedes et al., 2011, Weigelt and Banerjee, 2012). CTNNB1 gain of function mutations, however, are almost exclusively seen in EECs (Matias-Guiu and Prat, 2013).

DNA microsatellites are short segments of nucleotides that are repeated throughout the genome. Microsatellite instability (MSI) is characterised by defects in the mismatch repair genes responsible for fixing these errors. MSI, mainly due to MLH1 promoter hypermethylation occurs in up to 20% of EECs, compared with less than 5% of type II tumours (Duggan et al., 1994, Faquin et al., 2000, Lax et al., 2000). These defects have also been reported in complex and atypical hyperplasia, indicating a role in early carcinogenesis (Mutter et al., 1996).

TP53 is a tumour suppressor gene that is implicated in up to half of all human tumours. Mutations in TP53 are present in up to 90% of serous carcinomas but also found in 2-20% of EECs. TP53 mutations have been reported in 75% of cases of endometrial serous intraepithelial carcinoma (the putative precursor of serous carcinoma), suggesting a role in endometrial serous carcinogenesis (Tashiro et al., 1997).

Mutations in ARID1A or loss of expression of its protein product BAF250a were identified in all grades of EECs and in clear cell and serous carcinomas (Weigand et al., 2011). Reduced BAF250a expression has also been documented in complex and atypical hyperplasia. PPP2R1A mutations, a putative tumour suppressor gene were reported in 40% of serous carcinomas, while rarely identified in endometrioid tumours (McConechy et al., 2011).
Table 1: Common genetic alterations based on the dualistic classification of epithelial EC

<table>
<thead>
<tr>
<th>Common genetic alterations</th>
<th>Type I (endometrioid)</th>
<th>Type II (non-endometrioid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN mutation</td>
<td>52-78%</td>
<td>1-11%</td>
</tr>
<tr>
<td>PIK3CA mutation</td>
<td>36-52%</td>
<td>24-42%</td>
</tr>
<tr>
<td>KRAS mutation</td>
<td>15-43%</td>
<td>2-8%</td>
</tr>
<tr>
<td>ARID1A mutation</td>
<td>25-48%</td>
<td>6-11%</td>
</tr>
<tr>
<td>CTNNB1 mutation</td>
<td>23-24%</td>
<td>0-3%</td>
</tr>
<tr>
<td>TP53 mutation</td>
<td>9-12%</td>
<td>60-91%</td>
</tr>
<tr>
<td>PPP2R1A</td>
<td>5-7%</td>
<td>15-43%</td>
</tr>
<tr>
<td>HER2 amplification</td>
<td>0</td>
<td>27-44%</td>
</tr>
<tr>
<td>Microsatellite instability (MSI)</td>
<td>28-40%</td>
<td>0-2%</td>
</tr>
</tbody>
</table>

Table adapted from “Classification of endometrial carcinoma: more than two types.”(Murali et al., 2014). Incidence rates extracted from the following references: (Weigelt and Banerjee, 2012, Kandoth et al., 2013, Lax et al., 2000, McConchy et al., 2011, Urick et al., 2011, Kuhn et al., 2012, Le Gallo et al., 2012)

1.1.5 Genomic sub-classifications for refining prognosis

The development of technologies for next-generation sequencing and bioinformatics approaches for handling large-scale datasets has enabled unbiased characterisation of genome-wide genetic alternations. Genome-wide studies have re-enforced the understanding of the overlap between the molecular landscape of high-grade endometrioid and serous carcinomas. The greatest discriminators between the two types are the higher prevalence of PTEN mutations and TP53 mutations in endometrioid and serous carcinomas, respectively.

The Cancer Genome Atlas Research Network (TCGA) assessed a large set of endometrial cancers (endometrioid n=307, serous n=53, mixed endometrioid with serous n=13) with next-generation sequencing technologies, in combination with DNA methylation analysis, reverse-phase protein array and microsatellite instability (Kandoth et al., 2013).

Following the integration of mutation spectra, copy number aberrations and MSI status, tumours were categorised into four genomic classes: 1) POLE (ultramutated ) tumours characterised by hotspot mutations in the POLE exonuclease domain (Church et al., 2013), low copy number aberrations, PTEN, PIK3CA, FBXW7 and KRAS mutations; 2) an MSI
A further study by the TransPORTEC initiative used routine molecular analysis and DNA hotspot mutations to detect prognostic subgroups. Four subgroups were identified from 116 high-risk EC patients 1) TP53 mutant tumours; 2) microsatellite instable tumours; 3) POLE-mutant tumours and 4) a group with no specific molecular profile. Consistent with the TCGA study, the POLE mutant and MSI groups had improved progression-free survival (93% and 95%, respectively) compared with the group 1 and 4 (42% and 52%, respectively)(Stelloo et al., 2015).

The mutational characteristics observed in EC are complex. Heterogeneity exists between type I and II cancers, with a degree of overlap between the two categories. The prevalence of individual genetic aberrations varies between endometrioid and non-endometrioid tumours, however no mutation in any gene has been identified to be exclusive to either tumour type. New molecular sub-classification of high-risk cancers, irrespective of the traditional Bokhman classifications can be used to improve clinical management and guide
provision of adjuvant therapy. A better understanding of the molecular characterisation may also assist in directing targeted therapies in the research setting. The presence of multiple mutations within a single tumour, however, suggests that a single target approach may not be successful.
1.2 Obesity and its association with EC

Overweight and obesity are defined as an excessive accumulation of adipose tissue that may impair health (WHO, 2012). Obesity is no longer a problem, exclusive to the Western world; developing countries across Asia, the Middle East, the Caribbean and Pacific Islands have seen a dramatic increase in obesity rates (Figure 2)(Crosbie et al., 2016). The latest Health Survey for England has shown that 31% and 27% of women were overweight and obese, respectively (NHS, 2015). Obesity is a recognised risk factor in most common solid tumours, and an established risk factor for EC (Calle et al., 2003, Crosbie et al., 2010). A large meta-analysis found a 5kg/m$^2$ increase in BMI to be significantly associated with EC (RR 1.59, p<0.0001) (Renehan et al., 2008). The odds of the histological type being low grade endometrioid also increases with increasing BMI [ BMI>40kg/m$^2$ vs BMI <25kg/m$^2$, (RR 2.7, 95% CI 1.2-5.9, p<0.001)] (Crosbie et al., 2010).

![Figure 2: World map showing the prevalence of obesity across the globe (BMI >30kg/m$^2$).] Estimates from the World Obesity Federation and World Health Organization ©World Obesity 2015. Reproduced from BJOG Editor’s Choice 2016 (Crosbie et al., 2016)

1.2.1 Hormonal imbalance in obesity

It is likely that the adverse effects of obesity are mediated by a combination of hyperoestrogenism, hyperinsulinaemia (Pollak, 2012a) and an imbalance of adipokines (Renehan, 2011). The unopposed oestrogen hypothesis postulates that the risk of EC is increased in women with high bioavailable oestrogen and/or low progesterone (Siiteri, 1978). Obese women have an excess of circulating oestrogen secondary to chronic anovulation or increased peripheral androgen conversion in adipose tissue (Kaaks et al., 2010).
Oestrogen binds directly to endometrial cell DNA and interacts with several proliferative pathways including PI3K/AKT/mTOR (Matias-Guiu and Prat, 2013). Oestrogen also increases the expression of c-Myc and cyclin A, genes linked to endometrial proliferation. The effect on these genes is more pronounced in obese rats compared with lean controls, suggesting that oestrogen in obesity induces an abnormal ratio of pro-proliferative and anti-proliferative signals (Zhang et al., 2009). Uncontrolled endometrial proliferation leads to increased replication errors, and the accumulation of mutations in both proto-oncogenes and tumour suppressor genes. If apoptosis is also impaired, cells harbouring mutations can survive and expand clonally, leading to hyperplasia or malignancy.

1.2.2 Hyperinsulinaemia and diabetes in EC
Most obese women have a degree of insulin resistance (Samuel et al., 2010) and hyperinsulinaemia. According to a large meta-analysis, women with T2DM have a two-fold increased risk of EC compared with non-diabetic controls (RR 2.10, 95% CI 1.8-2.5) (Friberg et al., 2007). Patients with EC also have a high incidence of undiagnosed insulin resistance, which is significantly associated with increased BMI (p<0.001) (Burzawa et al., 2011). Large case control studies have additionally reported that the combination of diabetes and obesity have an enhanced effect on EC risk; compared with non-diabetic, non-obese women, the OR for EC was 1.4 (95% CI 0.9-2.4) for non-obese women with diabetes, but rose to 5.1 (95% CI 3.0-8.7) for obese diabetic women (Lucenteforte et al., 2007).

1.2.3 Insulin and insulin-like growth factor (IGF) signalling
Insulin and IGFs share common signalling pathways. Insulin and IGFs bind to the insulin-like growth factor -1 receptor (IGF1R) and insulin receptor (IR). The IR and IGF1R are members of the tyrosine kinase class of membrane receptors. The receptors can associate to form holoreceptors or heterodimerise to form heteroreceptors, comprised of a half insulin receptor and a half IGF1R (Benyoucef et al., 2007, Belfiore et al., 2009). At a cellular level, receptor activation triggers activation of insulin receptor substrate-1 (IRS-1), which in turn triggers the PI3K/AKT/mTOR and MAPK signalling pathways. Insulin is reported to stimulate breast and endometrial cancer cell proliferation through activation of the PI3K/AKT pathways in cell culture models (Shukla et al., 2009, Wang et al., 2012).

Both IR and IGF1R are widely expressed on neoplastic and normal tissue. In the normal endometrium, cyclic changes in IGF-1 expression and signalling play key roles in regulating the transition of the premenopausal endometrium through the proliferative, secretory and
menstrual cycles. These receptors also have increased expression in multiple solid tumour types (McCampbell et al., 2006); high IGF1R expression has been reported in EC with increased proportions as BMI increased (Joehlin-Price et al., 2015). Although receptor levels are higher in cancer than in normal tissue, gene amplification associated with large increases in receptor number is rare. The receptors are rarely constitutively activated by mutation, or in a ligand-independent fashion, thus, ligand-mediated activation is necessary to influence carcinogenesis.

1.2.3.1 Hyperinsulinaemia and T2DM
Insulin is produced by specialised pancreatic β-cells and under physiological conditions production is tightly regulated by the level of circulating glucose. Abnormal autocrine production of insulin by cancers is uncommon, and insulin functions as a classic hormone, influencing tissues remote from its site of production. Insulin-stimulated glucose uptake by target tissues (liver, muscle, adipose tissue) reduces circulating glucose levels. In T2DM and obesity, many tissues become insulin-resistant (often induced by excess calories and over-activated cellular feedback pathways, e.g. phosphorylation of IRS) (Tanti and Jager, 2009) leading to hyperinsulinaemia. Initially, the increased insulin levels are sufficient to avoid hyperglycaemia. Hyperglycaemia eventually occurs because of increased insulin resistance and deficient insulin production from the pancreas. It is likely that the neoplastic tissue remains insulin-sensitive in the context of insulin resistance and is growth stimulated by the surrounding hyperinsulinaemia (Algire et al., 2010).

The main site of production of IGFs is the liver, although both IGF-1 and 2 are widely expressed by many cell types. Chronic hyperinsulinaemia increases production of IGF-1 in the liver (Boni-Schnetzler et al., 1991). There is substantial variation between normal individuals in circulating levels of IGF-1, with the variation being determined both by genetic and lifestyle factors (Harrela et al., 1996). An additional level of control is provided by the high affinity IGF-binding proteins (IGFBPs). These proteins modulate the bioavailability of IGFs by isolating them from clearance pathways and affecting their binding to receptors. There is evidence that free IGFBPs have anti-proliferative activity that is independent from their IGF-binding capacity (Firth and Baxter, 2002). Elevated insulin levels also result in altered levels of IGF-binding proteins (IGFBPs). Higher levels of IGF-1 and lower levels of IGFBP-1 have been reported in postmenopausal EC patients, consistent with a role in endometrial carcinogenesis (Ayabe et al., 1997). The data is conflicting, however, as a retrospective case-control study reported an inverse relationship between EC and IGF-1 levels (Petridou et al., 2003).
Increased insulin has been shown to inhibit IGFBP-1, resulting in elevated IGF-1 levels in endometrial stromal cells (Lathi et al., 2005). Reduced IGFBP1 levels are also associated with an increased risk of colorectal cancer in women (Kaaks et al., 2000). EC risk is increased with decreasing levels of IGFBP2 (RR 0.56, 95% CI 0.35-0.90), however is not significantly associated with IGFBP1 level (Cust et al., 2007). The role of IGFBPs remains unresolved, as over expression may contribute to cancer development in some settings by maintaining high local insulin concentration and by promoting tumour cell proliferation (Hsieh et al., 2010).

1.2.3.2 IGFs interaction with steroid hormones
The biological activity of the IGF system is strongly associated with oestrogen status. Oestrogens increase IGF binding and IGF1R mRNA levels in breast cancer cells, suggesting that oestrogen may stimulate breast tissue proliferation by enhancing IGF1R concentrations and potentiating the mitogenic effects of IGF1s (Stewart et al., 1990). Oestradiol is also shown to cause uterine epithelial proliferation through increased oestrogen receptor α (ERα) transcription in a mouse model. This signalling is mediated by IGF1R expression, and stimulation of the PI3K pathway (Zhu and Pollard, 2007).

1.2.4 Endocrine and inflammatory signalling in obesity

1.2.4.1 Adipokines
Adipose tissue plays a role in endocrine signalling and secretes adipokines (adiponectin & leptin) and a number of inflammatory cytokines. Leptin, a central mediator of the feedback loop that regulates appetite and energy homeostasis, is also involved in the STAT3 (signal transducer and activator of transcription 3) and MAPK pathways. A number of epidemiological studies have examined the association between leptin and cancer risk with differing conclusions. A regression analysis of women in the USA found that leptin was not associated with increased carcinoma in situ of the breast (Mantzoros et al., 1999), while an analysis of Japanese women with colorectal cancer indicated that leptin was associated with increased risk, independent of BMI (Tamakoshi et al., 2005). Increased levels of leptin are also associated with increased risk of EC (RR 1.6, 95% CI 1.3-1.9), after adjusting for confounding factors, including BMI (Wang et al., 2014). Leptin has been reported to activate proliferative MAPK pathways, by JUN-N-terminal kinase activation (Onuma et al., 2003) and by stimulating expression and activity of aromatase (Catalano et al., 2007).

Adiponectin, conversely, is reduced in obesity and acts to regulate glucose and lipid metabolism. Epidemiological studies have suggested that adiponectin levels are inversely
associated with breast (Tworoger et al., 2007) and EC risk (Dal Maso et al., 2004). Low levels of adiponectin are a surrogate marker of insulin resistance and correlate strongly with EC risk, irrespective of BMI. Adiponectin inhibits the PI3K/AKT/mTOR pathway by activation of AMPK and also acts on peroxisome-proliferator activated receptor-\(\alpha\) (PPAR-\(\alpha\)) (Yamauchi et al., 2007).

These data together suggest that leptin may stimulate and adiponectin may inhibit carcinogenesis. Actions of these adipokines on PI3K and MAPK pathways are likely to contribute to their opposing roles in cancer development.

1.2.4.2 Inflammatory Cells

Adipose tissue secretes inflammatory cytokines including tumour necrosis factor alpha (TNF-\(\alpha\)) (Hotamisligil et al., 1995, Souza et al., 2003), interleukin-6 (IL-6) (Fried et al., 1998, Olefsky and Glass, 2010) and plasminogen activator inhibitor 1 (PAI-1) (Sawdey and Loskutoff, 1991). These cytokines contribute towards insulin resistance and cause chronic inflammation that affects other tissues in the body. It is increasingly evident that these cytokines are produced from monocytes and other immune cells that infiltrate adipose tissue in obesity (Weisberg et al., 2003, Ruan et al., 2003).

TNF-\(\alpha\) is involved in the development of several cancers through promotion of angiogenesis and metastasis. It has been implicated in the development of colorectal cancer in a mouse model and its neutralisation reduces activation of pathways including PI3K/AKT/mTOR and reduces the growth of tumours in obese mice (Flores et al., 2012). The EPIC study reports an increased risk of EC amongst women in the highest versus lowest quartile of circulating TNF-\(\alpha\) (OR 1.73, 95% CI 1.1-2.7) (Dossus et al., 2011). IL-6 signals to the nucleus through STAT3, an oncoprotein that in activated in a wide variety of cancer, including cancer stem-like EC cells. Increased levels of IL-6 are associated with poorer outcomes (Bellone et al., 2005) and targeting of IL-6 inhibition is reported to decrease tumour proliferation \textit{in vitro} and \textit{in vivo} (van der Zee et al., 2015).

A further acute phase cytokine C-reactive protein (CRP), has been positively associated with increased EC risk. CRP is synthesised primarily by the liver in response to IL-6 and the markers are positively correlated. A case-cohort study nested within the Women’s Health Initiative reported a positive correlation between CRP and EC risk (hazard ratio HR 2.29; 95% CI 1.13-4.65, \(p=0.012\)) (Wang et al., 2011). A further case-control study reported similar findings; elevated CRP to be associated positively with increased risk of EC after
adjustment for age and cancer type (Odds ratio OR 1.25, 95% CI 1.03-1.52) (Friedenreich et al., 2013).

PAI-1 is the primary inhibitor of plasminogen activators urokinase and tissue plasminogen activator. Plasminogen is the precursor of plasma, and a key component for metastasis and angiogenesis (Dano et al., 2005). Increased PAI-1 levels were associated with shorter progression-free survival in EC patients with early stage disease (Fredstorp-Lidebring et al., 2001).

The chronic inflammatory state of obesity is likely to be linked to the development of insulin resistance and hyperinsulinaemia. Pro-inflammatory markers have also been implicated in carcinogenesis by their effects on innate and adaptive immune systems as well as through disturbed tissue homeostasis. A study that assessed obesity-related risk factors for EC including inflammation, hyperinsulinaemia and elevated oestrogen concluded that CRP was associated with increased EC risk after adjusting for BMI, but the HR was attenuated from 2.29 to 1.70 and did not reach further statistical significance following further adjustment for oestradiol and insulin. Pro-inflammatory makers can induce insulin resistance (Hotamisligil et al., 1996), stimulate the activity of enzymes involved with oestrogen biosynthesis and increase levels of oestrogen (Simard and Gingras, 2001). Thus, inflammation can contribute to increased levels of insulin and oestradiol and indirectly lead to the development of EC.

1.2.5 The effects of obesity on the endometrium
In summary, obesity is thought to contribute to endometrial carcinogenesis through several pathways, including hyperinsulinaemia, sex steroids, adipokines and inflammatory cytokines. Multiple metabolic aberrations exist, all of which contribute to a toxic metabolic environment and to driving endometrial hyperplasia and cancer.

The WCRF/AICR report gives life-style recommendations for cancer prevention, including intentional weight loss and increasing physical activity (WCRF, 2015). Intentional weight loss causes an increase in sex-hormone binding globulin (SHBG) levels and a decrease in levels of insulin, CRP and sex steroids (Byers and Sedjo, 2011, Hooper et al., 2010). Large prospective cohort studies also consistently suggest that physical activity protects against EC, independent of BMI (Moore et al., 2010). Based on these recommendations, weight loss and physical activity appear to be crucial in preventing EC risk.
The reality, however, is that 74% of the European population is insufficiently active, and 34% sedentary, an emerging risk factor for EC (Friedenreich et al., 2010, Moore et al., 2010). Life-style modifications, even in the clinical trial setting with monitored dietary interventions report very modest weight loss (mean weight loss 1.1kg over 2 years) (Bhopal et al., 2014). An alternative approach to modulation of cancer metabolism and creating a cellular “energy-deficit” is the anti-diabetes drug, metformin.
1.3 Metformin
Metformin (1,1-dimethylbiguanide hydrochloride) is one of the most preferred and cost-effective oral treatments for T2DM (Nathan et al., 2009, Inzucchi et al., 2012). It is recommended as initial pharmacological therapy when lifestyle modifications fail. When taken orally, it is absorbed rapidly across the intestinal epithelium and conveyed via the portal vein to the liver, its primary site of action. Metformin is not metabolised and is excreted unchanged in the urine and the bile.

Metformin monotherapy, while associated with initial gastrointestinal side effects, is usually well tolerated and not complicated by hypoglycaemia. The most serious side effect, lactic acidosis, is rarely seen in patients with normal renal and hepatic function. It is reported to occur in just 3/100 000 patient-years of use (Howlett and Bailey, 1999, Lalau and Race, 2000, Salpeter et al., 2006). Epidemiological studies have linked metformin exposure to a reduced risk of cancer. Pre-clinical studies have identified metformin to have a cytostatic effect on several cancers including EC. A number of ongoing early phase clinical trials aim to explore its anti-cancer effects and investigate its potential as therapy for EC.

1.3.1 Pharmacokinetics of metformin
The ability of metformin to exert antidiabetic and antineoplastic effects depends on its ability to be taken up by the target tissue. Metformin is primarily a hydrophilic molecule and has limited passive diffusion through cell membranes (Graham et al., 2011). Its uptake is largely dependent on organic cation transporters (OCTs). As a major target of metformin, hepatocytes express high levels of both OCT1 and OCT3 (Nies et al., 2009) (40). OCT1 has been demonstrated in the normal human endometrial stromal cells (Shao et al., 2014) but there are no publications confirming the presence of OCT3.

In the circulation, metformin has a large volume of distribution, likely attributable to the considerable tissue uptake of the drug. In animal and human models, metformin concentrations are several fold higher than serum concentrations in a variety of tissue types, including endometrial tumours (Wilcock and Bailey, 1994, Bailey et al., 1994, Mitsuhashi et al., 2014). After multiple doses of 1g twice daily, the mean plasma concentration of metformin ranges between 0.4-1.3mg/L (3-10µMol/L) (Timmins et al., 2005).
1.3.2 The proposed mechanisms of action of metformin in normal and neoplastic tissue

1.3.2.1 Metformin’s effects on the mitochondria and oxidative phosphorylation

Despite its widespread use in the treatment of T2DM, the mechanisms of action of metformin were only recently elucidated and remain incompletely understood. It has been proposed that metformin exerts its anti-cancer effects through multiple mechanisms of action, some of which overlap with its actions in normal tissue (Pollak, 2012b).

![Figure 3: Putative mechanisms of action of metformin in obesity and cancer. Metformin reduces systemic insulin, IGF-1 and oestrogen, thus reducing tyrosine kinase signalling to downstream targets e.g. PI3K/AKT/mTOR. Metformin activates AMPK and induces phosphorylation of TSC1/2 and subsequent inhibition of mTORC1. This leads to decreased protein synthesis and increased autophagy and apoptosis. AMPK activation also inhibits ACC, decreasing fatty acid synthesis. Inhibition of mTORC1 can also occur through AMPK-independent pathways. Anti-inflammatory actions have been reported, including reduced cytokine levels. Anti-angiogenic effects and reduced reactive oxygen species are also observed after metformin treatment. Reproduced from: Metformin in reproductive health, pregnancy and gynaecological cancer: established and emerging indications (Sivalingam et al., 2014). Abbreviations: 4EBP1 4E binding protein-1, S6 Ribosomal protein S6, ACC acetyl co-a carboxylase, AMPK AMP-activated kinase, AKT protein kinase B, ER oestrogen receptor, IR insulin receptor, IGF1R insulin-like growth factor 1 receptor, IL-6 interleukin-6, IRS-1 insulin receptor substrate-1, MAPK/ERK mitogen activated protein kinase/ extracellular signal regulated kinase, mTORC-1 mammalian target of rapamycin complex -1, REDD1 regulated in development and DNA damage response 1, ROS reactive oxygen species, TNF-alpha tumour necrosis factor – alpha, TSC tuberous sclerosis complex, VEGF vascular endothelial growth factor. It is believed that the fundamental mechanism of biguanides involves action on the mitochondria. Metformin inhibits the mitochondrial respiratory complex I (El-Mir et al., 2000, Owen et al., 2000) in cells, leading to reduced oxidative phosphorylation and ATP]
production. The resultant increase in AMP: ATP ratio mimics cellular energy stress, inhibits gluconeogenesis and activates AMP-activated kinase (AMPK) (Figure 1). Inhibition of oxidative phosphorylation leads to reduced ATP production and reprogramming of cellular energy metabolism in a manner to restore ATP levels. In cancer cells, there may be a switch to glycolysis and downregulation of ATP-consuming processes.

1.3.2.1 Metformin acts directly on AMPK to inhibit mTOR
AMPK is a key metabolic regulator that is frequently inactivated in energy-rich environments such as obesity and insulin resistance. Activation of AMPK mitigates energy depletion by acting on key enzymes including acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) to downregulate lipid synthesis (Zhou et al., 2001). AMPK activation also inhibits mammalian target of rapamycin (mTOR) signalling, a pathway involved in protein synthesis and mRNA translation. This is mediated by phosphorylation and activation of TSC2 and subsequent inhibition of mTOR.

Multiple studies have demonstrated that metformin has direct effects on AMPK, resulting in inhibition of mTOR signalling and decreased cellular proliferation (Zakikhani et al., 2006, Cantrell et al., 2010). Metformin treatment in breast cancer cell lines has demonstrated decreased activation of AKT (Zakikhani et al., 2010). The PI3K pathway is a critical signal transduction pathway involved in cellular proliferation, protein synthesis, cell survival, cellular metabolism and angiogenesis. PI3Ks are activated through several mechanisms including binding of a growth factor ligand (e.g. insulin-like growth factor 1) to its receptor tyrosine kinase. Activation of PI3K generates phosphatidylinositol 3,4,5-triphosphate (PIP₃) from phosphatidylinositol 4,5-biphosphate (PIP₂), a process negatively regulated by PTEN. Loss of PTEN function results in uncontrolled PI3K activity, which may lead to uncontrolled cellular proliferation and cancer (Slomovitz and Coleman, 2012). Type I ECs have a high rate of PTEN and PIK3CA mutations, as discussed previously.

PIP₃ recruits AKT, the serine-threonine kinase to the plasma membrane, where further phosphorylation by mTOR-rictor (mTORC2) fully activates AKT as a central node in the PI3K pathway. Activated AKT initiates a cascade of downstream signalling events, which promote cellular growth, metabolism, proliferation, survival, apoptosis and angiogenesis. A major downstream effector of AKT is mTORC complex 1. Activated AKT inactivates TSC1/2 which releases its inhibition on the mTOR-regulatory protein of mTOR (Raptor) (mTORC1) complex. Raptor also recruits substrates to mTORC1 for phosphorylation including the
eukaryotic initiation factor 4E binding protein (4EBP) and p70 ribosomal S6 kinase (p70S6K), which promotes ribosome biogenesis and protein synthesis (Figure 1).

The initiation of mRNA translation is generally the rate-limiting step of protein synthesis. Cellular mRNAs contain a 5′inverted 7-methylguanosine cap that is a recognition site for the translation initiation mechanism. The cap is recognised by initiation factor eIF4G which is regulated by mTOR. mTOR phosphorylation of 4EBPs promotes cap dependent mRNA translation by inactivating their eIF-sequestering activities (Pause et al., 1994). The selective inhibition of translation of a specific group of mRNAs that encode protein involved in angiogenesis, survival and other oncogenic functions may contribute to the anti-cancer effects of metformin.

1.3.2.2 Metformin modulates circulating insulin and glucose.
Systemic effects of metformin in diabetic patients were studied before cellular mechanisms were fully understood. T2DM is characterised by insulin resistance and increased circulating insulin and glucose. In the liver, metformin reduces hepatic glucose production, stimulates insulin-mediated glucose uptake by the liver and skeletal muscles and reduces substrate availability for gluconeogenesis by lowering serum lipid levels. This action secondarily lowers insulin levels, and is recognised as the “indirect” mechanism of action of metformin. A large randomised placebo controlled study in breast cancer reported significant decreases in glucose (-3.8%) and insulin (-11.1%) following treatment of non-diabetic subjects with adjuvant metformin after six months. These results suggest that metformin can reduce insulin levels, even in the context of normoglycaemia.

Insulin and insulin-like growth factors (IGFs) are strongly mitogenic and have stimulatory effects on cancer cell growth and metastasis (Wang et al., 2012, Ferguson et al., 2013). While normal target tissue (liver, skeletal muscle and adipose tissue) becomes insulin-resistant in the context of hyperinsulinaemia, tumour cells may have heightened sensitivity and respond to insulin stimulation. High circulating insulin levels increase hepatic IGF-1 production, while reducing IGF-binding protein synthesis, resulting in increased bioavailable IGF-1. Metformin treatment leads to a decreased hepatic glucose secretion and increased glucose uptake, resulting in an overall decrease in circulating insulin and glucose; thus reducing mitogenic stimulation. Oral metformin in a lung cancer animal model reduced tumour size by up to 50% independent of AMPK activation. Small decreases in circulating insulin and IGF-1 were noted, with significant inhibition of IGF1R signalling detected by downregulation of downstream pathways, including mTOR (Memmott et al.,
2010). A prior treatment strategy using a somatostatin analogue, ocretotide in addition to adjuvant treatment in breast cancer to reduce insulin and IGF-1 resulted in a statistically significant change in IGF-1 and C-peptide measurements. There was no clinical benefit however, after two years of treatment and gallbladder toxicity shortened the additional administration of octreotide (Chapman et al., 2015). Metformin may represent a more acceptable therapeutic strategy with its favourable long-term safety profile.
1.3.3 Existing evidence to support metformin as an anti-cancer therapy

1.3.3.1 Pre-clinical studies of metformin as an anti-cancer therapy

Pre-clinical studies in multiple cell lines including Ishikawa and ECC-1 EC cells have reported that metformin potently inhibits cell proliferation in a dose-dependent manner, albeit at supratherapeutic concentrations (Cantrell et al., 2010, Sarfstein et al., 2013). In these studies, metformin is thought to act through AMPK-dependent pathways to reduced mTOR activation with reduced phosphorylation of S6. LKB1, a tumour suppressor gene and activator of AMPK also modulates the effect of metformin. LKB1-deficient cells are more sensitive to metformin treatment, owing to their impaired ability to activate energy-conserving mechanisms (Algire et al., 2011).

Other proposed mechanisms of action include inhibition of cell cycle progression by downregulating cyclin D1 in MCF7 breast cancer (Zakikhani et al., 2006) and prostate cancer cells (Ben Sahra et al., 2008). Only a moderate effect on cell proliferation has been noted on normal cells, suggesting that metformin may specifically target cancer cells, a rapidly proliferating population.

Recent studies have also demonstrated that metformin inhibits mTOR through AMPK-independent pathways, including the Rag family of GTPases (Kalender et al., 2010) and the hypoxia inducible factor (HIF) target gene, regulated in developed and DNA damage response I (REDD I) (Ben Sahra et al., 2011). Treatment of EC cells with metformin causes displacement of constitutively active KRAS from the cell membrane, resulting in uncoupling of the mitogen-activated protein kinase (MAPK) signalling pathway (Iglesias et al., 2013).

Angiogenesis is a key process in cancer growth and metastasis. Metformin treatment blocks lutenising-hormone induced vascular endothelial growth factor (VEGF) expression, which is associated with tumour angiogenesis in Hey ovarian cancer cells (Liao et al., 2012). Metformin has also been shown to inhibit aromatase expression in primary culture of breast adipose stromal cells, suggesting that metformin treatment may decrease circulating levels of oestrogen in obese women (Brown et al., 2010).

1.3.3.2 Epidemiological evidence for metformin as an anti-cancer drug

A seminal report which concluded that T2DM patients on metformin may have a reduced incidence of cancer (OR 0.86, 95% CI 0.73-1.02) sparked interest in its potential application as an anti-cancer therapy (Evans et al., 2005). Despite initial promise, the evidence from epidemiological data linking metformin use in T2DM patients with reduced cancer risk is inconsistent. One meta-analysis of over 4000 cancer events and 500 cancer deaths
concluded that metformin reduced cancer risk by one third, however, this effect was limited to pancreatic and hepatocellular cancers (Decensi et al., 2010). A meta-analysis of fourteen RCTS did not concur with these findings, reporting no association between metformin use and cancer risk. The authors acknowledged, however, that their findings were limited by heterogeneity of the included trials, absent cancer data from two studies and a relatively short follow-up period. This review also highlighted that other diabetes drugs may increase risk, rather than metformin reducing it. (Stevens et al., 2012). Several retrospective studies have specifically assessed the impact of metformin use on the incidence of EC with conflicting conclusions. A recent case-control analysis derived from the UK-based General Practice Research Database explored the association between metformin use and EC risk over a 17-year follow-up period. There was no effect of the ever use of metformin (adjusted OR 0.65, 95% CI 0.63-1.2), nor was long term metformin treatment (>25 prescriptions) associated with reduced EC risk (Becker et al., 2013). Similarly, Ko et al. reported no difference in risk of EC between metformin-users and non-users in a population based cohort study (Ko et al., 2015). Conversely, a large Taiwanese study identified a protective effect of metformin use with a HR of 0.68 (95% CI 0.61-0.74) in metformin ever-users (Tseng, 2015) (Table 2).

The data on the effects of metformin on EC survival are more promising. A retrospective cohort study of patients treated for Type II EC found improved survival rates among T2DM patients treated with metformin, compared with those not using metformin and those without diabetes. This association remained significant after adjusting for age, clinical stage, grade and adjuvant therapy. The authors attributed the lack of association between metformin use and overall survival in Type I tumours to the low death rate in this group; (14% of patients with Type I tumours died versus 52% of type II EC) (Nevadunsky et al., 2014). Metformin users with EC were found to have improved recurrence-free and overall survival in another study of similar design. Non-metformin users had 1.7-fold poorer recurrence-free survival (95% CI 1.3-2.6, p=0.01) and were 2.3-fold more likely to die (95% CI 1.3-4.2, p=0.005) (Ko et al., 2014b). In addition, a retrospective review of obese women with type I EC reported that metformin use was significantly associated with reduced recurrence (1.9%) versus obese non-users (10.3%), over a 24 month follow-up period (Hall et al., 2016). Metformin therapy has also been associated with improved RFS in patients treated with chemotherapy for high stage or recurrent EC (Ezewuiero et al., 2016) (Table 2)
Many epidemiological studies are recognised to be limited by their retrospective nature and selection bias, introduced by sampling from hospital registries. Studies may also include patients with prior diagnoses of cancer and rely on self-reporting of confounding lifestyle variable such as physical activity and tobacco use.
Table 2: Summary of epidemiological studies assessing the impact of metformin use on the incidence and prognosis of EC.

<table>
<thead>
<tr>
<th>Study design &amp; participants</th>
<th>Co-variates</th>
<th>Conclusions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrospective cohort study assessing incidence of EC in 478 921 patients.</td>
<td>Age, obesity co-morbidities, use of other medications</td>
<td>-285 916 never users and 193 005 ever users of metformin. -Incidence of EC was 60 and 122 per 100,000 person-years for metformin users and never users, respectively. -Incidence of EC decreased in ever-users compared to never-users (HR 0.68 95% CI 0.61-0.74)</td>
<td>(Tseng, 2015)</td>
</tr>
<tr>
<td>Retrospective cohort assessing incidence of EC in 541 128 patients with T2DM.</td>
<td>Age, contraceptive use, HRT use, endometrial hyperplasia, obesity</td>
<td>-84% on metformin. -Univariate analysis, metformin users had lower risk of EC (HR 0.81, 95% CI 0.67-0.97) vs sulphonylurea users. -Multivariate analysis, metformin use was not associated with reduced risk of EC ( HR 1.1, 95% CI 0.9-1.4)</td>
<td>(Ko et al., 2015)</td>
</tr>
<tr>
<td>Prospective cohort study assessing the incidence of EC in 88107 post-menopausal women.</td>
<td>Age, ethnicity, education, physical activity, alcohol intake, HRT use, parity, BMI</td>
<td>No difference in incidence of EC between metformin users vs never-users ( HR 1.1, 95% CI 0.8-1.6)</td>
<td>(Luo et al., 2014)</td>
</tr>
<tr>
<td>Case-control study assessing the incidence of EC in 2254 cases of patients with EC and 15324 matched controls.</td>
<td>Age, smoking, BMI, co-morbidities</td>
<td>-2445 cases of EC -Metformin ever-users did not have an altered risk of EC compared with never-users (OR 0.9, 95% CI 0.6-1.2)</td>
<td>(Becker et al., 2013)</td>
</tr>
<tr>
<td>Retrospective cohort study assessing OS in 349 patients who received chemotherapy for Stage III-IV &amp; recurrent EC.</td>
<td>BMI, age, race, smoking history, FIGO stage</td>
<td>-17% had diabetes, 50% on metformin -Median OS longer with metformin use; 45.6 months with metformin, 12.5 months in diabetics with no metformin, 28.5 months in non-diabetics (p=0.023).</td>
<td>(Ezewuiro et al., 2016)</td>
</tr>
</tbody>
</table>
Multivariate analysis metformin users had improved OS compared with never-users with diabetes. (OS HR 0.42 95% CI 0.23-0.78)

Retrospective cohort study assessing OS/PFS in 1303 patients managed surgically for stage I-IV EC.

Propensity scoring adjusted for age, BMI, grade, stage, myometrial involvement, operative complexity, lymph node dissection, adjuvant therapy.*

-21% had diabetes, 42% treated with metformin.
- No difference in OS or RFS between metformin users with non-diabetics (OS HR 1.03, 95% CI -0.6-1.9 RFS HR 1.1 95% CI(0.5-2.6)
or with other patients with diabetes (OS HR 0.6, 95% CI 0.3-1.2, RFS 1.1, 95% 0.3-3.3).

Retrospective cohort study assessing recurrence in 351 patients with EC and BMI > 30kg/m².

N/A

-18.2% on metformin.
- In type 1 EC, recurrence rate of metformin users was reduced compared with metformin never users; 1.9% vs 10.3% (p=0.05).
- No difference in OS.

Retrospective cohort study assessing prognosis in 1496 patients with EC.

Age, stage, histology, adjuvant treatment

-54% on metformin
- Non-metformin users had worse RFS (HR 1.8, 95% CI 1.1-2.9, p=0.02) and OS (2.3 95% CI 1.3-4.2, p=0.005) compared with metformin users.

Retrospective cohort study assessing prognosis in 985 patients with EC.

Age, BMI, stage, grade, adjuvant therapy

-12% on metformin.
- Diabetics on metformin had improved OS (HR 0.54, 95% CI 0.30-0.97, p=0.04) compared with non-diabetics (non-endometrioid histology only).

The box colours indicate whether the study supported the hypothesis that metformin use reduced incidence or improved survival completely (white) or partially (pale grey). Dark grey studies did not support a protective effect of metformin use. Abbreviations: EC Endometrial cancer, HR Hazard ratio, OS Overall survival, RFS Recurrence-free survival.

*Propensity scoring was used to balance observed covariates between patients in study groups and calculated using a multivariable logistic regression model including demographic and clinical covariates.
1.3.4 Proof of principle presurgical window of opportunity studies

Metformin has a well-established safety profile and its extensive use in patients with T2DM including those undergoing chemo/radiotherapy suggests that it is safe to be used in the cancer setting. Metformin is also used safely in combination with numerous other drugs for common diseases, including hypertension and hypercholesterolaemia. This has promoted bypassing of the traditional drug development paradigm in the repurposing of metformin as an anti-cancer drug and early translation of pre-clinical findings to patient studies.

Window of opportunity presurgical studies allow the testing of an established drug for a new purpose directly in the patient population. This is often carried out as a short-course of treatment in the neoadjuvant setting between diagnosis and surgical treatment. This setting is ideal, as it allows single agent metformin to be tested in an uncontaminated therapeutic window without compromising standard clinical care. Most early phase studies have compared serum and tumour samples taken before and after metformin treatment and used biomarkers of clinical response as surrogate primary end-points.

1.3.4.1 Developing surrogate end points in presurgical window studies

The most widely used primary outcome measure thus far has been an assessment of Ki-67 expression, an immunohistochemical measure of actively dividing tumour cells. The Ki-67 score is usually reported as the percentage of positively stained nuclei and a decrease in Ki-67 score is interpreted as a reduction in cellular proliferation. Proliferation is a recognised hallmark of cancer (Hanahan and Weinberg, 2011) and Ki-67 is a nuclear protein that is only expressed by actively proliferating cells. It has been extensively validated as a prognostic and predictive biomarker of clinical response in breast cancer both in the neoadjuvant and long-term adjuvant setting (Dowsett et al., 2011). The change in Ki-67 score has been used to assess response to short-term metformin in multiple tumour types, including breast, prostate and EC (Hadad et al., 2011, Bonanni et al., 2012, Niraula et al., 2012a, Joshua et al., 2014, Mitsuhashi et al., 2014, Schuler et al., 2015). Studies have reported both an absolute (post-intervention – pre-intervention Ki-67 score) and relative [(post-intervention-pre-intervention Ki-67 score)/pre-intervention Ki-67 score] change in Ki-67 expression. Findings from presurgical window studies of metformin in breast, prostate and endometrial cancer are summarised in Table 3.

In breast cancer, hormonal treatments have been shown to reduce Ki-67 expression in both the short (six weeks) and long term. Early changes in Ki-67, even after two weeks of treatment, have been found to correlate with changes after three to four months and are mirrored by clinical response (Clarke et al., 1993, Decensi et al., 2003, Dowsett et al., 2007,
DeCensi et al., 2011). Three studies have used short-term change in Ki-67 score as a measure of response in EC, but there is a lack of consensus on IHC methodology. There is also a need to establish a robust and reproducible scoring system for Ki-67 expression in EC and to establish its role as a prognostic biomarker in EC. A small number of studies have evaluated the significance of Ki-67 as a prognostic biomarker in EC with conflicting results.

Some window studies have measured the proportion of tumour cells undergoing programmed cell death, or apoptosis, before and after metformin treatment (Niraula et al., 2012a, Cazzaniga et al., 2013). Two measures have been used, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) and immunohistochemical assessment of cleaved caspase-3. The TUNEL assay is a standard technique for detection of apoptosis in tissue sections. This technique can be non-specific and label non-apoptotic nuclei and cells in necrotic areas, thus resulting in erratic findings (Ansari et al., 1993, Gavrieli et al., 1992, Kockx et al., 1998). The TUNEL assay can fail to distinguish reliably between apoptotic cell death and tumour necrosis (Duan et al., 2003).

Apoptosis is mediated by a proteolytic cascade, and upon activating through proteolytic processing, caspases trigger substrate proteolysis and other changes resulting in chromatic condensation, DNA fragmentation and ultimately, the apoptotic phenotype (Thornberry and Lazebnik, 1998, Slee et al., 2001, Goyal, 2001). Caspase-3 is the one of the primary executioners of apoptosis, necessary for the cleavage of a large number of proteins (Slee et al., 2001). The detection of activated, or cleaved caspase-3 can be a valuable and specific tool for identifying apoptotic cells in tissue sections, even before all the morphological features of apoptosis occur. The caveat to this technique is that apoptosis may occur without caspase activation or with alternative executioner caspases (Borner and Monney, 1999, Sperandio et al., 2000). In general, however, the percentage of apoptotic cells detected by cleaved-caspase 3 immunohistochemistry is very close to that counted on H&E-stained sections. Overall, the cleaved caspase-3 immunohistochemistry technique is a simple and reliable technique for quantification of apoptotic cells (Duan et al., 2003) and a reasonable method of measuring apoptosis as a surrogate end-point.
<table>
<thead>
<tr>
<th>Tumour</th>
<th>Study design and participants</th>
<th>Dose and duration</th>
<th>Change in Ki-67 score after metformin treatment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>Randomised: metformin (n=24) vs no drug (n=22)</td>
<td>1g twice daily; 14 days</td>
<td>Mean absolute decrease in Ki-67 [-3.4% (p=0.03)].</td>
<td>(Hadad et al., 2011)</td>
</tr>
<tr>
<td>Breast</td>
<td>Single arm: Metformin (n=39)</td>
<td>500mg three times daily; Median 18 days</td>
<td>Mean absolute decrease in Ki-67 [-3.0% (p=0.016)].</td>
<td>(Niraula et al., 2012a)</td>
</tr>
<tr>
<td>Breast</td>
<td>Randomised: Metformin (n=100) vs placebo (n=100)</td>
<td>850mg twice daily; 28 days</td>
<td>Overall: Mean proportional increase in Ki-67 with metformin[ +4% (p=0.4)]. HOMA-IR&gt;2.8, Mean proportional decrease in Ki-67 [-10.5% (p=0.045)]. BMI&gt;27kg/m², Mean proportional decrease in Ki-67 – [-8% (p=0.14)]. Proportional change adjusted for co-variates[^1].</td>
<td>(Bonanni et al., 2012)</td>
</tr>
<tr>
<td>Breast</td>
<td>Single arm: Metformin (n=35)</td>
<td>1500mg daily; 14 days</td>
<td>Invasive tumours: Mean absolute decrease in Ki-67 [-0.56% (p=0.06)].</td>
<td>(Kalinsky et al., 2014)</td>
</tr>
<tr>
<td>EC</td>
<td>Single arm: Metformin (n=31)</td>
<td>1500-2250mg daily; 28-42 days</td>
<td>Endometrioid tumours. Mean relative decrease in Ki-67 [-44.2% (p&lt;0.001)].</td>
<td>(Mitsuhashi et al., 2014)</td>
</tr>
<tr>
<td>EC</td>
<td>Single arm, obese: Metformin (n=20)</td>
<td>850mg daily; Mean 15 days</td>
<td>Endometrioid tumours. Mean absolute decrease in Ki-67 [-11.8% (p=0.008)].</td>
<td>(Schuler et al., 2015)</td>
</tr>
<tr>
<td>EC</td>
<td>Single arm: Metformin (n=11)</td>
<td>500mg three times daily; Mean 37 days</td>
<td>Endometrioid and non-endometrioid tumours. Mean relative decrease in Ki-67 [-9.7% (p=0.02)].</td>
<td>(Laskov et al., 2014)</td>
</tr>
<tr>
<td>Prostate</td>
<td>Single arm: Metformin (n=22)</td>
<td>500mg three times daily; Median 41 days</td>
<td>Mean relative decrease in Ki-67 [-29.5 (p=0.0064)].</td>
<td>(Joshua et al., 2014)</td>
</tr>
</tbody>
</table>

Studies presented both absolute and relative decreases in Ki-67 score, suggesting a decrease in cellular proliferation.[^1] Bonanni et al. used an analysis of co-variance adjusted for baseline Ki-67 and change in placebo-controlled patients. Single arm studies used paired t-tests or Wilcoxon signed rank tests to compare pre-and post-intervention Ki-67 scores.
1.3. 4.2 Findings from presurgical window studies of metformin
The most extensive research in the presurgical effects of metformin has been reported in breast cancer. In two trials, the absolute Ki-67 score in core breast biopsies reduced significantly following short-term metformin treatment (1-4 weeks) (Hadad et al., 2011, Niraula et al., 2012a) (Table 3). A randomised trial of metformin 1700mg versus placebo, however, only showed a significant decrease in Ki-67 score in women who were insulin resistant or overweight (BMI> 27kg/m²), not the overall metformin group (Bonanni et al., 2012). The single arm study in breast cancer reported increased apoptosis measured by TUNEL post-treatment in comparison to baseline biopsies (Niraula et al., 2012a). In contrast, the placebo-controlled trial only revealed an increase in apoptosis in non-insulin resistant women following metformin treatment (Cazzaniga et al., 2013). A placebo-control is an obvious strength, but Bonanni et al. report both an extended wash-out period and variability in time between the last metformin dose and obtaining the surgical specimen, a factor that could affect interpretation of findings. Taken together, these biomarker studies show alterations in surrogate end-points but do not establish if systemic or local actions of metformin underlie the observed effects.

Targeting tumour metabolism would seem a rational therapeutic intervention in EC, which is strongly associated with obesity and hyperinsulinaemia. The effect of presurgical metformin has also been assessed in small single-arm studies of patients with EC (Table 3). A high dose (1500-2250mg/day) of metformin was administered to women with type 1 EC over a four to six week period in a single arm Japanese study. This resulted in a significant decrease in Ki-67 score of 44% (95% CI 35-53, p<0.001) and significant decrease in topoisomerase IIa labelling (Mitsuhashi et al., 2014). Two smaller studies have reported similar trends of decrease in Ki-67 score following presurgical treatment (Schuler et al., 2015, Laskov et al., 2014). These studies also assessed the changes in phosphorylation of downstream mTOR pathways, however, findings are inconsistent with data from cell lines and animal models (Schuler et al., 2015).

In a prostate cancer single-arm study, metformin treatment (1500mg/day) resulted in a significant decrease in Ki-67 score and was well tolerated in the neoadjuvant setting (Joshua et al., 2014). A six-month study of differing doses of metformin in patients with rectal aberrant crypt foci (ACF) (a pre-malignant colorectal lesion) concluded that the number of ACF per patient was reduced in the high (1500mg/day) and medium (500mg/day) metformin-treated group, but unchanged in the low dose (250mg/day) and untreated controls (Zhao et al., 2015). More recently, a placebo controlled randomised
study of metformin 250mg once daily reported a significant decrease in colorectal adenomas in non-diabetic post-polypectomy patients [risk ratio 0.60 95% CI 0.39-0.92 (p=0.016)] after a 1 year follow-up (Higurashi et al., 2016). These findings suggest that low dose metformin may have an effect on pre-malignant lesions and a role in cancer prevention.

These early clinical studies suggest a biological effect of metformin in vivo (Table 3). They also raise important questions about variables that may modify the effect of metformin, including BMI, host insulin resistance and therapeutic treatment ranges.

1.3.5 Metformin in combination with neoadjuvant and adjuvant therapies in EC

If obesity and insulin resistance are important drivers for EC, metformin may inhibit tumour growth both by reducing its nutrient supply and hindering its growth-stimulatory environment. Thus, metformin may be useful both as primary treatment and in the adjuvant setting (post-hysterectomy) to prevent recurrence and improve long-term survival from EC. A number of studies have assessed the effects of metformin in combination with standard chemotherapy with varying results (Table 4). Simultaneous exposure of EC cell lines to metformin with paclitaxel resulted in significant synergistic anti-proliferative effects (Hanna et al., 2012). Combined treatment of Ishikawa EC cells with metformin and cisplatin had additive antiproliferative effects (Uehara et al., 2015). There are no prospective clinical studies assessing the combination of metformin with standard chemotherapy. Ezewuiro et al. report in a retrospective study that patients on metformin have an improved OS when receiving chemotherapy for advanced/recurrent EC compared with non-metformin users with diabetes (Ezewuiro et al., 2016). In contrast, a xenograft study of endometrioid EC in nude mice reported no decrease in tumour growth with metformin alone or in combination with carboplatin (Schrauwren et al., 2015).

The combination of metformin and Diane-35 (cyproterone acetate-ethinyloestradiol combined oral contraceptive) in five women with PCOS and stage 1a grade I EEC resulted in complete resolution of hyperplasia and carcinoma after six months (Li et al., 2014). A more extensive phase II study treated seventeen patients with atypical endometrial hyperplasia and nineteen with stage 1 EC with medroxyprogesterone acetate (400mg/day) and metformin (750-2250mg/day) for 24-36 weeks. Of the 36 eligible patients, 81% achieved complete remission within 36 weeks; 27 remained disease free for a median follow-up period of 38 months (Mitsuhashi et al., 2015) (Table 4). This study did not have a metformin or medroxyprogesterone acetate alone arm, making it impossible to assess if
the combination therapy was superior to single agent treatment. The prospective evidence for conservative treatment of EC remains limited. An open label, three-armed phase II study, however, aims to address these questions by randomising women with AEH or early stage EC to progesterone treatment (via a levonorgestrel intrauterine system) ± metformin ± weight loss intervention. The primary outcome is to achieve a pathological complete response at six months from study initiation (Hawkes et al., 2014).

Metformin may have a role both in primary and secondary prevention of EC. Two studies have reported that the addition of metformin to megestrol (a progestin) achieved superior complete response of hyperplasia, compared with megestrol alone (Shan et al., 2014, Tabrizi et al., 2014). An EC chemoprevention study which is comparing the effect of metformin 850mg with placebo in obese, postmenopausal women over a four month period is ongoing (NCT 01697566). The primary outcome of this study is the change in Ki-67 score and findings will inform on the role of metformin as primary prevention of EC. This study was supported by findings from an obese rat model which suggests that clinically relevant oral doses of metformin can attenuate oestrogen-induced pro-proliferative changes in the endometrium (Zhang et al., 2013).

Findings from these chemoprevention and primary treatment studies could benefit patients, particularly those who wish to conserve fertility and reduce health system costs associated with currently observed surgical standard of care. Identifying a drug which may modify or prevent EC carcinogenesis in obese women will be of utmost value.
Table 4: Current evidence from pre-clinical and clinical studies supporting or disputing the role of metformin in EC.

<table>
<thead>
<tr>
<th>Role of metformin</th>
<th>Current evidence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary prevention of EC for at risk populations e.g. obese women</td>
<td>- Metformin treatment attenuated oestrogen-induced pro-proliferative changes in the obese rat endometrium.</td>
<td>(Zhang et al., 2013)</td>
</tr>
</tbody>
</table>
| Secondary prevention of EC                                                       | - Metformin + megestrol acetate resulted in complete response in 75% of patient with AEH compared with 25% in the megestrol alone group after 12 weeks (n=16).  
   - Metformin cause resolution of AEH in 96% patient compared with 62% of those treated with megestrol after 12 weeks (n=43). | (Shan et al., 2014)  
   (Tabrizi et al., 2014)                                                                                                            |
| Primary treatment of EC                                                           | - Metformin treatment has significant anti-proliferative action on EC cell lines.  
   - Metformin + medroxyprogesterone acetate treatment achieved partial/complete regression in 94% of patients with AEH/EC over 36 months (n=36).  
   - Metformin + Diane-35 (containing cyproterone acetate + ethynloestradiol) led to complete resolution of G1 Stage 1A EC after six months of treatment on the background of PCOS (n=5). | (Cantrell et al., 2010)  
   (Mitsuhashi et al., 2015)  
   (Li et al., 2014)                                                                                                                   |
| Adjuvant therapy in combination with chemo/radiotherapy                          | - Combined metformin and paclitaxel have synergistic anti-proliferative effects in EC cell lines.  
   - Improved OS in patients who received chemotherapy for advanced/recurrent EC taking metformin compared with non-metformin users with diabetes. Retrospective cohort study n=349.  
   - Combined therapy with metformin and cisplatin had additive anti-proliferative effects of EC cells.  
   - Concurrent metformin during adjuvant radiotherapy was associated with increased incidence of acute locoregional toxicity compared with non-metformin users. Case-control study n=102.  
   - Metformin treatment did not result in enhanced anti-cancer effect in combination with carboplatin in a mouse-xenograft model of EC. | (Hanna et al., 2012)  
   (Ezewuiro et al., 2016)  
   (Uehara et al., 2015)  
   (Ferro et al., 2013)  
   (Schrauwen et al., 2015)                                                                                                           |

Presurgical window studies supporting the biological effects of metformin in EC are listed in Table 3. Abbreviations: AEH Atypical endometrial hyperplasia, EC Endometrial cancer, G1 Grade 1, OS Overall survival, PCOS Polycystic ovary syndrome.
1.3.6 Challenges in translating from pre-clinical studies
The current evidence from pre-clinical and clinical studies assessing the role of metformin in EC as primary prevention, treatment or in combination with adjuvant therapy is summarised in Table 4. There have been some important negative findings of clinical trials of metformin. The randomised-placebo controlled study in breast cancer reported an overall null-effect of metformin treatment on cellular proliferation (Bonanni et al., 2012), despite promising data from single-arm studies (Niraula et al., 2012a, Hadad et al., 2011). The contrasting findings from these studies cannot easily be explained, but variability between baseline insulin resistance and BMIs of patient groups, different metformin doses and longer drug cessation periods prior to final tumour sampling and the lack of control groups in some studies may account for this.

A recent open-label randomised phase II trial investigating the effects of metformin (2g daily) combined with standard chemotherapy in metastatic pancreatic cancer reported no survival benefit (p=0.61) with metformin. Overall, survival at six months was 53% (95% CI 33-69) in the control group and 42% (95% CI 42-59) in the metformin group (Reni et al., 2015). Similar null findings were reported from a trial in the Netherlands (Kordes et al., 2015).

There are several reasons why compelling pre-clinical evidence of a beneficial drug effect fails to translate into a clinically important difference in patients. Many pre-clinical studies have used supratherapeutic doses of metformin, which do not equate to the doses typical for glycaemic control used in cancer trials. Thus, the drug concentration in the target tissue (for e.g. tumour) necessary to observe a cytostatic effect may not be attainable with the doses used. Pre-clinical studies have primarily been carried out in cell lines, which are usually a homogenous population. In vivo tumours are often heterogeneous, and tumour response complicated by surrounding hypoxia. In vitro models are artificial in many aspects, cell culture media is supplemented with high levels of glucose and growth factors, thus cancer cell lines are often maintained and tested in non-physiological conditions, optimised for maximum growth. A host “response” is difficult to reproduce in vitro, and as a result the indirect effects of metformin on hyperglycaemia have not been thoroughly explored. Finally, if the anti-cancer benefits of metformin occur as a systemic reduction of hyperglycaemia and hyperinsulinaemia, the survival benefit may not translate to non-diabetic patients.
1.3.7 Understanding metformin’s effects for targeted benefit

The anti-cancer effects of metformin are associated with multiple mechanisms of action. These include modulation of the insulin and IGF-1 signalling pathways and direct targeting of the PI3K/AKT/mTOR pro-proliferative pathway through actions on the mitochondria.

The safety profile of metformin has allowed bypassing of the traditional drug development structure and has expedited its use in the patient population. There is a risk, however, that findings from small clinical trials may not illustrate the expected clinical benefit because of suboptimal trial design. Most trials have used a standard dose of metformin rather than a varied dosage regime, likely driven by the time and cost required to recruit more patients to adequately power each treatment group. It remains to be determined whether metformin at standard anti-glycaemic doses will improve survival from EC.

Presurgical window studies of short duration rely on carefully validated biomarkers as surrogates for clinical response. In longer studies, in addition to traditional clinical end points (e.g. progression-free survival, complete pathological response), it is vital that translational analysis is included in clinical trial design. As many presurgical studies are small, the patients may have heterogeneous responses to metformin. Further studies will aid understanding of the impact of this heterogeneity, both to identify characteristics or predictors of metformin benefit and to better understand the mechanisms of action of the drug. For example, if metformin primarily acts by insulin-mediated effects, individuals with high insulin levels and obesity are likely to derive benefit. In contrast, if direct action on the PI3K/AKT/mTOR pathway is the key mediator, tumours with PTEN and/or PIK3CA mutations are likely to respond to metformin treatment.

It is possible that the predominant mechanism of metformin will differ across types of tumours. There is a need for prospective trials to determine these modes of action and allow the targeted use of metformin, with an improved understanding of patient and tumour characteristics to derive optimum benefit.
1.4 Project Rationale, Hypotheses and Aims

At the time of this PhD project design, very little was known about the role of metformin in obesity-driven EC. *In vitro* data from EC cell lines suggested that metformin had a potent effect on inhibiting cellular proliferation, and a lesser effect on increasing apoptosis (Cantrell et al., 2010). EC has the strongest links to obesity and diabetes, and it would seem a likely tumour to respond to metabolic reprogramming via metformin treatment. The presurgical window design is ideal for EC, as in a majority of women a pre-treatment tumour biopsy can be obtained in the clinic with minimal discomfort. Most women are managed with surgical treatment, involving removal of the uterus and cervix, allowing a post-treatment biopsy to be obtained.

The hypothesis of this study was that metformin has biologically measurable effects in EC both *in vitro* and *in vivo*. It is hypothesised that short term metformin will result in a decrease in cellular proliferation in women with EC through actions on insulin resistance and the PI3K/AKT/mTOR pathway. The study also tested the hypothesis that metformin has effects on tumour metabolism through actions on the mitochondria.

An *in vitro* model was established using EC cell lines. Study aims were to

- a) investigate the effects of metformin as a sole agent, or in combination with conventional chemotherapy on EC cell viability, using *in vitro systems*
  - a. on cell viability.
  - b. on the PI3K/AKT/mTOR pathway.
- b) assess modifiers of response to metformin including surrounding glucose concentration and hypoxia.
- c) assess the effects of metformin on tumour mitochondria.

Change in Ki-67 score, a marker of cellular proliferation was the primary outcome of the planned presurgical window study. There was a need to

- d) establish a robust and reproducible scoring system for the immunohistochemical assessment of Ki-67 and
- e) assess its role as a prognostic biomarker in EC.
A presurgical window study was designed to investigate the effects of metformin in women with AEH or type I EC. The clinical study aimed to

f) assess the effects of short-term oral metformin on
   a. physical measures of adiposity including weight, BMI and waist circumference.
   b. EC cell proliferation and apoptosis.
   c. the PI3K/AKT/mTOR signalling pathway.
   d. circulating serum biomarkers including glucose, insulin, c-peptide, adiponectin, leptin and CRP.

g) investigate the safety and tolerability of metformin in women with AEH or type I EC.
2. A presurgical window study of metformin in obesity-driven endometrial cancer

2.1 Introduction
Endometrial cancer (EC) is strongly associated with obesity (Renehan et al., 2008, Crosbie et al., 2010) and T2DM (Friberg et al., 2007). The causative mechanisms and association between obesity, insulin resistance and endometrial cancer development are incompletely understood. Metformin is a well-tolerated oral therapy for T2DM with minimal serious side effects. Epidemiological data have suggested that patients with T2DM on metformin have a lower incidence of cancer compared with those taking other therapies (Evans et al., 2005, Libby et al., 2009). Pre-clinical and small clinical studies have shown metformin to have a modifying effect on cellular proliferation and apoptosis through actions on the PI3K/AKT/mTOR pathway (Cantrell et al., 2010, Zakikhani et al., 2010, Niraula et al., 2012a).

Presurgical window studies allow the testing of licensed therapies for novel use directly in patients. These studies use surrogate end-points to assess the biological effects of short-term interventions. The impact of metformin on tumour proliferation has been assessed in women with breast cancer by measuring the change in Ki-67 score in tumours before and after metformin treatment (Hadad et al., 2011, Niraula et al., 2012a, Bonanni et al., 2012). Three single arm studies have now reported that short-term metformin treatment reduced Ki-67 expression in endometrial cancers in the presurgical window prior to hysterectomy (Mitsuhashi et al., 2014, Schuler et al., 2015, Laskov et al., 2014). All three studies, however, lacked a contemporaneous control group and cannot decisively attribute these effects to metformin.

The aims of this study were to test the hypotheses that 1) short-term metformin reduces cellular proliferation in women with AEH and endometrioid EC 2) short-term metformin has a modulating effect on the PI3K/AKT/mTOR pathway and 3) metformin is well tolerated in the oncological setting.
2.2 Materials and Methods

2.2.1 Clinical Trial Study Design and Recruitment
This was an open-label non-randomised study of metformin or no drug taken during the presurgical window between diagnosis and hysterectomy. Women with biopsy proven AEH or endometrioid endometrial adenocarcinoma (EEC) were eligible for recruitment based on the diagnostic endometrial biopsy. The trial had a metformin treatment group and an opportunistically recruited control group (no drug) (Figure 4). Patients were identified from the St Mary’s Gynaecological Oncology multidisciplinary team meeting.

Eligible patients were ≥ 18 years with biopsy proven EEC or AEH and scheduled for hysterectomy in 7-30 days. Women with known diabetes on medication, those with non-endometrioid histology and those on concomitant progesterone were excluded from the study (Table 5). Untreated controls (no drug) were recruited when patients declined metformin treatment but were otherwise eligible or when the window period was too short between eligibility screening and planned study end point (surgery).

Table 5: Inclusion and exclusion criteria

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Non-endometrioid endometrial cancer</td>
</tr>
<tr>
<td>Age 18 years or over</td>
<td>Pre-existing diabetes on treatment</td>
</tr>
<tr>
<td>Biopsy proven AEH or EEC</td>
<td>Already taking metformin</td>
</tr>
<tr>
<td>Scheduled for surgery in 7-30 days</td>
<td>Current alcohol abuse</td>
</tr>
<tr>
<td>Able to provide informed consent</td>
<td>Clinically significant renal or hepatic impairment</td>
</tr>
<tr>
<td></td>
<td>Concomitant anti-cancer/trial drugs</td>
</tr>
</tbody>
</table>

Abbreviations: AEH Atypical endometrial hyperplasia, EEC Endometrioid Endometrial Cancer.

Approvals were received from the Medicines and Healthcare Products Regulatory Authorities (MHRA), the Central Manchester University Hospitals NHS Foundation Trust (CMFT) Research and Development (R&D) department, and the North West Centre for Research Ethics Committee (REC). The study was prospectively registered on the European (EudraCT 2011-001382-40) and UK (International Standard Randomised Controlled Trial Number Register: 81570194) databases.
Figure 4: Trial design and planned interventions. The metformin and control groups were non-randomised cohorts.

Abbreviations: AEH Atypical Endometrial Hyperplasia BD Twice Daily, EEC Endometrioid Endometrial Adenocarcinoma, FFPE Formalin-fixed paraffin-embedded.
2.2.2 Screening Assessment
Eligible patients were approached following diagnosis and provided with patient information sheets (Appendix 1). Following informed consent, a screening assessment was undertaken. This included a detailed medical history to ensure eligibility and blood tests to determine renal and hepatic function.

2.2.3 Physical measurements
Height and weight were measured to calculate the body mass index (BMI) [Weight (kg)/height (m)²]. Waist measurements were measured at the narrowest part of the waist, (midpoint between the lower margin of the last palpable rib and the top of the iliac crest). Hip measurements were taken at the maximum circumference around the buttocks (WHO, 2008).

2.2.4 Blood and tumour collection
Fasted blood samples were collected for markers of insulin resistance and adiposity before and after the window period. A blind biopsy was taken at recruitment using a plastic endometrial sampler. Samples were flash frozen or fixed in paraffin immediately. The diagnostic biopsy 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. Directed biopsies of endometrial tumour tissue were obtained by a specialist gynaecological histopathologist from the hysterectomy specimen and flash frozen on dry ice within thirty minutes of surgical excision. Tumour tissue superfluous to the requirements of standard histological analyses were embedded in paraffin blocks and used for trial analysis. If the patients’ surgery date was delayed beyond the four week period, patients were invited back to the outpatient clinic to have blood and further endometrial biopsies taken.

2.2.5 Sample storage and data handling
All trial samples were stored anonymously in the CMFT Biobank, with appropriate Human Tissue Authority licensing. Stored blood samples were separated in serum, plasma, buffy coat pellets at stored at -80°. All data created from analysis of these samples were handled and stored according to the Data Protection Act 1998.

2.2.6 Drug treatment, accountability, pharmacovigilance and tolerability
Metformin hydrochloride (metformin) was obtained from Apollo Generics, Liverpool, UK (PL31603/0004) and dispensed on a named patient basis by the Clinical Trials Pharmacy at CMFT. Patients received 850mg twice daily for up to four weeks. The drug was stopped the
night before surgery to reduce the risk of lactic acidosis during general anaesthesia (Jones et al., 2003). All patients had assessment of toxicity and compliance in the first week of treatment by telephone. In some cases, doses were withheld over a short period because of intolerable side effects and the treatment recommenced following resolution of symptoms. All unused tablets were returned to the hospital and counted for accountability and compliance. Unused medication was disposed of according to local pharmacy procedure.

An adverse event (AE) was the appearance or worsening of any undesirable sign, symptom or medical condition occurring after the study has commenced. Abnormal laboratory values were also recorded as AEs. All AEs were recorded and treated appropriately (Appendix 2- instructions for AE evaluation from the CRF). On the day of surgery, a short structured interview was conducted with patients based on a questionnaire (Appendix 3) assessing tolerability and acceptability of metformin. Participants were asked about a list of specific AEs and to quantify the tolerability of metformin based on a numerical 1-10 scale (1=worse side effects, 10= no side effects).

2.2.7 Serum markers of adiposity and insulin resistance
Plasma glucose, serum insulin, C-peptide, adiponectin, leptin and hsCRP were measured in fasting blood samples obtained pre-and post-intervention (within 36 hours of last metformin dose) (Table 6). Plasma glucose was measured using a standard automated clinical laboratory assay and processed contemporaneously. Whole blood samples for serum insulin and C-peptide were maintained at 4°C, processed within 30 minutes of collection and frozen in aliquots at -80°C. These were processed using a commercial (enzyme-linked immunoabsorbent assay) ELISA kit (Mercordia, Uppsala, Sweden) in batches. The above samples were processed by the Department of Laboratory Medicine, Central Manchester Foundation Trust (CMFT). Insulin resistance was also measured by the homeostasis model of assessment of insulin resistance index (HOMA-IR), a product of fasting glucose and insulin by 22.5 (Matthews et al., 1985).

Further whole blood samples were similarly processed and aliquoted as plasma and serum samples, without prior maintenance at 4°C. Adiponectin and leptin were measured using a DuoSet ELISA development kit (R&D Systems, Abingdon, UK). High sensitivity CRP (hsCRP) was measured by an in-house antibody sandwich ELISA technique with anti-human CRP antibodies, calibrators and controls from Abcam (Cambridge, UK). These tests were carried out by Dr Philip Pemberton, CMFT.
IGFBP-1 analysis was carried out on frozen serum samples at the SAS Peptide Hormone Lab, Royal Surrey County Hospital, Guildford using an immunoenzymometric assay kit (Medix Biochemica, Kauniainen, Finland).

Table 6: Normal ranges (where available) for circulating biomarkers.

<table>
<thead>
<tr>
<th>Circulating Biomarker</th>
<th>Units</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose</td>
<td>mmol/L</td>
<td>3.0-6.0</td>
</tr>
<tr>
<td>Serum insulin</td>
<td>mU/L</td>
<td>2.0-25.0</td>
</tr>
<tr>
<td>Serum C-peptide</td>
<td>pmol/L</td>
<td>350-1800</td>
</tr>
<tr>
<td>Serum adiponectin</td>
<td>mg/L</td>
<td></td>
</tr>
<tr>
<td>Serum leptin</td>
<td>ng/mL</td>
<td></td>
</tr>
<tr>
<td>Serum hsCRP</td>
<td>mg/L</td>
<td></td>
</tr>
</tbody>
</table>

2.2.8 Immunohistochemistry

Endometrial biopsies and surgical specimens were routinely fixed in formalin and paraffin-embedded (FFPE). All tumours were reviewed by a panel of consultant gynaecological histopathologists prior to inclusion into the study. Histological subtype, grade, stage, depth of myometrial invasion and presence of lymphovascular space invasion were assessed using the FIGO 2009 Endometrial Cancer Staging System. The tumour sections included in the study were selected by the same gynaecological histopathologist, Dr Rhona McVey (RM), CMFT.

2.2.8.1 Leica Bond Max

Automated immunohistochemistry was performed on 4µm-formalin fixed paraffin-embedded sections using the Leica Bond Max (Leica Biosystems, Wetzlar, Germany). Slides were de-parafinised, rehydrated and if required underwent heat-induced epitope retrieval (HIER) with a pH 6 or pH 9 antigen retrieval solution (Leica Biosystems). Sections were incubated with the primary antibody, and in some cases, preceded by a protein block with 10% casein. The primary antibody detection was performed using a post-primary solution and polymer detection followed by DAB staining (Refine Detection Kit, Leica Biosystems). Appropriate positive controls using tissue or cell lines known to express the protein of interest and negative isotype controls were included. Slides were counterstained with haematoxylin and dehydrated through graded acid alcohol (70-100%), cleared in xylene and mounted.
The protocol for Ki-67 staining had previously been optimised on the Leica Bond Max using breast cancer tissue within the research group. Positive (tonsil) and negative (mouse IgG1 isotype) controls were used on each run for quality assurance. All other antibodies were optimised using an optimisation tissue microarray (TMA) constructed from tumour cores of low and high grade endometrial tumours. Isotype negative controls were used on each run and additional positive controls were cell pellets constructed from HEC1A EC cells that had been shown to express target proteins on Western immunoblot. A range of primary antibody dilutions were chosen based on manufacturer recommendations and published literature and a variety of antigen retrieval steps used (no antigen retrieval, HIER at pH6 and HIER at pH9). Optimum staining conditions were selected with input from Garry Ashton, Cancer Research United Kingdom Manchester Institute (CRUK MI) Histology Core.

The primary antibodies were 1) Ki-67 MIB-1 Clone mouse monoclonal (DAKO, Carpinteria, CA) 2) phosphorylated-AKT (Ser 473) 3) phospho-S6 (Ser235/236) 4) phospho-acetyl-CoA Carboxylase (Ser 79) 5)phospho-4EBP1 (Thr 37/46) 6) PTEN Clone 6H2.1 (DAKO, Carpinteria, CA) 7) cleaved caspase-3 (cc3) (cc3) (Table 7). All antibodies were obtained from Cell Signalling, Beverley, MA, unless otherwise specified.

Table 7: Primary antibodies and conditions used on Leica Bond Max.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source</th>
<th>Additional Block</th>
<th>Antigen Retrieval</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 MIB-1</td>
<td>Mouse mAB</td>
<td>10% Casein</td>
<td>HIER pH 9</td>
<td>1:100</td>
</tr>
<tr>
<td>P-AKT (Ser473)</td>
<td>Rabbit mAB</td>
<td>Nil</td>
<td>HIER pH9</td>
<td>1:50</td>
</tr>
<tr>
<td>P-S6 (Ser235/236)</td>
<td>Rabbit mAB</td>
<td>10% Casein</td>
<td>HIER pH9</td>
<td>1:400</td>
</tr>
<tr>
<td>P-ACC (Ser 79)</td>
<td>Rabbit pAB</td>
<td>10% Casein</td>
<td>HIER pH9</td>
<td>1:300</td>
</tr>
<tr>
<td>P-4EBP1 (Thr 37/46)</td>
<td>Rabbit mAB</td>
<td>10% Casein</td>
<td>HIER pH9</td>
<td>1:800</td>
</tr>
<tr>
<td>PTEN</td>
<td>Mouse mAB</td>
<td>10% Casein</td>
<td>HIER pH9</td>
<td>1:400</td>
</tr>
<tr>
<td>cc3</td>
<td>Rabbit pAB</td>
<td>Nil</td>
<td>HIER pH6</td>
<td>1:200</td>
</tr>
</tbody>
</table>
2.2.8.2 Ventana BenchMark XT

p53 (Novocasta, Newcastle), ER and PR (Roche, Basel) staining were performed using a clinically validated assay at the CMFT Department of Laboratory Medicine by Catherine Keeling. The automated Ventana Benchmark XT IHC staining module was used with the XT Ultraview 3, 3’ diaminobenzidine (DAB) detection system (Ventana Co., Tucson, AZ). Tissue was deparaffinised and underwent a heat-induced antigen retrieval process using a TRIS-ethylenediamine tetracetic acid (EDTA)-boric acid pH 8 buffer for 30 minutes. An ultraviolet inhibitor blocking solution was used prior to incubation with the primary antibody (Table 8). Sections were then incubated with a horseradish peroxidase-linked secondary antibody, followed by sequential DAB and copper chromogens. Counterstaining was performed using haematoxylin and a bluing reagent.

Table 8: Primary antibodies and concentrations used on the Ventana BenchMark XT

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source</th>
<th>Antigen Retrieval</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER (SP1)</td>
<td>Rabbit mAB</td>
<td>pH8</td>
<td>RTU</td>
</tr>
<tr>
<td>PR (1E2)</td>
<td>Rabbit mAB</td>
<td>pH8</td>
<td>RTU</td>
</tr>
<tr>
<td>p53 (DO7)</td>
<td>Mouse mAB</td>
<td>pH8</td>
<td>1:50</td>
</tr>
</tbody>
</table>

Abbreviations: RTU Ready to use i.e. no dilution.

2.2.9 Ki-67 Scoring

Full slides were digitised using the Leica SCN400 Slide Scanner (Leica Microsystems, Wetzlar, Germany). Ki-67 scoring was developed using recommendations from scoring in breast cancer (Dowsett et al., 2011). Over 2000 nuclei were scored per section and in sections obtained from hysterectomy, scoring was confined to the outer or surface tumour rather than invasive (myometrial) edge. This approach was to address the different sampling methods and allow comparison of the directed tumour biopsy to the random endometrial biopsy which was likely to sample the outer tumour surface.

In order to minimise bias and heterogeneity within tumour tissue, equivalent areas to be scored were selected by a gynaecological histopathologist (RM). She was blinded to treatment arm and selected areas on the corresponding haematoxylin and eosin stained sections. An automated score from these areas was obtained by applying an algorithm formulated on Definiens Developer which scored the nuclei based on staining intensity. An absolute cut-off was used to define positive and negative nuclei and staining variation was disregarded. The areas were manually selected to ensure that only tumour tissue was
scored while stromal and inflammatory cells were disregarded. Scoring was repeated by two independent observers [VS & Dr Sarah Kitson (SK)] and discrepancies resolved by consensus review.

2.2.10 Tissue Microarrays

Ki-67 staining and scoring was performed on whole section IHC. Following Ki-67 scoring, TMAs were constructed by Hannah Gregson, Histology Technician. TMAs were used to investigate multiple phosphorylated proteins in a limited amount of endometrial tumour tissue. At least three representative 1mM cores were taken from each donor tissue block from the area previously identified by the histopathologist. These cores were then placed into a recipient block, baked at 37°C overnight and then on a cold plate of the tissue embedding station, with subsequent two to three one hour-cycles of hot/cold to temper the array.

2.2.11 TMA Scoring

All subsequent staining and scoring was performed on TMAs. When TMA cores were missing, the whole section was stained and scored on the previously identified areas. The scoring was done by two independent observers (VS and SK) blinded to time point of the samples. p-AKT, p-ACC, p-S6, p-4EBP1, ER and PR staining was assessed using a modified H-score, the product of area score (proportion positively stained core, scored 0-6) and staining intensity score (0=none, 1=weak, 2=moderate, 3=strong). This scoring method was used to maintain consistency in reporting throughout the research group (Mukhopadhyay et al., 2012).

p-AKT, p-ACC and p-S6 staining was assessed in the cytoplasm and nucleus, while p-4EBP1, ER and PR were nuclear. PTEN was scored as positive (strong staining in the entire tumour) or negative (<10% staining of malignant glands with strong positive staining of adjacent stromal cells)(Garg et al., 2012). P53 was scored “mutant-like” if >50% of tumour cells showed strong positive nuclear staining or when no nuclear p53 staining was evident within the entire tumour (Nout et al., 2012, McCluggage et al., 2011). All IHC scoring was completed by two independent observers (VS, SK) who were blinded to time point and treatment arm.

Cleaved caspase 3 was scored on TMAs by applying by an automated algorithm (Definiens Developer) to count positive and negatively stained nuclei. Malignant glands were selected to exclude stromal and myometrial structures by one observer (VS). All positive nuclei were
also manually counted to ensure accuracy. A cc3 positive index was obtained as a percentage of positively stained nuclei.

2.2.12 Mutational Analysis of FFPE tissue
A mutational analysis was performed to investigate the hypothesis that tumours displaying PIK3CA or PTEN mutations were more responsive to metformin treatment. DNA was isolated from FFPE tissue blocks from 50 [AEH (n=2) + EEC (n=48)] samples, referred to hereafter as the Manchester 2015 cohort. The DNA concentration was too low from seven samples, leaving 43 samples available for mutational analysis. The final sample set consisted of tumours from 24 metformin-treated and 11 control participants. Mutation genotyping was performed by Ellen Stelloo using the GynCarta Assay version 2.0 (Sequenom), a MALDI-TOF-based mutational panel designed to detect hotspot mutations. The samples were tested for the presence or absence of 159 hotspot mutations in 13 genes (BRAF, CDKNA2, CTNNB1, FBXW7, FGFR2, FGFR3, FOXL2, HRAS, KRAS, NRAS, PIK3CA, PPP2R1A and PTEN) as previously detailed (Spaans et al., 2014). The analysis was performed at the Molecular Pathology Department, Leiden University Medical Centre. The selected gene panel does not fully cover PTEN mutations; therefore loss of PTEN was reported based on immunohistochemical assessment of absent protein expression.
2.2.13 Statistical Analysis
The primary outcome measure was the change in Ki67 score. The sample size was powered to observe a 20% reduction in Ki-67 expression following treatment. This was based on the assumption of a median baseline Ki-67 score of 50%; a standard deviation of 20% and a correlation of 70% between pre- and post-intervention measurements. At 80% power and alpha=0.05, the estimated sample size was 29, with aimed recruitment of 30 patients in the treatment group (samps in STATA v10.1) and opportunistic recruitment of as many contemporaneous controls as possible.

Demographic results are reported as mean and SD as well as median and interquartile range (IQR) to best represent the non-parametric data. When IQR is reported in this text, it is presented as the first and third quartile, i.e. IQR (Q1, Q3). The association between baseline physical variables (age and BMI) with immunohistochemical and serum biomarkers was assessed in the entire cohort of patients (n=40) using Spearman correlations. The distribution of some variables was difficult to distinguish, while others were skewed or symmetric. For this reason, non-parametric Wilcoxon tests were mainly used for paired samples (signed rank test) and for group comparisons (rank sum test). When the data were normally distributed as assessed by a D’Agostino-Smirnov normality test, a paired Student’s t-test was used. Two-tailed comparison tests were used in all cases.

The main treatment effect on Ki-67, phospho-markers and serum biomarkers was tested using an analysis of covariance (ANCOVA) linear regression model with post-treatment score as the response variable. This model adjusted for covariates which included baseline score, age, BMI, insulin resistance (HOMA-IR) and change in controls. HOMA-IR was excluded as a covariate when assessing the serum biomarkers. This analysis provided a mean adjusted difference.

Tabulation of means, medians and further Spearman correlation analyses were used to differentiate the tumour and metabolic factors contributing to metformin-response (change in Ki-67 score). In all cases, statistical significance was determined as p<0.05.
2.3 Results: Participants and treatment

2.3.1 Recruitment

From 1st October 2012 to 28 February 2014, 101 women with AEH or EEC who attended for surgical management at St Mary’s Hospital, CMFT, the Royal Oldham Hospital and Tameside NHS Foundation Trust were assessed for eligibility. Thirty-six women were recruited to take metformin; 32 participants completed the study. The four study withdrawals were secondary to unacceptable gastrointestinal adverse effects. One woman failed at screening because of renal impairment, and three others in the metformin group were excluded from the final analysis (Figure 5).

The control group was women who declined metformin treatment but were happy to assist with research (n=7) or those with a short window (<7 days) between screening and scheduled surgery (n=7). One woman with a known renal impairment was included in the control group. Fifteen women were recruited as untreated controls and three were excluded from the final analysis. While the groups were not randomised, there were no significant differences in baseline age, weight or tumour grade (all p>0.05, Wilcoxon rank sum).

Reasons for exclusion in both groups were non-endometrioid histology on the final hysterectomy specimen and concomitant progesterone use. One participant was excluded as she discontinued metformin treatment over one week prior to the trial end point (surgical management). The final analysis included 28 metformin-treated participants and 12 untreated controls. The participant flow diagram is shown in Figure 5 (Consort Diagram).
CONSORT diagram for the study indicating patient screening and accrual for the course of the study.

**Metformin-treated**

101 patients assessed for eligibility between Oct 2012 and Feb 2014

36 patients recruited
- 35 received treatment
- 1 screening failure (renal impairment)

28 patients completed the study and included in final analysis
- 4 patients withdrew because of gastrointestinal side effects
- 1 patient excluded as she discontinued metformin > 1 week prior to surgery (endpoint)
- 1 patient excluded as final histology was non-endometrioid
- 1 patient excluded because of concomitant progesterone use

**Untreated Controls**

65 patients not eligible or declined metformin treatment
- 20 patients declined metformin treatment
- 19 patients with diabetes on treatment
- 9 patients with a presurgical window period <1 week
- 17 others excluded for other reasons

15 patients recruited
- 7 patients declined metformin treatment but consent to the untreated arm
- 7 patients had a presurgical window < 1 week
- 1 patient had known renal impairment

12 patients completed the study and included in final analysis
- 2 patients excluded as final histology was non-endometrioid
- 1 patient excluded because of concomitant progesterone use

*Figure 5: Modified Consolidated Standards of Reporting Trials diagram.*
2.3.2 Patients excluded from study inclusion at screening

The majority of women who were ineligible had pre-existing diabetes on treatment (n= 19; 42% of ineligible patients). The presurgical window between screening and surgery was < 7 days in nine women. Other reasons for exclusion included lack of fitness for surgery, concurrent tamoxifen treatment and a lack of capacity to consent, including a language barrier. As toxicity assessments and the provision of advice were conducted over the telephone, it was not feasible to include women who did not speak English (Figure 5).

The common reasons for declining participation included concerns about taking new medications and potential side effects. Some women were unable to attend for a second visit for blood to be obtained under fasting conditions and others stated that the recent diagnosis of cancer was a source of anxiety and they were unable to consider participating in research studies.

2.3.3 Participants’ clinical characteristics

Baseline characteristics are illustrated in Table 9. The two groups were evenly matched in age (mean 63±8.8 vs 66±12.4 years) and BMI (median 34 vs 33 kg/m²) in the treated and untreated groups, respectively. Approximately 80% of participants each group were overweight or obese with at least 60% obese (BMI≥30 kg/m²). A majority of recruited participants were White British. Two patients of South Asian ethnicity were recruited to the treatment group but withdrew secondary to side effects. There were three South Asian participants in the control group, one of whom was excluded from the final analysis because of concurrent progesterone treatment.

Forty-six percent of women in the treatment group and 42% of control patients were non-smokers, while 36% and 50% were ex-smokers. Alcohol consumption of 5.1 (metformin-treated) and 4.5 (control) units per week was similar to the average intake of British women aged 65 years and over (4.6 units) (ONS, 2010). Over 50% of patients in each group reported no alcohol intake.
Table 9: Baseline physical characteristics and demographic information of study participants.

<table>
<thead>
<tr>
<th>Baseline</th>
<th>Metformin, n=28 (%)</th>
<th>Untreated, n=12 (%)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>63</td>
<td>66</td>
<td>0.170</td>
</tr>
<tr>
<td>SD</td>
<td>8.8</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>Age, years ≤50</td>
<td>1 (3.6)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>51-60</td>
<td>10 (36)</td>
<td>2 (17)</td>
<td></td>
</tr>
<tr>
<td>61-70</td>
<td>11 (39)</td>
<td>7 (58)</td>
<td></td>
</tr>
<tr>
<td>71-79</td>
<td>5 (18)</td>
<td>2 (17)</td>
<td></td>
</tr>
<tr>
<td>≥80</td>
<td>1 (3.6)</td>
<td>1 (8.3)</td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td></td>
<td></td>
<td>0.307</td>
</tr>
<tr>
<td>Median</td>
<td>88</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Q1, Q3</td>
<td>70.5, 117.8</td>
<td>68.7, 94.1</td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m^2</td>
<td></td>
<td></td>
<td>0.520</td>
</tr>
<tr>
<td>Median</td>
<td>34.1</td>
<td>33.2</td>
<td></td>
</tr>
<tr>
<td>Q1, Q3</td>
<td>26.8, 42.9</td>
<td>27.0, 34.8</td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m^2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25 (%)</td>
<td>6 (21)</td>
<td>2 (17)</td>
<td></td>
</tr>
<tr>
<td>25-29.9 (%)</td>
<td>5 (18)</td>
<td>3 (25)</td>
<td></td>
</tr>
<tr>
<td>=&gt;30-39.9 (%)</td>
<td>8 (29)</td>
<td>5 (42)</td>
<td></td>
</tr>
<tr>
<td>=&gt;40 (%)</td>
<td>9 (32)</td>
<td>2 (17)</td>
<td></td>
</tr>
<tr>
<td>Waist girth, cm</td>
<td></td>
<td></td>
<td>0.401</td>
</tr>
<tr>
<td>Median</td>
<td>108</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Q1, Q3</td>
<td>92, 125</td>
<td>88, 125</td>
<td></td>
</tr>
<tr>
<td>Hip girth, cm</td>
<td></td>
<td></td>
<td>0.623</td>
</tr>
<tr>
<td>Median</td>
<td>116</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>Q1, Q3</td>
<td>104, 139</td>
<td>107, 127</td>
<td></td>
</tr>
<tr>
<td>Waist/hip girth ratio</td>
<td></td>
<td></td>
<td>0.370</td>
</tr>
<tr>
<td>Median</td>
<td>0.87</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>Q1, Q3</td>
<td>0.84, 0.93</td>
<td>0.82, 0.89</td>
<td></td>
</tr>
<tr>
<td>Smoking habits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmoker (%)</td>
<td>13 (46)</td>
<td>5 (42)</td>
<td></td>
</tr>
<tr>
<td>Ex-smoker (%)</td>
<td>10 (36)</td>
<td>6 (50)</td>
<td></td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>5 (18)</td>
<td>1 (8)</td>
<td></td>
</tr>
<tr>
<td>Daily alcoholic units</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14 (50)</td>
<td>8 (67)</td>
<td></td>
</tr>
<tr>
<td>&gt;2</td>
<td>11 (40)</td>
<td>3 (25)</td>
<td></td>
</tr>
<tr>
<td>≥2</td>
<td>2 (7)</td>
<td>1 (8)</td>
<td></td>
</tr>
</tbody>
</table>

* Differences between the metformin-treated and control groups were assessed using a Wilcoxon rank sum test.
2.3.4 Treatment and compliance

Participants were prescribed 850mg of metformin twice daily. In one case, the investigators reduced the prescribed dose to 850mg once daily in the context of borderline renal impairment which was not severe enough for exclusion. Women received metformin for a median of 20 (IQR 17, 24) days. Fifteen (54%) participants were perfectly compliant and took all the tablets prescribed (Table 10). Protocol deviations were in place to allow doses to be withheld and restarted using a ramp up approach of 850mg once daily, then increased to 850mg twice daily following resolution of side effects. Anti-emetics (cyclizine or metoclopramide) were provided to alleviate nausea. Ten participants had withheld doses because of bothersome side effects.

Three participants took a reduced dose (850mg once daily) for greater than half the prescribed treatment duration. A detailed pill count allowed an assessment of each participant’s total metformin dosage and average daily dose. The average daily dose was calculated by the following equation:

\[
\text{Average daily dose} = \frac{\text{No of tablets taken} \times 850\text{mg}}{\text{Prescribed treatment duration (days)} - \text{no of days with withheld doses}}
\]

The median daily dose was 1573 (IQR 1475, 1659) mg, while a participant who was perfectly compliant would have received 1700mg (Table 10).

Table 10: Details and compliance of metformin treatment

<table>
<thead>
<tr>
<th>Metformin treatment (n=28)</th>
<th>Median</th>
<th>Q1</th>
<th>Q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of metformin treatment (days)</td>
<td>20</td>
<td>16.8</td>
<td>24</td>
</tr>
<tr>
<td>Number of tablets</td>
<td>35</td>
<td>22</td>
<td>44.5</td>
</tr>
<tr>
<td>Total dosage (mg)</td>
<td>29750</td>
<td>18700</td>
<td>37825</td>
</tr>
<tr>
<td>Mean daily dose (mg)</td>
<td>1476.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median daily dose</td>
<td>1573.0</td>
<td>1475.2</td>
<td>1658.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compliance (n=28)</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully compliant</td>
<td>15</td>
<td>53.6%</td>
</tr>
<tr>
<td>Once daily dosage for &gt; 1/2 treatment duration</td>
<td>3</td>
<td>10.7%</td>
</tr>
<tr>
<td>Doses withheld</td>
<td>10</td>
<td>35.7%</td>
</tr>
</tbody>
</table>
2.3.5 Tolerability of metformin treatment
Patients on metformin were contacted at the start of treatment to assess compliance and adverse events. A 24-hour emergency access number was available for advice. AEs and tolerability were assessed using a semi-structured interview at the study end-point. AEs were assessed in all patients who received at least one dose of metformin (n=35). Grade was determined by the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 (NIH, 2010). All AEs without another sufficient explanation were recorded as probably or possibly drug-related.

There were no Suspected Unexpected Serious Adverse Reactions (SUSARs) or Serious Adverse Reactions (SARs) in the study. Seventy-seven percent of all patients developed adverse effects, of these 96% were grade 1 (Table 11). Gastrointestinal (GI) side effects (nausea and vomiting, diarrhoea and abdominal pain) were the most common AEs. Seventy-seven percent of patients reported nausea or vomiting and 69% reported diarrhoea. Most gastrointestinal symptoms were rated as Grade 1 or mild AES; however two episodes of diarrhoea and one of nausea were Grade 2 AEs.

Other reported adverse effects included headache, fatigue, skin changes including itching and abdominal bloating. One patient was diagnosed to have atrial fibrillation prior to induction of anaesthesia, which represented a Grade 3 AE. This event was unlikely to be caused by the study drug. She was excluded from the final analyses secondary to non-compliance after the event.

Patients were asked to rate the acceptability and tolerability of metformin on a ten-point scale as an adjuvant to their surgical treatment, with 0=not tolerable and 10=very tolerable; the mean score from 29 patients was 6.1±2.5.
Table 11: Adverse events (AEs) experienced by all patients who participated in the metformin-treatment group

<table>
<thead>
<tr>
<th>Summary of adverse events experienced by all patients who received metformin treatment</th>
<th>n</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients that received at least one dose of metformin</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>Patients that developed any adverse events</td>
<td>27</td>
<td>77</td>
</tr>
<tr>
<td>Number of adverse events</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>Grade 1 AE</td>
<td>94</td>
<td>96</td>
</tr>
<tr>
<td>Grade 2 AE</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Grade 3 AE</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**No of patients experiencing an adverse event**

<table>
<thead>
<tr>
<th>Adverse event</th>
<th>n</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of Appetite</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Nausea/Vomiting</td>
<td>27</td>
<td>77</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>24</td>
<td>69</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>12</td>
<td>34</td>
</tr>
<tr>
<td>Skin changes</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Headache</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Fatigue</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Bloating</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Abnormal baseline bloods</td>
<td>10</td>
<td>29</td>
</tr>
<tr>
<td>Others</td>
<td>11</td>
<td>31</td>
</tr>
<tr>
<td>Mean patient tolerability scores</td>
<td>29</td>
<td>6.1 (SD 2.5)</td>
</tr>
</tbody>
</table>

Abbreviations: AE Adverse events
2.3.6 Tumour Characteristics & Surgical Management
Participants in the final analysis all had endometrioid tumours. Recruitment was based on histology from the diagnostic endometrial biopsy. Two patients were excluded from the control and one from the metformin group for non-endometrioid histology on the final hysterectomy specimen. A majority of tumours were low grade and early stage. Three patients in the control group had grade 3 endometrioid tumours (25%), compared with one in the metformin group. While all tumours in the control group were Stage 1, lymphovascular space invasion was present in 60% of cases, but only in 30% of metformin-treated patients. This may reflect the higher percentage of grade 3 tumours in the controls.

Fifty-eight percent of all patients had a laparoscopic procedure, and in 87% of cases, this was a total laparoscopic hysterectomy.

Final adjuvant therapy and follow-up data were collected in April 2015, thirty-one months after trial commencement. The average follow up was 15±5 months for metformin-treated and 14±8 months for controls. All patients were alive at the time of final follow-up. There was one vault recurrence, 22 months after surgical management in a control participant with the primary diagnosis of a grade 2 stage 1A endometrioid tumour.

As most tumours were early stage, 60% of patients were managed solely with clinical follow-up. All patients who had stage 1B or worse disease were offered adjuvant radiotherapy and/or chemotherapy and 91% accepted. Five patients received adjuvant carboplatin and paclitaxel for further management of EC (Table 12).
### Table 12: Tumour characteristics, surgical management and follow-up treatment.

<table>
<thead>
<tr>
<th></th>
<th>Metformin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumour grade at hysterectomy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEH</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>G1</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>G2</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>G3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><strong>FIGO Stage at hysterectomy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>1B</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><strong>Lymphovascular space invasion present</strong></td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td><strong>Mode of hysterectomy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal hysterectomy</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Total laparoscopic hysterectomy</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Laparoscopic assisted vaginal hysterectomy</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Follow-up &amp; Adjuvant therapy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical follow up</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>Vaginal brachytherapy</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>External beam radiotherapy</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>External beam radiotherapy + chemotherapy*</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Chemotherapy alone*</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* Five patients received adjuvant carboplatin and paclitaxel for endometrial cancer for high grade or stage 2 or worse disease. Two patients also received chemotherapy for concurrent primary ovarian tumours.
2.4 Physiological Analyses

2.4.1 Serum biomarkers of insulin resistance and adiposity

The baseline serum biomarkers for participants in each treatment group are shown in table 13; the groups were comparable at baseline. Four patients in the metformin group had undiagnosed diabetes (fasting plasma glucose > 7.0mmol/L). In addition, 46% of treated and 42% of control patients were insulin resistant (fasting blood glucose 6.0-6.9mmol/L and/or HOMA-IR>2.8). A linear equation was used to calculate HOMA-IR \[ \text{(fasting glucose \times fasting insulin)/22.5} \] (Matthews et al., 1985).

Table 13: Baseline fasting serum biomarkers.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unit</th>
<th>Metformin</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>SD</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td></td>
<td>Q1, Q3</td>
</tr>
<tr>
<td>Glucose</td>
<td>mmol/L</td>
<td>6.0</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>mU/L</td>
<td>16.0</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>C-peptide</td>
<td>pmol/L</td>
<td>1076</td>
<td>896</td>
</tr>
<tr>
<td></td>
<td></td>
<td>482</td>
<td></td>
</tr>
<tr>
<td>HOMA_IR</td>
<td></td>
<td>1055</td>
<td>860</td>
</tr>
<tr>
<td></td>
<td></td>
<td>655, 1365</td>
<td>605, 1024</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>mg/L</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td>ng/mL</td>
<td>54.1</td>
<td>43.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42.6</td>
<td></td>
</tr>
<tr>
<td>Ln (hsCRP)</td>
<td>mg/L</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

*Baseline values between groups were compared using a Wilcoxon rank sum test.

Baseline fasting circulating biomarkers of insulin resistance including glucose (Spearman \( r = 0.56 \) [95% CI 0.28, 0.74]), insulin (\( r = 0.57 \) [95% CI 0.31, 0.75]) and C-peptide (\( r = 0.64 \) [95% CI 0.40, 0.80]) (all \( p < 0.001 \)) were positively correlated with baseline BMI (Table 14 and Figure 6). In addition, BMI was positively correlated to leptin (\( r = 0.85 \) [95% CI 0.72, 0.92]) and negatively correlated to adiponectin (\( r = -0.53 \) [95% CI -0.73, -0.25]) (both \( p < 0.001 \)). There was no significant association with natural log-transformed hsCRP (Table 7). Scatterplots of all these associations are shown in Figure 6. The markers of insulin resistance were correlated with each other (glucose, insulin and C-peptide) and leptin and there were negative correlations with adiponectin (Table 14).
Figure 6: Correlations between baseline BMI and fasting circulating biomarkers a) glucose b) insulin c) C-peptide d) adiponectin e) Leptin f) ln hsCRP. (r = Spearman correlation coefficient, 95% CI are presented). Abbreviations: hsCRP highly sensitive C-reactive protein.
Table 14: Correlation between fasting serum biomarkers and fasting insulin.

<table>
<thead>
<tr>
<th>Baseline serum biomarker correlations (n=40)</th>
<th>Spearman nonparametric correlation, r (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin vs Glucose</td>
<td>0.512 (0.229, 0.715)</td>
<td>0.0007</td>
</tr>
<tr>
<td>Insulin vs C-peptide</td>
<td>0.858 (0.741, 0.924)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Insulin vs adiponectin</td>
<td>-0.754 (-0.866, -0.568)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Insulin vs leptin</td>
<td>0.626 (0.379, 0.791)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Insulin vs hsCRP</td>
<td>0.287 (-0.041, 0.559)</td>
<td>0.0774</td>
</tr>
</tbody>
</table>
2.4.2 Effects on metformin on physical measurements.
The median values for weight, BMI, waist circumference and the waist-hip ratio (WHR) pre- and post-intervention for both groups are shown in Figure 7. Within group comparisons demonstrate statistically significant percentage decreases in weight (-0.4%), BMI (-0.4%) and waist circumference (-0.8%) following metformin treatment (all p<0.05 Wilcoxon signed rank test), but not in controls. The trends in decrease in physical measurements, however, did not approach statistical significance after adjusting for covariates including age, baseline values and the change in controls using an ANCOVA.

![Figure 7: Box and whisker plots illustrating the change in a) weight, b) BMI, c) waist circumference and d) waist/hip ratio in metformin-treated and control patients. The mean adjusted difference and 95% CIs presented are derived from an ANCOVA adjusting for age, BMI, baseline values and change in controls. “Pre” and “post” indicate pre- and post-metformin (usually at hysterectomy) measurements in the metformin group and baseline and post-window measurements in the control group.](image)

2.4.3 Changes in serum biomarkers following metformin treatment


The box and whisker plots in Figure 8 illustrate the median values of fasting glucose, insulin, C-peptide and HOMA-IR in the metformin-treated and control patients, pre and post-intervention. Metformin treatment resulted in significant within group median decreases in glucose, insulin, HOMA-IR, C-peptide, adiponectin and log-transformed CRP which was not observed in control patients (all $p<0.05$, Wilcoxon signed rank test). There were, however, no statistically significant changes in these circulating biomarkers after adjustment for covariates including the change in the controls using an ANCOVA (Figures 8 & 9).

**Figure 8**: Box and whisker plots illustrating the median change in a) glucose b) insulin c) C-peptide and d) HOMA-IR in metformin-treated and control patients. The mean adjusted difference and 95% CIs presented are derived from an ANCOVA adjusting for age, BMI, baseline values and change in controls. "Pre" and "post" indicate pre- and post- metformin measurements (usually at hysterectomy) in the metformin group and baseline and post-window measurements in the control group. Abbreviations: BMI Body mass index, CI Confidence interval, HOMA-IR Homeostasis model of insulin resistance.
Figure 9: Box and whisker plots illustrating the median change in serum adipokines a) adiponectin, b) leptin, c) In hsCRP in metformin-treated and control patients. The mean adjusted difference and 95% CIs presented are derived from an ANCOVA adjusted for age, BMI, baseline values and the change in controls. “Pre” and “post” indicate pre- and post- metformin measurements (usually at hysterectomy) in the metformin group and baseline and post-window measurements in the control group. Abbreviations: In hsCRP natural log-transformed high sensitivity C-reactive protein.
2.4.4 Endometrial Tumour Sampling
The study protocol aimed to obtain an endometrial tumour biopsy at recruitment i.e. prior to starting metformin treatment. It was recognised that this would not always be possible because of technical difficulties or patient refusal. In these cases, the FFPE sample obtained at diagnosis would be retrieved and used as the pre-intervention biopsy. The recruitment biopsy (on study entry) was used in 18 participants, while the diagnostic biopsy was the baseline sample in 22 participants. Ninety-five percent of pre-intervention samples were obtained using a plastic endometrial sampler.

As the study was non-randomised, it was important to ensure a balanced presurgical window between groups. The diagnostic biopsy was used as the baseline sample when a recruitment biopsy was not available or insufficient for analysis. The mean presurgical windows were comparable (Wilcoxon rank sum p=0.64); 34.4 (SD 24.7) days and 35.3 (SD 14.7) days in metformin-treated and control patients respectively.

2.5 Immunohistochemical Analyses
The change in the Ki-67 score in the epithelial endometrial tumour was the primary outcome used to assess the biological effects of metformin in vivo. The Ki-67 score was determined by the percentage of positively stained nuclei in over 2000 cells per tissue section. Semi-automated scoring using Definiens Developer was repeated by two independent observers and results were consistent with an interobserver intra-class correlation coefficient of 0.94.

2.5.1 Baseline Ki-67
The Ki-67 score was assessed in endometrial tumour biopsies from twenty-eight metformin treated and twelve controls. The mean baseline Ki-67 score was 50.9 ± 17.1% and 55.6 ±25.1% in metformin-treated and control patients respectively, with no difference at baseline between treatment groups (Wilcoxon rank sum, p=0.44) (Table 15).

Baseline Ki-67 (n=40) was positively correlated with tumour grade (Spearman r= 0.37 [95%CI 0.06, 0.62], p=0.018) (Figure 10), but not tumour stage (Spearman r=0.28 [95% CI -0.04, 0.55], p=0.28). There was no correlation with age (Spearman r=0.299 [95% CI -0.02, 0.56], p=0.52) or baseline BMI (Spearman r=-0.106 [95% CI -0.412, 0.222], p=0.516). In addition, there was a significant negative correlation between baseline Ki-67 index and baseline insulin resistance (HOMA-IR) (Spearman r = -0.431 [95% CI -0.660, -0.129]).
Table 15: Ki-67 score (%) by treatment group.

<table>
<thead>
<tr>
<th></th>
<th>Metformin (n=28)</th>
<th>Untreated (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Pre-treatment (%)</td>
<td>50.9</td>
<td>17.1</td>
</tr>
<tr>
<td></td>
<td>49.8</td>
<td>40.6, 60.4</td>
</tr>
<tr>
<td>Post-treatment (%)</td>
<td>37.4</td>
<td>20.9</td>
</tr>
<tr>
<td></td>
<td>34.9</td>
<td>25.8, 53.6</td>
</tr>
</tbody>
</table>

Figure 10: Positive correlation between baseline Ki-67 score and tumour grade (Spearman r= 0.372 [95%CI 0.059, 0.618], p=0.018). Abbreviations: AEH Atypical endometrial hyperplasia, G1 grade 1, G2 grade 2, G3 grade 3.
2.5.2 Change in Ki-67 following metformin treatment

The primary outcome of the presurgical window study was the change in cellular proliferation as measured by the Ki-67 score in endometrial biopsies pre- and post-intervention. The change in pre- and post-treatment Ki-67 score by treatment group in each individual patient is shown in Figure 11. Overall, short-term metformin treatment significantly reduced Ki-67 score by 17% (95% CI -27%,-7%, p=0.002) compared with untreated controls. The analysis of covariance (ANCOVA) adjusted for the baseline Ki-67 score, age, BMI, insulin resistance and the change in the controls. The diagnostic biopsy was used as the baseline sample when a recruitment biopsy was not available. When post-intervention change in Ki-67 score was assessed using the diagnostic biopsies as the baseline sample for all patients (n=40), the reduction in Ki-67 score following metformin treatment remained significant (mean-adjusted difference -16% (95% CI -27%, -5%, p=0.005).

Figure 11: A Line graph showing the adjusted mean difference in Ki-67 score in paired pre- and post-intervention (at hysterectomy) endometrial tumours from metformin treated and control patients. 12 B & C: Endometrial tumours stained for Ki-67 before (B) and after (C) treatment with metformin at x 20 magnification. Adapted from Sivalingam et al. 2016 (Sivalingam et al., 2016).
2.5.3 Responders and non-responders

Absolute changes in Ki-67 score (pre-treatment – post-treatment Ki-67 score) were used for the following analyses. There was a decrease in Ki-67 score in 23/28 metformin-treated patients (range -3.5 to -54.8%), termed “responders” and remained static or increased in five others (range 0.6 to 14.2%), “non-responders”. There was also a marginal decrease in Ki-67 score in 5/12 untreated patients (range -1.1 to -7.0%), but Ki-67 remained static or increased in approximately 60% of controls (1.1-28.0%). There is no published data on the clinical significance of short-term Ki-67 change in EC. In breast cancer, the risk of invasive disease recurrence has been reported as 2.2% (95% CI 0.9-5.0) per point increase in post-tamoxifen Ki-67 (p<0.001) following a two-week presurgical period, if patients continued on long-term adjuvant tamoxifen (DeCensi et al., 2011). This data suggests that even small changes in Ki-67 score in response to metformin may equate to survival benefit if the drug is continued.

The data were analysed to distinguish differences between responders and non-responders on metformin treatment to identify women more likely to benefit from metformin. The responders were classed as those who had any decrease in Ki-67 score (median change -15% IQR -23, -5.6%) while non-responders (n=5) had no change or an increase in Ki-67 score (median change 3.5% IQR 1.3%, 9.8%). The baseline Ki-67 was similar in both groups, while non-responders had a higher baseline BMI [non-responders (median 40 IQR 32, 51 kg/m²) vs responders (median 34 IQR 24, 42 kg/m²)]. Women with greater Ki-67 decreases were also less insulin resistant at baseline and a greater decrease in glucose and insulin following metformin treatment. These differences were not statistically significant between the two groups. The only significant difference between the groups was tumour grade, as all non-responders had grade 1 tumours, while 61% of responders had grade 2/3 tumours (Wilcoxon rank sum p=0.028).
2.5.4 Effect of dose and BMI on change in Ki-67 score

Analysis of the dose-response effect was complicated by the heterogeneity of the dosage taken and the duration of treatment. It was possible to calculate an average daily dose by using the end of study pill count and records of withheld doses for all patients. Average daily doses ranged from 850-1700mg. A larger daily metformin dose was associated with a lower in the post-treatment Ki-67 score (Spearman r =-0.373 [95% CI -0.66, 0.01], p=0.058) (Figure 12).

The greatest decrease in Ki-67 score following metformin treatment was seen in leaner patients (BMI <25kg/m²) (Figure 13) (Spearman r=0.368 [95%CI -0.017, 0.659], p=0.054). The mean absolute decrease in Ki-67 was greater in normal weight women (- 29%) compared with overweight (-16%) and obese (-8%) women (Table 16). These changes may be secondary to the bioavailability of metformin, which is hydrophilic and not bound to plasma proteins.
Figure 12: Correlation between absolute Ki-67 score change (defined as post-intervention Ki-67 - pre-intervention Ki-67) and the average daily dose of metformin (mg). There was a non-significant association between increased daily doses and a greater Ki-67 response or change. Spearman r = -0.373 (95% CI -0.66, 0.012) p=0.058

Table 16: Change in Ki-67 score according to baseline BMI.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>Q1</th>
<th>Q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>All metformin-treated</td>
<td>28</td>
<td>-14%</td>
<td>-16%</td>
<td>-12%</td>
<td>-19%</td>
<td>-4%</td>
</tr>
<tr>
<td>BMI &lt;25 kg/m²</td>
<td>6</td>
<td>-29%</td>
<td>-20%</td>
<td>-29%</td>
<td>-44%</td>
<td>-12%</td>
</tr>
<tr>
<td>BMI 25-29.9 kg/m²</td>
<td>5</td>
<td>-16%</td>
<td>-7%</td>
<td>-17%</td>
<td>-18%</td>
<td>-14%</td>
</tr>
<tr>
<td>BMI ≥30 kg/m²</td>
<td>17</td>
<td>-8%</td>
<td>12%</td>
<td>-6%</td>
<td>-15%</td>
<td>-1%</td>
</tr>
</tbody>
</table>
2.5.5 Apoptosis in endometrial tumours
The apoptotic index was determined by the percentage and number of positively stained cells for cleaved caspase-3 (cc3). The baseline rate of apoptosis in both metformin-treated (1.0%) and control participants (0.3%) was low (Figure 14 & Table 17). Overall, the mean index of apoptosis in metformin-treated and control patients were reduced at hysterectomy compared with the pre-intervention biopsy. In metformin-treated patients, the cc3 index reduced from 1% (SD3.2%) to 0.15% (SD0.3%) (p=0.0014) and from 0.35% (SD 0.58%) to 0.12 % (0.17%) (p=0.090) in the control patients. Despite the significant decrease in the apoptotic index, when comparing the paired pre- and post-intervention biopsies (Wilcoxon signed-rank test), metformin did not have a significant treatment effect on the apoptotic index using an ANCOVA (mean adjusted difference 0.00052, [95% CI -0.0015, 0.0025], p= 0.608), adjusting for baseline apoptosis, age, BMI, insulin resistance and the change in controls.

In addition, there was no correlation between higher grade tumours and increased apoptotic index (Spearman r 0.08, p= 0.63). This finding must be interpreted cautiously as there were only four high grade tumours in the study sample.

Table 17: Descriptive statistics for apoptosis in metformin-treated and control patients.

<table>
<thead>
<tr>
<th></th>
<th>Metformin, n=28</th>
<th>Control, n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive Nuclei (n)</td>
<td>cc3 Positive Index</td>
</tr>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Mean</td>
<td>10.5</td>
<td>8.5</td>
</tr>
<tr>
<td>SD</td>
<td>10.3</td>
<td>16.2</td>
</tr>
<tr>
<td>Median</td>
<td>7.8</td>
<td>2.3</td>
</tr>
<tr>
<td>Q1</td>
<td>3.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Q3</td>
<td>16.8</td>
<td>8.7</td>
</tr>
</tbody>
</table>

Both the number of positive nuclei per tissue section and the cc3 positive index are presented. The cc3 positive index is a percentage of positively stained cells from the total number of cells counted.
Figure 14: cc3 staining at 20x magnification. Endometrial tumour tissue from one metformin-treated patient: A) pre-intervention and B) post-intervention.
2.5.6 AMPK and PI3K/AKT/mTOR phosphorylation events

Immunohistochemical analysis of tumour AMPK and PI3K/AKT/mTOR phosphorylated protein expression were carried out. AMPK is a fundamental regulator of cellular energy, and activation leads to phosphorylation of acetyl coA carboxylase (ACC). Phosphorylated-ACC expression was used as a downstream surrogate for AMPK activation.

Activation of AMPK can also downregulate mTOR signalling by activating tuberous sclerosis complex 2 (TSC2), a negative mTOR regulator. The mTOR pathway can be activated through the insulin-receptor, and subsequent AKT activation. In turn, AKT activation, by decreasing the AMP/ATP ratio, leads to inhibition of AMPK, demonstrating a feedback system.

p-AKT expression was measured as a downstream target of insulin-receptor activation. mTORC1 specifically phosphorylates S6 and 4EBP1, thus phosphorylation of these downstream proteins are often used as a surrogate for mTORC1 activation. The mean immunohistochemistry scores for baseline and post-intervention p-AKT, p-S6, p-ACC and p-4EBP1 are presented in Table 18. The baseline and post-intervention scores for each phosphorylated protein were similar between treatment groups for all the proteins, apart from baseline p-AKT (Wilcoxon rank sum test, p=0.001). The mean baseline p-AKT was significantly higher in the metformin-treated (8.86±3.14) compared with the controls (5.41±2.95).

Table 18: Mean scores of key phosphorylated proteins pre- and post-intervention.

<table>
<thead>
<tr>
<th></th>
<th>Metformin, n=28</th>
<th>Control, n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Baseline p-AKT</td>
<td>8.86</td>
<td>3.14</td>
</tr>
<tr>
<td>Post-intervention p-AKT</td>
<td>3.63</td>
<td>2.05</td>
</tr>
<tr>
<td>Baseline p-S6</td>
<td>9.30</td>
<td>2.08</td>
</tr>
<tr>
<td>Post-intervention p-S6</td>
<td>7.35</td>
<td>3.44</td>
</tr>
<tr>
<td>Baseline-p-ACC</td>
<td>8.82</td>
<td>3.63</td>
</tr>
<tr>
<td>Post-intervention p-ACC</td>
<td>6.86</td>
<td>3.09</td>
</tr>
<tr>
<td>Baseline p-4EBP1</td>
<td>7.99</td>
<td>3.18</td>
</tr>
<tr>
<td>Post-intervention p-4EBP1</td>
<td>2.19</td>
<td>3.06</td>
</tr>
</tbody>
</table>
2.5.6.1 Baseline correlation between AMPK and mTOR pathway proteins

AKT, also known as protein kinase B, is an upstream inducer and positive regulator of mTOR. The relationship between baseline p-AKT and the downstream mTOR proteins, p-4EBP1 and p-S6 was assessed using correlation analysis. There was a positive correlation between baseline p-AKT and p-4EBP1 (Spearman \( r = 0.455 \) [95% CI 0.157, 0.676], \( p=0.003 \)) but not with p-S6. In addition, there was no correlation between the two downstream proteins, p-S6 and p-4EBP1, or baseline p-ACC and p-4EBP1 scores or p-S6 (Figure 15).

Figure 15: Correlation between baseline phospho-markers. There was a positive correlation between baseline p-AKT and p-4EBP1. There was no correlation between baseline A) p-ACC and p-4EBP1, B) p-AKT and p-S6 or D) p-4EBP1 and p-S6. Spearman correlation used and asterisk* indicates \( p<0.05 \).
2.5.6.2 Effect of metformin on mTOR phosphorylation proteins
There were global reductions in phosphorylation of both upstream and downstream targets of the mTOR pathway in both metformin-treated and untreated patients. Examples of phospho-marker immunohistochemical staining pre- and post-intervention are presented in Figure 17. The mean expression of A) p-AKT, B) p-ACC, C) p-S6 and D) p-4EBP1 was reduced in the post-intervention compared with the pre-intervention biopsy of metformin-treated patients, but also reduced in the control patients. An ANCOVA adjusted for baseline phospho-protein expression, age, BMI, baseline insulin resistance and the change in controls to assess the metformin treatment effect. Phospho-4EBP1 expression was reduced to a greater degree in metformin-treated patients, demonstrating a significant mean adjusted difference of -2.30 (95% CI -4.61, -0.06, p=0.045). The reductions in the other phosphorylated proteins were not statistically significant for treatment effect after adjusting for covariates (Figure 16).

Figure 16: Effect of metformin on AMPK and mTOR phosphorylation proteins. A, B & C) There were reductions in cells staining positive for p-AKT and p-ACC in post-intervention (hysterectomy) biopsies in both treatment groups. After adjusting for the baseline protein expression, age, BMI, insulin resistance and change in controls, using an ANCOVA, there was no metformin treatment effect on p-AKT, p-S6 and p-ACC. D) Expression of p-4EBP1 was significantly decreased in tumour tissue of metformin-treated patients compared with tissue from untreated controls post-intervention. ANCOVA: Analysis of Covariance.
Figure 17: Representative images taken pre- and post-intervention of immunohistochemical expression of 1) p-ACC 2)p-AKT 3)p-S6 4)p-4EBP1. All images were taken from pre- and post-intervention biopsies from one-metformin treated patient (grade 1 endometrioid adenocarcinoma).
2.6 Mutational Analysis

It was hypothesised that tumours with PIK3CA and PTEN mutations would be more sensitive to metformin treatment because of its actions on the PI3K/AKT/mTOR pathway. A mutational analysis was performed to identify common hotspot mutations in tumours from the presurgical window study (referred to hereafter as the Manchester 2015 cohort). Mutation genotyping was performed by E Stelloo at the Molecular Pathology Department, Leiden University Medical Centre using the GynCarta 2.0 panel, a MALDI-TOF-based mutational panel designed to detect hotspot mutations in gynaecological cancers on tumours included in the presurgical window study. The mutations detected were compared with endometrial cancer samples analysed by Spaans et al. (Spaans et al., 2014) in the creation of GynCarta 2.0 to assess if mutations expressed by the Manchester 2015 cohort were representative of a larger dataset. This cohort included 227 endometrial cancers, 206 of which were endometrioid histology. In both sample sets, PIK3CA and PTEN mutations were the most frequently detected, representing 74% of mutations in the Manchester 2015 study and 69% in Spaans et al. The other two commonly mutated genes were KRAS and CTNNB1. There were no BRAF, CDKN2A, FGFR3, FOXL2 and HRAS mutations detected in either sample set. The catalogue of somatic mutations in cancer (COSMIC) database based on whole genome sequencing was interrogated for endometrioid histology samples only (n=513). The frequency of mutations reported by COSMIC were PTEN (32%), PIK3CA (26.3%), CTNNB1 (15%), KRAS (11%), PPP2R1A (6%) and FGFR2 (8%) (Table 19).

In the Manchester 2015 cohort, 20 tumours had more than one mutation detected. In five cases, there was both a PIK3CA and a PTEN mutation detected. There were 4 high grade endometrioid tumours in the sample set, expressing KRAS, PTEN and FGFR2 mutations. There was a co-existing FGFR2 and PTEN mutation in one high-grade tumour.
Table 19: Comparison of frequencies of mutations with GynCarta 2.0 validation set (Spaans et al.) and COSMIC database.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Manchester 2015 (43*)</th>
<th>Spaans et al (227**)</th>
<th>COSMIC (513***)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>No mutations</td>
<td>6</td>
<td>14.0%</td>
<td>49</td>
</tr>
<tr>
<td>Multiple mutations</td>
<td>20</td>
<td>46.5%</td>
<td></td>
</tr>
<tr>
<td>PIK3CA</td>
<td>17</td>
<td>39.5%</td>
<td>67</td>
</tr>
<tr>
<td>PTEN</td>
<td>15</td>
<td>34.9%</td>
<td>89</td>
</tr>
<tr>
<td>KRAS</td>
<td>8</td>
<td>18.6%</td>
<td>39</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>7</td>
<td>16.3%</td>
<td>33</td>
</tr>
<tr>
<td>FGFR2</td>
<td>6</td>
<td>14.0%</td>
<td>13</td>
</tr>
<tr>
<td>FBXW7</td>
<td>3</td>
<td>7.0%</td>
<td>12</td>
</tr>
<tr>
<td>PPP2R1A</td>
<td>3</td>
<td>7.0%</td>
<td>18</td>
</tr>
<tr>
<td>BRAF</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
</tr>
<tr>
<td>FGFR3</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
</tr>
<tr>
<td>HRAS</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
</tr>
<tr>
<td>NRAS</td>
<td>0</td>
<td>0.0%</td>
<td>6</td>
</tr>
</tbody>
</table>

*The initial Manchester 2015 cohort included tumours that were excluded from the final presurgical window analysis. ** The absolute number of mutations are reported; > 1 mutation detected in some tumours. *** COSMIC database accessed April 2015, limited to endometrioid endometrial adenocarcinoma only.
2.6.1 PTEN Mutations and immunohistochemical expression
There were 15 PTEN (35%) mutations in total and the most common observed mutation was a deletion in exon 5. PTEN expression, however, was characterised by immunohistochemistry into three categories; negative, i.e. mutant, positive and heterogeneous in 44 cases. Cases scored as heterogeneous were reclassified as positive if more than 10% of tumour cells were positive, i.e. wild-type. There was poor concordance between the mutations detected and the PTEN immunohistochemical expression. Of the fifteen mutations detected, five were classified as PTEN mutant and ten expressed PTEN (four weak, four moderate and two strongly stained). A further nine tumours were classified as mutant by IHC, where no PTEN mutations was detected.

Immunohistochemistry is thought to be the best method for assessing PTEN functional loss, since loss of PTEN can be caused by gene mutations, gene methylation, post-transcriptional regulation and micro-RNA actions (Fata et al., 2012). Other studies using the same mutational panel have reported up 50% of cases with PTEN mutations, substitutions and frameshifts that have positive or wildtype PTEN expression (Stelloo et al., 2014).

2.6.2 TP53 mutations
The TP53 gene was mutated in 15% of endometrioid adenocarcinomas assessed on the COSMIC Database (accessed April 2014). The TP53 gene was not included in the GynCarta panel as it did not fulfil the criteria of a “hotspot” gene, as it has many mutations scattered widely and would not be suitable for interrogation with a MALDI-TOF approach.

TP53 mutations in the Manchester 2015 cohort were thus assessed by IHC. There were two tumours (5%) expressing mutant TP53 expression in the cohort, one with no nuclear p53 staining, and another with tumour cell positivity in a discreet geographical area.
2.6.3 Mutational analysis of tumours included in the presurgical window study.
Thirty nine tumours in the Manchester 2015 study were included in the final analysis of the presurgical window study of metformin. In four tumours (10%), the DNA concentration was too low for mutational analysis. The mutations expressed by tumours in the metformin treated (n=24) and control (n=11) groups are shown in Table 20. Multiple mutations were common in both treatment groups, occurring in twelve metformin-treated and five control patients. There were no detected hotspot mutations in three metformin-treated patients, and untreated control. Consistent with findings from the entire sample set described above, the most common mutations in the metformin arm were PIK3CA (50%), PTEN (29%), KRAS (21%) and CTNNB1(21%), while the most common mutation in the control group was in PTEN (50%).

Table 20: Mutational analysis by treatment group

<table>
<thead>
<tr>
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<th>Metformin</th>
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<th>Control</th>
<th>%</th>
</tr>
</thead>
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<td></td>
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<tr>
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</tr>
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</tr>
<tr>
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</tr>
</tbody>
</table>
2.6.4 Metformin response based on mutational analysis
Exploratory analyses were carried out to test the hypothesis that the decrease in Ki-67 score (or metformin response) would be greater in tumours with PIK3CA and PTEN mutations. A trend towards a decreased response to metformin was seen when comparing tumours that had PIK3CA and/or PTEN mutations comparing with wild-type (Figure 18). Tumours with PIK3CA and KRAS mutations also had a decreased response to metformin, compared with wild-type. Using an analysis of co-variance, adjusting for age and BMI, the presence of a PTEN mutation was associated with a greater decrease in Ki-67 [mean adjusted difference, -13.9% (95% CI, -28.3%, 5.3%), p=0.058] (Figure 18B). However, when PTEN mutational status was assessed according to IHC expression, there was no modifying effect noted on Ki-67 response to metformin. None of these trends were statistically significant.

![Figure 18: Metformin response based on absolute decrease in Ki-67 score according to mutational analysis. Tumours wild-type for A) PIK3CA, C) PIK3CA and/or PTEN and D) KRAS had increased Ki-67 response to metformin compared with mutant tumours. B) Tumours with PTEN mutations had a greater median response to metformin compared with wild-type (WT) (ns).](image-url)
2.7 Discussion
This is the largest study of presurgical metformin treatment in EC conducted to date. We found that Ki-67 score was decreased following metformin treatment but remained stable in the untreated controls. There were global reductions in phosphorylation of PI3K/AKT/mTOR targets both in metformin-treated and control groups. While phosphorylation of 4EBP1 was reduced following metformin treatment, the changes in phosphorylation markers may have been affected by tissue hypoxia and ischaemia at hysterectomy. In addition, metformin treatment did not result in significant improvement in insulin resistance, compared with controls. The study is strengthened by the contemporaneous control group, as variability of repeated serum and tissue biomarkers between diagnosis and hysterectomy has not been reported before. Decreases in insulin resistance and phosphorylation events have previously been attributed to metformin treatment by other single-arm studies (Mitsuhashi et al., 2014, Schuler et al., 2015). While the assignment of participants into treatment and control arm was not randomised, the two groups were evenly matched in terms of age, BMI, insulin resistance status, tumour grade and stage. Strikingly, almost eighty percent of our participants were overweight or obese and there was a marked prevalence of undiagnosed insulin resistance. These findings are consistent with published studies (Burzawa et al., 2011, Crosbie et al., 2012).

In this study, the decrease in Ki-67 score following metformin treatment was positively correlated with the average daily dose received. It is not known whether standard diabetic doses of metformin are sufficient for anticancer activity in vivo. It has been speculated that metformin may accumulate in tumour tissue and concentrations equivalent to 20% of circulating serum levels can suppress proliferation in EC (Mitsuhashi et al., 2014). The optimal anti-cancer doses of metformin have yet to be established, however, comparison with previous window studies using a range of metformin doses (850-2250mg daily) suggest that a dose-response effect may exist (Mitsuhashi et al., 2014, Schuler et al., 2015, Laskov et al., 2014).

The greatest decrease in Ki-67 score following metformin was seen in leaner patients (ns) with all patients prescribed the same daily doses. Metformin remains unbound to plasma proteins and has a very high volume of distribution (Tucker et al., 1981); the effective circulating dose may therefore be affected by BMI. It is interesting to speculate whether higher doses of metformin may be required to achieve anti-cancer effects, particularly in obese and morbidly obese women. In this study, baseline insulin resistance did not affect the response to metformin, contrary to findings in breast cancer, where women who were
insulin resistant (HOMA-IR>2.8) had greater Ki-67 decreases following metformin treatment (Bonanni et al., 2012). Over 60% of patients in our study were insulin resistant, compared with 33% in the breast cancer study, which may have limited the impact of the subgroup analysis.

It is not clear whether metformin is exerting its anti-proliferative effects directly on the tumour tissue through actions on the mitochondrial electron transport chain and AMPK activation or that these changes are a bystander effect secondary to improved insulin resistance and reduced signalling through the IGF1R/IR tyrosine-kinase signalling pathways. It is possible that short-term metformin may have direct actions on tumour tissue; in this study there was no significant improvement in insulin resistance, despite a measurable biological effect on cellular proliferation. This suggestion is supported by findings that metformin accumulates in tumour tissue as demonstrated by Mitsuhashi et al. (Mitsuhashi et al., 2014).

The baseline level of apoptosis was low in tumours included in this study and there was no correlation with tumour grade. Most tumours were low grade with inclusion of only four high grade tumours in the study. Rates of apoptosis are poorly documented in EC; however a window study assessing the effects of medroxyprogesterone acetate reported similar baseline values (Zaino et al., 2014). There was no evidence for a pro-apoptotic effect of metformin in EC. This is in line with pre-clinical studies where the main effect of cell viability is cytostatic, while apoptosis is only observed with much higher concentrations of metformin (Cantrell et al., 2010).

Phosphorylation of 4EBP1 was significantly reduced at hysterectomy in metformin-treated patients, following adjustment for the change in controls. This suggests a biological effect of metformin on the PI3K/AKT/mTOR pathway. Other changes in AMPK and PI3K/AKT/mTOR signalling proteins were non-significant. Schuler et al. report significant decrease in phosphorylation of AMPK following metformin treatment in a presurgical window study of metformin in EC. Conversely, multiple cell line and pre-clinical models have demonstrated that metformin treatment leads to phosphorylation of AMPK (Zakikhani et al., 2006, Zakikhani et al., 2008) suggesting that the global decrease in phosphorylation events is an artefact of tissue ischaemia at hysterectomy. Significant decreases in p-AMPK have also been reported in breast cancer patients following short-term metformin compared with control (Hadad et al., 2015). Hysterectomy specimens were bisected and immersed in formalin within 30 minutes of resection. The fixation protocol
used is standard for clinical care and achieves adequate preservation of tissue architecture. It is likely that the expression of stable proteins like Ki-67 is preserved, but unstable phosphorylation events may be lost. This key methodological issue highlights the need for an appropriate post-intervention biopsy, ideally prior to tissue devascularisation to facilitate understanding of phosphorylation events.

Improvement in biomarkers of insulin resistance and adiposity were observed between baseline and hysterectomy in both groups. Long term metformin treatment in patients with diabetes is associated with improved insulin resistance and moderate weight loss. It is possible that the moderate weight loss observed in both groups is secondary to anxiety-induced behavioural change or intentional weight loss in preparation for surgery. Significant changes in biomarkers of adiposity and insulin resistance have been reported by previous window studies in EC (Laskov et al., 2014) and breast cancer (Niraula et al., 2012b). Both were single arm studies and the lack of contemporaneous controls hinders interpretation of these data. Other long term studies have demonstrated a decrease in BMI and improvement in insulin resistance following six months of adjuvant metformin treatment in breast cancer studies (Goodwin et al., 2015) and euglycaemic obese women (Worsley et al., 2014). These data suggest that the lack of beneficial effect of metformin in this study is secondary to the short duration of treatment.

Metformin was generally well tolerated, although four out of thirty-six patients withdrew from the study because of intolerable gastrointestinal SEs. When metformin is commenced for T2DM, it is started at a low dose and increased to limit gastrointestinal toxicity. Metformin was commenced at full dose to maximise the exposure over the presurgical window. There is also considerable variation in glycaemic response to metformin between individuals with T2DM. These have been partly explained by genetic differences in organic cation transporter-1 (OCT-1) expression in target tissues (hepatocytes & skeletal myocytes) (Graham et al., 2011, Berstein et al., 2013). OCT-1 expression levels have not been measured in EC, but it is conceivable that differences may explain variation in both SEs reported and tumour response.

Mutational analysis was performed as a post-hoc study to identify germ-line mutations that would predict response to metformin. This confirmed that the tumours included in this study were representative of EECs in terms of commonly detected mutations. Many tumours displayed multiple mutations with PIK3CA and PTEN mutations being most common. The PI3K signalling pathway is important in EEC carcinogenesis, and metformin is
thought to act directly on this pathway to inhibit downstream signalling (Pollak, 2012b). It is clear that tumour response to metformin is heterogeneous. It would seem rational that tumours with PIK3CA and PTEN mutations would be more sensitive to metformin; however, the analysis was unable to detect any significant trends. The sample size analysed was small (n=35), and a much larger data set may be required to identify a molecular signature that can predict response to metformin. Additional challenges include the presence of multiple co-existing mutations in EEC and the pleotropic actions of metformin on multiple cancer pathways.

In summary, short-term presurgical metformin is associated with a significant drop in Ki-67 score. Changes in PI3K/AKT/mTOR phosphorylation markers and serum markers on insulin resistance are observed both in metformin and control groups, emphasising the need for a control group to control for the variability over time. This study adds to the growing body of evidence supporting the therapeutic potential of metformin in EC. It confirms the tolerability and acceptability of presurgical metformin to EC patients prior to surgery and identifies the window study design as a novel means of assessing the biological effects of licensed drugs in the oncology setting.
3. Developing Ki-67 as a surrogate biomarker in endometrial cancer

3.1: Introduction
Establishing benefit and risk is an important element of developing novel therapeutic interventions. The most reliable way to assess the clinical impact of an intervention is through clinically important endpoints including cancer-free and overall survival. Short-term proof of principle studies, such as the presurgical study of metformin (Chapter 2) rely on surrogate end-points as a substitute for clinical response.

3.1.1: Immunohistochemical assessment of Ki-67
A biomarker is defined as a “characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention” (BDWG, 2001). Ki-67 is a nuclear protein, which is universally expressed in proliferating tissue and absent in resting cells (van Dierendonck et al., 1989). These characteristics and its critical role in cell division (Starborg et al., 1996) has generated interest in its potential as a biomarker of cellular proliferation. Ki-67 expression also correlates positively with other markers of tumour proliferation including S-phase fraction (Salvesen et al., 1998) and mitotic count (Stefansson et al., 2004).

Immunohistochemical assessment of Ki-67 in formalin fixed paraffin-embedded tissue (FFPE) has been reported using the MIB-1 and MIB-3 mouse monoclonal antibodies (Cattoretti et al., 1992) and is greatly enhanced by heated activated antigen retrieval (Shi et al., 1991). Ki-67 expression is usually estimated as the percentage of tumour cells positively stained by the antibody (referred to as the Ki-67 score), with nuclear staining being the most common criterion for positivity (Dowsett et al., 2011).

The International Ki-67 in Breast Cancer Working Group proposed guidelines for the analysis, reporting and use of Ki-67 to ensure reproducibility, reliability and accuracy of studies which used Ki-67 as a primary outcome measure. (Dowsett et al., 2011). Recommendations from the group discuss a variety of factors that may affect Ki-67 immunohistochemistry (Table 21).
Table 21: Summary of relevant recommendations from the International Ki-67 in Breast Cancer Working Group (Dowsett et al., 2011).

| Pre-analytical | • Core-cut biopsies and whole sections from excision biopsies are acceptable specimens; however when comparisons are to be made, it is preferable to use the same type for both samples.  
|                | • Tissue microarrays are acceptable for clinical trial use. |
| Analytical     | • Known positive and negative controls should be included in all batches.  
|                | • Heat-induced antigen retrieval is recommended.  
|                | • The MIB1 antibody is currently endorsed for Ki-67 detection. |
| Interpretation and scoring | • In full sections, at least three high powered (x40 objective) fields should be selected.  
|                | • For prognostic evaluation, the invasive edge of the tumour should be scored.  
|                | • If pharmacodynamics comparisons must be made between core cuts and sections from the excision, assessment of the latter should be across the whole tumour.  
|                | • If there are clear hot spots, data from these should be included in the overall score.  
|                | • Only nuclear staining is considered positive. Staining intensity is not relevant.  
|                | • Scoring should involve the counting of at least 500 malignant invasive cells, and preferably at least 1000 cells.  
|                | • Image analysis methods for Ki-67 remain to be proven in clinical practice. |
| Data handling  | • The Ki-67 score should be expressed as the percentage of positively stained cells among the total number of invasive cells in the area scored.  
|                | • Statistical analysis should take account of the log-normal distribution followed by the Ki67 scores.  
|                | • The most appropriate endpoint in comparative studies of treatment efficacy is the percentage suppression of Ki-67 positive cells. |

3.1.2: The use of Ki-67 as a biomarker in breast cancer

In breast cancer, Ki-67 is frequently measured both as a marker of proliferative activity and by making repeated measurements during treatment, as a surrogate of treatment efficacy and predictive biomarker (Urruticoechea et al., 2005). Some studies in breast cancer have demonstrated the prognostic value of Ki-67; however in view of the retrospective nature of these studies and varying cut-offs to designate “positive” and “negative” or “high” and “low” Ki-67 populations, the American Society of Clinical Oncology (ASCO) Tumour Marker Guidelines Committee has suggested that there is insufficient evidence to support the
clinical utility of Ki67 as a routine prognostic biomarker in newly diagnosed breast cancer (Harris et al., 2007).

There is, however, a proven role for Ki-67 as a predictive biomarker to assess treatment response in breast cancer studies and identify subpopulations of patients most likely to respond. In the clinic, hormonal treatments in breast cancer have been shown to reduce Ki-67 score in both the short term (e.g. 6 weeks) (Clarke et al., 1993, Decensi et al., 2003) and the long term (e.g. 12 weeks) (Geisler et al., 2001, Ellis et al., 2003). Early changes in Ki-67, within two weeks of treatment, have also been found to correlate with changes at 12 to 16 weeks. Both short and longer term changes are concordant with clinical response (Dowsett et al., 2007), including improved recurrence-free survival (DeCensi et al., 2011). Even a modest increase of 2.7% in the post-treatment Ki-67 following pre-surgical tamoxifen can result in a 5.0% (95% CI 2.3-7.7) increase in recurrence of invasive disease after 7.2 years if the adjuvant treatment is continued (DeCensi et al., 2011).

3.1.3: Establishing the role of Ki-67 in endometrial cancer

In EC, short-term studies have used change in Ki-67 score as measure of response to novel therapies (Mitsuhashi et al., 2014, Laskov et al., 2014, Schuler et al., 2015, Thangavelu et al., 2013). There are no recommendations or consensus on staining and scoring protocols for Ki-67 in EC. Furthermore, its role as a prognostic or predictive biomarker in EC is yet to be established. Previous work demonstrates Ki-67 to correlate positively with grade and survival (Stefansson et al., 2004, Salvesen et al., 1998, Liu et al., 2014) but comparison of these studies is complicated by heterogeneity of Ki-67 positivity scoring, cut-off thresholds and variable follow-up periods.

By extrapolating from breast cancer studies, it is likely that an early response to treatment measured by change in Ki-67 is likely to result in longer term Ki-67 suppression and potential clinical benefit with ongoing treatment. An improved understanding of baseline Ki-67 as a prognostic biomarker in terms of survival may help interpret the change in Ki-67 observed following metformin treatment.

The aim of this study was to identify a reliable and reproducible method of Ki-67 staining, scoring and analysis to identify correlations between Ki-67 and established clinicopathological prognostic variables and to evaluate the role of Ki-67 as a prognostic biomarker.
3.2: Materials and Methods: Immunohistochemistry

Immunohistochemical staining for Ki-67 expression was performed as described in Chapter 2 on FFPE endometrial samples collected prospectively from women undergoing hysterectomy by the Manchester Biomedical Research Centre Biobank. Histological assessment prior to IHC was performed by consultant gynaecological histopathologist, RM. The primary antibody used was the Ki-67 MIB-1 Clone (DAKO). Positive control (tonsillar tissue) and negative isotype control were used for quality assurance.

3.2.1: Initial validation set and manual scoring

The initial scoring system was based on recommendations published on the assessment of Ki-67 in breast cancer (Table 1). The Ki-67 score was calculated by a manual count of >1000 cells per tissue section from three representative high powered fields (x40) using a light microscope. Nuclear staining at all intensities was considered positive, and membrane or cytoplasmic staining disregarded. Scoring was repeated by two independent observers [Dr Anita Merritt (AM) & VS], who were blinded to tumour histology. Discrepant scores were resolved by consensus, using a double-headed microscope.

3.2.2: Development of a semi-automated scoring system

Next, a semi-automated scoring system was developed to improve accuracy of scoring and improve operator time-efficiency. The slides were digitised using a Leica Scanner (Leica Microsystems) and analysed with Definiens Developer XD. This image analysis tool allows the development of solutions based on a subset set of images, which is then applied to the larger series of slides. Nuclear size was determined based on the average nucleus, and a step added to exclude smaller pieces of cell debris. The IHC and haematoxylin thresholds were then determined to differentiate positive and negative staining, thus providing a count of positive and negative nuclei. Once this solution was saved, it could be applied by multiple assessors to tissue sections to obtain reproducible results.

Whilst the software is intuitive and could be trained to recognise differences between different cell types, it did not consistently separate glands from the stroma. The solution was applied to regions of interest (ROIs) that were manually selected by the assessors (SK & VS) at 20x magnification (Figure 19B & C) scoring over 2000 nuclei to obtain a Ki-67 score (Figure 19D). All sections were scored twice by one assessor (SK) and once by the other (VS), allowing both intra- and inter-observer variability to be measured.
3.2.3 Follow-up data collection
Demographic, pathology and follow-up data were obtained from electronic and hard copy patient records. The detection of recurrent disease was by symptom enquiry and clinical examination, with imaging as required. Cause of death was determined by primary care and mortuary records. All cases without events were censored at the last follow-up visit.

3.2.4 Statistical analysis
Ki-67 was measured as a continuous score and data conformed to a negatively skewed distribution. Intraclass correlation coefficients were used to measure intra- and inter-observer variability. The association between Ki-67 and other pathological and clinical variables were tested by using Spearman rank correlation for continuous variables. Kaplan-Meier curves were constructed to estimate cancer specific and recurrence-free 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 EC, while RFS was the interval between date of surgery and first documentation of recurrent disease. A Cox proportional hazard regression model was used in both univariate and multivariate analyses of overall survival and RFS, after confirming that the data complied with the proportional hazards assumption using log-log curves. A p-value of <0.05 was regarded as being of statistical significance. The analyses were carried out using SPSS version 22 and GraphPad Prism.
3.3 Results

Initial validation of Ki-67 in benign endometrium and endometrioid cancer tissue

3.3.1: Ki-67 expression in the validation set and assessment of manual scoring

The initial validation set consisted of forty eight patients undergoing hysterectomy for endometrioid EC (n=35) or other reasons, with morphologically normal endometrium (n=13). Ki-67 score was significantly higher in tumour tissue, compared with benign postmenopausal endometrium (1-way ANOVA, 44.7 %± SD 20.3 versus 3.9 %± SD 1.7, p<0.0001) and positively correlated with grade (Spearman r=0.71 [95% CI 0.53, 0.83], p<0.0001) (Figure 20).

![Figure 20: Ki-67 expression in benign endometrial tissue and grade 1 (G1), grade 2(G2) and grade 3 (G3) endometrioid EC. Ki-67 score is positively correlated with grade, and significantly increased in tumour tissue compared with benign postmenopausal endometrium.](image-url)
3.3.2 Comparison between manual and semi-automated scoring

The manual and semi-automated scores of thirty-three whole sections were compared using Bland-Altman plots. One observer (VS) used both manual and semi-automated scoring methods, with most cases falling between the 95% limits of agreement between -18 and +24% (Figure 21A). The mean manual scores of two observers (VS & AM) were then compared with the automated scores of VS & SK (Figure 21B). Again, most cases fell between the 95% limits of agreement of -14% and +32%. When comparing both mean scores, the automated scoring appeared to provide higher Ki-67 proliferation indices, particularly in tumours with higher Ki-67 expression. Both manual (ICC 0.90) and automated scoring (ICC 0.97) methods had good interobserver reproducibility using a two-way random effects model.

Overall, the semi-automated scores compared well with conventional manual scoring, but had superior interobserver reproducibility. The automation was time-efficient and allowed a better assessment of the whole tumour with >2000 nuclei included compared with >1000 nuclei manually.

The semi-automated system was used for the final analysis of Ki-67 expression in both the presurgical window study and in the assessment of prognostic value.

Figure 21: A. Bland–Altman plot comparing the automated and manual Ki-67 scores of one observer (VS). The difference was obtained from the automated-manual scores and plotted against the average of both. B. Bland-Altman plot comparing the mean automated scores (VS & SK) and mean manual scores (VS & AM).
3.4 Assessing Ki-67 as a prognostic biomarker in endometrial cancer

The semi-automated scoring was next applied to a larger sample of sections to assess the role of Ki-67 as a prognostic biomarker in EC.

3.4.1: Established a scoring system for Ki-67 as a prognostic biomarker.

In order to identify the most reproducible method of scoring, three different scoring approaches were used and the Definiens solution applied. These were whole section (WS) which included a random selection of high powered fields across the whole slide, hot spot (HS) scoring, defined as areas of maximal Ki-67 staining and scoring the endometrial/myometrial border [the invasive edge of the tumour (IE)]. For WS section, at least five areas were scored at 20x magnification, while at least three areas were selected for HS and IE scoring.

3.4.2: Endometrial cancer cohort and baseline parameters

The cohort consisted of prospectively collected tumours from 120 patients undergoing hysterectomy for EC. These included the 35 samples in the initial validation set. Eleven patients were excluded because of a lack of follow-up data, non-endometrial primary tumour and poor technical quality of IHC staining.

Baseline demographics are shown in Table 22. The mean age and BMI were 65 (SD 12) years and 32 (SD 9) kg/m², respectively. Sixty one percent of patients had endometrioid disease. Fifty-five cases (50%) were grade I and II endometrioid tumours, while fifty-four others were classed as grade III (endometrioid, clear cell, serous and carcinosarcoma). Seventy-two cases (66%) were Stage 1, according to the FIGO 2009 classification. The median follow-up was 35 (IQR 26, 46) months, with twenty-two deaths (20%) and twenty disease recurrences (18%). There were eleven cancer-specific deaths (10%).
Table 22: Baseline parameters of the endometrial cohort (n=109).

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</tr>
<tr>
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<td>25-29.9</td>
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<td>&gt;30-39.9</td>
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<td>Distant recurrence</td>
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* Censored March 2015
### 3.4.3 Ki-67 scoring

Intraobserver intraclass correlation coefficient (ICC) for WS, HS and IE were 0.98, 0.98 and 0.87, respectively (two-way random effects). All scoring approaches were reproducible between the two observers (VS and SK). WS and HS scoring were most consistent with interobserver ICCs of 0.96 and 0.95, while the ICC of IE scores was 0.93.

### 3.4.4 Ki-67 expression

Median nuclear Ki-67 expression was 30 (IQR 13, 42), 37 (IQR 22, 50) and 29 (IQR 15, 42) %, using whole slide (WS), hotspot (HS) and invasive edge (IE) scoring, respectively. IE scoring was only possible in seventy four cases when the endometrial/myometrial border was sampled. All scoring approaches positively correlated with each other (all p<0.001), with the strongest correlation between WS and HS scores (Spearman r=0.94).

Ki-67 score correlated with tumour grade using all three measurement approaches (Spearman r =0.41, all p<0.0001). Increased Ki-67 score was associated with increased age (Spearman r=0.29, p=0.002-0.02) and greater myometrial invasion (Spearman r=0.23, p=0.007-0.03). Ki-67 score was not correlated to BMI, FIGO stage, tumour size or the presence of lymphovascular space invasion.

### 3.4.5 Univariate analysis of patient survival

Table 26 shows the survival impact of selected histological features, FIGO stage and Ki-67 score using the three measurement approaches. Both OS from cancer deaths and RFS were assessed using univariate survival analysis. Patient survival from cancer deaths were significantly affected by age, diabetic status, FIGO stage and the presence of LVSI. When RFS was assessed, in addition to these variables, there was a significant association with FIGO grade and non-endometrioid disease. Estimated RFS for these variables is illustrated in Kaplan-Meier curves in Figure 22.

Median nuclear Ki-67 score was 32% (IQR 20-49%) within the hot-spot areas. The patients were divided into categories (high versus low Ki-67 score), according to the median score. Patients with high Ki-67 score (>32%) had lower recurrence-free survival compared with those with low Ki-67 score (<32%) (Log-rank test, p=0.08) (Figure 22). This association was also present when Ki-67 score was assessed as a continuous variable using univariate Cox regression. High Ki-67 score using the hot-spot scoring method was associated with reduced recurrence free survival [HR 1.02 (95% CI 1-1.04) p=0.046] (Table 23). WS and IE scoring identified a similar trend and univariate analysis approached statistical significance.
3.4.6 Multivariate analysis of patient survival

In multivariate analysis of RFS, age, diabetic status, FIGO stage, grade, histological type and LVSI were included with Ki-67 score. Age (HR 1.08, p=0.02), and diabetic status (HR 4.31, p=0.01) showed independent significance, while FIGO stage approached statistical significance (Table 23). Using a multivariate model, tumour grade, histological type, presence of LVSI and Ki-67 expression did not have an independent prognostic effect on RFS.
Table 23: Univariate analysis for age, diabetic status, FIGO stage, histological type, grade, LVSI and Ki-67 expression.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted HR OS</th>
<th>95% CI</th>
<th>p</th>
<th>Unadjusted HR RFS**</th>
<th>95% CI</th>
<th>p</th>
<th>Adjusted HR RFS***</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>1.10</td>
<td>1.04-1.18</td>
<td>0.002</td>
<td>1.09</td>
<td>1.05-1.13</td>
<td>&lt;0.001</td>
<td>1.08</td>
<td>1.03-1.14</td>
<td>0.02</td>
</tr>
<tr>
<td>Diabetic</td>
<td>4.82</td>
<td>1.46-15.88</td>
<td>0.01</td>
<td>4.66</td>
<td>2.23-9.75</td>
<td>&lt;0.001</td>
<td>4.31</td>
<td>1.69-11.0</td>
<td>0.01</td>
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<tr>
<td>Non-diabetic</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>FIGO Stage</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0.00</td>
<td>0.00-0.00</td>
<td>0.985</td>
<td>2.18</td>
<td>0.71-6.69</td>
<td>0.172</td>
<td>5.38</td>
<td>.89-32.4</td>
<td>0.07</td>
</tr>
<tr>
<td>III</td>
<td>8.25</td>
<td>2.13-31.94</td>
<td>0.002</td>
<td>3.02</td>
<td>1.39-6.59</td>
<td>0.005</td>
<td>1.77</td>
<td>0.64-4.93</td>
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<tr>
<td>IV</td>
<td>46.43</td>
<td>7.52-286.8</td>
<td>&lt;0.001</td>
<td>11.53</td>
<td>2.57-51.78</td>
<td>0.001</td>
<td>5.24</td>
<td>0.79-34.8</td>
<td>0.09</td>
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<tr>
<td>Endometrioid</td>
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</tr>
<tr>
<td>Non-endometrioid</td>
<td>173.14</td>
<td>.89-33560</td>
<td>0.055</td>
<td>6.84</td>
<td>3.06-15.3</td>
<td>&lt;0.001</td>
<td>2.05</td>
<td>0.43-9.74</td>
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<td>Grade</td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>1.15</td>
<td>.00-14684</td>
<td>0.977</td>
<td>0.66</td>
<td>0.12-3.71</td>
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<td>0.2</td>
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<td>3</td>
<td>98.36</td>
<td>.172-56096</td>
<td>0.156</td>
<td>5.39</td>
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<td>0.002</td>
<td>0.98</td>
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<td>LVSI present</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4.73</td>
<td>1.27-17.6</td>
<td>0.02</td>
<td>2.93</td>
<td>1.40-6.13</td>
<td>0.004</td>
<td>1.2</td>
<td>0.43-3.4</td>
<td>0.73</td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
<td>Whole slide</td>
<td>1.02</td>
<td>.98-1.05</td>
<td>0.379</td>
<td>1.02</td>
<td>0.99-1.04</td>
<td>0.057</td>
<td>1.01</td>
<td>0.98-1.04</td>
<td>0.54</td>
</tr>
<tr>
<td>Invasive edge</td>
<td>1.01</td>
<td>.96-1.06</td>
<td>0.668</td>
<td>1.03</td>
<td>0.99-1.05</td>
<td>0.058</td>
<td>1.03</td>
<td>0.98-1.07</td>
<td>0.16</td>
</tr>
<tr>
<td>Hotspots</td>
<td>1.01</td>
<td>.98-1.05</td>
<td>0.412</td>
<td>1.02</td>
<td>1.0-1.04</td>
<td>0.046</td>
<td>1.01</td>
<td>0.98-1.04</td>
<td>0.52</td>
</tr>
</tbody>
</table>

* OS was defined as survival from cancer-deaths at the time censoring, and included those with recurrence.** RFS included patients who died of non-cancer causes, and those who were alive with no evidence of recurrence.*** Adjusted hazard ratios included all variables which had a significant effect of RFS ( p<0.05). Abbreviations: LVSI Lymphovascular space invasion, OS Overall survival, RFS Recurrence-free survival
Figure 22: Estimated RFS among patients with endometrial cancer, according to diabetic status (p<0.001), International Federation of Gynaecology and Obstetrics (FIGO) stage (p<0.001), grade (p<0.001), histologic type (non-endometrioid=serous, papillary, clear cell, carcinomas) (p<0.001) and LVSI (p=0.006) (Kaplan-Meier). Ki-67 score measured by the hotspot approach influenced RFS in this prospective study with 109 patients with endometrial carcinoma. Using the median value, patients with higher Ki-67 (54/11) had a lower recurrence-free survival time (Log-rank test, p=0.08). For each binary category, the number of cases/number of cancer recurrences or death is shown.
3.4 Discussion

This is the first study to compare manual scoring of Ki-67 with semi-automated scoring using Definiens Developer in EC. Scores from manual and automated methods were comparable, with the semi-automated scoring being superior in terms of reproducibility, time-efficiency and allowed sampling of greater areas of each tumour section. Hotspot (HS) scoring was a reproducible method for scoring Ki-67 and high Ki-67 expression was associated with an increased risk of disease recurrence using a univariate analysis; this association did not persist in the multivariate analysis. Only age and co-existing diabetes were significantly associated with increased risk of recurrence in the multivariate model.

Whilst the software was not able to reliably distinguish between tumour glands and stromal tissue, selection of areas to be scored by different observers produced accurate and reproducible results. The automated method yielded similar results to manual scoring allowing comparison with other publications using manual scoring methods. A presurgical window study of 11 patients with endometrial cancer comparing light microscopy manual scoring and automated nuclear counting also reported similar results with both approaches (Laskov et al., 2014).

Both WS and HS scoring were reproducible methods of scoring Ki-67, with high Ki-67 score by HS scoring being associated with increased risk of recurrence. There is no consensus as to whether Ki-67 scoring should be representative of all areas of tumour (WS), concentrate on areas of most intense staining (HS) or the invasive edge (IE), arguably the most biologically active part of the tumour. Hot-spots may also represent clones of biologically active and potentially more aggressive cancer cells (Weidner et al., 1994).

In a prospective study of 115 patients treated with EC, Salvesen et al. report that high expression of Ki-67 (>31%) was significantly associated with FIGO stage (p=0.0004), histological type (p=0.03) and grade (p=0.0001). A further retrospective analysis of 225 patients with endometrial cancer from this group reported a strong correlation of high Ki-67 expression with advanced FIGO stage, histological grade and recurrence (Stefansson et al., 2004). In contrast, increased Ki-67 expression was not associated with FIGO stage or tumour size in this study.
Other established clinicopathological variables such as increased age, diabetes, FIGO stage, high grade histology, non-endometrioid disease (Abeler and Kjorstad, 1991, Zaino et al., 1996) and the presence of LVSI had prognostic impact in the univariate analysis. High lymphovascular density is associated with aggressive endometrial carcinoma features like high histologic grade and necrosis (Stefansson et al., 2004). T2DM has been associated with worse recurrence-free and overall survival in both univariate and unadjusted models (RFS HR 1.6 (95% CI 1.1-2.2, p=0.02, OS HR 2.2, 95% CI 1.5-3.4, p=0.0002). This association persisted when analysis was restricted to low grade endometrial tumours (Ko et al., 2014a).

Patients with early stage, low grade EC usually have excellent long-term prognosis. The provision of adjuvant therapy is based on FIGO stage, histological grade, depth of myometrial invasion and the status of LVSI. Lymph node dissection is often reserved for patients with high risk disease (high grade or late stage) or when lymph nodes appear clinically abnormal (ASG et al., 2009). Disease recurrence in “low risk” patients however suggests a need for more discriminative biomarkers. In this study, while there was a significant association between Ki-67 score and tumour grade the scatter plot (not shown) of baseline Ki-67 expression shows a wide variance according to grade. It is interesting to hypothesise whether disease recurrence is more likely in patients with low grade disease/high Ki-67 score compared with low grade disease/low Ki-67 score.

Ki-67 expression is emerging as a significant prognostic indicator for endometrial carcinoma. This is the first study to consider Ki-67 score as a continuous variable, where for every 1% increase in baseline Ki-67 score, there was a corresponding 2% decrease in recurrence-free survival. Several other studies have shown Ki-67 expression scored by the hot-spot approach to be an independent prognostic marker (Salvesen et al., 1998, Stefansson et al., 2004, Liu et al., 2014). Salvesen et al. report that Ki-67 is shown to be superior as a prognostic marker to S phase fraction, another marker of tumour proliferation based on an estimation of cells in DNA synthesis prior to mitosis (Salvesen et al., 1998). Ki-67 expression is easily measured by IHC and does not involve the additional tumour processing required for S phase fraction assessment by flow cytometry.

Short term change in Ki-67 score is additionally a useful surrogate biomarker of clinical effect in short term intervention studies. In breast cancer, change in Ki-67 score is a predictive biomarker. Short term change during the neoadjuvant period correlates with
long term effects when adjuvant treatment is continued (Clarke et al., 1993, Geisler et al., 2001, Decensi et al., 2003, Ellis et al., 2003). Ki-67 score was measured in tumour biopsies taken before and after two weeks of presurgical treatment with anastrazole or tamoxifen or the combination of both. In this study, higher Ki-67 score after two weeks of endocrine therapy was significantly associated with lower recurrence-free survival (p=0.004). The same treatment given in the neoadjuvant period, with continued as adjuvant therapy and 5-year recurrence free survival rates were 85%, 75% and 60% for the lowest (<2.7%), middle (2.7-7.4%) and highest (>7.4%) tertiles of 2-week post-treatment Ki-67 expression, respectively (Dowsett et al., 2007).

Findings from this study suggest that measurement of Ki-67 can be performed easily and reproducibly on FFPE tissue, allowing easy translation to the clinical setting. The strength of this study was the detailed clinical follow-up and expert pathology review of all sections by a single gynaecological histopathologist. Ki-67 may have a prognostic impact on RFS, however, there were too few tumours to adequately power the assessment of Ki-67 in a multivariate analysis. A further analysis is planned with the inclusion of high grade tumours to better ascertain the prognostic impact of Ki-67. The aim of this study, however, was not only to identify the role of Ki-67 as a prognostic marker, but to determine its value as a biomarker to predict treatment response and to establish a reproducible and reliable staining and scoring protocol. Quantifying the impact of Ki-67 expression as a continuous variable on prognosis helps interpret the short term change in Ki-67 score following presurgical metformin treatment (Chapter 2) on clinical outcomes.

It remains to be established whether the change in Ki-67 score can predict treatment response in EC. Results from the feMMe trial, a phase II randomised trial of levornorgestrel IUS +/- metformin +/- weight loss intervention in non-surgical patients with early stage EC will provide crucial information about the change in Ki-67 over a three month intervention and allow comparison with longer-term follow-up. A reliable predictive biomarker has the potential to improve allocation of adjuvant treatment and identify patients who will derive maximal clinical benefit, while reducing unnecessary debilitating side effects associated with chemo/radiotherapy and clinical costs.

4.1: Introduction

Early stage EC is managed with surgical resection of the uterus, cervix and both fallopian tubes and ovaries. This is often associated with excellent prognosis; however, treatment options are limited for advanced and recurrent disease (Bradford et al., 2015). Epidemiological studies have demonstrated that diabetic patients treated with metformin have improved response to chemotherapy compared with patients receiving other hypoglycaemic treatments both in breast (Jiralerspong et al., 2009) and advanced EC (Ezewuio et al., 2016).

Metformin is thought to exert anti-cancer effects by decreasing circulating insulin and glucose through actions of hepatocytes and skeletal muscle (Shaw et al., 2005). An alternative proposed mode of action is through inhibition of complex I in the mitochondria, leading to activation of AMP-activated protein kinase (AMPK) (Dykens et al., 2008) and suspension of ATP-consuming processes such as fatty acid and protein synthesis. Metformin is thought to behave as a novel mTOR inhibitor and is shown to reduce cellular proliferation in multiple cell line models, including endometrial (Cantrell et al., 2010, Sarfstein et al., 2013), breast (Zakikhani et al., 2010), colorectal and prostate cancer cell lines (Zakikhani et al., 2008).

The PI3K/AKT/mTOR pathway is commonly altered in EC, often by PTEN tumour suppressor gene function loss and activating mutations in the PI3K subunit p110α encoded by the PIK3CA gene (Kandoth et al., 2013). Given the high prevalence of PI3K/AKT/mTOR pathway activation, drugs targeting this pathway hold tremendous potential for the treatment of EC. Pre-clinical studies with PI3K/mTOR inhibitors have shown encouraging results in EC cell lines (Shoji et al., 2012). In addition, a recent study of the mTOR inhibitor everolimus, used in combination with letrozole in women with recurrent EC, reported a clinical benefit rate of 40% compared with 21% when using single agent everolimus (Slomovitz et al., 2015).

mTOR inhibitors have been shown to enhance the effects of other chemotherapeutic agents (Wu et al., 2005, Mondesire et al., 2004). The mTOR inhibitor rapamycin has been demonstrated to potentiate the effects of paclitaxel in EC cell lines (Shafer et al., 2010). Patients with advanced/recurrent or metastatic EC are often treated with carboplatin and paclitaxel. Given the inhibitory effects of metformin on the mTOR pathway, the hypothesis
that metformin, similar to rapamycin would sensitise the inhibitory effects of established chemotherapies i.e. carboplatin and paclitaxel was tested. An in vitro cell culture system using commercially available EC cells was developed as a model to test the effects of a number of chemotherapeutics on cell viability. The effects of phenformin, a more potent biguanide, were also tested on cellular proliferation using the cell culture model. Phenformin was withdrawn from the markets of most countries in the 1970s because of increasing reports of lactic acidosis (Nattrass and Alberti, 1978), however, was included in this study to identify if biguanides had a class effect on cellular proliferation. In addition, as some ECs are driven by hyperglycaemia and insulin resistance, the cell culture conditions were varied to test the effect of different glucose concentrations in the viability model. Cultured cells were also used to demonstrate the effects of metformin treatment on PI3K/AKT/mTOR phosphorylation events.
4.2: Materials and Methods

In order to determine the potential effects of metformin on EC cell viability, a cell culture system was established to allow the testing of multiple chemotherapeutics, both as single agents and in combination. All experiments were performed using at least two different EC cell lines.

4.2.1 Cell lines and maintenance

The Ishikawa (Ish) cell line originated from an endometrial adenocarcinoma from a 39 year old woman. These cells are used as a model of well differentiated tumours. Ishikawa cells were chosen because of their native expression of oestrogen (ER) and progesterone receptors (PR) and are reported to be PTEN null. They also express insulin (IR) and insulin-like-growth factor 1 receptors (IGF1R). HEC1A cells were established from a moderately differentiated human endometrioid adenocarcinoma of 71 year old Japanese female. HEC1A cells do not express steroid receptors and are PTEN wild-type (Nishida, 2003). KLE cells were established from a uterine adenocarcinoma of a Caucasian female. These cells are homozygous for a p53 mutation (c524.G>A), but PTEN wild- type. KLE cells are IR/IGF1R positive but steroid receptor negative (Table 9). Stocks of Ishikawa cells were purchased from HPA Culture Collections (Salisbury, UK) and HEC1A and KLE cells were obtained from ATCC (Middlesex, UK). All three cell lines have been used extensively as model systems to study the effects of drugs in EC.

Table 24: Endometrial Cancer Cell Lines, abbreviations used and their characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Ishikawa (Ish)</th>
<th>HEC1A (HEC)</th>
<th>KLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiation</td>
<td>Well</td>
<td>Moderate</td>
<td>Poor</td>
</tr>
<tr>
<td>Oestrogen Receptor Status</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Insulin Receptor Status</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Organic Cation Transporter -1 (OCT-1) Status</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>PTEN Status</td>
<td>Deficient</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
All cell culture techniques were carried out using biological safety cabinets (Airstream Duo). Cells were maintained in DMEM growth medium (LTC, Paisley, UK), supplemented with 10% heat inactivated fetal bovine serum (Hi-FBS) (Sigma-Aldrich, Dorset, UK), GlutaMAX (LTC, Paisley, UK) and 10 000u/ml penicillin + 10 000ug/ml streptomycin (Sigma-Aldrich, Dorset, UK). Cells were grown in 162cm² or 222cm² culture flasks (Corning, Tewksbury, MA) in a humidified atmosphere containing 5% CO₂ (Thermo Forma).

4.2.2 Retrieving cells
Cells were removed from liquid nitrogen and rapidly thawed in a water bath at 37°C and re-suspended in growth media (Corning, Tewksbury, MA). The following day, media was replaced to remove DMSO.

4.2.3 Sub culture and freezing of cells
Cells were routinely passaged on reaching 80% confluence. The growth medium was aspirated and cells were washed with phosphate buffered saline (PBS) (Appendix 1). Pre-warmed trypsin/EDTA (0.25%) (Sigma-Aldrich, Dorset, UK) was added and flasks returned to the incubator for five minutes until all cells had rounded up and detached. Cells were then re-suspended in supplemented growth medium. Cell count was performed using an automated cell counter (TC20 Automated Cell Counter, BIO-RAD) and cells were reseeded in appropriate culture flasks.

Cells were harvested and counted at 80% confluence and pelleted by centrifugation at 1500rpm for five minutes. Growth medium was removed and the cell pellet thoroughly re-suspended in freezing medium (50% growth medium and 50% Hi-FBS + 10% DMSO) in labelled cryovials (Nunc, Roskilde, Denmark). Cryovials were transferred to an isopropanol gradient freezing pot (Nalgene, Roskilde, Denmark) and placed at -80°C overnight before being transferred to liquid nitrogen for long term storage.

4.2.4 Drug preparation
Metformin hydrochloride and phenformin hydrochloride were diluted in growth media to obtain stock solutions of 100mM. Carboplatin and paclitaxel stocks were prepared in growth media and DMSO, respectively to make up 10mM stock solutions. Final DMSO concentration in assays was consistent at 0.1%. All drugs were purchased from Sigma-Aldrich (Dorset, UK).
4.2.5 Cell Viability Assays: Growth conditions

Endometrial cancer cells in the logarithmic growth phase were seeded in 96 well plates at varying cell densities (1000, 3000, 5000, 8000 & 10000 cells/well) to determine the optimal growth conditions over five days. After 24, 48 and 72 hour incubations, cell viability was assessed using an MTS assay (Figure 23) to determine the optimum seeding density for logarithmic growth. Following this, a SRB assay was performed to determine the seeding density for logarithmic growth and SRB measurements in the optimum linear range (Figure 24) in all three cell lines. For the subsequent experiments, Ishikawa, HEC1A and KLE cells were plated and grown in 96 well plates at the concentrations of 5000 cells/well, 10,000 cells/well and 10,000 cells/well, respectively. Experiments were carried out in DMEM with 1.0g/L D-glucose (termed low glucose) or 4.5g/L D-glucose (termed high glucose).

Cells were grown for at least 24 hours prior to growth media being replaced with media supplemented with a drug. Cells were treated with increasing doses of drugs over 24, 48 and 72 hours for individual experiments and cell viability assessed. For each data point (drug dose or time point), at least three technical replicates were performed. Each experiment was repeated in triplicate.

Figure 23: Cell viability according to initial cell density over 24-72 hours using an MTS assay to determine optimum seeding density to achieve logarithmic growth throughout the experiment. For Ishikawa and HEC1A cells, an initial seeding density of 5000 and 10,000 cells were chosen.
Figure 24: Cell viability of EC cells cultured in high and low glucose media for 72 hours according to initial cell density using an SRB assay. Results are representative of mean from triplicate wells ± SEM.

4.2.6: CellTitre Aqueous One (MTS) Cell Viability Assay

Cell viability was assessed using a AQ-96 reagent assay (Promega, Southampton, UK). This is a colorimetric method for determining the number of viable cells in cytotoxicity assays. The reagent contains [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS] and an electron coupling reagent (phenazine ethosulfate; PES). The MTS compound is converted by metabolically active cells through mitochondrial respiration, presumably by NADPH or NADH produced by dehydrogenase enzymes.

Drug effects were measured by adding 20uL of the AQ-96 reagent to each well. Two hours post incubation, absorbance at 490nM was measured using a FLUOstar Omega Microplate Read (BMG Labtech, Ortenberg, Germany). Results were recorded as optical density measurements but presented as a percentage of viability compared with untreated controls.
4.2.7: Sulforhodamine B (SRB) Cell Proliferation Assay

This assay is based on the ability of the protein dye sulforhodamine B (SRB) to bind to basic amino acid residues of trichloroacetic acid (TCA)-fixed cells under acidic conditions. The dye can then be extracted from cells and solubilised for measurement under mild basic conditions, providing a colorimetric end point (Voigt, 2005). The assay assesses an increase or decrease in the number of cells (total biomass), resulting in a concomitant change in the amount of dye incorporated by the cells. This indicates the degrees of cytotoxicity caused.

Following drug treatments, the drug supplemented media was aspirated. The plates with live adherent cells were washed with PBS and fixed by protein precipitation with 10% TCA. Cells were left to dry overnight and then stained for 15 minutes with 0.4% SRB. Subsequent washes with 1% acetic acid removed unbound stain. After further air-drying, bound protein was solubilised with Tris base (tris(hydroxyl-methyl aminomethane) and measured on a FLUOstar Omega Microplate Read (BMG Labtech, Ortenberg, Germany) at 540nM absorbance. Results were presented as a percentage of viability compared with untreated controls.

4.2.8 Western Blot analysis for protein expression

Western blots were used to assess protein expression and phosphorylation changes in EC cells.

4.2.8.1 Preparation of protein extracts from cultured cells

Endometrial cancer cells were seeded in 10cm² dishes at densities of 0.75 x 10⁶cells/dish (Ishikawa) and 1.5 x 10⁶cells/dish (HEC1A & KLE) and incubated at 37°C for 24 hours prior to drug treatment in low glucose DMEM. The growth media was then replaced with fresh media containing drug. After the treatment duration, the media was aspirated and plates washed twice with ice-cold PBS. Whole cell lysates were collected with a lysis buffer (RIPA) supplemented with phosphatase and protease inhibitors (1:100)(Sigma-Aldrich, Dorset, UK). Following addition of the lysis buffer, cells were mechanically degraded with a cell scraper and collected into pre-cooled centrifuge tubes. The lysate was incubated on ice for 1 hour, and then centrifuged at 13000rpm for 15 minutes at 4°C. The supernatant was collected and stored at -80°C until use.

4.2.8.2 Determination of protein concentration

The protein concentrations of samples were determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL), colorimetric based protein quantification assay. The assay combines the reduction of Cu²⁺ to Cu⁺ by protein in an alkaline medium with the
detection of the Cu$^{+1}$ cation by a bicinchoninic acid containing reagent. Protein standards of 25, 125, 250, 500, 750, 1000, 1500, 2000 ug/mL were prepared by serial dilution of bovine serum albumin (BSA). Samples were diluted (1:2-1:10) in distilled water to ensure that the protein concentration would be within range of the standard curve (20-2000ug/mL). Protein sample (10uL) was added to the 200uL of BCA working reagent in a 96 well microplate, alongside the standards. Following incubation of the plate at 37°C for 30 minutes, absorbance was measured at 562nM.

A standard curve of protein concentration versus absorbance readings was constructed by linear regression (Figure 25). The protein concentration in each sample was derived from the equation and multiplied by the dilution factor used.

![Figure 25: Representation of BSA standard curve. The equation derived from the straight line allows interpolation of the protein concentration in samples.](image)

4.2.8.3 Preparation of polyacrylamide gel
Protein separation was achieved using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Pre-cast 10% Mini-PROTEAN polyacrylamide (Bio-RAD) gels were used for the final gel electrophoresis experiments presented in this thesis. The method for preparing polyacrylamide gels is detailed below.

A 10% resolving gel was made (Table 10) and loaded between two glass plates. The gel was then superimposed with 100% isopropanol to allow a smooth level and allowed to set. The isopropanol was removed prior to addition of the stacking gel (Table 11) over the resolving layer. Wells were formed by the addition of a comb in the stacking gel solution and allowed to set. The comb was removed and gels used immediately or stored in buffer at 4°C
overnight. For the final results presented in this thesis, pre-cast gels (Mini-Protean Gels, BIORAD) were used.

Table 25: Composition of a 10% resolving gel suitable for separation of proteins (20-300kDa)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>2.2</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>1.7</td>
</tr>
<tr>
<td>TrisHCl (pH 8.8)</td>
<td>1</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>0.05</td>
</tr>
<tr>
<td>Ammonium persulphate (20%)</td>
<td>0.05</td>
</tr>
<tr>
<td>Teemed</td>
<td>0.0042</td>
</tr>
</tbody>
</table>

Table 26: Composition of a stacking gel

<table>
<thead>
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<th>Solution</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>Acrylamide</td>
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<td>TrisHCl (pH 6.8)</td>
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<tr>
<td>SDS (10%)</td>
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</tr>
<tr>
<td>Ammonium persulphate (20%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Teemed</td>
<td>0.00168</td>
</tr>
</tbody>
</table>

4.2.8.4 Protein preparation, loading and SDS-PAGE for western blotting
Protein concentrations were standardised by dilution with dH₂O and denatured prior to loading at 95°C in a 1X Lamelli Solution. Samples were loaded at 20-30ug protein per well depending on the native expression of the proteins. A Kaleidoscope pre-stained molecular weight (MW) marker (Bio-Rad) was loaded alongside protein samples and separation achieved at 80-120V in an electrophoresis running buffer. Following electrophoresis, proteins were transferred onto a PVDF membrane using a seven minute transfer.

4.2.8.5 Antibody Probe
Membranes were kept moist in tris buffered saline (TBS) buffer prior to use and blocked with 5% BSA Block for one hour. Primary antibodies were diluted in a 1% BSA + 0.05%
Tween solution and incubated for one hour at room temperature or overnight at 4°C. Following incubation with the primary antibody, membranes were washed four times for five minutes with TBS + 1% Tween. Primary antibodies directed against AKT, phospho-AKT (Ser 473), AMPKα, phospho-AMPKα, 4EBP1, phospho-4EBP1 (Ser65), S6 ribosomal protein (RP) and phospho-S6 RP(Ser235/236) were used evaluate the effect of metformin on mTOR pathway phosphorylation events.

The membranes were then incubated with anti-mouse or anti-rabbit fluorescently labelled secondary antibodies (IRDye®) diluted in 1% BSA + 0.05% Tween. Membranes were washed again in TBS +1% Tween and once in PBS. Images were obtained using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, Nebraska). The membranes were protected from light after addition of the secondary antibodies.

Beta-actin expression was used as a loading control using anti-Beta-actin IgG (Sigma-Aldrich, Dorset, UK). PTEN and OCT-1 expression was determined in a similar fashion using primary antibodies directed against PTEN (Abcam, Cambridge, UK) and anti- SLC22A1 (Sigma-Aldrich, Dorset, UK).

4.2.9 Antibodies
Antibodies against AKT, AMPK, S6, 4EBP1 and the corresponding phosphorylated isoforms were obtained from Cell Signalling Technology (Beverley, MA). Antibodies against Beta-actin (AC-74) and OCT-1 (SLC22A1) were obtained from Sigma-Aldrich (Dorset, UK), while the anti-PTEN antibody was supplied by Abcam (Cambridge, UK). Primary antibodies and the dilutions used for western blotting are shown in Table 27.
### Table 27: Primary antibodies and the dilutions used

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Catalogue number</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT</td>
<td>#9272</td>
<td>1:1000</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>p-AKT (Ser 473)</td>
<td>#4060</td>
<td>1:1000</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>AMPKα</td>
<td>#2603</td>
<td>1:1000</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>p-AMPKα (Thr 172)</td>
<td>#2535</td>
<td>1:1000</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>S6 ribosomal protein</td>
<td>#2217</td>
<td>1:2000</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>p-S6 ribosomal protein (Ser 235.236)</td>
<td>#4858</td>
<td>1:2000</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>4EBP1 (S3H11)</td>
<td>#9644</td>
<td>1:2000</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>p-4EBP1 (Ser 65)</td>
<td>#9451</td>
<td>1:1000</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>PTEN</td>
<td>Ab31392</td>
<td>1:500</td>
<td>Abcam</td>
</tr>
<tr>
<td>OCT-1 (SLC22A1)</td>
<td>AV41516</td>
<td>1:250</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Beta-actin</td>
<td>AC74</td>
<td>1:4500</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>
4.2.10 Statistical analysis
All statistical analyses were performed using Graph Pad Prism version 6 (GraphPad Software, San Antonio, CA). IC$_{50}$ values were obtained from non-linear regression curve fitting to log-transformed data. Parametric data were analysed using unpaired Student’s t-tests and 1-way analysis of variance (ANOVA). Statistical significance was accepted at p<0.05.

Drug combination studies were tested using CompuSyn software to obtain a combination index (CI). The CI index method of Chou-Talalay is based on the multiple drug effect equation derived from the median effect principle of the mass action law (Chou, 2006). It allows determination of synergism (CI<1), additivity (CI=1) and antagonism (CI>1). The algorithm takes into account both the potency and the shape of the dose effect curve of each drug alone and their combination.
4.3: Results

4.3.1 Characterisation of the endometrial cancer cells
The organic cation-1 transporter (OCT-1) is responsible for organic cation uptake into hepatic cells through facilitated diffusion and active transport. OCT-1 has been shown to be highly expressed in epithelial ovarian cancers, while non-cancerous tissue shows a virtual absence of OCT-1 expression (Segal et al., 2011). OCT-1 expression was assessed in Ishikawa, HEC1A and KLE cell lines. Greatest expression was seen in HEC1A and KLE cells, while an additional lysate from a breast cancer cell line (as a negative control), MCF-7 did not demonstrate OCT-1 expression (Figure 24).

PTEN expression was also assessed using western blotting. Ishikawa cells are widely reported to be PTEN null, while HEC1A and KLE cells are thought to express PTEN. In these cells, both Ishikawa and HEC1A cells did not express PTEN, while KLE cells were PTEN wild-type. Lysates from MCF-7 breast cancer are reported to express PTEN, as seen in Figure 25 (Saal et al., 2008).

![Figure 24: OCT-1 expression in endometrial cancer cells and MCF-7 breast cancer cells, cultured in low glucose DMEM.](image)

![Figure 25: PTEN expression in endometrial cancer cells and MCF-7 breast cancer cells, cultured in low glucose DMEM](image)
4.3.2: Optimising treatment conditions and time-dependent effects

Metformin treatment resulted in decreased cell viability in Ishikawa, HEC1A and KLE EC cell lines in a time- and dose-dependent manner, using an MTS assay. Initial experiments used Ishikawa and HEC1A cells only, cultured in MEM (containing 1.0g/L D-Glucose) + 5%FBS and McCoy’s 5A Media (containing 3g/L D-glucose) + 10% FBS. The cells were treated with metformin (0.01-10mM) over 24-72 hours. Using these conditions, significant effects on cell viability were only observed with metformin concentrations > 4mM after 72 hours of treatment in Ishikawa cell lines (1-way ANOVA, p<0.001). In HEC1A cell lines, cell viability was reduced to 75% in cells treated with 8 & 10mM metformin, only (p<0.01, 1-way ANOVA).

A further series of experiments was carried out using Ishikawa, HEC1A and an additional cell line, KLE. In these experiments, all cells were cultured in a standard media, DMEM (D-glucose 4.5g/L, i.e. high glucose) + 10% heat-inactivated FBS (HiFBS). Cells were treated with 10mM metformin, as the effect on cell viability was modest with lower doses. MTS readings were taken at 24, 48 and 72 hours. In the high glucose media, Ishikawa cells were noted to be resistant to metformin treatment, even at 10mM. Time-dependent effects were noted in HEC1A and KLE cells, with the greatest effect following 72 hours of treatment (p<0.01, 1-way ANOVA) (Figure 26).

The experiment was repeated using an SRB assay with the same drug concentrations in low glucose media. Here, time-dependent effects were noted in all three cell lines, with the greatest effects observed at 72 hours (p<0.001, 1-way ANOVA) (Figure 26). The SRB assay was used as the primary cell viability assay, as it measures cell biomass directly and is not a measure of mitochondrial respiration.
Figure 26 (top): Metformin treatment has a time-dependent effect on HEC1A and KLE cancer cell viability in high glucose media using an MTS assay. Cells treated with 10mM metformin were compared with control (untreated cells) in high glucose media over 72 hours. HEC1A and KLE cells demonstrated time-dependent effects at 24, 48 and 72 hours (p<0.01, 1-way ANOVA), while Ishikawa cells were resistant to metformin treatment. Figure 26 (bottom): The effects of 10mM metformin on cell viability were assessed using an SRB assay in low glucose media. Here, time-dependent effects on cell viability were noted on all three cell lines. Results are representative of three biological triplicates ± SD. *denotes statistically significant differences.

4.3.3 Effect of metformin on cell viability in low and high glucose media
The cytostatic effect of metformin was noted to be influenced by glucose concentration of the media. Ishikawa cells grown in high glucose media (4.5g/L D-glucose) were resistant to cytostatic effects of metformin (p<0.001, 1-way ANOVA) after 72 hours of treatment. The effect on cell viability was also reduced in high glucose compared with low glucose (1.0g/L D-glucose) in HEC1A (p<0.001, 1-way ANOVA) and KLE cells (p<0.01, 1-way ANOVA). This modulating effect of glucose concentration was confirmed using both MTS and SRB cell viability assays in Ishikawa and HEC1A cells. Using the MTS assay, the mean IC50 in low glucose was 0.1mM and 0.7mM in Ishikawa and HEC1A cells (Figure 27). The mean IC50 in low glucose was 2.4mM, 1.6mM and 4.2mM in Ishikawa, HEC1A and KLE cells, respectively using the SRB assay (Figure 28).

When treated in low glucose, the cytostatic effects of metformin after 72 hours were significantly different from control cells at 0.5-10mM in HEC1A cells (p<0.01, 1-way ANOVA), while in KLE cells, significant differences were seen at 1-10mM treatment doses (p<0.01, 1-way ANOVA) (Figure 29). The initial high glucose (4.5g/L) media was used as it was the standard media used in the laboratory. The low glucose (1.0g/L) was then selected based on review of the literature and its similarity with normoglycaemia in patients.

Figure 27: The effect of metformin on cell viability in low and high glucose after 72 hours treatment using an MTS assay. In Ishikawa and HEC1A cells, the effect of metformin was significantly different at 1-20mM (p<0.001, 2-way ANOVA). Results are representative of three biological repeats± SD. *denotes statistically significant differences between high and low glucose at the same metformin concentration.
Figure 28: The effect of metformin on cell viability after 72 hours treatment in low and high glucose using an SRB assay. In Ishikawa and HEC1A cells, the effect of metformin was significantly greater at 1-10mM (p<0.001, 2-way ANOVA), while in KLE cells, the difference between the metformin effect in high and low glucose was only statistically significant at 5mM (p<0.01, 2-way ANOVA). Results are representative of three biological repeats± SD. *denotes statistically significant differences between high and low glucose at the same metformin concentration.

Figure 29: Metformin has dose-dependent cytostatic effects after 72 hours of treatment in low glucose media. The cytostatic effects are significant at 0.5-10mM in Ishikawa and HEC1A cells (p<0.001, 2-way ANOVA). In KLE cells, the cytostatic effects are significant at 1-10mM (p<0.01, 2-way ANOVA). The mean IC50 was 2.4mM, 1.6mM and 4.2mM in Ishikawa, HEC1A and KLE cells, respectively. Results are representative of three biological repeats± SEM. *denotes statistically significant differences compared with untreated cells.
4.3.4 Effect of phenformin on cell viability in low and high glucose media

The effect of phenformin on cell viability was assessed using MTS and SRB assays. With the MTS assay, phenformin treatment resulted in a decrease in EC cell viability in a dose-dependent manner in HEC1A cells cultured in low glucose (p<0.001, 1-way ANOVA). The IC50 of phenformin in HEC1A cells was 4.8uM, 300 fold less than metformin. There did not appear to be a dose-dependence in Ishikawa and KLE cells, as treatment at 25-200uM resulted in a similar decrease in cell viability. In Ishikawa cells, viability was reduced to <30% compared with control (p<0.0001, 1-way ANOVA) (Figure 30).

The experiment was repeated using an SRB assay. Ishikawa and HEC1A cells were cultured in low glucose and treated with phenformin at dose ranges from 25-500uM for 72 hours. Cell viability was reduced compared with control following 50-500uM and 25-500uM of phenformin, in Ishikawa and HEC1A cells, respectively (both p<0.0001, 1-way ANOVA) (Figure 31).

In Ishikawa cells, as seen with metformin, high glucose concentration in the media protected against the cytostatic effects of phenformin at dose ranges from 25 to 200uM (p<0.0001, 1-way ANOVA). High glucose had a less profound modulating effect on the other cell lines, with a difference in cell viability noted at 25-100uM (p<0.01, 1-way ANOVA) and 25uM only (p<0.01, 1-way ANOVA) in HEC1A and KLE cells, respectively (Figure 32).

These experiments demonstrate that the cytotoxicity of biguanides, both metformin and phenformin, is impaired in high glucose or hyperglycaemic conditions. The impact of surrounding high glucose on cell viability was explored further in Chapter 5.
Figure 30: Phenformin treatment resulted in dose-dependent cytostatic effects in low glucose in HEC1A cells. The dose-dependence was not observed in Ishikawa and KLE cells, but phenformin treatment did reduce cell viability compared with control. Results are representative of three biological repeats ± SD. *denotes statistically significant differences.

Figure 31: Phenformin treatment resulted in reduced cell viability in low glucose in Ishikawa and HEC1A cells at dose ranges of 50-500uM and 25-500uM, respectively using an SRB assay. Results are representative of three biological repeats ± SEM. **** denotes p<0.0001.
Figure 32: The effect of phenformin on cell viability after 72 hours treatment in low and high glucose media using an MTS assay. In Ishikawa cells, the effect of phenformin was greater at 25-200uM (p<0.0001, 1-way ANOVA), while in HEC1A and KLE cells, high glucose had a less pronounced protective effect. Results are representative of three biological repeats± SD. *denotes statistically significant differences.
The effects of metformin treatment on the PI3K-AKT-mTOR pathway

4.3.5 Metformin has time and dose-dependent effects of the PI3K-AKT pathway

Ishikawa and HEC1A cells were treated with metformin in low glucose media to investigate the effect on AMPK, and downstream signalling through the PI3K-AKT pathway. Phosphorylation of AMPK leads to inhibition of mTOR and decreased downstream phosphorylation of 4EBP1 and S6 ribosomal protein (S6). Metformin induced phosphorylation of AMPK in a dose-dependent manner in both cell lines following 72 hours of treatment. Metformin treatment also resulted in a dose-dependent decrease in phosphorylation of both 4EBP1 and S6 (Figure 33). Total-4EBP1 was noted to have two bands, with loss of the lower band likely to represent the Serine 65 phosphorylation event.

<table>
<thead>
<tr>
<th>Metformin (mM)</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>+</th>
</tr>
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<td>2</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 33: Western immunoblots following metformin treatment for 72 hours in low glucose media. In both cell lines, there was a dose-dependent increase in phosphorylation of AMPK-α and decrease in phosphorylation of S6 and 4EBP1.
The short and long-term effects of metformin were assessed using Ishikawa and HEC1A cells. For a short time-course, cells were treated with 2mM metformin for 0-120 minutes in low glucose media with no effect on phosphorylation of AKT, S6 or 4EBP1 (Figure 34). Cells were then treated with metformin 2mM over 24-72 hours to assess longer term effects. In both cell lines, increased phosphorylation of AMPK-α was not evident until 72 hours of treatment with metformin. Similarly, decreased phosphorylation of S6 and 4EBP1 was only seen following 72 hours of treatment in Ishikawa cell lines. In HEC1A cell lines, S6 and 4EBP1 phosphorylation was observed following 48 hours of treatment (Figure 35). It was interesting that phosphorylation of AMPK was a delayed event, compared with de-phosphorylation of S6 and 4EBP1, suggesting that these events occurred independently of AMPK activation. In addition, the lack of effect over the short-time course (0-120 minutes), suggests that metformin is not acting through immediate binding to cell surface target receptors and producing rapid changes in phosphorylation events. This may be secondary to the need for active uptake into cells through organic cation transporters.
Figure 34: Protein expression after short-term treatment with metformin. No effects were seen on phosphorylation of AKT, S6 or 4EBP1 with 2mM metformin treatment.

Figure 35: Protein expression following 24-72 hours of 2mM metformin treatment. In Ishikawa cell lines, there was increased phosphorylation of AMPK-α and decreased phosphorylation of S6 and 4EBP1 after 72 hours of treatment. In HEC1A cells, these effects were noted by 48 hours of treatment.
4.3.6 Cytostatic effects of metformin and carboplatin
The effects of metformin in combination with carboplatin on cell viability were examined in the Ishikawa, HEC1A and KLE EC cells lines. This combination was used to investigate if metformin could potentiate the response of cancer cells to standard chemotherapy. All cell lines were exposed to varying doses of carboplatin alone or in combination with 2mM metformin (=IC\textsubscript{50} of metformin) and increasing concentrations of carboplatin (0.0001 to 1mM). Lower doses of metformin were not used as there were no significant effects on cell viability as a single agent.

As anticipated, carboplatin treatment resulted in dose-dependent growth inhibition in all three cell lines with IC\textsubscript{50} values of 0.4, 0.25 and 0.25 mM for Ishikawa, HEC1A and KLE cells, respectively. The addition of 2mM metformin led to a greater inhibition of proliferation compared with carboplatin treatment alone in all three cell lines. The increase in inhibition was observed in Ishikawa, HEC1A and KLE cells with 0.001-0.5mM, 0.1-0.5mM and 0.01-0.1mM carboplatin (all \(p<0.01\), t-test), respectively (Figure 36). Single agent carboplatin levels required to achieve an inhibitory effect were approximately 100-fold higher than plasma concentrations in patients. The addition of metformin allowed for greater inhibition at lower carboplatin concentrations. Therapeutic carboplatin therapy in patients results in peak plasma levels of 1.8-5.4\(\mu\)M while trough concentrations range from 0.3-1.2\(\mu\)M (Mayo, 2016).

Synergism was assessed using median-effect plots and calculation of individual /combined drug effects with CompuSyn software (Chou and Talalay, 1984). Exposure to both carboplatin and metformin in Ishikawa, HEC1A and KLE cells lines resulted in a significant synergistic activity with a combination index (CI) <1, at carboplatin concentrations of 0.1-1mM, 0.5-1mM and 0.1-0.5mM, respectively (Table 28).
Figure 36: Carboplatin treatment resulted in a dose-dependent inhibition of cell viability in endometrial cancer cells. The addition of 2mM metformin led to increased inhibition in Ishikawa, HEC1A and KLE cells with 0.001-0.5mM, 0.1 & 0.5mM and 0.01-0.1mM (all p<0.01, t-test) carboplatin, respectively. Results are representative of three biological repeats± SD. *denotes statistically significant differences.

Table 28: Combination index (CI) values for the Ishikawa, HEC1A and KLE cell lines treated with metformin and carboplatin.

<table>
<thead>
<tr>
<th>Metformin (mM)</th>
<th>Carboplatin (mM)</th>
<th>CI for Ishikawa</th>
<th>CI for HEC1A</th>
<th>CI for KLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.001</td>
<td>2.60</td>
<td>1.00</td>
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<tr>
<td>2</td>
<td>1.0</td>
<td>0.20</td>
<td>0.67</td>
<td>1.30</td>
</tr>
</tbody>
</table>

Note: A CI of <1 indicates synergy between two chemotherapeutic agents (highlighted in grey).
4.3.7 Cytostatic effects of metformin and paclitaxel

The effects of metformin were also assessed in combination with single agent paclitaxel. All cell lines were exposed to varying doses of paclitaxel alone and in combination with 1mM metformin (<IC\textsubscript{50} of metformin) and increasing concentrations of paclitaxel (0.1 to 100nM). A lower dose of metformin was used because of the significant inhibitory effects of sole agent paclitaxel. As anticipated, paclitaxel treatment resulted in dose-dependent growth inhibition in all three cell lines with IC\textsubscript{50} values of 2.8, 6.4 and 6.4nM for Ishikawa, HEC1A and KLE cells, respectively (Figure 37). The addition of 1mM metformin led to a greater inhibition of cell growth compared with paclitaxel treatment alone in HEC1A and KLE cells.

A lower cell mass was noted at 0.1 and 1nM concentration paclitaxel (p<0.01, t-test), with a corresponding decrease in IC\textsubscript{50} to 5.0 in HEC1A cells. In KLE cells, there was a significant increase in inhibition in combination with 1-10nM concentration of paclitaxel (p<0.05, t-test)(Figure 39). Significant synergistic effect was noted with CI<1, in combination with paclitaxel at 50-100nm, 0.1-50nM and 0.1-100nM in Ishikawa, HEC1A and KLE cells (Table 29).

![Figure 37](image-url)

Figure 37: Paclitaxel treatment resulted in a dose-dependent inhibition of cell viability in endometrial cancer cells. The addition of 1mM metformin led to increased inhibition in HEC1A and KLE cells with 0.1-1nM and 0.1-10nM concentrations of paclitaxel (p<0.001, 2-way ANOVA), respectively. Results are representative of three biological repeats± SD. *denotes statistically significant differences.
Table 29: Combination index (CI) values for Ishikawa, HEC1A and KLE cells treated with metformin and paclitaxel.

<table>
<thead>
<tr>
<th>Metformin (mM)</th>
<th>Paclitaxel (nM)</th>
<th>CI for Ishikawa</th>
<th>CI for HEC1A</th>
<th>CI for KLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>1.26</td>
<td>0.44</td>
<td>0.84</td>
</tr>
<tr>
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<td>1.0</td>
<td>2.86</td>
<td>0.52</td>
<td>0.42</td>
</tr>
<tr>
<td>1</td>
<td>10.0</td>
<td>5.14</td>
<td>0.52</td>
<td>0.36</td>
</tr>
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<td>1</td>
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<td>0.76</td>
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</tr>
<tr>
<td>1</td>
<td>100.0</td>
<td>0.32</td>
<td>1.67</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Note: CI<1 indicates synergy between two chemotherapeutic agents (highlighted in grey).
4.3.8 Anti-proliferative effects of metformin, paclitaxel and carboplatin

In the previous analysis, synergistic effects have been demonstrated between the dual combinations of 2mM metformin and carboplatin (Table 28), and 1mM metformin with paclitaxel (Table 29). As paclitaxel and carboplatin are currently used together as adjuvant therapy in endometrial cancer, 1mM metformin was combined with these two agents in a triple combination.

In both HEC1A and KLE cell lines, cell viability was reduced in the triple combination, compared with carboplatin and paclitaxel alone at paclitaxel doses of 0.1 and 1nM (p<0.05-0.0001, unpaired Student’s t-test) (Figure 38). At higher doses of paclitaxel (10-100nM) and carboplatin (0.5mM), the addition of metformin did not cause increased effects on cell viability. Ishikawa cell lines were not used because of the profound effects on cell viability caused by paclitaxel alone at >10nmol concentrations.

The synergy/additive effects of the double and triple combinations were assessed using the Chou-Talalay method. Synergistic effects were observed with the combination of carboplatin and paclitaxel, particularly with the lower carboplatin dose of 0.01mM in HEC1A cells. The combination index obtained with the addition of metformin suggests that metformin has additive effects in the HEC1A cells in a triple combination. In the KLE cells, the double combination of paclitaxel and carboplatin appeared to be antagonistic. The addition of metformin to 0.1mM of carboplatin, however, displayed very synergistic effects at all concentrations of paclitaxel (Table 30).

![Figure 38: The triple combination of carboplatin, paclitaxel and 1mM metformin reduced cell viability more than carboplatin and paclitaxel alone, at paclitaxel doses of 0.1 and 1nM. At higher doses of paclitaxel, the addition of metformin did not have additional effects of cell viability.](image-url)
Table 30: Combination index (CI) values for HEC1A and KLE cells using a double combination of carboplatin and paclitaxel and a triple combination, with the addition of metformin.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>HEC1A</th>
<th>KLE</th>
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<tbody>
<tr>
<td></td>
<td>Carboxplatin (mM)</td>
<td>Paclitaxel (nM)</td>
</tr>
<tr>
<td>0.01</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>0.01</td>
<td>1.0</td>
<td>1</td>
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<tr>
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<td>0.01</td>
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<td>0.01</td>
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<td>0.1</td>
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<tr>
<td>0.1</td>
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<tr>
<td>0.1</td>
<td>10.0</td>
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<tr>
<td>0.1</td>
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<td>0.1</td>
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Note: CI<1 indicates synergy between chemotherapeutic agents (highlighted in grey)
4.4 Discussion
This is the first study that identifies the impact of glucose concentration on the effect of metformin on cell viability and mTOR phosphorylation in EC. Metformin potently inhibits EC cell viability and this effect on cell viability is attenuated by high glucose. Phenformin, a more lipophilic biguanide was shown to have more potent effects on EC cell viability, in a dose dependent manner suggesting that biguanides as a class have effects on cellular proliferation. Metformin treatment resulted in a dose- and time-dependent effect on activation of AMPK, and dephosphorylation of downstream targets, S6 and 4EBP1. A synergistic relationship was demonstrated between metformin, carboplatin and paclitaxel in regards of inhibition of cell viability.

The effects of single agent metformin on cell viability were confirmed using two assays; MTS which relies on viable cell mitochondrial activity and SRB, which measures the protein content of viable cells. The findings using both assays were similar, but the SRB assay was chosen as an assessment for viability as it was independent of mitochondrial activity. This is a key distinction, as metformin acts to reduce mitochondrial respiration. The IC50 of metformin and the corresponding effect on cell viability are in keeping with previously published findings (Cantrell et al., 2010). In addition to EC, metformin is recognised to have an inhibitory effect on cell viability on multiple cancer cell lines including breast, colorectal, ovarian and prostate cancer (Zakikhani et al., 2010, Zakikhani et al., 2008, Lengyel et al., 2015).

Phenformin was noted to be more potent, with a significant decrease in viability observed at concentrations 20x lower than metformin reflecting its more lipophilic structure (Goodarzi and Bryer-Ash, 2005). Phenformin exhibits higher affinity and transport activity with marked differences in uptake kinetics compared with metformin (Sogame et al., 2009). Phenformin was initially licensed for glycaemic control but taken off the US market in 1970 because of increased rates of lactic acidosis (0.6 cases per 1,000) that carried mortality rates of up to 40% (Luft et al., 1978). Multiple in vitro and xenograft models have confirmed that phenformin has similar anti-cancer effects to metformin, however is more potent than metformin (Orecchioni et al., 2015, Appleyard et al., 2012). With the existence of other less toxic hypoglycaemics, there is no justification for use of phenformin in the treatment of T2DM. These data, however, suggest that further investigations including safety studies of phenformin as a chemotherapeutic may be warranted. Often, treatments with a less appealing side effect profile are acceptable to patients with advanced or recurrent cancers.
Metformin can act to inhibit carcinogenesis by multiple mechanisms. In this analysis, the mechanistic focus was activation of AMPK and the PI3K/AKT/mTOR pathway, one of the most commonly dysregulated signalling pathways in EC. Treatment with metformin resulted in activation of AMPK, an immediate downstream target. In HEC1A cells, the decreased phosphorylation of S6 and 4EBP1 occurred prior to AMPK activation. These findings suggest that inhibition of the mTOR pathway has occurred and identify metformin as a novel mTOR inhibitor. Metformin, however, may be acting through AMPK-independent pathways, including decreased IR/IGF1R-receptor signalling. The cell culture model was limited, as the endocrine effects of metformin on reducing insulin and glucose by distant actions on hepatocytes and subsequent decreased IGF1R/IR signalling could not be assessed. Animal models using oral metformin are required to assess if metformin exerts its anti-proliferative effects by direct action on tumours or by reducing the available circulating substrate (e.g. insulin, glucose). An obese rat model has shown that oral metformin decreases serum glucose and reduces messenger RNA levels of endometrial pro-proliferative genes c-myc, c-fos, SFRP4 and RALDH2 (Zhang et al., 2013). It is likely, however, that metformin may be exerting its anti-cancer effects through multiple mechanisms.

The effect on the PI3K/AKT/mTOR phosphorylation events was dose and time-dependent. There were no significant effects on phosphorylation of upstream or downstream targets over a 120 minute time course, suggesting action through secondary messengers, rather than direct protein binding and immediate phosphorylation changes. Similar effects on the mTOR pathway have been reported in EC cell lines (Cantrell et al., 2010, Sarfstein et al., 2013). Oral metformin treatment has been demonstrated to reduce mean EC tumour weight in a nude mouse xenograft model with concurrent decrease in S6 phosphorylation (Iglesias et al., 2013).

The addition of metformin to paclitaxel and carboplatin as single agents resulted in significant synergistic inhibition of EC cell viability. Additionally, the combination of metformin, carboplatin and paclitaxel were synergistic. Synergy was quantified using the CI equation of Chou and Talalay which allows for the evaluation of two or more chemotherapeutic agents using different concentrations and effect levels (Chou and Talalay, 1984). This technique allows the analysis of synergy versus antagonism of various drug combinations to identify maximal anti-tumour effect. The synergistic activity of metformin and paclitaxel with regard to inhibition of cell proliferation and induction of
apoptosis has previously been reported (Hanna et al., 2012) and is in keeping with these findings.

The results from studies of metformin in combination with platinum chemotherapy in EC are conflicting. Uehara et al. report additive anti-proliferative effects when Ishikawa EC cells were treated with cisplatin and metformin (>2mM)(Uehara et al., 2015). In contrast, metformin treatment did not cause a reduction in tumour growth in a xenograft model of EC. This model was established from primary endometrioid and endometrioid/serous tumours. Only the endometrioid/serous xenograft showed a significant reduction in tumour growth, with no difference between carboplatin single agent or combined carboplatin and metformin treatment. Metformin treatment alone had no effect on tumour growth, despite use of a clinically relevant concentration of metformin. The endometrioid xenograft was resistant to both metformin and carboplatin treatment as single agents or in combination. The difference in the findings of Schrauwen et al. with those reported by Iglesias et al. may be secondary to increased metformin doses used by the latter (250mg/kg/day versus 1g/kg/day)(Schrauwen et al., 2015, Iglesias et al., 2013). In addition, primary cultures from only two tumours were used to create xenografts used by Schrauwen et al.; these may not sufficiently represent the heterogeneous clinical response of EC tumours. It has been demonstrated that the mutation profile of tumour cells can have influence on the response to metformin; specifically more potent effects on tumour cells harbouring homozygous inactivated mutations of the p53 gene (Buzzai et al., 2007) and cells harbouring KRAS mutations (Iglesias et al., 2013). It is feasible that the primary tumours used expressed metformin-resistant mutations or did not contain OCT-1 transporters, required for active uptake of metformin.

Carboplatin and paclitaxel are commonly used adjuvant therapies for stage III EC. Paclitaxel is a chemotherapeutic agent that stabilises the microtubules leading to mitotic arrest and cell death. Carboplatin is an alkylating agent that modifies DNA structure and synthesis by causing intra and inter-strand cross linkage of DNA molecules within the cell. Use of carboplatin and paclitaxel regimes have reported initial response rates of up to 71% but a median PFS of only 10 months in chemotherapy naïve patients with advanced EC. These regimes are associated with frequent treatment modifications secondary to toxicity (Vandenput et al., 2009). The combination of paclitaxel, doxorubicin and cisplatin has been reported to be superior to cisplatin and doxorubicin with progression-free survival or 8.3 months, versus 5.3 months (Fleming et al., 2004). However, these regimes are associated
with significant side effects include neutropenia, thrombocytopenia, peripheral neuropathy and neutropenic sepsis (Vergote et al., 2015).

This study is the first to test the combination of metformin, paclitaxel and carboplatin, chosen to reflect current standard adjuvant chemotherapy. The addition of metformin potentiated this chemotherapy regime and clinically, may allow decreased doses of carboplatin/paclitaxel, thus improving the side effect profile without significantly impairing efficacy. It is important to highlight that the cytostatic effect of metformin may interfere with the action of carboplatin, which targets rapidly dividing cells. In addition, the doses required for inhibition of cell viability of single agent carboplatin and paclitaxel were increased compared with clinically used doses in patients, reflecting the non-physiological setup of in vitro models.

Metformin has been shown to have synergistic effect with medroxyprogesterone acetate to overcome progestin resistance in EC cell lines (Xie et al., 2011). In addition, combinations of metformin and progesterone have been used for primary treatment of EC (Mitsuhashi et al., 2015) with encouraging results. Aromatase inhibitors have also been shown to have biological activity in EC. Anastrazole treatment in a presurgical window study has been associated with decreased cellular proliferation in EC (Thangavelu et al., 2013). Combinations of letrozole and everolimus in vitro have been reported to inhibit cell viability of Ishikawa EC cells as single agents and in combination (Lu et al., 2014). Replication of this study using the same Ishikawa cell line did not show an inhibitory effect of letrozole on cell viability despite using doses 1000 fold greater than those published (data not shown). Combination studies of metformin and letrozole were thus not performed. A clinical trial of letrozole in combination with everolimus showed a high clinical benefit ratio in comparison with single agent everolimus in patients with recurrent EC (Slomovitz et al., 2015). While no patient discontinued therapy as a result of toxicity, 55% had hyperglycaemia. Metformin may be a less toxic mTOR inhibitor, and the combination of everolimus, letrozole and metformin are currently being investigated in a group of patients with recurrent EC (Clinical Trial NCT01797523). Novel combinations of metformin and progesterone, or metformin and aromatase inhibitors, which act to modifying the obesity-driven toxic effects on the endometrium, may prove to be well tolerated alternative adjuvant therapies for EC.
5. The effects of metformin on endometrial cancer are modulated by hypoxia and hyperglycaemia.

5.1: Introduction
Metformin treatment has been associated with reduced cancer incidence and improved cancer-specific survival in patients with diabetes (Gandini et al., 2014). There is limited information on the benefits of metformin for cancer patients without diabetes. In T2DM, metformin increases insulin sensitivity and reduces serum glucose concentrations through inhibition of hepatic gluconeogenesis and enhanced glucose uptake in myocytes. If the anti-cancer benefits of metformin relate to improved glycaemic control in the context of background hyperglycaemia and hyperinsulinaemia (Pollak, 2012b), these benefits may not translate to non-diabetic patients. It is thus imperative to explore the effect of metformin both in normo- and hyperglycaemia.

Metformin directly affects the energy balance in cells through inhibition of complex I of the mitochondrial electron transport chain, leading to reduced oxidative phosphorylation (El-Mir et al., 2000, Owen et al., 2000). Most studies investigating the mechanisms of action of metformin in vitro have used supraphysiological doses of metformin (1-10mM) (Cantrell et al., 2010, Zakikhani et al., 2010, Sarfstein et al., 2013). It is likely that such doses are required because of the high glucose composition of growth media. Most commonly used growth media contain 25mM of D-glucose, a concentration comparable with extreme hyperglycaemia (normoglycaemia serum glucose range: 3.9-5.9mmol/L). In this chapter, the impact of glucose concentrations mimicking normo- and hyperglycaemia was assessed on cell proliferation and mitochondrial function cell culture models.

We and other authors have observed a growth static effect of short-term metformin given between diagnosis and hysterectomy in non-diabetic women with EEC (Mitsuhashi et al., 2014, Laskov et al., 2014, Schuler et al., 2015). In the presurgical study described in Chapter 2, the mean Ki-67 score by IHC was 17% lower at hysterectomy in the metformin-treated group, but individual patients varied in their response.

Tumour hypoxia arises as a result of morphologically and functionally inappropriate vascularisation, irregular blood flow and high oxygen demand of rapidly proliferating malignant cells. Hypoxic tumours display greater resistance to radio-and chemotherapy (Unruh et al., 2003). Cancer cells alter their metabolism to adapt to decreased oxygen availability in the microenvironment and certain genes are activated in response to hypoxia, including hypoxia-inducible factor-1α (HIF-1α) and its downstream target,
carbonic anhydrase IX (CA-9). Metformin acts as a mitochondrial poison in cancer cells, and reduces oxidative phosphorylation. Hypoxic tumour cells, however, are more likely to rely on glycolytic pathways, and thus be relatively immune to metformin. In this present study, the hypothesis that response to metformin varies according glucose concentration and background tumour hypoxia was tested. To approach this, cell viability and mitochondrial function cell culture models and IHC analysis on paired tumour samples from the presurgical study of short term metformin (Chapter 2) were used.
5.2: Materials and Methods

5.2.1 Flow Cytometric Analysis for detection of mitochondrial mass and function
Fluorescent-activated cell sorting (FACS) was used to assess mitochondrial mass and function. Ishikawa, HEC1A and KLE cells were plated in 6 well plates at fixed cell densities [Ishikawa (200,000 cells/well) HEC1A & KLE (300,000 cells/well)] in DMEM media supplemented with either 1.0g/L (low) or 4.5g/L (high) D-glucose. Low and high glucose concentrations equate to normoglycaemic (5.5mM) and extreme hyperglycaemic (25mM) conditions in patients. The media was changed 24 hours later to media containing metformin 2mM or media alone, and cells were grown for an additional 72 hours.

5.2.1.1 Mitochondrial staining
The cell-permeant MitoTracker® probes (Molecular Probes, Invitrogen) contain a mildly thiol-reactive chloromethyl moiety for labelling mitochondria. MitoTracker probes passively diffuse across the plasma membrane and accumulate in active mitochondria. MitoTracker Deep Red (M22426) is a far red-fluorescent dye (absorbance/emission 644/65nM) that stains mitochondria in live cells and is used for mitochondrial localisation and quantification of mitochondrial mass.

Mitotracker Orange CMTMRos (M7510) is an orange-fluorescent dye (absorbance/emission 544/576nM) that accumulates in live cells dependent upon membrane potential. It provides a quantitative measurement of mitochondrial membrane potential. Increase in mitochondrial membrane potential was used as a surrogate for quantification of mitochondrial function.

5.2.1.2 Preparation of stock solutions
The lyophilized MitoTracker product was dissolved in DMSO to a final concentration of 1mM. The 1mM stock solution was stored in aliquots at -20°C and thawed prior to use.

5.2.1.3 Staining adherent cells and FACS
A staining solution was prepared by diluting the 1mM stock to a working concentration of 50nM in prewarmed PBS with added MgCl₂ and CaCl₂ (PBS++) (D8662, Sigma). Media was aspirated and cells were washed with prewarmed PBS prior to incubating with the staining solution for 20 minutes at 37°C in the dark. The cells were then washed with PBS and trypsinised with 0.25%, and resuspended in PBS++ to neutralise the trypsin. The cell suspension was centrifuged at 1800rpm for three minutes. The supernatant was discarded and the cell pellet resuspended in 300μL of PBS++ on ice prior to FACS.
Flow cytometric analysis was performed on a Fortessa machine (BD Bioscience). Flowjo software was used to control for dead cells, couplets and debris, ensuring that cells analysed were live, singlet cells. All experiments were performed in triplicate and results are representative of mean + SEM of three biological repeats for consistency of response.

5.2.2: Cell viability assays

Ishikawa, HEC1A and KLE cells were cultured in high glucose DMEM as detailed in Chapter 5. For cell viability assays, cells were plated and grown in 96 well plates at the concentrations of 5000 cells/well, 10 000 cells/well and 10 000 cells/well, respectively. Experiments were carried out in DMEM with 1.0g/L D-glucose (termed low glucose) or 4.5g/L D-glucose (termed high glucose). Media was supplemented with 2mM L-glutamine (Sigma, Dorset, UK).

For hypoxia experiments, cells were transferred to a hypoxic incubator immediately following plating and incubated for 24 hours. The cabinet was equilibrated at 37°C in a humidified atmosphere of 5% CO₂ and oxygen restriction (1%). Cells were then treated for a further 24-72 hours in the hypoxia cabinet with metformin-supplemented media. Following treatment, an SRB assay was performed to assess cell viability (described in Chapter 5).

5.2.3 Seahorse XF Analyser

5.2.3.1 Basis for Seahorse Analysis

Cellular metabolism involves substrate uptake (oxygen, glucose, fatty acids) and energy production through series of controlled oxidation and reduction reactions. These reactions are executed through intracellular processes including glycolysis, Krebs cycle, electron transport and oxidative phosphorylation (OXPHOS). Energy production is measured by production of ATP and results in the release of heat and chemical by-products (lactate and CO₂) into the extracellular environment. The Seahorse XF Analysers measure the oxygen consumption rate (OCR) at five to eight minute intervals.

OCR is an indicator of mitochondrial respiration. The analyser allows real-time measurements of OCR by isolating a small volume of media (2μL) above a cell monolayer on a microplate. Cellular oxygen consumption or respiration and proton excretion secondary to glycolysis cause rapid measureable changes to the dissolved oxygen and lactate concentrations in this transient volume of fluid. Solid state sensor probes resting 200microns over the cell monolayer measure the oxygen and pH change every few seconds.
allowing the calculation of the OCR. The instrument has an integrated drug delivery system, allowing the pneumatic injection of compounds into wells at user-defined intervals.

5.2.3.2 Seahorse XF Assay: Growth Conditions
Ishikawa, HEC1A and KLE cells were maintained in high glucose DMEM. Cells were split and seeded into 75cc flasks in low and high glucose DMEM supplemented with L-glutamine. For 24 hour treatments, cells were split 24 hours later and seeded into the wells of an XF cell culture microplate in low or high glucose DMEM alone for untreated cells or with the addition of 2mM metformin. For 72 hour treatments, media in the flasks were replaced 24 hours later with low/high glucose media containing 2mM metformin for a further 48 hours. Cells were then seeded into the XF cell culture plate and treatment continued for a further 24 hours. The number of cells seeded into the XF cell culture microplate was kept constant for the untreated, 24 and 72 hour treatments; Ishikawa 16000 cells/well, HEC1A 10000 cells/well and KLE 15000 cells/well.

5.2.3.3 Seahorse XF Assay: Measurements
Following the drug treatment, the growth media was replaced with mitochondrial stress test bicarbonate-free media and incubated for one hour. The bicarbonate-free media matched the glucose and L-glutamine concentrations of treatment growth media used.

A mitochondrial stress test allows a quantitative assessment of the impact of metformin on mitochondrial respiration. This test was carried out in normoxia and hypoxia (3% O₂) by sequentially adding pharmacological inhibitors of oxidative phosphorylation (Figure 39) (Hill et al., 2012). Baseline OCR (pmol/min) represents the resting energetics of the cell and was measured three times prior to the automated addition of oligomycin, which inhibits ATP synthase, the protein that catalyses mitochondrial ATP production. Some respiration persisted and this reflects the rate of proton leak across the mitochondrial inner membrane. A mitochondrial uncoupler, FCCP was then added allowing measurement of the maximal mitochondrial respiration. Finally, rotenone, a complex I inhibitor and antimycin A, a complex III inhibitor were added to completely obliterate mitochondrial respiration. The oxygen consumption following this is derived from non-mitochondrial sources (non-mitochondrial-derived OCR).

The basal respiration was calculated by subtracting the non-mitochondrial derived OCR from the basal or baseline OCR, prior to oligomycin addition. The proton leak was obtained subtracting the non-mitochondrial OCR from the measured OCR after oligomycin addition. The respiration used to drive ATP production under basal conditions was calculated by
subtracting the proton leak from the basal respiration. The maximal respiration was measured after FCCP addition and by subtracting the non-mitochondrial-derived OCR. The spare respiratory capacity or reserve of the cell was the difference between the rate of basal and maximum respiration.

The metabolic rate of the same cell population can be measured repeatedly as the XF measurements are non-destructive. After the XF assay, the cell biomass was measured with an SRB assay to allow normalisation of the measurements to the viable cell mass. The XF experiments were designed by VS and Dr Ayse Latif (AL). The protocol was optimised and experiments carried out by AL.

![Diagram of OCR profile](image)

Figure 39: Representative OCR profile of the mitochondrial stress test using Ishikawa cells cultured in high glucose media. Following measurement of basal respiration, oligomycin (final concentration 1µM) was injected into each well to determine oxygen consumption resulting from proton leak. The difference between basal OCR and proton leak yields the amount of oxygen consumption linked to ATP production. FCCP (final concentration 0.5µM) injection allows determination of maximal oxygen consumption. The difference between maximal respiration and basal respiration yields an estimate of the spare respiratory capacity of cells. This broadly indicates the ability of the cells to deal with an energetic crisis. The rotenone/antimycin AA (Rot/AA) injection allows the determination on non-mitochondrial derived OCR, representing oxygen consumption by cellular enzymes including NADPH oxidase, cytochrome p450 etc.

5.2.4 Assessment of immunohistochemical markers (HIF-1α, CA-9 and TOMM-20) in endometrial tumour tissue
HIF-1α and CA-9 expression were assessed on tissue microarrays (TMAs). TMAs were created from triplicate cores taken from equivalent areas in the pre- and post-intervention biopsies by the study histopathologist (RM), who was blinded to treatment arm. The Pipelle endometrial baseline sample was a scrape of the tumour surface while the final hysterectomy specimen provided a full thickness tumour sample that included endometrial/myometrial junction and myometrial tissue. To reduce the bias inherent to comparing tumour from two different sampling methods, the TMAs were constructed from the luminal (surface) aspect of the tumour in the surgical specimen. Selection was not based on vascular “hot spots”, and instead provided tumour representative of final grade in the paired sample. Areas of tumour necrosis were avoided.

Automated immunohistochemical staining was performed on four-µm thick sections using the Leica Bond Max (Leica Biosystems, Wetzlar, Germany) with heat-induced epitope retrieval as detailed in Chapter 3. Primary antibodies used were HIF-1α (BD 610959) mouse monoclonal (BD Biosciences) 1:50 dilution and CA-9 (NB100-147) rabbit polyclonal (Novus Biological) 1:2000 dilution. Negative (isotype control) controls were used for quality assurance.

Translocase of the outer mitochondrial membrane 20 (TOMM20) is responsible for the recognition and translocation of mitochondrial pre-proteins synthesized in the cytosol. Expression of this protein has been shown to correlate with mitochondrial mass and function (Whitaker-Menezes et al., 2011).

TOMM20 (#sc-17764 Santa Cruz Biotech) staining was performed on full tissue sections in patients where sufficient pre- and post-intervention tissue was available (n=33). Tissue sections were prepared as detailed in Chapter 3. TOMM20 IHC staining had previously been optimised on the Leica Bond Max using breast cancer tissue. Re-optimisation using the same protocol was performed using endometrial cancer tissue. Of note, antigen retrieval and staining was reduced in slides that were pre-cut and stored at 4 degrees for one month. Sections were cut and stained immediately to maintain consistency of staining. Slides underwent heat-induced epitope retrieval at pH 6 in antigen retrieval solution for 20 mins at 98°C. Sections were then incubated with the primary antibody (1:250) for 30 minutes.

Full slides (TMAs and tissue sections) were digitised using the Leica SCN400 Slide Scanner. HIF-1α nuclear staining and CA-9 cytoplasmic and membrane staining were assessed using
a modified H-score, calculated as the product of the area score (proportion of positively
stained core, scored 0-6) and the intensity of staining score (0=none, 1=mild, 2=moderate,
3=strong). TOMM20 cytoplasmic staining was assessed using the same scoring system.

All scoring was performed by two independent scorers (VS, SK) who were blinded to
treatment group. The inter-observer intraclass correlation coefficient (ICC) was 0.98 (95% CI 0.95-0.99), 0.92 (95% CI 0.77-0.96) and 0.91 (95% CI 0.67-0.96) for HIF-1α, CA-9 and
TOMM20, respectively. Any discrepancies were reviewed together and resolved by
consensus agreement.

5.2.5 Statistical Analysis
All statistical analyses were performed using GraphPad Prism. Cell culture experiments
were presented as mean ± SD/SEM of at least three technical repeats and experiments
were repeated three times, unless otherwise stated. Parametric data were analysed using
unpaired Student’s t-tests , 1- and 2- way analysis of variance (ANOVA). The treatment
effect on Ki-67 and IHC markers was tested using an analysis of covariance linear model as
detailed in Chapter 2. Statistical significance was accepted at p<0.05.
5.3: Results

5.3.1 Modulating effects of glucose concentration on the effect of metformin

5.3.1.1: Mitochondrial biogenesis is increased in low glucose environments
Endometrial cancer cells were grown for 96 hours in media supplemented with low or high D-glucose to assess the effects on varying glucose concentrations on mitochondrial activity. Ishikawa and HEC1A cells grown in low glucose had increased mitochondrial mass, while KLE cells had lower mitochondrial mass, compared with cells grown in high glucose (all p<0.0001, unpaired Student’s t-test)(Figure 40A).

All three cell lines grown in low glucose had overall increased mitochondrial function compared to high glucose with a statistically significant increase in Ishikawa and HEC1A cells (p<0.0001, unpaired Student’s t-test) (Figure 40B). As the net mitochondrial function would be expected to increase with increased mitochondrial mass, the ratio between net mitochondrial function and mass was calculated to assess mitochondrial function per mitochondria (normalised mitochondrial function). The data suggests that there was no difference in normalised mitochondrial function in Ishikawa and HEC1A cells depending on surrounding glucose concentration. There was, however, an increase in mitochondrial function in KLE cells grown in low glucose media (p<0.0001, unpaired Student’s t-test) (Figure 40C).
Figure 40: A. EC cells grown in low glucose had increased mitochondrial mass compared with cells grown in high glucose for 96 hours. B. Overall mitochondrial function of cells was increased in low glucose, compared with high glucose, however, C. when function was normalised to mitochondrial mass, the increase was only significant in KLE cells. The bars represent the fold-change compared with high glucose. Results are representative of three biological repeats ± SD. **** indicates p<0.0001 using an unpaired Student’s t-test.
5.3.1.2 Metformin treatment affects mitochondrial biogenesis and function.
Endometrial cancer cells treated with 2mM metformin for 72 hours have increased mitochondrial mass compared with untreated controls (all p<0.0001, unpaired Student’s t-test). This increase was observed, irrespective of the surrounding glucose concentration (Figure 41A). The treatment dose, 2mM, was the IC50 required for reduced cell viability over the same treatment duration. Metformin treatment also resulted in decreased normalised mitochondrial function in all three cell lines (p<0.0001, unpaired Student’s t-test) (after adjusting for mitochondrial mass) (Figure 41B).

Surrounding high glucose attenuated the effects of metformin on the mitochondria. The trends observed were similar in both glucose concentrations, however, the increase in mitochondrial mass was greater in Ishikawa cells following metformin treatment in low glucose media (p<0.0001, unpaired Student’s t-test) (Figure 41A). The decrease in normalised mitochondrial function following metformin treatment was also more pronounced in low glucose in Ishikawa (p<0.0001) and HEC1A (p<0.01, Student’s t-test) cells, compared with high glucose (Figure 41B).

Figure 41: EC cells were grown and treated in high (4.5g/L) and low (1.0g/L) glucose media with metformin 2mM or untreated for 72 hours. The bars represent fold-change compared with untreated cells in high and low glucose. In Ishikawa and HEC1A cells, mitochondrial mass was increased in both high and low glucose (A), while mitochondrial function was decreased (B). The increase in mitochondrial mass was greater in Ishikawa cells, and decrease in function more pronounced in both Ishikawa and HEC1A cells in low glucose, compared with high glucose media. Results are representative of three biological repeats ±SD. * indicates p<0.05, ** indicates p<0.01 and **** indicates p<0.0001, using an unpaired Student’s t-test.
5.3.1.3 The effect of metformin on the mitochondria is time-dependent.

Endometrial cancer cells were treated with metformin 2mM or untreated for 24 or 72 hours in low glucose DMEM. Twenty four hours of metformin treatment resulted in increased mitochondrial mass in Ishikawa (p<0.001) and HEC1A cells (p<0.0001, unpaired Student’s t-test) (Figure 42A). The fold-increase in mass compared with untreated cells was greater after 72 hours of treatment in Ishikawa cells (p<0.0001, unpaired Student’s t-test) (Figure 44C). There was no added increase in mitochondrial mass in HEC1A cells at 24 hours, compared with 72 hours of treatment (Figure 42A & C).

Metformin treatment also resulted in a decrease in normalised mitochondrial function in all cell types at both 24 and 72 hours. The decrease in function was greater following 72 hours of treatment, compared with 24 hours in Ishikawa and HEC1A cells, suggesting a cumulative time-dependent effect (both p<0.01, unpaired Student’s test) (Figure 42 B &D).

![Graphs showing mitochondrial mass and function](image)

**Figure 42:** EC cells were grown and treated in low glucose media with metformin 2mM or untreated for 24 (A&B) and 72 (C&D) hours. Ishikawa and HEC1A cells treated with metformin had increased mitochondrial biogenesis, as demonstrated by increased mitochondrial mass at 24 (A) and 72 (C) hours. Mitochondrial function (normalised to mitochondrial mass) was decreased after metformin treatment in all three cell lines at 24 (B) and 72 (D) hours. Results are the average of three biological repeats ±SD. *** indicates p<0.001 and **** indicates p<0.0001.
5.3.2 The effects of hypoxia and glucose concentrations on the mitochondrial function of endometrial cancer cells

5.3.2.1 Mitochondrial respiration is suppressed in a high glucose, hypoxic environment in endometrial cancer cells.

**Figure 43:** Mitochondrial stress test on Ishikawa and HEC1A cells grown and treated in hypoxia and normoxia in high (HG) and low glucose (LG). C. Mitochondrial respiration was significantly decreased in Ishikawa cells grown in hypoxia and high glucose. Data represent the mean ± SEM of three biological replicates in normoxia and two in hypoxia experiments. *denotes p<0.05, using a 2-way ANOVA test.

Ishikawa and HEC1A cells were grown and treated in hypoxia and normoxia in high (HG) and low glucose (LG) for 24 hours prior to a Seahorse mitochondrial stress test. KLE cells were excluded from this analysis, as they behaved dissimilarly from the other two cell types and are more similar to type 2 or high grade EC. In normoxia, there was no difference in OCR at basal or maximal respirations in high or low glucose (Figure 43A & B). In hypoxia however, Ishikawa cells grown in high glucose had decreased basal and maximal respiration (2-way ANOVA p<0.05) (Figure 43C).
5.3.2.2 Mitochondrial respiration is suppressed in hypoxia, irrespective of surrounding glucose concentration.

Ishikawa and HEC1A cells were grown and treated in hypoxia (3% O₂) and normoxia, in both high(HG) and low glucose (LG). The effect of hypoxia on mitochondrial respiration was then tested using a mitochondrial stress test. In Ishikawa cells, the maximal respiration was significantly decreased in hypoxia, both in high and low glucose environments (2-way ANOVA, p<0.05) (Figure 44 A&B). This decrease was more pronounced in high glucose. Basal respiration was also suppressed in hypoxia in Ishikawa cells grown in high glucose. Basal and maximal respiration was suppressed in hypoxia in HEC1A cells grown in low and high glucose (2-way ANOVA, p<0.05) (Figure 44 C&D). A decrease in mitochondrial respiration suggests a switch to glycolytic pathways, triggered both by hypoxia and high substrate (glucose) availability.

Figure 44: Mitochondrial stress test on Ishikawa and HEC1A cells grown and treated in low (LG) and high glucose (HG) in hypoxia and normoxia. Mitochondrial respiration was suppressed in all cells grown in hypoxia. The greatest suppression was seen in Ishikawa cells in high glucose. Data represent the mean ± SEM of three biological replicates in normoxia and two in hypoxia experiments. *denotes p<0.05, using a 2-way ANOVA test.
5.3.2.3 Metformin treatment suppresses mitochondrial respiration in low and high glucose

Consistent with previous mitochondrial function experiments, metformin treatment over 24 hours significantly suppressed basal oxidative phosphorylation in both high and low glucose in normoxia and hypoxia (2-way ANOVA, all p<0.001). Metformin treatment also suppressed the maximal response to FCCP, representing maximal respiration in both high and low glucose. The effect of metformin on maximal respiration was greater in high glucose compared with low glucose in Ishikawa cells grown in normoxia. (72.1% ± 1.4 vs 61.5% ± 3.6, p=0.017, Student’s t-test) (Figure 45A). This was assessed using the percentage decrease in maximal respiration, compared with untreated cells. In hypoxia, both Ishikawa and HEC1A cells had increased maximal respiration in low glucose, compared with high glucose. Metformin treatment suppressed maximal respiration in both glucose environments in hypoxia (Figure 45 C&D). In Ishikawa cells, however, the treatment effect on maximal respiration was greater in low glucose (71.9% ± 4.7 vs 55.3% ± 3.3, p=0.019, Student’s t-test) (Figure 45 D). These findings suggest that in hypoxia, a high glucose
environment drives glycolytic processes and protects against drugs that target oxidative phosphorylation.

### 5.3.2.4 Metformin treatment results in decreased ATP production

![Graphs showing ATP production](image)

Figure 46: Ishikawa and HEC1A cells were treated with 2mM of metformin for 24 hours in high and low glucose, in hypoxia (3% O₂) and normoxia. ATP-linked OCR was lower in hypoxia (C&D) compared with normoxia (A&B). Cells treated with metformin in normoxia had significantly reduced ATP-linked OCR (A&B) in both high and low glucose. In hypoxia, there was no ATP-linked OCR in high glucose (C&D). Abbreviations LG low glucose, HG high glucose. Data represent the mean ± SEM of three biological replicates in normoxia and two in hypoxia experiments. *denotes p<0.05, using a 2-way ANOVA test.

The oligomycin injection blocks ATP synthase, allowing calculation of ATP-linked oxygen consumption. ATP-linked OCR was significantly reduced in both low and high glucose in Ishikawa and HEC1A cells treated with metformin (all p<0.001, Student’s t-test) (Figure 46 A & B). As expected, ATP-linked OCR was reduced in hypoxia, compared with normoxia, particularly in Ishikawa cells grown in high glucose. Metformin treatment in hypoxia further suppressed ATP production, with no ATP-linked OCR noted in high glucose either Ishikawa or HEC1A cells (all p<0.05, Student’s t-test) (Figure 46 C&D).
5.3.3 The effects of metformin on mitochondrial mass in vivo

5.3.3.1 Endometrial epithelial cancer cells show increased mitochondrial mass

TOMM20 is an established IHC marker of mitochondrial mass and biogenesis (Whitaker-Menezes et al., 2011), known to be overexpressed by epithelial cancer cells. We determined the distribution of mitochondrial mass, by staining endometrial cancer sections using an antibody directed against TOMM20.

Figure 47: TOMM20 selectively stains epithelial glandular hyperplasia and cancer cells and is largely excluded from the tumour stromal compartment. Staining is noted in all grades of endometrioid endometrial adenocarcinoma (EEC). Images are taken from representative tumours at 20x magnification.

Figure 47 shows that TOMM20 selectively stains epithelial endometrial cancer cells and is largely excluded from the adjacent tumour stromal compartment in both complex atypical hyperplasia and EEC. Thus, it appears that epithelial endometrial cancer cells harbour mitochondria and show a relative increase in mitochondrial mass, compared with stromal cells. Baseline TOMM20 expression may correlate positively with tumour grade; a
Spearman’s correlation approached statistical significance \([r=0.33, (95\% \text{ CI} -0.2, 0.63)\]
\[p=0.06\] as demonstrated in Figure 48.

![Figure 48: Correlation of baseline TOMM20 expression with final tumour grade \(n=32\). Spearman \(r= 0.33 (95\% \text{ CI} -0.2, 0.63)\), \(p=0.06\). G1=grade 1; G2=grade 2; G3=grade 3.](image)

5.3.3.2 Metformin treatment results in an increased mitochondrial mass in vivo.
Cytoplasmic TOMM20 expression was assessed using a modified H-score by two independent observers (VS & SK), who were blinded to treatment group and time-point. The scoring was comparable (interobserver ICC 0.91). The mean baseline scores of metformin treated \(n=25\) and controls \(n=8\) were similar, 11.6 ± SD 2.8 and 11.0 ±3, respectively.

Following metformin treatment, TOMM20 expression was increased by 2.1 (95\% CI 0.2-4.0, \(p=0.033\)) at hysterectomy compared with recruitment, after adjusting for baseline expression, age, BMI, tumour grade and change in controls. Short-term metformin treatment is associated with an increase in mitochondrial mass in women with EC, consistent with findings in vitro, using EC cell lines (6.3.1.2). Figure 51 demonstrates endometrial tumour stained for TOMM20 at recruitment and hysterectomy for metformin-treated and control groups.

Extrapolating from the in vitro experiments, we can postulate that metformin treatment results in decreased mitochondrial function, despite increased mitogenesis in vivo. Further experiments using tissue collected directly into a coating buffer are required to confirm this by measuring COX (cytochrome C oxidase) staining. This detects the activity of complex IV,
reflecting the capacity of cells to undergo mitochondrial electron transport and oxidative phosphorylation (OXPHOS) (Sotgia et al., 2012).

Figure 49: Endometrial tumour stained for TOMM-20, before and after metformin treatment and in the control group (at diagnosis and hysterectomy). Images were taken at 20x magnification.
5.3.4 Endometrial tumour hypoxia and the effect on metformin treatment

5.3.4.1 The cytostatic effects of metformin are reduced in high glucose and hypoxia
The effects of metformin on cell viability were assessed in Ishikawa and HEC1A endometrial cancer cell lines in hypoxia (1% O\textsubscript{2}) and normoxia. The experiments were conducted using high and low glucose concentrations and varying doses of metformin (0-10mM) for 72 hours. Metformin treatment resulted in dose-dependent growth inhibition in both cell lines in low glucose. The cytostatic effects of metformin were reduced in both cell lines in high glucose.

Ishikawa cells were less sensitive to the effects of metformin (p=0.05-0.001, 2-way ANOVA) in hypoxia in both glucose conditions. The attenuating effects of hypoxia were more pronounced in low glucose (Figure 50A). In HEC1A cells, the effects of hypoxia were only noted in cells treated in low glucose at metformin concentrations of 1 and 5mM (p<0.0001, 2-way ANOVA) (Figure 50B).

Figure 50: Hypoxia and high glucose reduce sensitivity of EC cells to metformin. A. Ishikawa cells grown in low and high glucose were more sensitive to the cytostatic effects of metformin in normoxia (72 hours treatment). B. HEC1A cells grown in low glucose were more sensitive to the effects of metformin in normoxia. The bars represent the percentage cell viability compared with control. Results are representative of three biological repeats ± SEM. **** indicates p<0.0001 using a 2-way ANOVA.
Previous experiments have shown that 24 hours of metformin was not sufficient to produce significant cytostatic effects even in low glucose (10mM) (Chapter 4). The cell viability experiment was repeated using very low glucose concentrations (0.5-10mM) to further investigate the impact of surrounding glucose. A dose response effect was seen in normoxia using very low glucose concentrations of 0.5 and 1mM D-glucose following 24 hours of treatment. This cytostatic effect was reduced when cells were treated in hypoxia, further emphasising that hypoxia and high glucose decrease the cytostatic effects of metformin (Figure 51 A&B).

Figure 51: Hypoxia and high glucose reduce sensitivity of endometrial cancer cells to metformin. Ishikawa cells grown in very low glucose (0.5 and 1mM) were sensitive to metformin following 24 hours of treatment. This effect was significantly reduced in hypoxia at higher concentrations of metformin (A & B). Metformin did not have significant cytostatic effects at higher glucose concentrations following 24 hours of treatment. There were no differences seen in cells treated in hypoxia or normoxia. The bars represent the percentage cell viability compared with control. Results are representative of three biological repeats ± SEM. * indicates p<0.05, **** indicates p<0.0001 using a 2-way ANOVA.
5.3.4.2 Endometrial tumour hypoxia assessed by immunohistochemistry was positively correlated to tumour grade
The baseline hypoxia of endometrial tumours from the presurgical study was assessed by IHC staining for HIF-1α and CA-9 expression. Baseline HIF-1α (r=0.423, p=0.07) and CA-9 (r=0.461, p=0.03) were positively correlated to tumour grade, using Spearman’s correlation analysis (Figure 52). HIF-1α and CA-9 expression were also positively correlated at baseline (r=0.451, p=0.004).

Figure 52: Positive correlation between tumour grade with A) HIF-1α and B) CA-9 positive expression, n=40. Abbreviation AEH: Atypical endometrial hyperplasia.

5.3.4.3 HIF-1α and CA-9 were not affected by metformin treatment or the operative technique used.
Metformin treatment did not increase expression of either HIF-1α (mean adjusted difference, -0.9 [95% CI -2.4, 0.56], p=0.21) or CA-9 (0.8 [95% CI -0.85, 2.46], p=0.33) (Table 31). This was tested using an ANCOVA, adjusting for baseline expression, age, BMI, grade and treatment group. However, both HIF-1 and CA-9 expression were lower in the surgical specimen compared with the pre-intervention biopsy, irrespective of treatment group. This was converse to initial expectation, as the surgical specimen would have been subject to a period of warm and cold ischaemia. It is likely that the period of surgical ischaemia was insufficient to trigger changes in CA-9. In cell culture models, eight hours of severe hypoxia (0.0% O₂) was required to obtain measurable mRNA levels of CA-9 (van den Beucken et al., 2009). HIF-1α, on the other hand, has a short half-life (~ 5 minutes) (Salceda and Caro, 1997) and exposure to normoxic room conditions prior to fixation of the hysterectomy specimen may have led to degradation of this protein.
Table 31: Expression of HIF-1α and CA-9 by treatment group.

<table>
<thead>
<tr>
<th></th>
<th>Metformin</th>
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<tbody>
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<td>Pre</td>
<td>Post</td>
<td>Pre</td>
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<tr>
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<td>6.8, 9.0</td>
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<tr>
<td>CA-9</td>
<td></td>
<td></td>
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<td>Mean, SD</td>
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<td>2.5</td>
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* p value calculated for treatment effect between groups with analysis of covariance adjusting for baseline protein expression, age, BMI, tumour grade and treatment group.

5.3.4.4: The modulating effect of baseline tumour hypoxia on Ki-67 response

Tumour hypoxia may modulate the effect of metformin on cellular proliferation. The response to short-term presurgical metformin was quantified using the change in the post-intervention Ki-67 score as detailed in Chapter 3. Tumour hypoxia was assessed using the pre-intervention biopsy taken prior to devascularisation and fixed in formalin immediately. The hypoxic changes noted are thus likely to relate to true tumour hypoxia, rather than tissue ischaemia from surgery.

The decrease in Ki-67 score (“response to metformin”) was lower in tumours with higher baseline HIF-1α (n=28), using an analysis of co-variance (mean adjusted difference 2.9% [95% CI 0.7, 5.1%], p=0.01) (Figure 53A). Covariates included baseline Ki-67, age, BMI and tumour grade. In addition, Ki-67 response to metformin was greater in tumours with lower baseline CA-9 (mean adjusted difference 10.9%, [95% CI -2.0, 23.7%], p=0.093) when using median CA-9 (modified H-score 3.01) to discriminate between high and low expression (Figure 53C). This association approached statistical significance. This association was not observed when using continuous CA-9 expression as a covariate (Figure 53B). Finally, lower grade tumours had a greater response to metformin. This effect was noted, independent of hypoxia.
Figure 53 A. Correlation between absolute KI-67 score change (defined as post-intervention KI-67–pre-intervention KI-67 score) and baseline HIF-1α. B Correlation between baseline CA-9 and the absolute KI-67 score change. C Correlation between baseline CA-9 (as a binary variable) and absolute KI-67 score change.
5.4 Discussion
Metformin has shown significant promise as an anti-cancer therapy in pre-clinical studies (Cantrell et al., 2010, Zakikhani et al., 2010, Sarfstein et al., 2013) and single arm clinical trials (Niraula et al., 2012a, Hadad et al., 2011, Mitsuhashi et al., 2014). A placebo-controlled randomised study of pre-clinical metformin in breast cancer, however, only reported a treatment effect in patients with insulin resistance and raised BMI (Bonanni et al., 2012). There are legitimate concerns raised about the translation of pre-clinical findings to routine practice and the potential benefit of metformin in patients without diabetes.

In this study, glucose concentration and hypoxia were demonstrated to modulate the effect of metformin. Metformin treatment was shown to increase mitochondrial biogenesis, but decrease mitochondrial function with the effects being more pronounced in low glucose using FACS and Seahorse metabolic assays. These findings were translated to clinical samples; tumours from patients on metformin treatment had a significant increase in TOMM20 staining, an IHC marker of mitochondrial mass. Additionally, tumours which were more hypoxic at baseline had a decreased response by metformin, as measured by a change in Ki-67 score.

Cell culture studies are imperfect models of human physiology because of high glucose growth conditions. Cells grown in low glucose have increased mitochondrial mass, compared with those in high glucose. Metformin treatment in low glucose resulted in more pronounced mitochondrial biogenesis and reduced function compared with high glucose. This suggests that high glucose or hyperglycaemia encourages cells to harness glycolytic pathways, thus protecting against metformin, a drug that targets oxidative phosphorylation. This mechanism may also rationalise why such high metformin doses are required to affect viability in cell culture models.

The mechanism of metformin as an anti-cancer agent is not fully understood, and a number of pathways have been explored (Pollak, 2012b). Metformin is recognised to act as a mitochondrial complex I inhibitor (El-Mir et al., 2000). The flow cytometry experiments using cultured cells demonstrate that metformin treatment leads to increased mitochondrial biogenesis (by measuring mitochondrial mass), but reduced mitochondrial function. An increase in mitochondrial mass was demonstrated in repeated tumour samples from patients in the metformin group of the presurgical window study. While it was not possible to directly measure mitochondrial function in the tumour samples available, it can be suggested that the increased mitochondrial mass following metformin treatment in
patients may be accompanied with reduced function, as seen *in vitro*. This mitochondrial dysfunction could contribute to inhibition of pro-proliferative pathways and decrease in cellular proliferation observed. Further studies using specifically collected tissues biopsies are required to accurately demonstrate the effects of metformin on tumour mitochondrial function, *in vivo*.

Mitochondrial function was additionally assessed in EC cell lines using the Seahorse XF metabolic assay.Mitochondrial respiration overall was suppressed in hypoxia, and this effect was more pronounced in high glucose, suggesting a switch in EC cell metabolism to glycolytic pathways. Metformin treatment suppressed mitochondrial respiration, both at the baseline and at extreme mitochondrial stress (maximal respiration). In these experiments, high glucose and hypoxia attenuated the effects of metformin.

These pre-clinical findings suggest that metformin treatment is less likely to benefit patients with hyperglycaemia and hypoxic tumours. In the setting of hyperglycaemia, cancer cells undergo a compensatory increase in glycolysis. Metformin treatment has been demonstrated to result in greater reduction in tumour weight in normoglycaemic mice, compared with hyperglycaemic mice (Litchfield et al., 2015). Higher doses of metformin or additional hypoglycaemic therapy may be required to improve glycaemic control, and subsequent effects on cancer cell proliferation. Alternatively, the addition of a glycolytic inhibitor, 2-deoxyglucose may improve metformin response. Pre-clinical studies have already shown that metformin and 2-deoxyglucose have improved cytotoxic effects (Cheong et al., 2011, Ben Sahra et al., 2010).

Further exploratory analyses on the impact of tumour hypoxia and surrounding hyperglycaemia were performed based on the pre-clinical findings. Tumour hypoxia and necrosis are recognised as poor prognostic indicators in EC (Bredholt et al., 2015). In our study, baseline HIF-1α (r=0.423, p=0.07) and CA-9 (r=0.461, p=0.03) were positively correlated to tumour grade (Figure 14), a finding that corresponds with other publications in EC (Mhawech-Fauceglia et al., 2012, Seeber et al., 2010). In contrast, two studies using archival hysterectomy specimens have reported that CA-9 expression in endometrial cancer is not related to tumour grade or stage (Pijnenborg et al., 2007, Sadlecki et al., 2014).

Ki-67 response to metformin was significantly lower in tumours with higher baseline HIF-1α, suggesting a resistance to metformin in hypoxia. While HIF-1α is an unstable protein, the baseline expression was measured on the diagnostic biopsy, which would have been
taken and immediately fixed in formalin with less risk of degradation. This finding corresponds with cell culture models, where hypoxia attenuates the cytostatic effects of metformin. Hypoxia markers have been suggested as prognostic biomarkers by a variety of studies for tumour “aggressiveness” and recurrence (Pansare et al., 2007, Pijnenborg et al., 2007). In addition, tumour hypoxia is recognised to contribute to resistance to radiation and chemotherapy (Gray et al., 1953).

These data suggest that metformin is likely to be of greater benefit to a subset of patients, with normoglycaemia and low grade, normoxic tumours. This is in contrast with findings from Bonanni et al. who reported significant Ki-67 score decrease following metformin treatment in patients who are insulin resistant (HOMA-IR>2.8) (Bonanni et al., 2012) The Italian patients included in this study, however, were much less insulin resistant (mean HOMA-IR 2.3 vs mean HOMA-IR 4.2 in this study in EC). In this study, while there was a trend towards lower insulin resistance in metformin “responders”, there was no significant association between baseline insulin resistance and response to metformin (as measured by change in Ki-67 score) (Section 2.5.3). This may reflect the high prevalence in insulin resistance (60% of all participants) in the EC population. Alternatively, it may be that the in vitro findings from cell culture models cannot be directly extrapolated to human disease and more extensive studies using both animal models and clinical trials are required to identify patients and tumours likely to respond to metformin treatment.

Hyperglycaemia and tumour hypoxia are likely to contribute to metformin-resistance. Our data again highlights the heterogeneous response to metformin, and a need to identify means of individualising therapy. As we move to an era of personalised medicine, giving patients presurgical neoadjuvant treatment, and planning long-term therapy based on initial response can help identify those likely to derive long-term survival advantage.
6. Concluding Remarks and Future Directions

EC is now the most common gynaecological malignancy in developed countries (Murali et al., 2014). Despite the considerable rise in incidence over the last thirty years, there have been relatively few major breakthroughs in the prevention and treatment of this disease. One approach in increasing the efficiency of new cancer drug development is the repurposing of drugs approved for non-cancer indications and building on the available data to develop the cancer therapeutic potential of the drug (Chong and Sullivan, 2007). Metformin has been a forerunner as a potential anti-cancer drug since 2005, and subject to multiple pre-clinical and clinical studies in several different cancer types.

In this study, the role of metformin in obesity-driven EC was investigated in a presurgical window study in patients and by using *in vitro* cell culture models. A non-randomised trial of short-term metformin versus no drug demonstrated a significant inhibitory effect on cellular proliferation as measured by Ki-67 score. Despite significant changes in Ki-67 score, metformin did not have a treatment effect on serum biomarkers of insulin resistance and adiposity, possibly reflecting the short duration of treatment. This further suggests that the changes in cellular proliferation are secondary to direct actions on AMPK activation, rather than a systemic decrease in hyperinsulinaemia and hyperglycaemia driving IR/IGF1R signalling. A robust staining and scoring protocol for Ki-67 score was established both as a tissue endpoint and a prognostic marker. Hot spot scoring was found to be closely associated with tumour grade and of significant clinical prognostic value.

Both metformin and phenformin were shown to have dose- and time-dependent effects in EC cell viability using an *in vitro* cell culture model. These effects were attenuated by high glucose and hypoxia. In the early stages on this study, one of the aims was to determine whether metformin acted directly on AMPK to inhibit mTOR, or indirectly by a net reduction in insulin and glucose and subsequent decreased activation of the IR/IGF1R tyrosine kinase signalling pathway. It was not possible to assess the indirect effects of metformin, using the planned cell culture models. The interpretation of phosphorylation markers on pre- and post-intervention endometrial tumour tissue was also adversely affected by inclusion of biopsies obtained from the devascularised hysterectomy specimens. Ki-67 is a stable protein, however it is likely that significant effects on mTOR phosphorylation events are masked by hypoxic-ischaemic change. Future studies should consider taking a random biopsy at hysterectomy prior to surgical treatment to allow
preservation of unstable phosphorylation events. A post-intervention endometrial sample obtained using a plastic endometrial sampler would strengthen the interpretation of findings, allowing “like with like” comparison of sequential biopsies.

It has been shown that metformin accumulates in tumour tissue using mass spectrometry (Mitsuhashi et al., 2014). Advanced imaging techniques including Raman microspectroscopy could be used to identify the mode and site of action on metformin on cancer cells. A recent publication by Hart et al. reports the identification of novel binding sites whose expression is altered by metformin through protein-protein interactions (Hart et al., 2016). It is feasible that an improved understanding of the mechanism of action of metformin will be possible using advanced structural pharmacology models.

Presurgical window studies are a useful model for testing the biological effects of a licensed drug for a new purpose directly in patients in line with routine clinical care. Like breast cancer, repeat sampling in EC is both possible in the outpatient setting and acceptable to most patients. This allows drug-induced changes in tissue samples to be correlated with effects on serum markers pre- and post-intervention during an uncontaminated neoadjuvant period. These studies rely on surrogate tissue end-points to assess treatment effects. To date, three presurgical studies have reported significant decreases in Ki-67 score associated with short term metformin (Mitsuhashi et al., 2014, Laskov et al., 2014, Schuler et al., 2015). It is important to include a control arm and appropriately collected blood and tumour samples, as discussed previously, to account for the inherent variability in biomarkers over time.

While window studies have the benefit of direct testing in patients, the bypassing of the traditional drug paradigm is not without its drawbacks. Translation of findings from cell line models to clinical studies can pose several challenges. Tumours are heterogeneous, while cell lines represent a homogenous population. The experimental environment used in vitro is not physiological and it is challenging to replicate the complex tumour microenvironment. Testing drugs in patients is expensive and time-consuming. Most presurgical studies were small and have not included a dose-escalation protocol. Preliminary data from this work suggests that metformin has a BMI- and dose-dependent effect of cellular proliferation in patients. This is contrary to findings in diabetes, where increasing doses of metformin over 2g daily does not improve glycaemic control, and in instead associated with undesirable side effects (FDA, 2002). There may be merit in either designing a clinical trial with dose escalations and titration to BMI or planning animal
studies to identify the optimum metformin dose for an anti-cancer effect with an acceptable side effect profile. Two other modifying factors of response to metformin were identified, surrounding hyperglycaemia and hypoxia. In this study, hypoxic tumours were resistant to metformin, possibly reflecting their switch to glycolytic metabolism.

Presurgical studies rely on surrogate end-points (change in Ki-67 score) to determine response to a drug. There is limited data available to help interpret the clinical significance of the change in Ki-67 score in terms of long-term prognosis. Assessment of Ki-67 as a prognostic marker confirmed prognostic impact in a univariate analysis in keeping with a number of other publications (Salvesen et al., 1998, Stefansson et al., 2004, Liu et al., 2014). A 1% decrease in Ki-67 expression is associated with a 2% improvement in recurrence free survival (p=0.046). This puts the adjusted mean difference of 17% in Ki-67 score following short-term metformin into clinical context. The role of Ki-67 as a predictive biomarker is yet to be determined; however, in breast cancer an initial drop of even 2% following short-term treatment with neoadjuvant chemotherapy is predictive of tumour responsiveness to that drug and long term survival advantage (DeCensi et al., 2011).

feMME, a phase II randomised clinical trial is assessing the benefit of metformin in combination with a levonorgestrel-releasing intrauterine system in non-surgical patients with AEH and EC. The study design includes repeated endometrial sampling (Hawkes et al., 2014) which may provide information to support the role of Ki-67 as a predictive biomarker.

Despite the initial focus on the PI3K/AKT/mTOR pathway, it became clear that metformin is a drug that alters metabolism through actions on the mitochondrial electron transport chain and oxidative phosphorylation. Cancer cells alter their metabolism to support the increased energy requirements due to continuous growth and rapid proliferation. OXPHOS upregulation is a common feature of human cancer, identifying cancer mitochondria as a potential therapeutic target. The reverse Warburg hypothesis suggests that epithelial cancer cells induce oxidative stress in adjacent stromal fibroblast, leading to catabolic activity. This in turn provides catabolised nutrients to fuel the growth of cancer cells by enhancing mitochondrial activity and energy generation via OXPHOS (Martinez-Outschoorn et al., 2010). By acting on OXPHOS, metformin treatment may lead to interruption of metabolic coupling between the cancer-stromal compartment.

It has been shown that cancer cells that are more sensitive to low glucose are deficient in OXPHOS and more sensitive to biguanides. The low glucose condition is advantageous for
cells displaying intact mitochondrial capacities, as they rely on mitochondrial respiration. This does mean that cells cultured in absent or low glucose display increase mitochondrial metabolism and are much more sensitive to the effects of metformin (Birsoy et al., 2014). In high glucose and hypoxia, it is possible that EC cells utilise glycolytic metabolism, even though this is an inefficient means of energy production, resulting in metformin resistance.

While it is recognised that metformin is a mitochondrial poison, this was the first study to document increased mitochondrial biogenesis in EC tumours following short-term metformin treatment. Many early repurposing studies have focused on metformin’s effect as a novel mTOR inhibitor. It is likely that the inhibitory effect on cellular proliferation and mTOR inhibition is caused by mitochondrial dysfunction. This may be the basis for combining metformin with other chemotherapeutics; the added energetic stress could make cancer cells more susceptible and improve response to chemotherapy.

This study has primarily explored the role of metformin as a therapy in established cancer. Another potentially more important role would be its effects as chemoprevention for women at risk of developing EC (e.g. obese and/or polycystic ovary syndrome). Data from this study suggests that metformin has biological effects on cellular proliferation in established EC. While it is not possible to extrapolate directly to primary and secondary prevention studies, it is interesting to postulate whether these biological effects would persist in the pre-malignant endometrium or even prevent hyperplastic changes in the context of hyperinsulinaemic or oestrogenic stimulation. A primary prevention study of metformin in obese postmenopausal women is ongoing (NCT01697566) with expected completion in 2019. In addition, a large phase III study of adjuvant metformin in 3649 women treated for breast cancer over 5 years will provide information on the chemopreventative role of metformin. While incidence of EC is not one of the planned secondary outcomes, it is likely to be incorporated as an important post-hoc analysis, as breast and EC share many aetiological factors including obesity and insulin resistance.

The role of metformin in obesity-driven EC has been explored in this study. Ultimately, the important clinical questions are “will metformin at clinically tolerated doses have a survival advantage in patients with EC?” and “does metformin have a role in preventing EC?” Further studies are required to establish the role of metformin as adjuvant therapy in EC and it is imperative that pharmacokinetic and translational studies are included to ensure that metformin is prescribed to patients most likely to derive clinical benefit.
Further Planned Work

- A gold-standard placebo-controlled randomised presurgical window study PREsurgical Metformin in Uterine Malignancy (PREMIUM), has been designed based on findings from this proof of concept study. PREMIUM is actively recruiting and expected to complete recruitment in 2017. Important modifications to the study design include a blind endometrial biopsy obtained prior to tumour devascularisation, allowing assessment of mTOR phosphorylation events.

- The PREMIUM study aims to recruit 90 patients and will be the largest study of presurgical metformin in EC. The larger numbers in this study may help identify patients likely to benefit from metformin and further assess the modulating effects of hyperglycaemia and hypoxia.

- The assessment of Ki-67 as a prognostic marker will be continued using a large cohort of endometrial cancers, including samples from the TransPORTEC consortium. These samples are predominantly high grade tumours thus providing a greater number of “events” (recurrences and death) to determine prognostic significance.

Suggested Work

- The combination of metformin and aromatase inhibitors should be assessed using an in vitro model prior to a presurgical window study. EC cell lines may need to be transfected with aromatase to allow measurable effects. The combination of metformin and aromatase may target both insulin and oestrogen-driven pro-proliferative pathways. All in vitro experiments should use physiological levels of glucose in media to allow the best translation to the clinical setting.

- Laboratory experiments could be planned to confirm the effect of metformin on oxidative phosphorylation. Supplementary experiments could include measurement of lactate and oxidative phosphorylation.

- Seahorse metabolic assays could confirm the switch to glycolytic metabolism in the presence of high glucose in EC cells. These assays could also investigate if metformin treatment triggers glycolytic compensation. Metabolic assays could be repeated using primary endometrial cancer cell lines to account for tumour heterogeneity. It would be interesting to investigate if EC cells from patients on
long-term metformin are metabolically divergent from normoglycaemia patients.

- A further presurgical window study could allow specific collection of tumour tissue to allow *in vivo* assessment of metformin’s effects on mitochondrial function.
References


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induced expression of carbonic anhydrase 9 is dependent on the unfolded protein response. J Biol Chem, 284, 24204-12.


Appendix 1: Patient Information Sheets

Central Manchester University Hospitals

Patient Information Sheet – Part 1

A study of metformin for the treatment of endometrial cancer

INVITATION

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We’d suggest this should take about 15 minutes. Talk to others about the study if you wish. (Part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study). Ask us if there is anything that is not clear.

WHAT IS THE PURPOSE OF THE STUDY?

This study will test the effect of metformin on endometrial cancer, or cancer of the lining of the womb. Metformin is one of the drugs used to treat diabetes. Recently, its role in the treatment of cancer has been explored. Diabetics who take metformin for many years are less likely to develop and die from cancer than those who do not. They are also more likely to respond well to chemotherapy if cancer does develop. Metformin has been shown to slow down the growth of breast, prostate, colon and endometrial cancers in the laboratory.

We want to test metformin as a treatment for atypical hyperplasia of the endometrium (pre-cancer) and endometrial cancer. Women taking part in this study will receive metformin for two to four weeks before surgery to remove the womb (hysterectomy). The effects of metformin will then be assessed by comparing the characteristics of an endometrial biopsy taken at diagnosis with those of an endometrial biopsy taken at the time of hysterectomy.
WHY HAVE I BEEN CHOSEN?

The gynaecology team looking after you have decided to perform a hysterectomy to treat your atypical hyperplasia (pre-cancer) or endometrial cancer. We would like to try metformin tablets, taken twice a day, as a new treatment for endometrial cancer whilst you are waiting for your surgery, which will take place in two- to four-weeks’ time.

DO I HAVE TO TAKE PART?

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

The study will be discussed with you in person by a member of the research team during the clinic. We will give you an information leaflet to read. If you are interested in participating in the study a member of the research team will answer any questions that you may have and take you through the consent procedure (which should take 5 to 10 minutes). If you would like more time to consider taking part in the study, a member of the research team will be happy to answer any questions that you may have by phone or in another clinic.

If you consent to take part in the study you will be asked questions regarding your past health, medications and alcohol intake; this should take no more than 10 minutes.

You will have pre-operative blood tests taken as routine before any operation. You will be asked to attend for a pre-admission clinic on a different day (usually the following morning) with a nurse as part of your clinical care. During this visit, we will take a research blood sample (30 ml or 2 tablespoons of blood) and an endometrial biopsy. We will ask you to attend “fasted” (i.e. nothing to eat or drink apart from water for eight hours before the blood test).

The endometrial biopsy sample is taken by placing a speculum inside the vagina to allow the doctor to see the neck of the womb clearly. You may have had this done before if you have had a cervical smear test in the past. A fine straw-like device is then placed inside the womb and a sample of the lining of the womb is taken. Sometimes we are not able to take a sample in the clinic for various technical reasons, and in those circumstances, we will ask the pathology department at St Mary’s, or your referring hospital, to send us any remaining tissue from the original biopsy that was taken to diagnose your endometrial cancer.
We will then provide you with metformin tablets (850mg each) to be taken twice a day until the evening before your surgery. It is quite common for patients to feel sick, experience an upset tummy (vomiting, diarrhoea, abdominal pain, loss of appetite, altered taste sensation) or to notice a skin rash or itchy skin with metformin, but these side effects are usually mild and short-lived. We will contact you by telephone after the first week to check if you have had any side effects and also give you a contact number to call if you have any concerns. Alcohol can interact with metformin and we suggest that you do not drink alcohol while you are taking metformin. In the event that you become acutely unwell for any reason, we ask that you stop the metformin tablets immediately and alert medical staff as well as a member of the research team.

We will ask you to take the metformin tablets twice a day right up until the day before your surgery. Please bring your tablet box into hospital when you come for your operation so that we can dispose of any left over tablets safely.

On the day of your surgery, we will take a further blood test from you (30ml or two tablespoons of blood). We will also take an endometrial biopsy at the time of hysterectomy and some of the endometrial tumour removed during surgery for research purposes once the pathology team have taken all the samples that they require for your clinical care.

You will take the metformin tablets for up to 4 weeks, depending on the date of your surgery. In the event that your surgery is delayed beyond 4 weeks, we will ask you to come back to the clinic before your operation to have your final blood test and endometrial biopsy taken. If this happens, we will reimburse your travel expenses.

**WHAT DO I HAVE TO DO?**

Please consider the contents of this information sheet. If you are interested in taking part in the study, we are happy to answer any queries that you have. If you do decide to take part, we will contact your GP on your behalf and let them know.

**WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART?**

If you choose to participate you will need to put aside thirty minutes or so to answer questions about your medical health and to have blood and endometrial samples taken. Blood tests occasionally cause discomfort or bruising. Endometrial biopsies can be uncomfortable and some patients find intimate examinations like this embarrassing. Metformin treatment can cause a tummy upset but this is usually mild and settles with time. You must avoid drinking alcohol whilst you are taking metformin. Occasionally metformin can cause a condition called lactic acidosis or hepatitis, inflammation of the liver. This is rare but can be serious. If you become unwell when you are taking metformin, we ask that you stop taking the tablets immediately and contact us. On the day of surgery, you will have a further blood test, which may cause discomfort or bruising.
WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

Metformin has shown promise as an anti-cancer drug in cancers of the breast, prostate and colon. It has also been tried in endometrial cancer in the laboratory with some exciting results. This is the first time that metformin has been tried in patients with atypical hyperplasia (pre-cancer) or endometrial cancer although it is a very common treatment for diabetes. If effective, metformin may stop cancer cells dividing and reduce the chance of the cancer spreading. These effects may be small and may not benefit everyone who takes metformin. Although we cannot promise the study will help you personally, the information we get from this study may help to improve the treatment of women with endometrial cancer in the future.

WHAT HAPPENS WHEN THE RESEARCH STUDY STOPS?

When the study stops no further blood tests for the study will be required from you. Your usual medical treatment and follow up will continue as usual.

CONTACT FOR FURTHER INFORMATION?

Dr Vanitha Sivalingam      Dr Emma Crosbie
5th Floor Research          5th Floor Research
St Mary’s Hospital          St Mary’s Hospital,
Oxford Road                 Oxford Road
Manchester, M13 9WL         Manchester, M13 9WL
Tel: 07876024034/ 0161 2763355 Tel: 0161 701 6942

Many thanks for considering taking part in this study.

Dr Emma Crosbie           Professor Henry Kitchener
Senior Lecturer and Honorary Consultant
Professor of Gynaecological Oncology
in Gynaecological Oncology 5th Floor
A study of metformin for the treatment of endometrial cancer

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

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

WILL ANY RESEARCH BE DONE?

The tissues we collect will be processed and stored in the Gynaecological Oncology Research Laboratory at St Mary’s Hospital. To try to improve our understanding of the biology of endometrial cancer we may in the future want to look at your genetic material (DNA/ RNA) for mutations, sequence variation and gene expression differences. This work may involve investigators from other research institutions. These investigators will not, however, have access to your clinical records or identity. Your samples will be part of a tissue bank and will be retained for future use after this study closes. Your clinical details will remain linked to your tissue samples for use in future studies. Any transfer of material or data to future collaborators/ investigators and institutions will be anonymised.

WHAT IF THERE IS A PROBLEM?

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

**WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?**

Your participation in this trial is confidential and a record of your participation will be kept with your hospital records. Only the study team at the St Mary’s Hospital and other healthcare professionals who require access to your medical records will know of your participation. We will also inform your GP of your participation in the study. Healthcare professionals and scientists with whom we may collaborate outside St Mary’s Hospital will not be able to identify you from the data we share.

**WHAT WILL HAPPEN TO ANY SAMPLES I GIFT?**

During your participation in the study, we ask that you gift to us the blood and tissue samples. As a gift, you understand that if this research leads to the development of a new treatment or medical test that you will not benefit financially.

All research samples will be collected, processed and stored in the Gynaecological Oncology Research Laboratory at St Mary’s Hospital (Custodian: Dr Emma Crosbie) and will be linked to data from your clinical records. Only the study team at St Mary’s Hospital will have knowledge of your identity linked to your clinical record and laboratory samples/ data. The study will use donated samples to improve our understanding of the biology of endometrial cancer. The results of these investigations are unlikely to have any implications for you personally.

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

**WHAT WILL HAPPEN TO THE RESULTS OF THIS CLINICAL TRIAL?**

We aim to widely publish the results of this study at international meetings and in medical journals.

**WHO IS ORGANISING AND SPONSORING THIS CLINICAL TRIAL?**

Dr Emma Crosbie, NIHR Clinical Lecturer in Gynaecological Oncology is the chief investigator. Co-investigators include: Professor Henry Kitchener, Professor of Gynaecological Oncology at St Mary’s Hospital, Dr Lynne Hampson, Lecturer in Gynaecological Oncology at St Mary’s Hospital and DR Andrew Renehan, Senior Lecturer in Cancer Studies and Surgery at The Christie NHS Foundation Trust.
The study is being sponsored by Central Manchester University Hospitals NHS Foundation Trust. If you have concerns about the conduct of the study you may wish to contact the Trust Research Office (0161 276 3565) or the Patient Advice & Liaison Service (0161 276 8686).

**WHO HAS Reviewed THE STUDY?**

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favorable opinion by the North-West Research Ethics Committee-Haydock.

**CONTACT FOR FURTHER INFORMATION?**

*Dr Emma Crosbie*

*Senior Lecturer and Honorary Consultant in Gynaecological Oncology*

*5th Floor*

*St Mary’s Hospital*

*Hathersage Road*

*Manchester M13 9WL*

*Phone. 0161 701 6942*

Many thanks for considering taking part in this study

Yours Sincerely,

Dr Emma Crosbie  
Senior Lecturer and Honorary Consultant in Gynaecological Oncology

Professor Henry Kitchener  
Professor of Gynaecological Oncology
Appendix 2: Instructions for Adverse Events Safety Evaluation

An adverse event occurring in this study whether or not deemed to be causally associated with the study drug is defined as:

- an abnormal change in physical signs or symptoms, or
- an abnormal result in laboratory test which results in change in concomitant medication, or
- any change in the results of a laboratory test which the investigator judges clinically significant.

The investigators will enter details of any adverse events on the case report form provided. Any adverse event will be graded as mild, moderate or severe. Its duration will be recorded, as well as any action taken and the investigators opinion of relationship to study drug.

The following are definitions for grading the severity of adverse events:

1. **Mild**
   - Awareness of signs or symptoms, but which are easily tolerated; are of minor irritant type; no loss of time from normal activities; symptoms would not require medication or medical evaluation; signs and symptoms are transient, disappearing during the study.

2. **Moderate**
   - Discomfort enough to cause interference with usual activities.

3. **Severe**
   - Incapacitating with inability to work or do usual activities. Signs and symptoms may be of systemic nature, and may require medical evaluation or treatment.

In addition to this grading, investigators must also decide whether each event constitutes a **SERIOUS ADVERSE EVENT** (defined as follows):

**Serious**
- Fatal
- Life threatening
- Requires or prolongs in-patient hospitalisation
- Results in persistent or significant disability/incapacity
- Congential abnormality/birth defect
- Other (e.g. important medical event which may jeopardise the patient)

All adverse events will be reported on an adverse events form. These will be summarised in the report produced at the conclusion of the study.

**Serious Adverse Events (SAE)** which are unexpected and attributed to the medicinal product being studied will be reported immediately to the study sponsor (or Chief Investigator) who must then inform the regulatory authorities (Ethics Committee and MHRA).
Appendix 3: Safety and Tolerability Questionnaire from Case Report Form.

Central Manchester University Hospitals

Proof of mechanism presurgical window trial of metformin in non-diabetic women with endometrial carcinoma: a feasibility study

METFORMIN TOLERABILITY AND SAFETY QUESTIONNAIRE

Date treatment started: __________________________ Date questionnaire completed: __________________________

Did you take the metformin tablets twice a day whilst waiting for your hysterectomy? Yes/No

If not, please give your reasons here ______________________________________________________

Did you have any side effects from the metformin tablets? Yes/No

Please describe what happened here ______________________________________________________

In particular, did you experience any:

- Loss of appetite? Yes/No
- Abdominal pain? Yes/No
- Nausea/Vomiting? Yes/No
- Diarrhoea or loose bowel habit? Yes/No
- Skin changes, including rashes, redness, or itch? Yes/No

Please describe any other side effects that you noticed here __________________________

Do you have any further comments that you would like to make regarding the metformin treatment? If so, please write them here

_______________________________________________________________________________________

_______________________________________________________________________________________

How tolerable were the side effects on a scale of 1-10? (1 = no side effects, 10 = worse side effects imaginable) ______________________________________________________

Thank you for your participation in the trial.
Appendix 4: Publications
Metformin in reproductive health, pregnancy and gynaecological cancer: established and emerging indications

Vanitha N. Sivalingam1, Jenny Myers2, Susie Nicholas3, Adam H. Balen3, and Emma J. Crosbie1,*

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3Leeds Centre for Reproductive Medicine, Leeds Teaching Hospitals, Seacroft Hospital, Leeds LS14 6UH, UK

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BACKGROUND: Metformin is an effective oral anti-hyperglycaemic drug used as first-line medical treatment for type 2 diabetes. It improves systemic hyperglycaemia by reducing hepatic glucose production and enhancing peripheral insulin sensitivity. It also stimulates fat oxidation and reduces fat synthesis and storage. The molecular mechanism of this drug is thought to be secondary to its actions on the mitochondrial respiratory chain.

METHODS: This paper reviews the relevant literature (research articles up to October 2013) on the use of metformin in infertility, polycystic ovary syndrome (PCOS), pregnancy and gynaecological cancers. We present a comprehensive discussion of the evidence supporting the efficacy of metformin in these clinical conditions.
**RESULTS:** Metformin is used clinically off-label in the management of hirsutism, acne and insulin resistance in PCOS, although the evidence for anti-androgenic effects is inconsistent. Metformin is also used to improve ovulation in women with PCOS both alone and in combination with clomiphene citrate. Trial findings are conflicting but metformin treatment in IVF/ICSI cycles may reduce the risk of ovarian hyperstimulation syndrome and increase live birth rates. Metformin also appears to be effective and safe for the treatment of gestational diabetes mellitus (GDM), particularly for overweight and obese women. Studies have shown that metformin is safe in pregnancy and women with GDM treated with metformin have less weight gain during pregnancy than those treated with insulin. One study with a 2-year follow-up demonstrated that babies born to women treated with metformin also developed less visceral fat, making them less prone to insulin resistance in later life. These findings have sparked interest in the use of metformin for pregnant, obese, non-diabetic women. On-going clinical trials are underway to determine if women treated prophylactically with metformin have a reduced incidence of GDM and demonstrate less weight gain during pregnancy. The hypothesis in these studies is that babies born to obese women on prophylactic metformin will also have better outcomes. Epidemiological studies have linked metformin exposure to a decreased risk of cancer. Pre-clinical experiments report that metformin has a growth-static effect on several cancers, including endometrial cancer, which may be partly due to the effect of metformin on the PI3K/AKT/mTOR signal transduction pathway. A number of on-going early phase clinical trials aim to explore the anti-cancer effects of metformin and investigate its potential as a chemopreventative or adjuvant treatment.

**CONCLUSIONS:** Obesity is on the rise in developing countries and is strongly linked to several reproductive health problems, including PCOS, GDM and endometrial cancer. Traditional lifestyle measures aimed at weight reduction are challenging to implement and maintain. Metformin may be a valuable alternative to, or adjunct for, modifying the toxic effects of obesity in these populations. This review will appraise the evidence for the use of metformin for the prevention and treatment of adverse health outcomes in obstetrics and gynaecology.

**Key words:** metformin / endometrial cancer / polycystic ovary syndrome / gestational diabetes / obesity in pregnancy

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**Introduction**

Insulin resistance occurs when insulin-responsive tissues (including liver, skeletal muscle and adipose tissue) become less sensitive to insulin (Lebovitz, 2001). Failure of pancreatic β islet cells to produce sufficient compensatory insulin results in chronic hyperglycaemia and hyperinsulinaemia. Insulin resistance comprises a spectrum of disease ranging from pre-clinical impaired glucose tolerance to overt type 2 diabetes mellitus (T2DM). T2DM is an increasing problem in elderly, overweight populations. Treatment of T2DM aims to normalize glycaemic levels as much as possible as this has been shown specifically to reduce microvascular complications (The Diabetes Control and Complications Trial Research Group, 1993). Lifestyle interventions that reverse or reduce obesity have a beneficial effect on glycaemic control (Pi-Sunyer et al., 2007) but are limited by poor compliance and high relapse rates. Drug therapy is initiated where lifestyle measures fail to achieve glycaemic targets.

Metformin is the preferred and most cost-effective first-line oral therapy for the treatment of T2DM (Nathan et al., 2009; Inzucchi et al., 2012) and is usually well tolerated and not complicated by hypoglycaemia. The most serious side effect, lactic acidosis, is rarely seen in patients with normal renal and hepatic function, occurring in just 3/100 000 patient-years of use (Howlett and Bailey, 1999; Lalau and Race, 2000; Salpeter et al. 2006). In contrast to most oral hypoglycaemic agents, metformin promotes weight loss. The UK Prospective Diabetes Study (UKPDS) found that metformin offers protection from the macrovascular complications of T2DM independently of its hypoglycaemic actions (UKPDS, 1998). Reduced atherogenesis, less oxidative stress and redistribution of visceral adiposity have all been described as potential mediators of this effect (Scarpello and Howlett, 2008). Metformin is also associated with reductions in total cholesterol, low-density lipoprotein-cholesterol and triglyceride levels (Nagi and Yudkin, 1993).

Metformin can have health benefits in non-diabetic patients at risk of the disease. The US Diabetes Prevention programme found metformin reduces the risk of frank diabetes in patients with impaired glucose tolerance by as much as 31% (95% confidence interval (CI) 17–43) when compared with placebo, although lifestyle interventions that achieve weight loss and regular exercise are even more effective (58% reduction in risk, 95% CI 48–66) (Knowler et al., 2002). Other studies have reported similar results (Ramachandran et al., 2006). Metformin can also improve histological and biochemical liver function in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (NASH), diseases associated with insulin resistance and the metabolic syndrome (Loomba et al., 2009; Nadieu et al., 2009). Improvements in systemic insulin and glucose levels are the likely mechanisms responsible here.

**The effect of metformin on insulin resistance**

Metformin is a synthetically derived biguanide that takes its origins from Galega officinalis, a medicinal herb used in medieval Europe (Bailey et al., 2008). Taken orally, it is absorbed rapidly across the intestinal epithelium and then conveyed via the portal vein to the liver, where it accumulates. The liver is its primary site of action. Metformin is not metabolized and is excreted unchanged in the urine and bile.

Metformin reduces hepatic glucose production, stimulates insulin-mediated glucose uptake by the liver and skeletal muscle, and reduces substrate availability for gluconeogenesis by lowering serum lipid levels. Its effects on glucose metabolism appear to be secondary to its actions on the mitochondrial respiratory chain. Metformin inhibits the mitochondrial respiratory complex I (El-Mir et al., 2000; Owen et al., 2000), leading to reduced oxidative phosphorylation and ATP production. The resultant increased AMP:ATP ratio inhibits gluconeogenesis and activates AMP-activated kinase (AMPK) (Fig. 1). AMPK is frequently inactivated in energy-rich environments such as obesity and insulin resistance. Its activation by metformin decreases lipid synthesis, increases fatty acid oxidation and inhibits gluconeogenesis (McGarry and Brown, 1997; Li et al., 2011). Metformin regulates AMPK via an upstream kinase, LKB1, a tumour suppressor gene product that controls cell growth. Metformin
also has AMPK-independent effects on glucose metabolism, since mice deficient in both LKB1 and AMPK show reduced serum glucose levels following treatment with metformin. These effects may be caused by the change in AMP:ATP ratio which modulates hepatic glucose output upstream of AMPK and inhibits cyclic-AMP-protein kinase A signalling.

Indications for metformin in reproductive health, pregnancy and gynaecological cancer

Metformin is currently used for the management of gestational diabetes mellitus (GDM). It is also prescribed off-label in polycystic ovary syndrome (PCOS) to treat insulin resistance and improve outcomes in assisted reproduction. Recently, epidemiological, pre-clinical and early phase clinical trials have suggested that metformin may be effective as an anti-cancer drug in tumours driven by insulin resistance and obesity. This article will summarize the current clinical uses of metformin in pregnancy and reproductive healthcare and discuss the emerging evidence from the research setting for potential novel applications of this ‘old’ drug.

Methods

PubMed and the Cochrane Library were searched for high-quality studies including randomized trials, systematic reviews and meta-analyses between 1 January 2000 and 1 October 2013 for the terms ‘endometrial cancer’, ‘endometrial hyperplasia’, ‘atypical endometrial hyperplasia’, ‘endometrioid’, ‘uterine cancer’, ‘ovarian cancer’, ‘polycystic ovary syndrome’, ‘polycystic ovary’, ‘ovulation induction’, ‘anovulatory infertility’, ‘in-vitro fertilisation’, ‘insulin resistance’, ‘type 2 diabetes’, ‘gestational diabetes’, ‘obesity in pregnancy’, ‘metformin’ and ‘biguanides’. In addition, a hand-search identified older publications that were considered to be important. Pertinent references from selected articles were also included.
PCOS

PCOS, insulin resistance, metabolic dysfunction and infertility

PCOS is the most common endocrine disorder to affect women during their reproductive years (ESHRE/ASRM, 2004). The symptoms of PCOS include menstrual cycle disturbance and features of hyperandrogenism (hirsutism, acne, alopecia), with associated fertility problems, obesity and psychological issues.

In PCOS, both extra-ovarian and intra-ovarian factors lead to dysregulation of normal follicular recruitment and ovulation. There is uncertainty as to the mechanisms that lead to an increased pre-antral follicle population in the polycystic ovary and there appear to be intra-ovarian disturbances in the expression of growth differentiation factor-9, anti-Müllerian hormone and androgen production by the theca cells (Chang and Cook-Andersen, 2013). These are further influenced by disturbances in gonadotrophin secretion (FSH and LH) secondary to abnormalities of the GnRH pulse generator and perturbations of ovarian-pituitary feedback.

LH stimulates androgen production by the ovaries and hypersecretion of LH in PCOS, which is seen predominantly in slim women, occurs secondary to both an increase in the GnRH pulse generator and abnormalities in ovarian-pituitary feedback. Hyperandrogenism is also amplified by hyperinsulinaemia secondary to insulin resistance, which in turn may be promoted by obesity (Cussons et al., 2008). Serine kinase activity leads to phosphorylation of the insulin receptor, blocking insulin signalling, and also phosphorylation of the cytochrome P450c17a enzyme (CYP17) to activate androgen production (Bremer and Miller, 2008). Excess insulin also binds to insulin-like growth factor (IGF)-1 receptors which enhances theca cells androgen production in response to LH stimulation (Bergh et al., 1993). Hyperinsulinaemia inhibits hepatic secretion of insulin-like growth factor binding protein-1 (IGFBP-1), leading to increased bioavailability of IGF-1 and 2, important regulators of ovarian follicular maturation (De Leo et al., 2000). This further augments ovarian androgen production, which contributes to anovulation by promoting follicular atresia. Sex hormone-binding globulin synthesis by the liver is also lowered by hyperinsulinaemia leading to an increase in bioactive serum free-testosterone concentration (Fig. 1). A number of additional defects within the polycystic ovary have been described (Chang and Cook-Andersen, 2013) but are beyond the scope of this review.

PCOS may also be associated with an increased risk of an individual developing T2DM, the metabolic syndrome and endometrial cancer (Cussons et al., 2008). Both obese and non-obese women with PCOS are more insulin-resistant and hyperinsulinaemic than age- and weight-matched women with normal ovaries, suggesting a tendency towards insulin resistance which is independent of obesity (Tsilchorozidou, 2004). Pancreatic beta cell dysfunction has been described in women with PCOS, where there is increased basal secretion of insulin yet an inadequate post-prandial response. This defect may remain even after weight loss, despite an improvement in glucose tolerance (Holte et al., 1995). Women with PCOS who are oligomenorrheic are more likely to be insulin resistant than those with regular cycles, irrespective of their BMI (Robinson et al., 1993).

There are many studies that have shown that women with PCOS have an increased rate of impaired glucose tolerance, T2DM and metabolic syndrome when compared with weight-matched controls (Moran et al., 2010). Cardiovascular risk factors for ischaemic heart disease and stroke include obesity, insulin resistance, glucose intolerance, diabetes, hypertension, dyslipidemia (in particular raised serum triglycerides) and various markers of inflammation (Fauser et al., 2012). Lifestyle advice and weight reduction should be the first-line approach for the management of PCOS, and options for treatment then include a range of therapies depending upon the constellation of an individual’s problems. These are too numerous to be elucidated (RCOG, 2010) and this review will focus on the use of metformin.

Metformin in the management of anovulatory PCOS

PCOS accounts for anovulatory infertility in ~80–90% of women. Algorithms for the induction of ovulation are well established (ESHRE/ASRM TEA-SPCWG, 2008) and include the use of clomiphene citrate as first-line therapy followed by either gonadotrophins or laparoscopic ovarian diathermy for those who fail to ovulate with clomiphene citrate (Balen, 2013). There may also be a role for aromatase inhibitors while IVF remains the last resort. Weight loss prior to treatment in the overweight improves the chance of success and decreases the risks of miscarriage, fetal abnormalities and maternal morbidity from GDM and pre-eclampsia (Clark et al., 1995; Fedorcsak et al., 2001; Balen and Anderson, 2007). In the UK, national guidelines suggest that ovulation induction should not be commenced in a woman with a BMI >35 kg/m² and preferably limited to women with a BMI <30 kg/m² (Balen et al., 2006; Balen and Anderson, 2007).

Insulin-sensitizing agents, such as metformin, were thought to have potential in the management of PCOS, and indeed early studies suggested an improvement in reproductive function and the possibility of benefits to long-term health (Stadtmueller et al., 2002). Metformin may act indirectly by reducing systemic insulin levels and directly within the ovary itself (Diamanti-Kandarakis et al., 2010), with a reduction in CYP17 activity and subsequent androgen production (Nestler and Jakubowicz, 1996; Attia et al., 2001). Metformin therapy may also lead to an increase in IGFBP-1 thereby reducing the availability of IGF-I (De Leo et al., 2000).

The results of large prospective, randomized studies have, however, failed to demonstrate benefit: significant weight loss is not achieved by metformin and whilst some biochemical parameters may improve this does not translate into a significant benefit in outcomes, whether for the dermatological manifestations of hyperandrogenism (Costello et al., 2007) or the enhancement of fertility (Tang et al., 2012).

The combined effects of lifestyle modification and metformin on obese anovulatory women (BMI >30 kg/m²) with PCOS were evaluated in a prospective randomized, double blind, placebo-controlled multicentre study (Tang et al., 2006a). All the patients had an individualized assessment by a research diettian in order to set a realistic goal which could be sustained for a long period of time with an average reduction of energy intake of 500 kilo calories per day. As a result, both the metformin-treated and placebo groups managed to lose weight, and the amount of weight reduction did not differ between the two groups. An increase in menstrual cyclicity was observed in those who lost weight but again did not differ between the two arms of the study, reinforcing the notion of weight reduction holding the key to improving reproductive function (Tang et al., 2006a).

Other large studies have explored the use of metformin combined with clomiphene citrate. In a Dutch multicentre trial, 228 women with...
PCOS were randomly allocated to receive either clomiphene citrate plus metformin or clomiphene citrate plus placebo (Moll et al., 2006). The ovulation rate in the metformin group was 64% compared with 72% in the placebo group (non-significant), and furthermore there were no significant differences in either the rates of ongoing pregnancy (40 versus 46%, respectively) or spontaneous miscarriage (12 versus 11%, respectively). The gastrointestinal side effects of metformin led to more women discontinuing therapy than in the control group (16 versus 5%). The pregnancy in polycystic ovary syndrome trial enrolled 626 anovulatory women with PCOS and randomized them to three different treatment arms for a total of six cycles or 30 weeks: (i) metformin 1000 mg twice daily plus placebo, (ii) clomiphene citrate 50 mg/day on Day 3–7 of cycle plus placebo, or (iii) combined metformin 1000 mg twice daily plus clomiphene citrate 50 mg/day (Days 3–7). Overall, live birth rates were 7.2% (15/208), 22.5% (47/209) and 26.8% (56/209), respectively, with the metformin alone group faring significantly worse than the other two groups. Pregnancy loss rates were also higher in the metformin alone group (40.0% versus 22.6% and 25.5%, respectively). The mean BMI of recruited patients was 35 kg/m², so the results of this study may not be applicable to all PCOS patients (Legro et al., 2007).

The most recent Cochrane review included 38 trials with a total of 3495 participants (ranging from 16 to 626 per study), with a median daily dose of metformin of 1500 mg and durations ranging from 4 to 48 weeks. This systematic review concluded that metformin is effective in achieving ovulation in women with PCOS when comparing metformin versus placebo (odds ratio (OR) 1.81, 95% CI 1.13–2.93; 16 RCTs, 1208 participants) as well as metformin and clomiphene citrate versus clomiphene citrate alone (OR 1.74, 95% CI 1.5–2.0; 18 RCTs, 3265 cycles) (Tang et al., 2012). The analysis of pregnancy rates showed a significant treatment effect for metformin and clomiphene citrate (111 RCTs, 1208 participants; OR 1.51, 95% CI 1.17–1.96); however, these benefits were not translated into live birth rates (7 RCTs, 907 participants; OR 1.16, 95% CI 0.85–1.56). Live birth rates were significantly improved in obese women with PCOS taking clomiphene citrate compared with those taking metformin alone (2 RCTs, 500 participants; OR 0.30 95% CI 0.17–0.52) (Legro et al., 2007; Zain et al., 2009). There was also a suggestion that those with clomiphene-resistance might benefit from the combined use of clomiphene citrate with metformin to improve the chance of ovulation, although the numbers studied were relatively small and this did not translate into an increase in live births. This review concluded that the benefit of metformin in women with anovulatory PCOS may be limited and did not demonstrate any benefit from metformin in improving weight loss, insulin sensitivity or lipid profiles (Tang et al., 2012). In contrast, a recent large study from Finland enrolled 329 women to receive metformin (1500–2000 mg/day) or placebo for 3 months prior to fertility treatment, for a further 9 months during fertility treatment, and until the 12th week of gestation if the patient conceived, showed an increase chance of pregnancy from 40.4 to 53.6% (OR 1.61, 95% CI 1.13–2.29), with the greatest benefit seen in obese women (Morin-Papunen et al., 2012). Whilst there was no reduction in miscarriage rate, the live birth rate was significantly increased in those who received metformin (41.9 versus 28.8%, P = 0.014). This latest study keeps the debate open and will have to be incorporated into the updated Cochrane review, which is currently in progress.

Metformin therapy in PCOS and assisted reproduction

Although the benefits of metformin in inducing ovulation in PCOS appear to be limited, there is more supportive evidence for its use in women with PCOS who are undergoing IVF. Metformin appears to act as a brake on the response of polycystic ovaries to exogenous stimulation, which pathophysiologically may explain its lack of efficacy in ovulation induction. Women with polycystic ovaries are at high risk of developing ovarian hyperstimulation syndrome (OHSS), which may cause serious morbidity and even death from intravascular volume depletion, thrombosis and adult respiratory distress syndrome. Risk reduction strategies include low dose stimulation protocols, freezing embryos for those at serious risk and the use of GnRH antagonist cycles with a GnRH agonist to trigger oocyte maturation (Nastri et al., 2010).

One of the first large RCTs to compare metformin with placebo in IVF cycles in women with PCOS, randomized 101 consecutive cycles using a conventional long GnRH agonist protocol and metformin 850 mg twice daily prior to egg collection. There was no difference in total FSH dose, number of oocytes retrieved or overall fertilization rate; however, there was a significant increase in clinical pregnancy rate beyond 12 weeks (38.5 versus 16.3%, P = 0.023) and a clinically significant reduction in severe OHSS (3.8 versus 20.4%, P = 0.023). Metformin was also shown to attenuate the ovarian secretion of vascular endothelial growth factor, which is thought to be key in the pathophysiology of OHSS (Tang et al., 2006b). Early work by Kjøtrod et al. (2004) had found similar benefit; lean women with PCOS had a higher live birth rate. Subsequent work from the same group provided further support. A total of 150 patients were randomized to either 2000 mg/day metformin or placebo for 12 weeks prior to IVF treatment. Implementing intention to treat analysis, the live birth rate was significantly higher in the metformin group (48.6 versus 32.0%; 95% CI 1.1–32.2; P = 0.0383) (Kjøtrod et al., 2011). A Cochrane meta-analysis concluded that the main benefit of metformin in the context of IVF therapy for women with PCOS is for the prevention of OHSS (OR 0.27, 95% CI 0.16–0.47) (Tso et al., 2009). The potential benefit of metformin therapy may also stretch into subsequent frozen embryo transfer cycles. A retrospective study found that for those who used metformin during the fresh attempt, the subsequent frozen cycle had a significantly increased live birth rate (28.6 versus 12.3%) (Brewer et al., 2010). This was most significant in those who had all embryos frozen due to OHSS risk, in whom a 9-fold increase in live birth rate was seen (Brewer et al., 2010).

Metformin therapy during pregnancy in PCOS

Some early studies, with inferior design, suggested that metformin might reduce the miscarriage rate in women with PCOS although this has since been shown not to be the case (Palomba et al., 2009; Morin-Papunen et al., 2012). Women with PCOS are at increased risks of pregnancy-related complications including GDM, pregnancy-induced hypertension, pre-eclampsia and neonatal morbidity (Boomanma et al., 2006). A large Swedish population-based cohort study found a strong association with pre-eclampsia (adjusted OR 1.45, 95% CI 1.24–1.69) and preterm birth in women with PCOS compared with those without PCOS. The risk of GDM was doubled (Roos et al., 2011). This group concluded that there was an increase in adverse pregnancy events for those
with PCOS not purely explained by increased use of assisted conception. In view of the favourable effects of metformin on thrombotic events in the diabetic population, it would seem feasible that microvascular dysfunction and subsequent downstream events could be improved in PCOS pregnancies with metformin. However, a large Norwegian multicentre RCT found no improvement in these complications with continued use of metformin from late first trimester to delivery (Vanky et al., 2010), although there appeared to be a reduction in late miscarriage and preterm delivery rates, which is now the subject of a large ongoing RCT. Women in the metformin group also gained less weight during pregnancy compared with the placebo group. It is reassuring that metformin has a good safety profile in healthy women and early pregnancy with no evidence of teratogenicity, although the gastrointestinal side effects that occur in ~10% are well recognized (Tang et al., 2012).

In summary, metformin may both reduce the risk of OHSS and increase ongoing pregnancy rates in women with PCOS undergoing IVF, although further research is needed to substantiate these findings.

## Metformin in pregnancy

### GDM

The tide has turned quite dramatically over the last decade in favour of metformin for the treatment of diabetes, both pre-existing (T2DM) and newly diagnosed in pregnancy (GDM) since the first study that used metformin in pregnancy in 1979 (Coetzee and Jackson, 1979). In 2001, in a critical appraisal of the published evidence describing treatment options for GDM, Dorman stated ‘It would be a brave, possibly foolhardy, person to do a controlled trial of metformin’ in diabetic pregnancy (Dorman and Hollis, 2001). Since then three retrospective studies, two non-randomized prospective studies and five RCTs have published pregnancy outcomes for around 1690 women treated with metformin during pregnancy (Ekpebegh et al., 2007; Rowan et al., 2008, 2011; Tertti et al., 2008; Balani et al., 2009; Goh et al., 2011; Ijas et al., 2011; Gandhi et al., 2012; Niromanesh et al., 2012; Spaulonci et al., 2013). Despite methodological issues with many of these studies and limitations associated with comparison populations due to differences in baseline characteristics, the overwhelming conclusion from these studies is that metformin is safe in pregnancy. There have been no reports of an increase in congenital abnormalities or deleterious effects on fetal growth or short-term neonatal health. In fact several studies have described a number of beneficial effects of metformin (Lautatzi et al., 2013).

A normal pregnancy is associated with a 50% reduction in insulin sensitivity in the third trimester, which is compensated by a 200–250% increase in the production of insulin in order to maintain a euglycaemic state (Catalano et al., 1991; Kuhl, 1998). Women who are at the highest risk of developing GDM are those with a pre gravid reduction in insulin sensitivity, including obese women, women with family history of T2DM and those who have previously been affected by GDM. A reduction in insulin sensitivity coupled with suppressed pancreatic insulin production in late pregnancy (Buchanan et al., 1990) results in both fasting and post-prandial hyperglycaemia with increased nutrient availability to the fetus (Catalano et al., 2003). The exact mechanisms by which insulin resistance develops in the second half of gestation are not completely understood but are likely attributable to placental hormones (e.g. human placental lactogen, human placental growth hormone) (Beck and Daughaday, 1967; Handwerger and Freemark, 2000) and a number of adipokines including leptin, adiponectin, tumour necrosis factor (TNF)-α, interleukin (IL)-6 and resistin (Barbour et al., 2007). Metformin treatment combats both of these effects by increasing insulin sensitivity and reducing basal hepatic glucose output (Catalano et al., 1991; Stumvoll et al., 1995) (Figs 1 and 2).

One of the most consistent observations from the metformin studies to date is the reduction in maternal weight gain during pregnancy (Rowan et al., 2008; Balani et al., 2009; Niromanesh et al., 2012) which is likely to be associated with long-term health benefits for women. Maternal hypoglycaemia is also much less prevalent in women treated with metformin alone and although 10–46% of women with GDM treated with metformin have required supplemental insulin therapy to achieve optimal glycaemic control, the total dose of insulin in metformin-treated women is lower than in women treated with insulin alone (Lautatzi et al., 2013). As demonstrated by the recent study performed by Tertti et al., women who require supplemental insulin are older and often require pharmacological treatment earlier in pregnancy. Interestingly, fructosamine was shown to be a useful predictor of the need for supplemental insulin in this study (Tertti et al., 2013). Metformin was well tolerated in all of the studies with around 2–7% unable to tolerate it (Rowan et al., 2008; Balani et al., 2009; Niromanesh et al., 2012). It was also an acceptable treatment in those studies that assessed this element (e.g. (Rowan et al., 2008)). The majority of the studies did not identify a significant difference in short-term neonatal outcomes in women treated with metformin compared with insulin although individual trials were not powered to demonstrate differences in severe perinatal morbidity or perinatal mortality. Neonatal birthweight, frequency of large for gestational age, small for gestational age, preterm deliveries and neonatal unit admissions were not different between groups in the majority of studies (Rowan et al., 2008; Tertti et al., 2008; Goh et al., 2011; Ijas et al., 2011; Niromanesh et al., 2012; Spaulonci et al., 2013). Neonatal hypoglycaemia was reduced in the metformin group in the metformin in GDM (MiG) trial (Rowan et al., 2008) and whilst a reduction in neonatal anthropometric measurements (head, arm and chest circumference) was demonstrated in the study by Niromanesh et al. (2012), this was not shown in the larger MiG trial (Rowan et al., 2008).

A common criticism of RCTs in pregnancy is the assessment of meaningful long-term child health outcomes. This is particularly important for interventions which could have a profound effect on the metabolic programming of the fetus/infant. There is now a wealth of evidence supporting the hypothesis that an adverse in utero environment has lasting effects on the metabolic and future cardiovascular health of the offspring. The children from the MiG trial have been followed up to 2 years (Rowan et al., 2011). Anthropometric assessments were performed on the children that did not identify differences between the groups in central fat measures, total fat mass, percentage body fat, or central-to-peripheral fat, however the children exposed to metformin in utero had larger upper arm circumference, and bigger biceps and subscapular skinfolds. The authors concluded that this would suggest that exposure to metformin in utero leads to a shift in fat deposition from visceral fat stores to subcutaneous sites, which has important implications for the development of insulin resistance in the future (Ali et al., 2011).

## Obesity and pregnancy

Maternal obesity in pregnancy is a significant and growing health problem. Obese women are at significantly higher risk of a number of pregnancy...
complications including miscarriage, GDM, pre-eclampsia and Caesarian delivery (Galtier-Dereure et al., 2000). Many of these complications have been attributed to insulin resistance, metabolic dysfunction, inflammation and oxidative stress affecting the mother and the placenta (Ramsay et al., 2002).

The effect of metformin on inflammation in pregnancy
In addition to its role as an insulin-sensitizing agent, metformin also has anti-inflammatory actions (Dandona et al., 2004; Isoda et al., 2006) which may be relevant to its beneficial effects in pregnancy for both the mother and fetus. Moderation of both metabolic dysfunction and inflammation may improve pregnancy outcomes by altering placental development and function, in addition to reducing the likelihood of vascular endothelial activation (Hattori et al., 2006; Isoda et al., 2006) and potentially reducing insulin resistance in the fetus (Fig. 2). Metformin crosses the placenta and therapeutic levels of the drug have been quantified in cord blood (Hague et al., 2003); therefore it is likely that there is some effect of metformin on placental and fetal metabolism.
Pre-clinical models for metformin use in pregnancy

Several animal studies have demonstrated the link between maternal obesity, inflammation and significant and lasting metabolic effects in the offspring, particularly insulin resistance (Ainge et al., 2011). In pregnant rats, a reduction in maternal inflammatory cytokines (IL-6, CCL2, TNFα) has been observed following treatment with metformin. This is particularly relevant as these cytokines have been implicated in the development of insulin resistance and T2DM (Fried et al., 1998; Sartipy and Loskutoff, 2003; Ainge et al., 2011). In addition, in vitro studies of placental cell lines pre-treated with metformin demonstrated a significant reduction in IL-6 production in response to TNFα stimulation (Desai et al., 2013), and placental levels of TNF α-6 were also significantly reduced in metformin-treated animals compared with obese animals. It has been proposed that obesity contributes to placental inflammation through regulation of the NFκB pathway (Zhu et al., 2010). In the study by Desai et al., inhibition of NFκB reduced IL-6 production in response to TNFα stimulation and metformin treatment suppressed TNFα-induced degradation of IkBα, the natural inhibitor of NFκB nuclear localization and activation (Desai et al., 2013). In other studies, metformin has been shown to reduce proteosomic activity which mediates IkBα degradation (Chen et al., 1995) and suppresses TNFα-induced NFκB activation in human vein umbilical endothelial cells (Hattori et al., 2006). Additional pathways by which metformin may alter inflammatory signals in the placenta include mediators of oxidative stress (Srividhya et al., 2002) and the AMP kinase/mammalian target of rapamycin (mTOR) pathways (Fig. 2) (Nerstedt et al., 2010) although these signalling pathways have not been directly tested in placental models. Animal studies clearly provide an important opportunity to investigate the potential impacts of metformin during pregnancy in the context of both obesity and diabetes.

Current clinical studies in obese pregnant women

A number of studies have investigated the impact of lifestyle interventions on pregnancy outcomes with limited success (Oteng Ntim et al., 2012); one such study is ongoing in the UK (UPBEAT). Whilst it seems logical that lifestyle interventions can impact favourably on pregnancy outcomes, it also seems likely that pharmacological interventions may be associated with a more significant metabolic effect. At time of writing two UK-based RCTs aim to investigate the effect of metformin on maternal and fetal outcomes in pregnancies complicated by maternal obesity. The ‘Metformin in Obese Non-diabetic Pregnant Women’ (MOP) (NCT01273584) trial is currently recruiting for a sample size of 546 women across seven centres. The primary outcome is a difference in birthweight centile and a number of secondary maternal and fetal outcomes in pregnancies complicated by maternal obesity. The ‘MiTy Kids Trial’ is a follow-up to the MiTy Trial, which will determine whether treatment with metformin during pregnancy in women with T2DM will lead to a reduction in adiposity and improvement in insulin resistance in the offspring at 2 years of age. However, definitive answers on the long-term effects of metformin treatment in pregnancy will only be determined by larger, longer term studies which follow children for much longer than 2 years.

Metformin as an anti-cancer drug

Several large epidemiological studies first pointed to a role for metformin as an anti-cancer drug. A flurry of prospective observational studies suggested that patients with T2DM taking metformin were not only at lower risk of developing cancer (Libby et al., 2009) but were also less likely to die from it (Evans et al., 2005). These studies sparked excitement from cancer research scientists, who set off to demonstrate the anti-neoplastic effects of metformin in animal and pre-clinical models. Metformin was found to have a growth static effect on breast, prostate, pancreatic, ovarian and endometrial cancer cell lines, amongst others, with demonstrable impact on glucose metabolism and PI3K-Akt-mTOR pathway inhibition (Fig. 1) (Zäkihi et al., 2006, 2008, 2010; Cantrell et al., 2010; Sarfstein et al., 2013). Metformin has a well-established safety profile and its extensive use in diabetic patients, including those diagnosed with and undergoing treatment for cancer, suggests that it is safe to be used in the anti-cancer setting. This has encouraged bypassing of the traditional drug development paradigm and early translation of laboratory-based research findings to the clinic.

Proof of principle window of opportunity studies

Promising data from pre-clinical research inspired proof of principle intervention studies that tested the effects of metformin in newly diagnosed cancer patients awaiting standard surgical care. This clinical setting was ideal as it allowed single-agent metformin to be tested in an uncontaminated therapeutic period without compromising standard patient care. Most of the early phase clinical studies have compared tumour biopsies taken before and after metformin treatment in the presurgical window period between diagnosis and surgery. These so-called ‘window’ studies offer a unique opportunity to test the biological effects of metformin in proof of principle analyses that use surrogate markers of early clinical response as primary end-points. The most widely used primary outcome measure in studies thus far has been the Ki-67 receptor expression and maternal brachial arterial endothelial dependent flow mediated dilatation will be assessed. Detailed measurements of neonatal body composition will also be obtained. This study aims to recruit 400 women.

Ultimately, in order to understand the effects of metformin treatment in pregnancy, long-term follow-up studies of the infants and children are necessary. Population studies have already demonstrated that pregestational maternal obesity is associated with a significant increase in fetal abdominal circumference and birthweight (Tanvig et al., 2013). It is hoped that studies such as the ‘Lifestyle in Pregnancy and Offspring—Comparison Between Children Born to Obese Women and Children Born to Normal Weight Women’ will provide valuable information related to the metabolic health of children, although studies such as these are likely to be confounded by other lifestyle factors. In addition to the MiG follow-up study (Rowan et al., 2011), ‘The MiTy Kids Trial’ will be a follow-up to the MiTy Trial, which will determine whether treatment with metformin during pregnancy in women with T2DM will lead to a reduction in adiposity and improvement in insulin resistance in the offspring at 2 years of age. However, definitive answers on the long-term effects of metformin treatment in pregnancy will only be determined by larger, longer term studies which follow children for much longer than 2 years.

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proliferation index, where the proportion of tumour cells actively dividing is measured by immunohistochemistry using antibodies directed against Ki-67. Proliferation is a hallmark of cancer (Hanahan and Weinberg, 2011) and Ki-67 is a nuclear protein that is only expressed by proliferating cells. Ki-67 has been extensively validated as a prognostic and predictive biomarker of clinical response in breast cancer (Dowsett et al., 2007), and subsequently extrapolated for similar uses in different cancer types. Other studies have examined the proportion of tumour cells undergoing programmed cell death, or apoptosis, before and after metformin treatment, as measured by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling). Reduced apoptosis is another classical feature of malignancy.

Most of the work published thus far has focused on breast cancer. Two small, uncontrolled pilot studies (n = 55 and n = 39, respectively) found a significant reduction in Ki-67 expression by breast tumours following the short-term pre-surgical administration of metformin (mean 15.9 days, range 13–21 days) and median 18 days (range 13–40), respectively (Hadad et al., 2011; Niraula et al., 2012). A larger placebo-controlled trial (n = 200) went on to show that metformin reduced tumour Ki-67 expression (by ≈10%) in overweight or insulin-resistant breast cancer patients, but not in the whole metformin-treated population (Bonanni et al., 2012). Niraula et al. additionally reported an increase in tumour apoptosis, as assessed by TUNEL, following short-term administration of metformin (Niraula et al., 2012). A placebo-controlled study was not able to replicate these findings, however, with increased tumour apoptosis seen only in non-insulin-resistant breast cancer patients following subgroup analysis (Cazzaniga et al., 2013), findings that were contrary to the conventional hypothesis. Such conflicting results may be explained by small patient numbers and study heterogeneity, particularly with respect to the histological subtypes included in the studies, the duration and dose of metformin treatment received, and the range of BMI and insulin resistance observed at baseline in the different patient populations examined.

Besides breast cancer, an early phase clinical study of prostate cancer treated pre-surgically with metformin has also yielded promising results. In a study involving 24 patients who received neoadjuvant metformin for a median duration of 41 days (range 18–81 days) prior to radical prostatectomy, metformin reduced the Ki-67 proliferation index by an average of 29% compared with the pretreatment biopsy (Joshua et al., 2013). Several studies are also underway to test the pre-surgical administration of metformin in endometrial cancer (NCT01911247, NCT01205672 and ISRCTN81570194). Preliminary findings from one group found a reduction in tumour Ki-67 expression in sixteen obese patients with endometrial cancer following short-term pre-surgical administration of metformin (mean 14.5 days). Metformin treatment was associated with a reduction in Ki-67 proliferation index (mean 19.5%) in 10 of 16 patients (Schuler et al., 2013). Two other groups with similar study designs are actively recruiting and read-outs from these studies are eagerly awaited.

Metformin to treat endometrial cancer

Targeting tumour metabolism would seem a rational therapeutic intervention in cancers linked to obesity and insulin resistance. A comprehensive systematic review and meta-analysis of obesity and risk of all cancers found that endometrial cancer is the most strongly associated with obesity (Renehan et al., 2008). Excess body fat increased endometrial cancer risk in a dose-dependent manner, with every 5 kg/m² increase in BMI conferring a 1.6-fold (95% CI 1.5, 1.68) increased risk. This effect was observed even at extremely obese BMIs; indeed, a woman with a BMI of 42 has a near 10-fold increased risk of type I (also known as endometrioid) endometrial cancer than her normal weight counterparts (BMI 20–25 kg/m²) (Crosbie et al., 2010). Insulin resistance is also a recognized risk factor for type I endometrial cancer. Women with T2DM have a 2-fold increased risk of endometrial cancer compared with non-diabetic controls according to a large meta-analysis of 16 studies (relative risk (RR) 2.10, 95% CI 1.75–2.53) (Friberg et al., 2007). One prospective study of endometrial cancer patients found 30% had T2DM and a further 36% had previously undiagnosed insulin resistance, which was independently and significantly associated with increasing BMI (P < 0.001) (Burzawa et al., 2011). Large case–control studies have also shown that diabetes has a supramultiplicative effect with BMI on endometrial cancer risk (Sivalingam and Crosbie, 2013). Using non-diabetic, non-obese women as the referent group, the endometrial cancer risk was 1.4 (95% CI 0.9, 2.4) for non-obese diabetic women, 2.3 (95% CI 1.8, 3.0) for obese non-diabetic women, and 5.1 (95% CI 3.0–8.7) for obese diabetic women (Lucente-forte et al., 2007).

If obesity and insulin resistance are important drivers of endometrial cancer, metformin may inhibit tumour growth both by reducing its nutrient supply (glucose) and by thwarting its growth-stimulatory environment (reduced insulin and IGF levels) (Fig. 1). Thus metformin may be useful post-hysterectomy, in the adjuvant setting, to prevent recurrence and improve long-term survival from endometrial cancer. Animal studies have shown that combining metformin treatment with conventional cytotoxic agents can overcome tumour chemoresistance and inhibit emergence of cancer stem cells (Hirsch et al., 2009). Indeed, diabetic patients with breast cancer on metformin were shown to have higher rates of pathological complete response to neoadjuvant chemotherapy than those not on metformin, according to a retrospective study of 2529 patients, including 68 and 87 diabetic patients taking and not taking metformin, respectively (Jiralserspong et al., 2009). Whilst there are currently no studies testing metformin for endometrial cancer in the adjuvant setting, a phase III randomized trial of adjuvant metformin versus placebo in 3649 early-stage non-diabetic breast cancer patients is underway and due to report in 2016 (Goodwin et al., 2011). This study is powered to look at recurrence-free survival as the primary outcome measure.

Metformin to prevent endometrial cancer

Besides possible applications in the adjuvant setting, it is logical that metformin, by reducing the carcinogenic effects of obesity and insulin resistance, could be used as a long-term chemopreventative in women at high risk of endometrial cancer. Identification of a high risk group is important, and this may include women with PCOS, morbid obesity, impaired glucose tolerance and/or endometrial hyperplasia. Several case reports and one small randomized open label study have demonstrated resolution of endometrial hyperplasia following treatment with metformin, including two separately reported individual cases of progestin-resistant atypical hyperplasia (Session et al., 2003; Shen et al., 2008). Metformin treatment in a rat model reverses endometrial hyperplasia (Tas et al., 2013) possibly through a reduction in S6, a downstream kinase of the mTOR pathway (Fig. 2) (Erdemoglu et al., 2009). These data are
encouraging but a definitive chemoprevention trial of metformin in high risk women would require thousands of patients engaged in long-term follow-up if a clinical end-point (e.g. incident endometrial cancer) were used as the primary outcome measure. Initial proof of principle studies are therefore using surrogate markers of early clinical response as primary end-points, specifically effects on insulin signalling and cellular proliferation. One such study plans to recruit 100 obese, insulin-resistant (although not frankly diabetic) post-menopausal women aged 50–65 years to a study that compares the effects of metformin plus lifestyle changes (dietary advice and supervised exercise); placebo plus lifestyle changes; metformin alone; and placebo alone on endometrial Ki-67 expression and biomarkers of endometrial mTOR inhibition and insulin resistance (NCT01697566).

**Epidemiological evidence for metformin as anti-cancer drug**

Despite initial promise, the epidemiological data linking metformin use in T2DM patients to reduced cancer risk are inconsistent. One meta-analysis of eleven retrospective studies, 4042 cancer events and 529 cancer deaths, concluded that metformin reduces cancer risk by one-third (DeCensi et al., 2010). This effect was limited to certain cancer types, specifically pancreatic and hepatocellular cancer, with a non-significant reduction also observed in colon, breast and prostate cancer. A meta-analysis of fourteen RCTs did not concur with these findings, however, reporting no association between metformin use and cancer risk. The authors acknowledge that their findings are limited by the heterogeneity of the included trials, absent cancer data from two, and a relatively short follow-up period (average 4.1 years). Many of the studies involved treatment with several glucose-lowering agents in combination, and it is unclear whether other hypoglycaemics actually increase cancer risk, rather than metformin reducing it (Stevens et al., 2012). Those studies that specifically tested metformin monotherapy did find a non-significant 16% reduction in cancer risk in patients taking metformin, but CIs were wide due to small numbers in the subgroup analyses. A recent case–control analysis derived from the UK-based General Practice Research Database specifically explored the association between metformin use and endometrial cancer risk over a 17-year follow-up period. Comparing 2554 endometrial cancer cases with 15,324 age-matched controls, there was no effect of ever use of metformin (adjusted OR 0.65, 95% CI 0.63–1.18), nor was long-term metformin treatment (>25 prescriptions) associated with reduced endometrial cancer risk (adjusted OR 0.79, 95% CI 0.54–1.17) (Becker et al., 2013).

Other studies have investigated the effects of metformin on cancer survival rates. A retrospective cohort study of patients treated for type 2 endometrial cancer found improved survival rates among diabetics treated with metformin compared with those not using metformin and those without diabetes. This association was significant after adjusting for age, clinical stage, grade and adjuvant therapy. The lack of association between metformin use and overall survival in type 1 endometrial cancers may be explained by fewer deaths in this group (14% of patients with type 1 endometrial cancer died versus 52% of patients with type 2 disease) (Nevadunsky et al., 2013). Another study of similar design found improved recurrence-free and overall survival in metformin users with endometrial cancer, after controlling for age, stage, grade, histology and adjuvant treatment. Non-metformin users had 1.7-fold worse RFS (95% CI, 1.3–2.6, \( P = 0.01 \)), and were 2.3-fold more likely to die compared with metformin users (95% CI 1.3–4.2, \( P = 0.005 \)) (Ko et al., 2013).

**Putative anti-cancer mechanisms**

The mechanisms that underlie metformin’s putative anti-cancer activity are incompletely understood. Insulin and IGFs are strongly mitogenic and their stimulatory effect on cancer cell growth and metastasis is well established (Wang et al., 2012; Ferguson et al., 2013). Although normal target tissues (liver, skeletal muscle and adipose tissue) show reduced sensitivity to insulin in the context of hyperinsulinaemia, tumour cells may remain exquisitely sensitive to it and continue to respond to its stimulation (Pollak, 2012). High circulating insulin levels increase hepatic IGF-I production but reduce IGF binding protein synthesis, resulting in a net increase in circulating, bioavailable IGF-1. Specific interactions between insulin, IGF-1 and their respective receptors triggers a cascade of downstream events that ultimately drive cellular proliferation through activation of the PI3K-AKT-mTOR pathway (Fig. 3) (Pollak, 2008). The PI3K-AKT-mTOR pathway is frequently up-regulated in many cancers and is associated with resistance to chemotherapeutic drugs.

Metformin activates AMPK, a potent inhibitor of the PI3K-AKT-mTOR pathway. This has been demonstrated in numerous pre-clinical cancer models, including endometrial cancer cell lines, where a supratherapeutic concentration of metformin stops cellular proliferation, induces phosphorylation of AMPK, and reduces S6 phosphorylation, a downstream target of the mTOR pathway (Fig. 3). Metformin was shown to induce GI cell cycle arrest, increase apoptosis and reduce human telomerase reverse transcriptase expression (Cantrell et al., 2010; Sarfstein et al., 2013). These findings were confirmed in a mouse xenograft model where metformin reduced S6 phosphorylation and decreased mean tumour weight (Iglesias et al., 2013).

Recent studies have demonstrated that metformin also inhibits mTOR through AMPK-independent pathways, including the Rag family of GTPases (Kalender et al., 2010) and the hypoxia inducible factor (HIF) target gene, regulated in development and DNA damage response 1 (REDD1) (Ben Sahra et al., 2008, 2011). Iglesias et al. (2013) showed that treating endometrial cancer cells with metformin causes displacement of constitutively active KRAS from the cell membrane resulting in uncoupling of the mitogen-activated protein kinase (MAPK) signalling pathway (Fig. 3) (Iglesias et al., 2013). Additional anti-cancer activity that may be especially relevant in endometrial carcinogenesis includes anti-aromatase activity (Brown et al., 2010), leading to a reduction in circulating estrogen levels in obese women, and increased progesterone receptor expression by endometrial cancer cells (Xie et al., 2011). Metformin also has anti-angiogenic effects (Liao et al., 2012), directly scavenges free radicals and can block endogenous reactive oxygen species. In an in vitro model, the latter effects significantly reduced DNA damage and mutation rates (Fig. 2) (Aligre et al., 2012), offering an explanation for the reduced risk of cancer seen in metformin users across several epidemiological studies.

**Metformin to treat ovarian cancer**

Whilst the association with obesity is less pronounced than it is for endometrial cancer, a systematic review found ovarian cancer was slightly more common in obese women (BMI > 30 kg/m²; OR 1.3, 95% CI 1.1–1.5) (Joly et al., 1974) and a prospective cohort study showed...
increased mortality from ovarian cancer amongst overweight (RR 1.16, 95% CI 1.04–1.30) and obese women (RR 1.26, 95% CI 1.07–1.48) compared with normal weight women (Rodriguez et al., 2002). Furthermore, a recent systematic review and meta-analysis of 19 studies found an increased risk of ovarian cancer in diabetic women (RR 1.17, 95% CI 1.02–1.33), which persisted after adjusting for age, BMI, smoking and alcohol intake (RR 1.55, 95% CI 1.11–2.19) (Lee et al., 2013).

The effect of metformin on ovarian cancer risk has been studied in a retrospective case–control analysis, where long-term use of metformin was associated with lower rates of ovarian cancer (OR 0.61, 95% CI 0.3–1.25) (Bodmer et al., 2011). A further case–control analysis found an association between metformin and improved survival from ovarian cancer (5-year disease-specific survival for cases versus controls, 73 versus 44%; \( P = 0.002 \)). When considering epithelial ovarian cancer alone, and after adjusting for age, stage, optimal cytoreduction, serous histology and platinum chemotherapy, women taking metformin had improved 5-year survival rates compared with controls (67 versus 47%; \( P = 0.007 \)) (Kumar et al., 2013).

Proof of principle window studies to assess the effects of metformin treatment prior to definitive surgery are more challenging in the context of ovarian cancer, where diagnosis is suspected but not confirmed in the majority until laparotomy. Taking a biopsy from an apparently early-stage ovarian cancer could result in spillage of malignant cells into the abdominal cavity, upstage the patient and mandate adjuvant chemotherapy, where this may have been avoided. However, a study using metformin in combination with (neo)adjuvant chemotherapy and
<table>
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<tr>
<th>Pathophysiological driver</th>
<th>Effect of metformin</th>
<th>Clinical impact</th>
<th>Proposed mechanism of action</th>
<th>References</th>
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<tr>
<td><strong>Insulin resistance</strong></td>
<td>Polycystic ovary syndrome</td>
<td>↓ Insulin resistance, ↓ Blood insulin and glucose</td>
<td>↓ Ovulation and live birth rate in obese women with PCOS treated with clomiphene and undergoing IVF, ↓ ↓ Risk of T2DM</td>
<td>Attia et al. (2001), Brewer et al. (2010), De Leo et al. (2000), Diamanti-Kandarakis et al. (2010), Morin-Papunen et al. (2012), Nestler and Jakubowicz (1996), Tang et al. (2006a, b)</td>
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<td>↓ CYPI7 activity and androgen production, ↓ IGF-1 by ↑ IGFBP1 production</td>
<td>Ali et al. (2011), Balani et al. (2009), Catalano et al. (1991), Hague et al. (2003), Lautatzi et al. (2013), Nerstedt et al. (2010), Niromanes et al. (2012), Rowan et al. (2008), Rowan et al. (2011), Studwoll et al. (1995), Bonanni et al. (2012), Cantrell et al. (2010), Erdemoglu et al. (2009), Hadad et al. (2011), Niraula et al. (2012), Session et al. (2003), Shen et al. (2008), Tas et al. (2013), Zakikhani et al. (2006), Tang et al. (2006a, b), Tso et al. (2009)</td>
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<td><strong>Pregnancy</strong></td>
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<td>↓ Maternal weight gain and neonatal hypoglycaemia in GDM and obesity, ↓ ↓ Insulin resistance in the infant of obese and diabetic mothers with possible metabolic reprogramming of the infant</td>
<td>Attia et al. (2001), Brewer et al. (2010), De Leo et al. (2000), Diamanti-Kandarakis et al. (2010), Morin-Papunen et al. (2012), Nestler and Jakubowicz (1996), Tang et al. (2006a, b)</td>
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<td>↓ Hepatic glucose output and substrate activation of IR/IGF1R, ↑ AMPK activation and mTOR inhibition</td>
<td>Ali et al. (2011), Balani et al. (2009), Catalano et al. (1991), Hague et al. (2003), Lautatzi et al. (2013), Nerstedt et al. (2010), Niromanes et al. (2012), Rowan et al. (2008), Rowan et al. (2011), Studwoll et al. (1995), Bonanni et al. (2012), Cantrell et al. (2010), Erdemoglu et al. (2009), Hadad et al. (2011), Niraula et al. (2012), Session et al. (2003), Shen et al. (2008), Tas et al. (2013), Zakikhani et al. (2006), Tang et al. (2006a, b), Tso et al. (2009)</td>
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<td><strong>Anti-cancer</strong></td>
<td>Prevention and treatment</td>
<td>↓ Tumour growth, ↑ Reverses endometrial pre-cancerous change</td>
<td>↓ PI3K/AKT/mTOR pathway activation, ↓ MAPK/ERK pathway activation</td>
<td>Attia et al. (2001), Brewer et al. (2010), De Leo et al. (2000), Diamanti-Kandarakis et al. (2010), Morin-Papunen et al. (2012), Nestler and Jakubowicz (1996), Tang et al. (2006a, b)</td>
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<td>↓ Response of polycystic ovaries to exogenous stimulation, ↓ Secretion of VEGF</td>
<td>Attia et al. (2001), Brewer et al. (2010), De Leo et al. (2000), Diamanti-Kandarakis et al. (2010), Morin-Papunen et al. (2012), Nestler and Jakubowicz (1996), Tang et al. (2006a, b)</td>
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<td><strong>Inflammation and oxidative stress</strong></td>
<td>Polycystic ovary syndrome</td>
<td>↓ Incidence of severe OHSS in women with PCOS undergoing IVF</td>
<td>↓ Response of polycystic ovaries to exogenous stimulation, ↓ Secretion of VEGF</td>
<td>Attia et al. (2001), Brewer et al. (2010), De Leo et al. (2000), Diamanti-Kandarakis et al. (2010), Morin-Papunen et al. (2012), Nestler and Jakubowicz (1996), Tang et al. (2006a, b)</td>
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<td>↓ Maternal inflammatory cytokines (IL-6, CCL2, TNFα), ↓ TNFα induced NFκB activation</td>
<td>Attia et al. (2001), Brewer et al. (2010), De Leo et al. (2000), Diamanti-Kandarakis et al. (2010), Morin-Papunen et al. (2012), Nestler and Jakubowicz (1996), Tang et al. (2006a, b)</td>
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<td><strong>Pregnancy</strong></td>
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<td>Alters placental development and function, ↓ Vascular endothelial activation, ↑ Improve pregnancy outcomes</td>
<td>Attia et al. (2001), Brewer et al. (2010), De Leo et al. (2000), Diamanti-Kandarakis et al. (2010), Morin-Papunen et al. (2012), Nestler and Jakubowicz (1996), Tang et al. (2006a, b)</td>
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<td><strong>Anti-cancer</strong></td>
<td>Prevention and treatment</td>
<td>↓ Migration and metastasis, ↓ DNA damage and mutation rates</td>
<td>↑ Anti-angiogenic effects and ↑ REDD1 expression, Blocks production of endogenous reactive oxygen species</td>
<td>Attia et al. (2001), Brewer et al. (2010), De Leo et al. (2000), Diamanti-Kandarakis et al. (2010), Morin-Papunen et al. (2012), Nestler and Jakubowicz (1996), Tang et al. (2006a, b)</td>
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<td>↑ Anti-angiogenic effects and ↑ REDD1 expression, Blocks production of endogenous reactive oxygen species</td>
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<td><strong>Cancer stem cells</strong></td>
<td>Anti-cancer drug Treatment</td>
<td>↑ Improves efficacy of existing chemotherapy, Overcomes chemoresistance</td>
<td>↑ Inhibits re-emergence of cancer stem cells</td>
<td>Attia et al. (2001), Brewer et al. (2010), De Leo et al. (2000), Diamanti-Kandarakis et al. (2010), Morin-Papunen et al. (2012), Nestler and Jakubowicz (1996), Tang et al. (2006a, b)</td>
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Abbreviations: AMP-activated kinase (AMPK), chemokine (CC-motif) ligand 2 (CCL2), cytochrome P450c17 enzyme (CYPI7), insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein-1 (IGFBP-1), insulin-like growth factor receptor 1 (IGFIR), interleukin-6 (IL-6), insulin receptor (IR), mammalian target of rapamycin (mTOR), mitogen-activated protein kinase /extracellular receptor kinase (MAPK/ERK), NF-kappa beta (NFκB), ovarian hyperstimulation syndrome (OHSS), polycystic ovary syndrome (PCOS), phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), regulated in development and DNA damage responses 1 (REDD1), tumour necrosis factor alpha (TNFα), type 2 diabetes mellitus (T2DM).
surgical debulking in ovarian cancer is actively recruiting (NCT01579812). The hypothesis is that metformin will act as an anti-cancer stem cell agent, enhancing the efficacy of (neo)adjuvant chemotherapy by preventing the emergence of chemoresistant clones.

Conclusions

Metformin has several established and emerging applications in reproductive healthcare, pregnancy and gynaecological cancer (Table 1). Its recognized impact as an insulin sensitizing drug has been exploited in the treatment of GDM and for cardiovascular risk reduction in PCOS and obesity. Whilst its effects on fertility in PCOS is uncertain, it remains a promising candidate for further scrutiny because of its affordability and low toxicity profile. Its extensive safety credentials make it an obvious choice for application in pregnancy, where it can be difficult to establish safety parameters of new drugs. In obese pregnant women, metformin may not only improve maternal outcomes, but also initiate favourable metabolic reprogramming in the fetus. In addition to these indications, epidemiological evidence as well as pre-clinical and early phase clinical trials have indicated a role for metformin in the prevention and treatment of cancer. Its potential in this setting is unproven but extremely promising.

Authors’ roles

All authors contributed to the conception and design of this review and drafted the manuscript. Critical revisions were finalized by V.N.S. and E.J.C. The final version has been approved by all authors.

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Conflict of interest

V.N.S., J.M., S.N., A.H.B. and E.J.C. report no conflicts of interest.

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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 progesterone 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 recommenced 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-url)
The effect of presurgical metformin in endometrial cancer

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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 homeostasis 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. p-AKT, p-ACC, p-4EBP1, ER and PR staining was assessed 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.

Enzyme-linked immunosorbent 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 assessed 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.
RESULTS

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 \(-0.66\) to \(-0.13\), \(P = 0.006\)), 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 \(-27.4\%\) to \(-7.0\%\)), \(P = 0.002\)) 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 observed in 23 out of 28 (82%) women in the metformin group (range \(-4\) to \(-55\%\)); the remaining five (18%) showed static or higher Ki-67 levels (range \(0.6\%\)–\(-14\%\)). There was some evidence of an association between the average metformin dose received and change in Ki-67 (Spearman’s correlation coefficient \(r = -0.37\), 95% CI \(-0.66\) to \(-0.01\), \(P = 0.051\); Supplementary Figure S3A). A nonsignificant correlation was also observed between baseline BMI and Ki-67 proliferation index change with metformin (Spearman’s correlation coefficient \(r = 0.37\), 95% CI \(-0.02\) to 0.66, \(P = 0.054\); Supplementary Figure S3B), with leaner women showing a greater Ki-67 change post-treatment with metformin than obese women. Five of 12 (42%) untreated women showed modest reductions in Ki-67 between baseline and hysterectomy (range \(-1\) to \(-7\%\)), but Ki-67 remained static or increased in most (7 out of 12 women (58%) showed a 1–28% increase in Ki-67).

The interval between baseline and hysterectomy was similar for metformin-treated and -untreated women (median 30 days (IQR 22, 43) vs 34 days (IQR 28, 39), respectively). The diagnostic biopsy was used as the baseline sample when a recruitment biopsy was not available. When postintervention change in Ki-67 was assessed using diagnostic biopsies as the baseline sample for all patients (n = 40), the reduction in Ki-67 in metformin-treated women remained significant (mean adjusted difference \(-16\%\) (95% CI \(-27\), \(-5\%\)), \(P = 0.005\)).

Effects of metformin on markers of obesity and insulin resistance. Short-term metformin was associated with improvements in serum markers of insulin resistance and adiposity (median 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).

Effects of metformin on phosphorylated mTOR proteins, markers of apoptosis and hormone receptor expression. There were global reductions in the expression of phosphorylated mTOR pathway proteins in both groups. Figure 3 shows phosphoryl-ATK, phospho-ACC, phospho-S6 and phospho-4EBP1 expression in metformin-treated and -untreated patients at hysterectomy compared with baseline. p-4EBP1 expression was significantly lower in the metformin-treated patients compared with the untreated group (mean adjusted difference in modified H-score of \(-2.30\) (95% CI \(-4.61\), \(-0.06\), \(P = 0.045\)). The change in expression of the other phosphorylated mTOR pathway proteins was not statistically significant for treatment effect.

The baseline rate of apoptosis was very low (mean positive index 0.01 and 0.003 in metformin-treated and -untreated patients, respectively). We found no correlation between apoptotic index and grade of tumour, but there were very few grade 3 tumours (n = 4 out of 40). The apoptotic index remained stable over time in both groups; there was no significant effect of treatment (mean adjusted difference 0.00052, 95% CI \(-0.0015\), 0.0025, \(P = 0.608\), not significant). Oestrogen receptor and PR expression was lower in the hysterectomy specimen compared with the diagnostic biopsies of women from both groups. There was no significant effect of treatment.

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 P13K-ATK-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; Croeslie 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
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<th>Table 1. Baseline patient and tumour characteristics at recruitment.</th>
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<td>61–70</td>
</tr>
<tr>
<td>&gt;80</td>
</tr>
<tr>
<td>Body mass index (kg m⁻²)</td>
</tr>
<tr>
<td>&lt;25</td>
</tr>
<tr>
<td>25–29.9</td>
</tr>
<tr>
<td>&gt;30–39.9</td>
</tr>
<tr>
<td>Waist/hip girth ratio</td>
</tr>
<tr>
<td>Smoking habits</td>
</tr>
<tr>
<td>Nonsmoker</td>
</tr>
<tr>
<td>Ex-smoker</td>
</tr>
<tr>
<td>Current smoker</td>
</tr>
<tr>
<td>Daily alcoholic units</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1–2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>HOMA-IR index</td>
</tr>
<tr>
<td>Insulin resistance (HOMA-IR &gt;2.8)</td>
</tr>
<tr>
<td>Tumour grade at hysterectomy</td>
</tr>
<tr>
<td>AEH</td>
</tr>
<tr>
<td>G1</td>
</tr>
<tr>
<td>G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
<tr>
<td>FIGO stage at hysterectomy</td>
</tr>
<tr>
<td>1A</td>
</tr>
<tr>
<td>1B</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>Lymphovascular space invasion present</td>
</tr>
<tr>
<td>Myometrial invasion</td>
</tr>
<tr>
<td>&lt;50%</td>
</tr>
<tr>
<td>&gt;50%</td>
</tr>
<tr>
<td>Follow-up and adjuvant therapy</td>
</tr>
<tr>
<td>Clinical follow-up*</td>
</tr>
<tr>
<td>Vaginal brachytherapy</td>
</tr>
<tr>
<td>External beam radiotherapy</td>
</tr>
<tr>
<td>External beam radiotherapy and chemotherapy</td>
</tr>
<tr>
<td>Chemotherapy alone*</td>
</tr>
<tr>
<td>ER expression</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>PR expression</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>PTEN expression</td>
</tr>
<tr>
<td>Wild type</td>
</tr>
<tr>
<td>Mutant</td>
</tr>
<tr>
<td>PS3 expression</td>
</tr>
<tr>
<td>Wild type</td>
</tr>
<tr>
<td>Mutant</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. The italic entries show that certain figures are the median and IQR, whereas the other figures are the mean and s.d.

* Wilcoxon’s rank-sum test used to compare baseline characteristics in metformin-treated and controls.

** Two control patients were excluded as the final histology was atypical endometrial hyperplasia.

*** Two controls did not have cancer in the final hysterectomy specimen and were discharged from clinical follow-up post-surgery.

**** Two 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.
The effect of presurgical metformin in endometrial cancer

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 μmol 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

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>
<th>Patients who received at least one dose of metformin</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients who received at least one dose of metformin</td>
<td>35</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Patients who developed any AEs</td>
<td>27</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Number of AEs</td>
<td>98</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Grade 1 AE</td>
<td>94</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Grade 2 AE</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Grade 3 AE</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Mean 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)

| Grade 3 AE | 1 | 1 |
| Grade 1 AE | 94 | 96 |

Table 3. Change from baseline following intervention

<table>
<thead>
<tr>
<th>Table 3. Change from baseline following intervention</th>
<th>Metformin</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>Post-treatment</td>
<td>Pretreatment</td>
</tr>
<tr>
<td>Tumour and metabolic parameters</td>
<td>Mean (S.d.)</td>
<td>Median (Q1, Q3)</td>
</tr>
<tr>
<td>K-Cr proliferation index</td>
<td>%</td>
<td>50.9 (1.1)</td>
</tr>
<tr>
<td>Body mass index</td>
<td>kg m⁻²</td>
<td>35.1 (11.3)</td>
</tr>
<tr>
<td>Waist/hip girth ratio</td>
<td></td>
<td>0.9 (0.1)</td>
</tr>
<tr>
<td>Glucose</td>
<td>mmol l⁻¹</td>
<td>6.0 (1.5)</td>
</tr>
<tr>
<td>Insulin</td>
<td>mU l⁻¹</td>
<td>16.0 (9.4)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td></td>
<td>4.4 (2.8)</td>
</tr>
<tr>
<td>C-peptide</td>
<td>pmol l⁻¹</td>
<td>1076.1 (482.3)</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>mg l⁻¹</td>
<td>3.3 (1.5)</td>
</tr>
<tr>
<td>Leptin</td>
<td>ng ml⁻¹</td>
<td>54.1 (42.6)</td>
</tr>
<tr>
<td>Ln (hsCRP)</td>
<td>mg l⁻¹</td>
<td>1.3 (1.3)</td>
</tr>
</tbody>
</table>

Abbreviations: ANCOVA = analysis of covariance; BMI, body mass index; CI, confidence interval; HOMA-IR = homeostatic 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, 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 interindividual 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

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