Racial differences in the molecular profile of endometrial carcinoma

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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April 2020

Abstract

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Endometrial cancer (EC) is the commonest gynaecologic malignancy in developed countries and its incidence is rising. Although excellent prognosis is associated with early stage disease, treatment for late stage cases is challenging and the treatment optionslimited. Racial differences have been shown in Black and Caucasian women diagnosed with EC however, evidence for other racial groups is limited with Asian patients being particularly under-represented in the literature.

The aims of the study were to propose effective targeted drug treatments suitable for subsequent testing in animal models of EC and to identify differences in the mutational profile of endometrial tumours between British White (BW) and South Asian (BSA) women.

CUDC-907, a PI3K and HDAC inhibitor, was the most effective monotherapy treatment in EC cells. Several combination treatments showed synergism and efficacy in EC cell lines with the most efficacious being CUDC-907 and S63845 (MCL1 inhibitor) combined with the MEK inhibitor PD0325901.

Although there was no significant difference in the overall mutation frequency of the 10 genes analyzed, numerous differences were observed between the two racial groups. PIK3CA and PTEN mutations were positively associated in the BSA but not in the BW group. On the contrary, BW women had co-existent ARID1A and PI3K pathway mutations, which was not shown in BSA women. BSA women had higher grade disease in our cohort and survival data are eagerly anticipated.

A range of targeted inhibitory drugs emerged from this study showing *in vitro* efficacy, alone or in combination, in endometrial cancer cells. Further validation of these therapeutic options in animal models is needed prior to confirming their suitability for use in EC. This study, the first to attempt a comparison between a Caucasian and South Asian cohort of women in genes frequently driving carcinogenesis in EC, showed several differences with translational and clinical relevance.

Acknowledgements

I'm submitting to you the results of my work during the previous four years. And although the final judgement of this work is deservedly yours, I must fulfil an important task; Thanking those who helped me to get through these challenging but fruitful years.

Firstly, my family and in particular my wife, Eva. She is the rock of my family, wonderful mother of our two children and the best partner I could ever imagine. I cannot recall a single time that she moaned at me for being isolated in a room, analyzing results and writing up my thesis. Always there for me and our little ones, both of whom born during these 4 years. I would have never made it without her. I also want to thank my 3-year old daughter, Eleni, for understanding that dad was busy and patiently waiting until he had some time to spend with her.

I'm grateful to my PhD supervisor, Dr Esther Moss. She was the first one to believe that a guy who has never been in a lab before, could be committed and dedicated enough to complete an ambitious project. She was always helpful, approachable and encouraging to me. She dealt with my insecurities and doubts and helped me to enter the next level of my clinical career, a Fellowship in Gynaecologic Oncology. Above all, she still is a great mentor to me.

I want to thank my PhD supervisor, Dr Salvador Macip for all the advice, the encouragement, the positive attitude and the knowledge he freely shared with me. I'm grateful for his patience and his generosity, both in terms of time spending with me and kind words. Maybe more importantly, I thank him for the philosophical, political and sociological discussions we had which taught me, amongst others, that a true scientist has a duty to share his knowledge and expertise to the many and especially to the young generation.

I owe a large debt to Dr David Guttery. Without his expertise in Next Generation Sequencing, it would be impossible to successfully complete this part of the project. His accessibility, politeness and knowledge had a great impact on this study.

I’m grateful to Professor Paul Symonds and Dr Meike Vogler, my committee members monitoring my progress and for their insight and advice.

I’m thankful to Dr Akang Ekpenyong-Akiba, my friend and colleague in the lab. We started our PhD almost simultaneously and since the first day she was teaching me experimental techniques and science methodology. I owe her a great deal.
I’m also grateful to my other labmates, Dr Ghalia Shelmani, Dr Miran Rada, Dr Yixiang Chen, Dr Mohammad Althubiti, Sandra Germano, Dr Marwah Maashi and Gabriella Kocsis-Fodor for all their support, guidance and advice.
I’m thankful to my newer colleagues, Victoria Smith, Antonella Tabasso, Marta Poblocka, Laura Castilla Vallmanya and Dr Diana Kluczna. Their hard work was inspirational and the pleasant atmosphere they created in the lab made it an ideal place to work. I’m grateful to Yoel Sanchez Molina, the brilliant student who helped me for a few months by diligently performing dozens of MTS assays for his and my project.
I want to thank Dr Richard Kelly, Dr Grace Hodson, Dr Emma Kelsall and Dr Claire Barnes for the great interaction we had over the course of these three years.

I want to express my gratitude to Dr Catherine Moreman, Dr Elizabeth Stannard, Dr Rachael Bishop and Dr Roger Hew from the Pathology department in Leicester Royal Infirmary for their unconditional support in providing tumour samples and reviewing immunohistochemistry slides for our study.
I want to thank Nalini Foreman, a skillful technician helping us with collection of plasma samples and DNA extraction for future parts of our project.

Finally, I’m grateful to my good friends Dr Aditya Chandru and Dr Pooyeh Lopu Farahmand for all their help and support which went beyond the boundaries of the lab and extended into a good friendship.

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AA	African American
AKT	Protein kinase B
AML	Acute myelogenous leukemia
APS	Ammonium persulfate
ASA	American Society of Anaesthesiologists
Bcl-2	B-cell lymphoma 2 protein
BET	Bromodomain and extraterminal
BMI	Body mass index
BSA	British South Asian
BSO	Bilateral salpingo-oophorectomy
BTK	Bruton's tyrosine kinase
BW	British White
CAH	Complex atypical hyperplasia
CDK	Cyclin-dependent kinase
CI	Calculation Index
CLL	Chronic lymphocytic leukemia
CR	Complete response
CT	Computed tomography
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
DSPc	Dual specificity phosphatase catalytic
EC	Endometrial cancer
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase

ESGO	European Society of Gynaecological Oncology
ESMO	European Society of Medical Oncology
ESTRO	European Society for Radiotherapy & Oncology
FBS	Fetal Bovine Serum
FFPE	Formalin Fixed Paraffin Embedded
GCLC	Glutamate-cysteine ligase synthetase catalytic
GOG	Gynecologic Oncology Group
GPCR	G-protein-coupled receptors
GSK	Glycogen synthase kinase
HDAC	Histone deacetylase
HNPCC	Hereditary non-polyposis colorectal cancer
HRT	Hormone replacement therapy
Hsp	Heat Shock Protein
IC50	The concentration of a drug that is required for 50% inhibition in vitro
IHC	Immunohistochemistry
JAK	Janus-associated kinase
LS	Lynch syndrome
LVSI	Lympho-vascular space involvement
MAPK	Mitogen-activated protein kinase
MCL1	Myeloid cell leukemia 1
MCT1	Monocarboxylate transporter 1
MI	Myometrial invasion
MIS	Minimally invasive surgery
MSI	Microsatellite instability
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
mTOR	mammalian target of rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing

NHL Non-Hodgkin lymphoma

NIDDM Non-Insulin Dependent Diabetes Mellitus

NSCLC Non-small cell lung cancer

ORR Objective response rate

PCOS Polycystic ovarian syndrome

PD-1 Programmed cell death protein 1

PDK1 Phosphoinositide-dependent kinase-1

PD-L1 Programmed death-ligand 1

PFS Progression-free survival

PI Propidium Iodide

PI3K Phosphoinositide 3-kinase

PIP2 Phosphatidylinositol 4,5-bisphosphate

PIP3 Phosphatidylinositol 3,4,5-triphosphate

PP2A Protein phosphatase 2

PR Partial response

RCT Randomized controlled trial

RFS Recurrence-free survival

RIPA Radio immunoprecipitation assay

RTK Receptor tyrosine kinase

SCNA Somatic copy number alterations

SDS Sodium Dodecyl Sulfate

SEER Surveillance, Epidemiology and End Results database

T-ALL T-cell acute lymphoblastic leukemia

TEMED Tetramethylethylenediamine

T-LBL T-cell lymphoblastic lymphoma

VEGF Vascular endothelial growth factor

Chapter 1 Introduction

1.1 Endometrial cancer

1.1.1 Epidemiology

Endometrial cancer (EC) is the most common gynaecologic malignancy in the developed world. In the UK, 9,538 new cases were diagnosed in 2016 (Cancer Research UK. 2019). Worldwide, there were an estimated of 382,069 new cases with 89,929 deaths in 2018 and EC is the second most common gynaecological cancer after cervical (Bray *et al.*, 2018). The incidence has risen in the UK by more than 50% in the last 20 years (Office for National Statistics, 2016) and it is projected to rise even more (Mistry *et al.*, 2011). Unlike most other cancers, in which mortality is falling with time, in the UK mortality rates from EC have plateaued over the last four decades (6.6 per 100,000 population in 1975 versus 7.1 in 2016) (Cancer Research UK. 2019) despite advances in the diagnosis and surgical management of the disease. This confirms data from the USA, which show that endometrial and cervical cancer are the only malignancies for which the five-year survival has decreased when comparing the 1975-77 period with the 2006-12 (Ahmedin Jemal et al, 2017).

1.1.2 Histological and Molecular Classification

The histological subtypes of endometrial cancer are shown in Table 1.1 below.
Traditionally, EC is classified as type I and type II (Bokhman, 1983). Type I is histologically an endometrioid adenocarcinoma and is associated with a hyper-estrogenic state. It accounts for more than 80% of cases. Patients with this type of cancer are typically obese and often have cardio-metabolic syndrome (diabetes, heart disease and dislipaedemia). It is usually preceded by endometrial hyperplasia and has a very favourable prognosis if diagnosed early. Type II is not associated with obesity and oestrogen exposure, hence the endometrium is usually atrophic. The histological subtypes are serous or clear cell and are associated with a poor prognosis.

Histological subtypes
Endometrioid adenocarcinoma
Mucinous carcinoma
Serous carcinoma
Clear cell carcinoma
Squamous cell carcinoma
Undifferentiated carcinoma
Mixed carcinoma
Metastatic carcinoma

Table 1.1 Histological subtypes of endometrial cancer (Gordon & Ireland, 2009).

Newer molecular studies have attempted to re-classify endometrial cancer. The most significant of them (TCGA, 2013) identified four distinct groups: a POLE ultra-mutated group, a group characterized by microsatellite instability (MSI) and two groups divided by the number of somatic copy number alterations (SCNAs) to low (where most of the endometrioid histology cases belong) and high (where most serous cases belong). The paradigm shift of this new categorization of endometrial cancer is that it 'cuts through' Bokhman's binary model and identifies previously regarded as 'Type 1' cases (endometrioid histology) which 'behave' similarly to 'Type 2' tumours (mostly serous histology) and encompasses them in the SCNAs high group. The remaining three groups, although they consist mostly of endometrioid tumours, have very different prognosis: Excellent for the POLE ultra-mutated cohort, much worse for the SCNAs low group. The implementation of this new molecular characterization of endometrial cancer in current clinical practice could eventually alter the treatment strategies used and will unavoidably lead to a more 'personalised' cancer therapy.

1.1.3 Aetiology-Pathophysiology

Obesity is the most significant risk factor contributing to the rise in the prevalence of EC. In the last 20 years, there has been a continuous increase in the incidence of obesity in the UK and currently 27% of women are obese (BMI>30) as compared to 16% in 1993 (more than 68% rise in the last 22 years) (Health survey for England. 2015). Obese women,

particularly post-menopausal, have higher circulating levels of oestrogens, causing chronic endometrial stimulation which subsequently leads to endometrial hyperplasia and cancer (Kirschner *et al.*, 1982). The mechanism behind the hyperestrogenemia observed in obese women is the peripheral aromatization of androgens to oestrogens, taking place in the adipose tissue.

Increased life expectancy plays a crucial role in the rising incidence of all cancers. Women in the UK live longer compared to the past, with life expectancy having increased from 76.8 years (1980-82) to 82.9 (2015-17) (Office for National Statistics, 2018). Cancer is, partly, a disease of aging, hence the projections are that EC will follow the trend of life expectancy and will increase even further in the future. Mistry projected that in 2030 there will be 45% more EC cases in the UK compared to 2007, an annual increase of 1.6% (Mistry *et al.*, 2011).

Tamoxifen use is another well-established risk factor for EC. Although it is used for the treatment of breast cancer due to its anti-estrogenic activity, it acts as an oestrogen agonist in the endometrium. The reason for this differential action is unclear but thought to be associated with the expression of its co-regulator proteins (Yongfeng Shang & Myles Brown, 2002). Its use in the UK has significantly increased over time, which correlates with the large increase in the incidence of breast cancer (27% increase between 1993 and 2014) (Cancer Research UK. 2019). Tamoxifen use increases the risk of EC by 2-3 times (Fisher *et al.*, 1998; Wickerham *et al.*, 2002).

Nulliparity is an independent risk factor for the development of EC and increases in the UK (18% of women born in the UK in 1971 have no children compared to 11% of women born in 1944) (Office for National Statistics, 2018). Recent studies (H. P. Yang *et al.*, 2015) have confirmed this causal association and large meta-analysis have shown that there is an inverse, non-linear relationship between the number of children a woman has and her lifetime risk of developing endometrial cancer (Q. Wu *et al.*, 2015). The likely reason behind this inverse association is the prolonged, protective progestogenic stimulus to the endometrium associated with pregnancy.

Oestrogen-only hormone replacement therapy (HRT) is a well-known risk factor for EC (Valerie Beral *et al.*, 2005a), although its use nowadays is almost non-existent in non-

hysterectomized women. Tibolone, a drug used for the management of menopausal symptoms, also increases the risk of EC. Continuous combined HRT has been shown to reduce the risk of EC in obese women (BMI>30) but does not have a similar protective effect in women of normal weight (Valerie Beral *et al.*, 2005b). Similarly, cyclic combined HRT also reduces the risk of EC in obese women but, unlike continuous preparations, actually increases the risk of EC in women of normal BMI (<25) (relative risk 1.54, CI 1.20-1.99) (Valerie Beral *et al.*, 2005b).

Hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) is an autosomal dominant condition which increases the risk of developing one or more of several cancers at young age, most notably colorectal and EC. The lifetime risk of developing EC is reported to range between 27-71% (Hendriks *et al.*, 2004) depending on the exact mutation of the mis-match repair (MMR) deficiency genes. The risk is higher with *MLH1* and *MSH2* mutations, followed by *MSH6* while it is lower with *PMS2* mutation (Steinke *et al.*, 2013). Patients with Lynch syndrome are diagnosed with endometrial cancer on average at the age of 53.4 years as opposed to 63.6 years for EC patients without MMR deficiency (Karamurzin & Rutgers, 2009).

Polycystic ovarian syndrome (PCOS) with its associated oligomenorrhea and anovulatory cycles renders the endometrium susceptible to long periods of oestrogenic stimulation without the protective influence of progesterone. This results in a three-fold increased risk of EC later in life (Haoula *et al.*, 2012).

The association of diabetes with EC is well established as the incidence of EC among diabetic women is higher (Jakob Starup-Linde *et al.*, 2013). Diabetic patients are more obese, which predisposes to EC. Whether there is a further causal relationship, though, is still debatable, as large studies failed to show higher incidence among diabetics when controlled for BMI (J Luo *et al.*, 2014). However, it has been shown that hyper-insulinaemia and the associated mitogenic activity increases the risk of endometrial cancer (Marc J. Gunter *et al.*, 2008).

The fall in the hysterectomy rates in the UK has been implicated, at least partly, for the increasing rates of EC. There was a 64% decline in the number of hysterectomies performed in the NHS for menorrhagia between 1989-90 and 2002-3 (Reid & Mukri,

2005) and the rates kept declining in the next decade, at a slower pace though (Mukhopadhaya & Manyonda, 2013).

1.1.4 Symptoms-Diagnosis

The most common symptom leading to the diagnosis of EC is unscheduled vaginal bleeding. This usually occurs in the menopause and prompts medical investigations which for most patients consist of an ultrasound scan that measures the endometrial thickness. The accuracy of the scan is higher when the transvaginal approach is being used as a transabdominal probe is at greater distance from the uterus, particularly in obese patients, which constitute the majority of EC cases. Depending on the scan result, a hysteroscopy and endometrial biopsy might be needed. In pre-menopausal women, common symptoms seen prior to diagnosis include intermenstrual bleeding, menorrhagia, abnormal vaginal discharge and rarely fatigue and anaemia. After diagnosis has been established, an MRI scan can be a useful tool in the preoperative staging of the disease as it can identify with great accuracy the degree of myometrial invasion and serosal, adnexal and pelvic nodal involvement. On the contrary, CT is not as sensitive as MRI in identifying the degree of myometrial invasion. Its use in EC is limited in the staging of patients with advanced disease as it can detect peritoneal spread and distant metastasis (Olawaiye et al, 2017).

1.1.5 Staging

The FIGO staging of EC has been amended (Pecorelli, 2009) and is presented below in Table 1.2.

Stage	Criteria
0	Carcinoma in situ
I • IA • IB	Tumour confined to uterine body • Tumour limited to endometrium or involves <50% of myometrium • Tumour invades >50% of myometrium
II	Tumour invades cervical stroma, does not extend beyond uterus
III • IIIA • IIIB • IIIC i) IIIC1 ii) IIIIC2	Local and/or regional spread of tumour • Tumour invades serosa of uterine body and/or adnexae • Vaginal and/or parametrial involvement • Metastases to pelvic and/or para-aortic lymph nodes i) Positive pelvic nodes ii) Positive para-aortic lymph nodes with/without positive pelvic lymph nodes
IV • IVA • IVB	Tumour invades bladder mucosa and/or bowel mucosa and/or distant metastases • Tumour invasion of bladder and/or bowel mucosa • Distant metastases, including intra-umbilical metastases and/or inguinal nodes

Table 1.2 Staging of endometrial cancer

In December 2014, a joint conference was held in Milan and representatives of the European Society of Medical Oncology (ESMO), European SocieTy for Radiotherapy &

Oncology (ESTRO) and European Society of Gynaecological Oncology (ESGO) defined new risk groups of EC in order to guide the use of adjuvant therapy (Colombo *et al.*, 2016). The groups are shown in Table 1.3 below.

Risk group	**Description**
Low	Stage I endometrioid, grade 1-2, <50% myometrial invasion (MI), LVSI negative
Intermediate	Stage I endometrioid, grade 1-2, ≥ 50% MI, LVSI negative
High-intermediate	• Stage I endometrioid, grade 3, <50% MI, regardless of LVSI status • Stage I endometrioid, grade 1-2, LVSI unequivocally positive, regardless of depth of invasion
High	• Stage I endometrioid, grade 3, ≥ 50% MI, regardless of LVSI status • Stage II • Stage III endometrioid, no residual disease • Non endometrioid (serous or clear cell or undifferentiated carcinoma, or carcinosarcoma)
Advanced	Stage III residual disease and stage IVA
Metastatic	Stage IVB

Table 1.3. Risk groups to guide adjuvant therapy use (Colombo *et al.*, 2016).

1.1.6 Prognosis

Survival significantly deteriorates with increased stage of disease (Table 1.4).

Stage of endometrial cancer	5-year survival
Stage I	95%
Stage II	77%
Stage III	39%
Stage IV	14%

Table 1.4. 5-year survival in different stages of endometrial cancer (Office for National Statistics, 2016).

Prognosis of endometrial cancer deteriorates in older patients. Five-year net survival is more than 85% for women younger than 60 years old and it drops to 73% for women aged 70-79 years and 57% for those older than 80 (Cancer Research UK. 2019).

1.1.7 Treatment

For most patients diagnosed in early stages, treatment involves a total hysterectomy with bilateral salpingo-oophorectomy (BSO). This can been performed through a laparotomy or minimal invasive surgery (MIS, either laparoscopically or robotically). MIS confers equal oncological outcomes and significantly improved quality of life along with less risk of surgical complications as compared to open surgery (Joan L. Walker *et al.*, 2012). In cases of young women with an early stage disease without any evidence of adnexal involvement, ovarian preservation can be offered and it might actually improve the overall survival (Jia & Zhang, 2017). For women with high grade disease, pelvic and para-aortic lymphadenectomy and omental biopsy are usually performed. In these latter cases, chemo-radiotherapy is usually offered post-operatively. For patients not amenable to surgery due to co-morbidities, radiotherapy and/or treatment with high doses of progestogens can be used.

Several chemotherapy regimens have been tried as adjuvant treatments in advanced EC and most of them are platinum-based. A recent Cochrane review (Galaal *et al.*, 2014) identified three main combinations: Cisplatin with doxorubicin, cisplatin with doxorubicin and paclitaxel and cisplatin with doxorubicin and cyclophosphamide. A randomized controlled trial (RCT) comparing the first two combinations showed that adding paclitaxel

does not add any benefit to the combination of cisplatin with doxorubicin (Homesley et al, 2008).

1.2 Molecular pathways involved in endometrial carcinogenesis

The most important property of cancer cells is their uncontrolled proliferation, which is achieved by a variety of different mechanisms. One of them is the activation of the various components of signaling pathways, bypassing the need for receptor mediated activation (Hanahan & Weinberg, 2011). In many cases this is achieved by somatic mutations which offer a selective growth advantage in the subclone of cancer cells harboring them. These mutations are called driver mutations and exert their action through a limited number of signaling pathways (Bert Vogelstein *et al.*, 2013). Cancer progression is therefore achieved by the expansion of those subclones containing these driver mutations. The acquisition of such mutations is usually a stochastic phenomenon although it can occur as part of an inherited phenotype (Hanahan & Weinberg, 2011). It is also known that such driver mutations increase the rate of other mutations (genomic instability), which in turn might lead to further clonal expansion and eventually to 'fitter' tumour cells (Greaves & Maley, 2012).

In 1971, Alfred Knudson proposed the 'two hit' model of carcinogenesis, which was based on his work in retinoblastoma (Knudson, 1971). He observed that cases with bilateral tumours were occurring earlier as these patients had inherited a germline mutation (first hit) and hence they only needed one somatic mutation at a later stage (second-hit) in order to develop the tumour. On the contrary, unilateral tumours (the majority), were occurring at an older age as they required two somatic mutations (one in each allele of the gene) in order for the tumour to develop (two-hit model of carcinogenesis). This hypothesis was later confirmed with studies on the retinoblastoma gene (Friend S. H. et al, 1986) and lead to the understanding of tumour suppressor genes. According to this model, when cancer arises as a result of biallelic inactivation of a gene, this gene can be called 'tumour suppressor gene'. A more generic definition was later proposed by Haber and Harlow according to whom tumour suppressor genes are 'genes that sustain loss-of-function mutations in the development of cancer' (Haber & Harlow, 1997).

There are exceptions to the above definition of tumour suppressor genes and the 'two-hit' model of carcinogenesis. Two of the most notable are loss of heterozygosity (LOH) and epigenetic silencing.

LOH refers to the loss of one of the two alleles of a gene in a cell. If this gene happens to be a tumour suppressor gene, it would be enough for just one somatic mutation ('one-hit model') occurring in the other copy of the gene in order to completely inactivate the gene and potentially lead to a cancer growth. This was clearly shown in the case of *CDKN1B* homozygous knockout mouse model, where all mice developed pituitary adenomas while their wild-type counterparts did not. Some (32%), but not all, heterozygous mice also developed pituitary tumours but it took longer for those tumours to develop (Fero M.L et al, 1998).

Epigenetic silencing is an alternative mechanism that leads to suppression of gene expression without the presence of a mutation. The first to be described and best-known change associated with epigenetic silencing is CpG methylation (the methylation of cytosine followed by a guanine nucleotide) (Holiday & Pugh, 1975; Riggs, 1975). Several tumour suppressor genes have been described as epigenetically silenced including *MLH1*, a gene particularly important in endometrial carcinogenesis. Germline mutation of *MLH1* leads to microsatellite instability and subsequent increased risk for colonic, endometrial and ovarian cancer (Lynch syndrome). *MLH1* can also be inactivated through promoter hypermethylation, leading to similarly increased risk of carcinogenesis. Interestingly, *MLH1* loss is much more frequently associated with promoter hypermethylation than a germline mutation (Buchannan DD et al, 2014).

To summarise, the cancer cell uses a variety of mechanisms in order to increase its proliferation and gain an advantage over neighbouring cells. Most of them exploit known pro-survival pathways. The two most important signaling pathways for endometrial carcinogenesis will be described below.

1.2.1 Phosphoinositide 3-kinase (PI3K) signalling pathway

PI3K is a family of kinases which consist of three classes. Class I is the best described and most involved in tumourigenesis. It has a catalytic and regulatory subunit. The catalytic subunit (p110a) is encoded by the *PIK3CA* gene, while the regulatory subunit (p85) is

encoded by the *PIK3R1-3* genes (Cheng *et al.*, 2009). Signals from tyrosine kinase receptors (RTK) and G-protein-coupled receptors (GPCR) in the cell membrane lead to PI3K activation which in turn phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) and thus converting it to phosphatidylinositol 3,4,5-triphosphate (PIP3) (Figure 1.1). PIP3 can be dephosphorylated by the PTEN phosphatase and reverted back to PIP2, which halts the downstream effects of PIP3. In the absence of this action, PIP3 recruits phosphoinositide-dependent kinase-1 (PDK1) and protein kinase B (AKT) in the inner cell membrane. PDK1 subsequently phosphorylates AKT (p-AKT). Its activity increases even further following phosphorylation by the mammalian target of rapamycin complex 2 (mTORC2) (Manning & Toker, 2017).

The activated AKT has several downstream effectors (Vanhaesebroeck & Alessi, 2000). One of the most important is the activation (phosphorylation) of the mammalian target of rapamycin complex 1 (mTORC1), which results in increased synthesis of proteins and survival (Martini *et al.*, 2014), an action that cancer cells take advantage of. Another one is the inhibition (via phosphorylation) of glycogen synthase kinase-3 (GSK3) which has an inhibitory function on many targets associated with cell survival and metabolism, including the anti-apoptotic myeloid cell leukemia 1 (MCL1) protein (Manning & Toker, 2017). GSK3 inactivation by pAKT leads to subsequent downstream activation of those factors previously inhibited by GSK3.

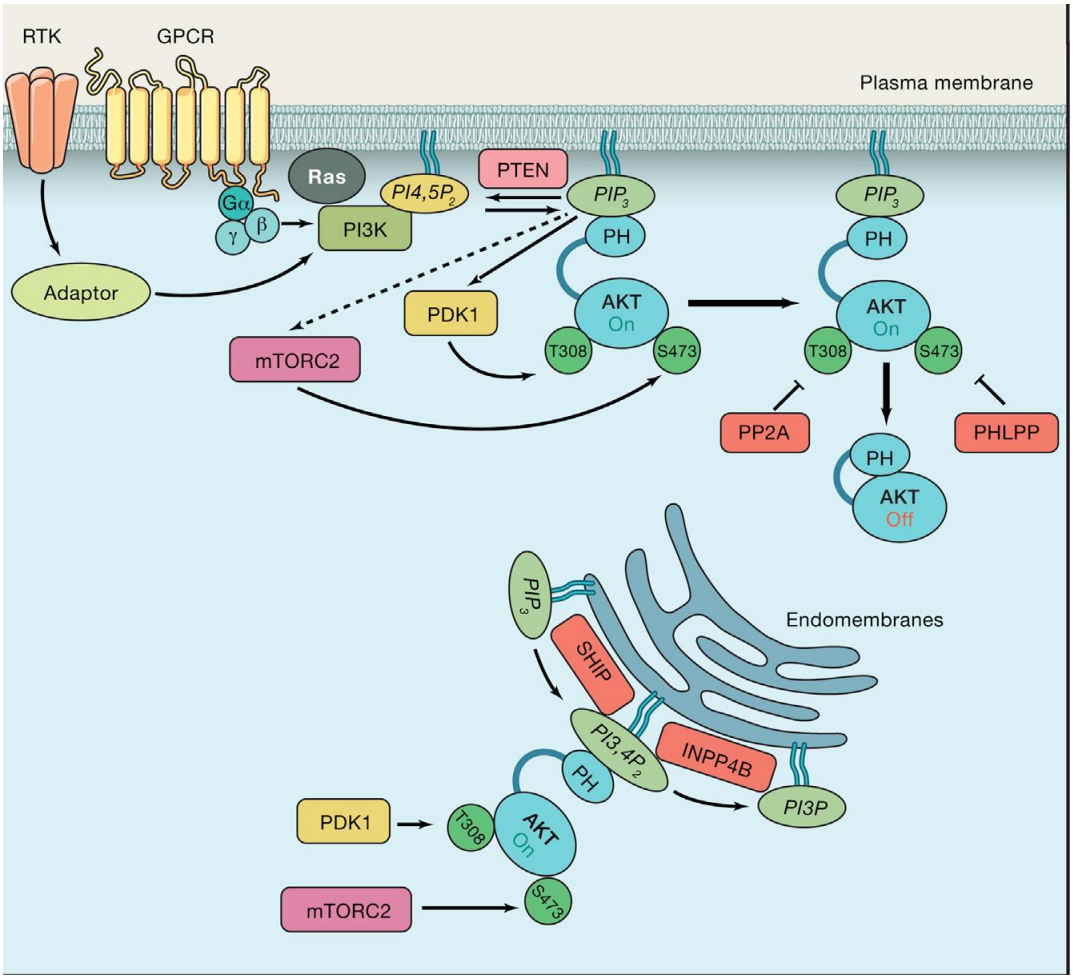


Figure 1.1 Schematic representation of PI3K/Akt pathway and the associations between its various components (Manning & Toker, 2017).

1.2.2 PI3K targeted therapies

Endometrial cancer has the highest rate of PI3K pathway mutations compared to any other cancer studied (TCGA, 2013). As this is one of the most amenable to targeted therapies pathway, these drugs could be potentially good candidates to be tested in advanced cases of EC.

1.2.2.1 Preclinical studies

Many inhibitors of PI3K and/or mTOR have been tested in vitro. Their efficacy depends on the endometrial cancer cell line in which they have been tested and, in particular, in the mutational status of this, along with the corresponding molecular pathway activation.

AZD8055 is an mTOR inhibitor that showed significant in vitro efficacy. It was tested in 24 EC cell lines and in all but one the surviving fraction 50% (SF50) was achieved with concentrations of <1μM (Weigelt *et al.*, 2013). Exactly the same applied to PF-04691502, a dual PI3K/mTOR inhibitor which was equally effective (Weigelt *et al.*, 2013). The same group also reported on Pictilisib (GDC-0941), a PI3K inhibitor found to be very efficient against half of the cell lines tested (13 out of 24) and in particular those harbouring *PIK3CA* mutations. Similar findings on Pictilisib were confirmed recently by a different group, which reported IC50 values of less than 1μM in 11 out of 13 cell lines (Aslan *et al.*, 2018). From the same study, excellent results were shown when Apitolisib (dual PI3K/mTOR inhibitor) was used (12 out 13 cell lines had an IC50 of less than 0.6μM). GSK2636771, a selective PI3K beta inhibitor, showed no efficacy in any of the 24 EC cell lines tested (Weigelt *et al.*, 2013). Dactolisib (NVP-BEZ235) was tested in 13 EC cell lines and showed significant efficacy as in 10 out of 13 cell lines had IC50 values of 100nM or less (as compared to everolimus which had similar efficacy in only 3 out of these 13 cell lines) (Keiko Shoji *et al.*, 2012). Voxtalisib (SAR245409) is another dual PI3K/mTOR inhibitor which was tested in twelve EC cell lines with modest results as only in two of them the IC50 values were <1μM. In the remaining ten cell lines, IC 50 values were between 2.2 and 7μM (Inaba et al, 2015).

Animal studies have tested a dual mTORC1/2 inhibitor (PP242, Torkinib) (Korets et al, 2013). The results were very promising with significant reduction of tumour volume after 20 days of treatment, better compared to everolimus and carboplatin alone. When PP242 was combined with carboplatin, the reduction in tumour volume was even more significant.

1.2.2.2 Clinical trials

Several studies have attempted to explore the efficacy of PI3K/mTOR inhibitors in advanced cases of endometrial cancer, with different results. The mTOR inhibitor Temsirolimus was tried in a phase 2 trial (Amit M. Oza *et al.*, 2011) showing some benefit, particularly in patients who had not received chemotherapy previously (14% partial response for 5.1 months and 69% stable disease for 9.7 months). The same research group reported on the use of another mTOR inhibitor (ridaforolimus) in previously treated EC with marginal benefit [progression free survival (PFS) of 3.6 months compared to 1.9

months for progestins or any other alternative] at the expense of more serious side-effects (Amit M Oza *et al.*, 2015). Another mTOR inhibitor tested in a phase 2 French study was everolimus (I Ray-coquard *et al.*, 2013). It showed some small benefit in refractory to first line chemotherapy cases with median duration of response 3.1 months.

Several PI3K inhibitors have been tested in vitro but only few of them made their way into clinical trials in the context of advanced endometrial cancer. Gedatolisib (PF-05212384) and PF-04691502 are dual PI3K and mTOR inhibitors which showed efficacy in preclinical models (Mallon *et al.*, 2011; J. Yuan *et al.*, 2011). They were subsequently evaluated in clinical trials which showed that PF-04691502 was not tolerated and had to be discontinued due to side-effects. Gedatolisib had some modest activity with a progression free survival (PFS) of 3.6 months and objective response rate (ORR) of 16% (Josep Maria del Campo et al, 2016). Minimal efficacy was shown with the use of Pilaralisib (PI3K inhibitor) in another phase II study, as the ORR was only 6% (Matulonis et al, 2014). Similar results published from a phase II trial testing Apitolisib (dual PI3K/mTOR inhibitor) in patients who had previously treated with one or two cycles of chemotherapy (Makker *et al.*, 2016). The ORR was 9% and PFS was 3.5 months with hyperglycaemia being the main side-effect. An AKT inhibitor (MK-2206) tested in a phase II trial has shown poor efficacy in patients with advanced EC and up to two lines of chemotherapy (Andrea P. Myers & et al, 2013). Buparlisib (BKM120) is a PI3K inhibitor that was evaluated in a phase II trial in patients with advanced or recurrent EC who had received up to one line of chemotherapy (P-E Heudel *et al.*, 2017). This trial was closed prematurely due to toxicity. There was no reported response and the PFS was 4.5 months. LY3023414 is a dual PI3K/mTOR inhibitor who was tested in a phase I trial in patients with advanced cancers, including 12 with EC. In this study, the only patient with a prolonged partial response (PR) was a patient with EC carrying a *PTEN* and *PIK3R1* mutation (Bendell Johanna *et al.*, 2018), while two other EC tumours harbouring *ARID1A* mutations had reduced tumour sizes. This relative success is currently being evaluated in a phase II trial (Memorial Sloan Kettering Cancer Center, NCT02549989).

1.2.3 Mitogen-activated protein kinase (MAPK) pathway

The MAPK pathways are some of the best-studied intracellular signaling pathways in humans. They are responsible for several key cellular functions, including proliferation,

differentiation and apoptosis (Morrison, 2012). Each MAPK pathway is a phosphorylating cascade that involves a minimum of three kinases (MAP3K, MAP2K and MAPK) (Shaul & Seger, 2007). Five MAPK cascades have been characterized in mammals (Qi & Elion, 2005). The most extensively characterized due to its involvement in the process of human disease is the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2, Figure 1.2). The pathway is triggered by cell surface receptor tyrosine kinases (RTK) stimulation, followed by Ras activation (Qi & Elion, 2005). Ras will then recruit Raf. There are three Raf genes, A-Raf, B-Raf and C-Raf (Raf-1). MEK1/2 phosphorylation subsequently ensues, followed by ERK1/2 phosphorylation (Figure 2). All Raf proteins can phosphorylate MEK1/2, but the potency of them varies with B-Raf being the most and A-Raf the least potent (McCubrey *et al.*, 2007). ERK1/2 has several downstream effectors including NF-κB, p53, Bcl-2 (Qi & Elion, 2005; Shaul & Seger, 2007). Bcl-2 phosphorylation, along with other pro- and anti-apoptotic molecules, regulates apoptosis.

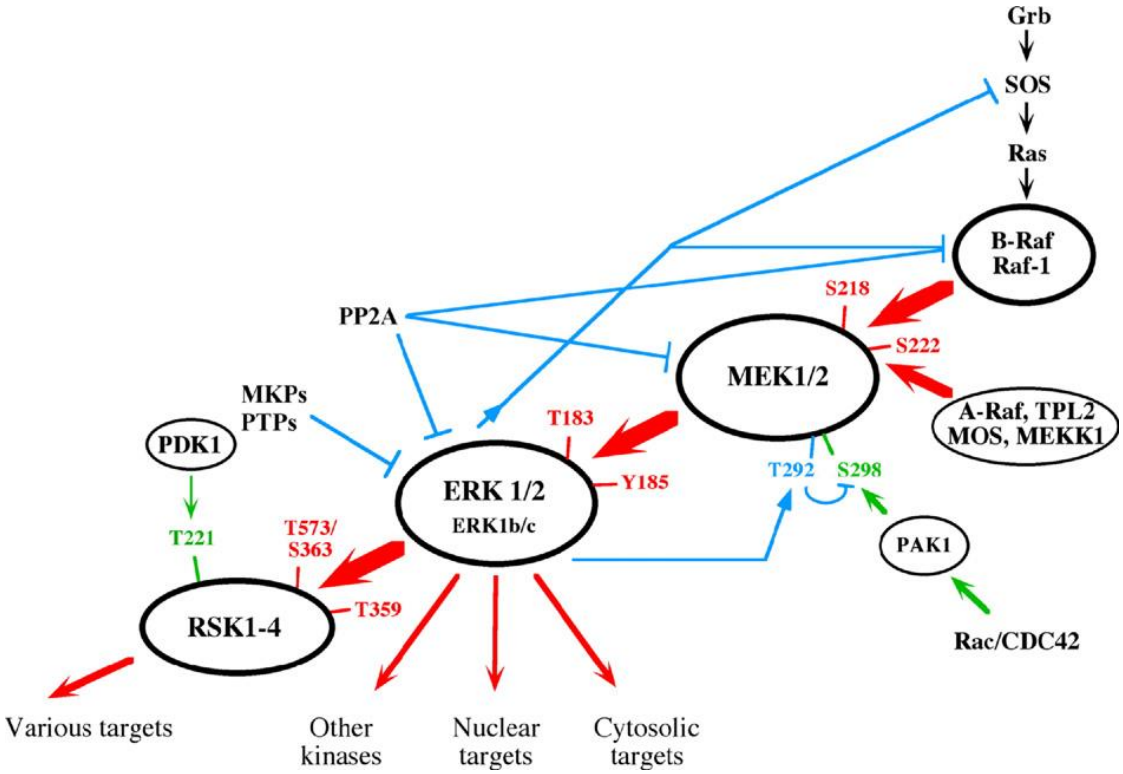


Figure 1.2 Schematic representation of ERK1/2 pathway (Shaul & Seger, 2007). Red arrows: Activating phosphorylation. Blue arrows: Inhibitory phosphorylation/de-phosphorylation. Small straight lines (i.e S218, S222 etc.) represent sites of phosphorylation. See text for more details.

1.2.4 MAPK targeted therapies

1.2.4.1 Preclinical studies

MAPK pathway inhibitory drugs have been tested *in vitro* although not to the same extent as PI3K inhibitors. Cobimetinib is a MEK inhibitor which was tested in 13 endometrial cancer cell lines (Aslan *et al.*, 2018). It showed some efficacy in 9 out of 13 cell lines (IC50<1μM) and particularly in three cell lines carrying a *KRAS* mutation. SB203580, a MAPK inhibitor, was effective against *PTEN*-deficient cell lines but not against those carrying a wild-type *PTEN* (Lan Xiao *et al.*, 2010). Pimasertib, a MEK1/2 inhibitor, showed good results in six out of twelve cell lines tested (IC50 values<5μM) (Inaba et al, 2015). MEK inhibitor UO126 was shown to have inhibitory effect and also induce senescence in three EC cell lines (Suga et al, 2006). Sorafenib is a B- and C-Raf inhibitor currently used in clinical trials. It was tested in EC cell lines and showed cytotoxic effect in all four of them but only in very high doses (>10μM), which probably is not clinically relevant (Llobet et al, 2009). These results were later confirmed by another group which tested Sorafenib in three EC cell lines and reported IC50 values between 4.2-8.6μM and significant reduction in viability only when high doses (10μM) were used (N. Sun *et al.*, 2013). The Ras inhibitor Salirasib was used in two EC cell lines with disappointing results as the IC50 values were above 50μM (Faigenbaum *et al.*, 2013). Selumetinib, a selective MEK1/2 inhibitor was tested in three endometrioid EC cell lines and showed no clinically relevant efficacy as monotherapy since the IC50 values were between 27 and 79.7μM (Schrauwen et al, 2015).

1.2.4.2 Clinical studies

Selumetinib was the only MEK inhibitor assessed as monotherapy in a Phase II trial in patients with recurrent EC (Coleman et al, 2015). The results revealed low efficacy with ORR of 6% and stable disease in 25% of the patients but the drug was well tolerated which suggests that it might be useful in combination treatments. Another MEK inhibitor (PD-0325901) was tested as part of a phase I trial in combination with PI3K/mTOR inhibitors (PF-04691502 and gedatolisib) in advanced solid tumours (Wainberg *et al.*, 2017). The arm assessing PF-04691502 was closed early due to concerns regarding safety while the

gedatolisib and PD-0325901 arm recruited 37 patients including one with advanced endometrial cancer. Interestingly, this patient achieved a partial response and remained on treatment for more than a year (376 days). She was carrying both a *PIK3CA* and *KRAS* mutation and she was the second best responder in the whole study.

1.3 Drugs under investigation

From the data discussed previously it becomes apparent that most targeted drugs either have limited efficacy when used as monotherapy or their use is limited due to toxicity. It would be reasonable that the most appropriate next step would be to try rational combinations of inhibitors, looking for synergistic effects. This could increase efficacy against cancer cells while reducing the total side effects. For my study I selected some of the most relevant pathways to be inhibited and some popular targeted drugs not yet tested in EC, which are presented below.

1.3.1 UO126

As mentioned previously, UO126 is a selective MEK1/2 inhibitor which showed in vitro efficacy in EC cell lines (Suga et al, 2006).

1.3.2 Idelalisib

PI3K inhibitor currently used against haematological malignancies. In particular, it is currently a second-line treatment for the treatment of chronic lymphocytic leukemia (CLL) and third-line treatment for the management of follicular lymphoma in the UK (Joint Formulary Committee, 2018). As of January 2019, there are 17 trials testing idelalisib which actively recruit patients with haematological cancers in several countries around the world and one american study testing the drug in lung cancer patients (U.S. National Library of Medicine, 2019). To our knowledge, this drug was never tested in EC previously.

1.3.3 Ibrutinib

It is a Bruton's tyrosine kinase (BTK) inhibitor currently used in the treatment of haematological malignancies. In the UK it is a second-line treatment for CLL and Waldenström's macroglobulinaemia (Joint Formulary Committee, 2018). It is a drug that is being investigated in a large number of trials, with 117 active ones recruiting patients around the world with haematological but also solid cancers (renal, melanoma, colorectal, gastro-oesophageal, glioblastoma and breast), either as monotherapy or, more commonly in solid tumours, in combination with other drugs (U.S. National Library of Medicine, 2019). To our knowledge, it was never tested in EC before.

1.3.4 IMD0354

IκB kinase-β (IKKβ) inhibitor that blocks NF-κB p65 nuclear translocation. Although it has not been tested in EC, pre-clinical data suggest its efficacy in CLL (Kanduri *et al.*, 2011) and breast cancer cells (Azucena Gomez-Cabrero *et al.*, 2013). It has also been shown to have anti-angiogenic properties in ovarian cancer cells and animal models (Kinose *et al.*, 2015). Encouraging results were also shown with this drug in malignant mesothelioma, both in vitro and in animal model (Nishikawa *et al.*, 2014). Similarly good results were achieved in adult T-cell leukemia cells and mice model (Uota *et al.*, 2012) and in pancreatic cancer cells (Ochiai *et al.*, 2008). Despite this relative success in pre-clinical studies, it has not made its way into a clinical trial as yet.

1.3.5 PF-03084014

It is a gamma secretase inhibitor which blocks the cleavage of Notch receptors and hence leads to inhibition of Notch signalling pathway. This can induce apoptosis, particularly in cancer cells overexpressing Notch. It showed promising efficacy in explant colorectal cancer model, but mainly in tumours showing highly activated Notch and Wnt pathways (Arcaroli *et al.*, 2013). It was also effective in hepatocellular carcinoma tumour models, both as monotherapy (C. X. Wu *et al.*, 2017) but also in combination with Sorafenib (X. Yang *et al.*, 2018). Similarly, in combination with the epidermal growth factor receptor (EGFR) inhibitor Erlotinib, it has shown synergistic efficacy against five head and neck squamous cell carcinoma cell lines, although the doses of PF-03084014 used to achieve

this were very high (30μM) (Zheng *et al.*, 2018). A phase II clinical trial tested its efficacy in patient with recurrent desmoid tumours. 29% of patients (5/17) had a confirmed partial response while another five patients had stable disease (Kummar *et al.*, 2017). Another phase I study evaluated PF-03084014 combined with docetaxel in advanced breast cancer (triple-negative). The combination was tolerated but the results were modest with 16% of patients achieving a confirmed partial response and PFS of 4.1 months (Locatelli *et al.*, 2017). The same combination showed promising results in prostate cancer cell lines (Cui *et al.*, 2015). A small, phase I clinical trial evaluated the efficacy of PF-03084014 in eight patients with refractory T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL), one of which achieved a complete response (Papayannidis *et al.*, 2015). In combination with the purine analog fludarabine, it exerts synergistic effect in CLL cells (López-Guerra *et al.*, 2015). A phase I trial also evaluated its efficacy in 64 patients with a variety of advanced solid tumours including three cases of endometrial leiomyosarcoma. Although the drug was well tolerated, its efficacy was poor with the exception of desmoid tumours in which five out seven patients achieved a partial response (Messersmith *et al.*, 2015). PF-03084014 has not been tested in EC.

1.3.6 RO4929097

RO4929097 is another γ secretase inhibitor, also called RG-4733. It has been extensively tested in pre-clinical and early phase clinical trials although not in EC. At a pre-clinical level, it showed efficacy in cervical cancer cell lines (L. Wang *et al.*, 2018) It was tested, in combination with Bevacizumab, in a phase I clinical trial involving 13 patients with malignant glioma with modest results as two out of twelve patients available for evaluation had response (one CR and one PR) (Pan *et al.*, 2016). Another phase I trial assessed its efficacy, in combination with the anti-metabolite Capecitabine, in advanced solid tumours (mostly colorectal). The results were poor, with only 10% of the thirty patients responding (including the only patient of the study with cervical cancer) (LoConte *et al.*, 2015). It was also tested in combination with Gemcitabine in a phase I trial involving 18 patients with advanced solid tumours, which showed that the combination is safe but with limited efficacy as only one patient responded (6%) and three patients (16%) had stable disease as best response (Richter *et al.*, 2014). Its combination with the mTOR inhibitor Temsirolimus was safe in a phase I trial but again there was no response in 17 patients

with advanced solid tumours although most of them had stable disease (73%) (Diaz-Padilla *et al.*, 2013). When combined with the vascular endothelial growth factor receptor (VEGF) inhibitor Cediranib in a phase I trial enrolling patients with advanced solid tumours, it was well tolerated, with one out of nineteen patients achieving a partial response (a patient with an endometrial stromal sarcoma) and 58% of them having stable disease (Sahebjam *et al.*, 2013). A phase II clinical trial evaluated RO4929097 in patients with recurrent, platinum-resistant ovarian cancer. The results were disappointing as none of the 40 patients available for evaluation responded (although a third of them had stable disease) with a PFS of only 1.3 months (Diaz-Padilla *et al.*, 2015). Equally poor results were shown on another phase II trial in patients with metastatic melanoma who had not received any prior chemotherapy. Although the drug was well tolerated, the response rate was only 3% (1/32), with 25% of patients having stable disease (S. M. Lee *et al.*, 2015). In a phase II trial in pancreatic cancer, none of the twelve patients responded to treatment with RO4929097 with three of them having stable disease (De Jesus-Acosta *et al.*, 2014). Finally, no efficacy was shown in a phase II trial of RO4929097 in patients with advanced colorectal cancer as none of the 33 patients had objective response (Strosberg *et al.*, 2012).

1.3.7 Dinaciclib

A cyclin-dependent kinase (CDK) inhibitor with activity against CDK 1, 2, 5 and 9. Various studies evaluated its efficacy. At a pre-clinical level, it was shown to be cytotoxic against squamous cell lung cancer cells (Jeong *et al.*, 2018), very efficient against T-cell acute lymphoblastic leukemia cell lines (Moharram *et al.*, 2017) and seven thyroid cancer cell lines along with a mice model of thyroid cancer (S. Lin *et al.*, 2017). It was also efficient in pre-clinical models of triple-negative breast cancer (Rajput *et al.*, 2016), neuroblastoma (Z. Chen *et al.*, 2016) and acute myeloid leukemia (Baker *et al.*, 2016). A study from our laboratory showed excellent results in CLL cell lines and primary CLL cells (Y. Chen *et al.*, 2016). Impressive results were also shown in eight pancreatic cancer models when Dinaciclib was combined with the AKT inhibitor MK-2206 (Hu *et al.*, 2015) and in ovarian cancer mouse model in combination with cisplatin (X. Chen *et al.*, 2015). A recent study on serous endometrial cancer cells modified to carry *FBXW7* mutations showed that these cells were very sensitive to Dinaciclib in clinically relevant low concentrations (Urick & Bell, 2018b). Dinaciclib entered several clinical trials which showed its tolerability, albeit with important side-effects. A phase I trial in patients with

various solid tumours showed no response in 61 patients (Mita *et al.*, 2017). Another small phase I study was conducted in nine patients with metastatic triple negative breast cancer in order to assess the combination of Dinaciclib with the anthracycline Epirubicin. The study closed early due to toxicity. No response was noted (Mitri *et al.*, 2015). A small RCT compared Dinaciclib with the anti-metabolite Capecitabine in metastatic breast cancer. The study was closed early due to inferiority of the Dinaciclib arm in PFS (Mita *et al.*, 2014). A phase III randomized controlled trial (RCT) comparing Dinaciclib with the monoclonal antibody Ofatumumab in patients with refractory CLL closed prematurely (unrelated to side-effects) but reported superiority of Dinaciclib in oncological outcomes with PFS of 13.7 months compared to 5.9 for Ofatumumab (Ghia *et al.*, 2017). A phase 1/2 study evaluated Dinaciclib in heavily pre-treated patients with multiple myeloma with 19% of patients (5/27) having some degree of response while the drug was shown to be tolerated (Kumar *et al.*, 2015). There are currently four clinical trials recruiting patients with haematological malignancies (2), advanced solid tumours and advanced breast cancer in combination treatments including Dinaciclib.

1.3.8 Palbociclib

An inhibitor of CDK 4 and 6. It has been extensively tested in many cancers, including EC. There are currently 102 clinical trials actively recruiting patients with various cancers in treatment arms involving Palbociclib (U.S. National Library of Medicine, 2019). Two thirds of them recruit breast cancer patients but the drug is currently under investigation for many solid tumours (pancreas, brain, colon, lung, head and neck and prostate) and haematological malignancies (leukemias and lymphoma). There is also a phase 2 RCT currently recruiting patients with advanced estrogen receptor positive (ER+) EC in treatment of Letrozole and Palbociclib versus Letrozole alone. Palbociclib has been approved in the UK for the treatment of locally advanced or metastatic breast cancer (Joint Formulary Committee, 2018). Recent study in EC *PTEN*-deficient mouse model showed reduction in tumour volume and cell proliferation along with better survival when Palbociclib was used compared to untreated animals. Similar results were obtained in vitro (3D cultures) (Dosil *et al.*, 2017). Contemporaneously, another group reported efficacy of Palbociclib in two out of the four EC cell lines tested along with reduction in tumour growth in a mouse model (T. Tanaka *et al.*, 2017).

1.3.9 Sorafenib

It is an inhibitor of many protein kinases, most notably C-Raf, B-Raf and VEGFR. It has been widely tested and is already licensed in the UK for the treatment of advanced renal cell carcinoma (where interferon treatment has failed), advanced thyroid carcinoma resistant to iodine treatment and hepatocellular carcinoma (Joint Formulary Committee, 2018). There are currently 114 clinical trials recruiting patients on Sorafenib treatment in many solid and haematological cancers, the majority of them being focused on hepatocellular carcinoma (U.S. National Library of Medicine, 2019). In EC, Sorafenib showed efficacy in two EC cell lines, albeit at high doses (>5-10μM), but these results were not reproducible in a *PTEN*-deficient EC mouse model as there was no effect in the tumour size following Sorafenib treatment (Mirantes *et al.*, 2016). Prior to that, Sorafenib had already been tested in a phase 2 clinical trial in patients with advanced endometrial carcinoma and carcinosarcoma. The results were poor as only 5% of patients with EC (2/40) and none of the 16 patients with carcinosarcoma achieved a partial response (although 42.5% of patients with EC had stable disease), with a PFS of 3.2 months (Nimeiri et al, 2010).

1.3.10 Selumetinib

A MEK1 and 2 small molecule inhibitor. It has been extensively tested in various cancers, including EC. As mentioned previously, the drug has already been tested in a phase 2 clinical trial in patients with advanced EC. It showed poor response rate (6%) as monotherapy although it has a favorable toxicity profile (Coleman et al, 2015). There are currently 23 clinical trials recruiting patients with haematological, breast, lung, gastrointestinal, thyroid and bladder malignancies in Selumetinib treatment, usually in combination with other drugs (U.S. National Library of Medicine, 2019).

1.3.11 Trametinib

Another MEK1 and 2 inhibitor which has already been licensed in the UK for use in advanced melanoma and non-small cell lung cancer (NSCLC) provided they carry a BRAF V600 mutation (Joint Formulary Committee, 2018). The treatment is either as a

monotherapy (in melanoma) or in combination with Dabrafenib (in NSCLC and melanoma). Trametinib has not been tested in endometrial cancer previously but it's under investigation in 72 clinical trials which currently recruit patients with a variety of cancers (U.S. National Library of Medicine, 2019).

1.3.12 PD0325901

MEK inhibitor which has been tested in pre-clinical studies and clinical trials, although not as extensively as Trametinib and Selumetinib. It appears to be an attractive drug for combination treatments in various solid tumours. In combination with Palbociclib, PD0325901 showed efficacy in pre-clinical model of colorectal cancer (Pek *et al.*, 2017). When combined with the Src inhibitor Saracatinib (AZD-0530) it had significant efficacy in the majority of NSCLC cell lines tested (Chua *et al.*, 2015). Similar efficacy was shown in pre-clinical studies on gastric (Sogabe *et al.*, 2014), papillary thyroid (Henderson *et al.*, 2010) and hepatocellular carcinomas (Hennig *et al.*, 2010). It was also efficient against melanoma cell lines (Ciuffreda *et al.*, 2009). A phase I study evaluated PD0325901 combined with the dual PI3K/mTOR inhibitor Gedatolisib in 36 patients with advanced solid tumours, including five patients with ovarian and one with endometrial cancer. The objective response rate was 11% (4/36) but interestingly these were three patients with ovarian cancer and the only patient with EC (Wainberg *et al.*, 2017). There is currently a Dutch phase 1/2 study recruiting patients with NSCLC in PD0325901 treatment combined with the EGFR inhibitor Dacomitinib (U.S. National Library of Medicine, 2019).

1.3.13 PLX-4720

A B-Raf V600E inhibitor who has never been tested in EC nor has ever been entered into a clinical trial. It showed no efficacy in a mouse model of colorectal cancer carrying microsatellite instability (MIS) (Rohde *et al.*, 2017). In combination with antibodies targeting the PD-1 and PD-L1 cell surface proteins significantly reduced tumour volume and prolonged survival in thyroid cancer mouse model (Gunda *et al.*, 2018). In combination with Cediranib, PLX-4720 proved effective in melanoma cell lines and lead to reduction in tumour size in animal model (Friedman *et al.*, 2015).

1.3.14 Dabrafenib

Small molecule BRAF kinase inhibitor. It has been licensed in the UK for the treatment of metastatic melanoma (with or without Trametinib) and advanced NSCLC (in combination with Trametinib), in tumours that carry a BRAFV600 mutation (Joint Formulary Committee, 2018). It has been tested in several clinical trials. It is currently the subject of investigation in 46 clinical trials recruiting patients, most of them with melanoma, but also with colorectal, brain, NSCLC, thyroid tumours and multiple myeloma (U.S. National Library of Medicine, 2019). This inhibitor has not been previously tested in EC although there is a case report in the literature of a patient previously treated for stage 1A, grade 1 EC and had disease recurrence 11 years later. After traditional chemotherapy failed and since her tumour was found to carry a BRAFV600E mutation, she was treated with a combination of Dabrafenib and Trametinib, which lead to a significant and maintained response (Moschetta *et al.*, 2017). This report highlights that such inhibitors might have a significant role to play in a subset of EC patients with specific mutational signatures.

1.3.15 GW5074

C-Raf inhibitor. It has been shown to reduce the rate of tumour proliferation in colorectal cancer mouse model (Borovski *et al.*, 2017) and to inhibit cell growth in two hepatocellular carcinoma cell lines (S. Lin *et al.*, 2013). There is currently an active, phase 1 clinical trial recruiting patients with solid tumours in combination treatment with GW5074 and Sorafenib (U.S. National Library of Medicine, 2019).

1.3.16 VER-155008

Adenosine-derived inhibitor of Heat Shock Protein 70 (Hsp70). It has been tested in vitro in four muscle invasive bladder cancer cell lines with some minimal efficacy as monotherapy, although the dose used was high (10μM). The efficacy improved when tested in combination with MAL3-101, another Hsp70 modulator, but still the doses required for a response were very high for clinical standards (Prince *et al.*, 2018). It was also tested in a prostate cancer cell line and decreased the proliferation rate but not the viability even at doses as high as 25μM (Kita *et al.*, 2017). When VER-155008 tested in

two breast and two colorectal cancer cell lines, it showed modest efficacy with IC50 values above 10μM for three out of four of them (and IC50 of 5.3μM for the HCT116 colorectal cell line) (Massey *et al.*, 2010). Similar findings were shown in two NSCLC cell lines as VER-155008 was efficient but only on very high concentrations (>15μM) (Wen *et al.*, 2014).

1.3.17 BX-912

BX-912 is a phosphoinositide-dependent protein kinase (PDK) 1 inhibitor. It has been shown to be effective in four mantle cell lymphoma cell lines (in two of them the IC50 values were less than 1μM) (Maegawa *et al.*, 2018). Results were not as promising in three diffuse large B-cell lymphoma (DLBCL) cell lines as the IC50 values were high (3.7-17μM) (Maegawa *et al.*, 2018). It has not been tested in endometrial cancer and it has not entered a clinical trial.

1.3.18 Fedratinib

Fedratinib is a janus-associated kinase (JAK) 2 inhibitor. It has been assessed in phase 2 clinical trials in patients with myelofibrosis with promising results in approximately half of the patients (55%) who received it as second line treatment (Harrison, Claire N et al, 2017). It also showed efficacy in six Hodgkin and mediastinal large B-cell lymphoma cell lines (Hao *et al.*, 2014). Preclinical efficacy was confirmed in a panel of 16 NSCLC cell lines (Pitroda Sean *et al.*, 2018).

1.3.19 AZD3965

Inhibitor of monocarboxylate transporter 1 (MCT1). The company who developed the drug tested it in a panel of 174 cell lines (Curtis *et al.*, 2017). The majority of them were resistant to treatment with AZD3965, especially those derived from solid tumours. A significant number of DLBCL cell lines though was very sensitive to this inhibitor, raising interesting clinical potential for its use in haematological malignancies. There is currently a phase 1 clinical trial recruiting in six UK hospitals assessing the safety of AZD3965 in

patients with advanced solid tumours, DLBCL and Burkitt lymphoma (U.S. National Library of Medicine, 2019).

1.3.20 Quercetin

Flavonoid which has been shown to inhibit PI3K/mTOR pathway (Castillo-Pichardo & Dharmawardhane, 2012) and have various effects in several other pathways. There are dozens of preclinical studies on a variety of cell lines in solid and haematological tumours showing Quercetin's efficacy, in pharmacologically high doses (Sak, 2014). It was previously tested in Ishikawa EC cells with an IC50 value of 10μM (Kaneuchi *et al.*, 2003).

1.3.21 ABT-199

Also called Venetoclax, a B-cell lymphoma 2 (Bcl-2) selective drug inhibitor showed to be effective in many CLL, acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma (NHL, including DLBCL) and acute myelogenous leukemia (AML) cell lines along with a mouse model (Andrew J Souers *et al.*, 2013). As part of the same study the drug was also tested in three patients with refractory CLL with very promising results. As a result of that, Venetoclax is currently licensed for the treatment of CLL (Joint Formulary Committee, 2018). There are currently 88 active clinical trials recruiting patients with various haematological malignancies around the world in treatment with Venetoclax, alone or combined with other drugs (U.S. National Library of Medicine, 2019). The drug was effective in xenograft mice model of breast cancer in combination with PI3K/mTOR inhibitors and/or Tamoxifen (Vaillant *et al.*, 2013). In pancreatic cancer cell lines, it improved the efficacy of gemcitabine (Zhou *et al.*, 2018). As monotherapy, ABT-199 had some modest efficacy in nasopharyngeal cancer cell lines (IC50 2-3μM) and xenograft mouse model (30% reduction in tumour volume) (Y. Wang *et al.*, 2018). In triple negative breast cancer cell lines, ABT-199 had no efficacy as monotherapy but showed promising results when combined with Doxorubicin and this was confirmed in a xenograft mouse model (Inao *et al.*, 2018). In combination with idasanutlin, ABT-199 showed synergistic activity in neuroblastoma cell lines and a mouse model of the disease (Van Goethem *et al.*, 2017). Combined with a bromodomain and extraterminal (BET) inhibitor (ABBV-075) showed synergistic effect in small-cell lung cancer cells (Lam Lloyd *et al.*, 2017).

1.3.22 S63845

Recently developed myeloid cell leukemia 1 (MCL1) inhibitor. Highly efficient against most of the multiple myeloma, lymphoma, CML and AML cell lines tested along with mouse models of these diseases (András Kotschy *et al.*, 2016a). It was not as effective when tested in solid tumours with 15-33% of NSCLC, breast and melanoma cell lines being sensitive to it when used alone (András Kotschy *et al.*, 2016a). In combination with ABT-199, it shows significant synergistic activity in T-cell ALL cell lines and zebrafish model (Zhaodong Li *et al.*, 2019) but also in nasopharyngeal cancer cells (Y. Wang *et al.*, 2018). Combined with Docetaxel, it acted synergistically in triple negative cells and xenograft model (Merino *et al.*, 2017). It has not yet been tested in endometrial cancer.

1.3.23 MG132

MG132 belongs to the family of proteasome inhibitors. Some of this class of drugs (Bortezomib, Carfilzomib and Ixazomib) have already been approved for the treatment of multiple myeloma. MG132 has only been tested in vitro in various cancers, usually in combination treatments. Recent examples include its combination with the PDK1 inhibitor GSK-470 which had synergistic efficacy in multiple myeloma cell lines (J. Zhang *et al.*, 2018). It also acts in synergy with cisplatin in osteosarcoma cell lines and xenograft model (F. Sun *et al.*, 2018) and with paclitaxel in breast cancer cells and mouse model (Y. Zhang *et al.*, 2018).

1.3.24 CUDC-907

PI3K and histone deacetylase (HDAC) inhibitor. It has been tested at a pre-clinical and clinical level. It was shown to be very effective in seven mantle cell lymphoma cell lines and eight patient samples with IC50 values less than 5nM (Guo *et al.*, 2018). It also showed promising efficacy in thyroid cancer cell lines and a mouse model of the disease (Kotian Shweta *et al.*, 2017). In a phase 1 clinical trials including patients with DLBCL, CUDC-907 was shown to be safe and with an encouraging response rate of 37% (Oki *et al.*, 2017). This group is currently conducting a phase 2 trial based on these preliminary results. In another phase 1 trial in patients with heavily pretreated lymphoma and multiple myeloma, modest efficacy and acceptable tolerability was confirmed (Younes et al, 2016). There are

currently two active clinical trials assessing the efficacy and tolerability of CUDC-907 in patients with solid tumours (mainly triple negative breast cancer and high-grade serous ovarian cancer) and children with lymphoma, neuroblastoma or brain tumours (U.S. National Library of Medicine, 2019).

1.4 Genetics of endometrial cancer

The endometrial cancer is the one with the highest number of significantly mutated genes across all cancers tested as part of the TCGA Pan-Cancer Atlas project (Michael S Lawrence *et al.*, 2014). The main genetic alterations involved in the pathogenesis of EC will be described and are shown in Table 1.5.

Genetic mutation	**POLE**	**MSI**	**SCNA low**	**SCNA high**
PTEN	94%	88%	77%	10%
TP53	35%	0%	0%	92%
KRAS	53%	35%	16%	0%
FBXW7	82%	11%	6%	22%
PPP2R1A	0%	0%	0%	22%
CTNNB1	0%	20%	52%	0%
POLE	100%	0%	0%	0%
PIK3R1	65%	42%	33%	13%
PIK3CA	71%	54%	53%	47%
ARID1A	0%	37%	42%	0%

Table 1.5 Genetic mutations associated with the four classification groups of the TCGA study and their respective incidence in its group.

1.4.1 *PTEN*

PTEN is a tumour suppressor gene located in the long arm of chromosome 10 (10q23). It appears to play a significant role in several important cell functions including survival, proliferation and metabolism (Song *et al.*, 2012). It exerts its role through the PI3K-AKT-mTOR pathway. In this pathway, the phosphatidylinositol 3 kinase (PI3K) phosphorylates

phosphatidylinositol 4,5-bisphosphate (PIP2) and converts it to phosphatidylinositol 4,5-triphosphate (PIP3). PIP3 will then activate AKT through recruitment of phosphatidylinositol-dependent kinase 1 (PDK1) and the mTOR complex (Milella *et al.*, 2015). *PTEN* exerts its action by converting PIP3 back to PIP2 (by dephosphorylation) and hence halting the PI3K-AKT cascade which leads to inhibition of cell growth and proliferation (Figure 1.3).

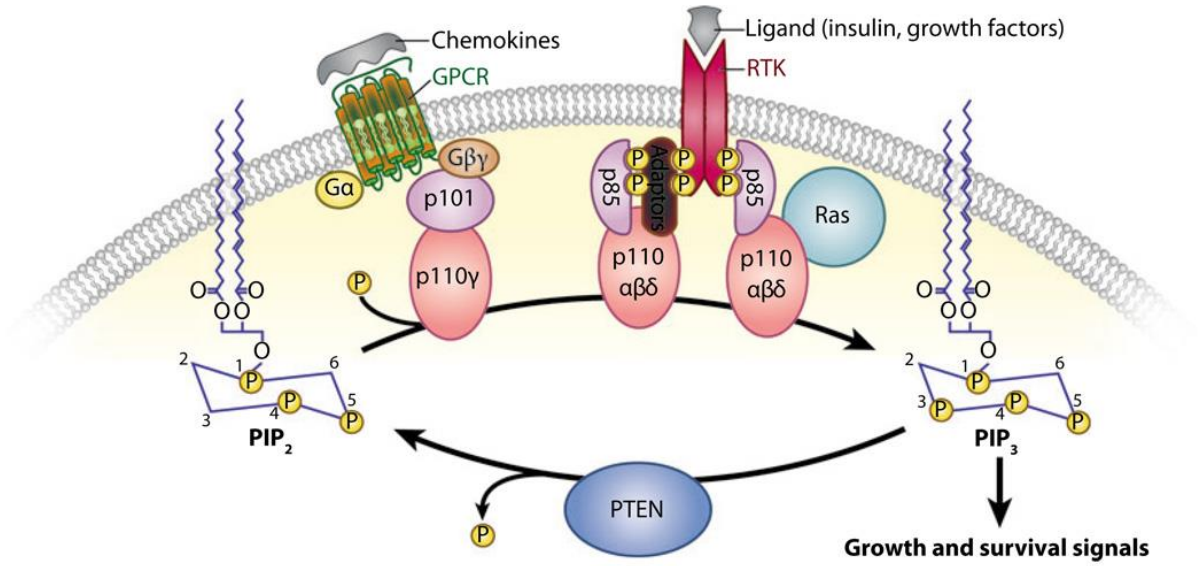


Figure 1.3 The action of *PTEN* within the PI3K pathway (see text for details) (Chalhoub & Baker, 2009).

Endometrial is the cancer with the highest *PTEN* mutational frequency (65.7%) amongst all 21 cancers tested in the TCGA Pan-Cancer Atlas project (Michael S Lawrence *et al.*, 2014). Their incidence is even higher amongst endometrioid EC (up to 83% incidence has been reported) (Mutter *et al.*, 2000). They are almost always present in the *POLE* ultra-mutated (94%) but also very frequent in the SCNA low groups of EC (77%), while they are less frequent in the SCNA high group (10%) (Piulats et al, 2016). *PTEN* mutations are also associated with microsatellite instability (MSI) (Bilbao *et al.*, 2006) and this was confirmed in the TCGA study which found that 88% of endometrial cancers in the MSI group were carrying *PTEN* mutations. The same study showed that *PTEN* mutations inactivate the gene, as shown by the reduced expression of its protein (TCGA, 2013). They

are considered to be an early event in the process of carcinogenesis as they are present in cases of complex atypical hyperplasia (CAH) of the endometrium (Levine *et al.*, 1998). This fact was confirmed in more recent, whole-exome sequencing study (William J Gibson *et al.*, 2016). The association between *PTEN* mutations and survival in EC is controversial. Older studies suggested that *PTEN* loss (usually associated with *PTEN* mutations) offer worse prognosis in advanced EC patients receiving post-operative chemotherapy (Terakawa *et al.*, 2003). More recent studies report the opposite, attributing favorable outcome to EC patients with *PTEN* mutations (A Akiyama-abe *et al.*, 2013).

1.4.2 *TP53*

TP53 is a tumour suppressor gene located in the short arm of chromosome 17. It has been called 'Guardian of the genome' (Lane, 1992) as it has a key role in preventing the proliferation of cells with damaged DNA. When DNA damage occurs, *TP53* will initiate a cascade of events leading to either cell cycle arrest and DNA repair or apoptosis.
TP53 mutations are well described in the majority of human cancers and EC is not an exception. Such mutations have been detected in 70-93% of type II EC (Moll *et al.*, 1996; Tashiro *et al.*, 1997; Lax *et al.*, 2000) and they are the most consistent genetic event in the non-oestrogen related type of EC. They do occur though in 12% of tumours of endometrioid histology (TCGA, 2013), which rise to 30% in grade 3 endometrioid tumours (Murali et al, 2014b). 92% of the endometrial tumours in the SCNA high group described in the TCGA study were harboring *TP53* mutations and only 1% in the SCNA low group (Le Gallo & Bell, 2014). *TP53* mutations were also present in the *POLE* ultra-mutated and MSI groups (35% and 8% respectively). Unlike *PTEN* mutations, which are inactivating, *TP53* mutations are activating as the protein expression is raised in EC (TCGA, 2013).

TP53 mutations have been shown to be present in 72-75% of endometrial serous intraepithelial carcinoma lesions, generally accepted as the precursor lesion of serous EC (Tashiro *et al.*, 1997; Lin Jia *et al.*, 2008). This provides evidence that genetic alterations in *TP53* are an early event in the pathogenesis of type II EC, which was also confirmed in a recent, whole-exome sequencing study (William J Gibson *et al.*, 2016). On the contrary, the absence of these mutations in the hyperplastic endometrium (the precursor lesion of type I EC) supports the idea that they occur late in the process of carcinogenesis in this type of EC (Lax *et al.*, 2000). The presence of *TP53* mutations is associated with much

worse survival and risk for distant recurrence of the disease (B G Wortman *et al.*, 2018). *TP53* mutations are correlated with increased age at diagnosis of EC (Gonzalez-Rodilla *et al.*, 2012)

1.4.3 *KRAS*

K-ras oncogene is a member of the RAS family of oncogenes and is located in the short arm of chromosome 12. It codes for the K-ras GTPase which is part of the RAS-RAF-MEK-ERK pathway. K-ras mutations have been identified in up to a third of endometrioid EC (Enomoto *et al.*, 1991; Fujimoto *et al.*, 1993; Lax *et al.*, 2000; Zauber *et al.*, 2015). They probably represent an early event in the process of tumourigenesis as shown by studies in which the rate of K-ras mutations in endometrial hyperplasias was very similar with that of carcinomas (Sasaki *et al.*, 1993; Zauber *et al.*, 2015). *KRAS* mutations are more common in endometrioid tumours with hyperplastic endometrium compared to similar tumours with atrophic endometrium (34.9% and 2.3% respectively, $p<0.01$) (Geels et al, 2015). They were also more common in the mucinous type of EC and in endometrioid tumours with significant mucinous differentiation (Xiong *et al.*, 2013). A direct correlation of *KRAS* mRNA levels and obesity was shown in cases of complex atypical hyperplasia which was not shown in EC cases, emphasizing again the role of these mutations in endometrial carcinogenesis among obese women (Anna Berg *et al.*, 2015). It was also shown that *KRAS* mutations were more prevalent in patients with BMI over 25 compared to those with normal weight and in those with grade 1-2 compared to high grade tumours (E Birkeland *et al.*, 2012). *KRAS* mutations are very rare in type II EC (Lax *et al.*, 2000), so many authors suggest that the presence of these mutations represent a good prognostic factor (Sasaki *et al.*, 1993) although more recent studies refute this (E Birkeland *et al.*, 2012). Others found that the presence of *KRAS* mutations is associated with longer disease free but not overall survival (Sara A Byron *et al.*, 2012a). They are frequently common (53%) in the *POLE* ultra-mutated and the MSI groups of EC (35%), less common in the SCNA low (16%) and rare in the SCNA high group (3%) (Le Gallo & Bell, 2014). Recently, *KRAS* mutations were shown to be more frequently present in sporadic MSI tumours compared to Lynch syndrome-associated EC (Libera *et al.*, 2018). *KRAS* mutations have been reported to occur rarely when *CTNNB1* mutations are present (mutual exclusivity) (Sara A Byron *et al.*, 2012a). This mutual exclusivity was particularly prominent in microsatellite stable tumours but not in the MSI ones and later confirmed by

the large TCGA study (TCGA, 2013). Older studies have suggested that *KRAS* mutations do not occur in conjunction with *PTEN* and *PIK3CA* mutations (mutually exclusive) (Ikeda *et al.*, 2000), although recent studies report a positive correlation between *KRAS* and *PTEN* (but not *PIK3CA*) mutations (William J Gibson *et al.*, 2016).

1.4.4 Microsatellite Instability

MSI refers to mutations in the microsatellite regions of DNA, which are caused by malfunctioning DNA repair mechanisms. Microsatellites are areas of DNA consisted of one or more nucleotides, being repeated in the genome. It has been proposed that MSI is an early event in the process of endometrial carcinogenesis (Zauber *et al.*, 2015).

MSI was previously reported to be present in 20-30% of cases of type I EC (Basil *et al.*, 2000; Bilbao *et al.*, 2006). 28% of tumours in the TCGA study were clustered in the MSI group (TCGA, 2013) with a 40% rate of MSI amongst endometrioid and only 2% among serous tumours (6% rate in the SCNA high cohort), while another study found the rate of MSI in clear cell EC to be 11.3% (Le Gallo *et al.*, 2017a). In the same study, 92% of MSI tumours were associated with promoter methylation of *MLH1*, pointing towards a sporadic origin rather than Lynch syndrome-associated EC (Libera *et al.*, 2018). An association between MSI and *PTEN* mutations was previously reported (Bilbao *et al.*, 2006). In this study, 58% of the MSI positive tumours had concomitant *PTEN* mutations as compared to 30% for those who were MSI negative (p=0.002). This association was later confirmed in a larger study (TCGA, 2013) which suggested that the rate of *PTEN* mutations in the MSI cluster was even greater, at 88% (Piulats et al, 2016).

MSI is also associated with hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome (LS), which is responsible for 2-6% of EC cases (Ring *et al.*, 2016) with a 39-50% lifetime risk of developing EC (Steinke *et al.*, 2013). This syndrome is associated with germline mutations in MMR genes (*MSH2*, *MLH1*, *MSH6*, *PMS2*). MSI can also be caused by methylation of the MLH1 gene promoter, leading to silencing of the gene and subsequent MMR deficiency (Esteller *et al.*, 1998), which is the case in sporadic cases of EC. This was confirmed by the TCGA study which showed lower levels of *MLH1* mRNA expression in the MSI cohort (TCGA, 2013). *MLH1* and *MSH2* gene mutations have a

higher impact on the DNA repair mechanisms and hence increased risk of carcinogenesis compared to *MSH6* mutation (Steinke *et al.*, 2013).

Sporadic cases of MSI (those associated with *MLH1* promoter methylation) occur in older patients compared to those associated with LS or those who do not have MMR deficiency (McMeekin *et al.*, 2016a; Cosgrove *et al.*, 2017). The same studies confirmed that those sporadic cases are associated with poor prognostic factors including higher stage and grade at diagnosis along with lymphovascular space invasion (LVSI). One of them also correlated these sporadic cases with larger tumour volume and higher rates of lymph node positivity compared to MMR proficient tumours (22% versus 10%, p=0.005) and also higher rates of needing adjuvant treatment (42% versus 29%, p=0.045) (Cosgrove *et al.*, 2017). Despite these poor prognostic features associated with MMR deficiency, the large Gynecologic Oncology Group (GOG) study did not confirm worse progression-free survival nor EC-specific survival on those patients (McMeekin *et al.*, 2016a). On the contrary, the more recent but smaller study showed that sporadic cases of MSI (associated with *MLH1* promoter methylation) have reduced recurrence-free survival in the endometrioid histology cohort (p<0.001) but not when non-endometrioid histologies were included in the analysis (p=0.054) (Cosgrove *et al.*, 2017). Interestingly, MMR deficient tumours with probable germline MMR mutation have lower BMI compared to sporadic cases of MMR deficiency or their MMR proficient counterparts (Cosgrove *et al.*, 2017).

Prognosis associated with MSI is excellent. In the TCGA study, the MSI cluster has 10-year recurrence free survival (RFS) of 80% (TCGA, 2013) with other studies reporting a 5-year RFS of 95% for MSI EC (Stelloo *et al.*, 2015).

Immunotherapy, and in particular immune checkpoint inhibitors [Programmed Death (PD)-1 and PD-Ligand 1 inhibitors], is an attractive option for MSI tumours (Naboush *et al.*, 2017). Studies have shown though that sporadic cases (those associated with *MLH1* promoter methylation), have different immune cell populations (Pakish Janelle *et al.*, 2017) compared to LS-related cases, and although the reason behind this observation is yet unclear, it might render the differentiation between the two quite important in terms of different response to immunotherapy.

1.4.5 FBXW7

FBXW7 is a tumour suppressor gene located in chromosome 4q32, encoding a member of the F-box protein family characterized by the F-box. This protein family is a component of a ubiquitin ligase which is responsible for the degradation of many proteins used by cancer cells for their survival and proliferation, including c-Myc, mTOR, Mcl-1, CDK-2 and many others (Cao *et al.*, 2016; Chien-Hung Yeh *et al.*, 2018). Hence, *FBXW7* mutation leads to inactivation of its ability to break down proteins which, when accumulated, increase cell proliferation and subsequently lead to carcinogenesis.

According to the COSMIC database, endometrium is the tissue with the highest percentage of *FBXW7* mutations amongst all tissues studied (9.26%) (Chien-Hung Yeh *et al.*, 2018), while the large TCGA study showed a 16% mutation frequency in EC. These mutations are extremely common (82%) in the *POLE* ultra-mutated cluster of the TCGA study (TCGA, 2013), they are common in the SCNA high cluster (22%) and less frequent in the MSI and SCNA low groups (9 and 6% respectively). Overall, the two largest studies to date on serous EC have shown remarkably similar results, confirming the incidence of *FBXW7* mutations in this group of patients being 29-30.2% (TCGA, 2013; Jones N et al, 2015). Similar rates of such mutations (25%) have been recently shown to be present in clear cell carcinomas of the endometrium (DeLair *et al.*, 2017).
It has been reported that *FBXW7* mutations were associated with lymph node metastasis in a large cohort (n=1063) of EC patients (Garcia-Dios, Diego A. et al, 2012).
Serous EC cell lines which have been edited to carry *FBXW7* mutations, show increased sensitivity to the CDK inhibitor Dinaciclib (Urick & Bell, 2018a).

1.4.6 *PPP2R1A*

The Protein Phosphatase 2 Regulatory Protein-1A (*PPP2R1A*) gene encodes a regulatory subunit of protein phosphatase 2 (PP2A), which is a serine threonine phosphatase. It has been suggested to have a tumour suppressor function (Janssens *et al.*, 2005).

PPP2R1A mutations have been shown to be present in 18.4% to 43.2% of serous EC (Haesen *et al.*, 2016). In the SCNA high cluster of the TCGA study, 22% of cases were harbouring such a mutation and, if serous cases only are taken into account, the rate of

PPP2R1A mutations was 36.6% (TCGA, 2013). Identical rates of such mutations (36%) have recently been reported in clear cell EC (DeLair *et al.*, 2017), although another larger study published at the same time reported a 15.9% mutation rate (Le Gallo *et al.*, 2017b). The rate of *PPP2R1A* mutations in the endometrioid type of EC is 5-7% (Shih, 2011; McConechy MK et al, 2011).

It was recently shown that *PPP2R1A* mutations coexist with *TP53* mutations and that they occur early in the pathogenesis of type 2 EC (William J Gibson *et al.*, 2016).

1.4.7 *CTNNB1*

The CaTeNiN Beta 1 (CTNNB1) gene encodes β-catenin, a protein of the Wnt signaling pathway which plays an integral role in cell-cell adhesion and the control of epithelial to mesenchymal transition (EMT) (Valenta *et al.*, 2012). EMT is the process during which an epithelial cell can obtain a mesenchymal phenotype through a complex pathway of biochemical alterations (Kalluri & Weinberg, 2009). It has been proposed that EMT is of paramount importance for the malignant transformation of epithelial tumour cells and their ability to invade and metastasize (Thiery, 2002).

The vast majority of *CTNNB1* mutations are missense and they occur in the 'hot-spot' area of exon 3. This area is responsible for the serine threonine phosphorylation sites where glycogen synthase kinase 3 beta (GSK3b) binds and commences the degradation of β-catenin. When it's mutated, degradation does not take place and β-catenin accumulates in the cytoplasm and the nucleus, which subsequently leads to its combination with transcription factors and the activation of cell proliferation and neoplastic transformation (C. Gao *et al.*, 2018). It has been suggested that these events occur relatively early in the process of endometrial carcinogenesis (Saegusa *et al.*, 2001).
Overall, 30% of EC carry *CTNNB1* mutations, with the rate increasing to 52% in the SCNA low cluster of the TCGA study, which includes most of the endometrioid cases (TCGA, 2013). The rate of *CTNNB1* mutations in the MSI group was 20% (Hong, 2014). No such mutation was identified in the 44 serous EC cases examined in the TCGA study, while another study which examined 30 cases of clear cell EC found that only two (7%) were *CTNNB1*-mutant but both of them had a concurrent *POLE* mutation (the remaining 28 cases with wt-*POLE* had no *CTNNB1* mutation) (DeLair *et al.*, 2017). Another study found

only one *CTNNB1* mutation (2%) among 47 non-endometrioid EC cases (Kurnit *et al.*, 2017).

Liu et al analyzed 271 cases of endometrioid EC from the TCGA study and a further 184 cases from MD Anderson Cancer Center (validation cohort) (Yuexin Liu *et al.*, 2014a). They proposed their classification in 4 clusters with separate genetic signatures. Cluster 2 was characterized by *CTNNB1* mutations in 87% of cases and younger age. This cluster was associated with worse PFS (although significance was not reached). As the overall rate of mutations in Cluster 2 was lower compared to all the other 3 clusters, the authors hypothesized that *CTNNB1* mutations are more likely to represent driver rather than passenger mutations. The presence of *CTNNB1* mutations in early grade (1-2) and stage (1-2) EC has been shown to be associated with a significantly worse prognosis, conferring an even worse RFS compared to *TP53* mutations (hazard ratio 5.97 and 4.07 respectively) in a more recent study (Kurnit *et al.*, 2017). The same study confirmed that *CTNNB1*-mutant patients are younger and, surprisingly, have lower rates of LVSI and deep myometrial invasion along with lower grade disease compared to their wild type counterparts.

CTNNB1 mutations are more frequently occurring in endometrioid tumours next to atrophic rather than hyperplastic endometrium (Putten, L. J. M. van der *et al.*, 2017) and they are more frequent in sporadic cases of EC rather than those associated with Lynch Syndrome (31% versus 7%, p=0.002) (Huang *et al.*, 2013).

A phase 2 trial using Temsirolimus (mTOR inhibitor) in advanced EC concluded that carriers of somatic *CTNNB1* mutations have a longer PFS compared to wt-*CTNNB1* patients (Myers, Andrea P. et al, 2016). Similar results were shown from a similar phase 2 trial testing another mTOR inhibitor (everolimus) combined with letrozole in advanced EC. Although the numbers were small (n=25) and significance was not reached (p=0.09), the five *CTNNB1*-mutant patients had a PFS of 26 months compared to 2 months for the wt-*CTNNB1* group (Brian M Slomovitz *et al.*, 2015). It has been shown that EC cell lines and mouse model carrying *CTNNB1* mutations are significantly more sensitive in treatment with TTK kinase inhibitors compared to wt-*CTNNB1* cells (Zaman Guido *et al.*, 2017).

1.4.8 *POLE*

Polymerase epsilon is an enzyme encoded by the *POLE* gene. Along with the other 2 polymerases (alpha and delta), it plays a crucial role in the process of DNA replication and repair (Garg & Burgers, 2005).

POLE mutations have been shown to be present in 7% of EC cases. They are associated with a hyper-mutability state (more than 4-times higher non-synonymous mutation rate compared to all other EC cases studied in the TCGA study, $7.2x10^{-6}$ compared to $1.7x10^{-6}$ mutations/megabase, $p<0.001$) (TCGA, 2013). It has recently been proposed that they represent early events in the process of both endometrial and colorectal carcinogenesis (Temko *et al.*, 2018). *POLE* mutations co-exist with *PTEN* (94%), PI3K mutations (*PIK3CA* in 71% and *PIK3R1* in 65% of cases), *ARID1A* (76%), *FBXW7* (82%) and *KRAS* mutations (53%), while they are characterized by microsatellite stability (TCGA, 2013; Piulats et al, 2016), although others have reported no difference between MSI and microsatellite stable tumours within the *POLE* cluster (Billingsley *et al.*, 2015). They are associated with higher grade at diagnosis but they are usually stage 1 and the prognosis is excellent (8-year RFS of >90% in the TCGA study, 10-year RFS of 100% in 16 *POLE*-mutant patients recruited in the PORTEC-1 trial) (TCGA, 2013; Van Gool Inge *et al.*, 2018). The latter study also showed *in vitro* that *POLE* mutations do not render EC cells more chemo- or radiosensitive and hence this cannot explain the excellent prognosis associated with them.

Interestingly, patients carrying somatic *POLE* mutations were shown to have the lowest BMI compared to any other cluster of the TCGA study (mean BMI 29.8 compared to 33, 35.8 and 32.2 for the other three clusters) (Roque D et al, 2016).

Theoretically, *POLE*-mutant tumours are good candidates for treatment with immune checkpoint (PD-1 and PDL-1) inhibitors as they are associated with significant immune response due to their large number of neoantigens (Gadducci & Guerrieri, 2017). In reality though, due to their good prognosis, it is unlikely that such treatments would be necessary in this group of patients.

1.4.9 *PIK3R1*

PhosphoInositide-3-Kinase Regulatory subunit 1 is a gene encoding the 85kDa regulatory subunit of the PI3K enzyme (PI3K function was described in 1.2.1). It is located in the long arm of chromosome 5 (5q13.1) (Volinia *et al.*, 1992).

Endometrial cancer is the one with the higher mutational rate of *PIK3R1* mutations amongst all cancers tested by the TCGA Pan-Cancer Atlas study, with one third of cases (33.9%) harbouring such a mutation (Michael S Lawrence *et al.*, 2014). As expected, these mutations are more common in the *POLE* ultra-mutated (64.7%), the MSI (41.5%) and the SCNA low (33.3%) clusters, where the vast majority (88%) of endometrioid cases belong. They are less common in the SCNA high group (13.3%), the group incorporating almost all serous cases (TCGA, 2013), and similar mutational rates (15.9-18%) were shown in clear cell EC (DeLair *et al.*, 2017; Le Gallo *et al.*, 2017b).

PIK3R1 mutations usually do not co-occur with *PIK3CA* mutations (mutual exclusivity) in all clusters of the TCGA study (TCGA, 2013), but they coexist with *PTEN* and *KRAS* mutations (Cheung *et al.*, 2011).

There was no impact of *PIK3R1* mutations in progression free survival in a large cohort of endometrioid EC (S. N. Westin *et al.*, 2015).

1.4.10 *PIK3CA*

PhosphatIdylinositol-4,5-bisphosphate 3-Kinase Catalytic subunit Alpha gene encodes the catalytic subunit of PI3K enzyme, the role of which has been explained in 1.2.1. It is located in the long arm of chromosome 3 (3q26.32) and its mutations have been involved in many cancers due its integral role in the PI3K pathway (Volinia *et al.*, 1992). The majority of *PIK3CA* mutations occur in two hotspot regions; the helical domain in exon 9 (E542K and E545K) and the kinase domain in exon 20 (H1047L and H1047R) of the *PIK3CA* gene (Samuels *et al.*, 2005).

Endometrial cancer has the highest *PIK3CA* mutational rate (53.6%) compared to any other cancer tested in the TCGA Pan-Cancer Atlas project (Michael S Lawrence *et al.*,

2014). They are spread evenly across all clusters of the TCGA molecular classification of EC. They are extremely common in the *POLE* ultra-mutated group (71%) but also in the MSI (54%) and the SCNA low clusters (53%), where most endometrioid cases belong (TCGA, 2013). Unlike other mutations which are common in the first three clusters but not in the SCNA high cohort (where the majority of serous cases belong), *PIK3CA* mutations occur in 47% of cases in this cluster (and 42% of serous cases) (Murali et al, 2014a). The rate of these mutations in clear cell EC is lower and has recently been reported to be 13-36% (DeLair *et al.*, 2017; Le Gallo *et al.*, 2017b; Baniak *et al.*, 2018).

There are conflicting reports on the effect of *PIK3CA* mutations in clinico-pathologic variables. Earlier studies reported that those mutations are associated with an invasive phenotype and they are relatively late events in the process of carcinogenesis (Monica Prasad Hayes *et al.*, 2006; Oda *et al.*, 2008). The opposite was shown in a recent, whole-exome sequencing study, which identified *PIK3CA* mutations in cases of CAH and confirmed that these mutations occur early in the process of carcinogenesis (William J Gibson *et al.*, 2016). Another recent study showed no association between the presence of *PIK3CA* mutations and age, BMI, stage, grade, LVSI, myometrial invasion, lymph node metastasis, hormonal receptor status and histological subtype in 280 EC cases (Mjos *et al.*, 2017). From the same study, no association was found between *PIK3CA* mutational status and disease-specific survival but exon 9 mutations were found to be associated with worse disease-specific survival compared to wt-*PIK3CA*. Similarly, another study showed no correlation between *PIK3CA* mutations and PFS in endometrioid EC (Westin, Shannon N et al, 2015). On the contrary, a previous study analyzing the 90 cases with endometrioid tumours from the TCGA database in the SCNA low cluster (most of which were low grade tumours), reported improved survival for *PIK3CA*-mutant patients, which was not seen in the other 3 clusters (D. I. Lin, 2015). Another study though reported that patients carrying somatic *PIK3CA* mutations (in exons 9 and 20) have worse disease specific survival compared to their 'wild type' counterparts in a group of grade 3 endometrioid tumours but the same was not observed in serous EC (McIntyre, John B. et al, 2013). Older study has suggested that exon 20 *PIK3CA* mutations are associated with higher grade disease and more myometrial invasion and LVSI while exon 9 mutations with low grade EC (Lluis Catasus *et al.*, 2009). Others have reported an association between *PIK3CA* mutations and higher grade disease and that the H1047R mutation was associated with reduced RFS (Garcia-Dios, Diego A. et al, 2012). A large study involving 464 tumours confirmed that

PIK3CA mutations are not associated with age at diagnosis, they are associated with low stage disease and they have no impact on disease-specific and overall survival (Sara A Byron *et al.*, 2012b).

It has been reported that *PIK3CA* mutations are more frequent in sporadic rather than Lynch Syndrome-associated cases of EC (40% versus 14%, p=0.015) (Huang *et al.*, 2013). They are also significantly coexist with *PTEN* mutations (Konopka, 2011). As mentioned previously, *PIK3CA* mutations are negatively associated (mutual exclusivity) with *PIK3R1* mutations (TCGA, 2013) and older studies have suggested mutual exclusivity with *KRAS* mutations as well (Velasco *et al.*, 2006; Kang, 2008), although others, including whole-exome sequencing studies, found the opposite (Ollikainen *et al.*, 2007; William J Gibson *et al.*, 2016).

Recently, a phase 1 clinical trial assessed the efficacy of Alpelisib (PI3Ka inhibitor) in patients with solid tumours harbouring *PIK3CA* mutations (Dejan Juric *et al.,* 2018). It was tolerable and showed promising efficacy (disease control rate of 58.2%) in a cohort of patients including a few with EC. CH5132799, a PI3K inhibitor, showed pre-clinical efficacy in cells harbouring *PIK3CA* mutations (H. Tanaka *et al.*, 2011) and entered a UK-based, phase 1 clinical trial in patients with advanced solid tumours which showed some efficacy in a patient carrying a *PIK3CA* mutation but not in those with wt-*PIK3CA* (Blagden *et al.*, 2014) Another PI3K and mTOR inhibitor (GDC-0980) was shown to be significantly more effective in serous EC cell lines carrying *PIK3CA* mutations compared to wt-*PIK3CA* cells (English, Diana P. et al, 2013).

1.4.11 *ARID1A*

AT-Rich Interaction Domain 1A gene encodes a protein which participates in many proteinic complexes of the SWI/SNF (SWItch/Sucrose Non-Fermentable) family. This family plays an integral role in chromatin remodeling and through that, in DNA repair and cell proliferation (Reisman *et al.*, 2009). *ARID1A* gene is a tumour suppressor located in the short arm of chromosome 1 (1p36.11).

ARID1A mutations are common in EC as they occur in 34% of cases overall. They are even commoner amongst endometrioid tumours as they occur in 42% of cases in the SCNA

low and 37% of the MSI clusters of the TCGA study, which contain most of these type of tumours. Similarly to *PTEN* mutations, they are inactivating since their proteinic expression is reduced (TCGA, 2013). As expected, they occur frequently (76%) in *POLE*-ultramutated cases (TCGA, 2013).

ARID1A mutations occur less frequently in clear cell EC (15.9-22%) (DeLair *et al.*, 2017; Le Gallo *et al.*, 2017b), which is in contrast with much higher rates of these mutations in ovarian clear cell cancer (46-57%) (Alldredge & Eskander, 2017). They are rarely present in serous cases of EC (6-18%) (Wiegand *et al.*, 2011; Matthieu Le Gallo *et al.*, 2012).

ARIDIA mutations frequently coexist with PI3K pathway mutations (Liang *et al.*, 2012) and a wild type *TP53* (Allo *et al.*, 2014). They are also correlated with MMR deficiency in high grade EC cases (Nelson, Gregg S. et al, 2013). *ARID1A* mutations are associated with MSI caused by *MLH1* promoter methylation (sporadic cases) but not with germline mutations on the MMR genes (75% versus 14%, $p<0.0001$) (Tjalling Bosse *et al.*, 2013) Very few studies have examined the association between *ARID1A* loss and overall survival (OS) in EC. A recent meta-analysis included only two studies and showed that *ARID1A* status does not affect OS (Liu *et al.*, 2017). Similarly, another study focusing on clear cell EC, showed no effect of *ARID1A* mutations to survival (Fadare *et al.*, 2013). One of the two studies included in the meta-analysis found no association between *ARID1A* mutations and stage, grade, myometrial invasion, LVSI, BMI, menopausal status, age and hormonal receptor status of the 111 endometrioid EC cases analyzed (Rahman, 2013).

1.5 Overview of approach to variant calling

The use of next generation sequencing (NGS) is rapidly increasing in the study of many diseases including cancer. This has brought new challenges in the field of variant calling and the significance of each variant. The American College of Medical Genetics in conjunction with the Association for Molecular Pathology and the College of American Pathologists recently produced guidance for the interpretation of those variants (Richards *et al.*, 2015).

In this document variants are classified as pathogenic, likely pathogenic, of uncertain significance, likely benign or benign. The word 'likely' is advised when there is a more than 90% chance of the variant being pathogenic or benign respectively.
This consensus document acknowledges the usefulness of publicly available variant and population databases in researchers analyzing NGS results along with computer softwares which help interpreting such results.
Criteria for classifying pathogenic and benign variants have been defined (Appendix 1) and the selected criteria are marked and categorized according to Appendix 1 tables.

1.5.1 The role of family history

The importance of family history in the interpretation of somatic variants cannot be overstated. Clinicians and laboratories should recognize germline mutations that are known to increase the risk of an individual developing cancer as this might have significant consequences for family members as well (BRCA gene mutations are a good example). The family history itself can assist in determining the pathogenicity of variants. For example, a negative family history for a genetic disease known to be inherited in a dominant manner, can help in the interpretation of a mutation found in an offspring with the disease as pathogenic and *de novo*. It is also useful to evaluate incidental mutations in the absence of disease under the prism of family history. The possibility of such mutation (in a healthy individual) being pathogenic is much less if the family history is free of the disease associated with this mutation (Richards *et al.*, 2015). On the contrary, as shown in Table A.1, a strong family history is a supporting evidence for classifying a variant as pathogenic, even if there is no phenotypic confirmation at the time of testing.

1.5.2 Genotype/phenotype interactions

It is now widely accepted that apart from genetic aetiology, many diseases (including cancer) have environmental causes. Chemical agents, radiation, diet, are only a few examples of such aetiologic factors for disease onset. What is now becoming more apparent is the interaction between genotype and environment and that the same individual might have a different susceptibility to a disease depending on the environment. An example is the higher risk of skin cancer on a fair-skinned human if living in an environment with significant sunlight exposure compared to one with less (Adami *et al.*,

2002). The study of genetic/environmental interactions carries great importance as it provides more accurate assessment of the population-attributable risk, it identifies new environmental causes of disease, it reveals disease mechanisms and reasons for susceptibility of particular individuals and through all this it allows the development of preventive recommendations and/or new therapies (Hunter, 2005). These studies though can be extremely challenging to perform especially due to the inherent difficulty in quantifying the environmental exposure. A typical example of this is alpha 1-antitrypsin deficiency, a condition caused by *SERPINA1* gene mutations and associated with lung disease and in particular emphysema (US National Library of Medicine, 2020). Lung disease can also be caused by several environmental factors like smoking, chemicals, dust (pollution). An individual with *SERPINA1* gene mutation who doesn't smoke has a modest risk of developing lung disease. Their risk is highest in the presence of smoking/environmental pollution. The evaluation though of these two factors can be difficult as it relies mainly on questionnaires and is subject to recollection bias (Hunter, 2005). In summary, genotype is not the only factor determining the risk of disease. Its interaction with the environment is a complex process which is difficult to be quantified but will ultimately define disease onset and its course.

1.5.3 Single Nucleotide Polymorphisms (SNPs)

SNPs refer to single nucleotide changes across the genome. They are very common and usually occur in the intronic areas of the DNA (US National Library of Medicine, 2020). Nevertheless, they can occur within a gene or its regulatory area and can be involved in the disease process. It is now commonly accepted that SNPs play a role not only in the pathogenesis of diseases but also in the individual susceptibility to them (Deng *et al.*, 2017) along with responses to treatment (Chaudhary *et al.*, 2015). The latter has lead to the development of 'pharmacogenomics', a field studying individual variations in drug responses due to genetic differences. Typical examples include the different efficacy of anti-asthmatic and anti-hypertensive drugs to patients due to polymorphisms in various areas of their genome (Chaudhary *et al.*, 2015).

Studies have shown that genetic heterogeneity among various populations exists and that even a small number of SNPs can be used to differentiate groups of different geographical origin (Rosenberg *et al.*, 2002; Allocco *et al.*, 2007). This can be a very useful tool in

genetic studies comparing distinct ethnic populations as it would allow researchers to confirm the correct allocation of study participants in their respective ethnic group.

In view of the fact that SNPs are inherited (Hemminki *et al.*, 2005), they are also used as genetic markers of diseases and in particular of the familial component of the risk for developing this disease. Along with family history, SNPs have been shown to predict the susceptibility of an individual to several common diseases, including coronary heart disease (Tada *et al.*, 2016), breast and colorectal cancer (Lilyquist *et al.*, 2018; Jenkins, M. A. et al, 2019). It is anticipated that in the future, as data from genome-wide association studies increase, SNPs will be incorporated in risk prediction algorithms along with clinical variables of the disease (including family history) and this will increase the sensitivity of these tools in identifying susceptible individuals (Do *et al.*, 2012).

1.6 Racial differences in endometrial cancer

Racial disparities in the incidence and disease profile have been shown in many cancers and endometrial cancer is not an exception. The issue of investigating ancestry and genetics in research is challenging due to the concept of race being confused or influenced by the interaction of environment and culture, as well as heterogeneity within populations (Yudell *et al.*, 2016). Although it has been proposed that the use of race as a surrogate marker for measurable genetic differences should be avoided (Bevan, 2004), it is acknowledged that there is utility when investigating the interplay of genes and environmental factors (Williams, 2002). Most studies examining the epidemiological differences between various racial populations have been conducted in the USA and mainly focus on the White versus African American (AA) comparison. The data for other groups are more limited and will be examined below.

1.6.1 Incidence

White women have been shown to have the higher incidence rates of EC in the USA (24.38 per 100,000 women) compared to women from all other races (M. -. Cote *et al.*, 2015).

The same study, which examined more than 120,000 patients from the Surveillance, Epidemiology, and End Results (SEER) database, diagnosed with EC between 2000-2011, showed that the incidence of the disease was 19% less in AA women (rate ratio 0.81 compared to reference 1 for White women), while older studies have reported differences as high as 35% (Sherman & Devesa, 2003). The incidence of EC was even less amongst Hispanics and Asian women (rate ratio 0.73 and 0.70 respectively). A previous large prospective study examining almost 47,000 patients of multiethnic background in the USA showed that the incidence rate of EC, after adjusting for risk factors, was 32% less in AA compared to White (W) women (relative risk 0.68), while it was 35% less for Latinas (relative risk 0.65) and 26% less for Japanese Americans [relative risk 0.74, 95% confidence interval (CI) 0.54-1.01], with the latter difference being just outside statistical significance (Setiawan *et al.*, 2007). This difference in the incidence rates (32% less for AA women in the older study versus 19% in the recent one) can be explained, at least partly, by the rapid increase in EC cases among AA women (annual percentage change of 2.5, compared to 0.6 for White women) (M. -. Cote *et al.*, 2015). Based on these data, future projections estimate that the gap will keep closing and might even be reversed after 2030 (Gaber *et al.*, 2017).

The fact that Asian patients living in the USA have reduced rates of EC compared to their White counterparts has been confirmed in other studies as well. Zhang et al showed that the age-adjusted incidence rate of EC amongst Asians was 16.8 per 100,000 women compared to 26.1 for White patients (36% less) and similar results were shown by Liao et al (C. Katherine Liao *et al.*, 2003; M. Zhang *et al.*, 2006). The latter study, interestingly, confirmed that amongst women of Japanese and Chinese origin, the rate of EC was higher for those born in the USA compared to those who immigrated to the country, although still much less compared to White women. The rate of EC in the Asian group is rising at a fast pace with an annual percent change of 2.48 between 2000 and 2007, the higher among all ethnicities studied (M. -. Cote *et al.*, 2015).

Perhaps surprisingly, a study comparing the incidence rates of EC in various ethnic groups in the UK concluded that AA women had a 16% higher incidence of the disease compared to their White counterparts (rate ratio 1.16, CI 1.03-1.31), which contradicts the previous data from the USA (Shirley *et al.*, 2014). The same study revealed that the incidence of

EC among South Asian women living in the UK is 10% less compared to White (rate ratio 0.90, CI 0.81-1.01), but this was just outside statistical significance.

1.6.2 Clinico-pathologic differences

Interestingly, AA women are more frequently diagnosed with Type 2 endometrial tumours (serous, clear cell), which by definition are more aggressive and therefore carry a worse prognosis. Wright examined the data of more than 80,000 women from the National Cancer Institute's SEER database and found that 38% of endometrial tumours were type 2 in AA women as opposed to only 14.9% in White women (Wright *et al.*, 2009). The same study revealed that AA women were younger at diagnosis but presenting with higher stage disease (73% of White patients were stage I versus only 54% of AA women, $p<0.001$). A more recent, larger study confirmed these findings and showed that AA women presenting with late stage disease (IIIC/IV) was double that of Whites (17.8% compared to 9.8%, $p<0.001$) (Fader, Amanda N. et al, 2016). AA women also present with higher grade disease compared to White women, with identical results being confirmed by two studies (32.7% versus 19.2%, $p<0.001$ and 32.8% versus 17.7%, $p<0.001$) (Setiawan *et al.*, 2007; Fader, Amanda N. et al, 2016). Similar results were shown for Latinas women, who also had higher rates of late stage, higher grade and type 2 tumours compared to White women (Setiawan *et al.*, 2007). The same study showed that AA and Latinas have higher BMI compared to White patients. AA women have also been shown to have increased rates of LVSI in cases of early stage endometrioid EC (Elshaikh, Mohamed A. et al, 2012).

Asian women have been shown to present with EC on average 7 years earlier than White women (58.4 compared to 65.1, $p<0.01$ in an older study, 57.7 years versus 64.3 in a more recent one) (M. Zhang *et al.*, 2006; Mahdi *et al.*, 2014). They also present more frequently with late stage disease (21.5% versus 15.4%, $p<0.01$ and 15.6% compared to 13.3%, $p=0.04$ in the two aforementioned studies respectively) and have higher rates of lymph node positivity compared to White women (13.8% versus 11.8%, $p<0.001$) (Mahdi *et al.*, 2014). A higher rate of type 2 tumours amongst Asian women (10.6% versus 9.6% for White patients, $p=0.041$) has also been shown (Mahdi *et al.*, 2014).

1.6.3 Molecular and genetic differences

PTEN mutations in endometrial cancer are associated with a more favourable histologic subtype (endometrioid) and better prognosis (Risinger *et al.*, 1998). These mutations were shown to be more prevalent in White women compared to their AA counterparts in older studies (22% vs 5%, p=0.004) (Maxwell *et al.*, 2000). A recent analysis of TCGA data from our group showed that *PTEN* mutations were the commonest in both White and Asian patients with an incidence of 63% and 85% respectively, while they were less frequent in AA women (39%) (Guttery *et al.*, 2018).

MSI has also been associated with endometrioid histology and better survival. Basil et al reported that White patients are 3 times more likely to have MSI in their uterine specimens compared to AA (Basil *et al.*, 2000). On the contrary, Maxwell et al did not report a statistically significant difference in MSI between White and AA women (16% vs 13%) (Maxwell *et al.*, 2000). An analysis of data from the TCGA study showed that 29% of White patients belong to the MSI cluster compared to 14.3% of Black patients (Dubil *et al.*, 2018).

As mentioned previously, *TP53* mutation is the commonest genetic mutation in type 2 endometrial cancer and hence it has been associated with worse survival. Studies have shown that the overexpression of *TP53* occurs more frequently in AA women compared to White women (34% vs 11%, p=0.003) (Clifford *et al.*, 1997) and this may be one of the contributory factors which might explain the aggressive phenotype of their disease. These results were confirmed by a TCGA data analysis from our group which confirmed that *TP53* was the commonest mutation in AA women as it was present in 49%, while it was less common in White (32%) and Asian patients (25%) (Guttery *et al.*, 2018). In another analysis of TCGA data, it was shown that 61.9% of Black patients were clustered in the SCNA high group compared to only 23.5% for their White counterparts (p=0.0005) (Dubil *et al.*, 2018).

It has been reported that AA patients with low-grade endometrioid tumours have higher rates of *KRAS* and *PIK3CA* mutations compared to White patients (M. Cote *et al.*, 2012). HER2 overexpression is another common feature of serous endometrial tumours. Santin has reported that this overexpression is significantly more common in AA compared to

White patients (70% vs 24%, p=0.04), although this study was based on only 27 patients with serous EC (Santin *et al.*, 2005). He also showed that survival for those who were heavily expressing HER2/neu in their tumour samples was significantly reduced compared to those who were expressing little or no HER2.

1.6.4 Access to health care- Socioeconomic discrepancies

It has been postulated that access to health care might be a contributing factor for the worse outcomes observed in AA women, as earlier studies showed a higher percentage of those women not receiving cancer-directed treatment compared to their White counterparts (9% versus 4%) and being treated with surgery less frequently (79% versus 91%) (Hicks *et al.*, 1998). However, later studies controlling for variables associated with treatment showed that even when the same treatment modalities are being used, AA women carry a worse prognosis, which cannot be explained by access to health care or treatment bias (Maxwell *et al.*, 2006). Finally, more recent epidemiological studies showed that insurance status partially contributed to the survival difference observed between AA and White patients, although this was not as significant as clinico-pathologic variables (with histology being the most prominent) (Fedewa *et al.*, 2011).

1.6.5 Prognosis

AA women are 60% more likely to die from their EC compared to White, after controlling for known prognostic factors (Wright *et al.*, 2009). There is a significant difference in the 5-year survival between AA and White women (61% for AA women versus 85% for White) (Ahmedin Jemal *et al.*, 2010). Even within the group of patients with early stage 1 disease and after controlling for clinical and socioeconomic variables, 5-year survival was 89.4% for White compared to 82% for Black patients (<0.001) (Fader, Amanda N. et al, 2016). This difference was even greater for patients with late stage disease (IIIC/IV), 42.8% for White versus 24.6% for AA (p<0.001). The authors hypothesize that this might be attributed to cultural beliefs (making treatment less accepted) or disease being biologically different and hence less responsive to treatment compared to other races. Similar results were confirmed in another study which showed that, across all histologic types, AA women have a 55% higher mortality compared to White women (mortality rate

ratio 1.55), and the difference becomes even bigger in the subgroups of serous and clear cell histology (mortality rate ratio of 2.6 and 2.4 respectively) (M. -. Cote *et al.*, 2015).

Asian and Hispanic patients with EC in the USA are less likely to die from the disease compared to White women. Age-adjusted incidence-based mortality across all histologies is 42% less in Asian women (mortality rate ratio 0.58) and 20% less in Hispanic (mortality rate ratio 0.80), compared to White women (M. -. Cote *et al.*, 2015). The Asian group in particular has an impressively low mortality across all histologies with the exception of clear cell tumours, where there is no difference with the White cohort. The greatest difference is evident in the low-grade endometrioid tumours where mortality is less than half compared to White women (mortality rate ratio 0.44). The same study showed that 5-year survival across all histologic types was identical for White and Asian patients. An older study had shown better 5-year survival for Asian patients (79.4% compared to 75.2% for White, $p<0.01$) but this was attributed to the younger age at diagnosis as, when controlling for age, there was no difference in survival rates (M. Zhang *et al.*, 2006). A recent study though showed 14% better overall survival amongst Asian patients compared to White women, even after controlling for several prognostic variables including age (Mahdi *et al.*, 2014). Interestingly, this study also showed that Asian immigrants to the USA have a 17% better overall and 34% better cancer-specific survival compared to US-born Asian women.

Chapter 2 Materials and Methods

2.1 In-vitro cell line studies

2.1.1 Endometrial cancer cell lines

The Ishikawa cancer cell line was obtained from Sigma-Aldrich (now Merck KGaA, Darmstadt, Germany) and the HEC-1-A cell line from ATCC (ATCC® HTB-112™).

2.1.2 Resuscitation of frozen cells

Cryo-vials (1ml) containing cells were transferred from liquid nitrogen tanks to a 37°C water bath for approximately one to two minutes. The content of the vial was then transferred under aseptic conditions to a 15ml Falcon tube and pre-warmed complete culture media was added slowly.

For Ishikawa cells, complete culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) which was supplemented with 5% Fetal Bovine Serum (FBS) and 1% Non Essential Amino Acids. For HEC-1-A cells, complete culture medium consisted of McCoy's 5A (Modified) medium, supplemented with 10% FBS.

The cells were subsequently transferred to a 60 or 100mm culture dish and incubated at 37ºC in a humidified incubator with 5% CO_2.

2.1.3 Cell splitting

When cells reached 70-80% confluency in the culture dish, they were passaged into new dishes. Cell splitting was achieved by discarding the old medium and washing the adherent Ishikawa cells with 1X Dulbecco's Phosphate Buffered Saline (DPBS, Gibco™) and the HEC-1-A cells with 0.25% Trypsin-EDTA (Gibco™). Trypsin–EDTA 0.25% was then added to both cell lines before incubating them for 5 minutes (longer incubation was usually needed for the dissociation of HEC-1-A cells as they were always more adherent to the dish). The dissociated cells were then washed with their respective medium and transferred to a 15ml Falcon tube before centrifuging them at 150g for 5 minutes. The supernatant was then discarded and the cell pellets were re-suspended in medium prior to

seeding the cells in culture dishes at the desired number. Both cell lines were splitted every two to three days depending on the cell confluency.
The culture media, supplements and reagents are shown in Table 2.1 below.

Medium/Supplement	**Producer**	**Catalog Number**
Dulbecco's Modified Eagle's medium	Gibco	61965026
McCoy's 5A (Modified) medium	Gibco	16600082
MEM Non-Essential Amino Acids Solution 100X	Gibco	11140050
Fetal Bovine Serum	Gibco	10500064
Dulbecco's Phosphate Buffered Saline (PBS)	Gibco	20012019
Trypsin-EDTA 0.25% (Phenol red)	Gibco	25200056
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	D8418-100ML

Table 2.1 Culture media, supplements and reagents used in cell culture.

2.1.4 Cryostorage

The cell pellet was obtained with the procedure described previously in 2.1.3. It was then re-suspended with 1ml of solution consisted of 90% FBS and 10% Dimethyl Sulfoxide (DMSO) for the Ishikawa cells and 95% complete culture medium supplemented with 5% DMSO for the HEC-1-A cells. The cells were then transferred to cryovials at a concentration of 2-3x10^6 cells/ml. Isopropanol freezing container (Thermo Scientific™ Mr. Frosty™, Cat. No 5100-0001) was then used to achieve slow freezing of the cryovials (about -1°C per minute) in -80°C freezer. 24–48 hours later the cells were transferred to liquid nitrogen storage vessel.

2.1.5 Cell counting

Cell counting was performed prior to passaging the cells in order to assess their viability and also prior to seeding them for performing MTS assay (see 2.1.6). It was also performed prior to cryo-storage in order to ensure that a minimum of $2x10^6$ cells were stored in each cryo-vial. Cells were counted by mixing 10μL of cell suspension with 10μL of 0.4% Trypan Blue Solution (Gibco™). Half of this mixture was added to cell counting slides (Bio-Rad™) and counted using the T20 automated cell counter (Bio-Rad™) in cells/ml.

2.1.6 Cell proliferation assay

The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega™) was used to assess cell viability following treatment with various inhibitory drugs. This assay uses a tetrazolium compound (MTS) and an electron coupling reagent (PES) which are combined together in one solution. It is a colorimetric method as the MTS is converted by metabolically active cells to a colored formazan product.

5,000-10,000 cells were transferred to 96-well, flat bottom plates, in triplicates. When reached 70-80% confluence, they were treated with serial concentrations (10, 1, 0.1, 0.01 and 0.001μM) of various inhibitory drugs. Three control wells were used by adding DMSO to it as all the drugs of our study were diluted in DMSO. Finally, three wells were filled with culture medium only in order to measure the background absorbance.

CellTiter 96® AQueous One Solution Reagent (PROMEGA) was kept at -20ºC freezer, protected from light, as per manufacturer's instructions. 48-hours after the drug treatment it was thawed and added to the cells at a concentration of 5:1 (for every 100μl of medium, 20μl of the reagent was added). The 96-well plate was then incubated for 2-3hr at 37°C in a humidified, 5% CO2 atmosphere.

The absorbance of the plate was read at 490nm using Infinite® F50 / Robotic ELISA plate reader (TECAN™) and the data recorded with the Magellan™ data analysis software. The percentage of cell viability was subsequently calculated with Microsoft Excel 2013 by dividing the absorbance (optical density) of the sample with the one of the control and multiplying this by 100. Graphs were created using GraphPad Prism 7.0 software.

2.1.7 Flow Cytometry using Propidium Iodide staining

Propidium iodide (PI) is a dye which does not pass through the cell membrane of live cells but it easily permeates the membranes of permeabilized cells and binds to double stranded DNA. Along with the ability of flow cytometry to measure fluorescence intensity in those cell populations, PI staining is used to perform cell cycle analysis.

Cells were cultured in 60mm culture dishes. When they reached 70-80% confluency, they were treated with a drug inhibitor and incubated at 37°C in a humidified chamber with 5% CO_2. 24 hours later, their medium was collected on a 15ml Falcon tube. Trypsin 0.25%-EDTA was used to detach the adherent cells, which were then added to the same Falcon tube. Following centrifugation at 150g for 5 minutes, the supernatant was discarded and the cell pellet washed with 1ml DPBS. The solution was centrifuged again and the supernatant disposed. The cell pellet was then re-suspended with 1ml cold Ethanol 70% to fix the cells and stored in the -20°C freezer until used (usually overnight incubation).

On the day of the staining, the Ethanol fixated solution was centrifuged for 5 minutes at 250g and the supernatant discarded. DPBS was used to wash the pellet and further centrifugation ensued. The pellet was re-suspended with 300-500μl of PI staining solution constituted of 50μg/ml propidium iodide and 40μg/ml RNAase A in 1XPBS. The solution was then transferred to 5 ml polystyrene round bottom FACS tubes (Falcon™) and incubated in the dark for 30 minutes, at 37°C. The samples were read in BD FACSCanto™ II fluidics system (BD Biosciences™) and the cell cycle analysis was done using the FACS Diva™ software (BD Biosciences™), version 6.1.3.

2.1.8 Protein analysis

2.1.8.1 Cell collection

For studying cell pathway activation, cells were plated in 60mm culture dishes and incubated in a humidified chamber at 37°C. When they reached 70-80% confluency, they were treated with a drug inhibitor. 24 hours later, the culture medium was collected in a 15ml Falcon tube. The cells were washed with DPBS (Ishikawa) and Trypsin 0.25%-EDTA (HEC-1-A) and this was also collected in the same Falcon tube. The cells were

detached after an incubation with Trypsin 0.25% for a minimum of 5 minutes at 37ºC. They were subsequently centrifuged at 150g for minutes and the supernatant was discarded. The cell pellets were then lysed (as described in 2.1.8.2) in order to extract whole cell protein.

2.1.8.2 Obtaining whole cell lysates

The pellets obtained in the previous step were incubated on ice with 100μL RIPA (radio immunoprecipitation assay) lysis buffer containing 1% protease inhibitor and 1% phosphatase inhibitor cocktails (Sigma-Aldrich™). RIPA lysis buffers contained 150mM NaCl, 50mM Tris HCl pH 8.0, 1% Tergitol-type NP-40, 0.1% Sodium Dodecyl Sulfate (SDS) and 0.5% sodium deoxycholate diluted in distilled water (dH_2O) and stored at 4ºC. After incubating the mix in 1.5ml Eppendorf tubes on ice for 20 minutes, the pellets were macerated with 21 gauge needles (BD Microlance™ 3). They were subsequently centrifuged at 14,000rpm for 15 minutes at 4ºC and the supernatant was transferred into new tubes and the pellets discarded. Bradford assay, a spectroscopic method of calculating proteininic concentration, was then used to determine the protein concentration of the sample.

4xSDS sample buffer had been prepared in advance. 10ml of stock consisted of 2.4ml 1M Tris-HCl pH 6.8, 0.8 grams SDS, 4ml 100% glycerol, 2ml β-mercaptoethanol, 4mg bromophenol blue and 1.6ml dH_2O. The protein sample was diluted 1:3 with 4x SDS buffer and boiled at 100ºC for 5 minutes. Samples were then ready to be used or stored at -20ºC.

2.1.8.3 SDS-PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gels were prepared using a Mini-PROTEAN® Tetra Cell Casting Module (Bio-Rad, Catalog Number #1658015) and the concentrations of the chemicals used are shown in Table 2.2 below. The casting and the glass frames were cleaned and assembled prior to making the gels. Gels were constituted of a 10% running (lower) and a 5% stacking (upper) gel. From the ingredients shown in Table 2.2, TEMED was added in the fume hood and APS was added last. The running gel was then poured between the glass frames

and it was topped up with dH_2O for the last 2cm to the top. It was then left to solidify for 30 minutes.

After the gel was solid, the excess water was removed and the stacking gel was added up to the top. A 15-well Mini-PROTEAN® Comb (1.5 mm, 40 µl, Bio-Rad™, Catalog Number #1653366) was inserted in the glass frame. The gel was left to solidify for another 30 minutes and was subsequently placed into a Mini-PROTEAN Tetra Cell electrophoresis tank (Bio-Rad™).

Running (lower) gel	10%	**Stacking (upper) gel**	5%
Bis Acrylamide (40%)	6ml	**Bis Acrylamide (40%)**	875µL
1.5M Tris (pH 8.8)	6ml	**0.5M Tris (pH 6.8)**	1.75ml
10% SDS	240µL	**10% SDS**	70µL
10% APS	120µL	**10% APS**	70µL
TEMED	12µL	**TEMED**	7µL
dH_2O	11.5ml	**dH_2O**	4.325ml

Table 2.2 Volume of chemicals used to prepare polyacrylamide gel for Western Blotting. APS: Ammonium persulfate (Sigma-Aldrich™).
TEMED: N,N,N′,N′-Tetramethylethylenediamine (Sigma-Aldrich™).

The whole cell lysates, obtained as described in 2.1.8.2, were brought to the boil (100°C for 5 minutes in a heating block) and they were allowed to cool prior to their loading in each well. The volume loaded to each well had been previously calculated using the Bradford assay. A 4µL PageRuler™ Plus Pre-stained protein ladder (ThermoFisher Scientific™, Catalog Number 26619) was also loaded on the first well. The tank was subsequently filled with 1X Running Buffer (100ml of 10X Running Buffer, 900ml dH_2O). In order to make 1 litre of 10X Running Buffer, 30.3g of Tris Base (Fisher Scientific™) were mixed with 144g of Glycine (Fisher Scientific™) and 10g of SDS (Fisher Scientific™), the remaining being dH_2O.

Electrophoresis was performed starting at 60V for 20min, followed by 100V for at least 90 minutes.

2.1.8.4 Western Blot analysis

Transferring buffer (1X) had already been prepared by mixing 700ml of dH_2O with 200ml of Methanol and 100ml of 10X Transferring Buffer. The mix was left in -20ºC until needed. 10X Transferring Buffer was prepared by mixing 30.3gr of Tris Base with 144gr of Glycine and dH_2O up to the volume of 1 litre.

The proteins from the polyacrylamide gel were transferred to nitrocellulose filter membrane (Amersham; GE) using a Mini Trans-Blot® Cell (Bio-Rad™). Whatman Filter Paper (Amersham; GE) and nitrocellulose filter membrane were cut in the size of the gel and were immersed in transferring buffer. The filter sponge was placed in the black side of the cassette followed by two filter papers, the gel, the membrane, two more filter papers and finally another filter sponge. The cassette was subsequently placed into the plastic case of the transferring tank and filled with transferring buffer. Ice was added both inside the tank and outside of it. Transfer of the proteins occurred under constant 350mA current for 90 minutes.

Following transfer, membranes were incubated in blocking solution consisted of 5% Bovine Serum Albumin (Sigma-Aldrich™) in 1XPBS with 0.1% Tween-20 (Thermo Scientific™) for 1 hour at room temperature with orbital shaking. Following this, the membranes were incubated overnight with the primary antibodies (Table 2.3), diluted in blocking solution as per their manufacturer's instructions (1/1000 being the commonest used).

Antibody	Origin	Dilution	Molecular Weight (kDa)	Supplier/ Catalog Number
Phospho-Akt (Ser473)	Rabbit	1:1,000	60	Cell Signaling Technology/#4060
AKT	Rabbit	1:1,000	60	Cell Signaling Technology/#9272
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit	1:1,000	44, 42	Cell Signaling Technology/#4370
p44/42 MAPK (Erk1/2)	Rabbit	1:1,000	42, 44	Cell Signaling Technology/#9102
β-actin	Rabbit	1:5,000	42	Abcam/ Ab8227
Actin (C2)	Mouse	1:250	43	Santa Cruz/ sc-8432
Phospho-MEK ½ (Ser217/221)	Rabbit	1:1000	45	Cell Signaling Technology/#9154
MEK 1/2	Rabbit	1:1000	45	Cell Signaling Technology/#9122

Table 2.3 Antibodies used for Western blotting. All antibodies are monoclonal with the exception of β-actin which is polyclonal.

The following day, the membranes were washed three times with 1X PBS with 0.1% Tween-20 (ten minutes each). They were then incubated in the dark for 60 minutes and under constant agitation with blocking solution in which the secondary antibodies had been added. Secondary antibodies were Goat anti-Rabbit for rabbit primary antibodies (IRDye® 680RD Goat anti-Rabbit IgG, Li-COR^{TM}, product number 926-68071) and Goat anti-Mouse for mouse primary antibodies (IRDye® 800CW Goat anti-Mouse IgG, Li-COR^{TM}, product number 926-32210). Both types of secondary antibodies were used in a concentration of 1 in 10,000. The membranes were then washed in the dark three times with PBS-0.1% Tween-20 and once with 1X PBS. Finally, membranes were read in the Odyssey imager (Li-COR^{TM}) and the protein bands analysed and quantified using Image Studio Lite software version 5.2 (Li-COR^{TM}).

2.2 Racial differences between British White and British South Asian women

2.2.1 Ethical approval and recruitment

Ethical approval for the study was granted by Yorkshire and The Humber - Bradford Leeds Research Ethics Committee (REC reference number: 15/YH/0510). The study's protocol was also approved by the University Hospital of Leicester's Research and Development department.

Women with a preoperative diagnosis of endometrial cancer or complex atypical hyperplasia on a biopsy were recruited at the University Hospitals of Leicester between February 2016 and February 2018. They all gave written informed consent prior to their primary surgery, after being given a patient information leaflet. Each patient had a unique study number which was subsequently used during experiments and data analysis.

2.2.2 Tissue and data handling

All patients underwent a hysterectomy as part of their treatment. Following surgery, the uterine specimen was processed in the Pathology laboratory of Leicester Royal Infirmary by trained NHS staff. Formalin Fixed Paraffin Embedded (FFPE) blocks of tissue were available for the study after formal diagnosis had been established.

Soon after patient recruitment had commenced, a decision was made to add lipid profile and glycosylated haemoglobin (HbA1c) testing in the routine pre-operative bloods obtained from each participant, with the exception of those who had those investigations performed (within the previous month).

Clinico-pathological data for the participants of the study were obtained from the patients' medical notes and from the hospital's online ICE system which stores laboratory results of investigations. Data were stored in password-protected Microsoft Excel 2013 datasheets and handled in accordance with University Hospital of Leicester's policies.

2.2.3 Patient and gene selection for sequencing

60 patients were selected for sequencing (30 WB and 30 BSA), after being completely matched in terms of risk of adjuvant therapy using the ESGO-ESMO-ESTRO classification (Colombo *et al.*, 2016). Based on this consensus classification, patients with EC are categorised based on their risk of developing a recurrence to low, intermediate, high-intermediate, high, advanced and metastatic groups. We selected 15 patients from each group of interest who were of low risk and 15 who were intermediate to high risk. The percentage of patients with type 2 tumours in this cohort was representative of these type of tumours in the general population of patients with EC.

Ten genes were chosen for analysis based on their frequency of somatic mutations in EC, their tumour driving potential and their likely therapeutic significance. Five out of the six most frequent mutations in EC identified in the TCGA study (TCGA, 2013) were selected (*PTEN*, *PIK3CA*, *ARID1A*, *PIK3R1* and *TP53*) along with genes shown to play a critical role in endometrial and other common cancers (*POLE*, *CTNNB1*, *PPP2R1A*, *KRAS* and *FBXW7*).

2.2.4 'Subbing' of glass microscope slides

Microscope slides were coated with a positively charged solution (3-aminopropyltriethoxysilane) in order to increase its adhesiveness to the negatively charged tissues.

Glass microscope slides were placed in racks and soaked in hot water containing an alkaline cleaning solution (5% Decon 90), and they were left overnight. The following day, they were washed for 30 minutes in hot running water followed by three washes in distilled water. They were then dried in the oven for 1 hour at 60ºC, while 5 tanks were placed in the fume hood. The first one was filled with subbing solution (consisted of 2% 3-aminopropyltriethoxysilane in acetone), the next two with acetone and the last two with distilled water. The slides were immersed in each tank for two minutes. They were finally dried in the oven at 60ºC.

2.2.5 Tissue cutting

FFPE endometrial tissue was manually cut with a Leica RM2235 Rotary Microtome in 5μm sections which were placed on a R A Lamb E65.2 Mounting Bath (Akribis Scientific Limited) at 36ºC, prior to being mounted on the glass slides prepared previously (see paragraph 2.2.4). Finally, the slides were dried in the oven for two hours at 37ºC.

2.2.6 Haematoxylin and Eosin stain

Tissue cuts mounted on glass slides (as described in 2.2.5) were deparaffinised in the fume hood after being submerged in Xylene for 10 minutes. This step was repeated twice. They were then rehydrated by using serial concentrations of Ethanol (100%, 90% and 70%), for 10 minutes in each one of them (twice for Ethanol 100%). The slides were then washed in water for 3 minutes and immersed in Haematoxylin (Sigma-Aldrich) for 5 minutes. The water wash was repeated for another 3 minutes and then the slides were de-stained for 10 seconds using a 1% acid solution in 70% Ethanol (1% HCL in 70% Ethanol). They were then rinsed in tap water and immersed in Eosin for 1 minute. They were washed again in water and dehydrated in 70% Ethanol for 30 seconds followed by 100% Ethanol for 5 minutes, with the latter step being repeated once more. Finally, they were placed on Xylene for 5 minutes and again this step was repeated prior to placing coverslips on top of the tissues using DPX mountant for histology (Sigma-Aldrich).

2.2.7 Immunohistochemistry

Endometrial tissue was cut and mounted on slides as described in 2.2.5. The tissue was deparaffinised in two tanks containing Xylene in the fume hood (submerged for 10 minutes in each one of them) and then dehydrated in two tanks of 100% Ethanol (submerged for 10minutes in each one of them). Endogenous peroxidase was blocked for 10 minutes using a solution of 30% Hydrogen Peroxide in Methanol (2.4ml of H2O2 30% were diluted in 400ml Methanol) (Table 2.4). In the meantime, a Citrate buffer solution was prepared using 2.94gr of Tri-sodium citrate dehydrate diluted in 1 litre distilled water along with 0.5ml of Tween 20. The pH was adjusted to 5.98-6.00 using acetic acid and the solution

was microwaved in a pressure cooker for 10 minutes at 900 Watts (W). The slides were subsequently microwaved in the Citrate buffer for 15 minutes and were then left to cool down for approximately 30 minutes prior to wash on 1X PBS (twice, 10 minutes each). After washing, the slide edges were dried and the edges of the tissue were marked using a hydrophobic pen. The slides were placed in acrylic trays and incubated for 30-60 minutes with 5% swine or goat serum (for rabbit or mouse primary antibodies respectively) in PBS. The slides were then drained and the primary antibody added, diluted in the aforementioned serum solution. The slides were placed in a humidified tray at 4°C and were left overnight.

Following overnight incubation, 3 washes with 1X PBS performed (5 minutes each). The slide edges were dried and a secondary biotinylated antibody was added after diluted 1:300 in 5% serum for 30 minutes (Polyclonal Swine Anti-Rabbit, Dako). Further three washes with 1X PBS were performed and the slides were incubated for 30 minutes with Horseradish Peroxidase Streptavidin (Vector Laboratories). After washing three more times with PBS, slides were incubated for 5 minutes in the dark with DAB Peroxidase Substrate (DAB substrate kit, Vector Laboratories) which has been made by adding 1 drop of buffer solution, 2 drops of DAB and 1 drop of H_2O_2 in 2.5ml of distilled water. Following a further wash with tap water, the slides were submerged to Haematoxylin (Sigma Aldrich) for 10-15 seconds and subsequently washed in tap water. Dehydration was then achieved using serial concentrations of Ethanol (70%, 90% and 100%, for 10 minutes each). Finally, the slides were cleared in Xylene (twice, 5 minutes each) and coverslips were added on top of them using DPX mountant. Finally, they were dried in the fume hood prior to taking photos of them using a light microscope (Leica Microsystems).

The slides were reviewed by a Consultant Pathologist in order to confirm the positivity or negativity of staining. The Pathologist was blinded to the patient's details and their clinicopathologic data.

Antibodies/ Reagents	Company	Catalog Number
Hydrogen Peroxide Solution 30% (w/w) in H2O	Sigma Aldrich	H1009-100ML
Goat Serum (Normal)	Dako	X0907
Swine Serum (Normal)	Dako	X0901
Polyclonal Goat Anti-Mouse IgG / Biotinylated	Dako	E0433
Polyclonal Swine Anti-Rabbit IgG / Biotinylated	Dako	E0353
MSH2 Antibody (FE11) Mouse	Thermo Fisher	33-7900
Anti-MSH6 antibody [EPR3945] Rabbit	Abcam	ab92471
MLH1 Mouse Antibody	Leica Biosystems	NCL-L-MLH1
Anti-PMS2 antibody [EPR3947]	Abcam	ab110638
R.T.U. Horseradish Peroxidase Streptavidin	Vector Labs	SA-5704
DAB Substrate Kit for Peroxidase	Vector Labs	SK-4100
Haematoxylin	Sigma Aldrich	GHS132-1L
Eosin 1% aqueous	Raymond Lamb	LAMB/100-D
DPX Mountant	Sigma Aldrich	06522

Table 2.4 Antibodies and reagents used in Immunohistochemistry experiments.

2.2.8 DNA extraction

2.2.8.1 Core biopsy

FFPE blocks of endometrial tumour was cut and mounted on glass slides as previously described. Haematoxylin and Eosin stain was performed (see 2.2.6). A Consultant

Pathologist reviewed the slide and marked the area which had the higher density of tumour with a pen. A 1.5mm core biopsy was subsequently performed with a needle in the corresponding FFPE block in this high density tumour area.

2.2.8.2 Extraction of DNA

The QIAamp® DNA FFPE Tissue Kit (QIAGEN, Catalog Number 56404) was used and the manufacturer's protocol was followed.

First, 1ml of Xylene was added to the Eppendorf micro-centrifuge tube containing the previously obtained core biopsy and it was vortexed and centrifuged at full speed for 2 minutes in order to remove the paraffin. After removing the supernatant, 1ml of 100% Ethanol was added to extract the residual Xylene from the sample and the tube was vortexed and centrifuged again for 2 minutes. The supernatant was then removed and the tissue was left to air dry for 10 minutes. The pellet was re-suspended using 180μL of buffer ATL (provided with the kit) in which 20μL of proteinase K was added and the tube was vortexed, prior to being incubated at 56°C for at least 1hr. The sample was subsequently incubated at 90°C for 1hr and centrifuged afterwards. It was then allowed to cool before adding 2 μl of RNase A (100 mg/ml) for an incubation time of 2min. 200 μl of Buffer AL (provided with the kit) was subsequently added to the sample and mixed by vortexing followed by another step in which ethanol was added (200 μl) and a further vortexing step. The sample was then centrifuged and the lysate was transferred to the QIAamp MinElute column (in a 2ml collection tube) and re-centrifuged at 6000 x g for 1 min.

The collection tube was then discarded. Buffer AW1 had been previously prepared by adding 25ml of Ethanol (100%) to the 19ml of the pre-supplied buffer. 500μL of this buffer was added and the column centrifuged at 6000g for 1 min. The column was placed in a clean 2 ml collection tube and the previous one was discarded. Buffer AW2 has been previously prepared by adding 30ml of Ethanol 100% to the 13ml of pre-supplied buffer. 500μL of this was subsequently added and the column centrifuged at 6000g for 1 min. The column was placed in a clean 2 ml collection tube and centrifuged at full speed (20,000g) for 3 min to dry the membrane completely.

The column was finally placed in a clean 1.5ml Eppendorf tube, prior to adding 100µL of pre-supplied buffer ATE to the center of the membrane and incubating for 5 minutes. It was then centrifuged at full speed for 1 min.

2.2.8.3 Next generation sequencing (NGS)

Dr David Guttery used the Ion AmpliSeq designer software (https://www.ampliseq.com) to generate a custom 190 amplicon Ion AmpliSeq panel for analysis of hotspot regions in 10 genes commonly mutated in EC (*ARID1A*, *CTNNB1*, *FBXW7*, *KRAS*, *PIK3CA*, *PIK3R1*, *POLE*, *PTEN*, *PPP2R1A*, *TP53*) based on publicly available databases (https://portal.gdc.cancer.gov/). The panel was designed to contain two pools, against FFPE tissue, with amplicons ranging from 125-175 bp. The previously extracted DNA was undertaken by Dr David Guttery, who performed NGS with the protocol presented in the Appendix. I was present during the sequencing process in order to have a better understanding of the technique and the processes involved along with the potential problematic and challenging areas.

Sequencing data were analysed by Dr Guttery using the Torrent Suite v5.6.0. Mutations with a quality score below 100 were omitted along with all variants detected in the first or last 10 bases of an amplicon as likely mispriming events (Guttery *et al.*, 2015). ANNOVAR (K. Wang *et al.*, 2010) was used to annotate all mutations with refGene ID, functional consequence and functional predictions using SIFT (Ng *et al.*, 2009), Polyphen-2 (Peshkin *et al.*, 2010) and MutationTaster (Jana Marie Schwarz *et al.*, 2014). Known germline variants without pathological consequence were omitted from the analysis. All variants detected were also manually confirmed across all samples using the Integrated Genomics Viewer 2.3 (Thorvaldsdóttir *et al.*, 2011; Thorvaldsdóttir *et al.*, 2013).

2.2.9 Statistical methods

IC50 values of inhibitory drugs were calculated after converting the cell viability values derived from MTS assay to percentage of inhibition. This was then converted to a logarithmic value and from the linear regression graph created with GraphPad Prism version 7.0, the IC50 value was calculated.

Calculation Index (CI) for combination drug treatments was calculated using the Chou-Talalay method (Chou & Talalay, 1983). The CI is calculated by the equation

$$CI = \frac{a}{IC50A} + \frac{b}{IC50B}$$

where a is the cell viability of a specific concentration of drug A and b the cell viability of a specific concentration of drug B, combined together. If CI<1, there is synergism, if CI>1 there is antagonism and when CI=1, there is additive effect. The mathematical rationale and explanation of this method is very complicated and beyond the scope of this thesis. CI though has prevailed in the literature over different methods of assessing synergism due to its simplicity and reproducibility, even when relatively small number of drug concentrations are combined. In this study, CI was calculated using CalcuSyn Version 2.0 software (Biosoft).

Unpaired Student's t-test was used to compare quantitative values between two groups (i.e age, BMI), while Fisher's exact test and Chi-square test were used to compare nominal and categorical variables (> 2 categories) respectively. Statistical significance was considered when p value was equal or less than 0.05.

This was an exploratory screening study that involved multiple testing to a relatively small number of comparisons between two groups. The limitations of multiple testing are acknowledged and any differences identified between the two groups would be validated in future larger studies.

Finally, as my null hypothesis was that BSA women with EC carry different somatic mutations compared to BW, it was of paramount importance to me to avoid a Type 2 error (which means to accept my null hypothesis when it is actually false). For all these reasons and based on Streiner and Norman's work (Streiner & Norman, 2011), I decided not to use the Bonferroni correction (Bonferroni, 1936) which is a statistical test used to adjust p values when multiple tests are being used in order to protect from Type 1 error (reject the null hypothesis when it is actually true).

Chapter 3 Results

Identifying the sensitivity of endometrial cancer cell lines treated with various inhibitory drugs and the response of the MAPK pro-survival pathway in these treatments was the primary goal of our experiments, aiming at proposing treatments which could be tested in patient samples or animal models.

Two endometrial cancer cell lines (Ishikawa and HEC-1-A) were chosen due to their entirely different properties and genetic background. Ishikawa has been previously characterised (Weigelt et al., 2013) and it is known to carry *PTEN* and *PIK3R1* mutations while HEC-1-A carry *KRAS* and *PIK3CA* mutations. Ishikawa has been derived from a well differentiated uterine adenocarcinoma of an Asian patient while the HEC-1-A was derived from a moderately differentiated papillary adenocarcinoma of a Caucasian background. As the main focus of our project was to elicit differences between Asian and Caucasian women, these two cell lines were chosen as an experimental model in order to try and help characterize differences between these two ethnic groups.

3.1 Effect of various inhibitory drugs on endometrial cancer cells

In order to explore the different sensitivity of these endometrial cancer cells with the distinct genetic background to a panel of specific inhibitors, they were treated with varying concentrations of the inhibitors and their viability was assessed using an MTS assay. A minimum of three independent experiments were performed for each inhibitor. The results are shown below (figure 3.1 for Ishikawa, figure 3.2 for HEC-1-A cells). The inhibitory concentration of 50% (IC50) values for these inhibitors are presented in table 3.1 below.

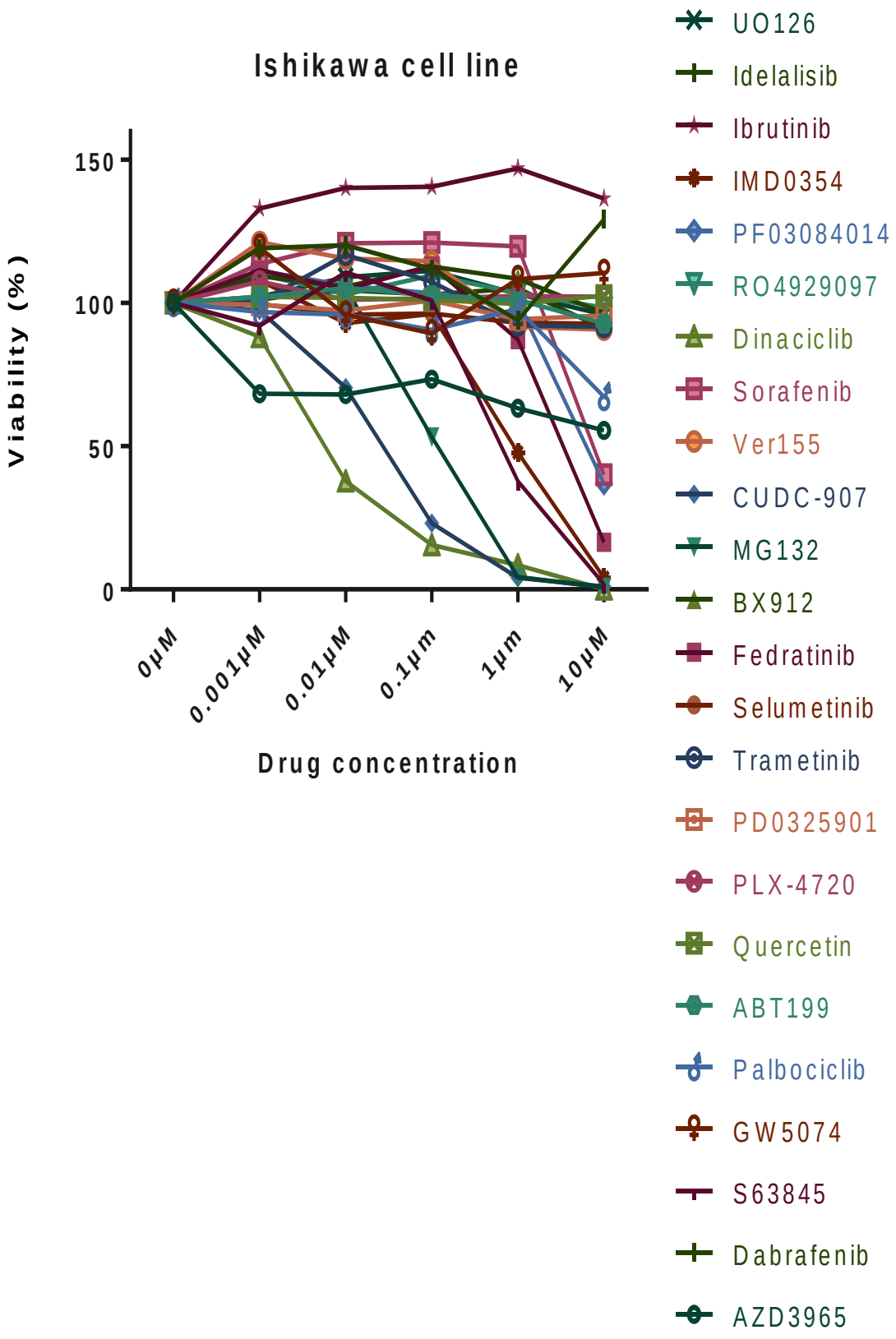


Figure 3.1 Assessment of viability using MTS assay after 48 hours of incubation with various inhibitory drugs on Ishikawa cells. **UO126**: MEK1/2 inhibitor. **Idelalisib**: PI3K

inhibitor. **Ibrutinib**: BTK inhibitor. **IMD0354**: IκB kinase-β (IKKβ) inhibitor that blocks NF-κB nuclear translocation. **PF03084014**: γ secretase inhibitor. **RO4929097**: γ secretase inhibitor. **Dinaciclib**: CDK1/2/5/9 inhibitor. **Sorafenib**: Raf-1, B-Raf and VEGFR-2 inhibitor. **Ver15508**: Adenosine-derived inhibitor of Heat Shock Protein 70 (Hsp70). **CUDC-907**: PI3Kα and HDAC1/2/3/10 inhibitor. **MG132**: Proteasome inhibitor. **BX912**: PDK1 inhibitor. **Fedratinib**: JAK2 inhibitor. **Selumetinib**: MEK1, ERK1/2 inhibitor. **Trametinib**: MEK1/2 inhibitor**. PD0325901**: MEK inhibitor. **PLX-4720**: B-Raf V600E inhibitor. **Quercetin**: Flavonoid, mTOR inhibitor. **ABT199**: Bcl-2 inhibitor. **Palbociclib**: CDK 4/6 inhibitor. **GW5074**: C-raf inhibitor. **S63845**: MCL1 inhibitor. **Dabrafenib**: Small molecule inhibitor of BRAF serine-threonine kinase. **AZD3965**: MCT1 inhibitor. Experiments were performed in triplicates and were repeated 3-10 times. Error bars are not shown in order to avoid confusion as the graph is already 'busy'.

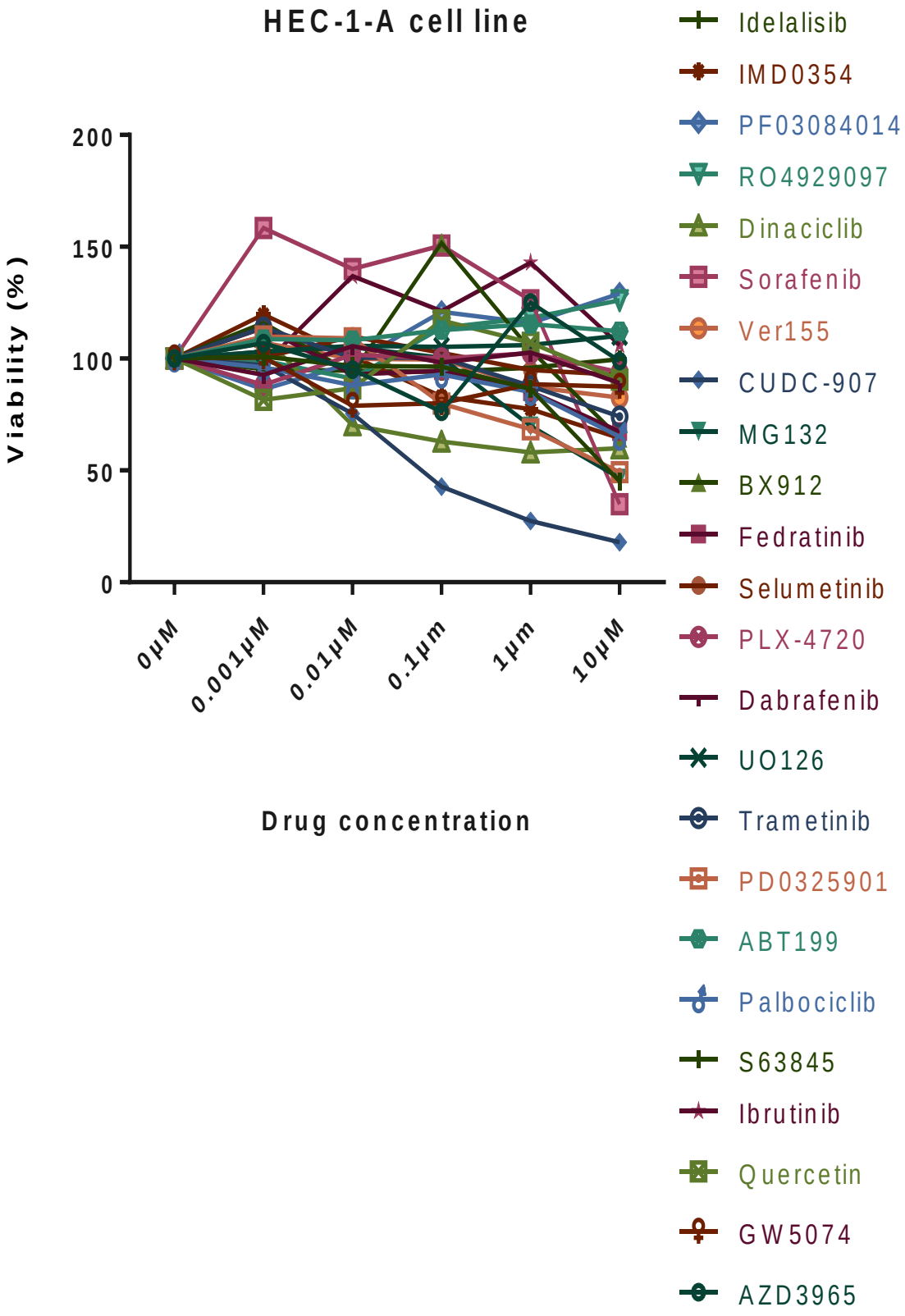


Figure 3.2 Assessment of viability using MTS assay after 48 hours of incubation with various inhibitory drugs on HEC-1-A cells. The names of the inhibitors and their mode of action has been described in Figure 3.1. All experiments were performed in triplicates and

repeated 3-10 times. Error bars are not shown in order to avoid confusion as graph is already 'busy'.

Drug	Ishikawa (PTEN, PIK3R1 mutations)	HEC-1-A (KRAS, PIK3CA mutations)	Drug	Ishikawa (PTEN, PIK3R1 mutations)	HEC-1-A (KRAS, PIK3CA mutations)
Dinaciclib (CDK inh.)	0.004	>15	*BX912* (PDK1 inh.)	>15	>15
CUDC907 (PI3Ka and HDAC inh.)	0.027	0.058	*GW5074* (CRAF inh.)	>15	>15
MG132 (proteasome inh.)	0.12	7.58	*Dabrafenib* (BRAF inh.)	>15	>15
S63845 (MCL1 inh.)	0.645	7.58	*RO4929097* (γ secretase inh.)	>15	>15
IMD0354 (IKKβ inh.)	0.912	>15	*PLX4720* (B-Raf V600E inh.)	>15	>15
Fedratinib (JAK2 inh.)	3.89	>15	*Ver15508* (Hsp70 inh.)	>15	>15
PF03084014 (γ secretase inh.)	6.165	>15	*ABT199* (Bcl-2 inh.)	>15	>15
Sorafenib (Raf and VEGFR-2 inh.)	7.41	6.91	*Ibrutinib* (BTK inh.)	>15	>15
Palbociclib (CDK inh.)	>15	>15	*Quercetin* (mTOR inh.)	>15	>15
PD0325901 (MEK inh.)	>15	9.33	*Idelalisib* (PI3K inh.)	>15	>15
Selumetinib (MEK inh.)	>15	>15	*UO126* (MEK inh.)	>15	>15
Trametinib (MEK inh.)	>15	>15	*AZD3965* (MCT1 inh.)	>15	>15

Table 3.1 IC50 values (μM) of inhibitory drugs tested on Ishikawa and HEC-1-A cells. Values were calculated using Graphpad Prism software. Colour code: Red (IC50< 1μM), yellow (IC50=1-10μM) and green (IC50>10μM).

The Ishikawa cells exhibited sensitivity to a variety of inhibitors, most notably Dinaciclib (CDK inhibitor), CUDC-907 (PI3K and HDAC inhibitor), MG132 (proteasome inhibitor), S63845 (MCL1 inhhibitor) and IMD0354 (NfkB inhibitor). Interestingly, each one of these inhibitory drugs has an entirely different mode of action, which suggests that these cells are relatively sensitive and can be inhibited using a variety of targeted approaches.

The most potent drug by far was Dinaciclib which had an IC50 value of just 4nM while CUDC-907 had an IC50 of 27nM. Two thirds of the inhibitory drugs tested (16 out of 24) had no clinically significant effect on the Ishikawa cells as their IC50 values were more than 15μM.

HEC-1-A cells proved more resistant compared to Ishikawa as it is obvious in the graph presented in Figure 3.2 and the IC50 values shown in Table 3.1.

HEC-1-A cells are sensitive only to CUDC-907 (IC50 58nM). 80% of the inhibitors tested (19 out of 24) had no effect on these cells while the remaining 4 inhibitors (MG132, S63845, Sorafenib and PD0325901) had some modest effect with IC50 values between 7 and 9μM.

The data shows that these two endometrial cancer cell lines exhibit different sensitivities to the inhibitors tested. For instance, Ishikawa were very sensitive to CDK inhibition (Dinaciclib) while HEC-1-A were very resistant since even when treated with 10μM of Dinaciclib, the viability remained at 60% (figure 3 below).

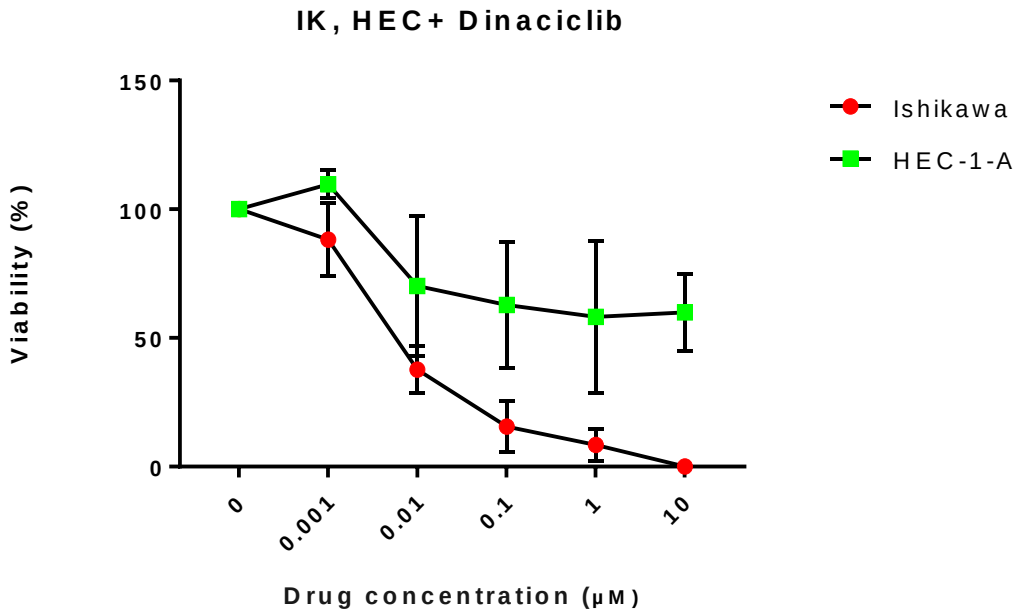


Figure 3.3 Effect of Dinaciclib treatment for 48 hours on two EC cell lines using MTS assay. Average of 5 experiments. Error bars represent standard deviation of the mean.

Other significant differences were seen with Nuclear Factor kappa B inhibition (IMD0354) as Ishikawa were sensitive (IC50<1µM) whereas HEC-1-A were very resistant. Also, Ishikawa cells were more sensitive to the proteasome inhibitor MG132 (IC50 0.12µM versus 7.58µM, figure 3.4) and the MCL1 inhibitor S63845 (IC50 0.645µM versus 7.58µM).

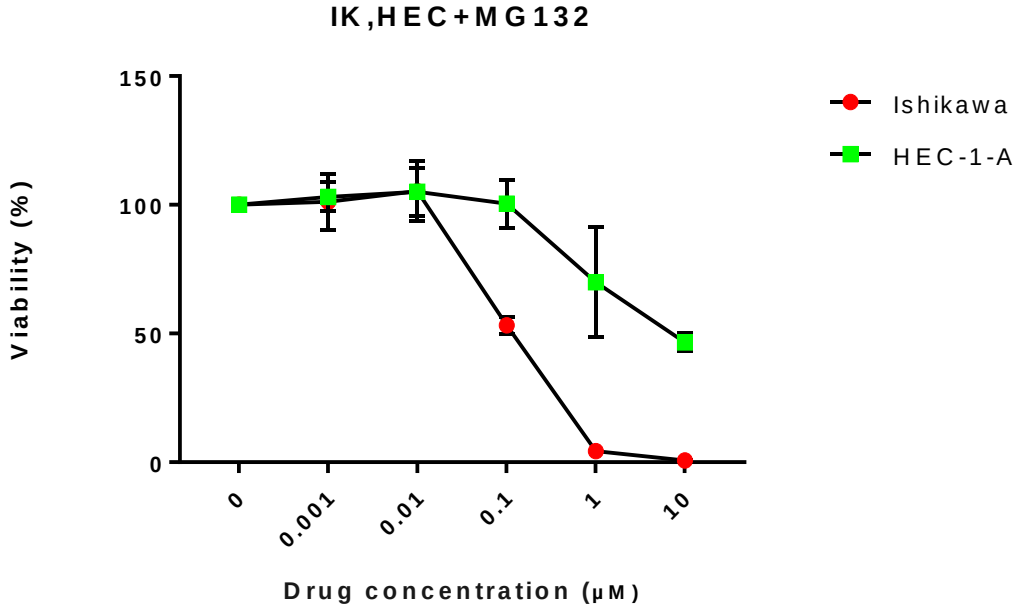


Figure 3.4 Effect of proteasome inhibitor MG132 treatment for 48 hours on two EC cell

lines using MTS assay. Average of two experiments. Error bars represent standard deviation of the mean.

Finally, Ishikawa cells were modestly sensitive to the Janus kinase 2 (JAK-2) inhibitor Fedratinib as opposed to HEC-1-A cells (IC50 3.89μM versus 77μM) and to the γ secretase inhibitor PF03084014 (IC50 6μM versus complete resistance for HEC-1-A cells). CUDC-907 was the only drug which had good efficacy in both cell lines (Ishikawa IC50 27nM versus 58nM for HEC-1-A). There were only two drugs in which HEC-1-A cells were slightly more sensitive: the Raf inhibitor Sorafenib (IC50 for HEC-1-A 6.9μM versus 7.4μM for the Ishikawa) and the MEK inhibitor PD0325901 (IC50 9.3μM for HEC-1-A cells versus 40μM for Ishikawa). Overall, there is a very clear distinction between these two cell lines, with Ishikawa being more sensitive to the panel of inhibitors tested.

3.1.1 MEK inhibitors

One of the main categories of inhibitors tested were MEK inhibitors (MEKi). The results of testing on both cell lines is shown on figure 3.5 below. These inhibitory drugs don't show any efficacy on Ishikawa cells when used alone. The results are similar for the HEC cells with a small exception the PD0325901 which slightly reduces viability on high concentrations. Our results suggest that these agents would not be good options for inhibiting endometrial cancer cells when used as monotherapies.

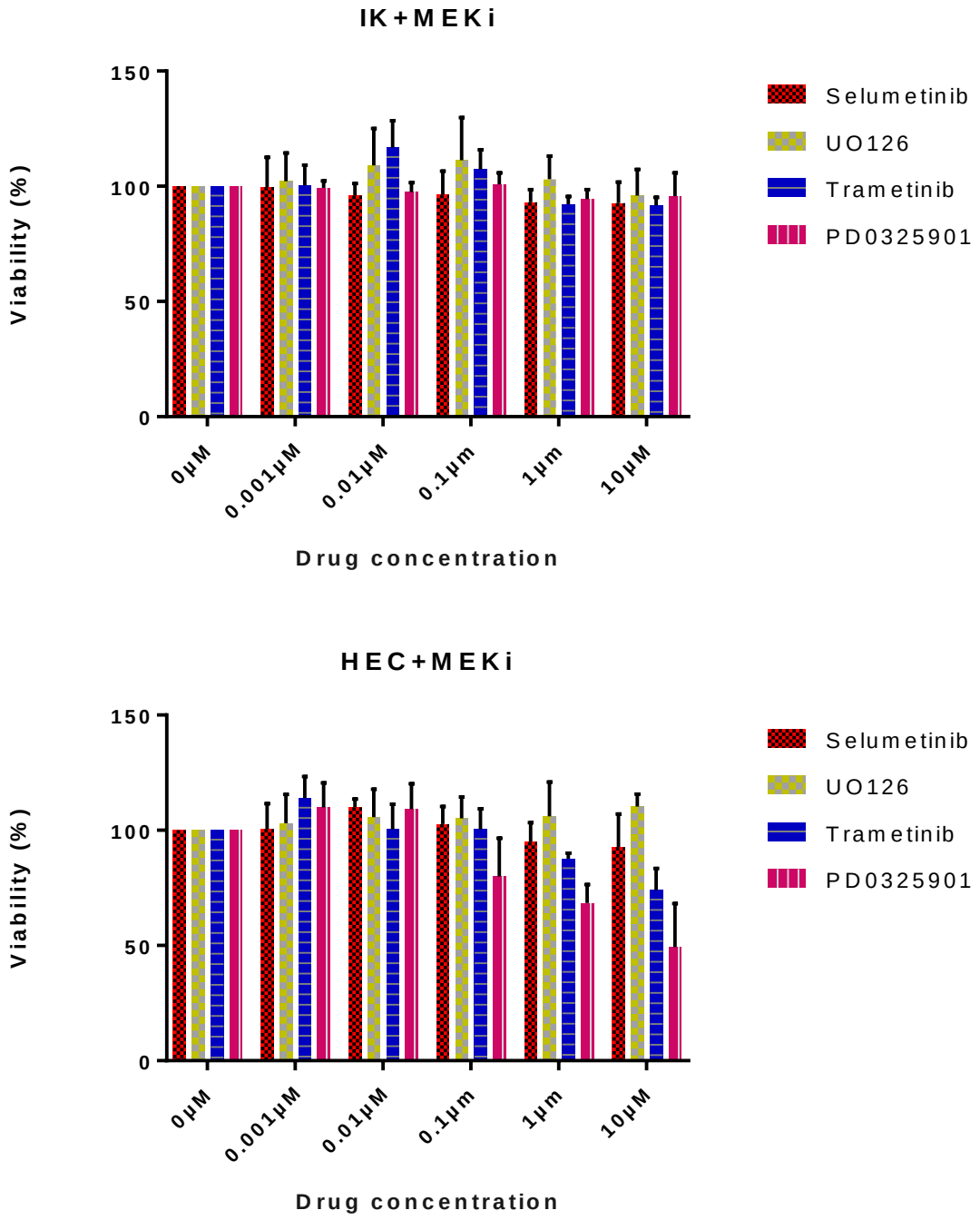


Figure 3.5 Effect of MEK inhibitors on viability of Ishikawa and HEC1A cells, after treating them for 48 hours. MTS assay used to determine viability. Experiments were performed in triplicates and repeated a minimum of three times. Error bars represent standard deviation of the mean.

In order to confirm the differences mentioned previously between the two cell lines, flow cytometry using Propidium Iodide (PI) was performed for some of the inhibitory drugs as shown in Figure 3.6 below. The treatments in this case were for 24 hours in order to detect any time-dependent discrepancies.

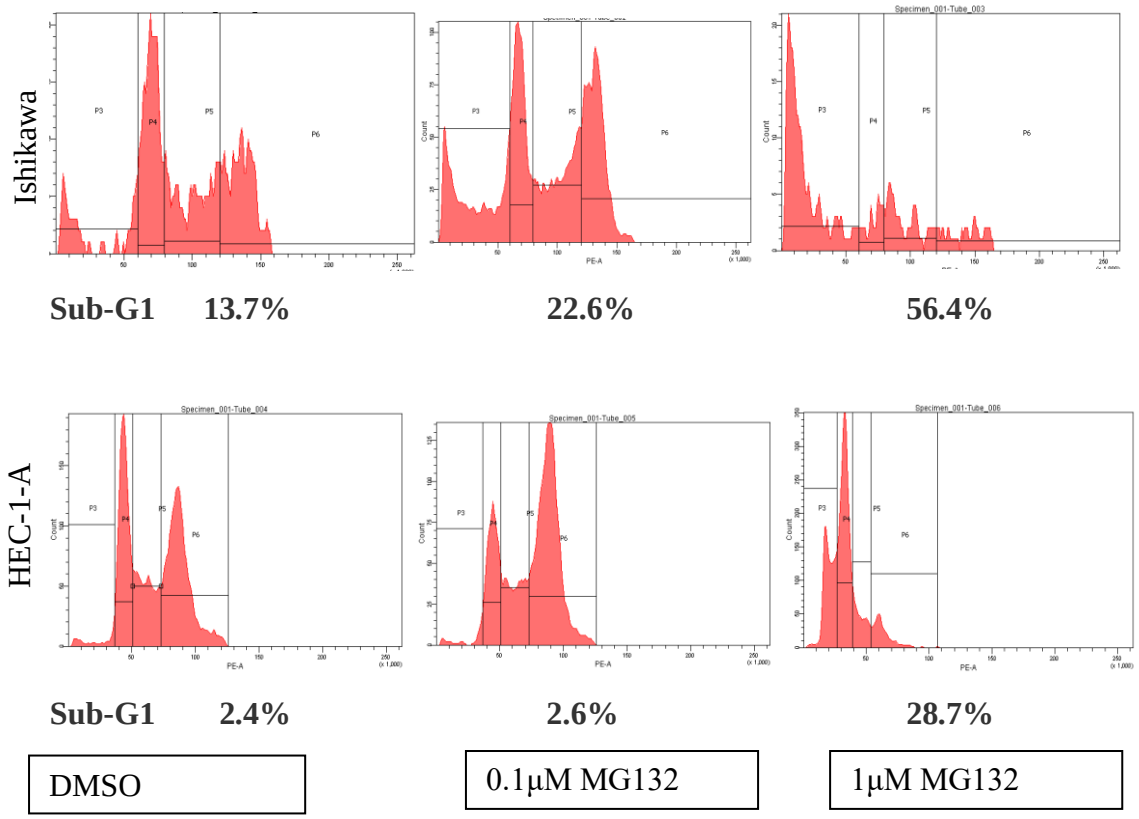


Figure 3.6 Flow cytometry analysis (PI staining) following proteasome inhibitor treatment (MG132) of endometrial cancer cell lines for 24 hours. Sub-G1 refers to the percentage of apoptotic cells.

As shown in Figure 3.6, the degree of cell death (percentage of cells in subG1) after 24hr of treatment with MG132 confirms the higher sensitivity of Ishikawa cells and is in line with the results shown earlier using MTS assay (Figure 3.4). Similarly, two cell lines were tested with various concentrations of CUDC-907 and the results are shown in Figure 3.7.

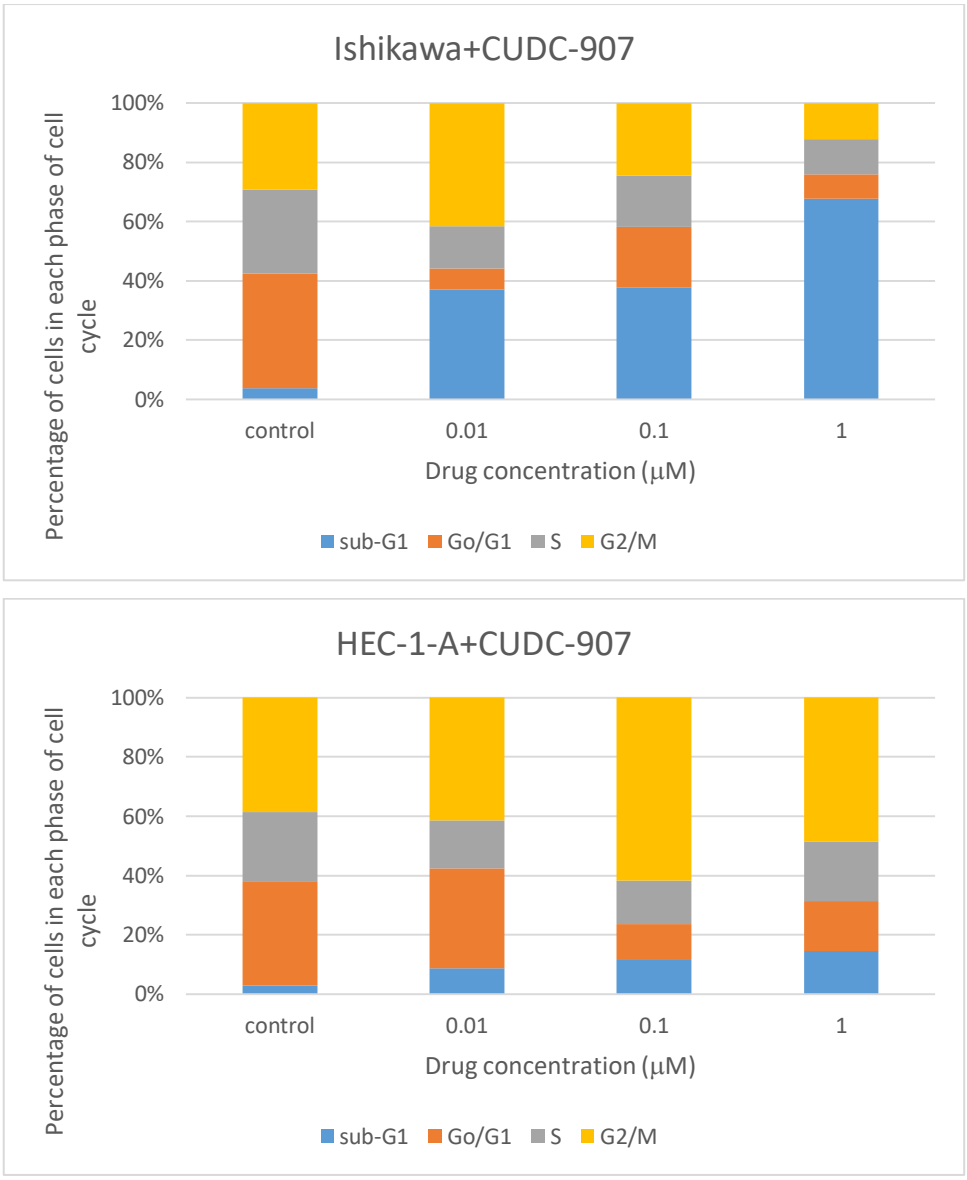


Figure 3.7 Effect of CUDC-907 treatment on endometrial cancer cell lines after 24 hours of incubation. The graphs present the percentage of cells in each stage of the cell cycle which were calculated using flow cytometry analysis with cells stained with PI. Average of 2 experiments.

As shown in Figure 3.7, Ishikawa cells are more sensitive than HEC-1-A cells. After 24 hours treatment with 1μM of CUDC, the percentage of cells in the sub-G1 phase is 68% versus 14% for the HEC cells. Similar differences are seen at lower concentrations as well. These results confirm the different sensitivities of the two cell lines as shown previously using a different technique (MTS assay).

3.1.2 PI3K and MAPK pathway activation

In view of the marked differences in the sensitivity of these two cell lines, further experiments were performed attempting to identify preferential molecular pathway activation which might suggest a possible mechanism of action of resistance.

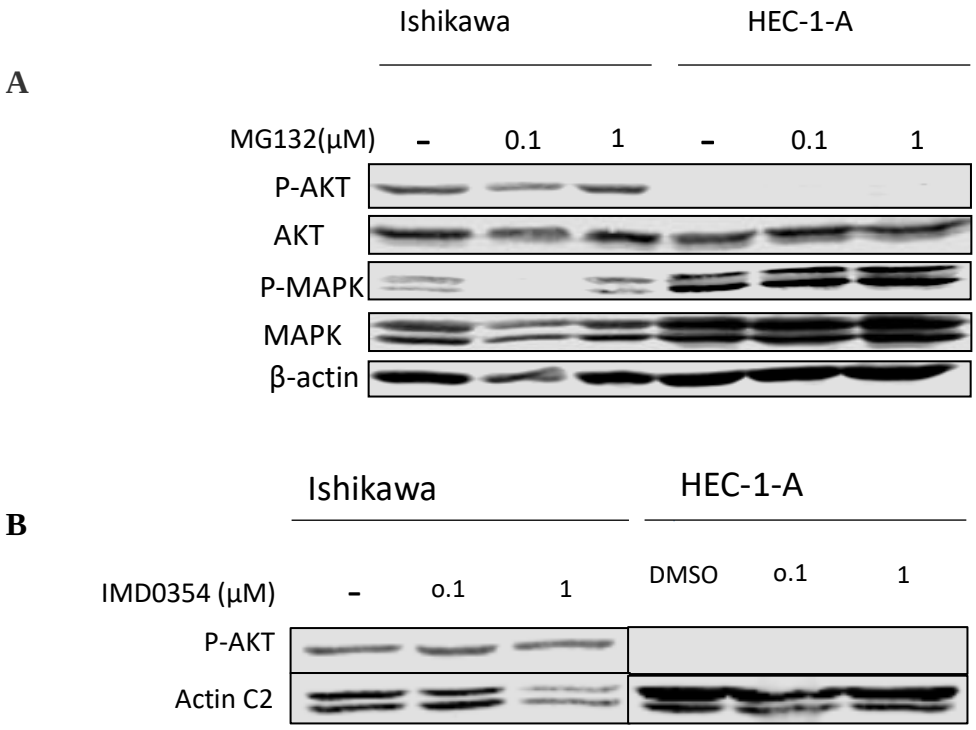


Figure 3.8 Western blot on endometrial cancer cell lines treated with A. Proteasome inhibitor (MG132). B. NFkB inhibitor (IMD0354) for 24 hours.

As shown in Figure 3.8A, the resistant cell line HEC-1-A, which carries a wild-type PTEN gene, did not have increased basal levels of phosphorylated AKT in contrast with the Ishikawa cells. This was confirmed with several other experiments during which HEC-1-A cells treated with various inhibitory drugs has shown no AKT phosphorylation (Figure 3.8B). On the contrary, HEC cells had high activation of the MAPK pathway. In order to explore the hypothesis that these resistant cells depend on activation of MAPK pathway for their survival, the two cell lines were tested with several MEK inhibitors to see whether they were able to suppress this pathway. The results are shown in Figure 3.9 below.

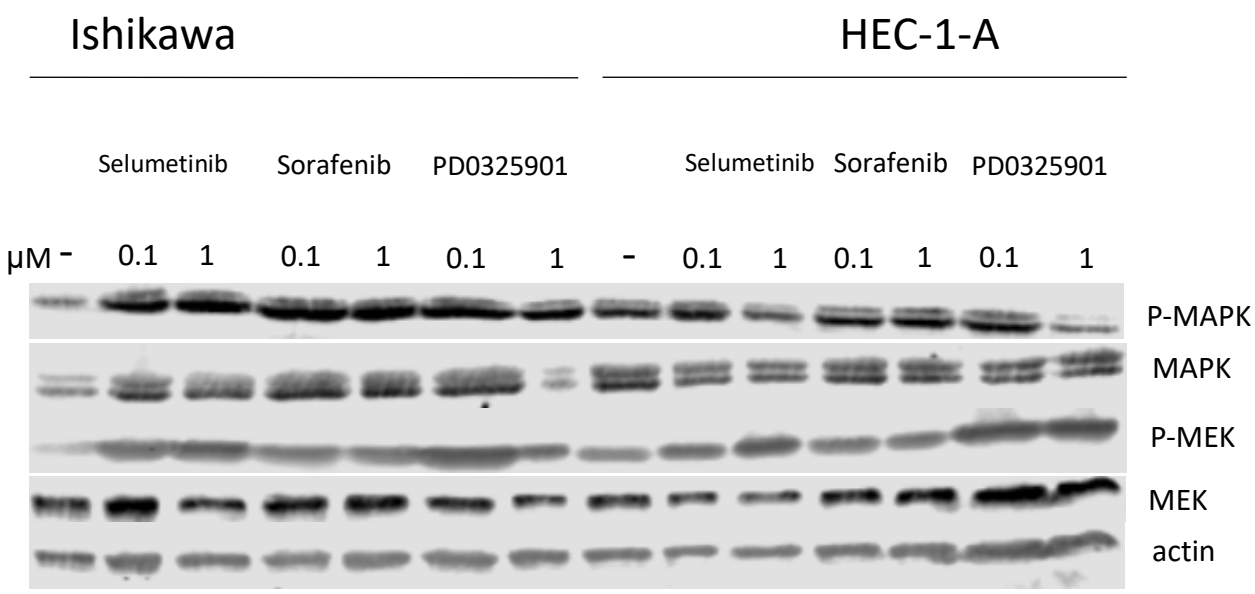


Figure 3.9 Western blot on endometrial cancer cell lines treated with MEK inhibitors Selumetinib and PD0325901 and Raf inhibitor Sorafenib for 24 hours.

The above shown MAPK pathway inhibitors have no effect on the viability of the two cell lines (in clinically relevant concentrations, see Figure 3.5 previously). Interestingly, there was an increase in the basal levels of MAPK and MEK phosphorylation on these EC cells, the opposite of the anticipated result (with the exception of high dose PD0325901 on HEC cells). This has been shown previously in other cancers (Y. Chen *et al.*, 2017) and might be associated with upstream reactivation of the pathway. It becomes obvious that MAPK inhibition alone is not a good therapeutic option since it does not reduce viability nor suppress this pathway even on relatively sensitive cells (Ishikawa).

Given that PI3K pathway is the most commonly mutated pathway in endometrial cancer, PI3K (and HDAC) inhibitor CUDC-907 was tested since it was shown to be very effective in both cell lines when viability assays were performed.

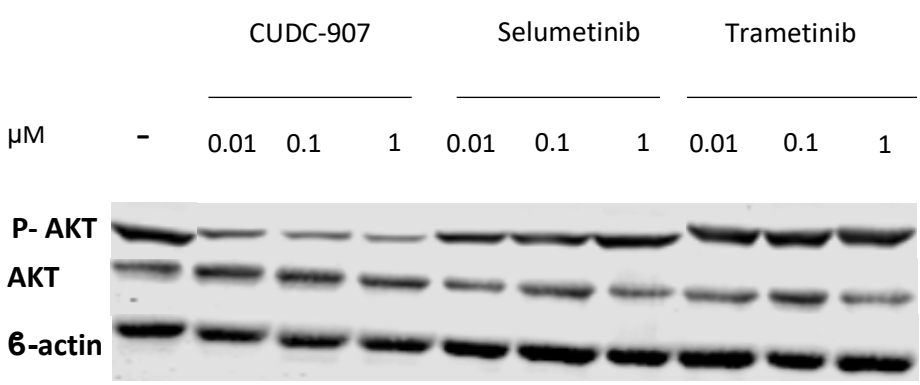


Figure 3.10 Western blot on Ishikawa cell lines treated with PI3K and HDAC inhibitor CUDC-907 and MEK inhibitors Selumetinib and Trametinib for 24 hours.

As expected, CUDC reduces the basal levels of AKT phosphorylation on Ishikawa cells (Figure 3.10), unlike the MEK inhibitors shown above (Selumetinib and Trametinib). Interestingly, when tested on both cell lines, CUDC seems to suppress MAPK phosphorylation (Figure 3.11). The effect was more pronounced on Ishikawa cells (Figure 3.11B) but was significant on HEC cells as well (Figure 3.11A). This supports our hypothesis that HEC cells may be dependent on the MAPK pro-survival pathway and drugs which manage to suppress it (like CUDC) should affect their viability.

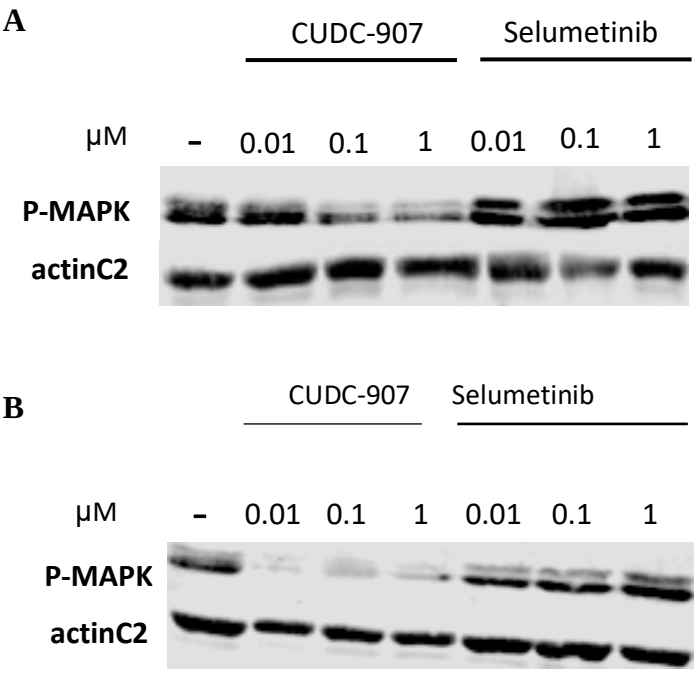


Figure 3.11 Western blot on HEC (A) and Ishikawa (B) cell lines treated with PI3K and HDAC inhibitor (CUDC-907) and MEK inhibitor Selumetinib for 24 hours.

3.1.3 Drug combination treatments

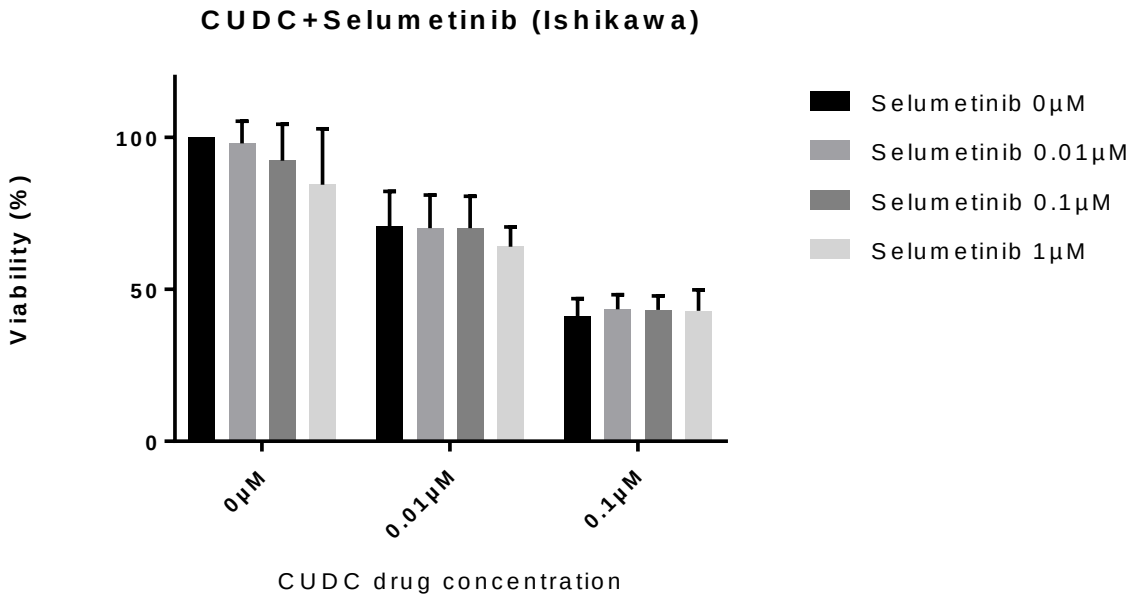
In order to further investigate molecular pathway activation and the effect on the viability of these two cell lines, several combination treatments were tested with emphasis on synchronous suppression of the MAPK pathway.

As the main objective of this part of the study was to propose effective treatments for endometrial cancer which can potentially be tested in animal models, the decision was made to focus combination treatments on drugs proved to be efficient when tested alone. For that reason, the majority of testing involved a combination of either CUDC907 or Dinaciclib since these drugs were the most potent during the previous experiments. The degree of synergism or antagonism for the combinations tested was calculated using the CalcuSyn Version 2.0 software (Biosoft). This is based on the Chou-Talalay method of calculating the combination index (CI) (Chou & Talalay, 1983) which was summarised in the methods.

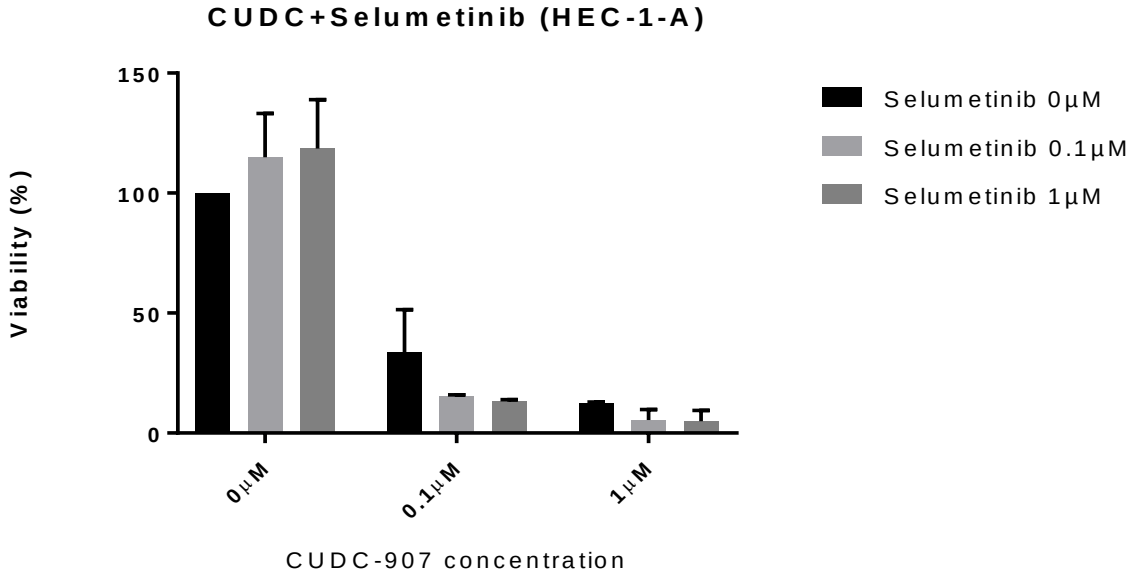
3.1.3.1 PI3K and MEK inhibition

The PI3K and HDAC inhibitor was tested in combination with MEK inhibitors Selumetinib, Trametinib and PD0325901 and the Raf inhibitor Sorafenib.

CUDC907+Selumetinib



A **CI=1.02**



B **CI=0.15**

Figure 3.12 MTS assay to assess viability of Ishikawa (A) and HEC (B) cells following treatment with combination of CUDC-907 and Selumetinib. The values shown are the average of 3 experiments. Error bars represent standard deviation of the mean. CI= Combination Index.

As shown above, adding Selumetinib (MEK inhibitor) to CUDC had a neutral result on Ishikawa cells, with no further reduction in viability (as reflected on the CI). On the contrary, the same combination had a synergistic effect in HEC cells, significantly reducing their viability (CI=0.15). This further enhances our hypothesis that HEC cells, although more resistant overall, depend significantly on the MAPK pro-survival pathway for their proliferation.

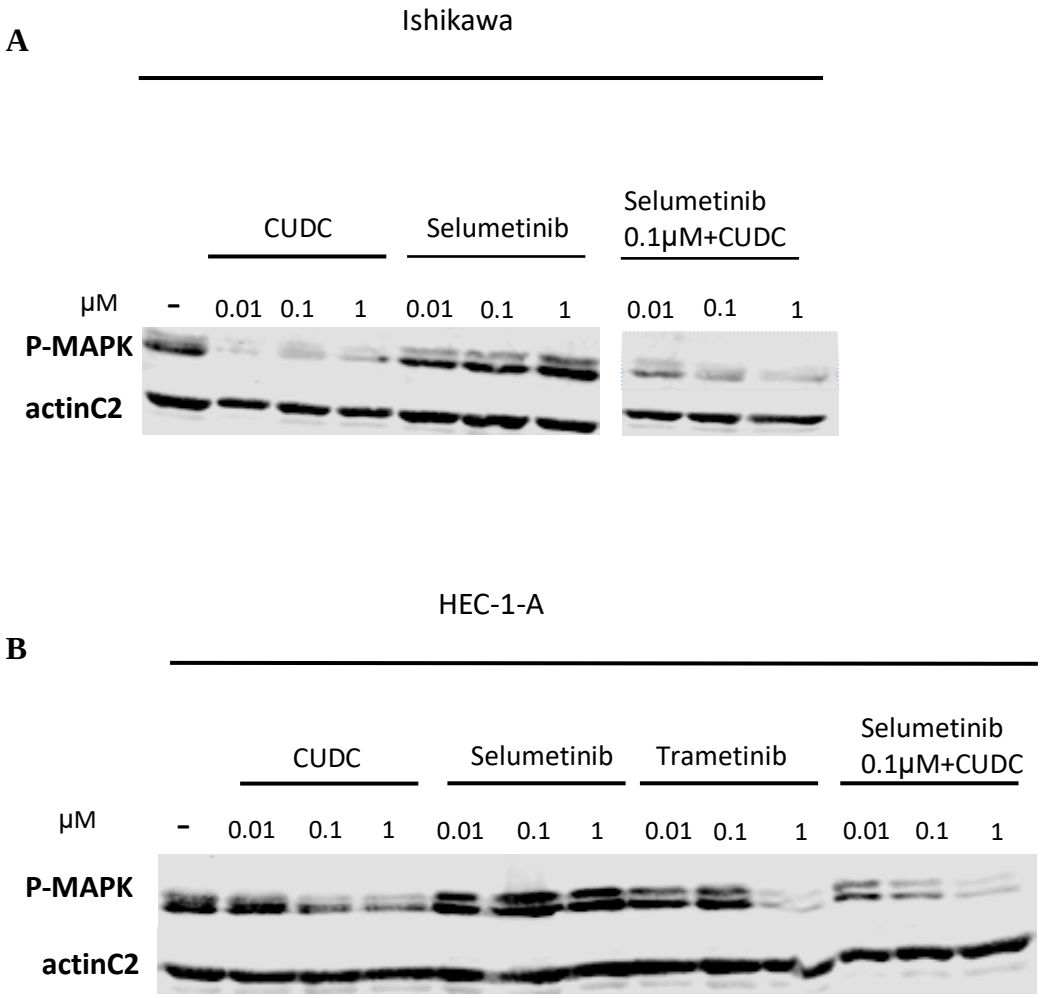
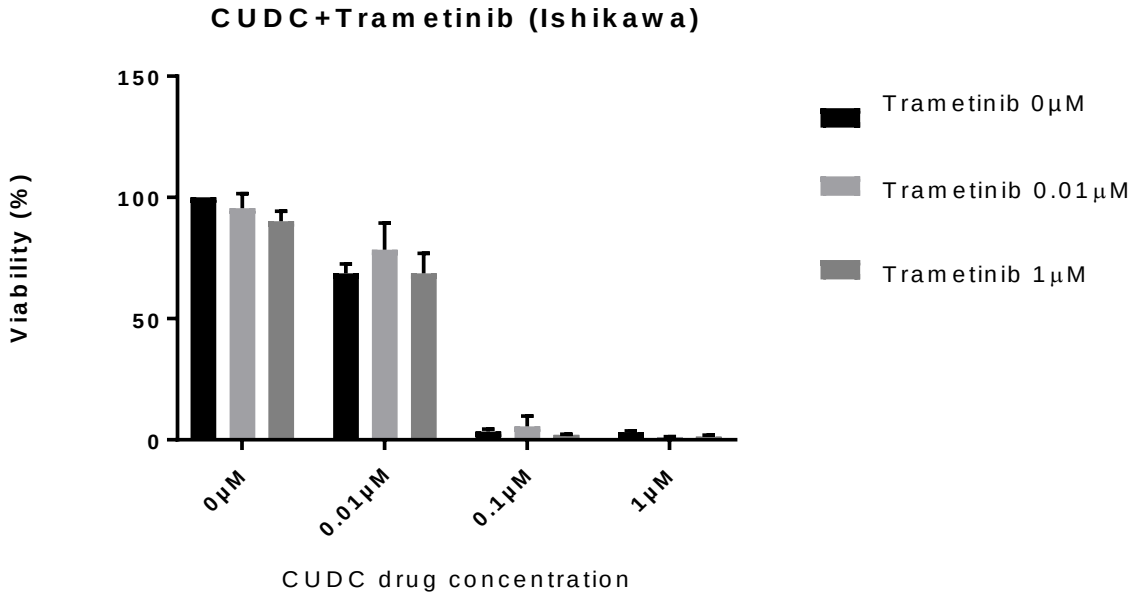


Figure 3.13 Western blot on Ishikawa (A) and HEC (B) cells treated with PI3K and HDAC inhibitor (CUDC), MEK inhibitors (Selumetinib and Trametinib) and combination of them for 24 hours.

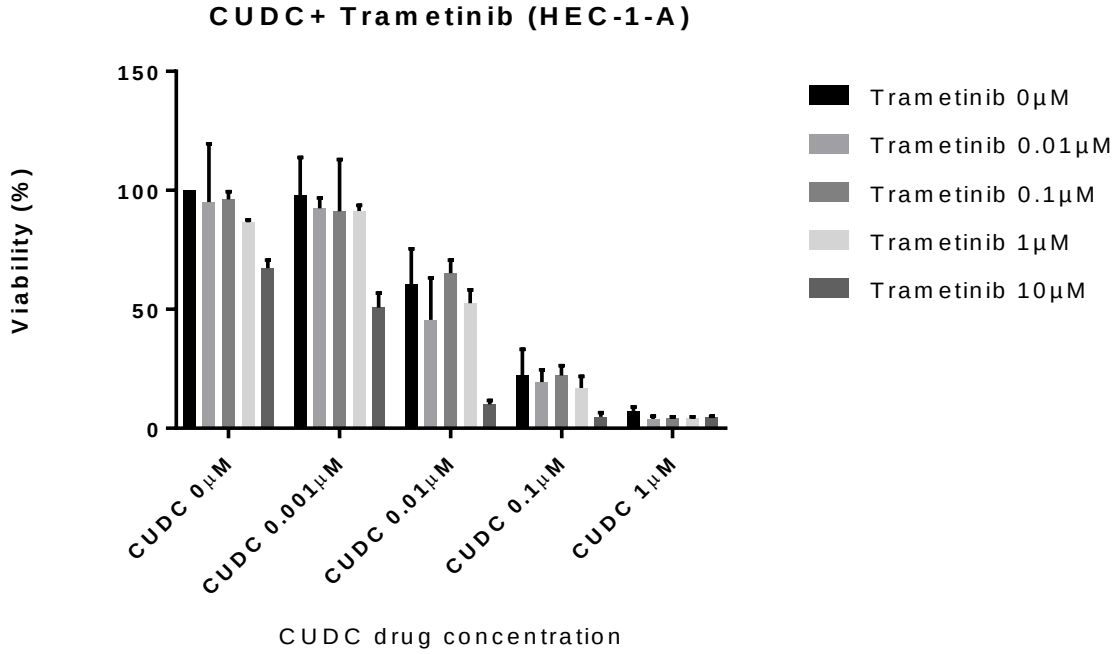
As shown in Figure 3.13A, adding Selumetinib to treatment of Ishikawa cells with CUDC does not add any further reduction to the basal levels of MAPK phosphorylation compared to CUDC alone. It is reasonable to assume that this is, at least partly, responsible for the lack of any synergistic effect when using this combination on Ishikawa cells.

The overall picture was quite different on HEC cells; when adding MEK inhibitor (Selumetinib) to cells treated with CUDC, basal levels of MAPK phosphorylation were reduced even further compared to CUDC or Selumetinib alone (synergistic reduction). This potentially explains the efficacy of this combination on HEC cells and the synergism shown previously but, more importantly, suggests that our hypothesis for the importance of MAPK pathway suppression on the resistant cell line (HEC-1-A) is more likely to be correct. Also, Trametinib (at least in concentrations higher than 1μM) suppresses MAPK phosphorylation in HEC1A cells better than Selumetinib (which paradoxically and consistently increases the P-MAPK levels). Due to this, I proceeded to investigate the combination of CUDC and Trametinib in both cell lines.

CUDC907+ Trametinib



A **CI=1.29**



B **CI=0.878**

Figure 3.14 MTS assay to assess viability of Ishikawa (A) and HEC (B) cells following treatment with combination of CUDC-907 and Trametinib. The values shown are the average of 3 experiments. Error bars represent standard deviation of the mean. CI= Combination Index.

Combining another MEK inhibitor (Trametinib) with CUDC-907 (PI3K and HDAC inhibitor) gave very similar results with the previous combination of Selumetinib and CUDC. There was no synergistic effect of this combination to Ishikawa cells while there was a mild synergism to HEC-1-A cells, not as profound though as shown previously with Selumetinib. This confirms the previous proposal that PI3K and MEK inhibition combined are a good therapeutic potential for resistant cells that carry mutations in both these pathways (like HEC-1-A). On the contrary, no synergism is seen on cells (like Ishikawa) which only carry mutations in the PI3K pathway, as these cells are already very sensitive to PI3K inhibition alone.

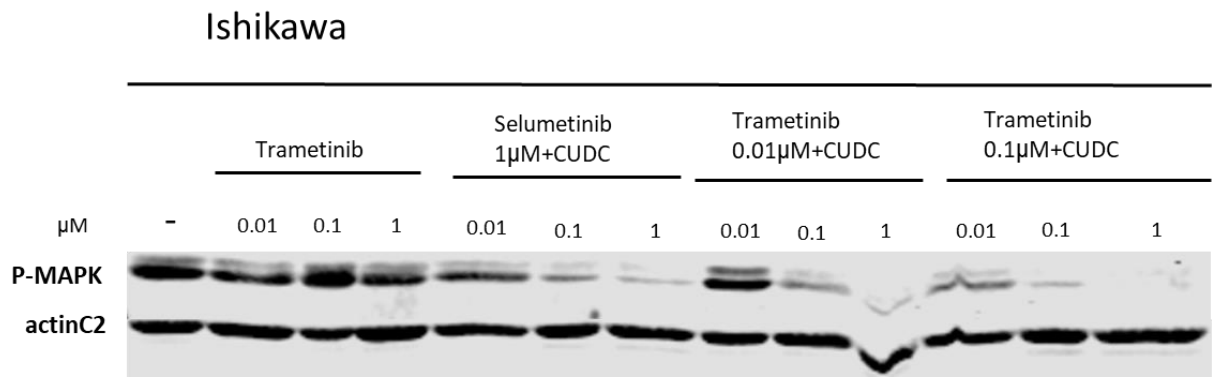


Figure 3.15 Western blot on Ishikawa cells treated with PI3K and HDAC inhibitor (CUDC), MEK inhibitors (Selumetinib and Trametinib) and combination of them for 24 hours.

The Western blot in figure 3.15 shows that although Trametinib alone does not suppress the basal levels of MAPK phosphorylation on Ishikawa cells (exactly as shown in figure 3.9 for three other MEK inhibitors), when combined with CUDC, it acts synergistically in suppressing this pathway. Despite this effect on pathway suppression, it does not reduce the viability of these cells even further, as they are already very sensitive to CUDC alone. Interestingly, it is apparent from the same figure that Trametinib, when combined with CUDC, is achieving better P-MAPK suppression with much smaller doses compared to Selumetinib and CUDC. This fact potentially makes this combination therapeutically more interesting due to the less risk of side-effects associated with using lower doses of inhibitory drugs.

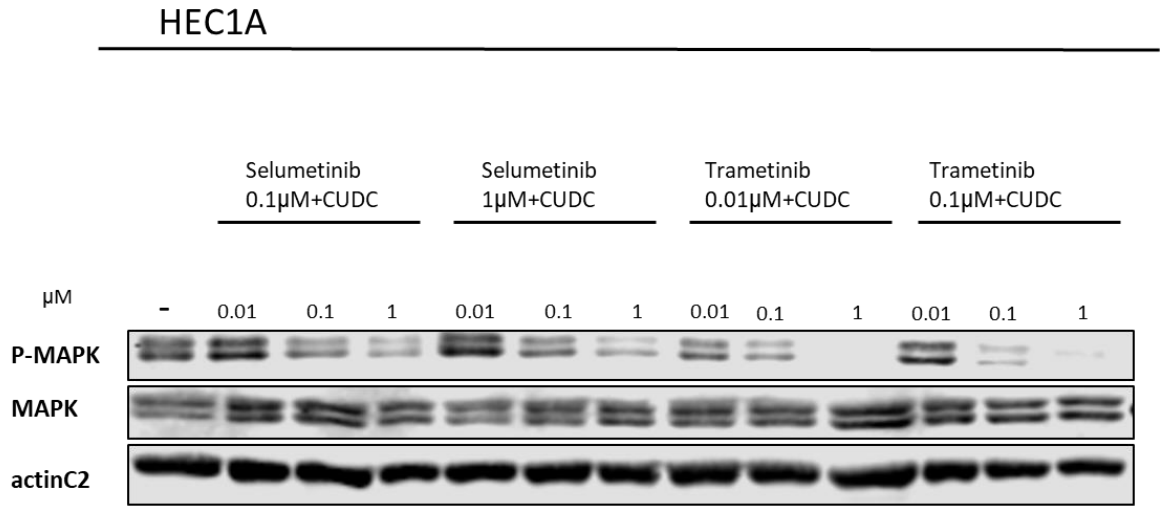
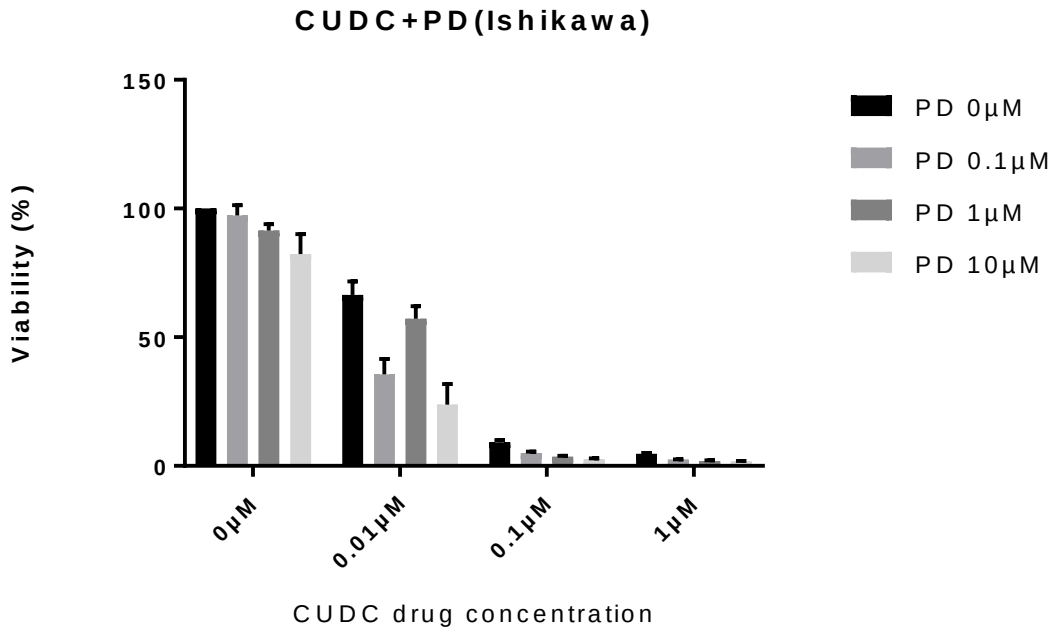


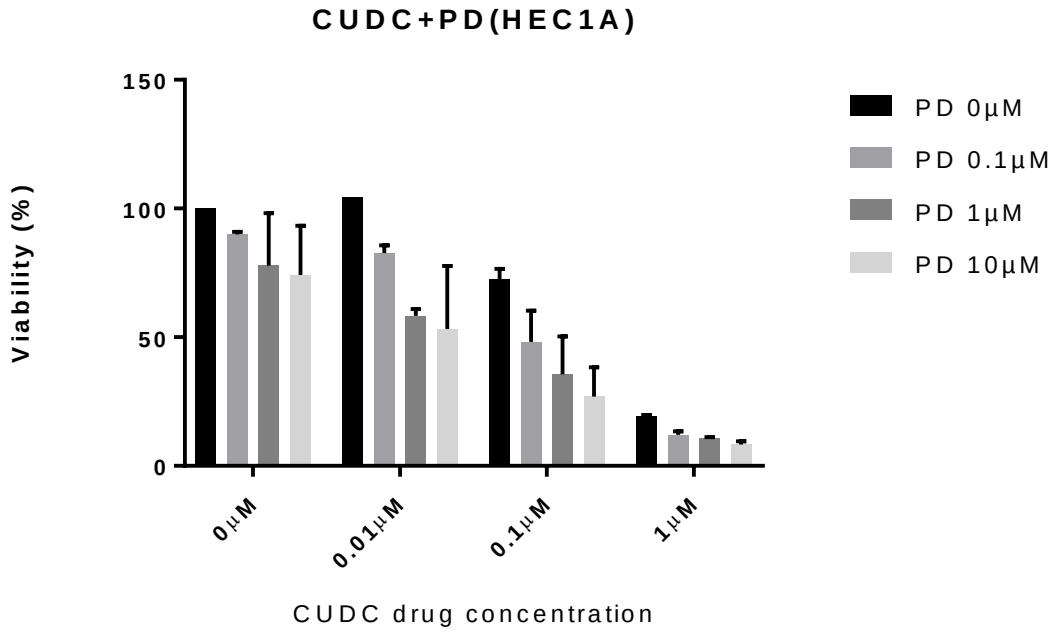
Figure 3.16 Western blot on HEC-1-A cells treated with PI3K and HDAC inhibitor (CUDC) combined with MEK inhibitors (Selumetinib or Trametinib) for 24 hours.

The fact that Trametinib achieves greater suppression of MAPK phosphorylation at much lower concentrations compared to Selumetinib is also apparent in Figure 3.16, where it can be seen that adding only 10nM of Trametinib to CUDC achieves greater P-MAPK suppression compared to 1μM of Selumetinib with the same concentration of CUDC. This renders this combination as a promising one due to the potential safer side-effect profile associated with the small doses necessary to achieve effective suppression of the MAPK pro-survival pathway.

CUDC907+ PD0325901



A **CI=0.381**



B **CI=0.401**

Figure 3.17 MTS assay to assess viability of Ishikawa (A) and HEC (B) cells following treatment with combination of CUDC-907 and PD0325901. The values shown are the average of 3 experiments. Error bars represent standard deviation of the mean. CI= Combination Index.

The next combination between CUDC-907 and a MEK inhibitor tested (PD0325901) showed significant synergism in both cell lines with very similar Combination Indices. Interestingly, this was the only combination of CUDC with a MEK inhibitor which showed synergism in the Ishikawa cells, making this combination a potentially interesting one. The result re-confirmed that combining PI3K and MEK inhibitors is a promising therapeutic strategy in endometrial cancer.

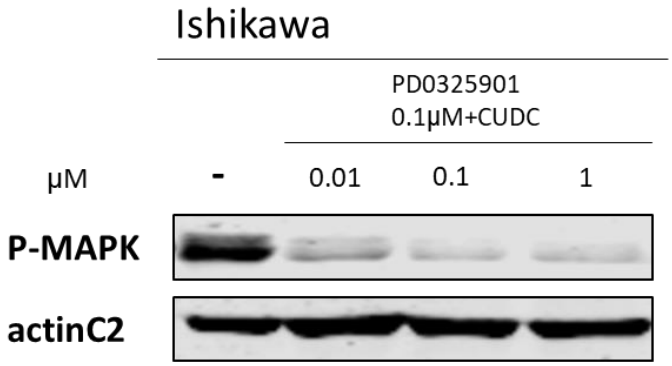
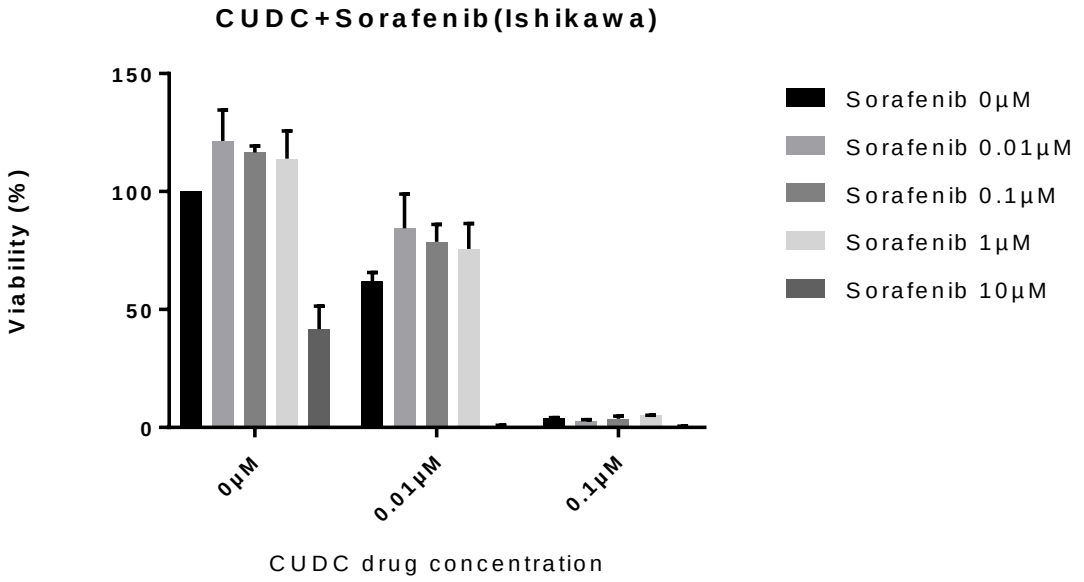


Figure 3.18 Western blot on Ishikawa cells treated with CUDC-907 (PI3K and HDAC inhibitor) and PD0325901 (MEK inhibitor) for 24 hours.

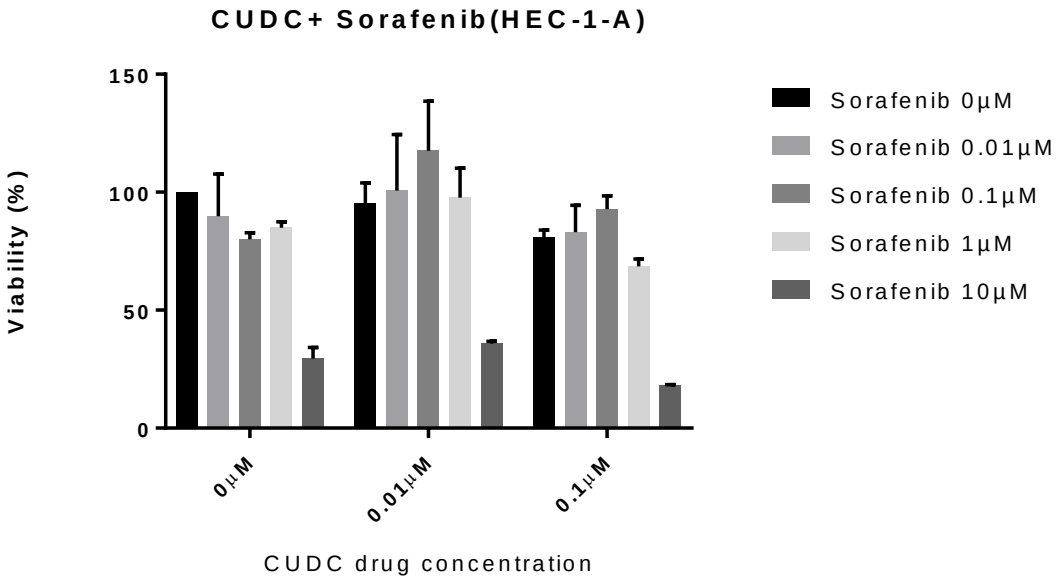
CUDC-907 has been shown to be very effective against Ishikawa cells and suppresses the MAPK phosphorylation as shown in figure 3.13A. Adding PD0325901 shows synergistic effect as it reduces viability from 66% (when Ishikawa cells are treated with CUDC 0.01μM alone) to 36% when adding 0.1μM of PD0325901 (CI 0.327) (Figure 3.17A). This synergism though, is not associated with further suppression of the MAPK pathway, as shown in figure 3.18, which confirms a very similar reduction to MAPK phosphorylation as CUDC alone (Figure 3.13A). This further enhances the previous hypothesis that Ishikawa cells do not significantly depend on the MAPK pathway for their survival.

CUDC907+ Sorafenib



CI=1.06

A



CI>10

B

Figure 3.19 MTS assay to assess viability of Ishikawa (A) and HEC (B) cells following treatment with combination of CUDC-907 and Sorafenib. The values shown are the average of 3 experiments. Error bars represent standard deviation of the mean. CI= Combination Index.

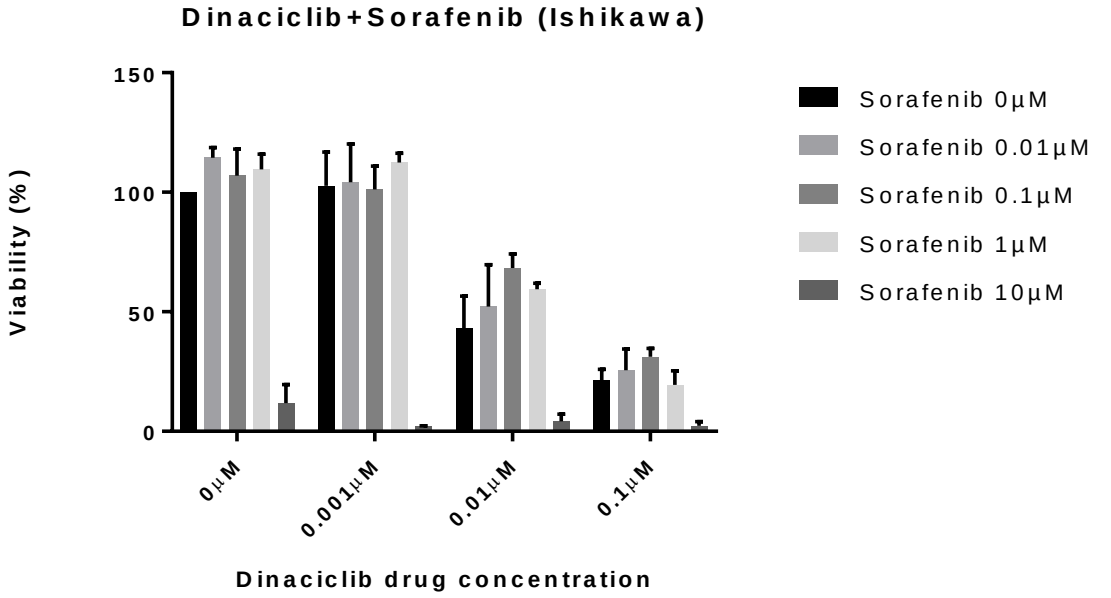
The next combination tested was this of CUDC and Sorafenib, a Raf inhibitor already used in the treatment of liver, kidney and thyroid cancer (Llovet *et al.*, 2008; Bernard Escudier *et al.*, 2009; Brose, Marcia S, Dr et al, 2014). The results shown in Figure 3.19, along with the results of using Sorafenib as monotherapy presented in Figures 3.1 and 3.2, confirm that this drug inhibitor is unlikely to be of therapeutic benefit in the context of endometrial cancer. As monotherapy, it is effective only in doses clinically irrelevant (10μM) and actually in smaller doses it appears to increase both the viability of the two cell lines but also the activation of the MEK-ERK pro-survival pathway as shown in Figure 3.9. This is particularly prominent in the Ishikawa cells who have relatively low basal levels of MAPK phosphorylation and when treated with Sorafenib, these levels are markedly increased (Figure 3.9). When tested in combination with CUDC, Sorafenib had a clear antagonistic action which was more prominent on HEC-1-A cells (Figure 3.19).

There were some concentrations were synergism was achieved but again this was the case only when Sorafenib was used in high concentrations (10μM), likely meaningless for use in clinical practice especially when used in combination with another drug which increases the risks of side-effects even further.

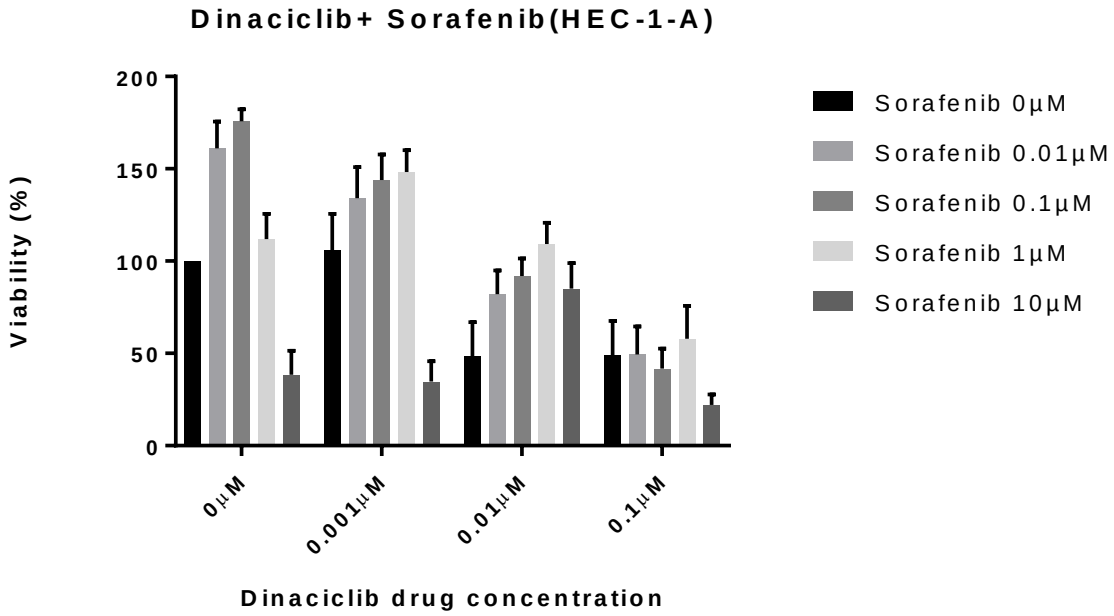
In order to confirm these findings, we decided to combine Sorafenib with Dinaciclib, a CDK inhibitor who was shown to be the most potent of the inhibitors tested in Ishikawa cells (but not shown the same efficacy in HEC cells).

3.1.3.2 CDK inhibition

Dinaciclib+ Sorafenib



A **CI=3.19**



B **CI=6.93**

Figure 3.20 MTS assay to assess viability of Ishikawa (A) and HEC (B) cells following treatment with combination of Dinaciclib and Sorafenib. The values shown are the average

of 3 experiments. Error bars represent standard deviation of the mean. CI= Combination Index.

The results from combining Dinaciclib with Sorafenib show a strong antagonistic effect in both cell lines. This confirms our previous findings that Sorafenib is unlikely to be of therapeutic advantage in endometrial cancer either as monotherapy or as a combination with CUDC and Dinaciclib, due to the fact that in both cases the cell viability is significantly reduced only when using very high concentrations of Sorafenib (10μM). The reasons for these paradoxical pro-survival action of Sorafenib in EC cells were studied using Western blotting.

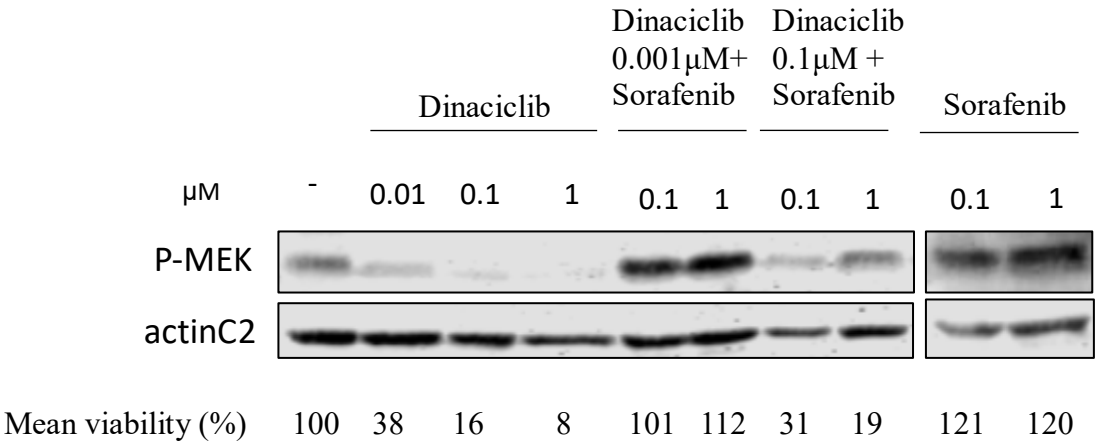


Figure 3.21 Western blot on Ishikawa cells treated with Dinaciclib (CDK inhibitor) and Sorafenib (MEK inhibitor) for 24 hours. The viability presented is the mean of at least 3 independent MTS experiments.

Western blotting (shown in Figure 3.21 and 3.22) confirmed previous findings (Figure 3.9) that Sorafenib increases the activation of the MEK-ERK pro-survival pathway in EC cells and this might explain the increased viability that these cells exhibit when treated with it. From Figure 3.21 it is evident that Dinaciclib, a very potent drug inhibitor against Ishikawa cells, significantly suppresses MEK phosphorylation when used alone, which is clearly reflected in the viability of these cells (16% when 0.1μM of Dinaciclib is used). When adding Sorafenib though, MEK phosphorylation is salvaged to a certain degree and this

could explain the obvious antagonistic effect (31% viability when adding 0.1μM of Sorafenib to the same concentration of Dinaciclib, CI=2.256).

Similar results are shown below (Figure 3.22) in HEC-1-A cells which are not as sensitive to CDK inhibition (Dinaciclib) as Ishikawa cells. As it can be seen, Dinaciclib suppresses the MEK phosphorylation when used alone with some reduction in cell viability (58% when used in a concentration of 1μM). Adding 1μM of Sorafenib to a similar concentration of Dinaciclib however, increases the MEK phosphorylation and adds no benefit as the viability is almost identical (55%).

We then proceeded to test Dinaciclib with a MEK inhibitor (Selumetinib), previously shown to act synergistically with CUDC in Ishikawa cells.

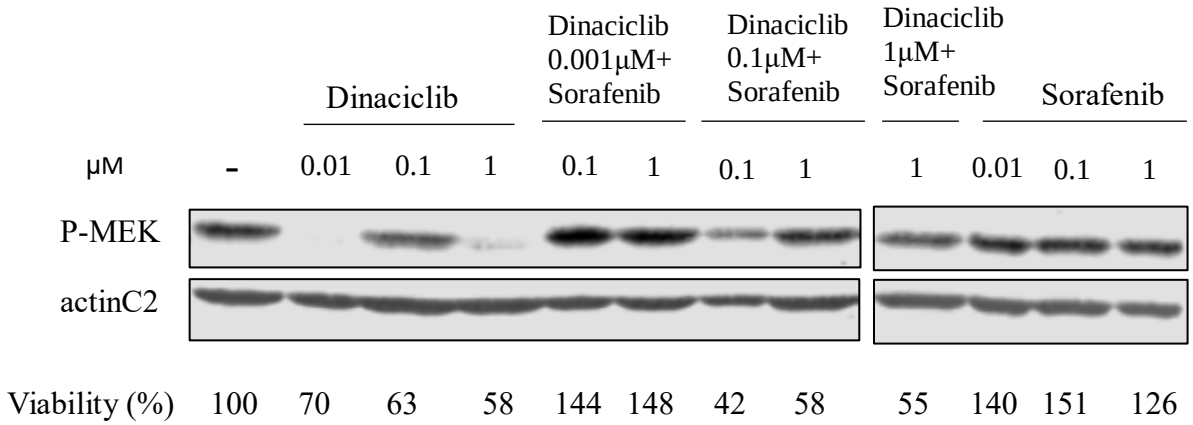
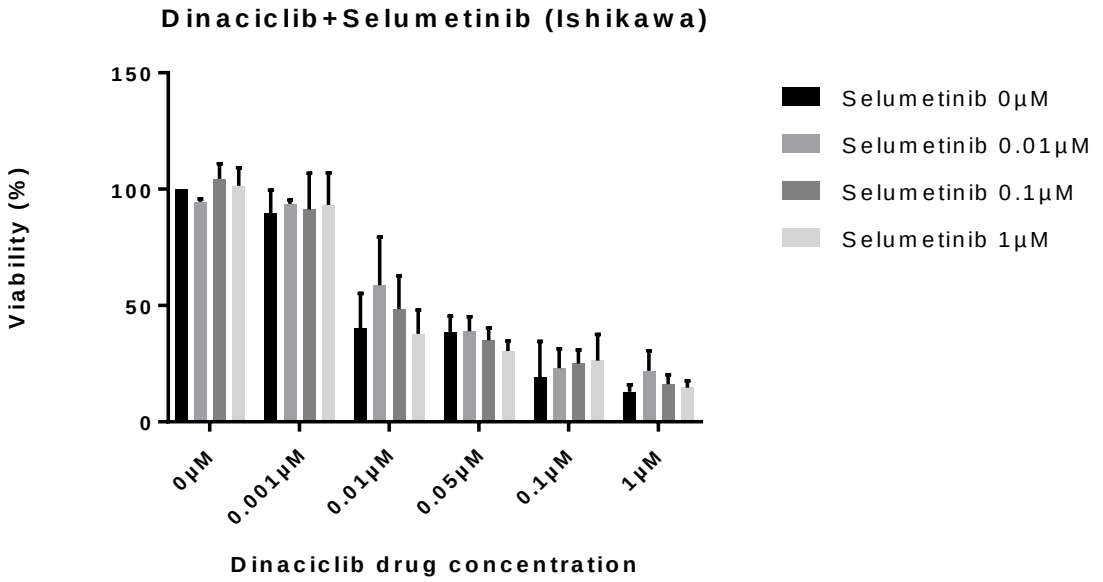
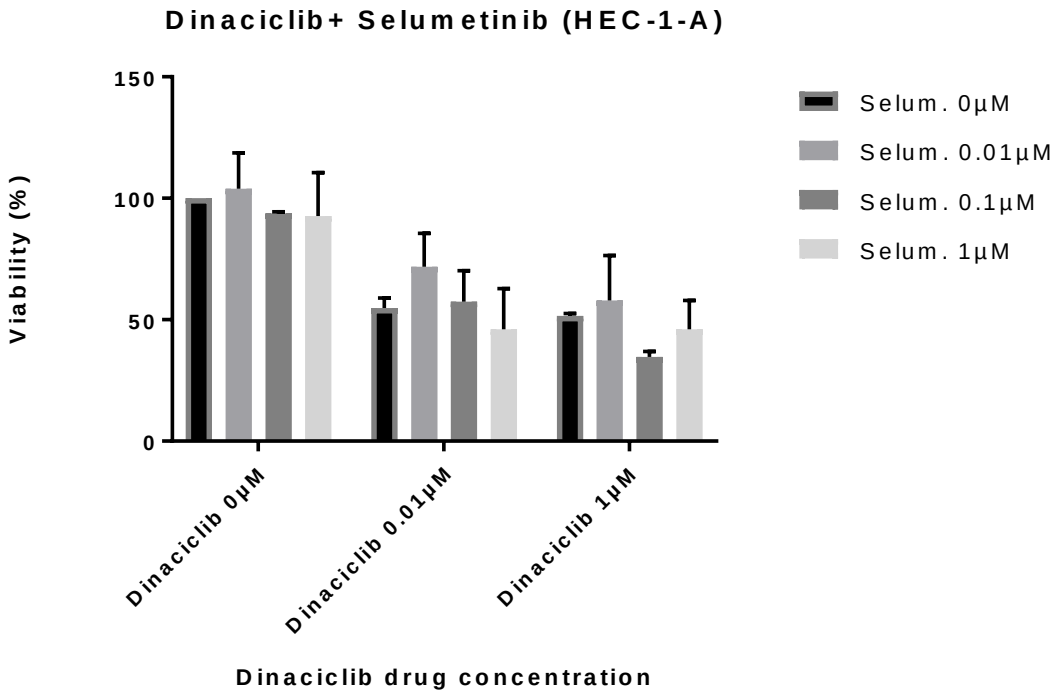


Figure 3.22 Western blot on HEC-1-A cells treated with Dinaciclib (CDK inhibitor) and Sorafenib (MEK inhibitor) for 24 hours. The viability presented is the mean of at least 3 independent MTS experiments.

Dinaciclib+ Selumetinib



A. **CI>10**



B

CI>10

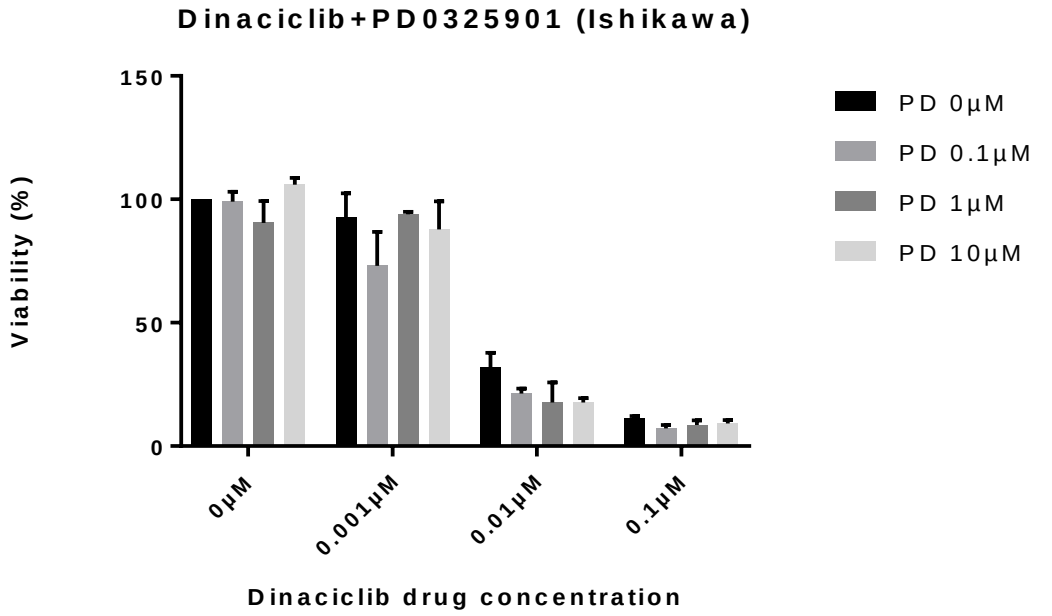
Figure 3.23 MTS assay to assess viability of Ishikawa (A) and HEC (B) cells following treatment with combination of Dinaciclib and Selumetinib. The values shown are the average of 3 experiments. Error bars represent standard deviation of the mean. CI= Combination Index.

As shown in Figure 3.23, combining Dinaciclib with the MEK inhibitor Selumetinib does not show any synergistic effect in neither of the two cell lines and gives similar results to the previous combination of Dinaciclib with Sorafenib. This is more obvious in the Ishikawa, as in these cells Dinaciclib is extremely potent when used alone and adding Selumetinib not only does not offer any therapeutic advantage but overall has significant antagonistic effect.

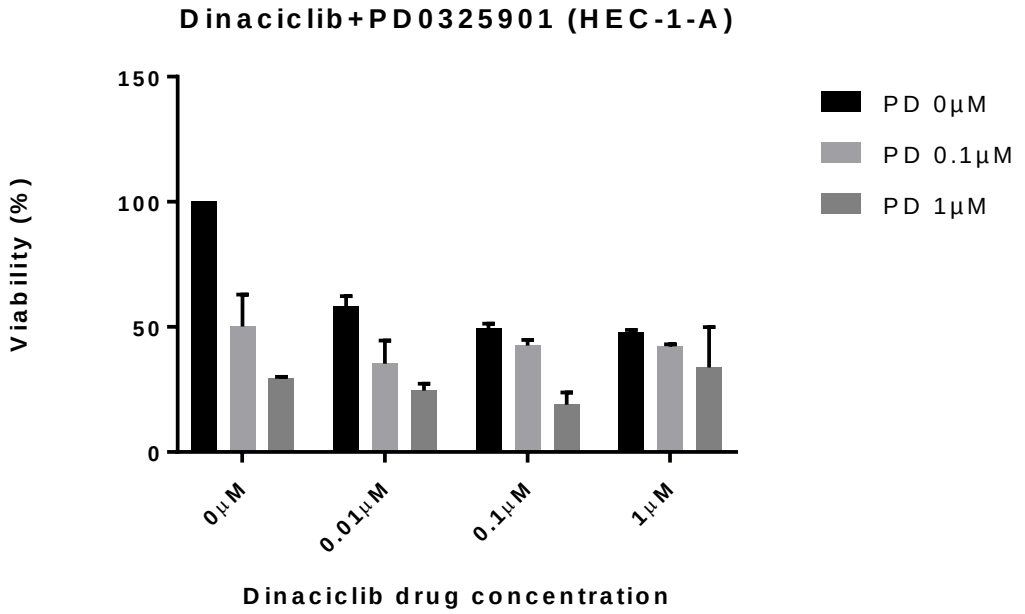
The results are more complex in HEC-1-A cells in which the addition of lower doses of Selumetinib has a large antagonistic effect while higher doses have some synergistic effect (CI=0.12 when adding Selumetinib 1μM).

In view of this result on HEC-1-A cells and taking into account the fact that PD0325901 was the only MEK inhibitor which was suppressing MAPK phosphorylation (at doses of 1μM, Figure 3.9), we tested the combination of Dinaciclib with PD0325901, in order to see whether CDK and MEK inhibition can be proposed for further testing.

Dinaciclib+ PD0325901



A **CI>10**



B **CI=0.61**

Figure 3.24 MTS assay to assess viability of Ishikawa (A) and HEC (B) cells following treatment with combination of Dinaciclib and PD0325901. The values shown are the average of 3 experiments. Error bars represent standard deviation of the mean. CI= Combination Index.

The results in Figure 3.24A show antagonism when Dinaciclib is combined with PD0325901 in Ishikawa cells. This confirms previous findings (Figures 3.20 and 3.23), which show that the combination of CDK (Dinaciclib) with MEK inhibition has antagonistic effect in Ishikawa cells. On the contrary, this latter combination of Dinaciclib with PD0325901 is a promising one when used in the more resistant HEC-1-A cells as synergism is clearly exhibited, even when relatively small doses of PD (0.1μM) are combined with Dinaciclib. This result also confirms that PD0325901 is the most effective MEK inhibitor from the ones we have tested in HEC-1-A cells, both as monotherapy and in combination with PI3K and CDK inhibitors (CUDC907 and Dinaciclib respectively).

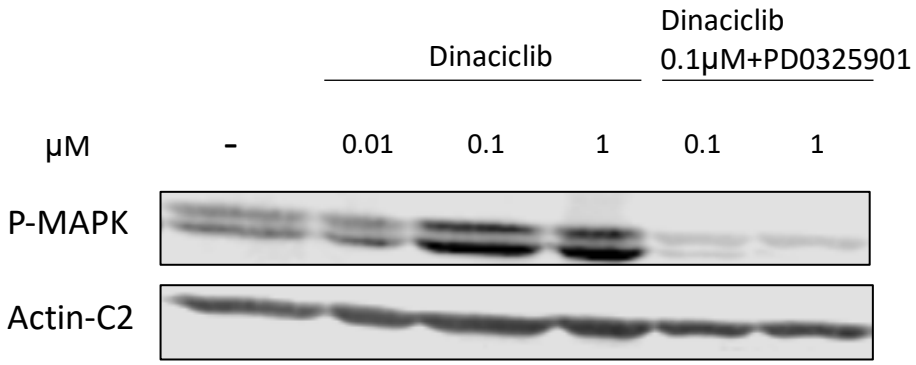


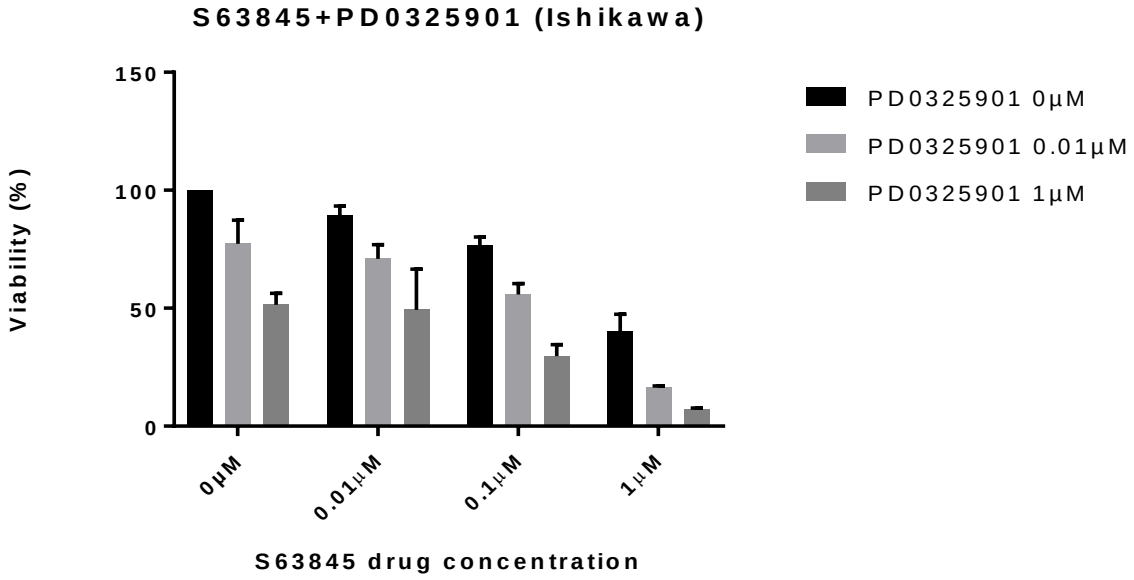
Figure 3.25 Western blot on HEC-1-A cells treated with Dinaciclib (CDK inhibitor) and PD0325901 (MEK inhibitor) for 24 hours.

Western blotting (Figure 3.25) showed that adding PD0325901 significantly suppresses the MAPK phosphorylation in HEC-1-A cells compared to Dinaciclib alone and this adds more evidence to the dependence of HEC-1-A cells to MAPK pathway activation for their survival.

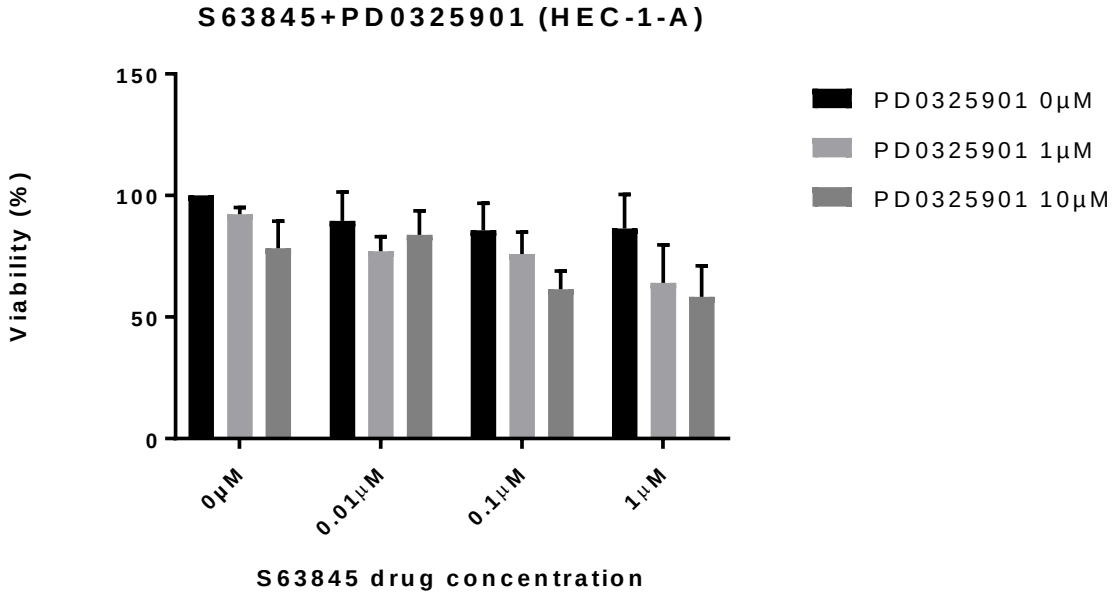
In view of the effectiveness of PD0325901 when used in HEC-1-A cells, we proceeded with testing it in combination with S63845, a promising MCL1 inhibitor in various cancer models (András Kotschy *et al.*, 2016b) which also showed great efficacy in Ishikawa cells and moderate efficacy in HEC-1-A cells when used alone (Table 3.1).

3.1.3.3 Other combinations

S63845+PD0325901



A **CI=0.257**

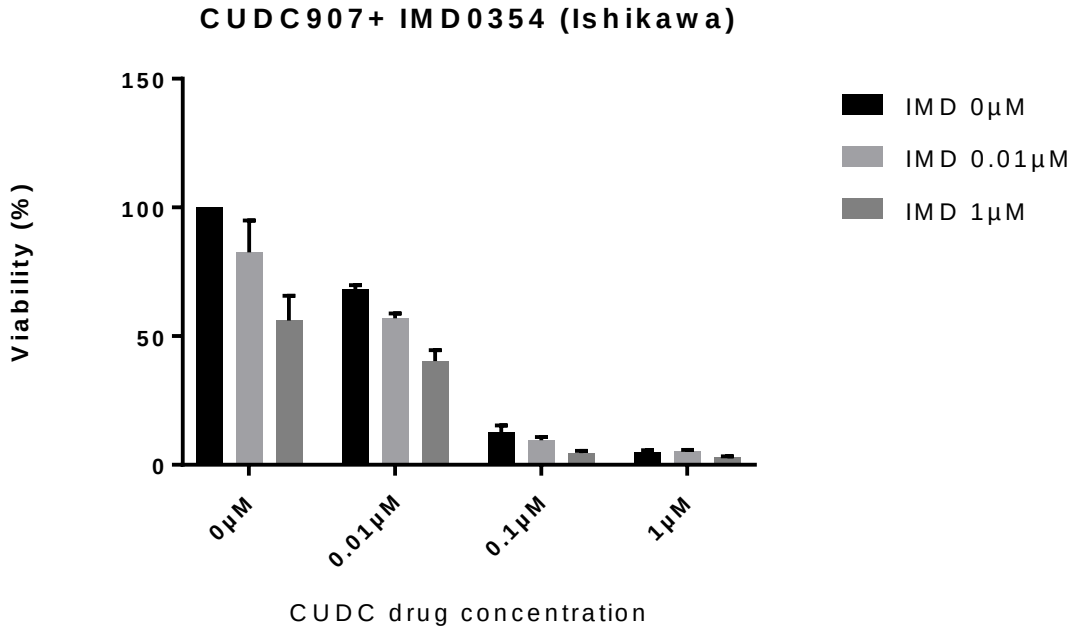


B **CI=0.421**

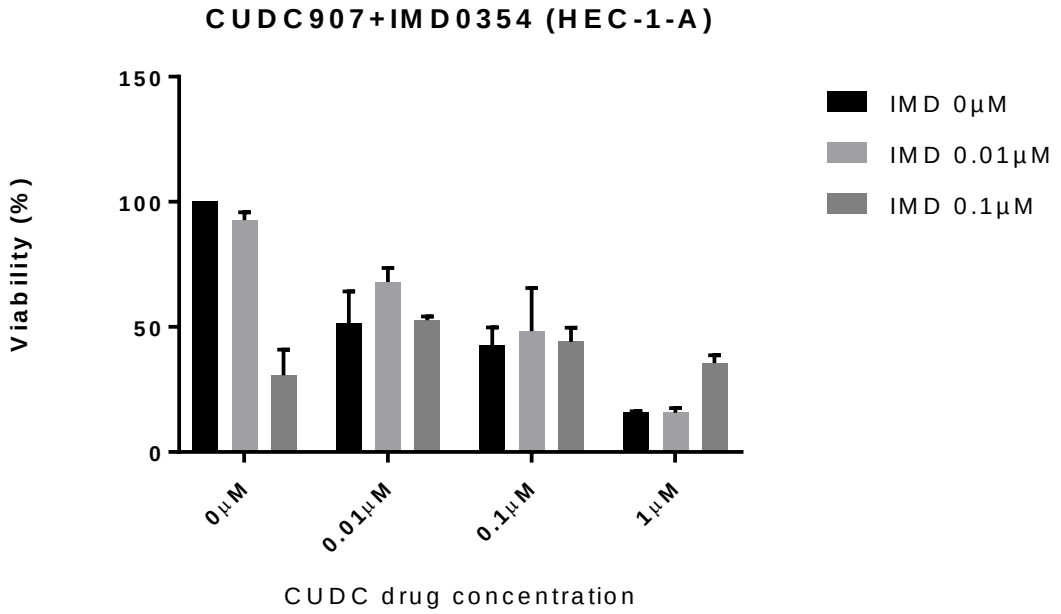
Figure 3.26 MTS assay to assess viability of Ishikawa (A) and HEC-1-A (B) cells following treatment with combination of S63845 (BCL-1 inhibitor) and PD0325901 (MEK inhibitor). The values shown are the average of 3 experiments. Error bars represent standard deviation of the mean. CI= Combination Index.

The results shown in Figure 3.26 reveal an interesting combination with synergistic action in both cell lines. The effect is more prominent in Ishikawa cells but it is significant even in the more resistant cell line (HEC-1-A). Unsurprisingly though, the doses of the MEK inhibitor needed in order to achieve this synergism were higher in HEC cells.
Finally, the combination tested was of IMD0354 (NFkB inhibitor), a very effective drug when tested in Ishikawa cells (Table 3.1), with CUDC907.

CUDC907+ IMD0354



A **CI=0.64**



B

CI=4.91

Figure 3.27 MTS assay to assess viability of Ishikawa (A) and HEC-1-A (B) cells following treatment with combination of CUDC-907 (PI3K and HDAC inhibitor) and IMD0354 (NfkB inhibitor). The values shown are the average of 3 experiments. Error bars represent standard deviation of the mean. CI= Combination Index.

The results shown in Figure 3.27 confirm the synergistic effect of NFkB and PI3K inhibition in Ishikawa cells. The same combination has a very antagonistic effect on the more resistant HEC-1-A cells, adding no benefit compared to treatment with CUDC-907 alone.

We showed previously that CUDC-907 suppresses the basal levels of MAPK phosphorylation (Figure 3.11). When an NFkB inhibitor is added, the MAPK phosphorylation is rescued and possibly enhanced (as shown in Figure 3.28 below). This further strengthens the hypothesis that these resistant cells are very dependent on the MAPK pathway and only inhibitory drugs and combinations which suppress it are effective in reducing their viability. The same combination suppresses the P-MAPK in Ishikawa cells (Figure 3.28) and this might be partly responsible for the great difference in viability seen between the two cell lines when this combination is being used.

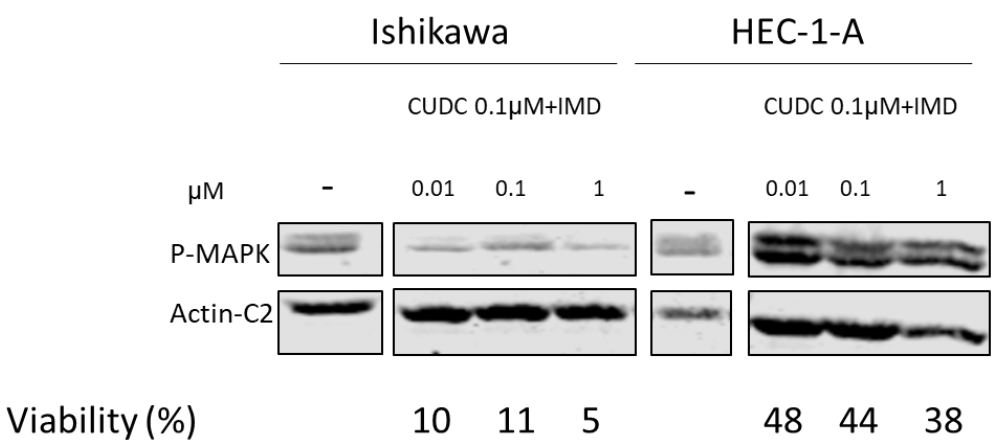


Figure 3.28 Western blot on Ishikawa and HEC-1-A cells treated with CUDC-907 and IMD0354 for 24 hours.

3.1.4 Summary

Table 3.2 below summarizes potentially efficient treatments which warrant further validation in pre-clinical models in order to confirm their in vitro efficacy.

Proposed treatments	Ishikawa	HEC-1-A
Single drug	Dinaciclib	
	CUDC-907	**CUDC-907**
	MG132	
	S63845	
	IMD0354	
Combination of drugs		CUDC-907+Selumetinib
		CUDC-907+ Trametinib
	CUDC-907+ PD0325901	**CUDC-907+ PD0325901**
		Dinaciclib+ PD0325901
	S63845+ PD0325901	**S63845+ PD0325901**
	CUDC-907+ IMD0354	

Table 3.2 Summary of effective treatments in two endometrial cancer cell lines. Single drugs were selected if IC50<1μM and combination of drugs if CI<1 (synergistic effect). In bold letters those drugs who were effective in reducing viability in both cell lines.

The results shown above confirm that CUDC-907 is the best monotherapy from all the inhibitory drugs tested as it is effective against both cell lines and it is the only drug shown to be effective in HEC cells when used alone. For the Ishikawa cells, there are a variety of inhibitory drugs which can be proposed as monotherapies. It is worth noting that Ishikawa cells carry *PTEN* mutations, which correlates with the majority of endometrial cancers (62% of EC tumours were carrying a *PTEN* mutation in the TCGA study) (TCGA, 2013). This means that treatment options effective against Ishikawa might have greater clinical relevance as they represent the majority of endometrial cancers.

In resistant cells (represented by HEC-1-A in our study), combination treatments might be the recommended approach. In particular, Table 3.2 shows that the combination of PI3K

and MAPK inhibitory drugs represent good therapeutic strategy as they exhibit synergistic effect in our in vitro model. The most effective of all the MEK inhibitors tested in HEC cells is PD0325901 as it worked synergistically not only with CUDC-907 but also with CDK inhibitor (Dinaciclib) and BCL1 inhibitor.

3.2 Differences between British White (BW) and South Asian (BSA) women

3.2.1 Clinico-pathological differences

In total, 207 patients were recruited as part of our study. One withdrew her consent at a later date for reasons unrelated to the study. Data from the remaining 206 patients were analysed. 78.6% (162) of them were BW women with the remaining 21.4% (44) being women of South Asian origin. The term BSA was used due to their geographical location of residing in the UK. Within the BSA cohort, the majority of women (61%, n=27) were born in India and later immigrated to the UK. There is also a number of women of Indian descent (14%, n=6), mainly Gujaratis originating from Western India who were born in African countries (Uganda, Kenya, Malawi and Mozambique) and followed the same journey. There were two patients of Pakistani origin (5%), three of Indian descent born in the UK (7%), one of Indian descent born in Singapore (2%) and five of South Asian descent (11%) for which we have no details on their place of birth.
The clinico-pathological data of the two groups of women are shown in Table 3.3 below.

As shown in Table 3.3 below, BW women are significantly older when diagnosed with endometrial cancer when compared with their BSA counterparts. Interestingly, BW women are also significantly more obese which is reflected in the rates of Class 3 (high-risk) obesity with 30% of them (48/162) having a BMI equal or higher than 40, compared to only 9% (4/44) in the BSA cohort, when overall, only 2% of Leicester population is morbidly obese (BMI>40) (Chris Rigby & Joe Wheeler, 2018). The higher rates of obesity among BW women confirm data from Leicester City Clinical Commissioning Group which show that obesity prevalence is higher among BW compared to BSA adults (22.5% versus 16%) (Atkinson *et al.*, 2016).

BSA patients are much more frequently diabetic with almost half of them (45.5%, 20/44) being diabetic at the time of diagnosis compared to 17% (28/162) rate of diabetes amongst BW women (p=0.0002). This result reflects the higher prevalence of diabetes amongst adult BSA patients compared to BW living in Leicester (13.2% versus 6.9%) (Benett *et al.*, 2016). Note that the rates of diabetes in Leicester are higher in the age groups in which

EC is more common (14.8% for the 45-64 age group and 26.2% for the 65-84 group) (Benett *et al.*, 2016). All 20 (100%) diabetic patients in the BSA group had type 2 diabetes which did not require insulin for its management (Non-Insulin Dependent Diabetes Mellitus, NIDDM). Similarly, 93% of BW women with diabetes (26/28) were type 2 diabetic. The difference is that, unlike the BSA cohort in which all patients were NIDDM, in the WB group, 31% (8/26) of type 2 diabetic patients were insulin-dependent ($p=0.0065$).

As mentioned previously, there is a proven association of diabetes with endometrial cancer although the causal relationship is less clear. We analyzed pre-operative blood testing of glycosylated Haemoglobin (HbA1c) levels in our patients in order to assess their glycaemic control. As HbA1c is a marker of glycaemia and reflects the average levels of glucose in the blood during the last three months, we wanted to assess the number of our non-diabetic patients who had either occult diabetes or pre-diabetes. After excluding patients who were known to be diabetic, we had data for 112 patients (92 BW and 20 BSA). There was no difference between the two groups with BSA women having a mean value of 5.91% versus 5.81% for the BW cohort ($p=0.64$). The interesting point of this analysis is that both groups had mean values placing them in the 'pre-diabetes' range (5.7-6.4%), raising interesting questions regarding the metabolic profile of EC patients.

	British White	British South Asian	p value
n (%)	162 (79)	44 (21)	
Mean age (range)	65.4 (35-91)	61.4 (36-77)	**0.034**
Mean BMI (range)	36.1 (16.9-65)	32.2 (22.7-52.2)	**0.0128**
Diabetic (%)	28 (17)	20 (45.5)	**0.0002**
Mean parity	2.1	2.8	**0.0024**
Hypertensive (%)	82 (51)	22 (50)	1
Histology (%)			0.326
Endometrioid	115 (71)	34 (77)	
Serous	9 (6)	5 (11)	
Clear cell	3 (2)	1 (2)	
Mixed	7 (4)	0	
Carcinosarcoma	3 (2)	1 (2)	
Mucinous	1 (1)	0	
Adenosarcoma	2 (1)	0	
Pre-cancerous	20 (12)	3 (7)	
Normal	2 (1)	0	
Stage			0.59
1	124	36	
2	8	4	
3	6	1	
4	2	0	
Grade (%)			**0.0024**
1	93 (67)	19 (46)	
2	16 (11)	14 (34)	
3	31 (22)	8 (20)	
MMR status (%)			0.174
Proficient	46 (85)	24 (73)	
Deficient	8 (15)	9 (27)	

Table 3.3 Clinico-pathological data of the 206 recruited patients of our study. Note that MMR status is not available for all patients. Age and BMI data were calculated based on

the patients with a cancer diagnosis, n=181 (pre-cancerous and normal histologies were excluded). P values were calculated using Fisher's exact test or Chi-square test (X^2) for categorical variables and t-test for independent variables.

There was no difference in the incidence of hypertension with half patients in both groups being hypertensive when diagnosed with EC (Table 3.3). Lipid profile blood tests were performed in 151 of our patients (115 BW and 36 BSA) as the addition of this test was commenced later in the recruitment process. Excluding 41 patients who were on statins prior to the blood test, we analyzed 110 patients (85 BW and 25 BSA). No difference was seen in the cholesterol levels between the two groups, with mean value for the BW group 5.4 mmol/L and the BSA group 5.2 (p=0.35). When we excluded patients with pre-cancerous histologies, we had 98 patients (75 BW and 23 BSA) but the results were identical with mean cholesterol level 5.5mmol/L for the BW and 5.2 for the BSA group respectively with no difference observed (p=0.325). Similar results were revealed after analyzing triglycerides levels between the two groups. After excluding patients who were receiving statins for treatment of dyslipidaemias, we analyzed 109 patients (85 BW and 24 BSA). There was no difference between the groups but the trend was the opposite observed with cholesterol as BSA women had a higher mean value (1.97mmol/L compared to 1.71mmol/L for BW, p=0.16). The results remained unchanged after excluding patients without a cancer diagnosis. 97 patients were analyzed (74 BW and 23 BSA) and BSA women had a mean value of 1.94mmol/L versus 1.69mmol/L for BW (p=0.19).

Increased parity is known to be protective against endometrial cancer (Raglan *et al.*, 2018). We identified a significant discrepancy in the mean parity between the two cohorts with BW women having on average 2.1 children while BSA women 2.8 (p=0.0024). Although the parity rate was significantly different, the nulliparity rate was very similar between the groups with 16% of BW women and 14% of BSA women having no children (p=1).

The majority of patients underwent a robotically-assisted hysterectomy for treatment of their endometrial cancer (55%), with 23% of patients undergoing a laparoscopic hysterectomy, bringing the total number of minimally invasive surgical (MIS) procedures to 78%, making this approach the standard of care in our unit. The remaining 22% of our patients had the traditional abdominal hysterectomy. Interestingly though, there was a

significant discrepancy in the rate of MIS between the two ethnic groups. 81% of BW women had a minimally invasive hysterectomy versus 66% of BSA women (p=0.04). Given that the rate of laparoscopic hysterectomies was identical between the two groups (24% for the BW versus 23% for the BSA), the main difference was in the robotically-assisted hysterectomies (57% in the BW cohort, 43% for the BSA). The explanation for this large discrepancy is the higher conversion rate among BSA women. Overall, the conversion rate was 5.3%, with 9 out of 169 patients undergoing a MIS being converted to abdominal surgery due to inability of the patient to tolerate the pneumoperitoneum or extensive adhesions, making access to the peritoneal cavity unsafe or completion of surgery impossible. Within the groups, 15% of cases (5/34) involving BSA women had to be converted from robotic/laparoscopic to open compared to only 3% (4/135) for BW women (p=0.017).

Although we were unable to control for previous abdominal surgery, another possible explanation for the higher conversion rate in BSA women could be the anaesthetic risk associated with the creation of pneumoperitoneum which is necessary for performing laparoscopic/robotic procedures. In order to investigate this possibility we analyzed data from the anaesthetic charts completed pre-operatively, focusing on the American Society of Anaesthesiologists (ASA) physical status classification system which classifies the patients in six categories (ASA PS 1-6). We retrieved data for 191 patients (152 BW and 39 BSA). There was no difference in the ASA status between BW and BSA women with 95% of women in both groups being classified as ASA grade 2-3 (Table 3.4 below).

ASA grade	**BW (%)**	**BSA (%)**	**p value**
1	6 (4)	2 (5)	0.37
2	88 (58)	27 (69)	
3	57 (37)	10 (26)	
4	1 (1)	0	

Table 3.4 ASA physical status criteria for the recruited patients of the study. ASA grade was defined by Anaesthetic assessment. p value calculated using X^2 test.

No difference was observed in the histological subtypes of endometrial cancer between the two groups with endometrioid histology representing 82% of EC cases in the BW

women (115/140) and 83% in BSA women (34/41). Interestingly, we were able to show a significant preponderance of higher grade tumours amongst BSA women (Table 3.5 below). Based on this, 54% of BSA patients had a grade 2-3 tumour versus a 34% in the WB cohort.

Grade of EC	**BW (%)**	**BSA (%)**	**p value**
1	93 (66)	19 (46)	*0.0021*
2	16 (12)	14 (34)	
3	31 (22)	8 (20)	

Table 3.5 Histopathological grading of our endometrial cancer patients in the two groups of interest. p value calculated using X^2 test.

There was no difference in the MMR status between the two groups of interest although there was a slight trend towards more MMR deficiency in the BSA group (Table 3.4). MMR status is not yet routinely tested in our unit. The local protocol states that only women younger than 60 or with a family history of endometrial or bowel cancer, raising the possibility of Lynch syndrome, are having Immunohistochemistry performed in order to identify Mismatch Repair deficient tumours. This was the case for 87 of our patients with EC and the cumulative rate of MMR deficiency was 20% (15% for BW patients and 27% for BSA, p=0.174).

Overall, there are significant differences, both clinical and pathological, between BW and BSA women in the cohort of patients recruited for our study. This further enhanced our hypothesis that genetic differences between those two populations in genes frequently driving carcinogenesis in EC might exist.

3.2.2 Gene differences

In order to test our hypothesis, tumour DNA derived from 60 patients was deeply sequenced for 10 genes commonly involved in endometrial carcinogenesis based on the protocols described in methods and in the appendix. Due to technical failure, we were

unable to obtain results for three BSA patients. The clinico-pathological data for the 57 patients who were successfully sequenced is shown in Table 3.6 below.

As described previously for our entire cohort, BW women have significantly higher BMI compared to the BSA group. Due to the relatively small number of patients in this sequenced cohort, there was no difference in the age at diagnosis of EC between the two groups. The incidence of diabetes was, as in the entire cohort of our study (Table 3.3), higher in the BSA group but didn't reach statistical significance. As expected, since our two sequenced cohorts were matched, there was no difference in the histology or the histopathological grading of their tumours.

Ethnic group	***British White***	***British South Asian***	***p value***
n	*30*	*27*	
Mean age (range)	*62.3 (48-84)*	*61.2 (36-77)*	*0.7*
Mean BMI (range)	*37.8 (19-65)*	*32.5 (23-52.2)*	***0.0253***
Histology			*0.4*
Endometrioid (%)	*28 (93%)*	*23 (85%)*	
Serous (%)	*2 (7%)*	*4 (15%)*	
Stage (FIGO)			*0.88*
1	*25 (83%)*	*23 (85%)*	
2	*3 (10%)*	*3(10%)*	
3	*2 (7%)*	*1 (5%)*	
Grade			*0.37*
1	*20*	*13*	
2	*7*	*10*	
3	*3*	*4*	
Diabetes (%)			*0.08*
Yes	*6 (20%)*	*12 (44%)*	
No	*24 (80%)*	*15 (56%)*	

Table 3.6 Clinical and epidemiological data for the 57 patients with EC that were sequenced as part of our study. p values were calculated using Student's t test (age and BMI), Fisher's exact test (Histology and Diabetes), X^2 test (stage and grade).

There was a total of 129 non-synonymous mutations identified in the 10 genes tested (an average of 2.3 mutations per patient, figure 3.29). There were five patients carrying five mutations or more in the 10 genes tested. Interestingly, all of them had a *POLE* mutation which is known to be associated with a hyper-mutated state (TCGA, 2013).

There was no difference between the two groups in the total number of non-synonymous mutations in the ten genes tested with BW women having 2.3 mutations per patient versus 2.2 mutations per patient for the BSA cohort.

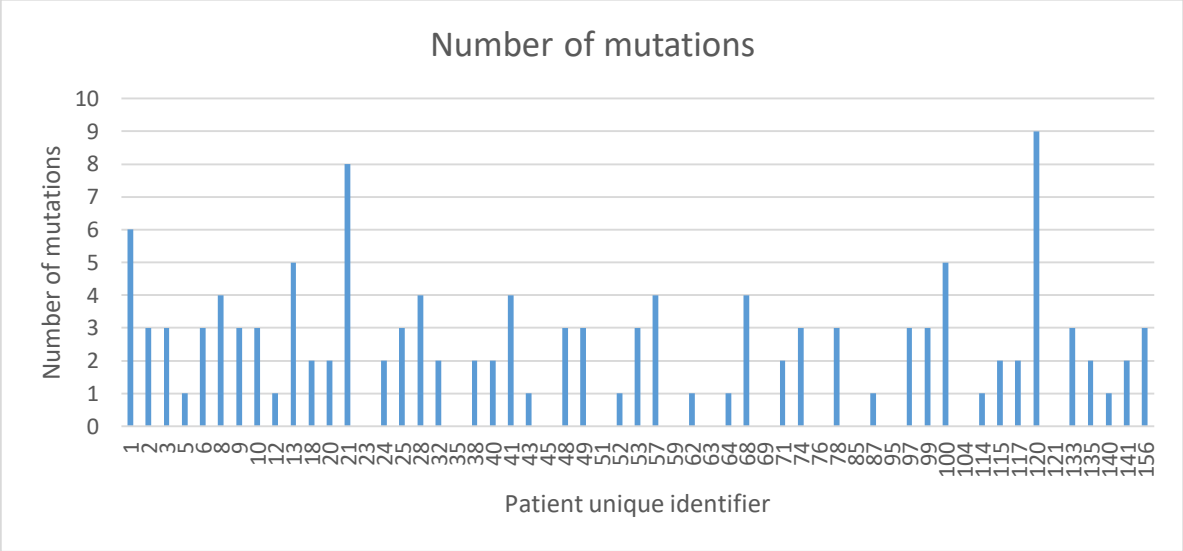


Figure 3.29. Total number of non-synonymous mutations in the 10 genes tested on each of the 57 patients of our study.

The total number of mutations in the ten genes tested, across the sequenced cohort, is shown in Figure 3.30 below.

Patient	ARID1A	CTNNB1	FBXW7	KRAS	PIK3CA	PIK3R1	POLE	PPP2R1A	PTEN	TP53
EC001	1				2		1		2	
EC002	1	1			1					
EC003				1	1				1	
EC005										1
EC006	1				2					
EC008	1	1			1				1	
EC009			1	1	1					
EC010					1				2	
EC012			1							
EC013	1					2	1		1	
EC018		1	1							
EC020	1								1	
EC021				2	4		1		1	
EC023										
EC024					1					1
EC025				1		1			1	
EC028	1				1		1			1
EC032					1				1	
EC035										
EC038		1							1	
EC040			1		1					
EC041					2				2	
EC043	1									
EC045										
EC048			1	1						1
EC049			1						2	
EC051										
EC052						1				
EC053			1			2				
EC057		1			2				1	
EC059										
EC062		1								
EC063										
EC064										1
EC068	1		1		2					
EC069										
EC071			1						1	
EC074			1		1				1	
EC076										
EC078	1								1	1
EC085										
EC087		1								
EC095										
EC097		1			1				1	
EC099		1			1				1	
EC100	1	1	1			1	1			
EC104										
EC114						1				
EC115	1								1	
EC117	1							1		
EC120		2	2		2	2	1			
EC121										
EC133	1				1				1	
EC135		1							1	
EC140			1							
EC141	2									
EC156	1				1				1	

Figure 3.30 Total number of non-synonymous mutations in each of the 57 patients of our cohort, in the 10 genes tested. The first column on the left represents each patient's unique study number. In bold letters and numbering are the BSA patients. White cells represent

no mutation while colored cells represent mutated gene. The number within colored cells shows the number of non-synonymous mutations identified in this gene.

The frequency of non-synonymous mutations in the ten genes tested is shown in figure 3.31 below.

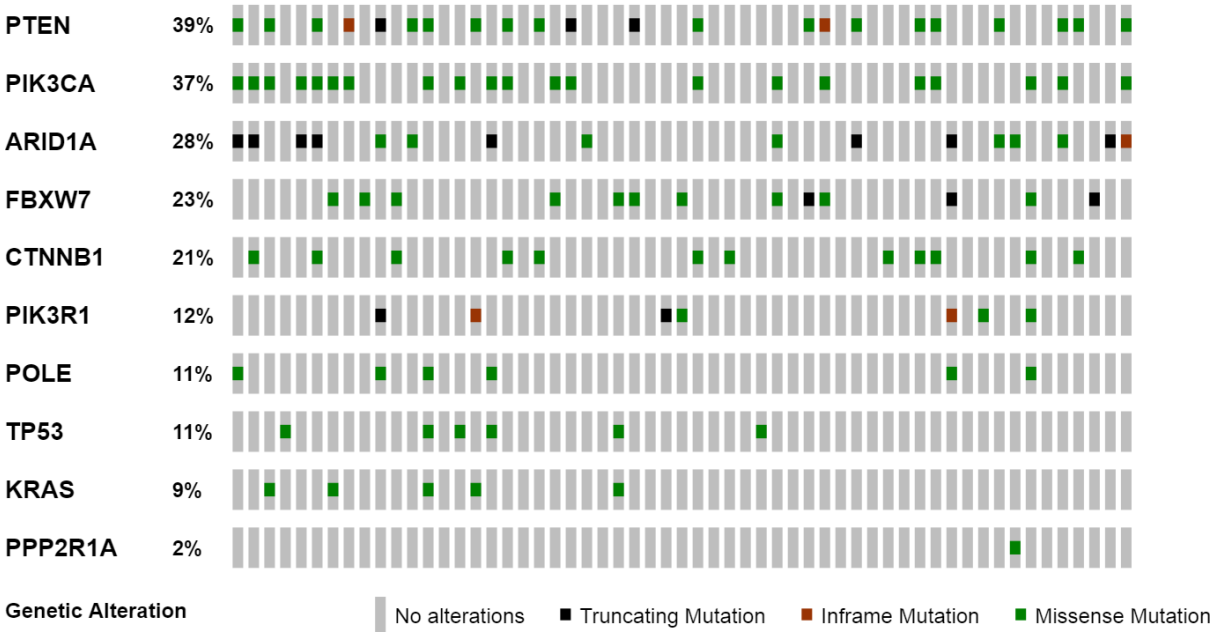


Figure 3.31 Frequency of mutations in the 57 patients sequenced for the 10 genes shown above. Figure created using Oncoprinter tool (Cerami *et al.*, 2012; J. Gao *et al.*, 2013).

As shown above, the most frequent mutations involve genes of the PI3K-PTEN pathway, more specifically *PTEN* and *PIK3CA* (39 and 37% respectively). Another gene of this pathway (*PIK3R1*), was mutated in 12% of our patients. Other common mutations include *ARID1A* (28%), *FBXW7* (23%) and *CTNNB1* (21%). Less frequent mutations include *POLE* and *KRAS* mutations (11 and 9% respectively). Given that the proportion of non-endometrioid tumours of our cohort is 11%, it is unsurprising that *TP53* and *PPP2R1A* mutations appear in 11 and 2% of patients respectively.

The incidence of mutations in the 2 groups of interest is shown in Table 3.7 below.

Gene	BW	BSA	p value
ARID1A	33%	22%	0.39
CTNNB1	13%	30%	0.19
FBXW7	20%	26%	0.75
KRAS	13%	4%	0.35
PIK3CA	40%	33%	0.78
PIK3R1	7%	19%	0.24
POLE	13%	7%	0.67
PPP2R1A	0%	4%	0.47
PTEN	43%	33%	0.59
TP53	10%	11%	1

Table 3.7 Incidence of non-synonymous mutations in British White and British South Asian women. The p value was calculated using Fisher's exact test.

BW women in our cohort had more *ARID1A* and *KRAS* mutations, although due to the relatively small number of sequenced tumours, no statistical significance was reached. BSA women had more *CTNNB1* (30% vs 13%, p=0.19) and *PIK3R1* mutations but again this was not statistically significant. There was no difference in the incidence of *FBXW7*, *PIK3CA*, *POLE*, *PPP2R1A*, *PTEN* and *TP53* between the two groups.

3.2.2.1 *ARID1A*

ARID1A mutations were identified in 28% of our patients (16 out of 57). There was a total of 17 mutations identified in 16 patients and these are shown in Figure 3.32 below.

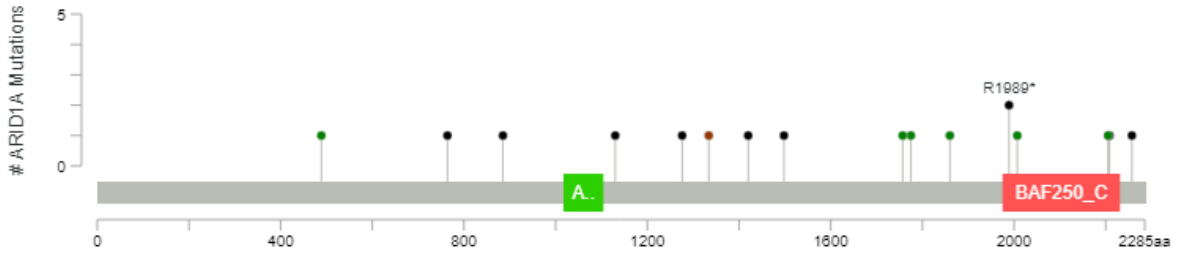


Figure 3.32 *ARID1A* mutations identified in our cohort and their localization on the gene's exones. Lollipop graph created using MutationMapper tool (Cerami *et al.*, 2012; J. Gao *et al.*, 2013). Colour code: Green: Missense mutations. Black: Truncating mutations. Brown: Inframe mutations. Purple: Other.

As seen in Figure 3.32, *ARID1A* mutations are scattered across the exons and no 'hot-spot' regions were identified.

There is a strong trend across the cohort showing co-existence of *ARID1A* with PI3K pathway mutations (as defined by mutation in one or more of the three genes belonging to this pathway, *PIK3CA*, *PIK3R1* and *PTEN*). 81% (13/16) of patients carrying *ARID1A* mutations had a co-existent PI3K mutation compared to 51% (21/41) for wild-type (wt) *ARID1A* tumours (p=0.069). This strong (but not significant) trend reached statistical significance in the BW cohort as 90% (9/10) of *ARID1A* mutant tumours had a synchronous PI3K mutation compared to only 50% (10/20) for the wt-*ARID1A* tumours (p=0.0485). There was no such trend in the BSA cohort (although the numbers were smaller) as 67% (4/6) of patient carrying an *ARID1A* mutation had a co-existent PI3K mutation compared to 52% (11/21) for the wt-*ARID1A* patients (p=0.66).

This positive association between *ARID1A* and PI3K mutations in the BW group is reflected in the co-existence of *ARID1A* and *PIK3CA* mutations in this group with 60%

(6/10) of *ARID1A*-mutant tumours having a concurrent *PIK3CA* mutation compared to 30% (6/20) of the wt-*ARID1A* tumours (p=0.14).

There was a significant association between *ARID1A* and *POLE* mutations across the cohort as 25% (4/16) of *ARID1A*-mutant tumours were also carrying a *POLE* mutation, compared to only 5% (2/41) for wt-*ARID1A* tumours (p=0.046). In subgroup analysis, the trend was similar in both groups but not significant as the numbers were small. 30% (3/10) of BW women carrying an *ARID1A* mutation had a concurrent *POLE* mutation versus only 5% (1/20) for the wt-*ARID1A* tumours (p=0.095). Similarly in BSA patients, 17% (1/6) had co-existent mutations in these two genes compared to only 5% (1/21) of wt-*ARID1A* patients carrying a *POLE* mutation (p=0.4).

There was no association between *ARID1A* mutations and the risk group of endometrial cancer as 34% of low risk patients (10/29) were carrying *ARID1A* mutations versus 21% (6/28) of high risk patients (p=0.38). Diabetes also has no association with the *ARID1A* mutational status as 22% of diabetic patients carry *ARID1A* mutations compared to 31% for the non-diabetic patients (p=0.75). There was no difference in the age at diagnosis between patients carrying *ARID1A* mutations (mean age 63 years) and those having a wt-*ARID1A* (61.2 years, p=0.58).

3.2.2.2 *CTNNB1*

CTNNB1 mutations are present in 21% of the patients tested (12/57). They were more prevalent among BSA women as 30% of them (8/19) were carrying a mutation as opposed to only 13% (4/30) of BW patients (p=0.19). There were a total of 13 mutations identified in 12 patients and these are shown in Figure 3.33.

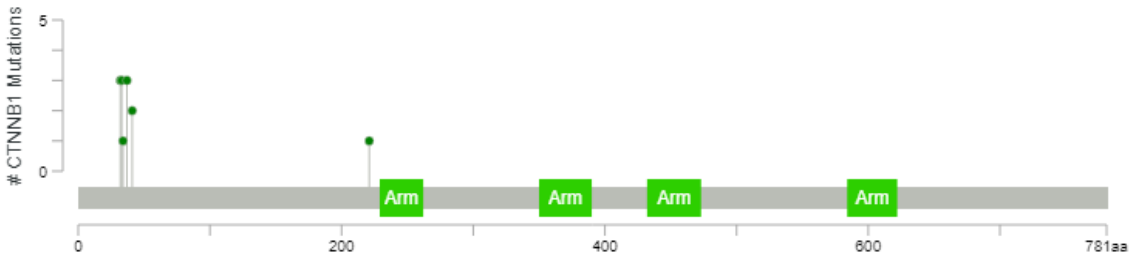


Figure 3.33 Frequency and location of *CTNNB1* mutations identified in our cohort. Lollipop graph created using MutationMapper tool. Colour code: Green: Missense mutations.

All *CTNNB1* mutations were missense. 12 out of 13 mutations (92%) were located in a 'hot-spot' area of exon 3, from codon 32 to 41. Two thirds of these alterations (67%, 8/12), affected Serine or Threonine residues (S33, S37 and T41).

Patients carrying *CTNNB1* mutations were younger compared to those having a wt-*CTNNB1* (58.2 versus 62.7 years, p=0.2) but this trend was not significant. In subgroup analysis though, the trend was even stronger among BSA women as those with *CTNNB1* mutation in their tumours were eight years younger on average (55.6 versus 63.5 years, p=0.077). This trend was not seen in the BW cohort (and if anything it was reversed, 63.3 years for patients carrying *CTNNB1* mutation versus 62.1, p=0.85).

Across the entire cohort, there was no significant association between *CTNNB1* mutations and any other gene mutation. In the BSA group though there was a strong, non-significant trend showing positive correlation between *CTNNB1* and *POLE* mutations as 25% (2/8) of *CTNNB1*-mutant tumours had a co-existent *POLE* mutation versus 0% (0/19) of wt-*CTNNB1* patients (p=0.0798). No such trend was shown in the BW cohort.

There was no association between *CTNNB1* mutations and the risk group of EC as the 12 patients with such mutations were evenly distributed between low and high risk cases. Diabetic status also had no correlation with the presence of *CTNNB1* mutations.

3.2.2.3 *FBXW7*

FBXW7 mutations were present in 23% of our patients (13/57). A total of 14 mutations were identified and are shown in Figure 3.34 below. 93% of these mutations (13/14) were missense.

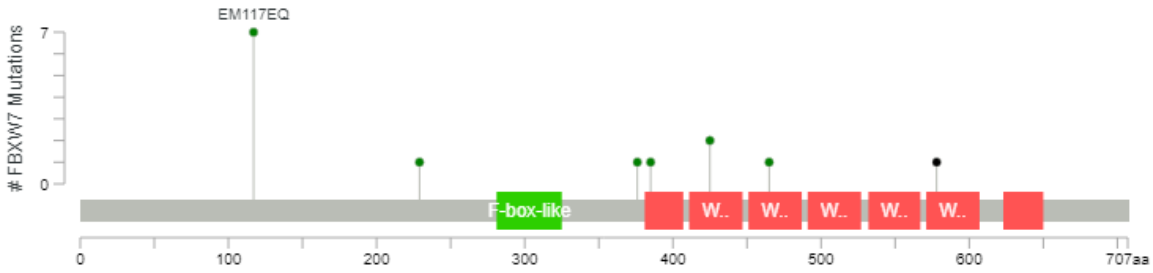


Figure 3.34 *FBXW7* mutations identified in our cohort and their localization and frequency on the gene's exones. Lollipop graph created using MutationMapper tool. Colour code: Green: Missense mutations. Black: Nonsense (truncating) mutation.

There was no difference between the groups of interest with 20% (6/30) of BW and 26% (7/27) of BSA carrying such a mutation (p=0.75).

No association was found between age at diagnosis and *FBXW7* mutations across the cohort (mean age 60 years for patients carrying mutation versus 62.3 for wt-*FBXW7*, p=0.51). Subgroup analysis yielded similar results for BW women (62.7 years for carriers of *FBXW7* mutation, 62.2 for non-carriers, p=0.92) and a non-significant trend towards younger age at diagnosis for BSA patients carrying an *FBXW7* mutation (mean age 57.7 years versus 62.4 years for wt-*FBXW7*, p=0.33).

There was a strong but not-significant trend positively associating *FBXW7* and *POLE* mutations in the BSA but not in the BW cohort. 29% (2/7) of BSA women carrying an *FBXW7* mutation had a concurrent *POLE* mutation compared to 0% (0/20) of those having a wt-*FBXW7* (p=0.0598). There was no such trend in the BW women as none of the 6 women carrying an *FBXW7* mutation had a coexistent *POLE* mutation [compared to 17% (4/24) for the wt-*FBXW7*, p=0.56].

Another strong (but again not significant) correlation identified in the BSA cohort was the coexistence of *FBXW7* with *PIK3R1* mutations as 43% (3/7) of *FBXW7*-mutant tumours had a concurrent *PIK3R1* mutation compared to only 10% (2/20) of wt-*FBXW7* tumours (p=0.09).

There was no association between the *FBXW7* mutational status and the risk group of endometrial cancer although *FBXW7* mutations where slightly more prevalent in low risk cases (28% versus 18% in the high risk group, p=0.53). No correlation between diabetes and *FBXW7* mutations was shown as the prevalence of these mutations was identical in diabetic and non-diabetic patients (22% and 23% respectively, p=1).

3.2.2.4 *KRAS*

KRAS mutations were identified in 9% of our patients (5/57). There was a total of 6 *KRAS* mutations and their localization is shown in Figure 3.35 below. All of them were missense and half of them resulted from a substitution of glutamine to histidine (Q61H), a mutation mainly reported in colorectal malignancies (Vaughn *et al.*, 2011).

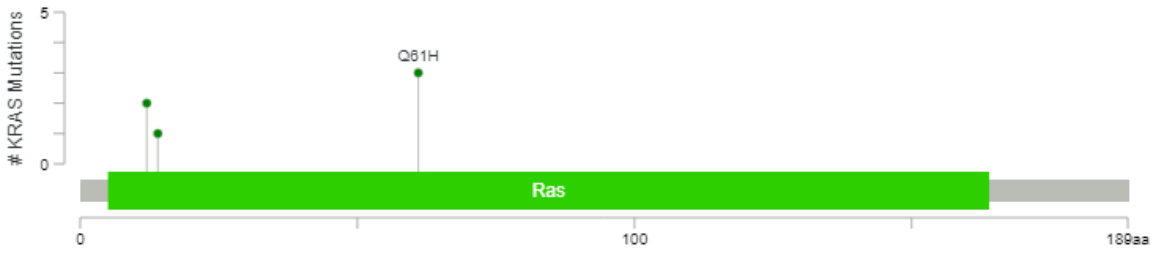


Figure 3.35 *KRAS* mutations identified in our cohort and their localization on the gene's exones. Lollipop graph created using MutationMapper tool. Colour code: Green: Missense mutations.

All four BW patients carrying *KRAS* mutations in their tumours had concurrent PI3K pathway mutations versus only 38% (10/26) of wt-*KRAS* patients (p=0.0365). As there was only 1 BSA patient with a *KRAS* mutation, analysis could not be performed for this group. No other association between *KRAS* and any other mutation was elicited from our analysis.

There was no association between *KRAS* mutation and age at diagnosis as the mean age of patients harbouring *KRAS* mutations was 64.2 years compared to 61.5 for wt-*KRAS* tumours (p=0.6). Similar results were shown within the BW cohort (63.3 years for *KRAS*-mutant tumours versus 62.1 for wt-*KRAS*, p=0.85). Again, there was no correlation between diabetes and the presence of *KRAS* mutations (10% prevalence in the non-diabetic cohort compared to 6% in the diabetic, p=1).

No correlation was found between *KRAS* mutations and the risk group of endometrial cancer. They were present in 7% (2/29) of low risk cases compared to 11% (3/25) of high-risk cases (p=0.67).

3.2.2.5 *PIK3CA*

PIK3CA was the second most frequent mutation across our cohort with 37% of patients harbouring one (21/57). There was a total of 30 non-synonymous mutations in these 21 patients ranging from one to four mutations per patient. There were seven patients with more than one *PIK3CA* mutations and frequently (three out of seven cases) this was associated with a concurrent *POLE* mutation.

The localization of these mutations is shown in Figure 3.36 below. All 30 mutations were missense with 33% of them (10/30) being located in the p85 binding domain. Out of these, 40% (4/10) were a gain-of-function R88Q mutation which represents 19% of *PIK3CA* mutant tumours (4/21). Interestingly, all these four mutations were identified in BW patients.

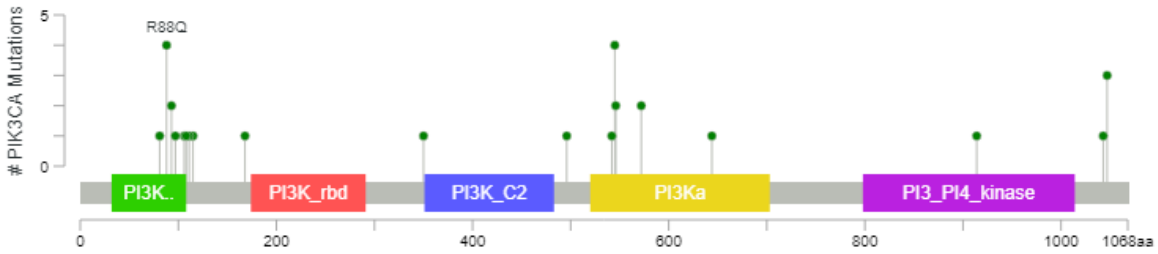


Figure 3.36 *PIK3CA* mutations identified in our cohort and their localization on the gene's exones. Lollipop graph created using MutationMapper tool. Colour code: Green: Missense mutations.

Another common mutation was the E545 in the helical domain with 19% of *PIK3CA* mutant tumours (4/21) carrying such a mutation.

Across the cohort, there was a positive association between *PIK3CA* and *PTEN* mutations. 62% (13/21) of patients harbouring *PIK3CA* mutations had co-existent *PTEN* mutations compared to only 25% (9/36) for wt-*PIK3CA* patients (p=0.01). Surprisingly, this correlation was not confirmed in both groups. It was very strong for the BSA group with 78% (7/9) of *PIK3CA*-mutant tumours also carrying a *PTEN* mutation versus only 11% (2/18) for the wt-*PIK3CA* cases (p=0.0012). On the contrary, no such association was confirmed in the BW group as 50% (6/12) of *PIK3CA*-mutant tumours had concurrent *PTEN* mutations compared to 39% (7/18) of the wt-*PIK3CA* ones (p=0.71).

There was no difference in the rate of these mutations among the two groups of interest (40% of BW compared to 33% for BSA patients, p=0.78).

PIK3CA mutations were more prevalent in high risk endometrial cancer cases with 46% of these cases (13/28) carrying such a mutation compared to 28% (8/29) of low risk tumours but this trend was not significant (p=0.17).

There was no association between such mutations and the presence of diabetes across the cohort as 36% of non-diabetic patients carried a *PIK3CA* mutation compared to 39% of diabetic ones (p=1). Subgroup analysis though has shown the existence of a non-significant trend within the BSA group as *PIK3CA* mutations were more frequent in diabetic patients (50% compared to 20% for non-diabetic patients, p=0.13). If anything,

the trend was the opposite in BW patients (although not as strong) with 46% of non-diabetic patients (11/24) carrying a *PIK3CA* mutation compared to 17% (1/6) for the diabetic ones (p=0.36).

Another interesting finding was the fact that patients harbouring *PIK3CA* mutations in their tumours were significantly older at diagnosis with a mean age of 65.9 years across the cohort compared to 59.3 for wt-*PIK3CA* patients (p=0.0245). This was even more significant in BW patients with a mean age of *PIK3CA*-mutant patients 68.7 years compared to 58 for patients having a wt-*PIK3CA* (p=0.0077). There was no such difference in the BSA group (mean age 62.2 years for *PIK3CA*-mutant patients versus 60.6 for wt-*PIK3CA*, p=0.72).

3.2.2.6 *PIK3R1*

PIK3R1 mutations were identified in 12% of patients (7/57). There was a total of ten *PIK3R1* non-synonymous mutations identified in these seven patients (as three patients had two mutations each. Two out of these three patients had a concurrent *POLE* mutation). The localization of these mutations is shown in Figure 3.37 below.

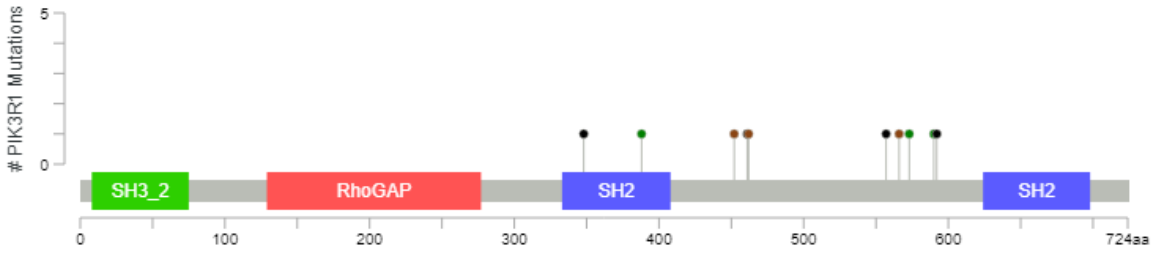


Figure 3.37 *PIK3R1* mutations identified in our cohort and their localization on the gene's exones. Lollipop graph created using MutationMapper tool. Colour code: Green: Missense mutations. Brown: Inframe deletions. Black: Truncating mutations (either stop-gain or frameshift variant).

As shown in Figure 3.37, *PIK3R1* mutations were scattered across the gene with no 'hot-spot' areas.

PIK3R1 mutations were more common in BSA women (19%, 5/27 compared to 7% for BW patients, 2/30) but this was not significant (p=0.24).

A significant correlation between *PIK3R1* and *POLE* mutations was seen across our cohort. 43% (3/7) of *PIK3R1*-mutant patients had co-existent *POLE* mutations versus 6% (3/50) for wt-*PIK3R1* patients (p=0.02). This was also significant in the BSA group where 40% (2/5) of *PIK3R1*-mutant patients had concurrent *POLE* mutations compared to 0% (0/22) for wt-*PIK3R1* patients (p=0.028). Due to the fact that there were only two patients in the BW cohort carrying *PIK3R1* mutation, significance was not reached in this group although the trend was similar.

As mentioned previously, there was also a non-significant (p=0.09) association between *PIK3R1* and *FBXW7* mutations in the BSA cohort with 60% of *PIK3R1*-mutant tumours (3/5) having a co-existent *FBXW7* mutation compared to 18% (4/22) for the wt-*PIK3R1* patients.

Interestingly, *PIK3R1* mutations were negatively associated with *PTEN* mutations in the BSA cohort as none of the five patients harboring such a mutation had a co-existent *PTEN* mutation (compared to 41% of the wt-*PIK3R1* patients, p=0.13) and although this was not significant, it is in contrast with the two BW patients carrying somatic *PIK3R1* mutations, as both of them had a co-existent *PTEN* mutation.

Patients carrying *PIK3R1* mutations were younger at diagnosis in our cohort (mean age 55.6 years at diagnosis compared to 62.6 years for wt-*PIK3R1* patients), but this trend did not reach significance (p=0.11). The same trend was shown in the BSA group (55.8 years for patients carrying *PIK3R1* mutations versus 62.4 years for the rest of the BSA group), but due to small numbers, significance was not reached.

There was no association between *PIK3R1* mutations and the risk status of endometrial cancer as these were evenly split between high and low risk cases.

3.2.2.7 *POLE*

Six *POLE* mutations were identified in the patients we tested (10.5% incidence rate across the whole cohort). All of them were missense mutations with most of them (4/6, 67%) in the exonuclease domain of the *POLE* gene (Figure 3.38). The commonest mutation was a P286R, a mutation recently reported to be important driver of carcinogenesis in colorectal malignancies (Ahn *et al.*, 2016).

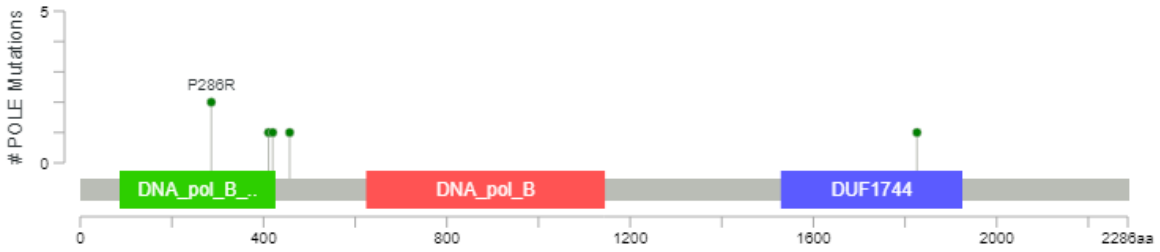


Figure 3.38 *POLE* mutations identified in our cohort and their localization on the gene's exones. Lollipop graph created using MutationMapper tool. Colour code: Green: Missense mutations.

There was no difference between the two studied groups in the incidence of *POLE* mutations (4/30, 13% for BW compared to 2/27, 7% for BSA patients, p=0.67).

Patients carrying somatic *POLE* mutations (n=6) had many co-existent mutations in the other nine genes tested. These patients had each an average of 6.2 non-synonymous mutations in the ten genes tested compared to only 1.8 for the remaining 51 patients who were carrying a wt-*POLE* gene (p<0.0001).

POLE-mutant patients are younger at diagnosis (mean age 57.2 years compared to 62.3 years for wt-*POLE* patients) but this was not significant (p=0.28).

POLE mutations were not associated with the risk status of endometrial cancer as the six cases were evenly distributed in the low and high risk group.

There was an interesting (although not significant) trend as all six mutations were identified in non-diabetic patients (6/39), with no mutations seen in any of the 18 diabetic patients of our cohort (p=0.16).

As mentioned previously, *POLE* mutations are significantly associated with *ARID1A* and *PIK3R1* mutations across our cohort. The former association was more prominent in the BW group (although not significant, p=0.095) and the latter in the BSA group (p=0.028).

3.2.2.8 *PPP2R1A*

There was only one *PPP2R1A* mutation identified in our cohort (incidence rate 1.8%) and its localization on the gene is shown in Figure 3.39 below. This mutation was in a 'hot-spot' area (R182-183) which has been shown to be mutated also in ovarian clear cell carcinoma (Siân Jones *et al.*, 2010), apart from endometrial cancer (Haesen *et al.*, 2016).

Figure 3.39 *PPP2R1A* mutation identified in our cohort and its localization on the gene's exones. Lollipop graph created using MutationMapper tool. Colour code: Green: Missense mutations.

This mutation was identified in a BSA patient with high risk endometrial cancer (stage 2, grade 2, endometrioid histology). This patient was also carrying an *ARID1A* mutation.

3.2.2.9 *PTEN*

PTEN was the most frequent genetic alteration identified in our cohort with 39% of patients (22/57) carrying at least one mutation in the gene. 18% of these patients (4/22) had two mutations on this gene bringing the total number of *PTEN* mutations identified to a total of 26. The localization of these mutations across the gene is shown in Figure 3.40 below.

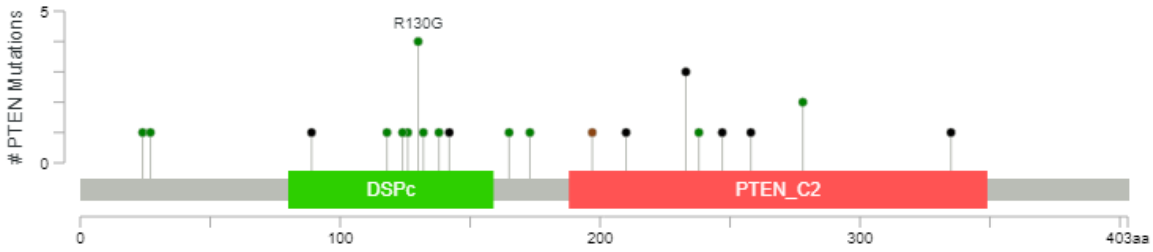


Figure 3.40 *PTEN* mutations identified in our cohort and its localization on the gene's exones. Lollipop graph created using MutationMapper tool. Colour code: Green: Missense mutations. Black: Truncating. Brown: Inframe deletion.

More than 60% (16/26, 62%) of these mutations were missense with most of the remaining (9/26, 35%) being truncating mutations, either frameshift (5/26, 19%) or nonsense mutations (stop-gain, 4/26, 15%). Finally, there was one inframe deletion (1/26, 4%). The mutations are evenly distributed between the dual specificity phosphatase catalytic domain (DSPc in Figure 3.40) and the C2 domain. These two areas have eleven mutations each (11/26, 42%) with only four mutations (4/26, 15%) lying outside these domains.

The most common mutation identified in 15% (4/26) of the *PTEN*-mutant tumours of our cohort was the substitution of arginine from glycine in position 130 of the *PTEN* gene (R130G). Interestingly, all these four mutations were identified in BW women. Another common mutation was the introduction of a stop codon in position 233 of the *PTEN* gene (R233*). There were three patients with such mutation across our cohort, and all of them were BSA.

PTEN mutations are slightly more prevalent among BW women as 43% of them carry one (13/30) compared to 33% incidence among BSA patients (9/27), but this was not significant (p=0.59). The localization of these mutations in various domains of the gene in each group are shown in Table 3.8.

***PTEN* domain**	**BW (%)**	**BSA (%)**	**p value**
Phosphatase catalytic	8 (53)	3 (27)	0.168
C2	4 (27)	7 (64)	
Other	3 (20)	1 (9)	

Table 3.8 Number of non-synonymous *PTEN* mutations identified in the two groups of interest and their localization on the gene's domains. p value calculated using X^2 test.

As shown in Table 3.8, BW women are more likely to have *PTEN* mutations in the phosphatase catalytic domain compared to their BSA counterparts which, in turn, are more likely to have mutations in the C2 domain of the gene. This trend did not reach significance possibly due to the small numbers of patients studied.

Although *PTEN* mutations are more prevalent in low risk cases (45%, 13/29 compared to 32%, 9/28 for high risk cases), this was not significant (p=0.42). There was also no significant association with diabetes despite the fact there was a trend towards more *PTEN* mutations among diabetic patients (50%, 9/18, compared to 33%, 13/39, for non-diabetic patients, p=0.25). Subgroup analysis revealed similar results with no association between *PTEN* mutation, race and diabetes or risk group of endometrial cancer.

As mentioned before, *PTEN* mutations significantly co-exist with *PIK3CA* mutations as 59% (13/22) of *PTEN*-mutant tumours have concurrent *PIK3CA* mutations compared to 23% (8/35) for wt-*PTEN* tumours (p=0.01). This correlation was mainly influenced by the BSA cohort in which the association was even stronger (p=0.001) and was not observed in the BW group.

There was no association between *PTEN* mutations and age at diagnosis (60.7 years for mutated tumours versus 62.4 years for wt-*PTEN*, p=0.58). In subgroup analysis, BW patients carrying a *PTEN* mutation tended to be younger (59.2 years compared to 64.6 years for their 'wild-type' counterparts) but this trend was not significant (p=0.19). On the contrary, BSA patients with *PTEN* mutations were slightly older (62.9 years compared to 60.3 years for wt-*PTEN*) but this was not significant (p=0.55).

3.2.2.10 *TP53*

The incidence rate of *TP53* mutations in our cohort was 11% (6/57). All six mutations were missense and their site on the gene is shown in Figure 3.41 below. All these mutations are localized in the DNA binding domain.

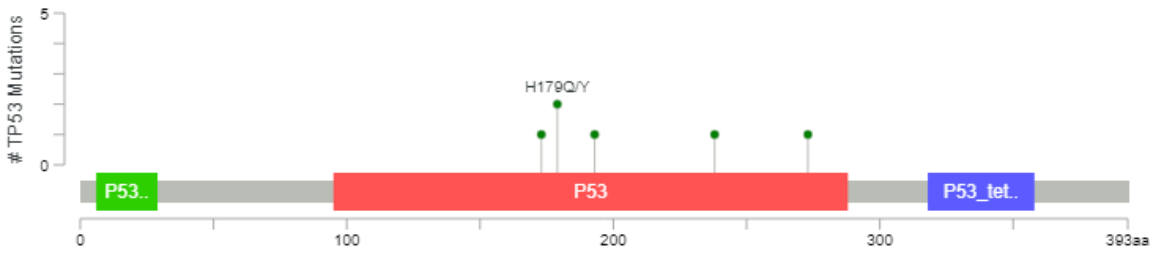


Figure 3.41 Site and frequency of *TP53* mutations identified in our cohort. Lollipop graph created using MutationMapper tool. Colour code: Green: Missense mutations.

The most frequent *TP53* mutation, identified in two patients, was the replacement of histidine from glutamine or tyrosine in position 179 (H179Q and H179Y). This position is part of the zinc coordination area and is important for the correct folding of the DNA binding domain (Olivier *et al.*, 2010). Both of them were in patients with a serous component on the histology of their tumours, older (70 and 77 years old) and with no other mutation in the other nine genes tested which likely means that this was the driver mutation of their endometrial cancer.

There was no difference in the incidence of *TP53* mutations between the two studied groups as this was identical (10%, 3/30 for BW and 11%, 3/27 for BSA women, p=1).

As expected, patients carrying somatic *TP53* mutations were more commonly high risk (18%, 5/28 compared to 3%, 1/29 for the low risk group) but due to the small number of cases studied this was not significant (p=0.10).

There was no association between *TP53* mutations and diabetes although five out of six mutations were found in non-diabetic patients (5/39, 13%), compared to one (1/18, 6%) which was found on a diabetic patient (p=0.65).

Patients with somatic *TP53* mutations were significantly older at diagnosis with a mean age of 72.5 years compared to 60.5 years for wt-*TP53* patients (p=0.008).

No significant correlation was shown between *TP53* and any other mutation in the other nine genes tested.

3.2.3 Mismatch Repair Deficiency analysis

As shown previously in Table 3.3, BSA patients with endometrial cancer (EC) were significantly younger at diagnosis compared to BW. The incidence of mutations in ten genes commonly mutated in EC was not different. We subsequently performed immunohistochemistry (IHC) on tumour samples obtained from the 57 patients of our cohort in order to check the presence of mismatch repair deficiency and any potential differences between the two groups. Antibodies for four of the most frequently mutated genes were investigated (*MSH2, MSH6, MLH1* and *PMS2*) using the protocol described previously in the 'Methods'. The results of the testing are presented in Table 3.9 below.

	MMR proficient	**MMR deficient**	**p value**
n	43	14	
Race (%)			0.54
BW	24 (56%)	6 (43%)	
BSA	19 (44%)	8 (57%)	
Mean age	59.8	67.8	0.014
Mean BMI	36.5	31.5	0.066
Diabetes status			0.51
Diabetic	15 (35%)	3 (21%)	
Non-diabetic	28 (65%)	11 (79%)	
Risk group (%)			0.55
Low	23 (53%)	6 (43%)	
Intermediate-High	20 (47%)	8 (57%)	
Grade (%)			0.16
1	27 (63%)	6 (43%)	
2	10 (23%)	7 (50%)	
3	6 (14%)	1 (7%)	

Table 3.9 MMR status in association with clinicopathological data. MMR proficient is defined as a tissue expressing all four proteins (MSH6, MSH2, MLH1 and PMS2), while MMR deficient a tissue lacking expression of at least one of the previously mentioned proteins. P values were calculated using Fisher's exact test (race, diabetes and risk group), chi-square test (grade) and Student's t-test (age and BMI).

The incidence rate of MMR deficiency in our cohort was 25% (14/57). There was no significant difference between the two ethnic groups of interest as BSA women had an incidence rate of 30% (8/27) versus 20% (6/30) for the BW group (p=0.54).

Interestingly, across the entire cohort, MMR deficient patients were significantly older at diagnosis (mean age 67.8 years compared to 59.8 years for MMR proficient patients, p=0.014). This was driven by a significant difference within the BW group in which MMR deficient patients had a mean age of 72.8 years as opposed to 59.6 years for MMR proficient patients (p=0.007). The trend was similar but less prominent in the BSA cohort (mean age 64 years for MMR deficient tumours compared to 60 years for MMR proficient) and hence did not reach significance (p=0.37).

Patients who had MMR deficient tumours across the cohort tended to have lower BMI (mean BMI 31.5 compared to 36.5 for MMR proficient patients), approaching significance (p=0.066). The same trend was noticed in both BW and BSA women. In the BW cohort, BMI was 38.8 for MMR proficient and 33.7 for deficient patients (p=0.28). In the BSA group, 33.7 for proficient and 29.85 for deficient patients (p=0.15). Since this finding was seen in both groups it suggests that this is a true finding although the results did not reach significance.

There was no association between diabetes and MMR deficiency as 17% (3/18) of diabetic patients were MMR deficient compared to 28% (11/39) of non-diabetic (p=0.51). Subgroup analysis revealed similar results with no statistical significance.

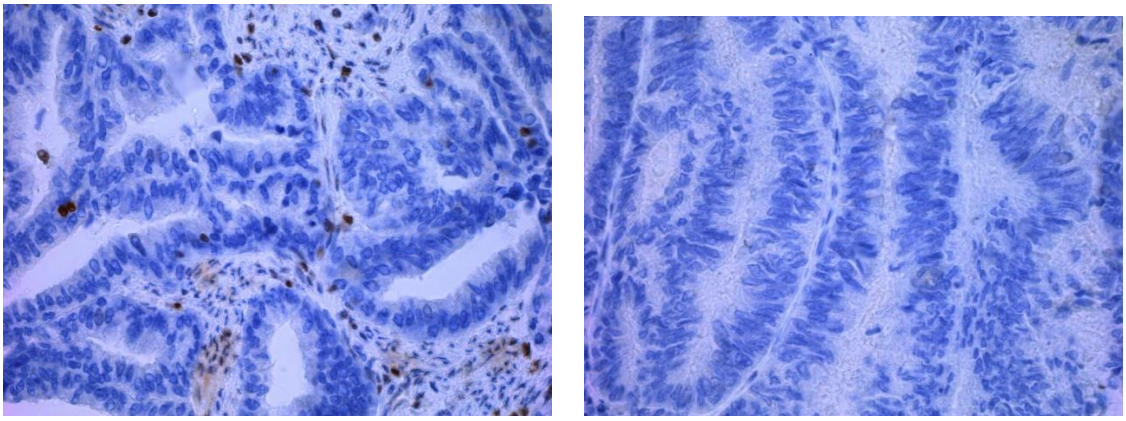
There was also no correlation between the risk and MMR status of the endometrial cancer cases studied. 21% (6/29) of low risk cases and 29% (8/28) of high risk cases were MMR deficient (p=0.55). In the BW 33% (5/15) of high risk cases were MMR deficient compared to only 7% (1/15) for the low risk patients (p=0.17). In contrast, in the BSA cohort most MMR deficient cases were identified in the low risk group (5/14, 36% compared to 3/13, 23% for the high risk group, p=0.68).

MMR deficient cases were more likely to be of higher grade, 57% (8/14) were moderately or poorly differentiated (grade 2-3) as compared to 37% (16/43) for the MMR proficient cohort but this trend was not significant (p=0.16).

The type of MMR deficiencies identified in the cohort is shown in Table 3.10 below. Almost 80% of these deficiencies (11/14, 79%) involved the *MLH1*/*PMS2* genes (Figure 3.42), two (14%) were *MSH6* deficiencies (Figure 3.43) and one (7%) was a deficiency of *MSH2*/*MSH6*.

MMR deficiency	**BW** n (%)	**BSA** n (%)	**p value**
MLH1*/*PMS2	4 (67)	7 (88)	0.54
***MSH2*/*MSH6* or *MSH6* alone**	2 (33)	1 (12)	

Table 3.10 Type of MMR gene deficiencies identified in our cohort. P value calculated using Fisher's exact test.



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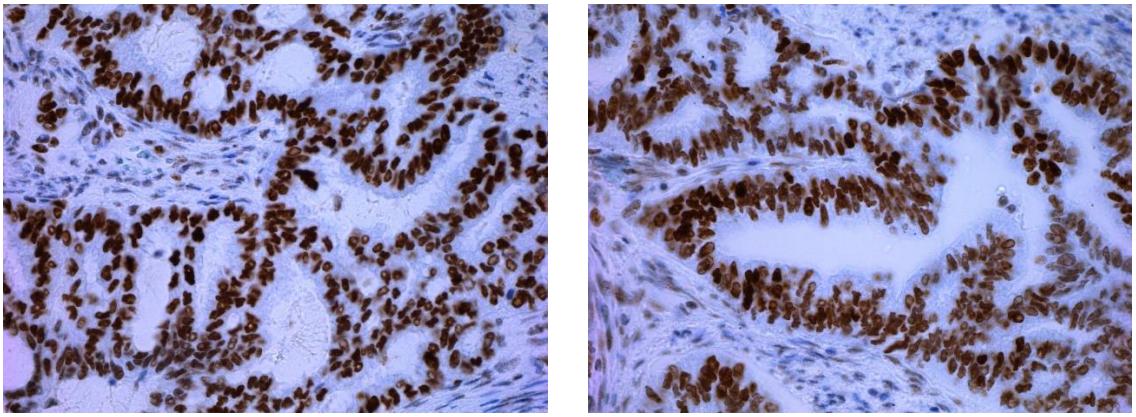

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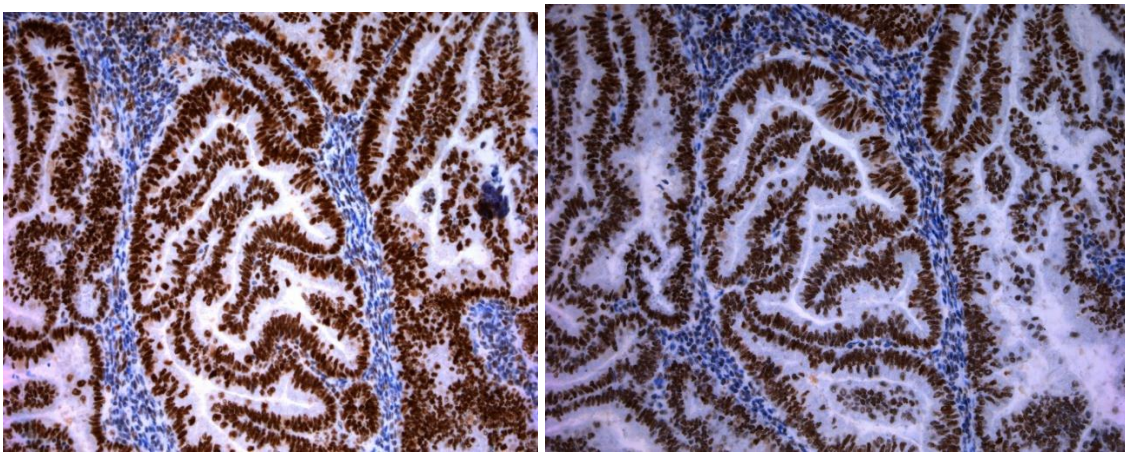
Figure 3.42 84 year old BW patient with stage 2, grade 2 endometrioid endometrial cancer. MMR deficient (MLH1/PMS2 negative). a) MLH1 negatively stained tumour (x40 magnification). b) PMS2 negatively stained tumour. c) MSH2 positively stained tumour. d) MSH6 positively stained tumour.



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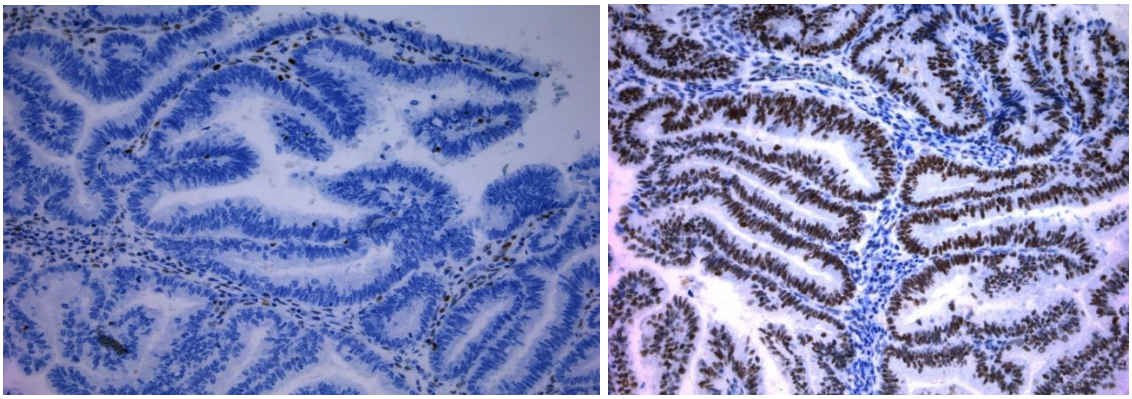

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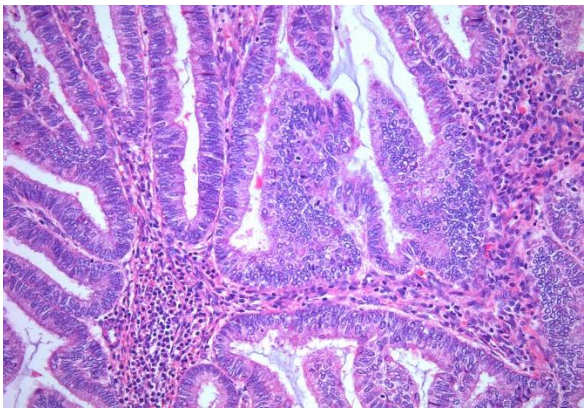

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Figure 3.43 76 year old BW patient with stage 1B, grade 2 endometrioid endometrial cancer. MMR deficient (MSH6 negative). a) MLH1 stained tumour (x20 magnification). b) MSH2 stained tumour (x20). c) MSH6 negatively stained tumour (x20). d) PMS2 stained tumour (x20). e) Haematoxylin and Eosin stained tissue from the same patient.

In the BSA cohort, there is a strong negative association between MMR deficient patients and *CTNNB1* mutations, raising the possibility of mutual exclusivity. In this group, none of the patients carrying a somatic *CTNNB1* mutation was MMR deficient (0/8, 0%). This was in contrast to 42% (8/19) of the wt-*CTNNB1* patients who was MMR deficient (p=0.06). Interestingly, there was no such trend among BW women as 25% (1/4) of patients with a somatic *CTNNB1* mutation was MMR deficient compared to 19% (5/26) of wt-*CTNNB1* patients (p=1).

A similar negative correlation was identified in the BSA cohort between MMR deficiency and *FBXW7* mutations. None of the seven patients carrying a somatic *FBXW7* mutation was MMR deficient (0/7, 0%) as opposed to 40% (8/20) rate of MMR deficiency for the wt-*FBXW7* women. This trend, although strong, was not significant (p=0.068). Again, no such negative association was seen in BW women as 17% (1/6) of *FBXW7*-mutant patients were MMR deficient compared to 21% (5/24) of wt-*FBXW7* women (p=1).

No other significant association was shown between MMR status and genetic alteration in any of the other eight genes tested. There was also no correlation between PI3K pathway mutations and MMR status.

Chapter 4 Discussion

4.1 Endometrial cancer: A neglected disease

Endometrial cancer is the commonest gynaecological cancer in the Western world. It is usually diagnosed at an early stage and its surgical management is associated with very good prognosis. Nevertheless, extrauterine disease (stage 3 or above) is associated with poor outcome, not greatly different to ovarian cancer, the disease frequently known as the 'silent killer' of women. And although it is true that the number of deaths from ovarian cancer in the UK is 80% more compared to endometrial cancer (4,227 versus 2,360 for 2016) (Cancer Research UK. 2019), the clinical trials recruiting patients with advanced endometrial cancer are disproportionately low. We are proposing the 'Research Fairness Ratio (RFR)' as a marker of this imbalance where disease impact is the annual- number of deaths from the disease in the UK.

$$\text{Research Fairness Ratio}=\frac{Disease\ impact\ (annual\ mortality)}{Recruiting\ clinical\ trials\ worldwide}$$

The lower the numerical value of RFR, the greater the number of active, recruiting clinical trials and hence extensive research on the field. The RFR for some of the common cancers are shown in Table 4.1 below. These numbers clearly show how poorly investigated EC is compared to other common cancers and justify efforts to propose new targeted treatments.

Type of cancer	Mortality UK	Recruiting clinical trials	RFR
Breast	11,563	1,781	6.5
Ovary	4,227	511	8.3
Cervix	854	513	1.7
Endometrium	2,360	157	15
Lung	35,620	1,487	24
Colon	16,384	835	19.6

Table 4.1 Common cancers affecting women in the UK. Data for mortality are for 2016 and they represent deaths from disease (Cancer Research UK. 2019). Recruiting clinical trials worldwide (209 countries). Data obtained April 2019 (U.S. National Library of Medicine, 2019). RFR: Research Fairness Ratio.

4.2 PI3K pathway: The main battlefield

No other cancer has more PI3K mutations than endometrial cancer (TCGA, 2013). It is unsurprising that most novel treatments for recurrent or advanced EC with targeted therapies focus on this pathway. Several PI3K and/or mTOR inhibitors have been tested in vitro with encouraging results however, unfortunately most of them failed to show efficacy when used in clinical trials as monotherapies. In view of this extensive testing in PI3K and mTOR inhibitors, we adopted a screening strategy with only 3 out of the 24 drugs tested inhibiting the PI3K pathway (Idelalisib, CUDC-907 and Quercetin, the former two had never been tested in EC). The other inhibitory drugs had different mode of action as I was hoping to discover alternative ways of inhibiting EC cell growth.

However, we can conclude that the most interesting drug whose action we investigated was CUDC-907, a PI3K and HDAC inhibitor. This was the only monotherapy treatment which was extremely effective in reducing cell viability in both cell lines, with IC50 values of 27nM for Ishikawa and 58nM for HEC-1-A cells. Given the fact that it has not previously been tested in EC but also has been proven to have tolerable toxicity profile as it is currently under investigation in a phase 2 clinical trial (on diffuse B-cell lymphoma)

(U.S. National Library of Medicine, 2019), I propose it as an attractive therapeutic option for further evaluation in endometrial cancer in vivo models.

I have shown that CUDC-907, not only suppresses activation of the PI3K pathway, but exerts its action through suppression of the MAPK pathway as well (Figure 3.11). This is in agreement with similar findings in NSCLC, breast, melanoma and, in a study of our lab, CLL cell lines (Qian *et al.*, 2012; Y. Chen *et al.*, 2019). The same study (Qian et al.) concluded that histone deacetylase inhibition was responsible for the effect of CUDC-907 on the MEK-ERK pathway. Interestingly, in this study, CUDC-907 was tested on 29 solid cancers cell lines and the IC50 values obtained are remarkably similar to the results of my study.

Another recent study has shown that treating *KRAS* mutant EC cells with Apitolisib (dual PI3K and mTOR inhibitor), although it achieves suppression of phosphorylated AKT, it increases MEK and MAPK phosphorylation (Aslan *et al.*, 2018). My results showed a superior effect when combining PI3K and HDAC inhibition as this combination (CUDC) suppresses both pathways (PI3K and MAPK), even on *KRAS* mutant cells (HEC-1-A).

4.3 MAPK pathway

This was the most extensively tested pathway in my project, with a third of the drugs used inhibiting molecules of this pathway. The rationale was based on the fact that this pathway is commonly involved in many cancers (Dhillon *et al.*, 2007). None of the drugs, however, proved effective when used alone. The most interesting member of this group is PD0325901, which showed mild activity in HEC-1-A cells (IC50 value of 9.33μM). Although this concentration is high for clinical purposes, the importance of the drug lies in its potential to be used in combination treatments, especially when taking into account that it has been shown to have a favourable toxicity profile. This was shown from its use in a phase 1 clinical trial in combination with a PI3K/mTOR inhibitor (Gedatolisib) (Wainberg *et al.*, 2017).

An interesting finding was the paradoxical activation of MEK-ERK pathway when EC cells were treated with MEK inhibitors, as shown by the increased basal levels of MEK

and MAPK phosphorylation in both cell lines, particularly at modest concentrations (0.1μM) (Figure 3.9). This was shown previously in CLL in a study of our lab which proposed as possible explanation an upstream reactivating loop (Y. Chen *et al.*, 2017). This paradoxical activation was more prominent in Ishikawa cells, possibly as a result of the wild type genes controlling their MAPK pathway, which under normal circumstances renders it in relative quiescence compared to their highly active PI3K pathway (see control samples in Figure 3.9). On the contrary, HEC-1-A cells, which carry *KRAS* mutations, have a constitutively more active MAPK pathway, and hence this activation is more subtle and even reversed at treatments with higher concentrations of MEK inhibitors.

The association between *KRAS* mutations and resistance to MEK inhibitors has been studied previously. In colorectal cancer, studies have shown that the stronger the ERK1/2 signalling is, the greater the sensitivity of colorectal cells to treatment with MEK1/2 inhibitors (Balmanno *et al.*, 2009). This is in keeping with my results which confirmed that the *KRAS*-mutant HEC-1-A cells, which have strong MEK1/2 signalling, are more sensitive to MEK inhibitors than the Ishikawa cells (wild type MEK-ERK pathway). Similarly, a study on 13 EC cell lines concluded that *KRAS*-mutant cell lines (including HEC-1-A), were more sensitive to the MEK inhibitor Cobimetinib compared to the *PTEN*-mutant cell lines (Aslan *et al.*, 2018), which again is in keeping with our results.

4.4 Combining treatments

As described in paragraphs 1.2.2 and 1.2.4, the efficacy of targeted treatments as monotherapy in advanced or recurrent endometrial cancer is limited. It becomes obvious then that the way forward in heavily pre-treated and hence highly mutated and aggressive tumours is attack from various angles.

In this study, several combination treatments were tested with some of them proving effective and synergistic.

4.4.1 PI3K and MEK inhibition

Four combinations of CUDC-907 with MEK and Raf inhibitors were tested and the results confirmed that the cell line which lacked mutations in the MEK-ERK pathway (Ishikawa) does not benefit from combined PI3K and MEK inhibition, as these treatments show no synergistic efficacy when used in such cells. This is in keeping with an earlier study combining PI3K/mTOR inhibitor with MEK inhibitor (Pimasertib), which showed antagonism in Ishikawa cells (Inaba et al, 2015). The only exception to this was PD0325901, which showed good synergistic efficacy when combined with CUDC in both cell lines, with CI values of 0.38 and 0.4 respectively. On the contrary, in cells carrying mutations in MEK-ERK pathway (HEC-1-A), the addition of MEK inhibitor (three out of four inhibitors tested) synergistically reduced viability. The notable exception for both cell lines was Sorafenib, which in both cell lines acted antagonistically to CUDC in therapeutically relevant concentrations. These results with Sorafenib are in line with study showing that the drug inhibits EC cell proliferation only on high doses (>5μM) and does not have activity in mouse model of the disease (Mirantes *et al.*, 2016).

The results of my work lead me to propose the combination of CUDC-907 with PD0325901 as the best combination treatment for the suppression of PI3K and MAPK pathways due to its efficacy in both cell lines but also its tolerability, as proven in a phase 1 clinical trial (Wainberg *et al.*, 2017). Overall, the combination of CUDC-907 with Selumetinib and Trametinib should probably be reserved for endometrial tumours harbouring MEK-MAPK pathway mutations.

4.4.2 CDK inhibition

CDK inhibitors have been extensively tested in clinical trials in various cancers, more notably breast, lung, melanoma and lymphoma (Asghar *et al.*, 2015). In this study I examined two of the most prominent members of this family (Dinaciclib and Palbociclib). Dinaciclib was extremely efficacious on Ishikawa but not HEC-1-A cells, while Palbociclib did not reduce cell viability significantly on either of the two cell lines. The Palbociclib result of the current study contradicts two recent, contemporaneous studies. The first one focused on *PTEN*-deficient mouse model and 3D cultures and showed

reduction in tumour volume following treatment with Palbociclib (Dosil *et al.*, 2017). The second one examined the effects of the drug in four EC cell lines and found it effective in two of them, including the HEC-1-A with a reported IC50 value of 0.65μM (T. Tanaka *et al.*, 2017). One might assume that the in vivo effects of a prolonged administration of a drug (15 days in the Dosil et al., study) might be different from its in vitro efficacy, which might potentially explain the results of the first study. As for the second study, the authors assessed cell viability after 72 hours (compared to 48 hours in the current study) and this might have contributed, at least partly, to the great difference observed.

Studies in *KRAS*-mutant colorectal cell lines have shown synergistic efficacy between CDK (Palbociclib) and MEK inhibitors (M. S. Lee *et al.*, 2016). The *KRAS*-mutant cells used in my study (HEC-1-A) confirmed this reported synergism when Dinaciclib was combined to PD0325901 (CI=0.61). This combination (CDK and MEK inhibitors) was antagonistic in the *KRAS*-wild type (Ishikawa) cells, irrespective of the type of MEK inhibitor used. A possible explanation might be that these cells are already very sensitive on CDK inhibition alone and adding MEK inhibitors does not confer any extra therapeutic advantage.

4.4.3 MCL1 inhibition

MCL1 overexpression has been shown in endometrial cancer (Hiroshi Hirata *et al.*, 2010). It is also common in most cancers and this is associated with cancer cell resistance to chemotherapy through apoptotic evasion, while many pathways, including PI3K and MAPK, control its transcription (Hird & Tron, 2019). For these reasons, I tested a novel MCL1 inhibitor (S63845). As monotherapy, it showed good efficacy in Ishikawa and modest efficacy in HEC-1-A cells (IC50 values of 0.6μM and 7.5μM respectively). This efficacy was improved when combined with MEK inhibitor (PD0325901) with exhibited synergism resulting in reducing viability in both cell lines, making this combination an attractive one for further evaluation as it was never tested before in EC.

4.5 Future directions for drug testing

Several treatment modalities, which have been explored in our project, worth further investigation in animal studies first and, if their efficacy is confirmed, in phase 1 clinical trials. Many rodent models of EC have been described and cover a broad spectrum of the biology of endometrial tumours, enabling a variety of experiments with translational interest (Van Nyen *et al.*, 2018). As monotherapy, CUDC-907 should be explored due to its efficacy in both cell lines. Nevertheless, previous experience from several studies exploring PI3K/mTOR inhibitors (described in 1.2.2.2), predict that it is unlikely to achieve significant clinical response rates in these recurrent, advanced cases with just one drug. Hence, it becomes apparent that the emphasis of future work should be on combination treatments, which have the ability to target several pathways of the cancer. In EC, we propose CUDC-907 combined with PD0325901. This combination showed high synergistic activity in both cell lines, while both drugs have been shown to be tolerable in clinical trials, as mentioned earlier. CUDC can be combined with other MEK inhibitors (Selumetinib, Trametinib) but only on patients carrying *KRAS* mutations. Finally, another interesting combination is S63845 (novel MCL1 inhibitor) with PD0325901, although no data are yet available for the safety profile of S63845 as it has not been tested in clinical trials.

4.6 Race: Does it matter in science?

'when we talk about the concept of race, most people believe that they know it when they see it, but arrive at nothing short of confusion when pressed to define it'

Dr Evelyn Higgenbotham- historian

The term race is very controversial in the medical literature. Many prominent scientists and scholars believe that race is a social construct which should not be used in science as genetic heterogeneity does not correlate with people's appearance. Others point out that it perpetuates false beliefs that people are fundamentally different when science has proved that the similarities we share are far more than the differences. How do we then define and compare different ethnic population, which have different cultural, environmental,

financial and social traits? Is the problem just about terminology? And how do we address the issue when studies confirm that, even when matched for everything else, AA women for example have worse prognosis and more aggressive endometrial tumours than White women (see paragraph 1.5)?

It is appropriate to use better terms than race, more inclusive and less politically and socially charged. Ancestry or origin are two of them. But nevertheless, we still need to keep studying people with different origin, culture, environment and behaviour, as all these factors can affect the biology of a disease. Without such studies, we would have never known the higher incidence of melanoma in Caucasian patients, the worse survival in prostate cancer among AA men, the higher rate of triple negative breast tumours among AA women and the higher rate of *BRCA1* mutations in Ashkenazi Jewish patients (Özdemir & Dotto, 2017).

4.7 South Asian women, endometrial cancer and Leicester

Leicester is the UK city with the largest proportion of people self-declaring Asian origin (37.1%), with 31.8% of them being of South Asian origin (Office for National Statistics., 2013). Previous work from our department has shown that British South Asian (BSA) women in Leicester present with endometrial cancer at significantly younger age compared to British White (BW) women, while they have much higher rates of diabetes (Morris et al, 2014). In view of these differences, we decided to investigate matched cohorts of BW and BSA patients in order to see whether genetic differences exist in common genes associated with endometrial carcinogenesis. We also thought that if such differences exist, the translational relevance would be huge, given the fact that South Asia is a very densely populated area of the planet, with approximately 1.7 billion inhabitants (United Nations, 2014). And although we acknowledge that South Asian may be too broad a term when we talk about such ethnic and cultural diversity in this part of the planet, at least we should try to differentiate somehow these ethnicities from the Caucasian.

4.7.1 Epidemiology

Our results in the cohort of 206 patients prospectively recruited as part of the current study confirmed previous work from our department and showed that BSA patients are an average of 4 years younger at diagnosis (Table 3.3). This is in line with previous epidemiological studies from the USA which showed that Asian women are younger at diagnosis with EC compared to White patients (M. Zhang *et al.*, 2006; Mahdi *et al.*, 2014), although the definition of Asian in these US studies is broadened to include women from the whole continent and East Asia in particular (Japan, China, Korea etc.).

We found BSA women to have significantly lower BMI but higher rates of diabetes compared to BW (Table 3.3). This paradox is also in keeping with large epidemiological studies which suggest that Asian women, even when matched for BMI, have higher rates of diabetes and insulin resistance compared to White women (Shai *et al.*, 2006). The lower BMI effect in women of Asian origin is well reported in the literature and has been confirmed in both Indian and Chinese populations (Misra & Vikram, 2004). Interestingly, South Asian women, when matched for BMI with their White and Black counterparts, have higher rate of body fat. Dyslipidaemias are also more common in women of Indian origin and all these factors combined confirm the increased prevalence of metabolic syndrome in these women (Misra & Khurana, 2009). Large epidemiological studies have confirmed that women with metabolic syndrome carry a 37-39% higher risk of developing EC (Tone Bjørge *et al.*, 2010; Trabert *et al.*, 2015). Based on these data, it is reasonable to assume that lifestyle changes (weight loss, exercise and diet adjustments), which are shown to improve the metabolic profile, would have much greater impact in reducing the prevalence of EC in BSA compared to BW women.

4.7.2 Clinico-pathological data

Previous reports have shown that Asian women present with EC at higher stage and grade compared to Non-Hispanic Whites (M. Zhang *et al.*, 2006; Mahdi *et al.*, 2014). Our study confirmed that BSA women have significantly higher grade at presentation compared to BW (54% of BSA women were stage 2-3 compared to 33% for BW, Table 3.3). However, there was no difference in the stage of the disease between the two populations. Whether

this is a reflection of our small sample size or more fundamental differences between South Asian patients of our cohort and the epidemiologically different Asian patients of the aforementioned American studies, remains to be clarified in future studies which will incorporate more South Asian patients.

The conversion rate of minimal invasive surgery (MIS) to laparotomy in our study (5.3%) is comparable to recent studies (Keurentjes *et al.*, 2018). Interestingly, the conversion rate was much higher in BSA women (15% compared to 3% for BW, p=0.017). This has been reported before in a study on robotic hysterectomies for Gynaecological cancers (Jones, 2014). According to this study from a large tertiary US hospital, non-White ethnicity was a risk factor for conversion to laparotomy, even when adjusted for pre-operative risk factors (OR 2.43, 95% CI 1.24-4.76, p<0.001). This study included 20 Asian patients and the conversion rate was higher compared to their White counterparts (10% versus 6%) but did not reach significance (p=0.36). The authors hypothesised that body habitus and presence of fibroids in Black women might be responsible for the results. It has been previously shown that Asian Indian women are generally shorter and with higher levels of abdominal adiposity compared to White women (Misra & Vikram, 2004). We hypothesize that these two factors significantly contribute to our reported higher conversion rates from MIS to laparotomy among BSA women.

Concerns were raised recently from a large randomized trial regarding oncological outcomes following MIS for cervical cancer as these were shown to be inferior compared to open surgery (3-year overall survival 93.8% compared to 99% for the open surgery group, hazard ratio for death from any cause, 6.00; 95% CI, 1.77 to 20.30) (Ramirez *et al.*, 2018). Although the same has not been shown in endometrial cancer (Janda *et al.*, 2017), it is important to remain vigilant and continue auditing oncological outcomes in EC as the underlying aetiology behind this difference observed in cervical cancer might be common.

4.8 Genetic differences between British South Asian and White women

In view of the aforementioned epidemiological and clinical differences, we proceeded to investigate mutational differences in 10 genes commonly altered in EC.

We found no difference in the non-synonymous mutation rate between the two groups of interest in the 10 genes tested. As expected, mutations of the PI3K pathway were the commoner in both groups, with 60% of patients carrying a *PIK3CA*, *PIK3R1* and/or *PTEN* mutation. This is in line with large studies confirming that PI3K is the most commonly altered pathway in EC (TCGA, 2013).

4.8.1 *ARIDIA*

The rate of *ARID1A* mutations in our cohort (28%) is similar to previously published reports, along with the significant co-existence between them and mutations in the PI3K pathway (Liang *et al.*, 2012). This co-existence was significant in the BW group but not in the BSA. The rate of *ARID1A* mutations in the two groups was not significantly different.

It was recently shown that *ARID1A*-mutant cancer cells were sensitive to glutathione and glutamate-cysteine ligase synthetase catalytic subunit (GCLC) inhibition (Ogiwara *et al.*, 2019). In particular, these cells were sensitive to PRIMA-1 (selective re-activator of mutant p53 activity) and APR-246 (PRIMA-1 analog), which renders *ARID1A* a druggable mutation. It is already known from previous work in breast and ovarian clear cell cancer cells that *ARID1A*-mutations increase the sensitivity of cells to PI3K inhibitors (Samartzis *et al.*, 2014). It is hence reasonable to assume that combining PI3K inhibitors with PRIMA-1 or APR-246 could be very effective in about a third of endometrial cancers carrying *ARID1A* mutations. This is more likely to be the case in BW compared to BSA women, as these patients have significantly more co-existence of these mutations.

4.8.2 *CTNNB1*

The rate of *CTNNB1* mutations in our cohort (21%) correlates with previous studies in which the reported rates are 23-24% (Murali et al, 2014a) and, although we found them to be more frequent in BSA women, this did not reach significance.

92% of the mutations identified (12/13) where in exon 3, with most of them affecting the serine threonine phosphorylation sites (S33, S37, T41 and S45) (8/12). This is in keeping with previous large studies confirming the presence of this ‘hot-spot’ area in exon 3

(Yuexin Liu *et al.*, 2014b). Interestingly, a third of the *CTNNB1* mutations in this study affected codons 32, 34 and 35, which are not phosphorylation sites, which is identical with what we observed (as 4 out of 12 exon 3 mutations were located in codons 32 and 34). Further studies are warranted to identify the importance of these mutations in the function of the Wnt signalling pathway.

The same study, examining data from the TCGA project, confirmed that *CTNNB1* mutations occur in younger patients and are associated with worse prognosis, the latter was also confirmed in a more recent study (Kurnit *et al.*, 2017). In our study, we confirmed a strong trend in the BSA cohort, with patients carrying *CTNNB1* mutations being much younger at diagnosis compared to wt-*CTNNB1*. Interestingly, this was not shown in BW women. As for the prognosis and although our survival data are not mature yet, we already noticed that one of the *CTNNB1*-mutant BSA women of our study had disease recurrence very soon after her 'curative' surgery for a stage 1A, grade 2 endometrioid tumour and sadly passed away just one year after her original diagnosis, giving an early indication that those mutations indeed offer a worse prognosis.

The clinical relevance of identifying somatic *CTNNB1* mutations has been confirmed in a phase 2 clinical trial examining the effect of an mTOR inhibitor (Temsirolimus) in advanced EC (Myers, Andrea P. et al, 2016). In this study, *CTNNB1* mutations were associated with better PFS survival, which confirmed the trend seen in a previous study using another mTOR inhibitor (Everolimus) combined with Letrozole (aromatase inhibitor) (Brian M Slomovitz *et al.*, 2015). All these suggest that BSA women in particular might be benefited more from treatment with mTOR inhibitors as they harbour *CTNNB1* mutations more frequently compared to BW women.

4.8.3 *KRAS*

Our reported rate of *KRAS* mutations (9%) is somewhat lower to recent large studies (20.7% in the TCGA project) (TCGA, 2013). 98% of *KRAS* gene mutations in cancer are located in the three 'hot-spot' areas, codons 12, 13 and 61 (Hobbs *et al.*, 2016). This is in line with our results as five out of six mutations (83%) identified in our cohort were found in codons 12 and 61.

Interestingly, the commonest mutation in our cohort was Q61H (3/6, 50%). This is in contrast with the large TCGA study which identified only 2 such mutations (2/52, 4%). It might be that the prevalence of specific *KRAS* mutations varies in different populations. In general, Q61 mutations are rare in cancer as they represent only 2% of the total *KRAS* mutations, with G12 mutations being the commoner (83%) (Hobbs *et al.*, 2016). They are more common in colonic and pancreatic adenocarcinomas. In the latter ones, it was shown that Q61 mutations are associated with much better survival compared to codon 12 mutations (Agnieszka K Witkiewicz *et al.*, 2015).

The only BSA patient in our cohort harbouring *KRAS* mutation had a G12D mutation. These mutations are the most common in codon 12 (41%) (Hobbs *et al.*, 2016) and have been shown to be associated with worse survival in pancreatic adenocarcinoma (Ogura *et al.*, 2013; Faris *et al.*, 2014) and increased AKT phosphorylation in NSCLC cells (Ihle *et al.*, 2012), rendering the carriers of such mutations potentially good candidates for treatment with PI3K inhibitors. This is particularly relevant in view of our other finding that in the BW group, *KRAS* mutations significantly correlate with PI3K pathway mutations which again raises the possibility of increased sensitivity to such treatments.

In summary, it would be interesting to see large studies in EC addressing the biological impact of specific *KRAS* mutations like the ones mentioned previously and how they are linked with treatment potential and survival.

4.8.4 *PIK3CA*

The rate of *PIK3CA* mutations across our cohort (37%) is comparable to recent reviews (Murali et al, 2014a). In total we identified 30 mutations, with a third localised in the p85 binding domain and another third in the helical domain (exon 9). It has been reported that exon 9 mutations are associated with worse prognosis (McIntyre, John B. et al, 2013; Mjos *et al.*, 2017). As previously mentioned, it would be interesting to analyse survival data from our study (when they mature) in order to confirm such observations in our population. We also had three patients with H1047R/L mutation (exon 20). These mutations have been previously shown to be associated with better response to PI3K/mTOR inhibitors

compared to other *PIK3CA* mutations (Janku *et al.*, 2014), which emphasizes the importance of identifying these mutations in advanced cases prior to allocating these patients in various therapeutic arms.

We observed a strong correlation between *PIK3CA* and *PTEN* mutations across our cohort, which is in line with previous studies (Oda *et al.*, 2005). In subgroup analysis though, this association was only confirmed in the BSA and not the BW group. The role of double mutations in the PI3K pathway (*PTEN* and *PIK3CA*) is intriguing. Older studies showed that both mutations can activate the PI3K pathway on their own but when combined, this triggers an even greater activating cascade (Oda *et al.*, 2005). Still, many would argue that a cancer cell does not need two mutations doing the same thing, unless they offer a selective advantage for the survival of the cell. The hypothesis is that, when both these mutations coexist, they either activate other pathways or inhibit negative feedback pressure on the PI3K pathway (T. L. Yuan & Cantley, 2008). Another hypothetical model summarised in this review by Yuan is based on the observation that *PTEN* loss leads to p53 loss and subsequent growth arrest due to senescence in mouse model of prostate cancer (Cordon-Cardo *et al.*, 2005). The author hypothesizes that these cells later acquire *PIK3CA* mutations in order to re-activate the PI3K pathway and overcome the growth arrest. Another large study examined almost 20,000 solid tumours and concluded that this co-existence is not uncommon, with 14% of *PIK3CA*-mutant tumours carrying a *PTEN* mutation (Millis *et al.*, 2016). An interesting finding from this study is the association of *PIK3CA* mutations with hormonal receptor status as 44% of *PIK3CA*-mutant tumours had estrogen receptor (ER) and 33% progesterone receptor overexpression (compared to 23% and 13% respectively for wt-*PIK3CA* tumours). To summarize, it becomes apparent that BSA women, who frequently harbour both *PIK3CA* and *PTEN* mutations, are excellent candidates for treatment with PI3K inhibitors, especially in combination with other targeted therapies, in order to overcome resistance from other activating pathways. The role of combining PI3K inhibitors with hormonal treatments in this cohort of patients warrants further investigation.

Our data show a significant correlation between the presence of *PIK3CA* mutation and older age at diagnosis across our cohort, which was driven by the BW group in which there was a more than 10 years mean difference between *PIK3CA*-mutant and wild type patients

(68.7 versus 58 , p=0.0077). This is in contrast with large studies which showed no correlation between *PIK3CA* mutations and age at diagnosis (Sara A Byron *et al.*, 2012b; Mjos *et al.*, 2017). Both studies were methodologically different than ours as they examined only exons 9 and 20 of the *PIK3CA* gene, which might have played a role in the clinico-pathological analysis of their results. In any case, more studies are needed in the BW population to confirm our reported association between older age and *PIK3CA* mutations.

4.8.5 *PTEN*

PTEN mutations were the commonest genetic alterations in our study with 39% of our patients harbouring one. This is still significantly lower than recent large studies, suggesting that two thirds of endometrial tumours (65.7%) carry such mutations (Michael S Lawrence *et al.*, 2014).

The mutations were equally distributed between the two 'hot-spot' areas of the gene, the dual specificity phosphatase catalytic (DSPc) and the C2 domains. The commonest mutation was the R130G located in DSPc, which was found in 4 BW women. This mutation has been associated with higher levels of AKT phosphorylation in glioblastoma and endometrial cancers (Papa *et al.*, 2014), which potentially suggest greater sensitivity to PI3K inhibitors in patients harboring these mutations. On the contrary, truncating PTEN mutations in the C-terminal region, which are more common in BSA patients of our cohort, were shown to be more sensitive to Doxorubicin and PARP inhibitors in breast cancer cells (Gong *et al.*, 2015). However, the authors hypothesize that patients with such truncated mutations in the C-terminus are not good candidates for PI3K inhibitors as the phosphatase activity of the *PTEN* gene is preserved and still able to suppress AKT. A recent study in glioblastoma showed that certain *PTEN* mutations are associated with resistance to immunotherapy (Junfei Zhao *et al.*, 2019), and more specifically, to PD-1 inhibitors (Nivolumab and Pembrolizumab). Interestingly, some of these mutations detected in non-responders (G132D, R173C and R233), have been identified in our cohort, raising questions about a similar lack of efficacy of these therapies in endometrial cancer.

4.8.6 Other genes

The rate of *FBXW7* mutations in our study (23%) is comparable with the large TCGA study which showed a rate of 16% amongst all EC tumours studied (TCGA, 2013). Such mutations are more prevalent in serous and clear cell EC (30% and 25% respectively) (Jones N et al, 2015; DeLair *et al.*, 2017). Surprisingly, none of the 6 serous/clear cell cases of our cohort was harboring such a mutation. *FBXW7* mutations have been shown to be associated with poor prognosis in gastric, colorectal and hepatocellular carcinoma (Cao *et al.*, 2016), so it would be interesting to assess our survival data in view of this association.

POLE mutations are of particular interest in EC in view of the excellent prognosis associated with their presence. 10.5% of our patients were carrying these mutations and this is similar to much larger cohorts (7% in the TCGA study). The strong coexistence of *POLE* with PI3K pathway and *ARID1A* mutations shown in the TCGA study was confirmed in our cohort as all 6 *POLE*-mutant tumours were harboring a PI3K pathway mutation and two thirds had *ARID1A* mutations as well. It would be interesting to confirm excellent prognosis in these 6 patients and especially in the two patients presented with stage 2 and 3 disease respectively.

Our reported rate of *PIK3R1* mutations (12%) is significantly lower compared to the TCGA dataset (33.9%) (Michael S Lawrence *et al.*, 2014). Within our two ethnic cohorts, BSA women had more *PIK3R1* mutations (19%) compared to BW (7%), although this was not significant (p=0.24). Given that 75% of sequenced patients in the TCGA study were Caucasian, this marked difference in the prevalence of somatic *PIK3R1* mutations raises questions about genetic heterogeneity even within group of patients with similar ancestry. On the contrary, our study confirms the significant coexistence between *POLE* and *PIK3R1* mutations, previously reported in the TCGA study (Piulats et al, 2016). We identified a patient with an R348* *PIK3R1* truncating mutation, which is of functional importance as it was shown to activate the ERK pathway (Cheung *et al.*, 2011) and hence rendering EC cells more sensitive to MEK inhibitors.

TP53 mutations are very common in Type 2 EC and this was also shown in our study, with four out of six serous/clear cell tumours carrying such a mutation. The prognosis associated with these mutations is notoriously worse (B G Wortman *et al.*, 2018) and it would be interesting to analyze survival data from the six patients of our cohort carrying those mutations. We confirmed the previously reported (Gonzalez-Rodilla *et al.*, 2012) association between the presence of *TP53* mutations and older age at diagnosis of EC (72.5 years versus 60.5, p=0.008). Age has been shown to be a predictor of poor survival in most cancers (Quaresma et al, 2015). Whether this fact is associated with most aggressive biological phenotypes (like *TP53* mutations) or factors including later access to healthcare and more comorbidities, which prohibit aggressive treatment modalities, warrants further research but is likely to be multifactorial. In the previous mentioned study (Gonzalez-Rodilla *et al.*, 2012), based on their findings, the authors favor the former hypothesis of a more aggressive EC tumour profile among elderly patients.

PPP2R1A mutations have been reported to occur frequently (36%) in serous and clear cell EC (TCGA, 2013; DeLair *et al.*, 2017), while only occur in 5-7% of endometrioid tumours (Shih, 2011; McConechy MK et al, 2011). We only identified one such mutation in an endometrioid tumour (2% rate among endometrioid EC) but none in the six serous/clear cell cancers, which is in contrast with previous large studies but probably is attributed to the very small numbers of such tumours sequenced in our study.

4.8.7 Mismatch Repair deficiency

Our reported rate of MMR deficiency across the sequenced cohort (25%) is similar to recent large studies giving an incidence of 24-27% (Stelloo *et al.*, 2017; Dillon *et al.*, 2017). In University Hospitals of Leicester, patients diagnosed with endometrial cancer under the age of 60, those with personal history of bowel cancer or family history of bowel and/or EC undergo IHC testing to confirm their MMR status. Those who test positive are deemed at increased risk of having Lynch syndrome and are referred to a clinical geneticist. Apart from the 57 patients of our sequenced cohort, we have MMR data on 30 more patients recruited in our study (Table 3.3). In total, we identified 17 patients with MMR deficiency and our cumulative rate of deficiency is 20% (17/87). Between the two

groups of interest, there was no difference in the rate of MMR deficiency (15% for BW versus 27% for BSA women, p=0.17). In view of this non-significant trend for higher rates of MMR deficiency among BSA women in our cohort and a recent study examining 385 Thai women and reporting unusually high rates of MMR gene defects (55%) (Tangjitgamol *et al.*, 2017), further studies are warranted to assess MMR status in South Asian populations.

We clearly demonstrated that MMR deficiency is associated with higher age at diagnosis (67.8 versus 59.8 years, p=0.014) and this was particularly prominent in the BW cohort (72.8 years for MMR deficient versus 59.6 for MMR proficient, p=0.007). The association between older age at diagnosis and MMR deficiency has been previously shown in studies on colorectal cancer and confirms our findings (Frank A. Sinicrope *et al.*, 2012; He *et al.*, 2016).

The concept of universal screening of tumours for MMR deficiency has long been debated in colorectal cancer. Current guidelines recommend screening for all colorectal cancers up to the age of 70 (Giardiello, Francis M. et al, 2014). In endometrial cancer though there is even more equivocacy, as American recommendations promote screening in patients younger than 50, while European experts recommend screening for all patients under the age of 70 (Giardiello, Francis M. et al, 2014; Vasen *et al.*, 2013). This is in line with studies suggesting that screening in EC under the age of 70 is cost-efficient (Goverde et al, 2016). Given the mean age of diagnosis of MMR deficiency in our cohort (67.8), I propose increasing our 'cut-off' for checking MMR protein expression from 60 to 70 years of age.

Our study reports a trend associating obesity with decreased rates of MMR deficiency (mean BMI 36.5 for MMR proficient tumours compared to 31.5 for MMR deficient, p=0.066), and although this trend is not significant, is in keeping with studies in both colorectal and endometrial cancer which confirm this association (Frank A. Sinicrope *et al.*, 2012; Joehlin-Price et al, 2014). The reasons behind this correlation are obscure although previous studies in colorectal cancer had shown that hyperestrogenemia reduces the risk of MMR deficiency, and this can explain the higher incidence of MSI in thin and elderly patients (Slattery *et al.*, 2001). The mechanism behind this might relate to the fact

that with aging, the estrogen receptors (ER) in colonic cells hypermethylate and this leads to reduced ER expression, which subsequently increases the carcinogenic potential of these cells (Issa *et al.*, 1994). Whether the same mechanism applies to endometrial cells should be examined in future studies.

There was a non-significant association between MMR deficiency and higher grade disease in our study (57% of MMR deficient tumours were grade 2-3 compared to 37% of MMR proficient, p=0.16). This correlation has been previously confirmed in both endometrial and colorectal cancer (Kim *et al.*, 2018; James H Park *et al.*, 2016). Despite this finding, MMR deficiency is associated with improved RFS and OS in colorectal malignancies, in both stage 2 and 3 disease as shown in randomized controlled trials (Klingbiel *et al.*, 2015; Sinicrope *et al.*, 2017). This association has not been confirmed in endometrial cancer. A meta-analysis reported a strong but not significant trend favoring worse OS in MSI tumours (HR=2.02; 95% CI=0.85–4.83) (Diaz-Padilla et al, 2013), while other studies showed no difference in OS between MMR deficient and proficient tumours, although the former ones were associated with higher grade and LVSI rates (McMeekin *et al.*, 2016b). This discrepancy in the findings between endometrial and colorectal cancer might represent different biologic behavior between different tumour sites and larger studies are needed to shed light to the reasons behind these differences.

Combined loss of MLH1 and PMS2 was the commonest abnormality in our cohort (19.3%), while isolated loss of MSH6 was identified in 3.5% of cases and combined loss of MSH2 and MSH6 in one case (1.8%). These numbers are in keeping with large, recent studies reporting respective rates of 15.7%, 3% and 1.9% (Joehlin-Price et al, 2014).

In the BSA cohort, there was an inverse correlation between *CTNNB1* mutations and MMR status (none of the *CTNNB1*-mutant patients was MMR deficient versus 42% of wt-*CTNNB1*, p=0.06). Interestingly, this was not shown to be the case in the BW cohort. This negative association between *CTNNB1* mutations and MSI has been reported previously in a large cohort of 466 endometrioid EC patients (Sara A Byron *et al.*, 2012a) and is in contrast with earlier findings in colorectal cancer which reported a positive association between *CTNNB1* mutations and MSI and no correlation in EC (Mirabelli-Primdahl *et al.*,

1999). The discrepancy between these results might be attributed to the different biologic behavior of endometrial and colorectal cancer but it is certainly worth investigating in future studies.

In conclusion, assessment of MMR status becomes increasingly important in endometrial cancer. In 2017, FDA approved Pembrolizumab, an antibody targeting PD-1 receptor in human lymphocytes (FDA News release, 2017). For the first time the approval was given to a drug for use in all MMR deficient solid tumours, irrespective of their location (tissue agnostic immunotherapy), emphasizing the importance of assessing the MMR status and officially entering the era of personalized medicine (Boyiadzis *et al.*, 2018).

4.8.8 Summary

Figure 4.1 summarizes the most important findings of our study. It proposes two novel combination treatments for further testing in animal models of EC and calls for larger studies in the two ethnic groups studied in order to explore the genetic associations shown in the graph and explained previously.

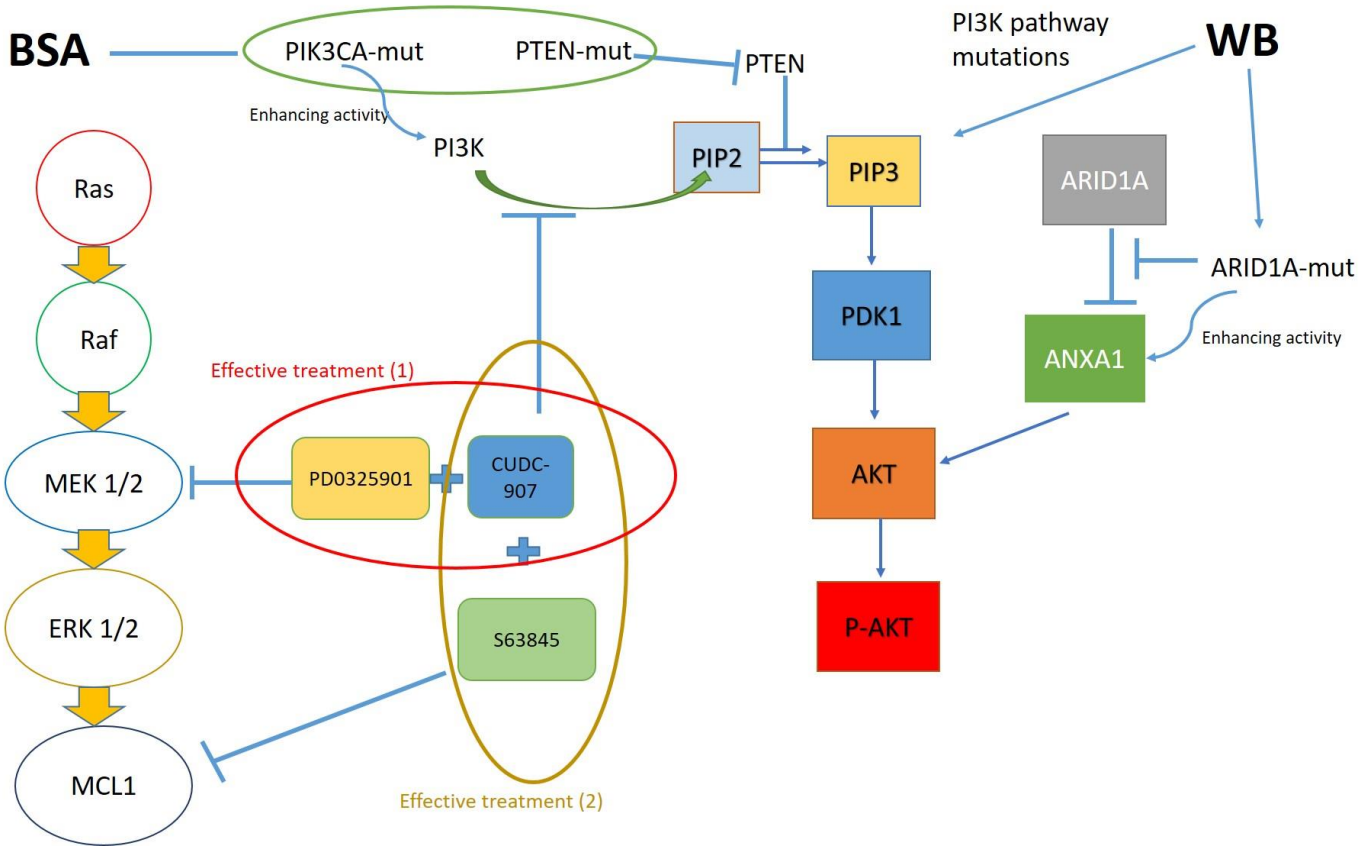

Figure 4.1 Molecular pathways (PI3K and MAPK) studied in the current thesis along with effective combination treatments in EC cell lines. The various elements of them have been explained in the previous chapters. Next Generation Sequencing showed the co-existence of *PIK3CA* and *PTEN* mutations in BSA women, while WB harbored *ARID1A* and PI3K pathway mutations (defined as either *PIK3CA*, *PIK3R1* or *PTEN* mutations). The function of these mutations is depicted on the graph. ANXA1: Annexin A1 gene.

4.8.9 Limitations

4.8.9.1 Cell line work

One limitation of my study was the fact that I used two endometrial cancer cell lines in order to test a panel of inhibitory drugs and although these cell lines had entirely different mutational profiles and suited the screening nature of the study, it would arguably be better to perform the experiments in a larger panel of cell lines which would be more representative of the disease.

The main limitation of any cell line work though is the uncertainty of the value of cell lines as cancer model. The debate is longstanding and ongoing.

Proponents of cell line work emphasize on the fact that it is a cheap and easy to grow screening tool available to almost every laboratory. They can undergo extensive testing which would be impossible to ever attempt on human beings or even animal models without huge ethical and regulatory issues. Work on cell lines has lead to therapeutic successes and although the failures have been more than the success stories, this supports the fact that cultured cells are a valid tool for cancer research (Gillet *et al.*, 2013). Many cell lines have been molecularly profiled (Barretina *et al.*, 2012) and hence extensive testing of their molecular pathways can lead to comparisons with tumours carrying similar profiles as defined by large studies (TCGA, 2013).

On the contrary, the disadvantages of cell line experiments are well described. Many of the properties that cancer cells possess in human beings (including multidrug resistance) depend on their microenvironment which immortalized cells cultured in vitro can not capture (Weinstein, 2012; Katt *et al.*, 2016). In vitro cells are also a very poor model in assessing the toxicities associated with anticancer drug treatments as these can only be reliably assessed in in vivo models (Weinstein, 2012).

In summary, with all the flaws associated with it, cell lines remain a useful experimental tool for cancer studies. The development of more sophisticated in vitro models (spheroids, hybrid and tumour microvessel models) is already allowing experiments on various parameters of the cancer phenotype and provide a wealth of information that the traditional Petri dishes could never imagine (Katt *et al.*, 2016).

4.8.9.2 Ethnic differences

The main limitation of our study is the relatively small number of sequenced patients and the subsequent lack of power to detect more subtle differences between the two ethnic groups of interest. Also, due to lack of resources, we were unable to sequence a larger number of genes which would have generated more information about the two studied populations.

Still, our cohort had more Asian patients (27) compared to the paradigm shifting TCGA study, which only included 20 Asian patients. The main strength of the study is that, to our knowledge, this is the first attempt to compare somatic mutations between a Caucasian and a South Asian cohort of women with EC. We also acknowledge the lack of germline molecular data on the participants of the study. Lack of resources and uncertainty of the effects of germline mutations for some of the genes tested in the offsprings, would have made the ethical approval of our study more difficult and therefore it was not included. We also do not present data on family history of the recruited patients as we deem the data we hold less robust due to the fact that many patients were uncertain of their family history and we did not have ethical approval to request access to the participants' families medical records.

The effect of the environment in the process of carcinogenesis is well established (Lesley Rushton, 2003). Our study compared South Asian patients, mostly of Indian origin, who are first or second generation immigrants to the UK. It is fair to say that the influence of the environment cannot be measured and it would be worth repeating a study in which endometrial tumours of Caucasian women would be compared with those of native South Asian patients, in order to assess differences, which might be attributed to environmental factors.

The reported in our study higher conversion rate of minimal invasive surgery to laparotomy among BSA women (15% versus 3% for BW, p=0.017) should be interpreted with caution as we have not controlled for the presence of previous surgery in these two groups. A larger comparison is already planned in order to control for factors which might affect the operability of a patient.

4.8.10 Future directions

Next generation sequencing is a valuable tool in providing accurate data on the mutational landscape of cancer. For a long time, the cost associated with it prevented its widespread use in every day clinical practice.

As shown in Figure 4.1 below, this cost is rapidly falling. In 2001, DNA sequencing for a whole human-sized genome costed 95 million US dollars compared to 1,121 dollars in 2017. This reduction will soon enable the transfer of this technology's capabilities in routine clinical care.

The previous discussion in 4.8 clearly demonstrates that the future of oncology in advanced cancer cases will be a detailed analysis of the tumour's mutational landscape. Depending on each patient's mutational burden, a combination of targeted therapies will be used (personalized medicine) rather than 'blanket' chemotherapy for tumours which might be in the same organ but have completely different biological behavior (current strategy).

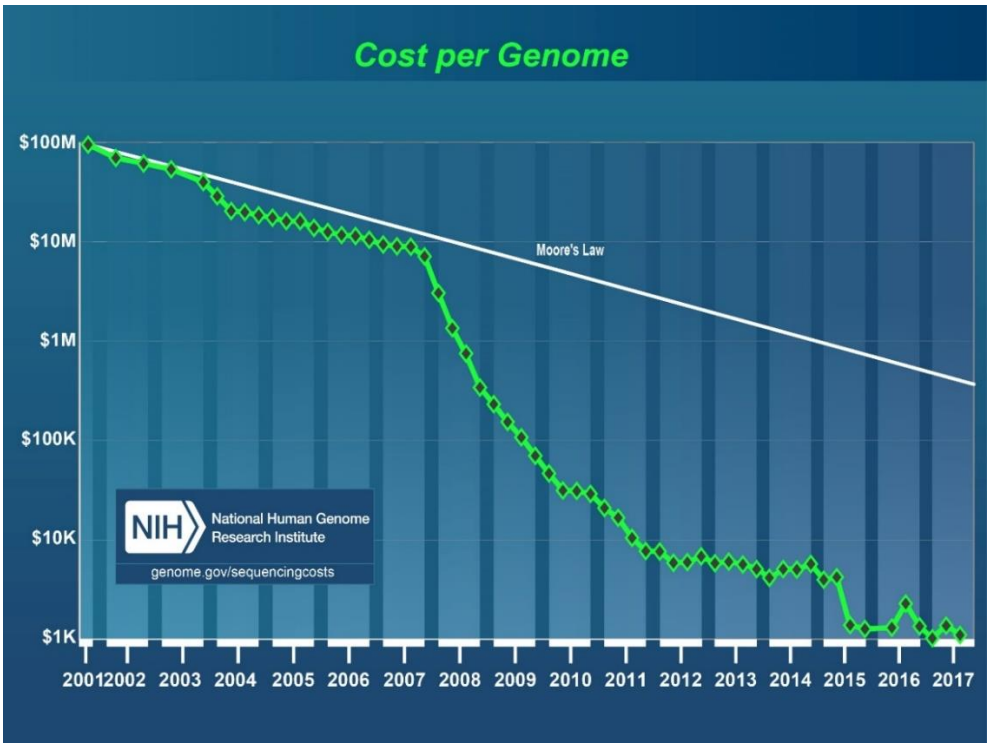

Figure 4.2 Cost of DNA sequencing technologies per human-sized genome in time (Wetterstrand,). The line for Moore's Law represents the trend in computing power of

doubling every two years and is shown for comparative reasons in order to show that DNA sequencing technologies progress at a much faster pace.

Many research groups, including ours, are experimenting on the clinical applications of detecting circulating tumour DNA (ctDNA) on patient's plasma. The so called 'liquid biopsies' have several advantages over traditional tissue biopsies; Less risks, easier to obtain, no need for local/regional/general anaesthetic. It is conceivable that, in the near future, these 'liquid biopsies' will be used to monitor the response to treatment and to predict recurrence of the disease, might help to select targeted treatments based on the mutational profile of the tumour, in specifying prognosis and inform on disease burden (Clare Fiala & Eleftherios P Diamandis, 2018).

For all those reasons, it becomes apparent that efforts to identify differences in the molecular profile of tumours and factors influencing those differences are important. Ethnic origin has been shown to play a role in many cancers. Still, there is a complete lack of data for people representing ethnicities of the developing world, which consist the larger proportion of the world's population. Our study attempted to shed some light in a previously uncharacterized cohort of patients. Larger and more powerful studies are needed in order to better characterize those populations in order to identify potential differences with translational relevance.

APPENDIX 1

Table A.1. Criteria for classifying pathogenic variants (American College of Medical Genetics) (Richards *et al.*, 2015). Across the tables, cis refers to variants being located on the same copy of the gene (same chromosome) and trans refers to variants located in both copies of the gene (both homologous chromosomes).

Criteria for classifying pathogenic variants		
Very strong evidence of pathogenicity	PVS1	Null variant in a gene where loss of function is a known mechanism of disease
Strong evidence of pathogenicity	PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change
	PS2	De novo in a patient with the disease and no family history
	PS3	Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product
	PS4	The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls
Moderate evidence of pathogenicity	PM1	Located in a mutational hot spot and/or critical and well-established functional domain without benign variation
	PM2	Absent from controls in Exome Sequencing Project, 1000 Genomes or ExAC
	PM3	For recessive disorders, detected in trans with a pathogenic variant
	PM4	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants

	PM5	Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before
	PM6	Assumed de novo, but without confirmation of paternity and maternity
Supporting evidence of pathogenicity	PP1	Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease
	PP2	Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease
	PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product
	PP4	Patient's phenotype or family history is highly specific for a disease with a single genetic etiology
	PP5	Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation

Table A.2. Criteria for classifying benign variants (Richards *et al.*, 2015).

Criteria for classifying benign variants		
Stand-alone evidence of benign impact	BA1	Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes, or ExAC
Strong evidence of benign impact	BS1	Allele frequency is greater than expected for disorder

	BS2	Observed in a healthy adult individual for a recessive, dominant, or X-linked disorder with full penetrance expected at an early age
	BS3	Well-established in vitro or in vivo functional studies shows no damaging effect on protein function or splicing
	BS4	Lack of segregation in affected members of a family
Supporting evidence of benign impact	BP1	Missense variant in a gene for which primarily truncating variants are known to cause disease
	BP2	Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed in cis with a pathogenic variant in any inheritance pattern
	BP3	In-frame deletions/insertions in a repetitive region without a known function
	BP4	Multiple lines of computational evidence suggest no impact on gene or gene product
	BP5	Variant found in a case with an alternate molecular basis for disease
	BP6	Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation
	BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site and the nucleotide is not highly conserved

Table A.3. Variant classification criteria. See Tables A.1 and A.2 for details on the criteria used (Richards *et al.*, 2015).

Pathogenic	1 PVS1 and one of	≥1 PS1-PS4
		≥2 PM1-PM6
		1 PM1-PM6 and 1 PP1-PP5
		≥2 PP1-PP5
	≥2 PS1-PS4	
	One PS1-PS4 and one of	≥3 PM1-PM6
		2 PM1-PM6 and ≥2 PP1-PP5
		1 PM1-PM6 and ≥4 PP1-PP5
Likely pathogenic	1 PVS1 and 1 PM1-PM6	
	1 PS1-PS4 and 1-2 PM1-PM6	
	1 PS1–PS4 and ≥2 PP1–PP5	
	≥3 PM1–PM6	
	2 PM1–PM6 and ≥2 PP1–PP5	
	1 PM1–PM6 and ≥4 PP1–PP5	
Benign	1 BA1	
	≥2 BS1–BS4	
Likely benign	1 BS1–BS4 and 1 BP1–BP7	
	≥2 BP1–BP7	

APPENDIX 2

NGS protocol

Generation of DNA library

The DNA, which was extracted as described in paragraph 2.2.8.2, was subsequently quantified using a nanophotometer. 20ng of genomic DNA was used for generating the library. The Ion AmpliSeq™ HiFi Master Mix was thawed and kept on ice during the procedure. In a 0.2ml PCR tube, the following mixture (Table A.4) was prepared for each DNAsample to a total volume of 20μl.

Component	**Cap colour**	**Volume**
5x Ion AmpliSeq™ HiFi Master Mix	Red	4μl
5x Ion AmpliSeq™ CHPv2 Panel	Clear	4μl
Genomic DNA, 10ng	N/A	Xμl
Nuclease-free Water	N/A	12- Xμl
Total		**20μl**

Table A.4. PCR mix using the Ion AmpliSeq™ HiFi Master Mix.

The pipette volume was set to 10μl and the mixture was pipetted up and down 5 times to mix. The PCR tubes were placed in a thermal cycler and the program shown below in Table A.5 was run in order to amplify the target genomic regions.

Step	Temperature	Time	Stage
Enzyme activation	99°C	2 min	Hold
Denaturation	99°C	15 sec	22 cycles
Annealing and extension	60°C	4 min	22 cycles
Final incubation	10°C		Hold

Table A.5. PCR program for amplifying the target genomic regions.

The PCR products were stored at -20°C until used.

The FuPa reagent was thawed and kept on ice during the next step. 2μl of FuPa was added to the PCR products and the sample was mixed. The PCR products were then placed in a thermal cycler and the program shown in Table A.6 was run.

Temperature	Time
50°C	10 min
55°C	10 min
60°C	20 min
10°C	Hold (for up to 1hr)

Table A.6. PCR program after adding the FuPa reagent.

A barcode was assigned to each library by using the Ion Xpress™ Barcode Adaptors 1-16 kit. A mix of Ion P1 Adapter and Ion Xpress™ Barcode was prepared at a final dilution 1:4 for each adapter as shown in Table A.7 below.

Component	Volume
Ion P1 Adapter	2µl
Ion Xpress™ Barcode	2µl
Nuclease-free water	4µl
Total volume	8µl

Table A.7. Mixture using the Ion Xpress™ Barcode Adaptors 1-16 kit for barcode assignment.

The Switch Solution was thawed and re-suspended and the mixture shown in Table A.8 was made.

Component	Volume
Switch Solution	4µl
Diluted barcode adapter Mix	2µl
Digested samples	22µl
Total volume	**28µl**

Table A.8. Switch solution mixture.

2µl of DNA ligase was then added to each tube and the sample was mixed by pipetting. The PCR tubes were then placed again in the thermal cycler and the program shown in Table A.9 was run.

Temperature	Time
22°C	30 min
72°C	10 min
10°C	Hold (for up to 1 hour)

Table A.9. PCR program after adding DNA ligase to the Switch solution mixture.

Purification of DNA libraries

The AMPure® beads were brought to room temperature, vortexed and pipetted. The ligated samples from the previous step were transferred to DNA Lo-Bind Eppendorf tubes and 45µl of AMPure® XP Reagent was added to each tube and mixed. The mixture was incubated at room temperature for 5 minutes. The tubes were then placed on a magnetic rack and incubated for 2 minutes. The supernatant was discarded and 200µl of 70% Ethanol (prepared with nuclease-free water) was added to each tube. The tubes were stirred while on the magnetic rack in order to wash the beads and they were left for 2 minutes to incubate. A second wash was subsequently performed after discarding the supernatant. The pellet was then left to dry for 5 minutes in the magnetic rack. 50µl of Platinum® PCR SuperMix High Fidelity and 2µl of Library Amplification Primer Mix (Ion AmpliSeq™ Library Kit) were added to each pellet and mixed by pipetting. The tubes were placed back in the magnet for 2 minutes. 50µl of the supernatant were then transferred to a PCR tube and the tubes were placed in a thermal cycler, programmed to run the cycle shown in Table A.10.

Step	**Temperature**	**Time**	**Stage**
Enzyme activation	98ºC	2 minutes	Hold
Denaturation	98ºC	15 seconds	7 cycles
Annealing and extension	60ºC	1 minute	
Final Incubation	10ºC	∞	Hold

Table A.10. PCR cycle after adding the SuperMix and the Primer Mix to the library.

The AMPure® beads were brought to room temperature, vortexed and pipetted. The samples from the previous step were transferred to DNA Lo-Bind tubes and 25µl of AMPure® XP beads added to each tube and mixed. The mixture was left for 5 minutes at room temperature. The tubes were placed on a magnetic rack for 5 minutes and subsequently the supernatant was transferred to a new DNA Lo-Bind tube. A further 60µl of AMPure® XP beads were added to the supernatant from the previous step and mixed by pipetting. The mixture was incubated for 5 minutes at room temperature. They were then

placed on the magnetic rack for 3 minutes. The supernatant was then discarded and 150µl of freshly prepared Ethanol 70% was added to each tube and the tubes were moved round in order to wash the beads. They were then returned to the magnet and incubated for 2 minutes. After discarding the supernatant, a second wash was performed. The supernatant was again discarded and the tubes were left to air-dry for 5 minutes. The tubes were removed from the magnet. 50µl of Low TE was added to each tube and the mixture was pipetted. The tubes were placed back on the magnet for 2 minutes. The supernatant was then transferred to a new DNA Lo-Bind tube. The DNA libraries were then quantified using the Qubit® 2.0 Fluorometer. 3µl of each library was finally diluted with sterile water to 100pM (0.013ng/µl) and stored at -20°C.

DNA template preparation

The Ion One Touch 2 system was set up as per manufacturer's instructions.
The reagents were prepared as followed:
Ion PGM™ Template OT2 200 Reagent Mix: The solution is thawed, vortexed for 30 seconds and centrifuged for 2 seconds.
Ion PGM™ Template OT2 200 PCR Reagent B: The solution is vortexed for 1 minute and centrifuged for 2 seconds. If the solution is cloudy or has crystals, it was heated for 1 minute at 75°C.
Both reagents were kept at room temperature.
Ion PGM™ Template OT2 200 Enzyme Mix: The mix was centrifuged for 2 seconds and placed on ice.
Ion PGM™ Template OT2 200 Ion Sphere™ Particles (ISPs): The tube was allowed to reach room temperature and vortexed.
Diluted DNA library: 2µl of the 100pM library was diluted in 23µl nuclease-free water, mixed, centrifuged and placed on ice.
In a DNA Lo-Bind tube, the reaction mixtures were prepared by adding the reagents in the order and volume shown in Table A.11 below.

Order	Reagent	Volume
1	Nuclease-Free Water	25µl
2	Ion PGM™ Template OT2 200 Reagent Mix	500µl
3	Ion PGM™ Template OT2 200 PCR Reagent B	300µl
4	Ion PGM™ Template OT2 200 Enzyme Mix	50µl
5	Diluted DNA library	25µl
-	**Total volume**	900µl

Table A.11. Reaction mixture.

The solution was vortexed at maximum speed for 5 seconds, centrifuged for 2 seconds and kept on ice. The Ion PGM™ Template OT2 200 ISPs were vortexed at full speed for 1 minute and then centrifuged for 2 seconds. The spheres were re-suspended and 100µl of the solution was added to the reaction mixture shown in table A.11. The new solution (volume 1ml) was vortexed for 5 seconds and kept on ice.

The amplification solution was inserted to the sample port of the Ion PGM™ One Touch™ Plus Reaction Filter Assembly. 1ml of Ion PGM™ One Touch™ Reaction Oil was subsequently added and after changing the tip, a further 500µl of Oil was added. The Assembly filter was connected to the Ion One Touch™ 2 instrument and the amplification run was commenced. At the end of the run, a 10 minute centrifugation step was performed. Both Ion One Touch™ Recovery Tubes were removed from the instrument and placed on a rack. The Recovery Solution was carefully removed from each tube apart from 50µl, taking care not to disturb the ISP pellet. The pellet was subsequently re-suspended with the Recovery Solution and the 2 solutions (50µl each) were mixed in a single DNA Lo-Bind® tube.

Quality control: 2µl of the final solution were transferred to a 0.2ml PCR tube along with 19µl of Annealing Buffer and 1µl of Ion Probes from the Ion Sphere Quality Control Kit, and they were mixed by pipetting. The tube was loaded to a thermal cycler and incubated at 95ºC for 2 minutes and 37ºC for the same time. The unbound probes were then removed

by adding 200µl of Quality Control Wash Buffer to the tube, mixing and centrifuging at 15,500x g for 2 minutes. The supernatant was discarded all but 10µl, taking care not to disturb the pellet and the wash was repeated two more times. After the last wash, 190µl of Quality Control Wash Buffer was added and the entire sample was transferred to a Qubit® assay tube. A negative control consisted of 200µl of Quality Control Wash Buffer was used and the Relative Fluorescence Units (RFU) were measured in the Qubit® instrument for both Alexa Fluor® 488 and 647 options (aiming at a count over 100 as a proof of the presence of ISPs in the assay). All these readings were entered in the respective section of the Qubit® 2.0 Easy Calculator along with the lot-specific conversion factor for the ISPs used in the Ion PGM™ Template OT2 200 Kit (obtained from the Ion Torrent Community website). The percentage of templated ISPs was subsequently calculated with optimal amount considered to be a 10-30%.

*Enrichment of template-positive ISPs***:** An 8-well strip was obtained from the Ion OneTouch™ Supplies Kit and the square-shaped tab of the well strip was kept on the left. In each well, the solutions shown in Table A.12 below were added. Well 1 was the well near the square-shaped tab of the strip.

The filled 8-well strip was placed in the right-hand end of the slot of the Ion OneTouch™ ES instrument tray The instrument was prepared and switched on as per manufacturer's instructions. Prior to starting the run, the contents of the first 2 wells were pipetted in order to re-suspend the beads, taking care not to introduce any bubbles. The run was then performed and the enriched ISPs were stored at 4ºC (after being inverted 5-10 times) until sequencing.

Solution	Preparation Procedure	Well number
130µl Dynabeads®	Vortex the beads for 30 seconds and centrifuge the tube for 2 seconds. Pipette the beads thoroughly and transfer 13µl of them to a 1.5ml DNA Lo-Bind® tube. The tube was placed on a magnetic rack for 2 minutes and the supernatant removed. 130µl of MyOne® Beads wash solution was then added and the pellet re-suspended. The solution was transferred to Well 2	Well 2
300µl Melt solution	The Melt solution was prepared fresh (280µl of Tween mixed with 40µl of freshly made 1M NaOH) and stored for 1 day. 300µl of the solution was transferred into well 7	Well 7
100µl ISPs sample mixture from previous step	The mixture was pipetted thoroughly and transferred to well 1	Well 1
300µl of Ion OneTouch™ Wash solution		Well 3, 4 and 5
Empty		Well 6 and 8

Table A.12. Composition of solutions needed to be added on each well of the 8-well strip.

Sequencing

A planned run was created on the Ion Torrent browser as per the system's instructions. The Ion PGM™ was cleaned every time the instrument was initialised using either 18MΩ water or a chlorite solution as per manufacturer's instructions.

The 3 wash bottles were prepared (after making 0.5ml of 100mM NaOH) as follows:

Wash 2 bottle: The bottle was washed 3 times with 200ml of 18MΩ water, filled with water and Ion PGM™ sequencing 200 v2 W2 solution (the whole bottle provided in the kit), while 70µl of 100mM NaOH was also added and the solution was mixed by inverting the bottle.

Wash 1 and 3 bottles: The bottles were washed 3 times with 50ml of 18MΩ water. 350µl of 100mM NaOH were added to the Wash 1 bottle while the Ion PGM™ sequencing 200 v2 1x W3 solution was added to the Wash 3 bottle and both bottles were capped. The system was then initialised and the on-screen instructions were followed including installing new sipper tubes in W1-3 positions and attaching Wash bottles 1-3 respectively. The dNTPs were prepared (dGTP, dCTP, dATP and dTTP) in their corresponding bottles and the bottles were attached to their corresponding port. After initialisation is successful, the chip was set up as follows:

Sample preparation: The control ISPs were vortexed and then centrifuged for 2 seconds. 5µl of control ISPs were added to the enriched, template-positive ISPs in a PCR tube, mixed and centrifuged for 2 minutes at 15,500x g. The supernatant was carefully removed, leaving 15µl in the tube. 12µl of Sequencing Primer was added and the sample was pipetted to disrupt the pellet. The sample was then incubated on a thermal cycler (95°C for 2 minutes followed by 37°C for 2 minutes). After removing the sample from the cycler, 3µl of Ion PGM™ sequencing 200 v2 polymerase were added to the ISPs, the sample was pipetted and incubated at room temperature for 5 minutes.

Chip preparation: The chip was placed on the Ion PGM™ grounding plate and the on-screen instructions were followed after pressing 'Run' on the main menu. After scanning the barcode, 'Chip Check' was pressed and the on-screen instructions were followed.

Sample loading: After tilting the chip by 45°, a pipette tip was inserted into the loading port in order to remove as much liquid as possible from it. The chip was then centrifuged in the MiniFuge for 5 seconds. The sample prepared in the previous step was then loaded to the chip slowly, ensuring that no bubbles were inserted and the chip was subsequently

centrifuged in the MiniFuge for 30 seconds. Following this, a pipette was firmly attached to the loading port and the sample was mixed by pipetting. This process (centrifuging and mixing) was repeated 3 times. After the final centrifugation step, with a pipette firmly attached in the loading port, as much of the liquid as possible was removed from the chip and discarded.

Performing a sequencing run: The run settings were selected from the dropdown lists. The chip was loaded and clamped. Following calibration, the on-screen instructions were followed and the run commenced. A water clean was performed after the end of the run.

Analysis: Sequencing data were accessed and analysed by Dr Guttery through the Torrent Suite v5.6.0. All mutations with a quality score below 100 were omitted and all variants detected in the first or last 10 bases of an amplicon were omitted as likely mispriming events. Known germline variants without pathological consequence were omitted from the analysis.

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