Investigating the role of poly (ADP-ribose) polymerase inhibitors in the treatment of endometrial cancer

Dominic Adam Blake

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Supervisors

Dr Rachel O'Donnell

Prof. Nicola J Curtin

Newcastle University

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Abstract

Introduction: Endometrial cancer is the most common gynaecological malignancy in the UK. Whilst most women have low grade indolent cancers, 20% have high grade tumours which result in a disproportionate number of deaths. Following first line treatment, there is no standard of care and response rates are poor. In line with ovarian cancer treatment, where PARP inhibitors have resulted in better survival, it is anticipated that a proportion of endometrial cancers are homologous recombination DNA repair deficient (HRD), providing opportunity to exploit this with PARP inhibitors.

The work in this thesis included pre-clinical and translational studies to assess the effect of cisplatin, ionising radiation and PARP inhibitor (Niraparib) on endometrial cancer cell lines and ex-vivo patient derived cultures, as well as assessing HRR status and other pathology markers as biomarkers to stratify the use of PARP inhibitor therapy.

Methodology: Cytotoxicity, growth inhibition, PARP assays and immunohistochemistry studies were undertaken in 6 endometrial cancer cell lines, along with functional HRR assay measuring RAD51 foci formation by immunofluorescence.

Direct endometrial tumour biopsies were taken from 54 patients (REC: 12/NE/0395) and the method optimised for ex-vivo culture. Where feasible, the RAD51 foci HRR assay alongside growth inhibition with Niraparib was undertaken.

A tissue microarray was constructed from available FFPE patient samples and cell line thrombin clots. Common IHC markers (p53, ER, PR, p53, MMR proteins) and PTEN were quantified and analysed stratified by HRR function and clinicopathological data.

Results: The diverse panel of endometrial cell lines were all HRR competent using the RAD51 foci functional assay, with similar growth and PARP activity levels. No significant differences in sensitivity to single agent IR, cisplatin were detected but trends in sensitivity were consistent (AN3CA most sensitive and HEC1A least sensitive). PARP activity was inhibited by 90% with Niraparib 1 μM, with no significant differences in cytotoxicity or growth inhibition across the cell lines. Radiopotentiation with Niraparib at the lethal concentration 50 (PF50) ranged from 1.00 to 1.84 in the Ishikawa cell line. Higher doses of IR (4 Gy) yielded greater potentiation in the endometrial cancer cell lines (PF up to 2.41). Minimal potentiation was

seen when increasing concentrations of cisplatin was combined with Niraparib 1 μM (PF50 ranging from 0.77- 1.44).

54 endometrial cancer biopsies were obtained with consent. 30 samples were successful in culture of which 18 completed RAD51 foci HRR assay. 3/18 were HRD and 15/18 were HRR competent (HRC). Niraparib growth inhibition studies were completed on 10 samples. The 2/10 samples that were HRD had lower mean GI50 when compared to 8/10 HRC samples (1.5 μM vs 20 μM, p=0.047). There were no clinicopathological or IHC markers which accurately correlated with HRR status in this small cohort.

Conclusion: Endometrial cancer cell lines are a limited resource with a lack of DNA repair dysfunction. There was modest potentiation when Niraparib 1 μM was combined with IR, and no potentiation in combination with cisplatin.

It is possible to develop ex-vivo endometrial cancer cultures, but the lifespan of cultures is short. 3/18 patients were HRD with corresponding sensitivity to Niraparib.

This study justifies further exploration to determine the incidence of HRD in endometrial cancer, with initial studies suggesting a potential role in radiosensitisation and as a single agent treatment.

Further optimisation of primary cell culture is needed along with better models to test biomarkers for appropriate stratification of therapy. The results support taking forward PARPi into xenograft models and early phase clinical trials to determine exact role of PARPi in endometrial cancer.

Dedications

To Dr Wendy Dirks, one of life's true scientists. To my parents (David and Karen Blake) for always encouraging me to pursue my career aspirations without hesitation and giving me my very sociable side. And my brother (Daniel Blake), who inspires me every single day.

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Thanks to Rachel O'Donnell, Nicola Curtin and Ali Kucukmetin for your supervision, unwavering support, and dedication in getting me through this MD. I will always be grateful to Rachel for her insane enthusiasm and positivity which I will take forward with me. I have found her supervision at the pub as equally supportive and would consider her a friend. So much to say about Nicola Curtin. Her knowledge, commitment and passion for science knows no bounds and whilst she is not directly patient facing, everything she does has the many thousands and millions of women she's helped with her research. Her patience has had some testing with my MD, but I am forever grateful for her input. I think I've just about understood that negative results are, if not more exciting than positive ones!

Thanks to the team of consultants at the Northern Gynaecological Oncology Centre, Gateshead for their support and tolerating my whining about balancing clinical time and research (Christine Ang, Ann Fisher, Nithya Ratnavelu, Ali Kucukmetin, Stuart Rundle and Porphyrios Korompelis). Particularly Chris (this MD thesis was a bit of a JFDI moment!). Thanks to the pathology consultants at the QE (Angela Ralte and Bronia Slana) without whose support, over a chapter of my thesis wouldn't have been possible. Thanks to Holly Buist and the pathology team at the RVI for helping me interpret my TMA and undertaking the staining, without hesitation.

Thanks to the wonderful people I have met in the lab (Lucy Gentles, Hannah Smith, Santu Saha, Sweta Saha and Vicky Chamberlain). I think I've worked out it's an incubator not a 'hot fridge'.

To the main scientific role models in my life growing up, Wendy and Don, thanks for your encouragement to go on and do research.

I am extremely grateful to my family (Sophie, Reggie and Penny, the rest of the Blake's and the Booy family) who bring calm to my crazy work life. Finally, thanks to Olaf (my future husband), without him, I don't think I'd have the self-belief to be who I am today. I'm excited to have our evenings back ☺.

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Abbreviations

Abbreviation	Full terminology
BER	Base excision repair
DDR	DNA damage response
DSB	Double strand break
EBRT	External beam radiotherapy
EC	Endometrial cancer
ER	Oestrogen receptor
FIGO	International federation of gynaecology and obstetrics
HNPCC	Hereditary nonpolyposis colorectal cancer
HRR	Homologous recombination
HRD/C	Homologous recombination deficient/competent
IHC	Immunohistochemistry
LVSI	lymphovascular space invasion
MMR	Mismatch repair
MSI	Microsatellite instability
NAD	Nicotinamide adenine dinucleotide
NAT	No adjuvant therapy
NER	Nucleotide excision repair
NHEJ	non-homologous end joining
OS	Overall survival
PARP	Poly (ADP ribose) polymerase
PCOS	Polycystic ovarian syndrome
PFS	Progression free survival
PR	Progesterone receptor
PTEN	Phosphatase and tensin analogue
ROS	Reactive oxygen species
SSB	Single strand break
TCGA	The cancer genome atlas
VBT	Vaginal brachytherapy

DNA	Deoxyribonucleic acid
RAD51	Rad 51 homologue
SRB	Sulforhodamine B
UV	Ultraviolet
XRCC	X-ray cross complementing
BSO	Bilateral salpingoophorectomy
TAH	Total abdominal hysterectomy
ATCC	American type culture collection
DAPI	4’,6-diamidino-2-phenylindole
Gy	Gray
HRC	Homologous recombination competent
IR	Irradiation
LC50	Lethal concentration 50
LD50	Lethal dose 50

Chapter 1. Introduction

1.1 Incidence

Endometrial cancer is the most common gynaecological malignancy. It is the fourth most common cancer in women with 9700 women diagnosed every year (2016-2018) in the UK and a prevalence of 320, 000 worldwide (Macmillan, 2018). It predominantly affects post-menopausal women with a peak incidence between 75-79 years. The incidence has increased by 59% from 1993 to 2018 with the largest increase seen in the 70-79 (85%) age group. This increase in incidence has been shown to be due to rising levels of obesity, increased life expectancy and the use of adjuvant hormonal therapy in the treatment of receptor positive breast cancer (Raglan et al., 2019) (Figure 1.1).

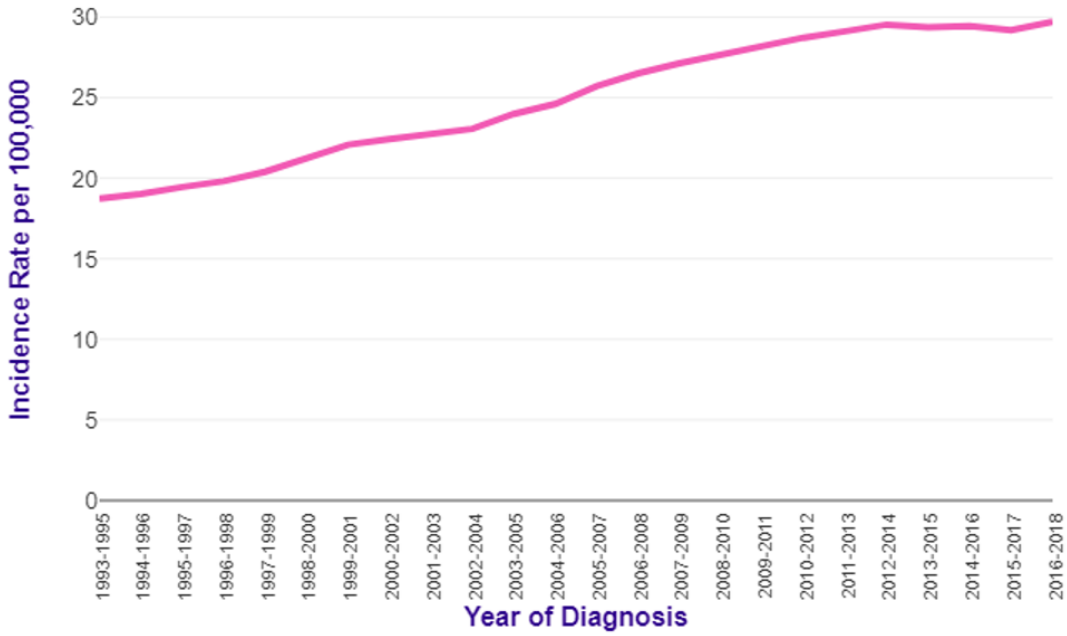


Figure 1.1.**Incidence of endometrial cancer** . Upward trend in the incidence of endometrial cancer in the UK(from (CRUK, 2022)) from under 20 per 100,000 women between 1993-1995 to 30 per 100,000 women between 2016-2018.

1.2 Survival

The overall five-year survival of endometrial cancer is 76%, with most women presenting with low grade tumours confined to the uterus (Figure 1.2). However, in those women who present with advanced disease or those with aggressive tumour histological subtypes, response rates to conventional treatments are poor (Crosbie et al., 2022). Whilst this high-risk group only represent 28% of endometrial cancer, it accounts for 78% of endometrial cancer related deaths (Boruta et al., 2009) and there is therefore urgent need for better treatment options for these women.

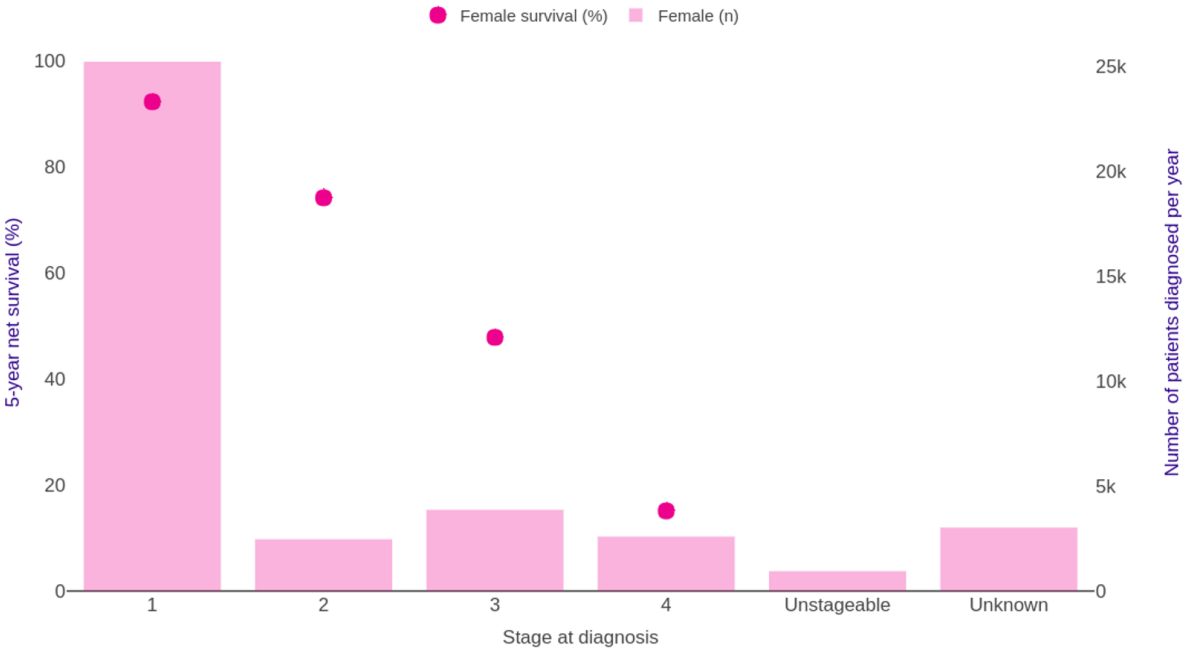


Figure 1.2. **Endometrial cancer survival by stage** . The 5-year survival in patients diagnosed with endometrial cancer in the UK (CRUK, 2018) from almost 100% in patients with stage 1 disease to around 15% in patients with stage 4 disease. It is worth emphasising that the majority have low grade disease which reflects the excellent survival, however patients with high grade tumours have a significantly poorer prognosis. Stage descriptions given in table 1.3.

1.3 Aetiology

Endometrial cancers are broadly subdivided into 5 main histological subtypes (see Section 1.4.1), of which endometrioid adenocarcinoma is the most common. Endometrioid endometrial cancer is associated with unopposed oestrogen stimulation with a recognised

precursor lesion, endometrial hyperplasia. The remaining histological subtypes are commonly described as oestrogen independent, arising within atrophic endometrium. Risk factors for endometrial cancer are outlined in Table 1.1.

Endometrial cancer risk factors
Advancing age Smoking Type 2 Diabetes mellitus Pelvic irradiation Oestrogen • Obesity through peripheral aromatisation of androgens to estrone • Hormone replacement therapy • Selective oestrogen receptor modulators (SERM, ie Tamoxifen) • Polycystic ovarian syndrome (PCOS through chronic anovulation) • Phyto-oestrogens • Nulliparity • Early menarche, late menopause • Oestrogen secreting tumours Genetic • Lynch syndrome • Cowden's syndrome • Family history of endometrial cancer (without germline mutations)

Table 1.1. **Risk factors for developing endometrial cancer**. The main driver for the most common type (endometrioid) is unopposed oestrogen therapy. Obesity and type II diabetes contribute as part of the metabolic syndrome with adipose tissue converting peripheral circulating androgens to oestrogen by aromatase (Raglan et al., 2019).

Around 2-5% of endometrial cancers are due to an underlying genetic predisposition. The most common of which is Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC) which is associated with 3% of endometrial cancers (Ryan et al., 2019). Lynch syndrome results in a greater risk of colorectal cancer, as well as endometrial, stomach, pancreatic and breast cancer (Kwon et al., 2011). It is an autosomal dominant syndrome which is characterised by germline mutations in at least one mismatch repair (MMR) gene which encode for the proteins MLH1, MSH2, PMS2 or MSH6. These proteins are responsible for genomic integrity by correcting DNA base mismatches resulting from replication errors (discussed in Section 1.7.5). Loss of function of MMR proteins is termed mismatch repair deficiency (MMRd) which leads to microsatellite instability (MSI), a hypermutated phenotype which results in increased cancer susceptibility (Ahadova et al., 2018). Women with germline MLH1 mutations have the greatest cumulative risk of developing endometrial cancer with a 60% lifetime risk of developing endometrial cancer. However, loss of MLH1 is most often due to epigenetic silencing by promoter hypermethylation and is therefore sporadic rather than part of Lynch syndrome (Constantinou and Tischkowitz, 2017).

A large proportion of endometrial tumours have been shown to harbour a high burden of MSI, the majority of which are due to somatic mutations in MLH1. Recent evidence suggests that these women may harbour mutations in genes involved in the repair of double stranded DNA breaks (such as MRE11) (Giannini et al., 2002, Koppensteiner et al., 2014).

Similarly, some families with endometrial cancer have been found to harbour germline mutations in DNA polymerase δ and ε (Burgers, 2009, Hindges and Hubscher, 1997), both of which are involved in the recognition of mispaired bases during replication and utilise the MMR system. The mutation in POLε encodes the catalytic subunit of DNA polymerase ε and POLδ1, a gene that encodes the catalytic subunit of DNA polymerase δ. POLδ1 mutations predisposes to early onset endometrial cancer as well as colorectal cancer (Palles et al., 2013).

Recent research has focused on genetic polymorphisms associated with endometrial cancer. Although many polymorphisms have been identified, their clinical significance remains unclear (Shai et al., 2014). Cowden's syndrome is a rare condition resulting from a mutation

in the tumour suppressor gene phosphatase and tensin homolog (PTEN). This autosomal dominant condition results in a 5 times higher risk of endometrial cancer as well as increased risk of thyroid and breast cancers (Tan et al., 2012, Eng, 2003).

There is conflicting evidence of the association of BRCA mutations and serous endometrial cancer, with recent genomic data supporting a potential link (Goshen et al., 2000, Shu et al., 2016, Moslehi et al., 2000, Thompson et al., 2002).

1.4 Classification of endometrial cancer

1.4.1 Histological subtypes

Epithelial endometrial cancer can be classified broadly histologically into endometrioid, clear cell, serous, mucinous, carcinosarcoma, mixed cell and undifferentiated, each with their own typical morphology and natural history (Table 1.2). Endometrioid histology accounts for around 80%. Tumours are graded using the International Federation of Gynaecology and Obstetrics (FIGO) classification system with an increasing proportion of solid growth pattern described from Grade 1 (well differentiated) to Grade 3 (poorly differentiated). In endometrial cancer grades 1-2 are low grade and grade 3 is considered a high grade tumour, grouped with the remaining histological subtypes. The majority of endometrioid tumours express both oestrogen (ER) and progesterone receptors (PR).

Serous cancers are the second most common (10%) subtype and are associated with a poorer prognosis, as there is often occult extrauterine spread to peritoneal surfaces, not dissimilar to high grade serous ovarian cancer. Clear cell endometrial cancer is uncommon with an incidence of 3%, often presenting at an advanced stage. Carcinosarcomas are a rare type of endometrial tumour which contain both epithelial and malignant stromal components but it is still regarded and managed as an epithelial tumour.

Gynaecological sarcomas are rare and account for 2% of uterine cancer. They are subdivided into mesenchymal tumours (leiomyosarcoma and endometrial stromal tumours) and mixed tumours such as adenosarcomas.

1.4.2 Pathogenic subtypes

On the basis of differences in histology and clinical outcomes, Bokhman separated endometrial cancers into two groups, type I and 2 tumours (Bokhman, 1983). Type II tumours are less differentiated and have a poorer prognosis in comparison to type I tumours, and they account for a disproportionate number of endometrial cancer deaths (40%). The genetic alterations found in type I and type II cancers consistently differ suggesting that they have distinct aetiologies (Sherman, 2000, Hecht and Mutter, 2006, Setiawan et al., 2013).

In clinical practice, establishing the correct histological subtype and therefore pathogenic subtype on pre-operative biopsies remains difficult with recognised inter-observer variability (Scholten et al., 2004). A review of serous cancers in two tertiary gynaecological cancer units found variable expression of p53, ER and WT1 status in serous carcinomas, concluding that immunohistochemistry (IHC) alone is not reliable in pre-resection diagnostic biopsies (Murali et al., 2019). Several studies have further demonstrated wide interobserver variability and poor reproducibility using morphology alone (Han et al., 2013, Nedergaard et al., 1995, Gilks et al., 2013). Inaccuracies in the interpretation of diagnostic biopsies has implications regarding extent of surgery and discrepancies between diagnostic and resection pathology makes recommendation for adjuvant therapy difficult (Trimble et al., 1999).

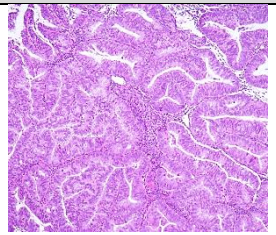
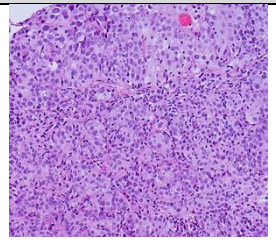
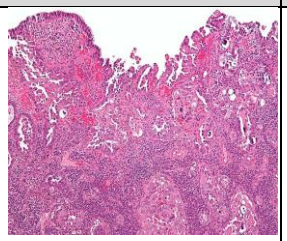
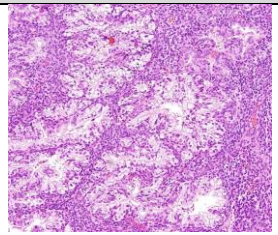
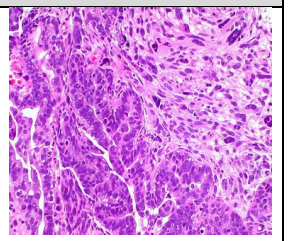
	Endometrioid		Serous	Clear cell	Carcinosarcoma
Morphology					
% of endometrial cancers	>70%	5%	5-10%	5%	<5%
Bokhmann class	1	2			
FIGO Grade	1/2	3			
5-year survival	85%	50%			
Genomic stability	Diploid (MSI high 40%)	Aneuploid			
Common mutations	PI3K, K-RAS, PTEN, MSI, β-catenin	P53, P16, HER2, ARID1A (clear cell)			
Hormone receptor status	Positive (ER/PR)	More commonly negative			

Table 1.2. **Endometrial cancer classification** into Type I and Type II tumours showing histology subtypes with representative image, frequency, genetic alterations, hormone receptor and survival (adapted from (Morice et al., 2016)). Whilst the common mutations are seen largely within one 'type', there is varying degrees of crossover. PTEN, PI3K mutations are seen across all subtypes, KRAS mutations are not seen in clear cell cancers.

1.4.3 Molecular subtypes

The Cancer genome atlas (TCGA) proposed four discrete groups within high grade (type II) endometrial cancer based on tumour biology (Cancer Genome Atlas Research et al., 2013) which has been subsequently confirmed in large cohorts in Canada and the Transportec group (Edmondson et al., 2017, Kommoss et al., 2018a, Auguste et al., 2018).

The TCGA studied 373 endometrial cancers comprising grade 3 endometrioid and serous subtypes. Integrated genomic, transcriptomic and proteomic characterisation enabled characterisation into four distinct groups (Figure 1.3 and 1.4);

1) copy number high, serous like (based on the somatic copy number alterations) (26%)
2) copy number low (39%)
3) microsatellite instability (MSI) hypermutated (28%) and,
4) POLε ultra-mutated (7%).

This categorisation revealed a proportion of endometrial cancers that behave more aggressively (copy number high) in comparison to other groups with a more indolent behaviour (POLε ultra-mutated).

The copy number high, serous-like group show high prevalence of TP53 mutations and copy number alterations, and had the worse overall survival. When examined more closely they have specific alterations in for example, IGF1R and FGFR1/3 genes that encode for tyrosine kinases, which when activated trigger a cascade that leads to the activation of multiple signal transduction pathways such as RAS/RAF/MAPK/PI3K promoting cell proliferation, survival, invasiveness (epithelial-to-mesenchymal transition) and angiogenesis (Li et al., 2018a). Serous uterine cancers appear to share similar genomic features with ovarian serous and triple negative breast cancers, both of which harbour defects in homologous recombination DNA repair (HRR) (see Section 1.7.9).

The POLε group, which have a mutation exonuclease domain of the catalytic subunit of DNA polymerase epsilon (POLε), have a high mutation load but an excellent prognosis. The TCGA study also demonstrated that high grade endometrioid cancers are a heterogenous group with 25% harbouring TP53 mutations, associated with poor prognosis, whilst 7% have POLE mutation with a more favourable prognosis. This molecular classification, with more accurate prognostic prediction, is forming the basis for clinical trials stratifying patients into

treatment groups based on endometrial cancer biology. The Royal College of Pathology have a minimal requirement of reporting in endometrial cancer which includes, ER, PR, p53 and MMR protein IHC status. With NICE recommending POLε studies as well as Lynch syndrome testing in selected individuals (NICE, 2019), this will facilitate enrolment into clinical trials, provide prognostic information, epidemiological analysis as well as identify those at risk of genetic syndromes.

1.4.4 Molecular classification in clinical practice and implications

Following the identification by the TCGA of 4 molecular subtypes in endometrial cancer, the **Pro**active **M**olecular R**is**k Classifier for **E**ndometrial Cancer **(ProMisE)** was developed and validated for clinical use. It includes a combination of IHC marker staining (p53 and MMR proteins) and targeted sequencing of 11 known pathogenic variants in the POLE exonuclease domain to assign endometrial cancers into POLE ultramutated, dMMR, NSMP and p53abn (León-Castillo et al., 2020a, McAlpine et al., 2018). Attempts have been made to classify into these groups using clinicopathological data alone (Karnezis et al., 2017, Kommoss et al., 2018b, Talhouk et al., 2015, Talhouk et al., 2017). Whilst most of the serous cancers are p53abn and low-grade cancers fit into the NSMP groups, it is difficult to accurately classify high grade cancers without specific targeted testing. In 2020, the 5th edition of the WHO Female Genital Tract Tumours recommended the integration of the molecular classification in the diagnosis of all endometrial cancers (McCluggage et al., 2022). MMR IHC testing has been widely integrated, due to its implication on germline testing and available immunotherapy options in these patients. The cost and speed of POLE testing has been a major barrier to its introduction. A more targeted approach to testing has been suggested by the British Association of Gynaecological Pathologists where it would alter management (Betella et al., 2022).

The order of testing is important as there is a 3-5% risk of an endometrial cancer having multiple classifiers (León-Castillo et al., 2020b). It has been identified that POLEmut-dMMR and POLEmut-p53abn endometrial cancers have morphology and clinical behaviour aligned with POLEmut tumours and should therefore be considered as such. Similarly, dMMR-p53abn tumours have similar clinical behaviour as the dMMR only group and therefore should be considered as dMMR tumours.

The tumours in each molecular subgroup are likely to require different approaches to adjuvant therapy and is currently being investigated in clinical trials. POLEmut endometrial cancers, particularly in the early stage, have an excellent prognosis and this is independent of the adjuvant therapy received and are likely to allow for treatment de-escalation. dMMR tumours are likely to be less chemosensitive and are known to respond well to immunotherapy, whereas p53mut cancers are aggressive and are likely to have a better outcome with the addition of systemic chemotherapy, even in early stage disease (McAlpine et al., 2021).

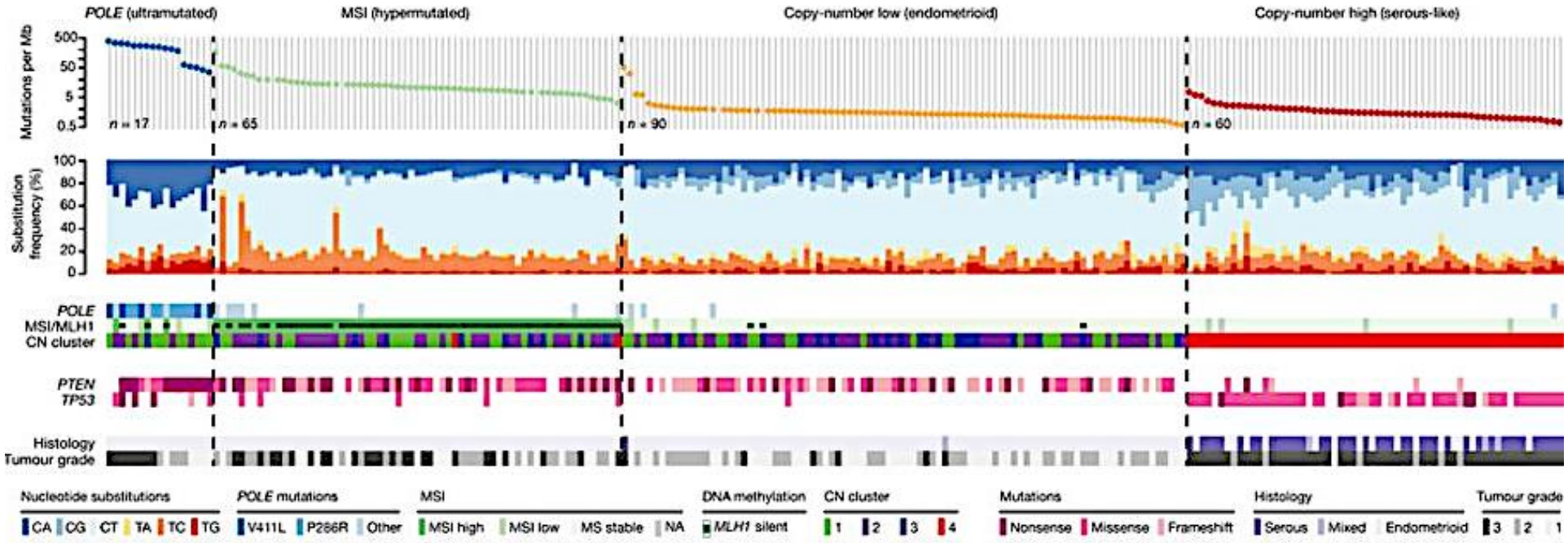


Figure 1.3. **The Cancer Genome Atlas (TCGA) study in endometrial cancer** undertook integrated genomic, transcriptomic and proteomic characterisation using array and sequencing in a cohort of all endometrial cancers types Similarities were noted between different tumours in terms of copy number and genomic alterations. The TCGA clustered endometrial cancers into four categories: POLε ultramutated, microsatellite instability hypermutated, copy-number low, and copy-number high, with PFS worsening across the groups. Image from Levine et al (Cancer Genome Atlas Research et al., 2013).

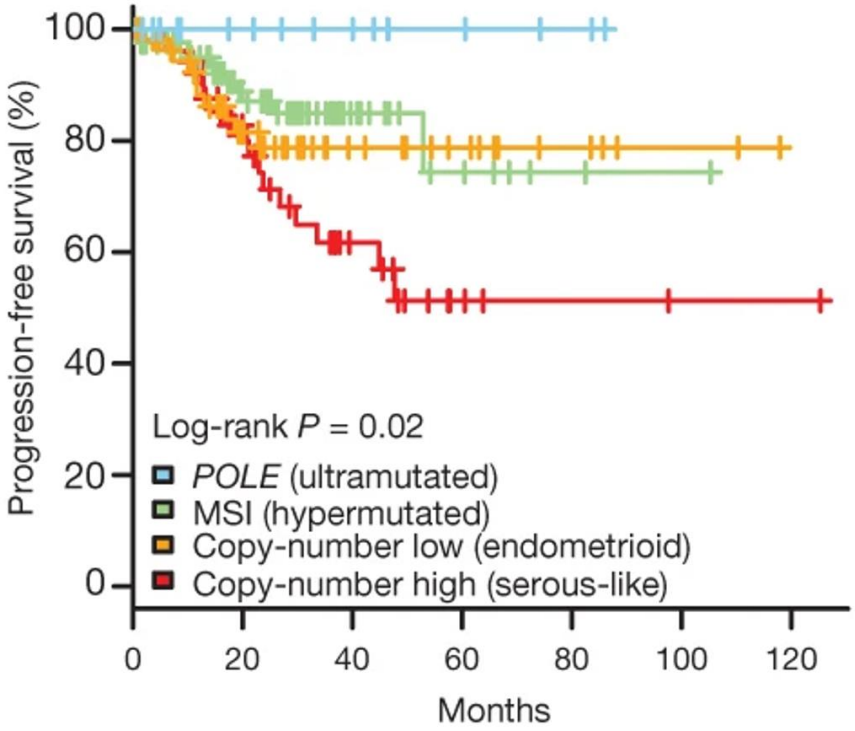


Figure 1.4. **The progression free survival between endometrial cancer TCGA subgroups**. Image from Levine et al(Cancer Genome Atlas Research et al., 2013). A clear difference in progression free survival was noted between each group in terms of copy number alteration and mutation profile.

1.5 Endometrial cancer staging

The International Federation of Gynaecology and Obstetrics (FIGO) classifies endometrial cancer into four stages (Table 1.3). This encompasses all epithelial subtypes but not endometrial sarcomas.

FIGO Stage		Description	5-year survival
1	1A	<50% myometrial invasion, confined to uterus	92%
	1B	>50% myometrial invasion, confined to uterus	
2		Cervical stromal involvement, confined to uterus	74%
3	3A	Invasion to uterine serosa and/or adnexal involvement	48%
	3B	Vaginal and/or parametrial invasion	
	3C1	Metastases to pelvic lymph nodes	
	3C2	Metastases to para-aortic lymph nodes	
4	4A	Invasion to bladder and/or bowel mucosa	15%
	4A	Distant metastases e.g. abdomen, liver, lungs, bone, inguinal nodes	

Table 1.3. **FIGO staging for endometrial cancer** 2018 with 5-year survival. Adapted from Creasman (2009).

1.6 Treatment

1.6.1 Surgery for apparent early-stage disease

Surgery is the cornerstone of treatment of early-stage endometrial cancer and is necessary in the majority to accurately stage the cancer. For most patients who present with Stage 1A disease (endometrial cancer confined to the uterus), surgery alone is curative. Surgery for endometrial cancer consists of removal of the uterus, cervix, fallopian tubes and typically ovaries. For Bokhman type II tumours (serous, clear cell, carcinosarcoma or G3 endometrioid subtypes), confined to the uterus on pre-operative cross sectional imaging, the addition of lymph node assessment is recommended (Creasman et al., 1987). A minimally invasive surgical approach (laparoscopic or robotic) reduces morbidity, in comparison to a traditional laparotomy, without impacting on survival (Janda et al., 2006). The role and extent of lymphadenectomy has been contentious with two randomised trials demonstrating no survival advantage but a more recent study showing a benefit of removal of all lymph nodes in the pelvic and para-aortic regions (Benedetti Panici et al., 2008, Todo et al., 2010). Sentinel lymph node biopsy has almost entirely replaced full systematic lymphadenectomy in those patients with non-bulky lymph nodes, with an associated reduction in morbidity without missing the lymph node most likely to be involved with cancer (Rossi et al., 2017, Nagar et al., 2021, Sozzi et al., 2020, Persson et al., 2019).

1.6.2 Adjuvant therapy in endometrial cancer

The aim of adjuvant therapy following staging surgery in endometrial cancer is to reduce the risk of recurrence. As most patients present with Bokhmann type I Stage 1A endometrioid endometrial cancer, surgery alone is sufficient, and they do not require adjuvant therapy. Stratification of adjuvant radiotherapy and/or chemotherapy is based upon histological subtype, stage, presence of lymphovascular space invasion (LVSI), surgical margin and molecular classification if available. Table 1.5 summarises the adjuvant therapy of offered to patients with endometrial cancer according to their risk stratification.

1.6.3 Radiotherapy

Adjuvant therapy in offered to patient according to risk factors in endometrial cancer and can include vaginal brachytherapy (VBT) with or without external beam radiotherapy (EBRT).

VBT is a form of internal irradiation using a small radioactive applicator delivered directly to the vaginal vault following hysterectomy. Addition of brachytherapy has been shown to reduce the rate of local recurrence from 14% to 7% (Nout et al., 2010, Creutzberg et al., 2000). EBRT is considered for the intermediate groups and involves provision of 50 Gy across 25 fractions with 5 fractions delivered per week. Use of EBRT has been shown to reduce risk of local recurrence from 14% to 4% (Keys et al., 2004) (Table 1.4). Whilst there is a reduction in local recurrence, adjuvant radiotherapy does not impact on overall survival in those women who have no treatment vs. adjuvant radiotherapy. EBRT can also be used for the treatment of inoperable advanced stage disease to reduce tumour burden and control symptoms (Colombo et al., 2013).

Trial	Patients Group	Surgery	Comparison	Locoregional recurrence	Survival difference
Norwegian (Aalders et al., 1980)	Stage I	TAH BSO	VBT vs VBT and EBRT	7% vs 2 %	None
PORTEC-1 (Creutzberg et al., 2000)	1b G2/3 Ic G1/2	TAH BSO	NAT vs EBRT	14% vs 4%	None
GOG-99 (Keys et al., 2004)	1b-2	TAH BSO and lymphadenectomy	NAT vs EBRT	12% vs 3%	None
ASTEC/EN5 (Group et al., 2009)	1a/b G3, IC, Stage II, Serous and clear cell	TAH BSO ± lymphadenectomy	NAT vs EBRT	7% vs 4%	None
PORTEC-2 (Nout et al., 2010)	>60 1b G3, 1c G1-2	TAH BSO	VBT vs EBRT	2% vs 2%	None

Table 1.4. **Randomised trials of adjuvant radiotherapy in endometrial cancer** and their results. They conclude that the risk of locoregional recurrence is lower when certain intermediate-high risk women are given adjuvant radiotherapy, but this does not impact on overall survival. 75% of recurrences occur at the vaginal vault, the PORTEC-2 trial set out to establish whether (vault brachytherapy) VBT was as good as (external beam radiotherapy) EBRT as well a quality-of-life indicators. Overall, there was no difference in locoregional recurrence between the two groups and no impact on overall survival. However, patients had a more favourable side effect profile with VBT. TAH BSO: Total abdominal hysterectomy and bilateral salpingoophorectomy. Table adapted from (Creutzberg and Nout, 2011). NAT =no adjuvant treatment. VBT= vault brachytherapy. EBRT= External beam radiotherapy.

Risk group	Molecular classification unknown	Molecular classification known	Adjuvant treatment
Low	Stage IA endometrioid + low-grade* + LVSI negative or focal	Stage I-II POLεmut endometrial carcinoma, no residual disease Stage IA MMRd/NSMP endometrioid	No adjuvant treatment
Intermediate	Stage IB endometrioid + low-grade* + LVSI negative or focal Stage IA endometrioid + high-grade* + LVSI negative or focal Stage IA non-endometrioid** without myometrial invasion	Stage IB MMRd/NSMP endometrioid carcinoma + low-grade* + LVSI negative or focal Stage IA MMRd/NSMP endometrioid carcinoma + high-grade* + LVSI negative or focal Stage IA p53abn and/or non-endometrioid** without myometrial invasion	Vaginal brachytherapy Surveillance is an option

High - intermediate	Stage I endometrioid + substantial LVSI, regardless of grade and depth of invasion Stage IB endometrioid high-grade*, regardless of LVSI status Stage II	Stage I MMRd/NSMP endometrioid carcinoma + substantial LVSI, regardless of grade and depth of invasion Stage IB MMRd/NSMP endometrioid carcinoma high-grade*, regardless of LVSI status Stage II MMRd/NSMP endometrioid carcinoma	External beam radiotherapy +/- VBT particularly for no nodal staging, extensive LVSI or Stage II Vaginal brachytherapy is an option if node negative Consider chemotherapy only if no nodal staging and extensive LVSI
High	Stage III-IVA with no residual disease Stage I-IVA non-endometrioid** with myometrial invasion, and with no residual disease	Stage III-IVA MMRd/NSMP endometrioid carcinoma with no residual disease Stage I-IVA p53abn endometrial carcinoma with myometrial invasion, with no residual disease Stage I-IVA NSMP/MMRd serous, undifferentiated carcinoma, carcinosarcoma with myometrial invasion, with no residual disease	EBRT +/- VBT Chemotherapy with carboplatin and paclitaxel

Advanced / Metastatic	Stage III-IVA with residual disease Stage IVB	Stage III-IVA with residual disease of any molecular type Stage IVB of any molecular type	Chemotherapy with carboplatin and paclitaxel

Table 1.5. **Risk categories for adjuvant treatment in endometrial cancer**. They are divided into low, intermediate, high-intermediate, high and advanced/metastatic. They are defined based on stage, grade, histological subtype, myometrial invasion and presence or lymphovascular space invasion (LVSI). The recent ESMO/ESTRO classification of risk in endometrial cancer now includes molecular profiling. The risk categories can be defined in the presence of the molecular classification or its absence. Possible adjuvant therapies are shown according to endometrial cancer risk. *=grade 1 or 2. POLε mut= POLε mutation, MMRd=Mismatch repair deficiency, NSMP=non-specific molecular profile/copy number low (tumours fall into this category when they do not fall into another), p53abn=p53 mutant staining pattern. This table was adapted from the 2020 ESMO/ESTRO/ESP guidelines (Concin et al., 2021).

1.6.4 Low risk endometrial cancer

Multiple studies have failed to demonstrate a survival benefit of adjuvant therapy in patients with low-risk endometrial cancer. Recent British guidance suggests omitting adjuvant therapy in patients with stage I-II disease if patients have a POLε mutation (Morrison et al., 2022).

1.6.5 Intermediate risk endometrial cancer

The addition of VBT in patients with intermediate risk disease is recommended to reduce risk of recurrence and consideration can be given to omitting it in younger patients. Whilst there is an improvement in progression free survival, there appears to be no survival advantage.

1.6.6 High-intermediate endometrial cancer

Management of high-intermediate endometrial cancer depends on whether nodal staging has been performed. If nodal staging has been performed and there is no LVSI, consideration should be for brachytherapy alone otherwise EBRT is recommended. However, if nodal staging has not been performed adjuvant EBRT is recommended, and consideration should be given to adjuvant chemotherapy if substantial LVSI is present. It should be noted that if molecular markers are known i.e. POLεmut or p53abn, consideration should be given to treating them as low risk or high risk respectively.

1.6.7 High risk endometrial cancer

EBRT with concurrent adjuvant chemotherapy or sequential chemotherapy (carboplatin/paclitaxel) and radiotherapy is recommended. If systematic lymphadenectomy has been performed, then consideration of adjuvant chemotherapy alone or with VBT should be given.

1.6.8 Adjuvant chemotherapy

The evidence for adjuvant chemotherapy in surgically staged early endometrial cancer is conflicting, as this is largely based on 9 heterogenous studies which included a variety of drugs and dosing regimens (Table 1.6). A Cochrane review demonstrated a small survival benefit (4%) in those women who were given adjuvant chemotherapy indiscriminately following surgery for endometrial cancer irrespective of whether they have had previous

radiotherapy (Johnson et al., 2011). However, the results of 3 recent randomised controlled trials taken together support the use of carboplatin-paclitaxel as first line standard of care in stage 3-4a disease and stage 1-2 serous endometrial cancers with myometrial invasion along with either concurrent or sequential radiotherapy (Concin et al., 2021).

Study, trial name	Stage	Arms	PFS	OS
Randall et al. (2006) GOG 122	3-4	Doxorubicin/cisplatin vs. Whole abdominal irradiation	Improved with chemotherapy	Improved with chemotherapy
Maggi et al. (2006)	1C-3	Cisplatin/doxorubicin/cyclophosphamaide vs. pelvic +/- para-aortic radiotherapy	No difference	No difference
Susumu et al. (2008)	1C-3	Cisplatin/doxorubicin/cyclophosphamide vs. pelvic irradiation	No difference	No difference
Kuoppala et al. (2008)	1A/B grade 3, 1C-3A	Pelvic irradiation, cisplatin /epirubicin /cyclophosphamide vs. pelvic irradiation	No difference	No difference
Hogberg et al. (2010) NSGO-EC-9501/EORTC-55991 and MaNGO ILIADE-III	1-3	Radiotherapy/chemotherapy (various chemotherapy regimens) vs. radiotherapy	Improved with chemotherapy and radiotherapy	No difference but a trend in pooled analysis towards improvement
Morrow et al. (1990)	1-3	pelvic +/- para-aortic radiotherapy /Doxorubicin vs. pelvic +/- para-aortic radiotherapy alone	No difference	No difference
Wolfson et al. (2007)	1-4 uterine carcinosarcoma	Cisplatin /ifosfamide /mesna vs. whole abdominal irradiation	No difference	No difference

Table 1.6. **Clinical trials in advanced endometrial cancer**. With chemotherapy +/- radiotherapy in high risk or locally advanced endometrial cancer, along with progression free or overall survival. The trials demonstrate the heterogeneity in studies and lack of consistent evidence.

1.6.9 Treatment of advanced stage/metastatic disease

Traditionally women with stage III/IV disease, receive a combination of palliative chemotherapy and radiotherapy without surgery. However, akin to the management of advanced ovarian cancer, there are reports of favourable survival with surgical cytoreduction (excision of all visible tumour) followed by adjuvant therapies, however high-quality data are lacking (Bristow et al., 2001, Bristow et al., 2002, Memarzadeh et al., 2002). A meta-analysis included 10 studies with patients undergoing cytoreductive surgery at primary presentation demonstrated that there was a 9-month survival benefit with every 10% increase towards complete cytoreduction. Cytoreduction in endometrial cancer demonstrates similar survival patterns to ovarian cancer in that those patients who have no residual disease after surgery (complete cytoreduction), have a survival benefit of 48 months, compared to disease <1 cm remaining (optimal cytoreduction) with survival of 23 months and worst is seen in those with residual disease > 1 cm after surgery (suboptimal cytoreduction) with survival of only 14 months (Eto et al., 2012).

The role of neoadjuvant chemotherapy (NACT) in advanced endometrial cancer is controversial due to lack of assessment in randomised controlled trials. However, there are several retrospective cohort studies describing the use of NACT followed by interval debulking surgery (IDS) in patients not suitable for upfront surgery, the largest studies demonstrating a response rate of 76% with carboplatin/paclitaxel (de Lange et al., 2019).

Chemotherapy typically consists of platinum agents, taxanes and anthracyclines. Historically doxorubicin (anthracycline) was used as a single agent but no overall survival advantage was seen until the introduction of taxanes (Bestvina and Fleming, 2016). The best response rates were seen in patients given paclitaxel/doxorubicin/cisplatin (TAP) when compared to doxorubicin/cisplatin (Homesley et al., 2009) (Table 1.7). However, the combination resulted in significant rates of myelosuppression. Results from GOG209, a non-inferiority trial comparing cisplatin/paclitaxel and TAP, which proved non-inferior with less myelosuppression and resulted in its widespread use (Miller et al., 2020a).

There is a lack of efficacious second line treatment regimes in those women who progress on first line treatment or recur out with the platinum sensitive window. Rates are disappointing with oxaliplatin showing the most promise since taxanes have been

transferred to the first line (Fracasso et al., 2006). The phenomenon of 'platinum sensitivity' or response is also seen in some patients in endometrial cancer. A multicentre retrospective cohort study demonstrated that the greater the time from original platinum chemotherapy, the greater the response rate, PFS and OS when re-challenged with platinum. Response rates of 25% were seen if within 6 months and 65% if greater than 24 months (Nagao et al., 2013). The phenomenon is seen in ovarian cancer and is used as a surrogate marker of homologous recombination repair deficiency (HRD).

Study	Arms	Progression free survival	Overall survival
Thigpen et al. (1994)	Doxorubicin Doxorubicin/cyclophosphamide	N/A	Improved with combination treatment
van Wijk et al. (2003)	Doxorubicin Doxorubicin/cisplatin	No difference	Trend towards improvement with combination treatment
Thigpen et al. (2004)	Doxorubicin Doxorubicin/cisplatin	Improved with combination treatment	N/A
Fleming et al. (2004b)	Doxorubicin/cisplatin Doxorubicin/Paclitaxel	No difference	No difference
Fleming et al. (2004a)	Doxorubicin/cisplatin Doxorubicin/cisplatin/paclitaxel	Improved with triple therapy	Improved with triple therapy
Miller et al. (2020a)	Carboplatin/paclitaxel Doxorubicin/cisplatin/paclitaxel	No difference	No difference

Table 1.7. **Phase III trials of chemotherapy used in the advanced or recurrent setting** and whether there was an improvement in progression free and/or overall survival. NA where information is not available.

1.6.10 Treatment of recurrent disease

Treatment of recurrent endometrial cancer depends on prior treatment modalities and extent of disease with no standard of care. However, it is agreed that an MDT approach should be used in these patients, often taking a multimodal approach. Women with an isolated vaginal recurrence, without prior irradiation, should be considered for radical radiotherapy which appears to result in better additional survival compared to surgery. For those women who have had prior radiotherapy, surgery should be considered if complete resection with clear margins is possible.

Patients with < 5 sites of disease (oligometastatic disease) should be considered for targeted radiotherapy or surgery with or without the addition of systemic therapy (Dhanis et al., 2022). Patients with multisite, widespread disease are however best considered for palliative systematic therapy. There is emerging evidence of the potential benefit of secondary cytoreductive surgery in selected patients, but the evidence is not of high quality.

Selected patients with grade 1-2 endometrioid cancer, with oestrogen/progesterone receptor positive tumours may benefit from hormonal treatment. Systemic therapy with the first line agents' carboplatin/paclitaxel in chemo naïve patients or those patients who are more than 6 months following platinum-based chemotherapy is another option. As mentioned above there is no standard of care in the second line setting in those patients who have relapsed <6 months following platinum chemotherapy. However, those patients who carry a POLε mutation or are MMRd, PD-1/PD-L1 inhibitors should be offered. Dostarlimab is available through the cancer drugs fund in those patients with dMMR/MSI-H tumours following progression on or following platinum chemotherapy (Oaknin et al., 2020).

1.7 DNA damage response (DDR)

Genomic instability (often due to dysregulation of the DDR) and tumour promoting inflammation is an enabling characteristic of cancer (Hanahan and Weinberg, 2011) as it results in mutations such as those described in Section 1.4.2 in endometrial cancer. Genomic instability is due to naturally high endogenous DNA damage, mostly by reactive oxygen species (ROS), that are increased in inflammation (another enabling characteristic) (Hanahan and Weinberg, 2011) combined with dysregulation of the DNA damage response (Hoeijmakers, 2009, Curtin, 2012). The DNA damage response (DDR) is a coordinated system of repair and cell cycle checkpoint signalling pathways which maintain genomic integrity. This allows for cell cycle arrest to ensure time for effective repair to take place and prevent damage transferring to daughter cells. The repair mechanism required depends on the type of damage incurred. Broadly, there are six pathways that respond to DNA damage: direct repair, single stranded DNA repair by single strand break repair/base excision repair (BER) for base damage and single strand breaks (SSBs) following e.g. ROS-induced damage, nucleotide excision repair (NER) of e.g. UV radiation damage, double strand break (DSB) repair comprising homologous recombination repair (HRR) that deals with stalled replication and DSBs in S/G2 and non-homologous end joining (NHEJ) of DSBs e.g. resulting from IR and from trapped Topoisomerase II (Hoeijmakers, 2001a, Bernstein et al., 2002). Dysregulation of the DNA damage response (DDR) is common in cancer presenting both challenges and opportunities. Accumulation of DNA damage can result in cancer but can also provide targets for anticancer treatments (Kennedy and D'Andrea, 2006, Lengauer et al., 1998). Upregulation of DNA repair mechanisms can lead to resistance to genotoxic therapy. inhibitors of upregulated pathways have the potential to sensitise cancer cells to standard treatment (Curtin, 2012). DDR defects can be exploited by the use of genotoxic therapy (e.g. loss of NHEJ confers radiosensitivity). Importantly, dysfunction of a DDR pathway can lead to dependence on compensatory mechanisms in cancer cells offering opportunity to target the relevant paired mechanism to selectively kill the DDR defective cancer cells with minimal host toxicity. The introduction of PARP inhibitors (PARPi) for breast and ovarian cancer, associated with BRCA mutations or other defects in HRR, is a recognised success story for DDR-targeted agents, which is now being extended to other cancer types. A detailed understanding of the DDR in endometrial cancer is critical for development of stratified targeted therapies needed to impact survival in high-risk endometrial cancer.

1.7.1 Base Excision Repair (BER) and single strand break repair

Single strand breaks are the most common endogenous lesions. They can be the result of several insults. Reactive oxygen species (ROS) are probably the most common cause. They cause lesions (Base oxidation (8-OHdG) and SSB) and can make SSB directly by oxidising the ribose-phosphate backbone as well as post damaged base removal.

Damaged bases are first removed by DNA glycosylases to form apurinic or apyrimidinic (AP) sites (globally known as abasic sites) which are then cleaved by an AP endonuclease (e.g., APE1) resulting in a SSB. SSBs can also arise directly through hydrolysis of the ribose-phosphate backbone. PARP1 binds to the SSB which results in its activation to form long negatively charged homopolymers of ADP-ribose (PAR) attached to itself and histone proteins in the vicinity of the break. This results in recruitment of XRCC1, PNPK and DNA polymerase as well as loosening the chromatin to allow repair. The repair pathway then splits into short patch repair depending (one nucleotide) and long patch repair (2-13 nucleotides replaced) depending on the nature of the 5' and 3' ends. Breaks with 3'OH and 5' phosphate can be repaired by short patch repair and breaks that require processing (e.g by PNPK if they have a 5'OH and 3') or with e.g. topoisomerase I bound are repaired by long-patch repair. In short patch repair, PolB replaces the missing nucleotide and ligase 3 (Lig3) joins the ends. PNPK is required if the break contains a 3'phosphate and 5' OH as this dephosphorylates 3' and phosphylates 5' end. In long patch repair POLd and POLe replace several nucleotides before FEN1 removes the resulting flap and Lig1 joins the ends(Lord and Ashworth, 2012) (Figure 1.5).

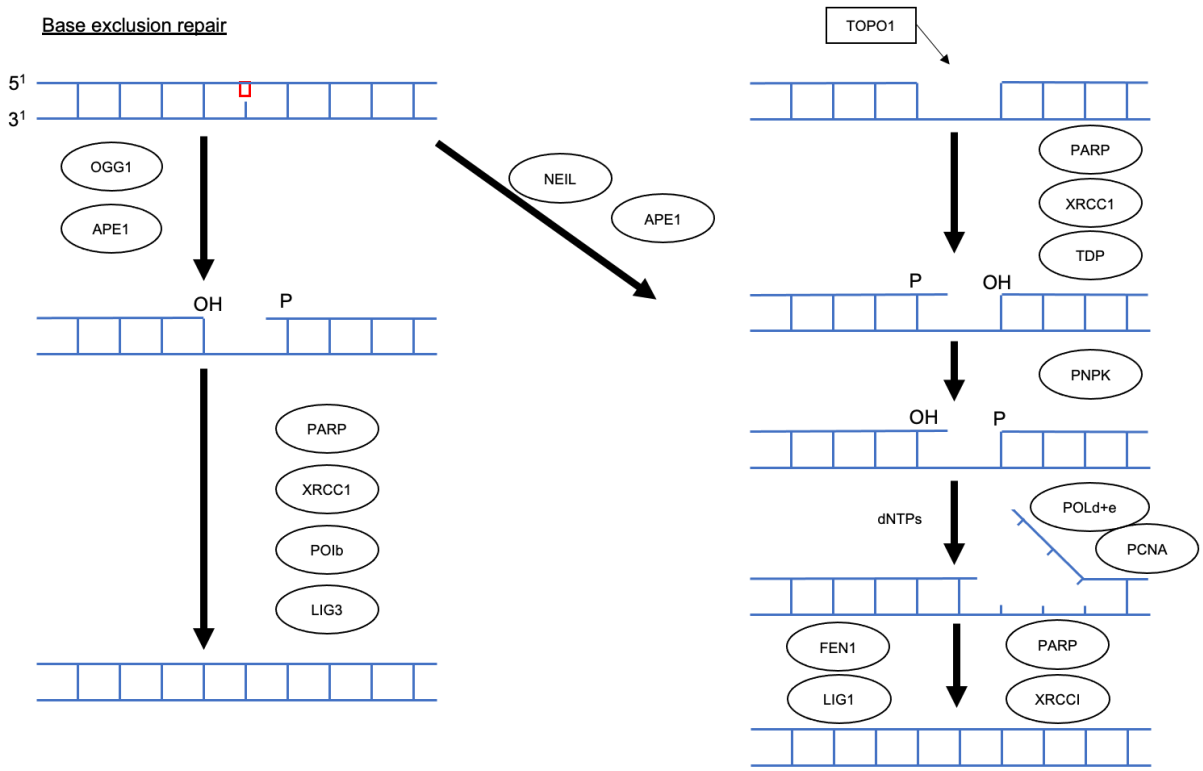


Figure 1.5. **Base excision repair (BER)/single strand break repair (SSBR) pathway**. Damaged bases are first removed by BER glycosylates forming abasic sites (OGG1 and NEIL are examples). BER endonucleases (such as APE1) hydrolyse the AP site causing a 'nick' resulting in a single strand break (SSB). The SSB can be repaired by short or long patch repair. PARP1 and XRCC1 facilitate both short and long patch repair by recruiting enzymes to aid repair and provide the scaffolding. ADP ribose polymers have a high negative charge this allows attraction of XRCC1 which can recruit DNA polymerase and polynucleotide kinase phosphatase (PNPK). PNPK can modify the broken ends to allow them to join g. DNA polymerases catalyse the repair of the breaks. Pol β in short-patch repair and Polδ and Polε in long-patch repair. DNA ligase III along with its co-factor XRCC1 repair the 'nick' in short-patch repair, whilst DNA ligase I ligates the break in long-patch repair (Baute and Depicker, 2008). Image from Curtin (2012).

1.7.2 BER aberrations in endometrial cancer

Oxidative damage is prevalent in endometrial cancer, predominantly because of increased metabolism, inflammation, and oncogenic signalling (Heidari et al., 2019), with upregulated BER promoting cancer cell survival (Abbotts and Madhusudan, 2010). Therapeutically, induced base damage caused by DNA alkylating agents, IR and topoisomerase 1 poisons

(TOPO1) are used in endometrial cancer. Alterations in BER can result in resistance to several anti-cancer therapies (Plo et al., 2003). These can be caused by germline and tumour-specific polymorphisms and mutations in BER genes (Larsen et al., 2007). BER is therefore an attractive target to modulate the effect of chemotherapy and radiotherapy. However, some studies outline how BER might contribute to endometrial cancer development. Analysis of TCGA data has shown that increased mRNA expression of XRCC1 in endometrial cancer is associated with a poor prognosis, which may be related to resistance to treatment(atlas, 2021). 15% of endometrial cancer harbour APE1 mutations suggesting inactivation may be part of carcinogenesis (Pieretti et al., 2001). High APE1 expression is associated with platinum and radiotherapy resistance in endometrial cancer alongside other cancers (Abbotts and Madhusudan, 2010). APE1 is overexpressed in high grade hormone insensitive endometrial cancer cell lines (Pandita, 2019). Polymerase β overexpression reduces the efficacy of IR and agents such as cisplatin. Several small studies have shown that up to 30% of cancers have a mutation in pol β including endometrial cancer (Canitrot et al., 1998). Endometrial cancer cell lines have been found to overexpress pol β protein and mRNA with siRNA mediating knock-down of pol β resulting in increased sensitivity to cisplatin (Albertella et al., 2005). PARP expression and activity in tumours is often high because of the high cell turnover and DNA damage in tumours or DNA repair defects that are compensated for by BER (Li et al., 2018b).The resultant high levels of PARP result in resistance to chemotherapy and IR, with inactivation of PARP shown to reverse this resistance (Rouleau et al., 2010). 85% of carcinosarcomas have been shown to overexpress PARP1 with a significant proportion of other endometrial cancer subtypes also with high expression (Ossovskaya et al., 2010). PARP2 overexpression in endometrioid grade 2 and 3 cancers is associated with shorter disease-free survival (Lawrence et al., 2020). Upregulation of FEN1 in endometrial cancer is associated with tumour progression and negatively effects prognosis (Zhang et al., 2020). However, up-regulation of LIG1 and 3 in endometrial cancer were not shown to be prognostic in the TCGA dataset.

1.7.3 Nucleotide excision repair (NER)

Bulky single-stranded lesions that distort the structure of the DNA double helix are repaired by NER. These lesions can be caused by UV radiation and tobacco smoke but also by chemotherapy drugs that cause intrastrand cross-links, e.g. cisplatin and NER contributes to

the repair of intra- and interstrand crosslinks such as those caused by platinum chemotherapy (Hoeijmakers, 2001a). Cisplatin causes crosslinks between adjacent (or adjacent plus one) guanines on the same strand (intrastrand crosslinks) or guanines of opposite DNA strands (interstrand crosslinks). Intrastrand crosslinks are repaired by NER but interstrand crosslinks are repaired by the Fanconi Anaemia (FA) pathway that includes both NER and HRR proteins (Rocha et al., 2018). NER is subclassified into 2 different pathways, Global genomic NER (GGR) which repairs throughout the genome and transcription-coupled NER (TCR) which addresses lesions at actively transcribing genes. The pathways differ in the components of their initial steps in GGR the recognition of the DNA lesions occurs with XPC-RAD23B and DDB, whereas in TCR initial detection occurs with CSA and CSB. XPA and TFIIH are common in both pathways and combine thereafter with XPG and ERCC1-XPF removing the damaged oligonucleotide, followed by resynthesis with DNA polymerase δ and ε using the undamaged strand as a template to fill the gap. The repair is completed by DNA ligase 3 re-joining the DNA to reform a DNA double strand (Figure 1.6).

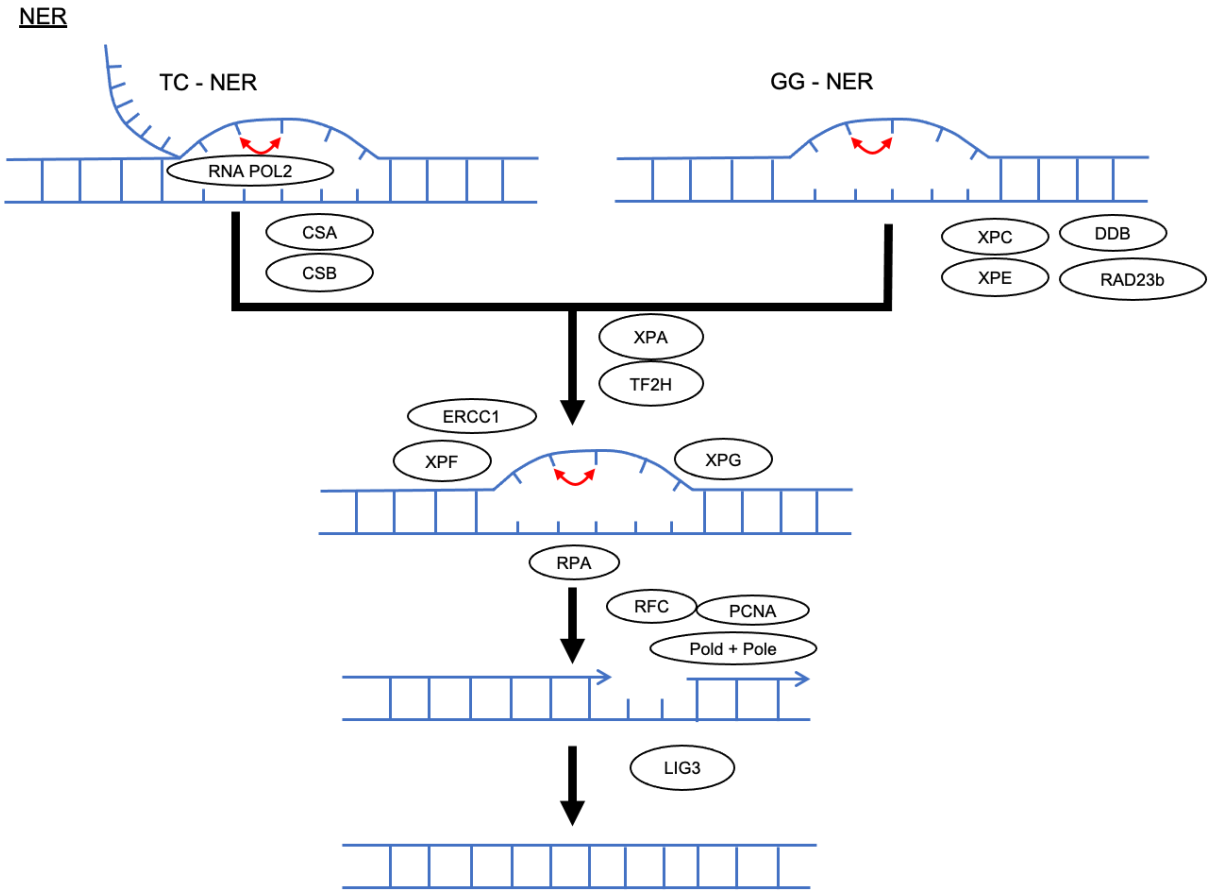


Figure 1.6. **Nucleotide excision repair (NER) pathway**. The NER process is a mechanism used to replace up to a 30 nucleotide strand of DNA containing the lesion (Curtin, 2012). The main

proteins involved include Cockayne syndrome WD repeat protein A and B (CSA and CSB) in TC-NER, the seven Xeroderma Pigmentosum Complementation group proteins (XPA to XPG), some exclusively for G-NER and some common of both pathway), the excision repair cross complementing group 1 protein (ERCC1), human Homolog of yeast (RAD23), Transcription Factor that possess Helicase activity (TFIIH) and Replication Protein A (RPA) (Curtin, 2012). Lesions that stall transcription use the transcription-coupled nucleotide excision repair pathway (TC-NER) (Gee et al., 2018). Lesions affecting the genome elsewhere use the global NER pathway (GG-NER). Initially these pathways differ in the recognition step. In TC-NER RNA polymerase II (RNA Pol II) senses the helix-distortion and is displaced by CSA and CSB. In GG-NER the damage is recognised by the XPC-RAD23 complex. The subsequent steps are common to both pathways. Both pathways use XPA and RPA which allow stabilisation of the heterodimer ERCC1-XPF and the transcription factor TFIIH (Doherty et al., 2011). TFIIH is then responsible for unwinding the DNA around the lesion, it does this using the helicases XPB and XPD (de Laat et al., 1999). The damaged oligonucleotide is cleaved by XPG in the 3' position and endonuclease ERCC1-XPF at the 5' end. DNA polymerase δ or ε resynthesizes and LIG3 ligates the gap with PNCA and Replication factor C (RFC) (Gee et al., 2018).

1.7.4 NER aberrations in endometrial cancer

Polymorphisms in the NER pathway have been observed to have associations with endometrial cancer risk. Polymorphisms in XPC, XPA, XPG and LIG1 have been shown to be associated with endometrial cancer (Doherty et al., 2011). Weiss J et al however found no association between risk of endometrial cancer and XPD or XPG (Weiss et al., 2006). Little is known regarding NER defects in endometrial cancer. A small number of studies have shown conflicting evidence of ERCC1 expression and chemotherapy response to platinum chemotherapy. Further studies are needed to explore NER in endometrial cancer.

1.7.5 Mismatch repair (MMR)

The mismatch repair (MMR) pathway detects and resolves incorrect paired bases and insertion/deletion errors that occur during replication The frequency of nucleotide misincorporation occurs at a relatively high rate ranging from 10^4 to 10^5 insertions. Misincorporations are proof-read by DNA polymerases in order to correct the wrongly incorporated nucleotides, but this is not infallible. The MMR pathway activity occurs post-replication and targets replication errors that have escaped proof-reading. These include

single nucleotide misincorporations and small insertion/deletion loops (IDLs) (Jiricny, 2013). MMR involves recognition and excision of a base mismatch in the newly synthesised strand followed by high-fidelity resynthesis and ligation of the strands, summarised in Figure 1.7.

MMR defects are associated with genomic instability, with high mutation rates and microsatellite instability (MSI). Microsatellites are repetitive sequences of nucleotides or dinucleotides in DNA e.g. a run of thymidines or CACACACA, these are sites where replication errors can occur and are repaired by MMR. In microsatellite instability, these errors are not corrected resulting in an accumulation of errors, which results when microsatallites are encountered, and DNA polymerase slippages occur resulting in deletions or insertions. This results in a microsatellite high (MSI-H) phenotype. While the exact mechanism of tumourigenesis is not clear, loss of post-replication proof-reading results in 100 to 1000-fold greater rate of accumulation of mutations, which not only increases the overall tumour mutation burden (TMB) but also enhances the risk of acquiring loss of function mutations in tumour suppressor genes (Loeb, 1991). MMR is important from DNA repair, cell cycle arrest and apoptosis. MSI is associated with cancer development, given the high mutational burden but particularly, if microsatellites in DDR proteins, caused by defective MMR, may also confer sensitivity to DNA-damaging agents used in cancer therapy (Li, 2008).

MMR is a strand specific stepwise process involved in the repair of replication errors which cause a wrong nucleotide (a mismatch) to be incorporated into DNA or nucleotide insertions and deletions (INDELs), particularly in microsatellites. Repair takes place during S phase (Tomkinson et al., 2006). MMR corrects the daughter strand and is therefore important in the correction of replications errors across from the 'normal' template strand. The repair system is split into 4 broad phases: recognition, recruitment of repair enzymes, excision and resynthesis using the parental strand by DNA polymerase. MSH2/MSH6 (MutSα) heterodimers recognise mis paired nucleotides and MSH2-MSH3 (MutSβ) detect deletions and insertions and at the site of DNA mismatches, downstream PMS2 and MLH1-MLH3 hetrodimers are required for processing. Exonuclease 1 excises the mismatch with replication protein A (RPA), proliferating cell nuclear antigen (PCNA), replicating factor C (RFC) and DNA polymerases δ and ε and Ligase 1 implicated in the system (Boland and Goel, 2010, Wu and Vasquez, 2008).

MMR defects are also associated with replication bypass of platinum adducts (intrastrand crosslinks) and is a mechanism for platinum resistance in cell lines.

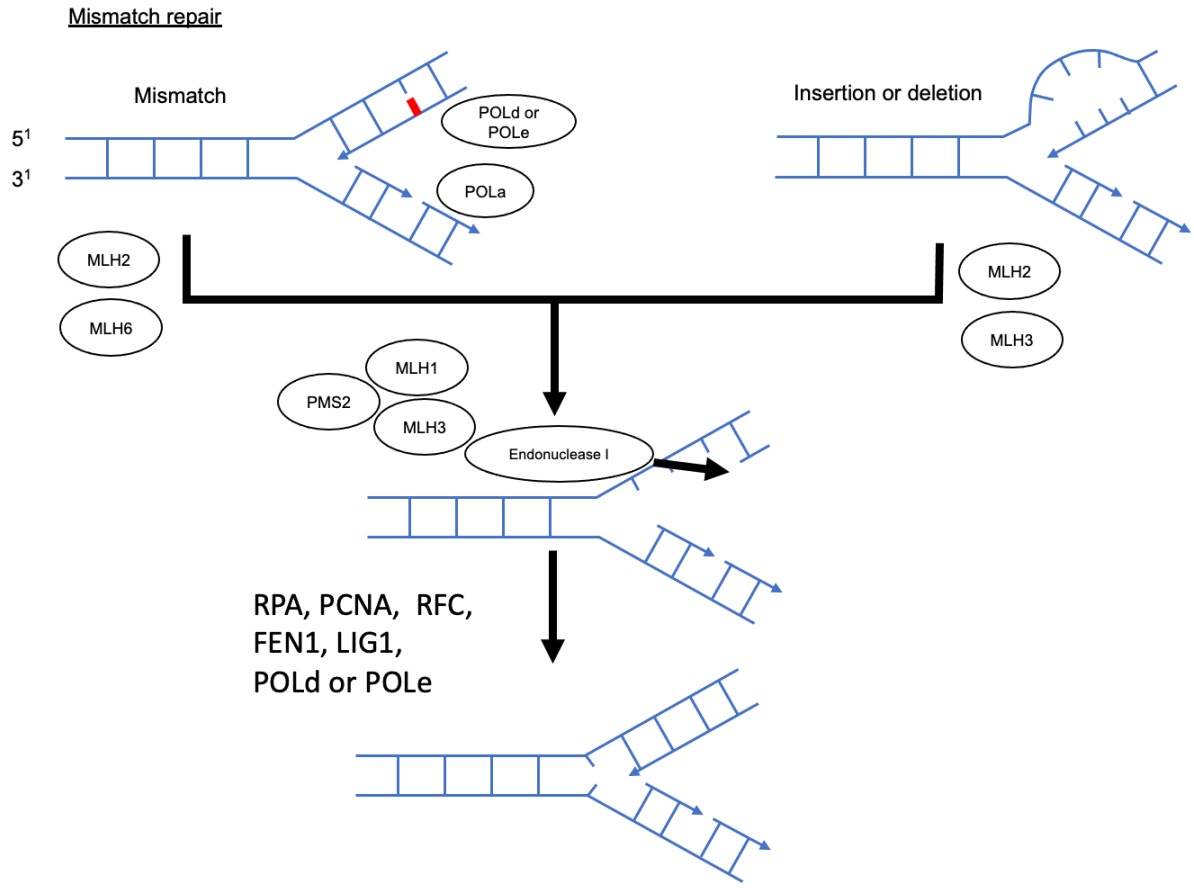


Figure 1.7. **Mismatch repair pathway (MMR) pathway**. Initially, MutS homologs recognise the error. MutSα (a MSH2/MSH6 heterodimer) recognises and initiates repair of single base mispairs and small INDELs of 1 - 2 nucleotides. MutSβ (MSH2/MSH3 heterodimer) is involved in repairing larger INDELs ranging from 1 - 15 nucleotides. Binding of MutS drives ATR-dependent conformational change in DNA and the recruitment of MutL (a protein complex comprising MLH1, MLH3 and PMS2). MutL binds to the site of damage resulting in influx of other DDR proteins including Endonuclease 1, resulting in excision of the damaged bases followed by influx of proliferating cell nuclear antigen (PCNA), replication factor C (RFC), DNA polymerase-δ (Pol δ) or Pol ε and flap endonuclease 1 (FEN1). Strand repair is achieved by DNA looping with repair initiated by the high-fidelity DNA polymerases (Pol δ/ε) and the break removed by DNA ligase (LIG1) (Jiricny, 2006).

1.7.6 MMR aberrations in endometrial cancer

The TCGA reported that 40% of endometrial tumours in their cohort had a MSI-H phenotype with the majority found in endometrioid histological subtype. This phenotype is predominantly due to MLH1 loss/silencing (somatic mutation) but may result from Lynch syndrome as previously discussed.

In the TCGA, MSI-H endometrial endometrial cancers harboured mutation frequencies as high as 18 x 10^{-6} mutations per Mb of DNA, which was found to be 10-fold greater than the microsatellite stable (MSS) tumours. These MSI-H endometrial cancers are also characterized with low frequency of somatic copy number alterations and a higher frequency of mutations in genes including KRAS, FBXW7, CTNNB1, PPP2R1A and TP53. Frame-shift deletions in RPL22 are found to be exclusively present in MSI-tumours. ARID5B is another gene, which is more frequently mutated in the MSI cluster of endometrial cancers with a frequency of ~23%. MSI clusters also show a characteristic activation of PI3K signalling pathway with high phospho-Akt and low phosphatase and tensin homolog (PTEN) expression. Therefore, the loss in MMR function could have implications in response to chemotherapy (Kandoth et al., 2013). Several DDR genes have microsatellites, for example, MRE11 (also known as MRE11A) and ataxia-telangiectasia mutated (ATM) and could be mutated in MSI-H cancer, potentially conferring sensitivity to some DNA-damaging agents (Giannini et al., 2002, Koppensteiner et al., 2014, Kristeleit et al., 2016).

Another subgroup from the TCGA study demonstrated that a proportion of endometrial cancers have somatic mutations in the catalytic subunit of polymerase epsilon (Pole). They represent around 7% of endometrial tumours and have a high mutation burden of 232 × 10^{-6} mutations per Mb. Loss of function of this polymerase leads to a high frequency of C → A transversions, few copy number alteration and microsatellite stability (Church et al., 2014, Meng et al., 2014, McConechy et al., 2016).

Platinum agents, used as the first-line therapy for advanced stage or recurrent endometrial cancers, form DNA adducts, which are recognised by the MutSb protein complex of MMR. This provokes a cell death response and therefore the cells with dMMR function are generally resistant to platinum agents such as carboplatin and cisplatin as a result of replication bypass (Brabec and Kasparkova, 2002). This was demonstrated in HEC59

endometrial cancer cell lines, deficient in MSH2, which were 1.5-fold more resistant to cisplatin and carboplatin as compared to a subline of HEC59 with wildtype MSH2 and is similarly seen in dMMR colorectal and ovarian cancer cell lines (Strathdee et al., 1999).

Despite high frequencies of MSI-H phenotype in endometrial cancer, the association between dMMR and disease prognosis is not clearly established. There is conflicting evidence as to whether defects in MMR confer resistance to IR (Resnick et al., 2010, Reijnen et al., 2019, McMeekin et al., 2016) or sensitivity (Fritzell et al., 1997, Brown et al., 2003, Franchitto et al., 2003, Fountzilas et al., 2019).

It is now standard of care in all patients with endometrial cancer to have their tumour tested for MMR protein loss by immunohistochemistry (IHC), as this has treatment implications in the recurrent setting as well as for screening for Lynch syndrome.

Studies of the tumour microenvironment in dMMR tumours have demonstrated prominent tumour lymphocyte infiltrates (TILs) consistent with an immune response to the tumour with frequent PD-L1 overexpression and POLE mutated endometrial cancers (Howitt et al., 2015, Piulats et al., 2017). Subsequently, there have been several trials with a response seen in endometrial cancer (Ott et al., 2017, Herbst et al., 2014, Le et al., 2017).

Immune evasion is a hallmark of cancer development (O'Connor, 2015). In normal circumstances, immune cells identify and kill cancer cells by recognising tumour antigens. The programmed death-1 (PD-1) pathway is one such example of an immune checkpoint involved in the regulation of T cell differentiation and apoptosis (Roche and Cresswell, 2016, Chen and Flies, 2013). PD-1 is a protein receptor expressed on immune cells and its interaction with programmed death-ligand-1 and 2 (PD-L1, PD-L2) inhibits the activation of cytotoxic T cells. PD-L1 is up-regulated in cancer cells and is responsible for tumour immune escape (Pardoll, 2012, Wu et al., 2019). Inhibitors of PD-L1 circumvent this have shown good anti-tumour activity (Topalian et al., 2012, Patel and Kurzrock, 2015).

Despite no direct clinical relationship in several cancer types, dMMR/MSI-H phenotype has been successfully exploited in cancer therapy due to the effect of this phenotype on tumour mutation burden (TMB). Increased TMB has been associated with a greater response to

immune checkpoint blockade (ICB) therapies, particularly those targeting PD-1 and PD-L1 immune checkpoint molecules, which block anti-tumour immune response (Yi et al., 2018). These findings led to the approval of pembrolizumab (PD-L1 inhibitor) as a second and higher-line choice for the treatment of patients with unresectable or metastatic dMMR/MSI-H solid tumours, irrespective of their tumour site (Marcus et al., 2019).

Given the success of immune checkpoint blockade therapies in CRCs (Le et al., 2015), several clinical trials have been initiated to test the efficacy of different ICBs such as pembrolizumab (NCT04014530) and avelumab (NCT02912572) in patients with MSI-H/dMMR phenotype in comparison to the MSS/pMMR EC patients with either metastatic or recurrent disease and dostarlimab (NCT02715284) which is now available through the cancer drugs fund in the UK for the treatment of those patients with advanced or recurrent endometrial cancer who may have progressed on or following platinum chemotherapy with MSI-H/dMMR tumours.

Anti-PD1 therapies are now approved in the second line in endometrial cancer. Results from the GARNET trial have resulted in the approval of dostarlimab as second line standard of care for patients with MSI-H/dMMR and MSS/MMRp. (Ana et al., 2022)Similarly, in advanced endometrial cancer the KEYNOTE-775 trial has shown a benefit of combination treatment with pembrolizumab and lenvatinib in both groups following relapse treated with platinum chemotherapy (Makker et al., 2022). The KEYNOTE-158 study has shown a benefit in the second line with pembrolizumab in recurrent dMMR endometrial cancers (O'Malley et al., 2022).

Interestingly, clinical evidence has suggested a strong correlation between PARP inhibition and (PD-1/PD-L1) up-regulation in different types of cancer. Therefore, the combined use of PARPi and anti-PD-L1 agents could show synergistic effects (Domchek et al., 2020, Chabanon et al., 2019).

1.7.7 Double strand break (DSB) repair

Double stranded breaks are highly lethal DNA lesions. Without successful repair they could ultimately result in cell death or mutation to daughter cells (Hoeijmakers, 2001a). SSBs can be converted to DSBs when they encounter replication forks. DSBs can also arise directly from ROS damage and can be caused therapeutically by IR and chemotherapeutic agents

such as topoisomerase II poisons (Curtin, 2012). The DDR process relies on the detection of DSBs and depending on the phase of the cell cycle and availability of certain proteins the DSB will either be repaired via non-homologous end joining (NHEJ) or homologous recombination repair (HRR). Both pathways rely on sensors, particularly the phosphatidylinositol 3-kinase-related protein kinase family (PI3K), including DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related protein (ATR). These proteins coordinate the initiation of cell cycle checkpoints and DSB repair (Helt et al., 2005). HRR can only take place in S and G2, as a complementary sister chromatid is required). NHEJ predominates in G1. In addition, 53BP1 and BRCA1 directly influence DSB repair choice by regulating 5' end resection. In G1 the resection of the 5' end is halted by the 53BP1/Rif1 proteins allowing Ku to bind and for NHEJ to predominate (Chapman et al., 2013). However, synthesis of BRCA1 in S and G2 phases inhibits Rif1 and allows 5' end resection with subsequent inhibition of NHEJ and repair by HRR (Feng et al., 2013).

1.7.8 Non-homologous end joining (NHEJ)

NHEJ repairs double stranded breaks throughout the cell cycle but predominantly in G0/G1 without the need of a sister chromatid (Curtin, 2012). Although efficient, Repair is not error free as broken ends are directly ligated without a homologous template (Lieber et al., 2003), which can result in loss of sequence and potential genomic instability. NHEJ is responsible for the repair of up to 85% of DSBs induced by IR (Mahaney et al., 2009, Beucher et al., 2009). ATM and DNA-PKcs play important roles in NHEJ by detecting the damage and signalling cell cycle arrest and repair by NHEJ respectively (Drouet et al., 2006). NHEJ is initiated by the binding of the Ku heterodimer (Ku70 and Ku80) to the DNA ends. Subsequently DNA dependent protein kinase catalytic subunit (DNA-PKcs) recruitment by the Ku heterodimer occurs. The collective unit (holoenzyme) of Ku70, Ku80 and DNA-PKcs is known as DNA-PK. DNA-PK stabilises and aligns the ends and facilitates recruitment the XRCC4/LIG4 complex to ligate the broken ends (Martensson and Hammarsten, 2002) (Figure 1.8). Defects in NHEJ have been associated with the development of cancer, as well as response to treatment in various tumours. Studies have shown that by inhibiting the effects of key components of NHEJ, this has resulted in profound radiation sensitivity. Studies in ovarian (Jin et al., 2016) and cervical (Hayashi et al., 2012) tumours have found that reduced Ku70 expression increases radiosensitivity.

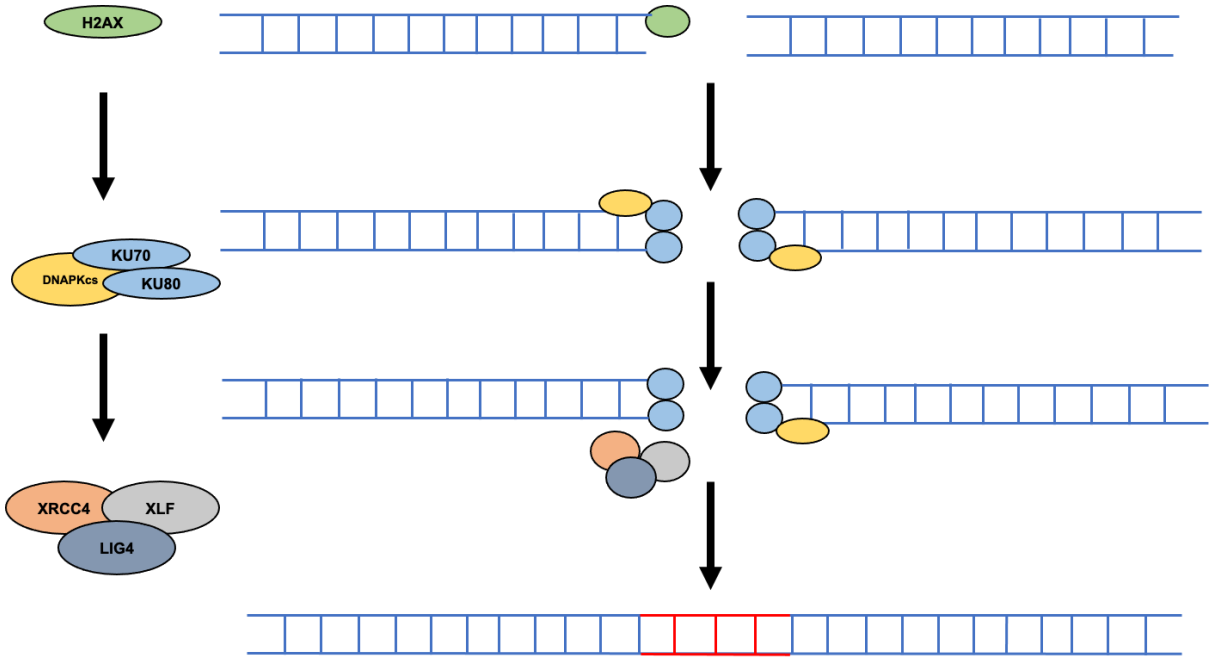


Figure 1.8. **Non-homologous end joining (NHEJ) pathway**. MRN nuclease complex is recruited to site of DNA double strand break and thought to promote bridging of the DNA ends. H2AX is phosphorylated and is located at the site of the double strand break. Repair requires the Ku heterodimer (composed of Ku70 and Ku80) to bind with DNA PKcs to form DNA-PK which binds to the DNA end, activating its serine/threonine kinase activity. DNA-PK holds the DNA together in a synapse and aligns the strands. DNA PKcs phosphorylates histone H2AX and itself which allows dissociation. Artemis processes the DNA ends which is then ligated by DNA ligase 4 (LIG4) and stabilised by XRCC4-XLF.

1.7.9 NHEJ in endometrial cancer

Defects in NHEJ are associated with profound radiation sensitivity. IR is used as part of standard care in those at increased risk of recurrence with localised disease. However, there is little known about NHEJ defects in endometrial cancer. There is some evidence that core NHEJ factors are downregulated in endometrial cancer. Lomnytska et al. (2012) compared protein expression in the normal endometrium to endometrial cancer. Whilst expression of Ku70 was abundant in the endometrium, levels were low in endometrial cancer, suggesting loss of NHEJ may play a role in the development of cancer and may be thus make endometrial cancers more sensitive to IR. Saygili et al. (2004) found a significant negative correlation between Ku70 expression and disease free survival (DFS) in irradiated patients with endometrial cancer. It has been suggested that DNAPKcs mutations may be present in

up to 34% of endometrial cancers with MSI (Bilbao et al., 2010). However, Doxorubicin, a topoisomerase II poison that induces DSBs is used in the second line in patients with advanced/metastatic disease, but response is poor with as little as 10% seeing any improvement which suggests defects in NHEJ are perhaps not so frequent or critical.

1.7.10 Homologous recombination repair (HRR)

HRR repairs DSBs as well as re-starting stalled replication forks. It occurs exclusively in S and G2 phases of the cell cycle as the process requires a sister chromatid to function as a template allowing robust high-fidelity repair (Saleh-Gohari et al., 2005). Whilst HRR repairs a small proportion of DSBs, it deals with stalled replication folks, single-ended DSBs and processing of inter-strand cross-links (along with NER). Tumours with HRR defects are highly sensitive to cross-linking agents such as platinum chemotherapy, IR and topoisomerase I poisons. HRR involves the resection of the break by endonucleases the invasion of the complementary sequence on the sister chromatid and re-synthesis to result in error-free repair as described in Figure 1.9.

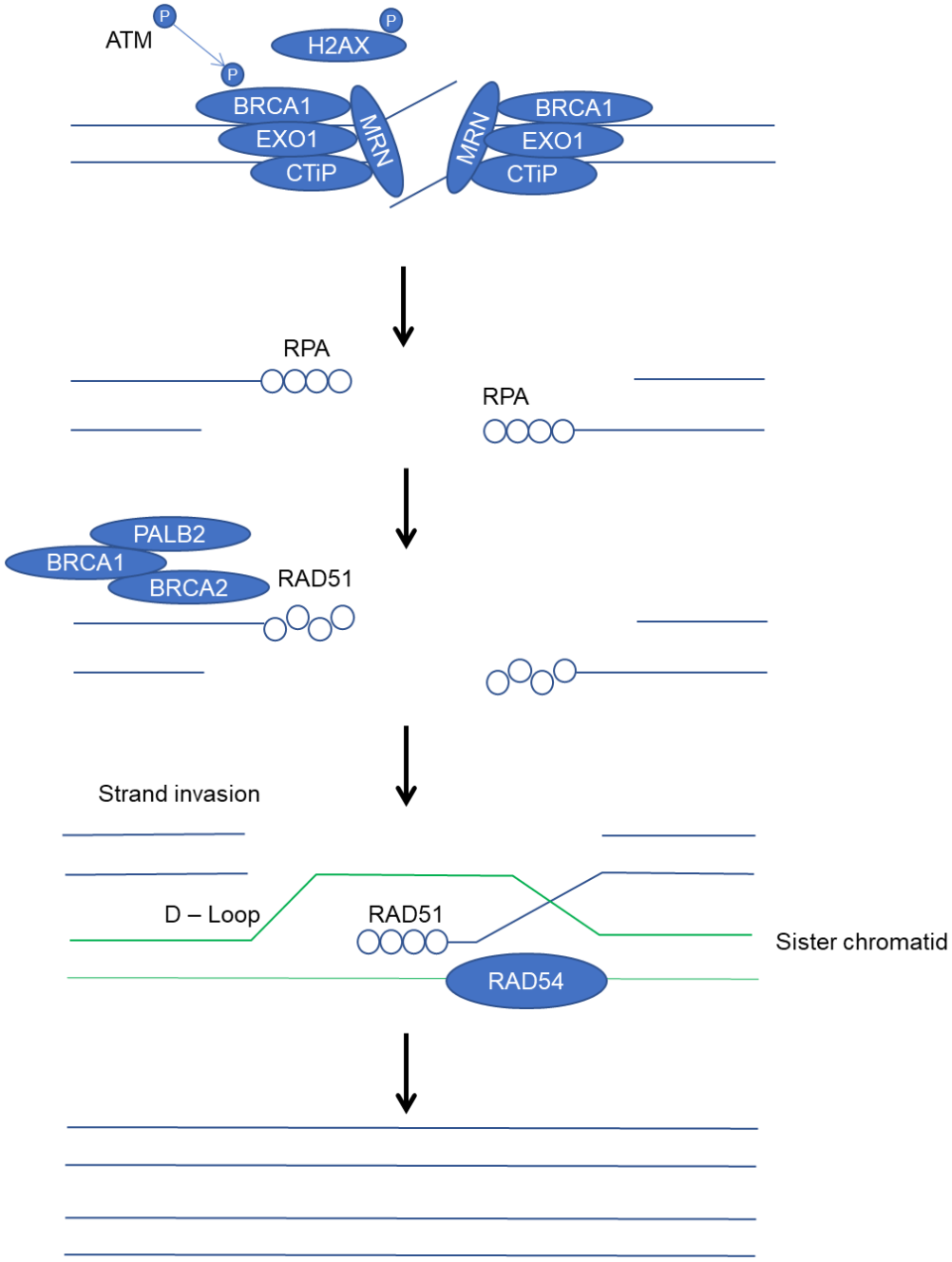


Figure 1.9. **Homologous recombination repair (HRR) pathway**. The DNA DSB is recognised by the MRN complex (a combination of MRE11, RAD50 and NSB1) and undergoes a series of conformational changes to attract, activate and increase affinity of ATM to the DSB (Balmus et al., 2019). MRN, along with CtBP-interacting protein (CtIP) and exonuclease 1 (EXO1) resects the DNA ends creating a single strand DNA overhang (Qiu and Huang, 2021). This is promoted by BRCA1. ATM stimulated NSB1, CtIP and EXO1 by phosphorylation and also phosphorylates H2AX which aids recruitment of DNA repair proteins such as BRCA1 (Kinner et al., 2008). RPA then binds to the DSB to facilitate the unwinding of the DNA secondary structure allowing access for DNA repair substrates and preventing DNA degradation of the

ends. BRCA2 (along with BRCA1 and PALB2) deliver RAD51 onto the RPA coated single strand ends (Chen and Wold, 2014). After identification of the homologous sister chromatid, the damaged 3' end invades the complementary DNA sequence of the sister chromatid creating a 'D loop' intermediate and primes DNA synthesis (Kennedy and D'Andrea, 2006, Moynahan et al., 2001, Davies et al., 2001, Cortez et al., 1999, Helleday et al., 2008). The complementary strand provides the template for error free DNA synthesis (Sung and Klein, 2006, Hartlerode and Scully, 2009). In DSB repair if both ends of the DSB are involved by invading the complementary sequence this creates holiday junctions (Sung and Klein, 2006, Hartlerode and Scully, 2009). During post-synapsis holiday junctions can be processed in one of three ways: either into non-crossover or cross over products or processed exclusively to non-crossover products by BLM-mediated branch migration and TOPOIIIα (Li and Heyer, 2008). After replication has extended past the region of the DSB, strand replication continues to the end of the chromosome (Hoeijmakers, 2001b, Khanna and Jackson, 2001, Bast and Mills, 2010). Image from (Cerbinskaite et al., 2012).

1.7.11 HRR and endometrial cancer

Data from TCGA demonstrated that copy number high endometrial cancers (predominantly serous subtype) have a similar genomic profile to high-grade serous ovarian cancers, triple negative breast cancer. These cancers share defects in the HRR proteins, particularly BRCA1/2, and there is some evidence from small case series to suggest that serous endometrial cancers may harbour germline mutations (de Jonge et al., 2017, Shu et al., 2016). However, there are several components in HRR which may have alterations.

Mutations in other proteins involved in HRR have been demonstrated in endometrial cancer, including RAD51c, PALB2, EMSY and ATM (Ring et al., 2016, Dedes et al., 2011). Similarly, MSI endometrial cancers may have resultant loss of the key HRR proteins. An example of this is MRE11, which is reported in 30% of endometrial cancer due to microsatellites in its promotor (Koppensteiner et al., 2014).

PTEN (frequently mutated in endometrial cancer), a tumour suppressor gene, functions as an antagonist of the PI3K/AKT pathway, promoting anti-proliferation and survival in the cell cycle(Shen et al., 2007). Furthermore, PTEN has been implicated in HRR by regulating the

expression of RAD51 (Shen et al., 2007) although the evidence is conflicting (Fraser et al., 2012).

More recently, further studies have demonstrated 24% of all high-grade endometrial cancer were HRR deficient (HRD) with the remainder being HRR competent (HRC). This was as the result 50% of non-endometrioid endometrial cancers (largely serous) in this cohort, harbouring a BRCA1 mutation or high-copy-number loss in other HRR genes, particularly RAD51 (de Jonge et al., 2018, Jönsson et al., 2021, Heeke et al., 2018). Another cohort study of serous cancers has shown that up to 40% had mutations in HRR genes (RAD51c, RAD50, ATM) and were sensitive to platinum agents, supporting the notion that platinum sensitivity could be a marker of HRD (Frimer et al., 2016). It is expected that around 4% of endometrial cancers harbour BRCA mutations (Gasparri et al., 2022). Understanding that there are a proportion of endometrial cancers that are HRD provides a rationale for treatment with a PARPi.

1.7.12 Methods of detecting HRD

There are broadly 3 ways to investigate whether a tumour is HRD. HRR gene level testing, genomic scars and signatures, and functional assays (Miller et al., 2020b). Firstly, sequencing of individual genes involved in HRR. BRCA 1 and 2 are the most common (and deriving the greatest benefit in clinical trials with PARPi) but there are a variety of genes involved in HRR that could confer HRD phenotype and are much less common (Qing et al., 2021). However, these genes can also develop reversion mutations which reinstate homologous recombination repair (Murciano-Goroff et al., 2022). The negative predictive value of these tests is also poor, as some BRCAwt patients also gained some benefit(Miller et al., 2020b).

Genomic scars are an alternative method of detecting ‘signs’ of HRD in cancers (Watkins et al., 2014). Cancers that have BRCA (and other HRR gene) mutations, exhibit genomic instability, demonstrated by large copy number variations (CNV) across the genome. These assays can predict HRD by quantifying large scale transitions (LST), loss of heterozygosity (LOH) and telemetric allelic imbalances (TAI) and are given a genomic instability score (Pacheco-Barcia et al., 2022). Current commercially available tests use a combination of BRCA mutation status GIS. It is helpful at identifying those patients who do not have a BRCA mutation but will derive benefit from PARPi. However, this method relies on good quality

tumour tissue for analysis, to obtain a meaningful result. It is also difficult to define a cut off value at which tumours are not HRD. An additional limitation of both mutational signatures and genomic scars is that they reflect of 'history' of HRD and do not provide information on current HRR status which could be reinstated through different mechanisms (Rempel et al., 2022).

Whole genome sequencing of cancers will reveal thousands of somatic mutations. The mutational patterns reflect historical mutational processes that have occurred in the cell (Alexandrov et al., 2020). Each mutational process may contain components of the DNA damage, repair and replication and can generate a characteristic mutational signature that can be detected. In high grade serous ovarian cancer, these mutational signatures correlate with survival and platinum response. Signature 3 is associated with BRCA mutation and BRCA1 promoter methylation in breast, ovary, pancreatic and stomach cancers (Batalini et al., 2022). They are likely to show promise, however they lack specificity and determining thresholds of derived benefit are difficult.

Functional assay, such as the RAD51 assay, have the potential to provide a real time assessment of HRR status (Miller et al., 2020b, Drew et al., 2011, Mukhopadhyay et al., 2010). However, they are temperamental and are not readily available for hospital practice. Currently tumour and germline BRCA testing is undertaken in clinical practice, along with assays that incorporate an assessment of genomic instability to determine which patients will derive benefit from PARPi therapy in ovarian cancer (Heitz et al., 2023).

All HRD tests fail to consistently identify those patients who will and will not derive benefit from PARP (Stewart et al., 2022)i.

1.8 PARP as a novel therapeutic target in Endometrial cancer

1.8.1 PARP structure and function

PARP1 is the founding and most abundant member of a family of 17 similar proteins. Only PARP1 and PARP2 are involved in BER/SSBR (Figure 1.5) PARP1 is composed of a DNA binding domain (two zinc finger motifs), an automodification domain and a catalytic domain. It also has a caspase cleavage site effectively separating the automodification and catalytic domain from the DNA binding domain and hence inactivation during apoptosis (Mégnin-Chanet et al., 2010). At the site of DNA damage, PARP1's DNA binding domain attaches inducing a conformational change and activating the enzyme several hundred-fold (Ray Chaudhuri and Nussenzweig, 2017). It catalyses the transfer of ADP-ribose residues from NAD^+ onto target substrates, building a poly (ADP ribose) (PAR) chain. This structural activation and production of PAR chains results in a strong negative change which attracts proteins for DNA repair and relaxes chromatin to allow for better access for these proteins. PARP2 has a catalytic domain but does not contain zinc finger motifs. Overall, the structure of PARP2 is like that of PARP1, however PARP2 binds less effectively to SSBs but instead recognises gaps and flap structures. This is reflected in its smaller DNA binding domain and lack of zinc fingers. Figure 1.10 shows the structure of PARP1 and 2.

The main role of PARP1 and 2 is to detect SSB's and initiate repair by signalling enzymes involved in SSB repair. PAR recruits XRCC1 which is a scaffold protein that then recruits PolB to replace missing nucleotide and Ligase 3 joins the ends. The cascade either results in repair via BER or leads to parthanatos.

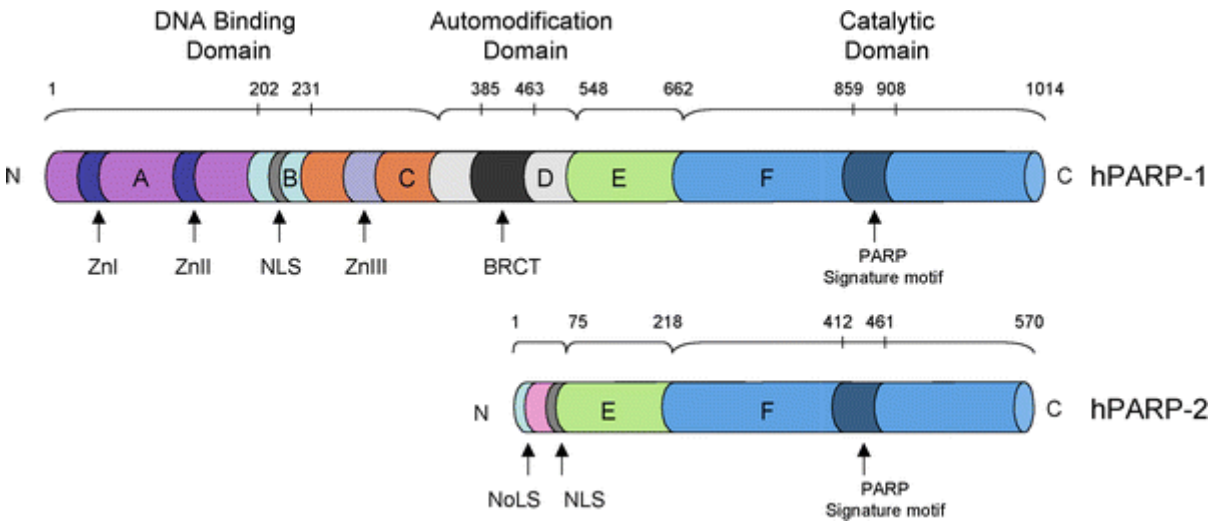


Figure 1.10. **Structure of PARP1 and PARP2** demonstrating their DNA binding, automodification and catalytic domains. Image from Mégnin-Chanet et al. (2010).

1.8.2 PARP inhibition

PARP inhibitors exhibit their response in two ways: Firstly, by inhibiting the catalytic activity of PARP, preventing autoPARylation and secondly by preventing its PARP's dissociation, so-called 'PARP trapping' (Pommier et al., 2016). When PARP is inhibited unrepaired SSBs collide with replication forks, causing them to stall and collapse, resulting in DSBs which can only be repaired by HRR. Each inhibitor of PARP has varying degrees of PARP trapping or ability to inhibit polymer formation which is likely to affect how each drug performs as a single agent or in combination (Curtin and Szabo, 2020).

1.8.3 Synthetic lethality and homologous recombination repair deficiency

If PARP is inhibited, SSB are converted to DSBs during replication. Alone, the resultant DSBs are not sufficient to cause cell death as repair can be undertaken by the high-fidelity HRR pathway. However, if the cancer cell is deficient in HRR repair genes, such as in BRCA1 and 2 mutated cells, cell death occurs. This phenomenon whereby two non-lethal mutations are combined to result in cell death is termed synthetic lethality (Dobzhansky, 1946) (figure 1.11). Similarly, defects in other proteins involved in HRR such as RPA, RAD51/4, MRE11, NBS1 ATM and ATR (McCabe et al., 2006) confer sensitivity to PARPi (Wang et al., 2004). Normal cells will not be affected if using PARPi alone as they exploit tumour HRD whilst sparing HRC normal cells. HRD also confers sensitivity to platinum chemotherapies (Mukhopadhyay et al., 2012) as well as PARPi (Ledermann et al., 2014, Farmer et al., 2005)

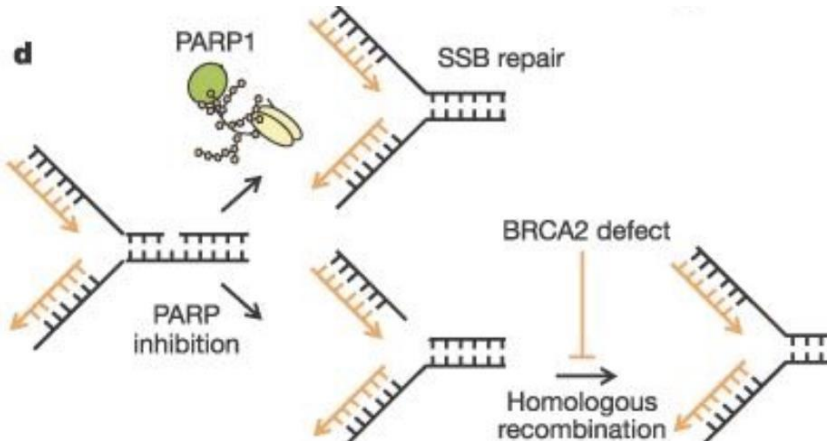


Figure 1.11**. Synthetic lethality** PARP inhibitors inhibit BER, trapping PARP to SSBs resulting in DSBs and collapsed replication forks. In cells with intact HRR DSBs can be repaired effectively. However, those cells defective in HRR are unable to repair these DSBs resulting in cell death (Bryant et al., 2005).

1.9 PARPi in cancer therapy

PARPi were initially approved for the use in the setting of patients with deleterious mutations of BRCA1 and 2. Since then further studies have highlighted additional patients where there is benefit of a PARPi, particularly those patients how have a defect in other HRR genes as well as patients who have responded to standard lines of chemotherapy with a platinum agent. Platinum response is seen as a biomarker to response to PARPi. PARPi are currently approved for use in the following cancers: Ovarian, breast, pancreatic and castrate resistant prostate cancer. There are currently 4 FDA approved PARPi; Rucaparib, Niraparib, Olaparib and Talazoparib. See Table 1.8 for list of approvals.

PARPi are well established in the treatment of epithelial ovarian cancer. Their benefit was demonstrated in all-comers with relapsed platinum sensitive patients following 2 previous lines of chemotherapy, with greatest benefit seen in those with germline and somatic BRCA mutations or HRD (Ledermann et al., 2014, Mirza et al., 2016). Subsequently, PARPi have been approved as maintenance in the first line setting with patients with response to first line platinum chemotherapy with and without BRCA mutation or in combination with bevacuzimab if HRD (González-Martín et al., 2019, Moore et al., 2018, Ray-Coquard et al., 2019).

Cancer type	Indication	Mutation or biomarker	PARP inhibitor			
			Niraparib	Rucaparib	Olaparib	Talazoparib
Tubo-ovarian stage 3-4	Maintenance following response to platinum	Germline or somatic BRCA1/2			X	
Tubo-ovarian stage 3-4	Maintenance following response to platinum	Response to platinum	X			
Tubo-ovarian stage 3-4	Maintenance following response to platinum. PARPi given with Bevacuizumab	HRD as defined by high GIS score and/or BRCA mutation			X	
Recurrent Tubo-ovarian	Second line maintenance following response to platinum	Response to platinum	X	X	X	
Recurrent Tubo-ovarian	Third line treatment	Germline or somatic BRCA1/2		X		
Recurrent Tubo-ovarian	Fourth line + Treatment	Germline BRCA1/2			X	
Recurrent Tubo-ovarian	Fourth line + treatment	Germline or somatic BRCA1/2 or HRD	X			
Metastatic breast cancer	Previous treatment with chemotherapy and or hormones	Germline BRCA1 or BRCA2 and HER2 negative			X	
Metastatic breast cancer	Treatment of metastatic breast cancer	Germline BRCA1 or BRCA2 and HER2 negative				X

High risk Early-stage breast cancer	maintenance follow treatment (not FDA approved but National Comprehensive Cancer Network (NCCN)	Germline BRCA1 or BRCA2 and HER2 negative			X	
Metastatic pancreatic cancer	Maintenance if not progressed <16 weeks from platinum chemotherapy	Germline BRCA1/2			X	
Metastatic prostate cancer	Maintenance if not progressed <16 weeks from platinum chemotherapy	Germline BRCA1/2			X	
Metastatic castrate resistant prostate cancer	Progressed following hormone treatment	Germline or somatic BRCA1/2 or other HRD gene			X	
Metastatic castrate resistant prostate cancer	Progressed following hormone treatment and taxane	Germline or somatic BRCA1/2		X		

Table 1.8. **PARP inhibitor approvals in cancers** , their indication, the mutation or biomarker required for their use and which PARPi can be used in that circumstance.

1.10 PARPi in endometrial cancer

Despite not knowing the true incidence of HRD in endometrial cancer, there is emerging pre-clinical and translational evidence for the role of PARPi in endometrial cancer with recruitment into clinical trials being undertaken (NCT05255653). As previously discussed, 24% of all and 50% of non-endometrioid/serous endometrial cancers are reported to be HRD and are therefore predicted to be sensitive to PARPi. There is conflicting evidence regarding the role of PTEN and whether it is linked with HRD, this may be important given that 80% of all endometrial cancer have PTEN mutations. Endometrial cancer cell lines have demonstrated a significant sensitivity to PARP inhibition in those with a loss of PTEN function. PTEN mutant cell lines were found to be more sensitive than wild-type. Furthermore, when wildtype PTEN cell lines had PTEN silenced, a significant increase in sensitivity was noted. Similarly, PTEN null cell lines with PARPi response were transfected with wild-type PTEN with associated reduction in PARPi sensitivity (Dedes et al., 2010, Dedes et al., 2011). This was further supported by increased sensitivity to Olaparib in PTEN mutated endometrial cell lines AN3CA and Ishikawa. The sensitivity was further increased with the use of the PI3K inhibitor BKM-120 which appeared to reduce RAD51 foci formation (Philip et al., 2017). However, subsequent studies appear to have refuted this (Bian et al., 2018) with no association found in a Japanese study between PTEN null and PTEN wildtype endometrial cancer cell lines when exposed to olaparib. The group did detect sensitivity within one cell line but they were unable to determine the mechanism (Miyasaka et al., 2014). Mouse models have demonstrated a response to olaparib in those PTEN null endometrial cancer xenografts- most notably in those mice in a hypoestrogenic state (Janzen et al., 2013).

MMRd may result in microsatellites in promotor region of HRR genes (e.g. MRE11, ATR among others), are unstable which results in indels that result in loss of gene expression. A study explored PARPi sensitivity in endometrial cancer cell lines and concluded that a cell line lacking in MRE11 expression was the most sensitive. As a result, the other cell lines were rendered MRE11 depleted, which resulted in PARPi sensitivity (Koppensteiner et al., 2014). However, there appear to be no studies that involve primary endometrial cancer culture with PARPi. As discussed previously, defective DDR results in a higher high proportion of TILs at the site of tumours, this has been demonstrated in BRCA mutated breast and ovarian

cancers (Jiao et al., 2017). The breast cancer cell lines and xenograft models also demonstrated an upregulation of PD-L1 with PARP inhibition and when combined with anti-PD1 agent, demonstrated greater tumour response. The was further supported by the phase 3 MEDIOLA study which showed a superior response to Olaparib and durvalumab in combination in platinum sensitive relapse ovarian cancer with germline BRCA mutations (Domchek et al., 2020). It is understandable that several trials have been commenced to explore this in endometrial cancer given that a proportion of these patients will be HRD.

There is emerging evidence of PARPi use in clinical trials. The phase 2 NRG-GY012 trial randomised patients 1:1:1 to cediranib (tyrosine kinase inhibitor) alone, Olaparib alone or a combination of the 2. In this unselected group with recurrent endometrial cancer having previously received at least one line of platinum chemotherapy, there was no significant improvement in progression free survival (Rimel, 2021). Another phase 2 trial assessing effect of Niraparib alone or in combination with anti-PD-1 dostarlimab in a similar setting, did show a modest benefit in both groups (Madariaga et al., 2021). The ENDOLA trial phase I-II trial presented at AACR 2022, has shown promise of the triplet therapy, olaparib, cyclophosphamide and metformin (You et al., 2022). However, further studies and biomarkers are needed to determine the patient group likely to receive the greatest clinical benefit.

1.11 Niraparib

Niraparib (MK-4827) (Jones et al., 2009) is an oral potent, selective inhibitor of PARP1 and 2 with IC50 of 3.8 nM/2.1 nM and half-life of 36 hours. It has a molecular mass of 320 g mol^{-1} and formula $C_{19}H_{20}N_4O$ (Figure 1.12). Inhibition results in the formation of PARP-DNA complexes, leading to DNA damage, apoptosis and cell death (Mirza et al., 2016). Dose is dependent on body weight and platelet count, For most patients It is taken orally at a dose of 200 mg daily, usually until disease progression of a maximum of 2 years. Most common adverse effects include low blood count, namely thrombocytopaenia, anaemia and neutropaenia. Other milder side effects include fatigue, nausea and constipation. 12% of patient in one study discontinued Niraparib due to adverse effects (Pagkali et al., 2022). Niraparib is licenced as maintenance after first line chemotherapy and for the management

of platinum sensitive, recurrent ovarian cancer as a third line agent. It first approval in the landmark NOVA trial then followed by the PRIMA trial It was initially shown to be effective in women with BRCA mutations. However, the benefit has been seen regardless of gBRCA mutations or HRD status. Quality of life studies have shown it to be a well-tolerated drug (Oza et al., 2018). Table 1.9 shows ongoing trials with Niraparib in endometrial.

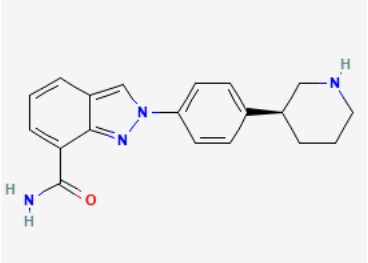


Figure 1.12. **Chemical structure of Niraparib** (NCBI, 2022).

NCT	Title	Status	Inclusion	Intervention	Trial type
NCT04716686	Niraparib monotherapy as maintenance and recurrent treatment of endometrial serous carcinoma	Recruiting	Stage 3 or 4 serous endometrial cancer • Completed 6 cycles of chemotherapy or, • Platinum sensitive recurrence or, • > 2 lines of platinum chemotherapy or a BRCA mutation	Niraparib as maintenance or monotherapy in recurrent disease	Non-randomised phase 2 trial
NCT04885413	An open-label, single Arm, phase II trial of Niraparib in combination with an-PD1 (programmed cell death protein 1) antibody in recurrence/advanced stage endometrial cancer patients	Recruiting	Recurrent or advanced high grade endometrial cancer Received at least first line chemotherapy with progression Progression of stage 3 or 4 disease following primary chemotherapy within 6 months Patients who cannot tolerate first-line chemotherapy	Niraparib + Sintilimab	Open label, single Arm, phase 2 trial

NCT03016338	Study of Niraparib and TSR-042 in recurrent endometrial cancer	Active, not recruiting	Recurrent endometrial cancer following first line platinum chemotherapy Not carcinosarcoma, clear cell	Niraparib + TSR-042 (anti-PD1 inhibitor)	Open label, phase 2 trial
NCT03586661	Niraparib and copanlisib in treating patients with recurrent endometrial cancer	Recruiting	Recurrent endometrial cancer in whom no curative treatment option is available	Niraparib + copanlisib (PI3K Inhibitor)	Open label phase 1b trial
NCT03651206	A multicentric randomised phaseII/II evaluating TSR-042 (anti-PD1 inhibitor) in combination with Niraparib vs. Niraparib alone compared to chemotherapy in recurrent endometrial or ovarian carcinosarcoma	Recruiting	Progressive or recurrent endometrial carcinosarcoma following at least 1 line of platinum chemotherapy	TSR-042 + Niraparib vs. Niraparib alone vs. Doxorubicin/paclitaxel/gemcitabine	Randomised phase 2 trial
NCT04080284	Trial of maintenance with Niraparib – uterine serous carcinoma	Recruiting	Advanced stage (3 or 4) or platinum sensitive recurrent uterine serous carcinoma	Niraparib maintenance following chemotherapy	Phase 2 trial
NCT03981796	A study to evaluate Dostarlimab plus carboplatin-paclitaxel verus placebo plus carboplatin-paclitaxel	Recruiting	Advanced stage (3-4) or first recurrence with low potential for cure	TSR-042/carboplatin/paclitaxel vs.	Phase 3 trial

	in participants with recurrent or primary advanced endometrial cancer			placebo/carboplatin/paclitaxel vs. TSR-042/carboplatin/paclitaxel/Niraparib vs. placebo/carboplatin/paclitaxel	
NCT04178460	A study Niraparib combined with MGD013 in patients with advanced or metastatic solid tumor who failed prior treatment	Recruiting	Advanced or metastatic endometrial carcinoma with disease progression or relapse following >1 cycle of standard chemotherapy	Niraparib + MGD013 (anti-PD1 and anti-LAG3 antibody)	Phase 1 trial
NCT00749502	A study of MK4827 in participants with advanced solid tumours or haematological malignancies	Completed	Persistent or recurrent endometrial cancer following >1 cycle of chemotherapy	Niraparib alone	Phase 1
NCT04159155	A study of various treatments in serous or p53 abnormal endometrial cancer	Recruiting	Pure serous endometrial cancer as part of front-line treatment	Carboplatin/paclitaxel vs. Carboplatin/paclitaxel/cisplatin/ERBT vs.	Phase 2/3 trial

				Carboplatin/paclitaxel/Niraparib	
NCT05169437	Niraparib in the treatment of patients with advanced PALB2 mutated tumours	Recruiting	Progressive/recurrent endometrial cancer with pathogenic mutation in PALB2 and exhausted all standard treatments	Niraparib alone	Phase 2 trial
NCT03221400	PEN-866 in patients with advanced solid malignancies	Recruiting	Progressive disease following >1 line of anticancer therapy where standard of care is deemed inappropriate treatment	PEN-866 (heat shock protein) + fluorouracil + folinic acid + Niraparib	Phase 1b trial

Table 1.9 **Ongoing clinical trials with Niraparib in endometrial cancer.**

Chapter 2. Hypothesis and aims

2.1 Hypothesis

The role of PARP inhibitors (PARPi) is firmly established in ovarian cancer but many other malignancies also harbour defects in homologous recombination DNA repair (HRR) and may therefore benefit from PARP inhibitors. We hypothesise

- a portion of endometrial cancers will have defective HRR function
- Defective HRR (HRD) is a biomarker for selective sensitivity to PARPi therapy in endometrial cancer.

2.2 Aims

2.2.1 Pre-clinical

In a panel of established endometrial cancer cell lines:

- Characterise for
    - HRR function using the functional RAD51 foci assay
    - PARP activity
    - Expression of diagnostic proteins using immunohistochemistry (IHC).
- Determine cell survival and growth inhibition in cell line panel to single-agent Niraparib, cisplatin, and irradiation.
- Determine survival and growth inhibition in cell line panel to combination therapies.
- Determine the concentration of Niraparib that inhibits cellular PARP activity by ≥90%.

2.2.2 Translational

- Establish and optimise a method of primary culture of endometrial cancer cells obtained from patient endometrial biopsies.
- Characterise successful cultures for
    - HRR function using the RAD51 foci assay
    - MMR/p53/ER/PR/PTEN by IHC
- Explore biomarker potential of common markers by IHC stratified by functional HRR status.

Chapter 3. Materials and methods

3.1 General laboratory practice

Laboratory practice was undertaken with the appropriate training and university regulations for safe working. This included completion of appropriate risk assessment and control of substances hazardous to health (COSHH) forms. Chemicals and reagents that were frequently used in experiments are presented in the tables below for reference. Below in the relevant experiment sections, preparation of specific reagents for investigative work is included.

3.2 Chemicals and reagents

Reagents were purchased from Sigma-Aldrich (UK) unless stated otherwise. Cisplatin (cis-Diammineplatinum (II) dichloride) was dissolved in sterile 0.9% w/v sodium chloride, passed through a sterile 0.2-micron filter and made to a stock concentration of 1 mM, aliquoted and stored at -20 °C. Niraparib tosylate monohydrate (PARP1 and 2 inhibitor) was a kind gift from Tesaro® Inc, (USA) and was dissolved in dry dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM, aliquoted and stored at -20 °C.

3.3 Cell lines

Six endometrial cancer cell lines have been used in experimental work. Cell lines were chosen based on what was commercially available and to reflect the variety of histological subtypes, including serous endometrial cancers, where we would expect to see HRD from the TCGA data. It was important to include cell lines that were known to have p53 mutations and MSI-H (see cell line classification chapter 4). Other cell lines were used as controls in experimental work (Figure 3.1). Cell lines were purchased from ATCC cell bank except for ARK1 and ARK2, which were purchased from Yale University. All cell lines were authenticated by analysis of short tandem repeats and microsatellites and confirmed to be mycoplasma free with 3 monthly mycoplasma testing (MycoAlert Mycoplasm detection kit, Lonza).

Cell line	Cell type	Medium	Reference	Source
Ishikawa	Grade 2 Endometrioid endometrial	MEM 2 mM L-Glutamine + 1% NEAA + 5% FCS	(Nishida et al., 1985)	ATCC
HEC1A	Grade 2 Endometrioid endometrial	McCoy's 5a + 10% FCS	(Kuramoto, 1972)	ATCC
AN3CA	Grade 3 Endometrioid endometrial	MEM 2 mM L-Glutamine + 1% NEAA + 10% FCS	(Dawe et al., 1964)	ATCC
RL95-2	Grade 2 Adenosquamous endometrial	DMEM:F12 + 2 mM Glutamine + 0.005 mg/ml insulin + 10% FCS	(Way et al., 1983)	ATCC
ARK1	High grade serous endometrial	RPMI 1640, 10% FCS	(Cross et al., 2010)	Yale
ARK2	High grade serous endometrial	RPMI 1640, 10% FCS	(Abdel-Fatah et al., 2013)	Yale
L1210	Murine leukaemia lymphocyte cell line. Used as internal control for PAR assay	Dulbecco's Modified Eagle's medium (DMEM) supplemented with 20 mM L-glutamine, 10% FCS	(DelloRusso et al., 2007)	ATCC

Table 3.1. **Panel of cell lines used in experimental work** used along with their histological type, the medium they were grown in and the associated mutations of interest.

3.4 Cell culture

Media was stored at 4 °C and warmed in a bead bath to 37 °C prior to use. All cells were handled separately with their own reagents. Experiments were undertaken at early passage (<30) and grown at 37 °C, 5% CO_2, 95% humidified air and maintained in exponential growth. Cell passage was performed using an aseptic technique in a containment level II laminar flow cabinet. Cells were kept at around 60-70% confluence for experimental work. Media was aspirated, cells were washed with PBS before incubation with 3 ml 0.25% trypsin-EDTA at 37 °C until they detached from the flask. The cell suspension was centrifuged at 250 g for 5 minutes to form a cell pellet. The supernatant was then discarded, and the cell pellet re-suspended in 10 ml cell line specific culture medium. 10 µl of media containing cell suspension was loaded onto a Neubauer Haemocytometer for cell counting. Cells were seeded into flasks/culture dishes as required at the appropriate cell number, to allow exponential growth to continue for further experimentation. Early passage cells were frozen down for future experimental use in cell line specific freezing media (media supplemented with 10% DMSO). Cells were aliquoted and frozen at a density of 1 x 10^6 cells/ml, initially for 24 hours at -80 °C before transferring into liquid nitrogen for storage.

3.5 Sulforhodamine B (SRB) growth assay and growth inhibition

3.5.1 Assay principle

The SRB assay measures the protein content of adherent cells to estimate cell density. Comparison between density of adherent cell therefore allows for estimation of growth. SRB is an anionic aminoxanthene dye which binds to basic amino acids under acidic conditions (Lillie and Conn, 1991). It can then be extracted under basic conditions; the resultant unbound dye can be used as a proxy for cell mass (Vichai and Kirtikara, 2006), this is because it produces a colour change which can be read by a photospectrometer. The dye concentration is directly proportional to the cell mass measured. It has been widely used for cell proliferation and growth inhibition studies with cytotoxic agents, is relatively simple, inexpensive, and reproducible. SRB assay can be used to assess response on cell proliferation to cytotoxic agents (Plumb et al., 1989).

The limitation to SRB studies is there may be an increase in cell size (unbalanced cell growth) response to cytotoxic agents, which could be interpreted as an increase in cell number, due

to an increase in protein content and therefore is only accurate if the cell size remains constant (Ross, 1983).

3.5.2 Assay protocols and data analysis

Cells were harvested in exponential growth and seeded at 500, 1000 and 2000 cells per well in 100 µl of media on seven 96 well plates, with six repeats at each seeding density. An individual plate was fixed every 24 hours with the addition of 25 µl methanol:acetic acid 3:1 and then stored at 4 °C until ready for analysis. The first plate (fixed after 24 hours) known as day 0 control, provides a baseline cell density measurement following cell attachment. Following 7 days of fixation plates were washed 5 times with distilled water and air dried overnight. 100 µl 0.4% SRB was added to each well at room temperature for 30 min before washing 5 times in 1% acetic acid and placed into a drying cupboard. 100 µL of 10 mM Tris was then added to each well and placed onto a shaker platform before reading absorbance on a FLUOstar® omega plate reader measuring absorbance at a wavelength 570 nM. Cell doubling time was then calculated using Graphpad prism software.

In order to estimate the growth effects caused by different drugs, the concentration of drug that inhibits cell growth by 50% (GI_{50}) in each cell line was calculated using SRB assay for cisplatin and Niraparib alone, as well as cisplatin + Niraparib 1 µM.

Cells were harvested in exponential growth phase and seeded into a 96 well plate in 100 µl of media at a concentration of 1000 cells per well, with 6 replicates for each concentration (plate 1) with an additional plate, of 6 replicates (plate 2) as day 0 control. Cells were incubated for 24 hours for attachment. Plate 2 was fixed with 25 µl of carnoy's fixative and stored at 4 °C as a day 0 control. Media was replaced in each well of plate 1 with increasing concentrations of cytotoxic agent, each with 6 replicates, alongside untreated controls, corrected for 0.5% DMSO. Plate 1 was incubated for 3 cell doubling times before fixing with 25 µl of Carnoy's fixative. The plates were then washed, dried, stained with SRB and absorbance read at 570 nm on an omega plate reader as above.

The mean optical density of the wells treated with each drug concentration was calculated and expressed as a percentage of treated and untreated control after day 0 subtraction. Growth inhibition studies were undertaken with 3 experimental repeats, control and

incremental drug concentrations had 6 replicate repeats to ensure differences were less likely to be related to contamination or error. Data from each experiment were summarised as mean and standard error of the mean (SEM) of the replicates at each concentration. The growth inhibition 50% or GI_{50} was calculated in each experiment as the concentration at which cell proliferation was inhibited by 50% at a given drug concentration. The data were then displayed in a point-to-point graph showing growth inhibition at increasing concentration of drug. Differences in survival between cell lines and within different conditions within cell lines were investigated with ANOVA and Students t-test.

3.6 Colony cell survival assay

3.6.1 Assay principle

The colony formation assay is a cell survival assay used to assess a single cell's ability to proliferate and form a colony (cluster of 30 or more cells). It was originally devised as a means to determine cell death following explore to IR (Puck and Marcus, 1956) and can be used to study the effects of various cytotoxic agents such as cisplatin and niraparib, irradiation alone as well as in combination on 'colony formation'. Results from colony survival assays have be shown to correlate with in-vivo tumour response to chemotherapy (Meyskens et al., 1984). There are some limitations to clonogenic assays in that not all cell lines form colonies at low seeding densities, particularly relating to autocrine and paracrine factors (Mittal, 2012). Whilst cell lines in this colony survival assay were seeded at low density, the cells were harvested and drugged in this assay when cells were in exponential growth phase and not in the lag phase.

3.6.2 Assay protocol

Exponentially growing cells were harvested as previously described. Cells were seeded at three cell densities per treatment in 6 well plates estimated to given reliably countable colonies (20-150 colonies >30 cells each/well) after incubation, based on the plating efficiency of an individual cell line. Plates were incubated at 37 °C for 24 hours to allow attachment. Cell media was aspirated and replaced with increasing concentrations of media containing cytotoxic agent with 0.5% DMSO. Control wells were exposed to growth media containing 0.5% v/v DMSO to correct for the effect of 0.5% DMSO on the cells. After 24 hours of treatment media was then replaced and incubated for 37 °C until visible colonies

were seen (ranging from 10-14 days). Media was then aspirated, wells gently washed with PBS and fixed with 2 ml of 70% methanol: acetic acid 3:1 per well for 5 minutes before removing and staining with 2 ml of 0.4% w/v crystal violet solution per well for a further 5 minutes. Plates were washed with water and dried overnight. Colonies of more than 30 cells were then counted, an average was calculated at each concentration and expressed as a percentage of cells seeded in the control well to show the cloning efficiency (CE) at each drug concentration. Survival was calculated by using the following formula:

Survival % = (CE treated cells/ CE control) x 100

3.6.3 Single agent cytotoxicity

Serial drug concentrations for colony survival assays were determined according to results of SRB growth inhibition assays. Stock solutions of cisplatin and Niraparib were thawed to room temperature prior to serial dilution in sterile conditions in either 0.9% w/v sodium chloride (cisplatin) or 0.5% DMSO (Niraparib). Final dilutions were achieved by adding drug to cell line media. Concentration range used is displayed in table 3.2.

Cisplatin (µM)	Niraparib (µM)	Ionising radiation (Gy)
0	0	0
0.03	0.3	2
0.1	1	4
0.3	3	6
1	10	
3	30	
10		

Table 3.2. **Concentration ranges used for single agent experiments** for cisplatin and Niraparib, along with dose range for ionising radiation. Diluent was cisplatin was 0.9% sodium chloride w/v and 0.5% DMSO for Niraparib.

For colony formation assays investigating the potentiation of the cytotoxic effects of cisplatin by Niraparib, a single concentration of Niraparib was used to potentiate the effects of a range of cisplatin concentrations. Niraparib 1 µM was chosen for combination

experiments with IR and cisplatin in clonogenic and SRB growth inhibition studies following growth inhibition studies with single agent Niraparib and PARP functional assay described in the results chapters.

Serial dilutions of cisplatin stock solution in 0.9% sodium chloride were prepared as described above. Final dilutions were prepared in duplicate in growth media containing either: Niraparib 1 μM; or control with equivalent 0.5% DMSO.

Identical series of 6-well plates were prepared with 3 duplicates per concentration before incubating, fixing, and analysing, as previously described. The potentiation factor 50 (PF50) was calculated and survival at Cisplatin 3 μM was displayed. Data were normalised to DMSO control.

3.6.4 IR combination with Niraparib

Niraparib 1 μM was used to investigate possible potentiation of the cytotoxic effect of ionising radiation (IR) in colony formation assays. Growth media containing: niraparib 1 μM in 0.5% DMSO; 0.5% DMSO only as control were added to each well of a series of 6 well plates. Each plate contained 1 row of three wells containing niraparib 1 μM, and one row of three wells as control containing 0.5% DMSO only. The 6-well plates containing known seeding densities of cells was incubated for 24 hours to allow cell attachment, media was then replaced containing, niraparib or DMSO in growth media. The plates were immediately exposed to increasing doses of ionising radiation and incubated for 24 hours. Media was then replaced without drug and allowed to grow in the incubator until visible colonies were seen. The plates were then fixed and analysed as described previously. The potentiation factor 50 (PF50) was calculated and survival at 4 Gy was shown.

3.6.5 Data analysis

Following fixation and staining, colonies were counted manually, and the percentage survival calculated in Microsoft Excel. Percentage survival results for each cell for each biological repeat were copied to Graphpad Prism and the mean and standard deviation of survival for each individual condition calculated. Survival curves were plotted. For cisplatin and IR potentiation experiments, two-way ANOVA was conducted to detect significant potentiation effects.

3.7 Homologous recombination repair (HRR) functional assay

3.7.1 Assay principle

The HRR assay is a functional study of a cells ability to repair DNA damage using the homologous recombination DNA repair pathway. There are three overarching principles of this assay. Firstly, inhibition of PARP (by rucaparib) leads to an accumulation of DNA SSB that result in replication stress, stalled replication forks, DNA double strand breaks resulting in γH2AX foci (marker of damage) and RAD51 locates to the site of damage to repair the DSB. The number of γH2AX foci has been shown to correlate with the number of DSBs (Paull and Lee, 2005, Rogakou et al., 1998). This accumulation at the site of DSBs allows for quantification using a directed antibody to γH2AX and thus DNA DSBs. Downstream repair by HRR is identified by quantification of RAD51 foci formation. RAD51 is an essential downstream protein involved in HRR. It localises to the nucleus in response to DSBs and has a vital role in DNA repair as described in section 1.7.9. RAD51 forms nucleoprotein filaments at the site of DNA breaks (Thacker, 2005). The RAD51 coated filaments then carry out homology search on the sister chromatid and undertake strand invasion to execute repair (Sung, 1994, Lundin et al., 2003). Quantification of RAD51 foci therefore can be used as a marker of HRR function and can determine whether cells are HRR proficient or defective (Mukhopadhyay et al., 2010, RL et al., 2014). In this functional assay, rucaparib 10 μM (a potent inhibitor of PARP1 and 2) was used to inhibit the repair of SSBs, resulting in DSBs. Identification of γH2AX foci therefore identifies induction of DSBs and RAD51 repair by HRR.

Whilst Niraparib was the PARP inhibitor chosen for cytotoxicity studies in this thesis, there are currently no validated studies using it in a functional HRR assay. Therefore, the PARP inhibitor chosen for the functional HRR assay in this thesis was Rucaparib at a concentration of 10 μM, which has been extensively studied and validated by our research group and published in cell line and in primary culture (solid tumour and ascites) (Drew et al., 2011, Gentles et al., 2019, Mukhopadhyay et al., 2010). Rucaparib 10 Um was chosen in the HRR assay as it was the concentration that inhibited PARP by >90% across all cell lines studied in several cancer types and DSB induction as demonstrated by H2AX foci formation. A cut off of a 2-fold increase in RAD51 foci formation to determine if a cell line was HRC was determined based on fold increase in RAD51 in known BRCA1/2 or XRCC3 mutations in cell lines, as compared to cell lines without mutations in HRR genes. This was further supported with increased sensitivity to PARP inhibitors in those cell lines with <2 fold increase in RAD51 foci

formation and gene mutation in HRR protein. However, setting a firm cut off at a 2-fold increase to determine HRR status is likely to be over simplistic and it should be recognised that HRR defects are more likely to be on a spectrum (Ngoi and Tan, 2021). This and the fact that the assay is quite labour intensive, limit its use in clinical practice (Miller et al., 2020b).

3.7.2 Assay protocol

Exponentially growing cells were harvested and seeded into a 6-well plate at a density of 0.5 x 10^5 cells/ml. Plates were incubated for 24 hours to allow attachment before replacing with media containing 0.5% DMSO or 10 μM rucaparib to induce DNA DSBs. After a further 24-hour incubation, cells were washed twice with PBS and fixed with 100% ice cold methanol for 30 min. Coverslips were then washed twice with 0.2% PBS-Triton-X100 before incubating with 200 μl blocking buffer (2% w/v bovine serum albumin (BSA), 10% w/v skimmed milk powder, 10% v/v goat serum in 0.2% PBS-Triton-X100) for one hour at room temperature. Coverslips were then incubated with 200 μl anti-RAD51 rabbit monoclonal antibody 1:500 in the blocking buffer overnight at 4 °C. Coverslips were then washed in 0.2% PBS-Triton-X100 before incubating with anti-phospho-histone γH2AX IgG mouse monoclonal antibody (1:1000) in PBS-Triton-X at room temperature for one hour. Following further washes secondary antibodies were then added and incubated for one hour (Alexa Fluor 488 goat anti-rabbit and 546 goat anti-mouse) before washing. Following DAPI wash coverslips were mounted onto slides, stored in dark conditions, and allowed to dry before imaging on a Leica DM6 fluorescence microscope. Antibodies used are shown in the table below. Representative images were captured and analysed using image J software before statistical analysis was performed in GraphPad prism. Antibodies used in HRR assay are displayed in table 3.3.

Target	Antibody	Manufacturer	Concentration	Secondary antibody	Manufacturer	Secondary antibody concentration
RAD51	Anti-RAD51 antibody [EPR4030(3)] rabbit monoclonal	Abcam	1:500 in 2% BSA, 10 % goat serum (v/v), 10% powdered milk (w/v) in PBS (in cell line work) or KCl 0.2% Triton-X-100 (in primary culture)	Alexa Fluor 488 Goat anti-rabbit Ab	Abcam	1:1000 in 2% BSA
Anti gH2AX	Anti- gH2AX antibody (SER139) [JBW301] mouse monoclonal	Millipore	1:1000 in 2% BSA	Alexa Fluor 546 Goat anti-mouse Ab	Abcam	1:1000 in 2% BSA
Vectashield®	Hardset™ mounting media (H100-10) with DAPI	Vector laboratories	4 µL	N/A	N/A	N/A

Table 3.3. **HRR assay antibodies.**

3.7.3 Data analysis

The number of γH2AX and RAD51 foci in the control were compared to the treated group in at least 50. Within image J a macro was created to identify foci within the nuclei only above a standardised threshold and then quantified. The mean number of foci per cell was expressed in the control and treated cells and a fold-increase expressed. Semi-automated counting has previously been validated (Mukhopadhyay et al., 2010, RL et al., 2014) and was used in this case. A two-fold increase in γH2AX foci in the treated group was considered to indicate adequate induction of DNA DSB (Mukhopadhyay et al., 2010). More than a two-fold increase in RAD51 foci, denoted HRR competence (HRC), and those with less than a 2-fold increase considered HRR deficient (HRD).

3.8 PARP assay

3.8.1 Assay principle

Permeabilised cells are maximally stimulated in order to quantify poly (ADP-ribose) (PAR), as well as baseline measurement of PAR and PAR inhibition by Niraparib. This 6-minute reaction requires a mixture of oligonucleotide (to simulate DNA DSBs) to activate PARP in the permeabilised cells when added and excess NAD+, PARP's substrate. The reaction is stopped by saturating the mixture with an excess of the PARP inhibitor Rucaparib and placing the cells on ice. Cells are then blotted onto a membrane that is incubated with an anti-PAR primary antibody followed by the addition of a conjugated secondary antibody conjugated by HRP. ECL is then added and chemiluminescence measured.

3.8.2 Assay protocol

Cells were harvested in exponential growth. 1 ml of media containing cells was then centrifuged and washed with ice cold PBS and then permeabilised with 100 µL Digitonin (0.15 mg/ml) at room temperature for 5 minutes before adding 900 µL of isotonic buffer (0.4 mM EGTA, 7 mM HEPES, 26 mM KCL, 0.1 mM dextran, 0.5 mM $MgCl_2$, 45 mM sucrose dissolved in distilled water at pH 7.8). Cells were then counted on a haemocytometer by combining 15 µL of cell suspension and trypan blue 1:1. The cell suspension was then diluted to an appropriate cell number for baseline (35,000-100,000 cells) and activated PARP (250-500 cells) experiments. Duplicate samples, alongside unstimulated controls (blanks) in the

absence of oligonucleotide and NAD+ were exposed to NAD+ (7 mM) and oligonucleotide (200 µg/ml) in a reaction buffer (100 mM Tris-HCl, 120 mM $MgCl_2$ at pH 7.8) for 6 minutes at 26 °C to establish optimal PARP activity. Duplicate samples were also exposed to a mixture including increasing concentration of Niraparib with DMSO control to estimate the IC_{50} of niraparib in each cell line. The PARP reaction was then stopped with the addition of rucaparib 10 µM. The reaction mixture was then loaded onto a nitrocellulose Hybond-N membrane through a plastic manifold. PAR standards were added to the manifold (0-25 pmol) to generate a standard curve to allow for comparison. The samples were then drawn through the membrane using a suction pump connected to the manifold. 400 µl 10% trichloroacetic acid/2% sodium pyrophosphate was then drawn through the manifold followed by 800 µl 70% ethanol. The membrane was then washed 3 times in PBS before incubating at room temperature with a blocking buffer PBS-MT (5% powdered milk, 0.0005% Tween-20 in PBS) for one hour. The membrane was then before incubated with primary antibody, anti-PAR 10H (1:1000), overnight at 4 C in PBS-MT on a rocking platform overnight. The following morning, the membrane was washed three times for 5 minutes in PBS-T and then incubated with goat anti-mouse HRP conjugated secondary antibody in PBS-MT for an hour at room temperature on a rocker. The membrane was then washed every 5 minutes for one hour with PBS-T and then Amersham ECL detection fluid was added and chemiluminescence recorded by Fujifilm LAS 3000 detection unit. The image was then analysed using a circular analysis over the blotted wells and densitometry measured and expressed per unit area (luminescent arbitrary units/mm^3). Blanks were subtracted from the stimulated samples to estimate the PAR formed during the reaction.

3.8.3 Data analysis

A standard curve was constructed using a non-linear regression curve generated by the densitometry measurements of the PAR standards in the blot using graphpad prism. Statistical analysis of PARP studies was undertaken following results of 3 independent experiments. Data were represented as percentage inhibition (compared to control with no drug) at a given concentration. Inhibitory concentration 50% or IC_{50} was calculated, which is the concentration at which 50% of PARP activity is inhibited in permeabilised cell. Students t-test and ANOVA were used to understand differences between cell lines.

3.9 Thrombin clot

3.9.1 Assay principle

Small volume cytology specimens can be processed using a cell block technique to improve the diagnostic yield such as those seen from fine needle aspiration (FNA) sampling in breast cancers (Nakayama et al., 2016). Cell pellets or small tissue FNA biopsies can be processed paraffin blocks, this allows the ability to perform multiple tissue sections and immunohistochemical stains like paraffin sections in diagnostic histopathology (Shidham and Layfield, 2021). Thrombin has can be used to 'cluster' pelleted cells together and thus an appropriate method to undertake IHC staining of endometrial cell lines used to this project alongside simultaneous assessment of patient samples in tissue microarrays (TMAs) (Newman et al., 2021, Gorman et al., 2012, Sauter et al., 2016).

3.9.2 Assay protocol

Cells were harvested in exponential growth, trypsinised and transferred into a 15 ml universal tube. The universal was centrifuged at 4 °C for 10 minutes at 200 g to form a cell pellet. The supernatant was then discarded, and the cell pellet resuspended in 50 µg thromboplastin/thrombin. 50 µl of circulating plasma was then added before the pellet was allowed to clot for 15-20 minutes. The clot was then transferred into a histology cassette. Excess moisture was removed by dabbing with the corner of a paper towel, the cassette closed and fully emersed into formalin for 1 hour. The cassette was then processed though Ventana IHC processor to form a formalin fixed paraffin embedded (FFPE) block by gradually dehydrating the contents with increasing concentrations of ethanol and then placing into xylene before fixing in cooled liquid paraffin.

3.10 Tissue microarray

3.10.1 Assay principle

Formalin fixed paraffin embedded blocks (FFPE) are used to preserve tissue samples taken from patients in formaldehyde and then embedded into a paraffin blocks. It preserves proteins, DNA and the structure of the tissue for further testing. Immunohistochemistry straining from tissue sectioning is used to determine antigen/protein expression in tissues. It's uses range from morphological diagnosis to the use of staining as adjuncts to inform diagnosis. Tissue microarrays (TMA) are paraffin blocks that contain cylindrical cores, usually

from different donor paraffin blocks, placed into one single block in an array fashion. Depending on the core and block size, this technique can secure up to 1000 cores into a single block and a therefore high throughput. This allows for many small representative tissue samples to be sectioned onto one slide for assessment, staining and analysis. Advantages of this method include significant saving of cost and time, reduction in amount of reagents used and reduction in inter-assay variation. However, construction is labour intensive and TMA blocks may require multiple cores from the same recipient to ensure full representation. TMA suffer from core loss and slippage during sectioning and slide preparation.

3.10.2 Assay protocol

Women consented to the DDR biobank (REC:12/NE0395) at Newcastle university who underwent surgery for confirmed on suspected endometrial cancer were identified between 2017 and 2020. FFPE blocks were identified and retrieved for slide sectioning and Haematoxylin and Eosin (H&E) staining in the QEH laboratory. A total of 50 patients were identified for inclusion in the TMA. Slides were reviewed alongside a consultant gynaecological pathologist to identify suitable areas within FFPE with representative tumour for core extraction. Once identified, anonymised blocks were transferred and store securely in the Newcastle Biobank ready for core sampling and TMA construction. TMA was designed on Galileo TMA CK3500 to assign random core locations ready for assembly. Random assignment was used to reduce the risk of core loss and bias interpreting the TMA. A total of 3 x 1 mm cores were taken from each of the 50 FFPE blocks. 1 mm donor core holes were drilled into the TMA recipient block, core placed in and sealed. Positive controls for antigens studied were added to ensure quality control. figure 3.1 shows final TMA layout and figure 3.2 shows image of constructed TMA.

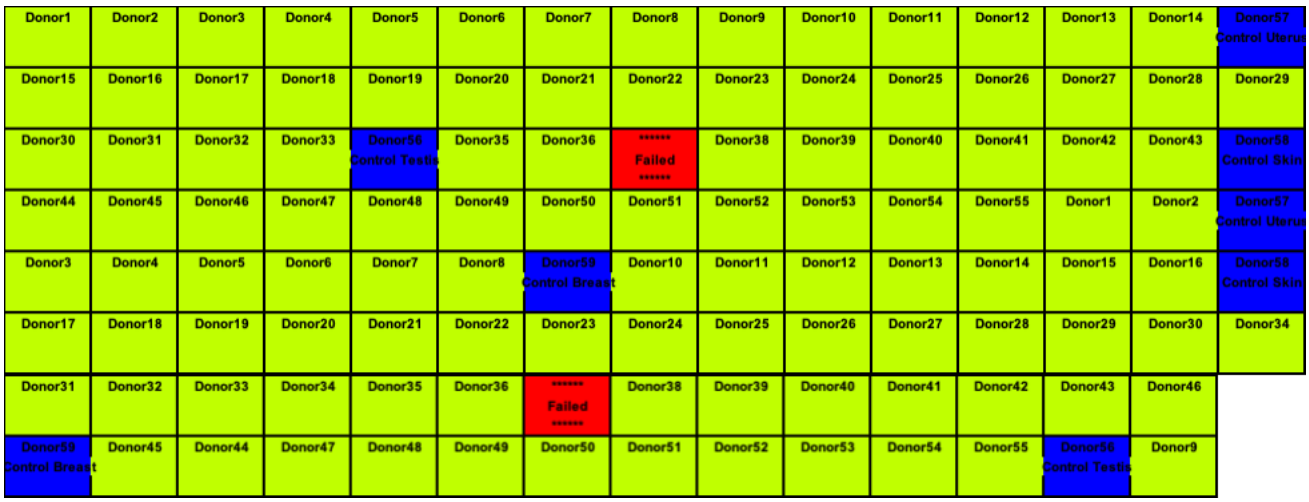


Figure 3.1. **TMA layout.**

3.11 Immunohistochemistry

Once the TMA was made, individual sections could be taken for immunohistochemistry staining. 5 µm sections were taken using a microtome and positioned onto glass prior to staining. The TMA sectioning, slide mounting and H&E staining was undertaken by NICR dedicated IHC technician according to local standard operating procedures. Primary and secondary antibodies used in experimental work are shown in table 3.4. IHC dilutions used were optimised by Newcastle Hospitals NHS Foundation trust's cellular pathology laboratory by a technician to obtain IHC staining whilst the university lab was closed during the covid pandemic. Concentrations above and below those used in diagnostic work were used given that TMA construction was not standard place in the lab to ensure that appropriate staining was obtained.

3.11.1 Assay protocol

Slides are deparaffinised with xylene, hydrated through graded ethanol (100-50%) and washed with distilled water. Primary antibody is then used to target epitope for antigenic staining. All were stained on the Ventana benchmark automated IHC platforms. The IHC detection kit used varied between antibodies (either UltraView DAB or OptiView DAB). A signal amplification step was used with all MMR antibodies and counter staining was with haematoxylin II and bluing reagent.

Antibody (clone)	Manufacturer	Dilution	Detection
ER (SP1)	Ventana	Predilute	UltraView
PR	Ventana	Predilute	UltraView
P53 (DO-7)	Ventana	Predilute	UltraView
MLH1 (M1)	Ventana	Predilute	OptiView + amp
MSH2 (G219-1129)	Ventana	Predilute	OptiView + amp
MSH6 (EPR3945)	Abcam	1:550	OptiView + amp
PMS2 (EP51)	Dako	1:200	OptiView + amp
PTEN (SP219)	Ventana	Predilute	OptiView

Table 3.4. **IHC antibody table** with reference to dilutions (where known) and detection method for clinical TMA. Antigen retrieval and staining was undertaken on Ventana benchmark IHC platforms according to manufacturer's guidelines.

3.11.2 Slide scanning and scoring

Slides were scanned by Dr Holly Buist, consultant gynaecological pathologist at Royal Victoria Infirmary, Newcastle upon Tyne using Leica SNC400 slide scanner. All slides were then available on the university slidepath platform for viewing and analysis. Given the volume of cores taken and the number of antibodies, the antibody score for each core was given as positive, weak positive or negative, or in the context of MMR proteins, proficient or deficient, for P53 either overexpressed, null or wild type. The scoring was undertaken by 2 independent reviewers and any discrepancies were resolved by a 3rd reviewing pathologist.

3.12 Statistical analysis

Statistical analysis was undertaken using a combination of raw data stored in Microsoft excel format and then transferred for analysis on graphpad prism 9.

3.12.1 Survival analysis

Overall survival and progression free survival were calculated using diagnosis date, date of first progression and date of least follow up or death. Kaplan meier survival analysis was undertaken, and univariate analysis was undertaken to determine patient and clinicopathological factors which impacted on progression and overall survival.

3.12.2 Correlation analysis

Correlation analysis was undertaken using Spearman's test. Correlations were expressed with an r value with statistical significance set at $p<0.05$.

Chapter 4. Endometrial cancer cell line characterisation

4.1 Introduction

Cell lines are a useful resource for studying cancer cells(Masters, 2000). Most are commercially available, although some are limited to academic institutes. Cell lines are immortalised offering researchers reproducibility and high cell yield which is not often possible with primary cancer cell cultures (Neve et al., 2006). However, caution needs to be exercised when interpreting results from cancer cell lines as their genomic signature can change following multiple passages through the acquisition of mutations (Liu et al., 2014). Another disadvantage of cell lines is that researchers can only study a single clonal cell type. We now understand that there is an interplay between different cell types in the tumour microenvironment (Hanahan and Weinberg, 2011, Joyce and Pollard, 2009, Egeblad et al., 2010) thus limiting value of cell lines n tumours with high proportion of heterogeneity. Nevertheless, cell lines offer a convenient way to investigate the impact of cell biology on response to therapy that can be validated and used in more complex pre-clinical models or taken forward clinically.

Contamination of cell cultures with other cell lines or microbes, particularly mycoplasma infection that can alter cell biology and behaviour (Nelson-Rees et al., 1981, Hay et al., 1989, Capes-Davis et al., 2010). To avoid these problems cancer cell lines used here were obtained directly from a commercial authenticated cell bank or from the originators, used individually in a laminar flow hood with their own reagents, regularly tested for mycoplasma infection, and limited to investigation below 30 passages.

A limited number of endometrial cancer cell lines are commercially available with many only available with restricted (non-commercial) use. Six endometrial cancer cell lines (Table 4.1) were studied as part of experimental work: AN3CA, ARK1, ARK2, HEC1A, Ishikawa and RL95-2. The cell lines chosen for the current study have distinct histological and biological characteristics that represent endometrial cancer subtypes that may differ in their DNA damage response and response to therapy. HEC1A and Ishikawa cell lines are type 1 endometrial cancers endometrioid subtype, with the remaining cancers being type 2

endometrial cancers, as classified by Bokhmann. AN3CA and RL95-2 are grade 3 endometrioid cancer and ARK1/2 representing serous endometrial cancers.

Alterations of components in the DDR affect the response to DNA damaging anticancer therapy. Adjuvant therapy in endometrial cancer usually consists of radiotherapy with or without platinum-containing chemotherapy, dysregulation of BER, NHEJ and HRR pathways may affect response to radiotherapy whilst defects in HRR confer sensitivity, and defects in MMR confer resistance to platinum therapy, see Section 1.7. As discussed in Section 1.7.11, it is anticipated that a proportion of endometrial cancers are likely to be HRD. This is thought to predominate in the serous and grade 3 endometrioid subtypes of which a significant proportion have p53 mutations. There is also emerging evidence that serous cancers may harbour BRCA mutations (Gasparri et al., 2022). BRCA mutations result in HRD, which can also be caused by epigenetic changes or mutations in other genes associated with the HRR pathway. PARPi are approved for other cancers with BRCA mutations or HRD phenotype and the possibility that these relatively non-toxic drugs could be applied to endometrial cancer is therefore worth exploring. Several commercial kits are available to identify HRD based on a combination of mutations in key genes and gross genomic changes but are relatively expensive and the results represent genomic scarring and will not detect acquired PARPi (or platinum) resistance (Miller et al., 2020b). Functional assays, such as the one developed in our laboratory (Drew et al., 2011, Mukhopadhyay et al., 2010) that can be applied to both cell lines and viable tumour samples may be more useful.

4.2 Aims and objectives

The aims to be investigated in this chapter are:

1. To determine the growth (doubling time) of the cell lines as this may be a determinant of sensitivity to cisplatin, IR and Niraparib
2. To determine the optimal seeding density for growth inhibition studies for endometrial cell lines and primary culture
3. To determine the cloning and plating efficiency for use in colony survival (cytotoxicity) assays
4. To determine the homologous recombination repair (HRR) status of each cell line

5. To determine the baseline and activated PAR in the cell lines as it may be a determinant of sensitivity to cisplatin, IR and to the PARP inhibitor Niraparib.

4.3 Materials and methods

Methods for cell line culture alongside descriptions of individual cell lines, chemical reagents used are discussed in section 3.3 and 3.4. Details of methods for calculating cell doubling time by SRB, plating efficiency by colony formation assay, homologous recombination repair functional assay (HRR) and PARP activity assay are also included in section 3.5-3.8.

4.4 Results

4.4.1 Morphology

All cell lines formed an adherent monolayer in culture medium and have a distinct morphological appearance. Cell line origin, characteristics, images with description of morphology, doubling time, plating efficiency, known protein expression along with common genetic mutations are displayed in Table 4.1.

4.4.2 Cell proliferation and plating efficiency

Accurate assessment of the growth rate of the cells was required because many anticancer agents are more toxic to rapidly dividing cancer cells. Growth inhibition studies identifying the cell seeding densities needed to ensure exponential growth until the end of the experiment. For colony forming cytotoxicity assays the plating efficiency (i.e. the number of colonies obtained/100 cells seeded) and the time needed to form colonies of >30 cells was established (Table 4.1).

Representative growth curves for AN3CA and HEC1A cell lines are shown in Figure 4.1. Data points in the lag and plateau phase were omitted from the doubling time calculation. Each growth experiment was undertaken 3 times in each cell line to calculate the average doubling time with the standard error of the mean. The doubling times are represented in Table 4.1

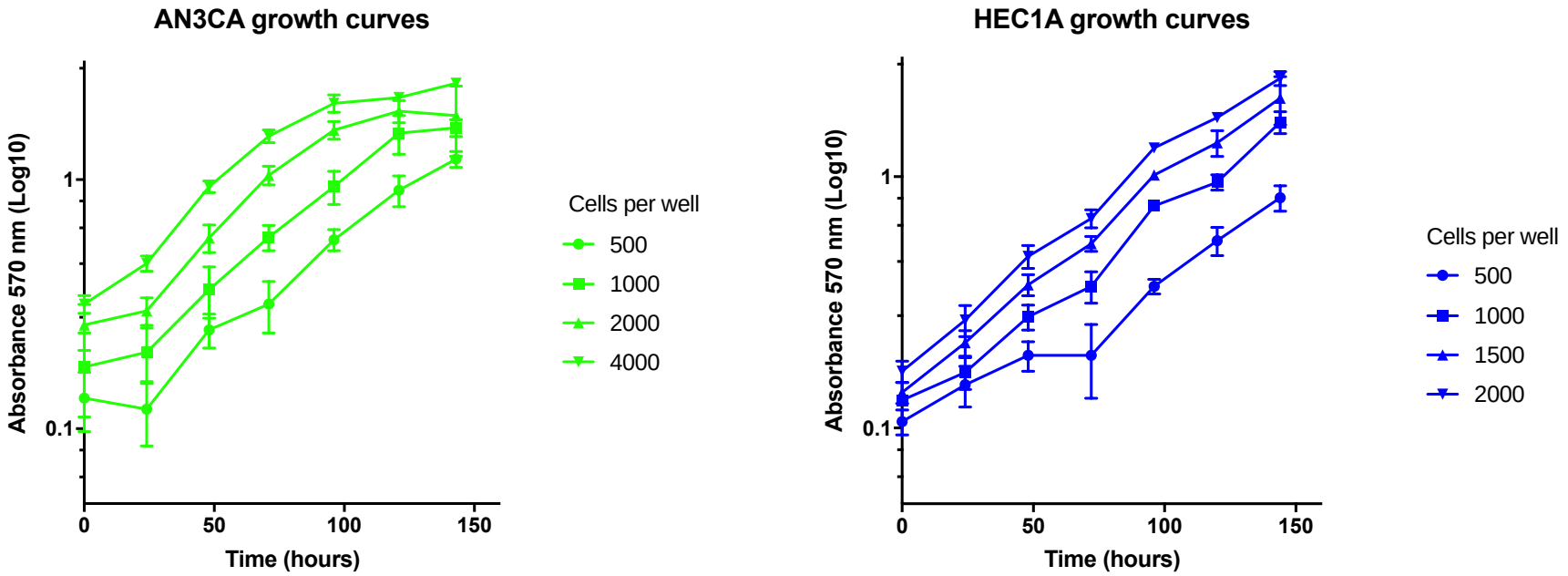
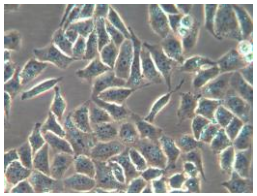
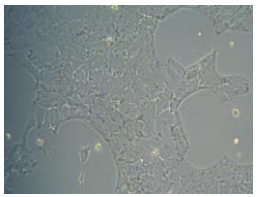
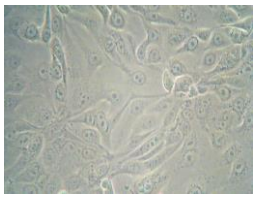


Figure 4.1. **Cell line doubling times for AN3CA and HEC1A**. Cell in exponential growth were seeded into 96 well plates at different cell densities. After 24 hours the first plate was fixed (day 0) and the others fixed daily for the subsequent 6 days. All plates were stained with SRB then optical density was read at 570 nm. Data shown are the mean and SD from a single experiment with 6 replicates at each cell density with time in hours after fixing from day 0 plate.

Cell line	Origin	Representative Image	Morphology	Cell doubling time, hours ± SEM (n)	Plating efficiency	Protein expression				
						ER	PR	PTEN	P53	Other alterations
AN3CA (Dawe et al., 1964)	55-yr-old endometrial adenocarcinoma from lymph node metastasis		Epithelial, forms monolayer culture with high nuclear: cytoplastmic ratio	40 ± 2 (3)	56 ± 2 (3)	-	-	Mut	Mut	MSI-H
ARK1 (El-Sahwi et al., 2010)	62-yr-old with stage 4A serous endometrial cancer		Epithelial, forms monolayer culture with high nuclear: cytoplastmic ratio, disorganised cell alignment	22.3 (1)	49 ± 1.5 (2)	+	-	Wt	Wt	PIK3CA
ARK2 (El-Sahwi et al., 2010)	63-yr-old with stage 4B serous endometrial cancer		Epithelial, forms monolayer culture with high nuclear: cytoplastmic ratio	21.9 (1)	76 ± 8 (2)	-	-	Wt	Wt	

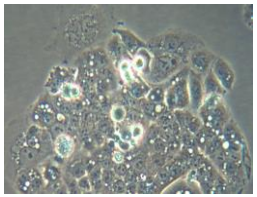
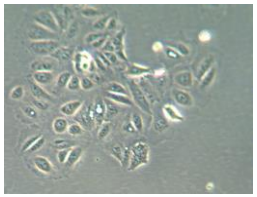
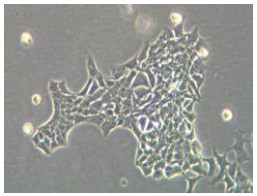
HEC1A (Kuramoto, 1972)	71-yr-old, stage 1A moderately differentiated endometrial adenocarcinoma		Epithelial, forms clumps which pile up, disorganised cell alignment, high nuclear: cytoplasmic ratio	37 ± 2 (3)	68 ± 4 (3)	-	-	Wt	Wt	PMS2 MSH6 KRAS MLH1
Ishikawa (Nishida et al., 1985)	39-yr-old, well differentiated endometrioid adenocarcinoma		Epithelial, forms clumps which pile up, disorganised cell alignment, high nuclear: cytoplasmic ratio	22 ± 1 (3)	54 ± 3 (3)	+	+	Mut het	mut	POLE
RL95-2 (Way et al., 1983)	65-yr-old, moderately differentiated adenosquamous carcinoma		Epithelial, monolayer culture with elongated appearance	46 ± 2 (3)	31 ± 3 (3)	+	-	Mut het	Mut het	MSI-H

Table 4.1**. Cell line characteristics** with name, origin of cell line, Images captured with Leica DM6 upright microscope x 40 magnification, as well as description of cell line, doubling time, plating efficiency, protein expression of common markers and known mutations are listed above. Mut=mutated. Wt=wild type. Het=heterozygous mutation. + = high expression. - = low expression . A limitation of the cell line characterisation for MMR status, P53, ER, PR and PTEN is that their status was not confirmed within experimental work in this thesis. It may be expected that

none of the above cell lines would be expected to be HRD based on the TRANsportec groups'(Stelloo et al., 2016) simplified classification of molecular classification, particularly when there are combination defects in POLE, MSI and p53

Doubling times were variable ranging from 21.9 (ARK2) to 46 hours (RL95-2). Interestingly the rapidly growing ARK1 and ARK2, are serous endometrial cancers, representing the more aggressive subgroup of cancers that typically respond better to platinum chemotherapy. HEC1A, and RL95-2 cell lines derived from the less aggressive low-grade endometrial cancers that are more likely to be resistant to chemotherapeutic, grew more slowly. However, the Ishikawa cells, also of low-grade origin grew rapidly and the AN3CA cells derived from a grade 3 endometrioid endometrial cancer, which are typically more aggressive tumours grew quite slowly so growth in cell culture does not always reflect the clinical experience.

Most of the cell lines had good plating efficiencies of > 50%. The RL95-2 cell line was an outlier at 31% observed plating efficiency. Interestingly the high-grade endometrial cancer cell lines (AN3CA, ARK1 and ARK2), all had relatively good plating efficiencies.

4.4.3 Homologous recombination repair functional status

HRR functional status was determined for each cell line using RAD51 foci assay as previously described (Mukhopadhyay et al., 2010). A more than a two-fold increase in mean yH2AX foci count per cell indicates sufficient induction of DNA DSBs. A more than 2-fold increase in mean RAD51 foci count per cell indicates HRR competence (HRC), whilst <2 fold indicated HRD.

All cell endometrial cancer cell lines were found to be HRC as demonstrated by a greater that 2-fold increase in RAD51 foci formation after 24-hour treatment to PARPi. Results are summarised in Figure 4.2 and Table 4.2. An example of the immunofluorescent images produced in the HRR assay is shown for the AN3CA cell line in Figure 4.2A. During initial HRR functional assays in the selected endometrial cell lines, an isogenic paired HRD/HRC cell line UWB ovarian cancer cell lines. A representative image has been provided below from Hannah Smith who provided the cell lines Figure 4.2B.

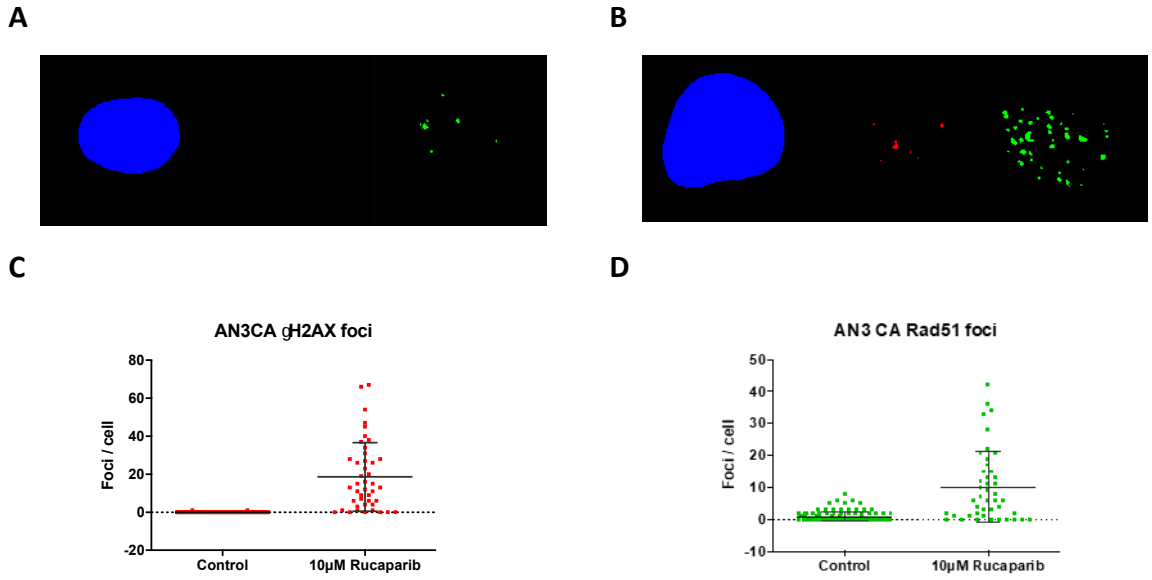


Figure 4.2A. **Functional HRR assay for AN3CA cell line** from a single representative experiment. A) example nucleus, stained with DAPI, in control cells (without rucaparib exposure) with few green RAD51 foci, B) example nucleus containing green RAD51 foci in the HRC nuclei of the AN3CA cell line; C) Scatter plot of γH2AX foci count per cell with median and SD in black bars in the control cells without treatment with PARPi rucaparib and cells treated with rucaparib 10 μM demonstrating the generation of DSBs 10; D) Scatter plot of RAD51 foci count per cell with median and SD in black bars. The graphs demonstrate a 2-fold increase in the average foci of both γH2AX and RAD51, showing that rucaparib has induced DSBs and RAD51 has been recruited downstream to repair the DSB. This shows that they are HRR competent. HRR assay methodology is discussed in Section.3.7.

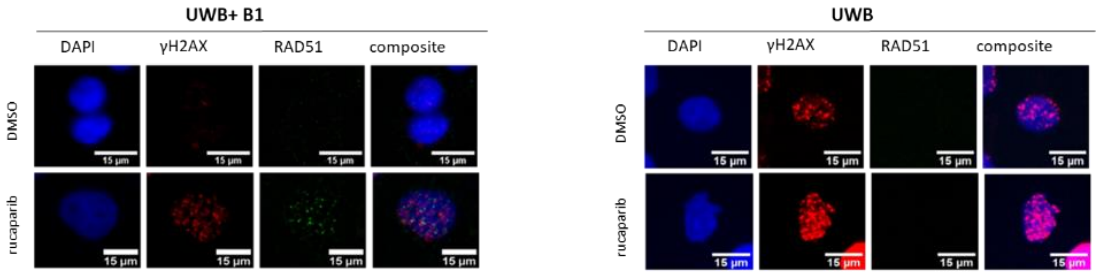


Figure 4.2B. **Isogenic paired cell line used in HRR assay.** Above we have representative HRR assay images (with permission and credit to Hannah Smith) of the BRCA1 mutated HRD cell line (UWB) on the right and its paired BRCA1 corrected HRC cell line (UWB+B1). In both cell lines, you can see a greater than 2-fold increase in H2AX foci, identifying DNA DSBs but we

see a greater than 2 fold increase in RAD51 in the UWB+B1 cell line only, signifying repair by HRR, whereas this is not seen in the UWB and is therefore HRD.

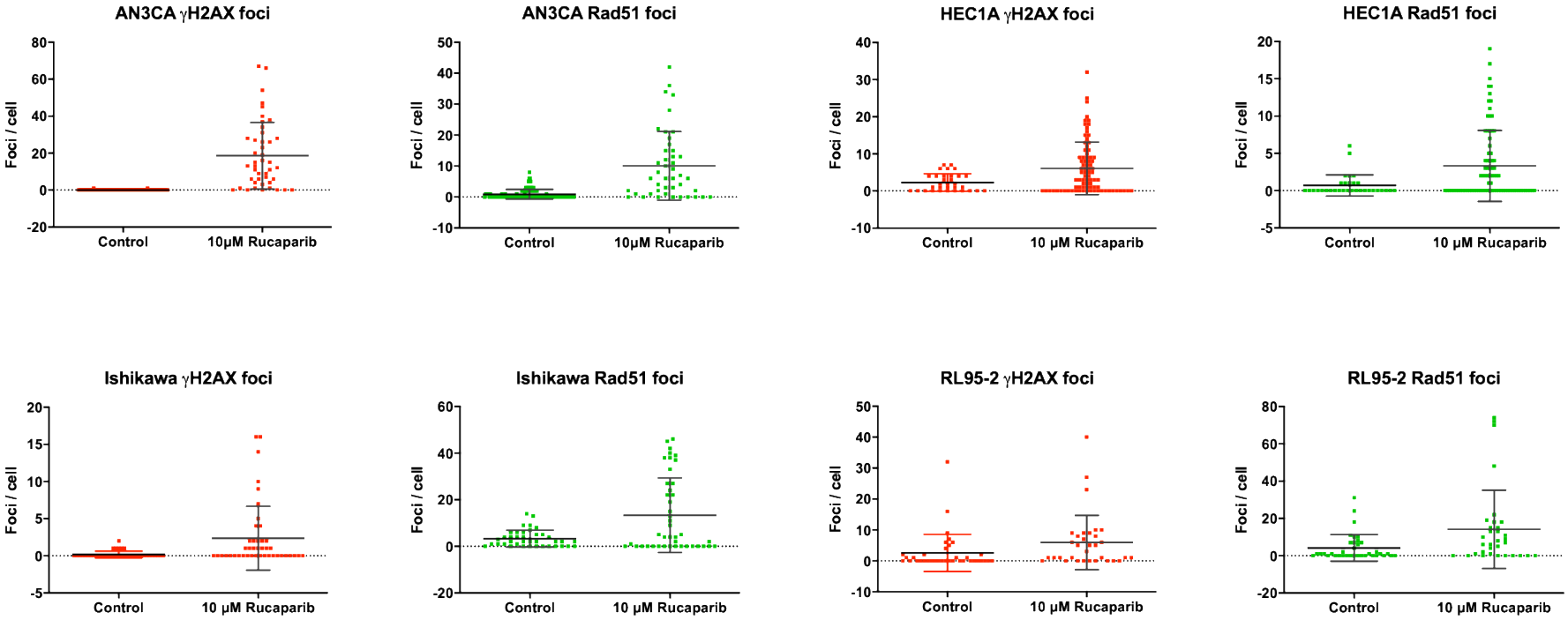


Figure 4.3. **Determination of HRR status of for EC cell lines**. Cells were exposed to 10 μM rucaparib for 24 hours or not (control) and the accumulation of DNA DSB / replication stress and ongoing repair by HRR were measured by immunofluorescence microscopy of yH2AX and RAD51 foci, respectively. Data are number of foci in individual cells with mean and standard deviations given for control and treated cells.

Cell line	Control γH2AX (mean Foci/cell)	Treated γH2AX (mean Foci/cell)	Fold change	Control RAD51 (mean Foci/cell)	Treated RAD51 (mean Foci/cell)	Fold change	HRR status
AN3CA	2.0 ± 1.0	15.0 ± 3.0	7.5	1.5 ± 0.5	7.5 ± 2.5	5.0	HRC
HEC1A	2.7 ± 0.3	6.0 ± 0.8	2.2	2.0 ± 1.0	7.0 ± 3.6	3.5	HRC
Ishikawa	2.3 ± 1.3	29.6± 24.2	12.8	4.3 ± 2.4	20.0 ± 3.7	4.7	HRC
RL95-2	1.7 ± 0.7	4.7 ± 1.3	2.8	2.7 ± 0.7	8.3 ± 2.8	3.1	HRC
ARK1	1.8	4.2	2.3	9.5	28.0	2.9	HRC
ARK2	3.8	76.3	20.0	2.3	27.1	11.8	HRC

Table 4.2. **Summary data for functional HRR assay in 6 EC cell lines**. Data are mean and standard deviation from 3 independent experiments or, where no SD is given for the ARK1 and ARK2 cell lines, from a single experiment (further experimental work was not possible due to lab shut down during the COVID-19 pandemic.

4.4.4 Correlation between fold increase in H2AX and RAD51

There was a statistically significant positive correlation between fold increase in γH2AX and RAD51 (Figure 4.4.) suggesting that the increase in RAD51 was in response to the induction of replication stress/DNA DSBs.

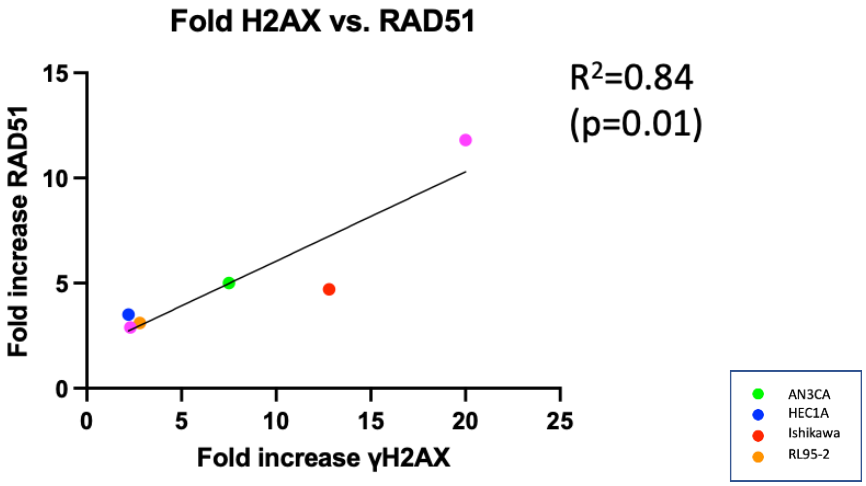


Figure 4.4 **The increase H2AX and RAD51 in the endometrial cancer cell lines**. All experiments, apart from those undertaken with ARK1 and ARK2 cell lines, were the result of 3 independent experiments. The mean value was used from these experiments.

4.4.5 Determining baseline and activated PARP

To understand the role of PARP in the repair of these endometrial cancer cell lines it was important to understand the baseline PAR activity of the cell line and the maximum ability of the cell line to activate PARP in exponentially growing cells. Doing this enables us to understand the relationship between PARP and sensitivity to common treatments used in endometrial cancer as well as the different characteristics of the chosen cell lines. Description of the methodology for the PARP assay to determine baseline and activated PARP is discussed in chapter 3. Since the response to PARP inhibitors may be affected by a cell's PARP activity at baseline and capacity for activation in response to DNA damage, the endogenous level of PARylation was determined and its increase following optimum activation by an oligonucleotide mimicking DNA damage in the presence of excess substrate, NAD^+.

4.4.6 Endogenous PAR

Data for individual measurements of the endogenous and stimulated PARP activity are given in figure 4.5 with mean and standard deviations in Table 4.3. Endogenous PAR expression was lowest in the RL95-2 cells and highest in the HEC1A cells.

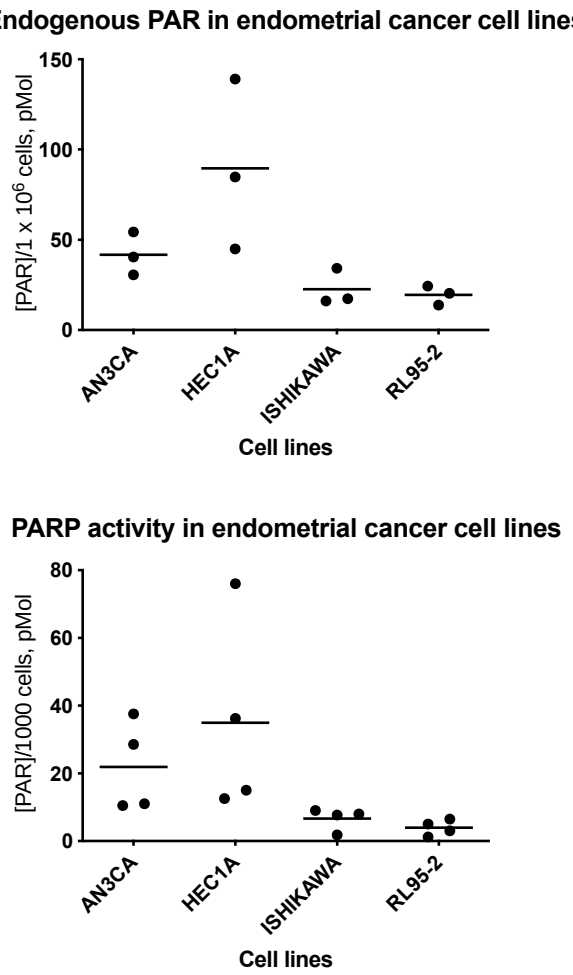


Figure 4.5. **Endogenous and activated PARP in endometrial cancer cell lines**. level of PARylation in permeabilised cells in the absence (endogenous) and presence (PARP activity) of exogenous NAD and activating DNA was determined by immunoblot measurement of PAR as described in the Methods Section 3.8. Following addition of NAD+ and an activating oligonucleotide, PARP activity was stimulated 200 to 500-fold. Cell lines ranked in the same order from lowest to highest expression in both activated and baseline PARP.

Cell line	**Endogenous PAR, [PAR]/1 x 10^6 cells, pMol ± SEM (n)**	**Activated PARP, [PAR]/1000 cells, pMol ± SEM (n)**
AN3CA	41.73 ± 6.90 (3)	21.90 ± 6.70 (4)
HEC1A	89.59 ± 27.27 (3)	34.96 ± 14.68 (4)
Ishikawa	22.53 ± 5.85 (3)	6.64 ± 1.63 (4)
RL95-2	19.48 ± 3.06 (3)	3.95 ± 1.15 (4)

Table 4.3. **Summary data for endogenous and activated PARP** . Mean and SEM from (n) independent experiments of endogenous and activated PARP in endometrial cancer cell lines.

4.4.7 Correlation between baseline and activated PAR

There was a positive correlation between baseline and activated PAR although this was somewhat dependent on HEC1A having the highest value in both and RL95-2 having the lowest.

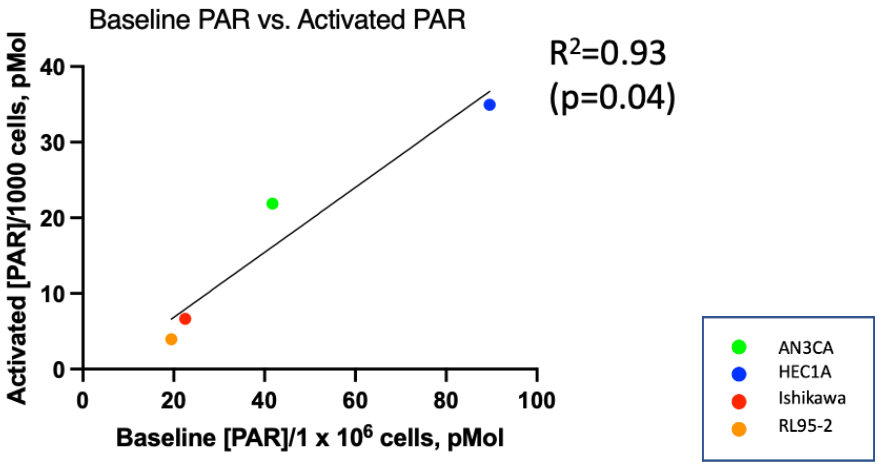


Figure 4.6. **Correlations between baseline and activated PARP** baseline PAR vs. activated PAR in the endometrial cancer cell lines. Data taken from the mean values in table 4.3.

4.4.8 Correlations between cell doubling time and HRR status

Cells with faster replication cycles may accumulate more replication stress and be more dependent on HRR so the increase in γH2AX foci RAD51 foci were investigated in relation to cell doubling time. Surprisingly there was no significant correlation between cell doubling time and either γH2AX or RAD51 foci formation in this small panel of cells figure 4.7.

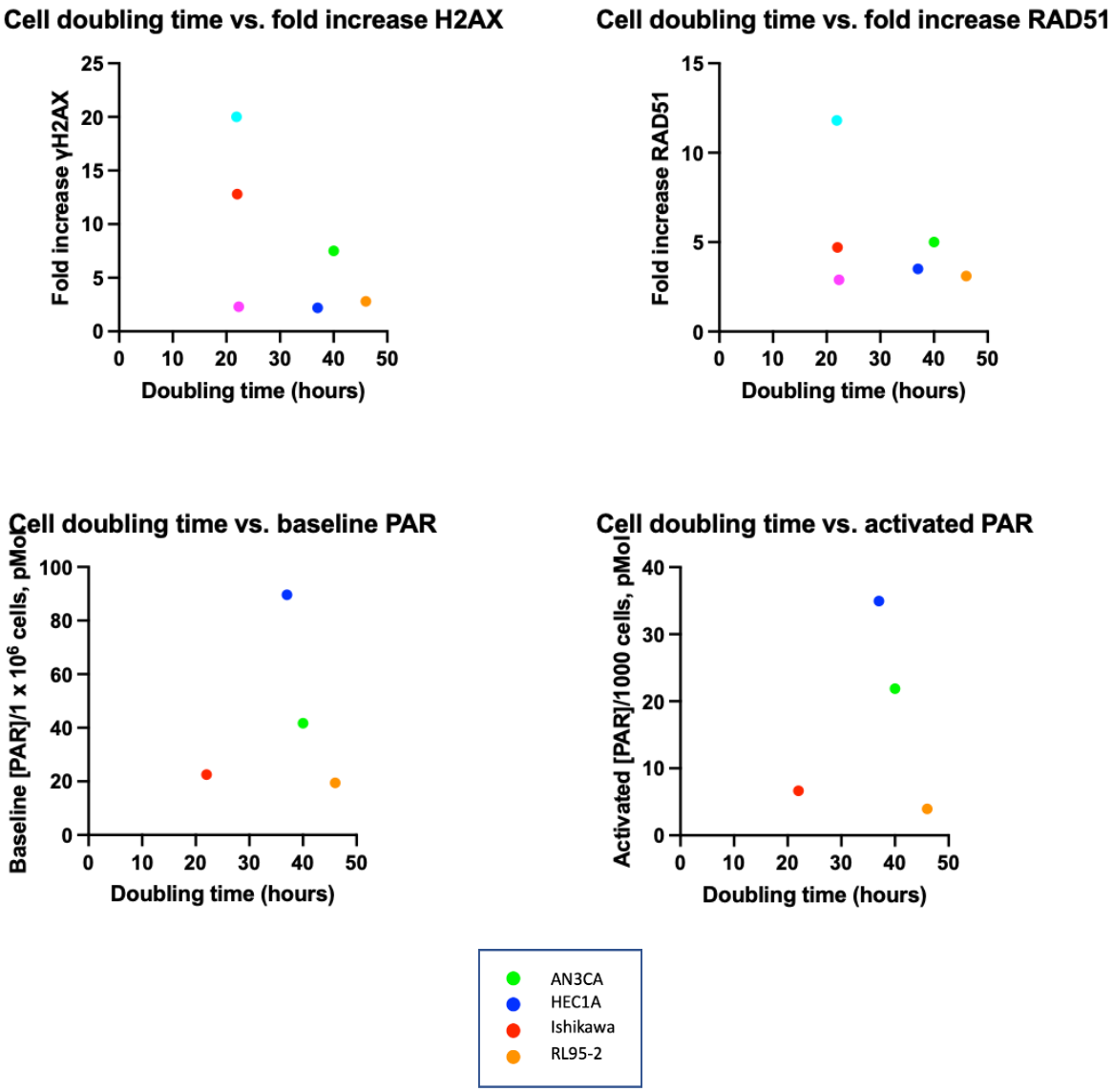


Figure 4.7. **Correlations between cell doubling time, H2AX and RAD51 foci formation, baseline and activated PARP** . Scatterplots of cell doubling timevs. other key cell line characteristics including fold increase in H2AX foci formation, fold increase in RAD51 foci formation during HRR assay, baseline PAR and activated PAR. Whilst all comparisons demonstrate no correlation, there is a trend towards negative correlation when comparing cell doubling time to fold increase in H2AX and RAD51. There was no trend between cell doubling and baseline or activated PAR. It is worth noting that the sample size is small to draw any definitive conclusions.

4.4.9 Correlations between endogenous PAR, activated PAR and cell doubling time

Cells growing more rapidly might be expected to have greater PARP activity, however there was no correlation between baseline PAR vs. cell doubling time, or between activated PAR vs. cell doubling time.

4.5 Discussion

In this Chapter the baseline characteristics of the endometrial cancer cell lines in terms of morphology, growth rate, HRR status, baseline and activated PAR were established. The growth rate of the cells determined here was similar to published proliferation rates (Qu et al., 2019). We expected to see a difference in proliferation rates between type 1 and type 2 endometrial cancer cell lines. Since low grade cancers behave clinically in a more indolent manner their growth rate was expected to be slower. This was true for HEC1A and RL95-2 but it was not the case for Ishikawa. Conversely, high grade and serous-type cancers tend grow rapidly but the high grade ANC3A cells were slow growing although the two serous cell lines (ARK1 and 2) did grow rapidly. This imperfect reflection of the clinical situation in monolayer culture is not entirely unexpected. High grade cancers have several cancer hallmarks, beyond proliferation rate, which make them behave in a more aggressive manner, such as growth signalling within the tumour microenvironment, and an ability to avoid detection by immune cells (Hanahan and Weinberg, 2011). Nevertheless, the growth rate may still affect the sensitivity to common agents used in the treatment on endometrial cancer such as cisplatin, IR and PARPi.

All 6 endometrial cancer cell lines studied were found to be HRR competent. This is the first time that a functional homologous combination repair assay has been undertaken in these cell lines. Interestingly, we hypothesised that p53 mutated endometrial cancers, those that are MSI-H and PTEN mutated, may be HRD. However, in this small cell line cohort we are yet to establish a link. Whilst being able to detect the most common causes of HRD, i.e., those upstream of RAD51 –mediated strand invasion, defects downstream cannot be excluded. Recently our team had noted an ovarian cancer cell line (NIH-OVCAR3) that was particularly sensitive to PARPi but was HRC by RAD51 assay and absence of HRR gene mutation. On deeper investigation of that cell line, the team demonstrated that there was functional loss of HRR identified by the plasmid re-joining assay (Bradbury et al., 2020).

Cell lines were chosen to reflect the broad disease of endometrial cancer including a variety of histological subtypes. They also included cell lines with known mutations in p53, MSI-H, those with non-specific molecular profile (NSMP) and POLE. HRD would be expected in the serous endometrial cancers (largely p53 mutated) or other histological subtypes that are p53

mutated as per TCGA, transportec and other groups (Bosse et al., 2018, Cancer Genome Atlas Research et al., 2013, Kommoss et al., 2018a); however, 3-5% of endometrial cancers will have multiple TCGA classifiers (e.g., dMMR and p53 mutation or POLE and p53 mutations coexisting) (León-Castillo et al., 2020b). Hierarchical testing is therefore employed in the instance of multiple classifiers (Alexa et al., 2021). Endometrial cancers with dual mutations in POLEmut-dMMR or POLEmut-p53abn have clinical behaviour aligned with POLEmut tumours and are considered POLEmut. dMMR-p53abn tumours have similar clinical behaviour as the dMMR and should be classified as such. The classification of dMMR-p53abn as dMMR tumours might suggest that dMMR and HRD do not coexist, which has been suggested in genomic studies in ovarian cancer (Rempel et al., 2022). Given that some of the chosen cell lines have multiple classifiers; AN3CA would be classified as dMMR (p53abn and MSI-H tumour), ARK 1 and 2 as NSMP, HEC1A as dMMR (MSI-H tumour), RL95-2 as dMMR and Ishikawa as POLEmut (POLE and p53abn) see table 4.1. This could be seen as a limitation in the choice of endometrial cancer cell lines as no cell line would be truly classified as p53abn and therefore may not be expected to be HRD.

However, it should not be assumed that dMMR and HRD are mutually exclusive. The classification system used in clinical practice, particularly with multiple molecular classifiers, attempts to simplify clinical decision making in patients. There is evidence that microsatellites in the promoter region of HRR genes give HRD phenotype in endometrial and other cancer types (Gaymes et al., 2013, Merentitis et al., 2019). In addition, MSI-H ovarian tumours are known to coexist with defects in HRR with associated PARPi sensitivity (Brandt et al., 2017). It was prudent to include such cell lines in this experimental work given that 30-40% of endometrial cancers are dMMR and previous publications in endometrial and colorectal cancers have demonstrated MRE11 mutations are driven by dMMR tumours (Koppensteiner et al., 2014, Giannini et al., 2004). It is therefore possible that MSI-H tumours could result in mutations in HRR genes and confer sensitivity to PARPi.

It may have been useful to attempt to include other cell lines in this work. However, there are only a limited number of commercially available endometrial cancer cell lines, particularly serous cell lines. These are limited to academic institutions and as a result there were barriers in accessing such cell lines due to this project's affiliation with the pharmaceutical industry.

4.6 Conclusions

- Cell doubling time and optimum seeding density for cell lines for use in growth inhibition studies by SRB had been calculated.
- All endometrial cell lines studied form colonies and have a good plating efficiency for cytotoxicity studies with colony survival assay.
- Endometrial cell lines were characterised for their HRR status, all cell lines were HRC.
- Baseline and activated PAR was similar in all cell lines studied with a positive correlation between baseline and activated PAR.
- There was no correlation between cell doubling time and HRR status or between baseline and activated PAR vs. cell doubling time.
- Further experimental work focused on understanding how these baseline cell line characteristics impact upon sensitivity to common agents used in endometrial cancer such as cisplatin, IR and Niraparib, along with understanding the impact that PARPi Niraparib has on the activity of PARP in endometrial cancer cell lines for combination studies.

Chapter 5. Radiation, cisplatin and Niraparib cytotoxicity and growth inhibition in endometrial cancer cell lines

5.1 Introduction

The 6 endometrial cancer cell lines have been characterised for their doubling time, cloning efficiency, HRR functional status, baseline and activated PARP in the previous chapter to aid interpretation of subsequent experimental work.

Radiotherapy is the mainstay of adjuvant treatment in early-stage endometrial cancer. Risk factors for disease recurrence dictate sequential chemotherapy and radiotherapy in stage 3 disease with chemotherapy alone being reserved for patients with stage 4 disease (Concin et al., 2021). Platinum based chemotherapy in the form of carboplatin is one of the principal agents used clinically, due to its reduced renal toxicity in patients (Vermorken et al., 1993). Cisplatin has been used in experimental work and has the same mode of action as carboplatin. Platinum chemotherapy and radiotherapy cause a variety of DNA damage and are repaired as part of the DNA damage response (see Section 1.7). Platinum chemotherapy causes intra- and inter-strand crosslinks which result in single strand breaks and double strand breaks during replication. This damage is repaired by NER and ICL/FANC pathway, and therefore has links with HRR. Radiotherapy induces both single and double strand breaks. These are repaired by BER/SSBR and NHEJ. Most defects confer sensitivity to treatment, however, dMMR and p53 mutations are known to confer platinum resistance and p53 mutations are also linked with IR resistance (Zhu et al., 2020, Vaisman et al., 1998). In the TCGA endometrial cancer study p53 mutated endometrial cancers were suspected to be the group in which, patients would be HRD and sensitive to PARPi. A significant proportion of endometrial cancers have PTEN mutations which is reflected in the cell line panel studied. There is conflicting evidence of the role of PTEN mutations in HRD in cancers. HRR defects are exploited using PARPi through synthetic lethality and therefore it is prudent to explore PARPi in endometrial cancer. HEC1A, Ishikawa and RL95-2 cell lines all demonstrated dMMR/MSI-H and AN3CA and Ishikawa cell lines have p53 mutations, however, all 6 cell lines were not functionally shown to be HRD despite this. There is little data on the cytotoxicity to these agents in the literature and therefore this study is novel in that it allows for direct comparison in cell lines in terms of response to cisplatin, IR and PARPi. In this

chapter we aim to explore sensitivity of the 6 endometrial cancer cell lines to IR, cisplatin and the PARPi, Niraparib, alone with reference to their cell line characteristics determined in Chapter 4.

5.2 Aims and Objectives

The aims to be investigated in this chapter are as follows:

1. To determine single agent cytotoxicity of cisplatin, Niraparib and IR in the endometrial cancer cell lines
2. To determine the growth inhibition by cisplatin and Niraparib in the endometrial cancer cell lines
3. To determine the IC50 of PARP inhibition by Niraparib in endometrial cell lines.

This will enable to following hypotheses to be tested:

- Doubling time correlates with sensitivity to cisplatin, Niraparib or IR
- Baseline or activated PAR correlate with single agent cytotoxicity
- RAD51 or γH2AX act as a biomarker for single agent cytotoxicity or growth inhibition in endometrial cancer cell lines
- GI_{50} can be used as a surrogate marker of cytotoxicity for use in primary culture experiments if primary endometrial cancer cultures do not form colonies
- Deficient MMR, p53 and PTEN mutation status are associated with sensitivity to cisplatin, IR and Niraparib.

5.3 Materials and Methods

Methods are described in chapter 3. Growth inhibition and cytotoxicity studies are discussed in sections 3.5 and 3.6, PARP activity assay is discussed in section 3.8.

5.4 Results

5.4.1 Determining PARP inhibitory concentration 50 (IC50)

PARP activity was measured in permeabilised exponentially growing cells in the presence and absence of increasing concentrations of Niraparib (Figure 5.1). The inhibitory concentration 50 (IC50) i.e., the concentration of Niraparib which inhibits the activity of PARP in the cell line by 50%, and inhibitory concentration 90 (IC90) were calculated. The methodology for the PARP assay is summarised in Section 3.8.

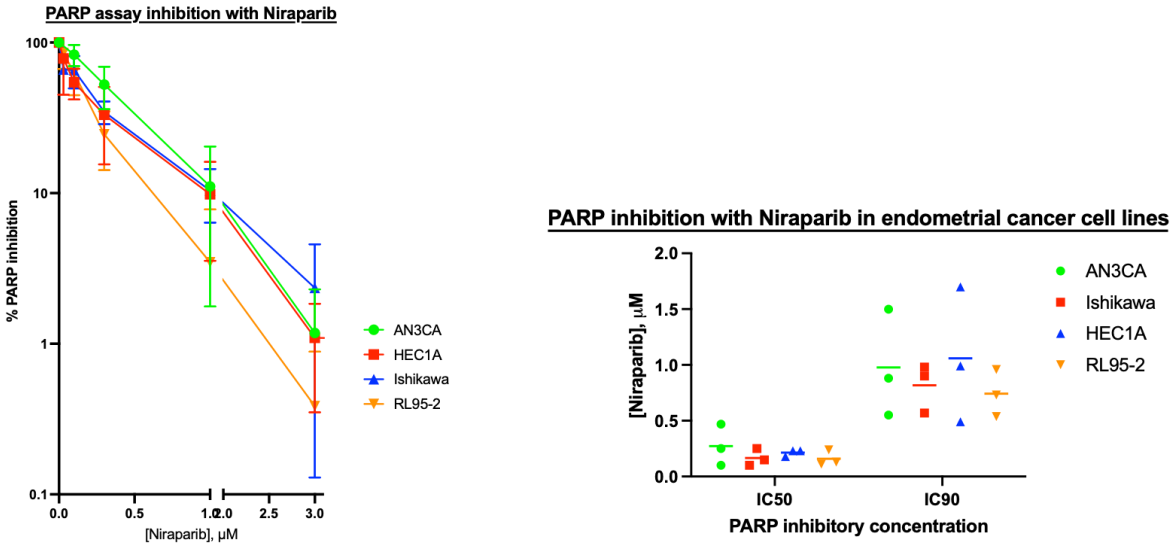


Figure 5.1. **PARP inhibition in endometrial cancer cell lines**. PARP activity was measured in permeabilised exponentially growing cells in the presence of activating oligonucleotide and excess NAD (as described in Section 3.8.2) and increasing concentrations of Niraparib. A) Pooled concentration response curves for the 4 cell lines, data are mean and SD from 3 independent experiments. B) Concentrations of Niraparib required to inhibit PARP by 50% (IC50) or 90% (IC90). Data are from independent experiments with means given as horizontal lines.

There was no significant difference in the PARP inhibition in the endometrial cancer cell lines with similar IC50 and IC90 values in all cells. This is not surprising as it is a biochemical assay and the cells had been permeabilised to permit oligonucleotide and NAD entry into the cell so transport of Niraparib into the cell is not an issue. Importantly, in all cell lines Niraparib 1 µM inhibits ≈90% of PARP activity

5.4.2 Single agent cytotoxicity: Cisplatin, Niraparib, IR

To determine the cytotoxicity of both the standard of care agents, cisplatin, IR and the novel agent Niraparib cell survival following incubation with single agent cisplatin, Niraparib or irradiation (IR) was determined by colony formation assays in all EC cell lines. LC50 values were calculated and comparison between cell lines made using GraphPad prism Figure 5.2 and table 4.1.

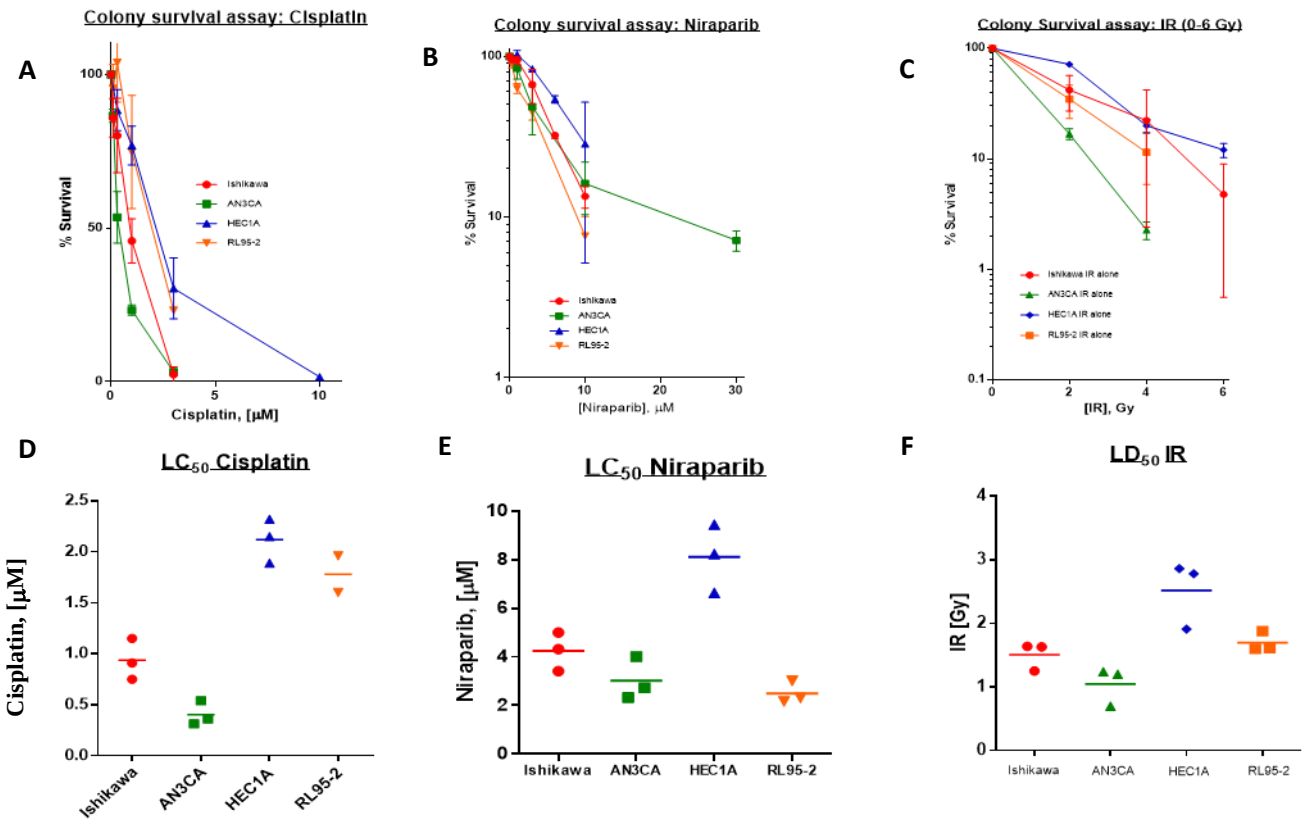


Figure 5.2. **Cytotoxicity of cisplatin, Niraparib and IR by colony formation assay.** Colony formation assays were undertaken as per section 3.6.2. 10-14 days after cells were treated with increasing concentrations of cisplatin (A) or Niraparib (B) for 24 hours or exposed to increasing doses of IR (C). Data are mean and SD from 3 independent experiments. The concentration (LC50) of cisplatin (D) or Niraparib (E) or radiation dose (LD50) (F) required to inhibit survival by 50% was determined by interpolation (GraphPad Prizm) on concentration/dose response curves where data points are from individual experiments with the mean given as a horizontal line.

Cell line	LC50 Cisplatin (μM ± SEM) -	% Colony survival at 1 μM cisplatin ± SEM	LC50 IR (Gy ± SEM)	% Colony survival at 4 Gy ± SD	LC50 Niraparib (μM ± SEM)	% Colony survival at 3 μM Niraparib ± SEM
AN3CA	0.40 ± 0.07	22.11 ± 1.83	1.05 ± 0.17	2.27 ± 0.24	3.02 ± 0.51	48.55 ± 9.32
Ishikawa	0.94 ± 0.12	45.77 ± 4.19	1.51 ± 0.13	22.21 ± 1.43	4.24 ± 0.46	66.86 ± 9.4

RL95-2	1.78 ± 0.34	61.65 ± 1.24	1.70 ± 0.09	11.43 ± 3.22	2.49 ± 0.26	45.14 ± 2.92
HEC1A	2.12 ± 0.13	76.83 ± 3.66	2.52 ± 0.30	19.99 ± 1.56	8.11 ± 0.81	83.75 ± 1.25

Table 5.1. **Summary of cytotoxicity data** Shown in Figure 5.3. Summary of LC50/LD50 data as shown in Figure 5.3 D, E and F alongside survival at 1 µM cisplatin, 3 µM Niraparib and 4 Gy. Data are mean +/- SD for 3 independent experiments

The spectrum of cisplatin, Niraparib and IR cytotoxicity across the panel of cells was relatively narrow. There was a 5-fold range of sensitivity to cisplatin (LC50 0.4 to 2.1 µM), a 4-fold difference in Niraparib (LC50 2.49 –8.11 µM) and only 2.5-fold range in IR sensitivity (LD50 = 1 to 2.5 Gy). In general, ANC3A were the most sensitive and HEC1A were the most resistant to all treatments. ARK1 and ARK2 cell lines were also sensitive to Niraparib (LC50 1.01 and 0.43 µM, respectively) but due to time constraints were not studied further.

In general, the LC50 appear to rank in a similar order with cisplatin and Niraparib and IR. It is worth noting that RL95-2 were resistant to cisplatin but sensitive to Niraparib and had intermediate sensitivity to IR.

5.4.3 Correlations between LC50 cisplatin, Niraparib and LD50 IR

Correlation analysis was undertaken to determine if there was any relationship between cytotoxicity to Cisplatin, Niraparib and IR. Individual scatter plots are shown below between Cisplatin LC50, Niraparib LC50 and IR50 in figure 5.3. There was no statistically significant correlation between Cisplatin and Niraparib, Cisplatin and IR, and Niraparib and IR. This was limited due to the small sample size in the endometrial cohort. However, the rank order in the cell lines between Cisplatin LC50 and IR LD50 is the same (AN3CA-> Ishikawa-> RL95-2-> HEC1A). There appears to be a trend towards a positive correlation. However, when cell lines were treated with Niraparib RL95-2 was the most sensitive (ranking 3rd when treated with Cisplatin and IR). This does not consider the sensitivity of ARK1 and ARK2 to cisplatin and IR was which not undertaken and is therefore a limitation.

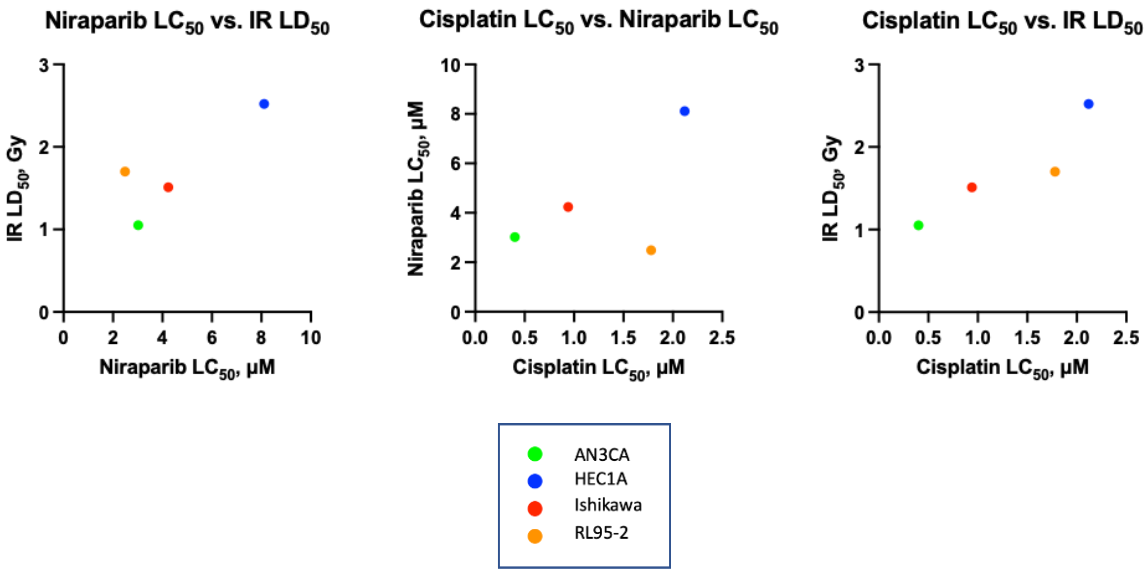


Figure 5.3. **Scatter plot of LC/D50 to Cisplatin, Niraparib and IR**. Data are mean values of three experimental repeats and are shown in table 5.2.

5.4.4 Single agent growth inhibition with cisplatin and Niraparib

Whilst colony forming assays are the most reliable method to determine the cytotoxicity of an agent it is not always possible to perform if the cells in question will not form colonies. Growth inhibition assays using a surrogate measure of cell number are a convenient alternative, but it should be noted that different surrogate measures of cell number, e.g. measurement of cellular reductase versus ATP, can result in very different estimates of drug sensitivity (Haibe-Kains et al., 2013). Here we sought to determine if growth inhibition using protein content, as measured by SRB staining, was a sufficiently reliable indicator of drug-induced cell killing. Growth inhibition by single agent cisplatin or Niraparib was performed in all cell lines (Figure 5.4, Table 5.2). It is not possible to do the same studies with IR due to "shadowing" by the walls, in relation to the small surface area, of the wells in the irradiator.

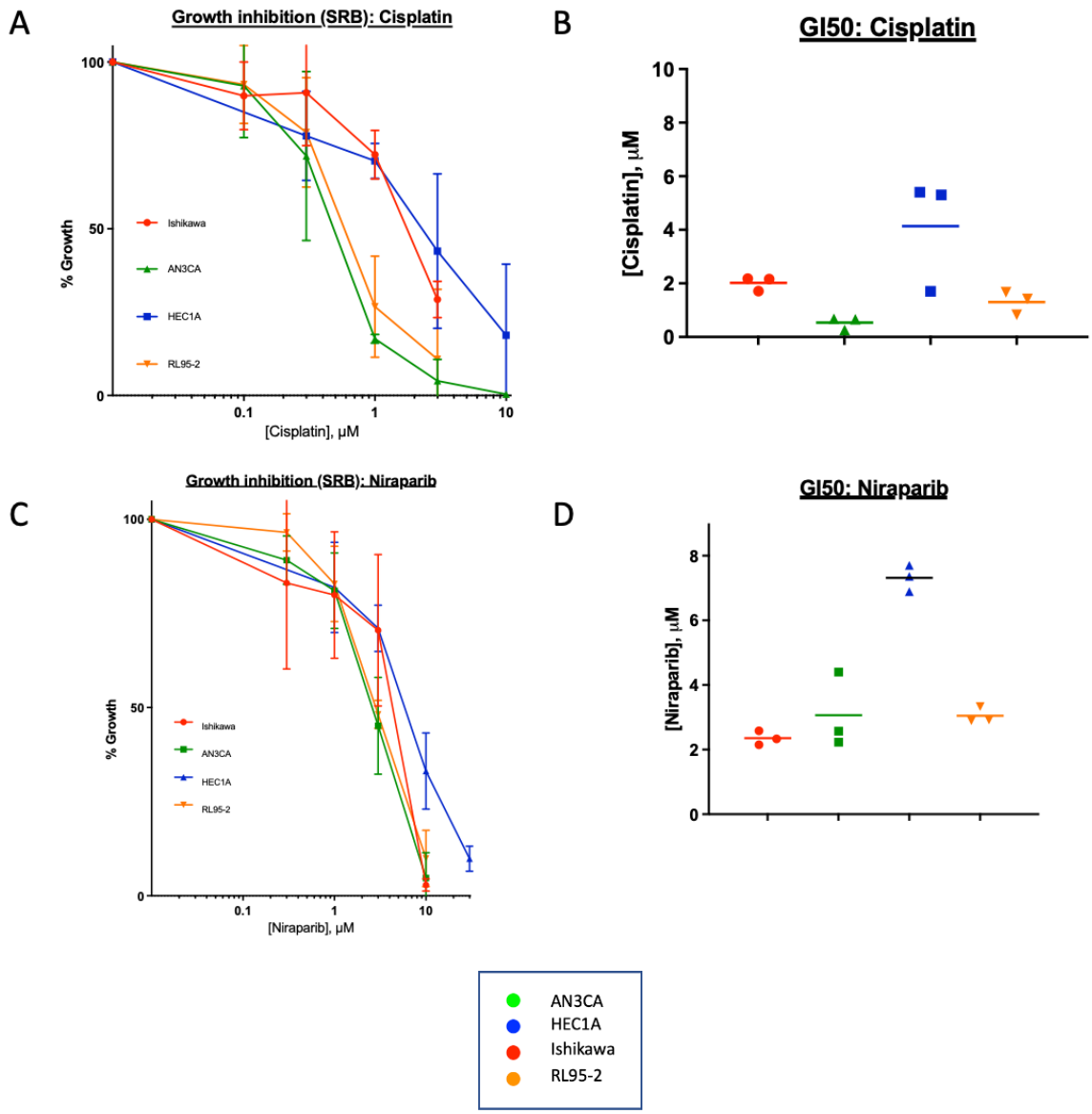


Figure 5.4. **Growth inhibition with single agent cisplatin and Niraparib by SRB assay**. Exponentially growing cells in 96-well plates were exposed to increasing concentrations of cisplatin (A) or Niraparib (C) for 7 days prior to fixing and staining with SRB, and cell growth compared to vehicle alone-treated cells was compared. Data are mean and SD from 3 independent experiments. The concentration required to inhibit growth by 50% (GI50) determined by interpolation (GraphPad Prizm) of the concentration response curves is given for cisplatin (B) and Niraparib (D) where data points are from individual experiments with the mean given as a horizontal line.

Reassuringly, the growth inhibition GI50 values were in a similar concentration range to the cytotoxicity LC50 values (0.4-4 µM for cisplatin and 2-8 µM for Niraparib), suggesting that these assays could be a convenient substitute for cells that do not form colonies. There was

a greater spectrum of growth inhibition than cytotoxicity by cisplatin with an 8-fold difference between the highest and lowest GI_{50}. As with the cytotoxicity experiments HEC1A were the most resistant (GI_{50} =4.13 μM) and AN3CA were the most sensitive ($GI_{50=}$ 0.53 μM). Growth inhibition by Niraparib was similar in AN3CA, Ishikawa and RL95-2 (GI50 = 2.4 – 3.1 μM) but as in the cytotoxicity studies HEC1A cells were 2-3 x more resistant.

Cell Line	Cisplatin: GI_{50} μM ± SEM	% Growth Cisplatin 1 μM	Niraparib: GI_{50} μM ± SEM	% Growth Niraparib 3 μM
AN3CA	0.53 ± 0.14	17.00 ± 0.76	3.07 ± 0.67	45.14 ± 7.42
Ishikawa	2.02 ± 0.15	72.24 ± 4.20	2.35 ± 0.13	70.55 ± 11.61
RL95-2	1.25 ± 0.42	39.95 ± 28.42	3.05 ± 0.14	48.18
HEC1A	4.13 ± 1.22	70.35 ± 3.05	7.32 ± 0.24	71.05 ± 3.55

Table 5.2. **GI_{50} values of cisplatin and Niraparib alone** Summary of the mean GI_{50} and growth inhibition to single agent cisplatin and Niraparib along with percentage growth inhibition in each cell line at a given concentration with means and standard error of the mean for 3 independent experiments. Percentage growth inhibition is also displayed at cisplatin 1 μM and Niraparib 3 μM.

5.4.5 Correlations between single agent growth inhibition and single agent cytotoxicity

Correlation analysis was undertaken to determine if there was a relationship between growth inhibition values and cytotoxicity in the endometrial cancer cell lines to Cisplatin and Niraparib. The individual scatter plots for Cisplatin GI50 vs. Cisplatin LC50 and Niraparib GI50 vs. Niraparib LC50 are shown in figure 5.5. There was not statistically significant correlation by Pearson's correlation coefficient, however, there appears to be a positive trend between GI vs. LC50 for both cisplatin and Niraparib. To probe this further the growth vs survival of cells at each drug concentration was evaluated (Figure 5.6). There is a clear positive trend between growth inhibition (%) and cell survival (%) by colony survival assay. Whilst the correlation is not perfect, it demonstrates that growth inhibition could be used as a surrogate for cytotoxicity in experimental work when cell cultures do not form colonies.

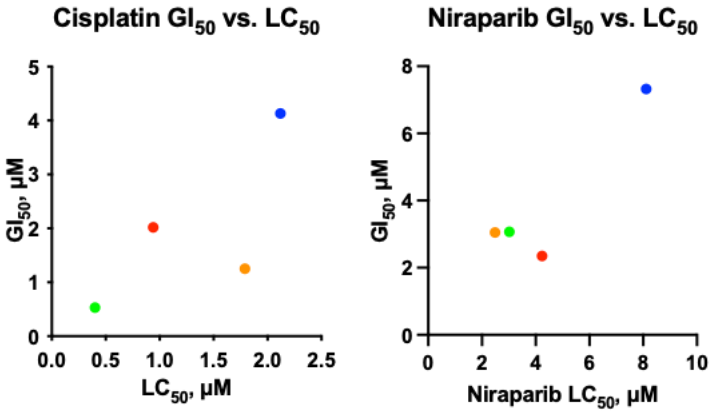


Figure 5.5. **Scatter plots of Cisplatin GI50 vs. LC50 and Niraparib GI50 vs. LC50**. Data are the mean of three experimental repeats and are shown in tables 5.1 and 5.2.

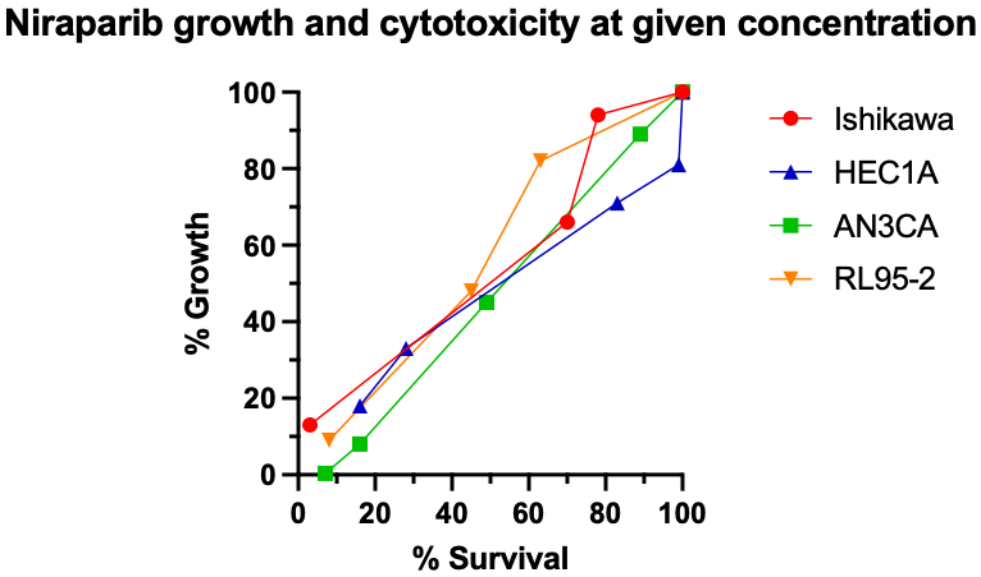


Figure 5.6 **Percentage growth vs. percentage survival at a given concentration of Niraparib**. Percentage growth was plotted against Percentage survival for each concentration of Niraparib in all 4 cell lines. Each data point is the mean growth and mean survival for a single drug concentration after 3 experimental repeats.

5.4.6 Correlations between doubling time, fold increase H2AX and RAD51 vs. cytotoxicity to Cisplatin, Niraparib and IR

There was no correlation between baseline cell characteristics (doubling time, fold increase in H2AX and RAD51) and LC50 values for cisplatin, Niraparib or IR. However, baseline characteristics in those compared to cisplatin LC50 and IR LD50 ranked similarly but were not statistically significant (figure 5.7). It is difficult to draw any firm conclusions in this small cell line panel.

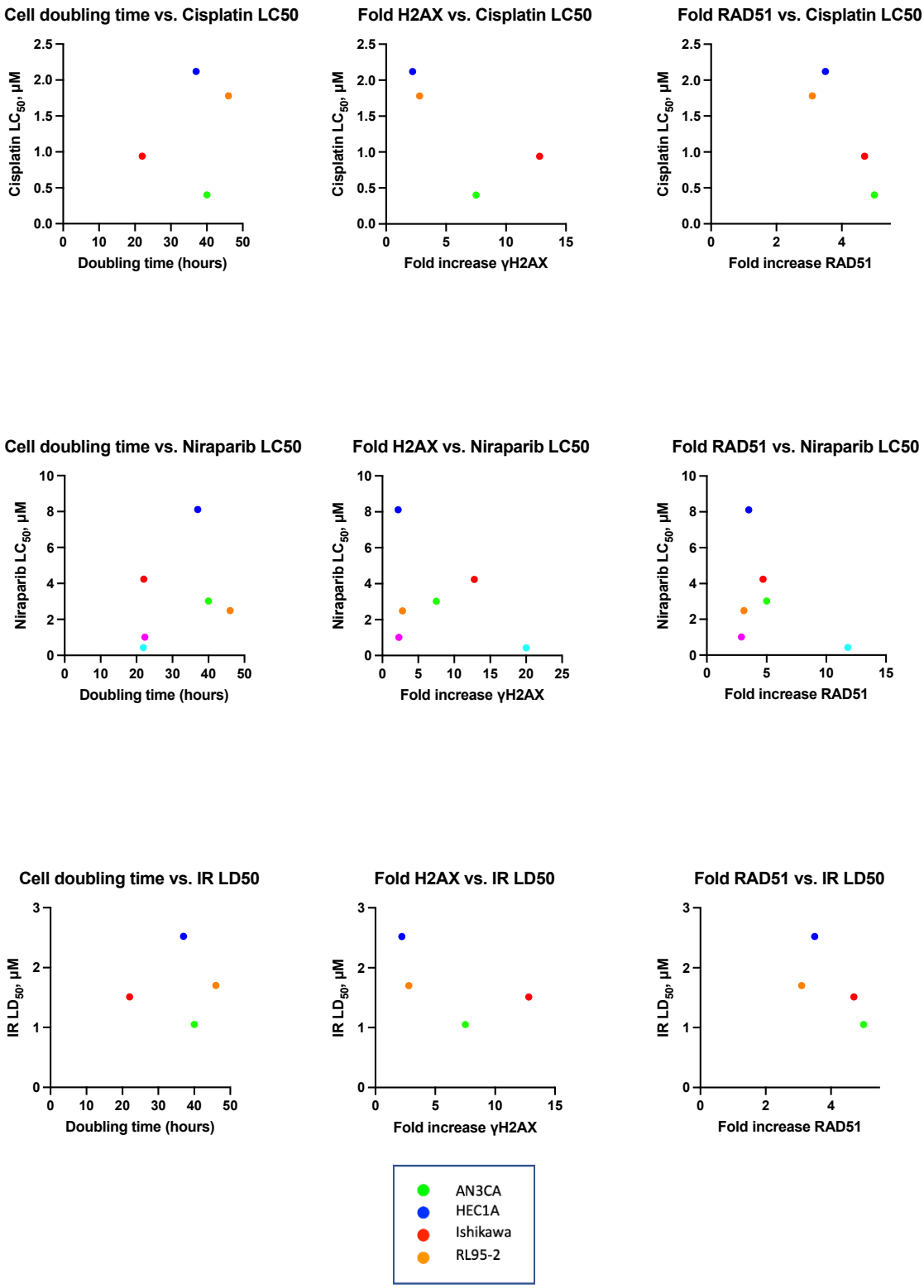


Figure 5.7. **Scatter plot of cell doubling time, fold increase in H2AX and RAD51 vs. Cisplatin LC50, Niraparib LC50 and IR LD50**. There was no statistically significant correlation between any of the variables. Data are mean values of 3 replicates calculated and displayed in tables 4.1, 4.2 and 5.1.

5.4.7 *Correlations between cell line characteristics, HRR status, and single agent cytotoxicity and growth inhibition*

Correlation analyses were also performed to determine if growth inhibition was related to baseline cell characteristics such as doubling time, HRR status, baseline and activated PAR. Although cell growth rate and induction of replication stress would be expected to correlate with both growth inhibition, no such correlation was observed between cell doubling or H2AX focus induction and either cisplatin or Niraparib-induced growth inhibition (Figure 5.8) neither was there a correlation with RAD51 focus induction, although all cell lines were found to be HRR competent.

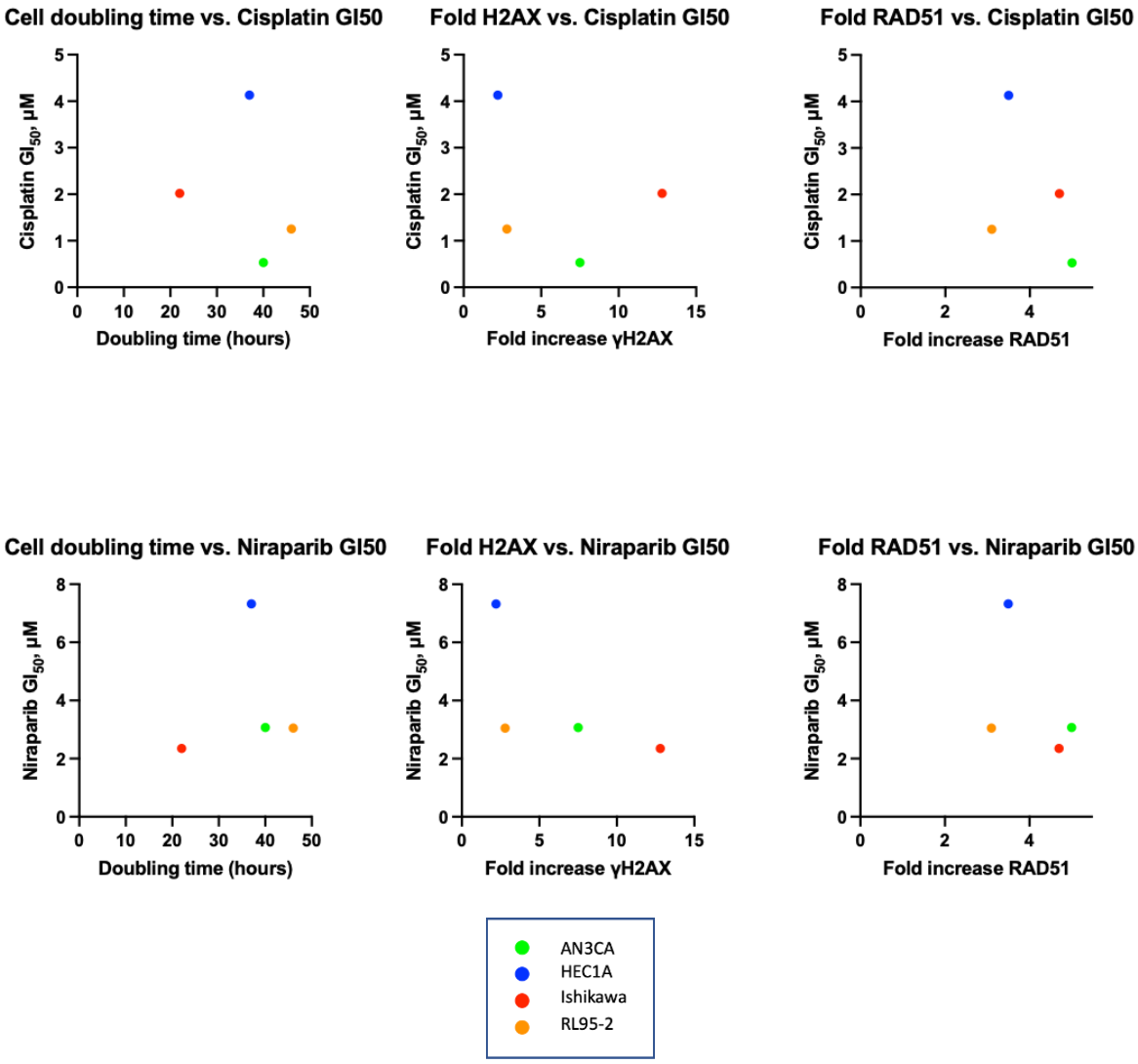


Figure 5.8. **Correlations between cell doubling time, fold increase H2AX and fold increase RAD51** vs. Cisplatin GI50 and Niraparib GI50. Data are mean values which are displayed in tables 4.1 and 5.1.

5.4.8 *Correlations between baseline PAR, activated PAR, PARP inhibition and single agent cytotoxicity to Niraparib*

Correlation analysis was undertaken to understand if there was any relationship between baseline PAR levels and PARP activity and inhibition by Niraparib with Niraparib cytotoxicity in the 4 studied cell lines (figure 5.9). No statistically significant correlation was observed between any of these parameters in this small number of cell lines. As PARP inhibition was very similar in all cell lines (section 5.4.1) it was not possible to relate the differences in Niraparib cytotoxicity or growth inhibition to PARP inhibition.

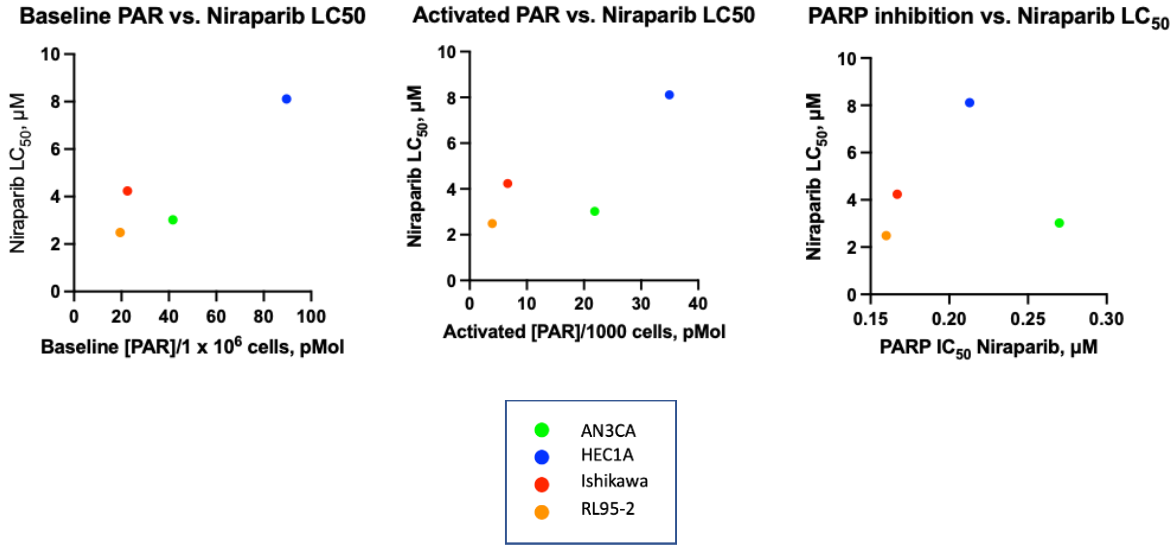


Figure 5.9. **Scatter plot of baseline PAR, activated PAR and Niraparib IC50 (PARP inhibition) vs. Niraparib LC50.** Data are mean values of three experimental repeats and are displayed in tables 4.3 and 5.2.

5.5 Discussion

In this chapter we examined the cell survival and growth inhibition to cisplatin, IR and Niraparib. We sought to explain the differences by comparing these to baseline cell line characteristics. We found a relatively narrow spectrum of sensitivity in all cell lines studies to cisplatin, IR and Niraparib, two of which are used in the standard of care in endometrial cancer management. There was a trend to sensitivity with HEC1A cell line being the most resistant to IR, cisplatin and Niraparib, and AN3CA being the most sensitive. This is the first time that this panel of endometrial cell lines have had sensitivity to cisplatin and IR to allow for direct comparison of sensitivity. We would expect low grade endometrial cancers, like HEC1A to be more resistant to platinum chemotherapy and IR, and for a high-grade endometrial cancer to be more sensitive to platinum (Bestvina and Fleming, 2016). This is nicely reflected in our data series. This is the first time that this panel of endometrial cell lines have had sensitivity to cisplatin and IR to allow for direct comparison of sensitivity. We might expect that p53 mutated cancers be more resistant to cisplatin and IR, as referenced in studies in other cancer sites (Chee et al., 2013, Reles et al., 2001), however we found that not to be the case with HEC1A being most resistant without p53 mutation and found that AN3CA were the most sensitive, with a p53 mutation. Interestingly, HEC1A and RL95-2 were more resistant to cisplatin, compared to other cell lines. Both cell lines are known to harbour defects in MMR. This is in keeping with the notion that dMMR tumours are more resistant to platinum agents because of replication bypass (Vaisman et al., 1998). This was not reflected in AN3CA which is also known to be MSI-H. However, it is difficult to form firm conclusions given the small number of cell lines studied. A similar trend in sensitivity to Niraparib as for cisplatin and IR which suggests sensitivity is not due to the classic determinant of PARPi sensitivity, rather that some cell lines may have more pro-survival factors that render them more resistant than others. Other aspects of the cell's biology didn't affect sensitivity to these drugs such as growth rate either. Cancer cell biology is complex with multiple determinants of resistance and sensitivity likely to be present making it difficult to define predictive biomarkers for selection of personalised therapy (Saeed et al., 2018). Other aspects of the cell's biology did not affect sensitivity to these drugs such as growth rate, fold H2AX, RAD51 and baseline or activated PARP.

This is the first time that Niraparib sensitivity and growth inhibition has been performed in endometrial cancer cell lines which makes this data novel. However, a panel of endometrial cancer cell lines has previously been treated with Olaparib to determine sensitivity in endometrial cancer cell lines by colony survival assay (Miyasaka et al., 2014). Interestingly, Ishikawa, RL95-2 and AN3CA cell lines were found to be more sensitive to Olaparib compared to the more resistant HEC1A cell line. All these cell lines harbour PTEN mutations, however in the cell line panel in this thesis, PTEN mutation status could not be linked to PARP inhibitor sensitivity, raising the question as to whether PTEN effects HRR function as seen in previous studies (Shen et al., 2007, Dedes et al., 2011).

Additionally, for the further studies described in subsequent chapters we identified that Niraparib 1 μM inhibited ~ 90% PARP activity without affecting cell growth or survival to a great extent (see table 5.3 below), thereby allowing this concentration to be selected for chemo- and radio-sensitisation studies. We also concluded that growth inhibition can be used as a surrogate for cytotoxicity in primary cell culture, which tend not to grow in colonies.

Cell line	**% PARP inhibition at Niraparib 1 μM**	**% Cell survival with Niraparib 1 μM**
AN3CA	88.9 (4)	81.05 ± 10.06
HEC1A	90.2 (2)	81.89 ± 11.99
Ishikawa	89.6 (3)	79.88 ± 16.80
RL95-2	96.5 (1)	82.83 ± 10.03

Table 5.3. **Demonstrating PARP inhibition at Niraparib 1 μM and the associated growth inhibition at Niraparib 1 μM in growth inhibition studies by SRB.**

5.6 Conclusion

- PARP inhibition by Niraparib was similar in all cell lines tested
- Growth inhibition correlated well with cytotoxicity in the cell lines
- No significant correlations were seen between baseline cell characteristics (doubling time, HRR functional status) vs. GI50, LC50 and PARP inhibition
- Cell lines ranked in a similar order of sensitivity when treated with cisplatin, IR or Niraparib.

Chapter 6. Potentiation of Cisplatin or IR by Niraparib

6.1 Introduction

Results from the previous chapter identified the growth characteristics, PARP activity, HRR status, growth inhibition and cytotoxicity by cisplatin, Niraparib and IR.
The overall aim of this thesis was to study the role of PARP inhibitors in endometrial cancer including their role as chemo- and radiosensitisers. The standard of care of for those with advanced disease or high risk of recurrence is either sequential chemotherapy and radiotherapy or chemotherapy alone. Carboplatin is used as first line standard of care when chemotherapy is required, usually in combination with paclitaxel. In this Chapter the combination studies have used cisplatin which causes the same DNA damage as carboplatin.

PARPi are well known to potentiate the effects of some anticancer treatment. PARP null mice are hypersensitive to IR (Masutani et al., 2000). Another argument for their use in combination is that they improve the effectiveness of standard treatments and may allow for lower doses of toxic agents to be used, therefore reducing the damage to normal surrounding tissue in IR or reduce the systemic effects on normal tissue from chemotherapy. PARPi have been shown in pre-clinical studies to be radiosensitisers, their sensitisation is thought to be between 1.3-2-fold (Curtin, 2005, Griffin et al., 1995, Chalmers, 2004, Powell et al., 2010). Radiosensitisation appears to be related to use at concentrations where PARP is inhibited by >90% (Weltin et al., 1997). IR induces both SSBs and DSBs, with the DSB's being more lethal, as described in section 1.7.7. The underlying mechanism for this radiosensitisation is thought to be inhibition of the repair of SSBs (Banasik et al., 1992), but PARP has also been implicated in DSB repair though an interaction with NHEJ and direct involvement with alt-NHEJ. In addition, it is also thought that PARPi may cause an improvement in perfusion of tumours which leads to a reduction in hypoxia and an improvement in the toxic effects of IR (Calabrese et al., 2003, Ali et al., 2009, Senra et al., 2011). PARPi have also been shown to have both cardio- and renal-protective effects in patients, both of which can be caused by cytotoxic agents (Saha et al., 2022, Korkmaz-Icöz et al., 2018, Ali et al., 2009). There are conflicting reports as to the effect of combination treatments with platinum and PARPi, with sensitivity appearing to be compound and cell line specific (Bernges and Zeller, 1996, Lu et al., 2018). The DNA damage caused by platinum

result in intra- and interstrand crosslinks, which are repaired by NER and HRR, and not be SSBR/BER, which would be inhibited by PARPi (Bürkle et al., 1993, Guggenheim et al., 2008, Curtin, 2005). The cell-specific synergy between PARPi and cisplatin may reflect the dependence on functional HRR for resistance to both agents as synergy between Olaparib and cisplatin was only observed in BRCA mutated cells (Evers et al., 2008). In this chapter we aim to explore combination therapies with Niraparib to determine whether Niraparib could sensitise endometrial cancer cells to irradiation or cisplatin. Following on from results in the previous chapter it was decided to use Niraparib 1 μM in combination therapy.

Niraparib was chosen as the PARP inhibitor of choice in this thesis as the project was kindly sponsored by GSK. However, Niraparib is a potent PARP1/2 inhibitor with middle ranking PARP trapping and half-life (Krastev et al., 2021). The available PARPi (Talazoparib, rucaparib, Niraparib and olaparib) have different biochemical and pharmacological characteristics which determine their dosing, scheduling, and their effects in combination (Bruin et al., 2022a) . These differences include their half-life, PARP selectivity, potency, durability, and PARP trapping capabilities. In general, talazoparib has the greatest PARP1 inhibition potency, half-life and greatest PARP trapping and Veliparib the least. Rucaparib despite having the second longest half-life and PARP1 inhibition, has the longest durability (Smith et al., 2022).

6.2 Aims and Objectives

The aims of experimental work in this chapter were:

- To determine whether Niraparib sensitises endometrial cancer cell lines to IR.
- To determine whether Niraparib sensitises endometrial cancer cell lines to cisplatin.
- To determine if p53/PTEN/MMR status or baseline cell line characteristics are associated with radio- or chemosensitisation.

6.3 Materials and methods

Details of methods for undertaking chemopotentiation with colony survival assay and by growth inhibition and radiopotentiation in colony survival assay, with Cisplatin alone or in

combination with Niraparib 1 μM are discussed in chapter 3. In Short, in the colony survival assay, cells were seeded into 6 welI plates for 24 hours, they were then exposed to increasing concentrations of cisplatin alone or in combination with Niraparib 1 μM for 24 hours, media was then replaced and allowed to incubate until visible colonies were seen. Plates were then fixed as discussed in section 3.6. For colony survival combination treatment, cells were seeded and allowed to attach for 24 hours. Media was then replaced media containing 0.5% DMSO control or with Niraparib 1 μM. Wells were then irradiated immediately with increasing doses of IR and continued in culture for 24 hours. Media was then replaced and incubated until visible colonies were seen. For combination SRB treatments, cells were seeded into 96 well plates, incubated for 24 hours to allow cell attachment. Media containing increasing concentrations of cisplatin +/- Niraparib 1 μM and allowed to incubate for 3 doubling times and then fixed and processed as discussed in section 3.5. Data were then normalised to either DMSO or Niraparib alone control.

6.4 Results

6.4.1 Radiation potentiation with Niraparib by colony formation assay

Results of colony formation assays are shown in Figure 6.1 and summarised in Table 6.1. Niraparib caused a modest radio-potentiation in all EC cell lines, which increased at higher IR doses. The greatest radio-potentiation was seen in Ishikawa cells with a potentiation factor at their LC50 (PF50) of 1.83. There was modest potentiation seen at the LC50 for; HEC1A (PF50 of 1.31) and RL95-2 (PF50=1.20) but no potentiation observed in AN3CA. However, there appeared to be a greater potentiation at 4 Gy with sensitisation ranging from 2.41-fold in the Ishikawa cells 1.16 in HEC1A. However, two-way ANOVA failed to detect a difference between IR alone and IR plus Niraparib in all but the AN3CA cell line (p=0.01). This may relate to the inter-assay variation.

A

B

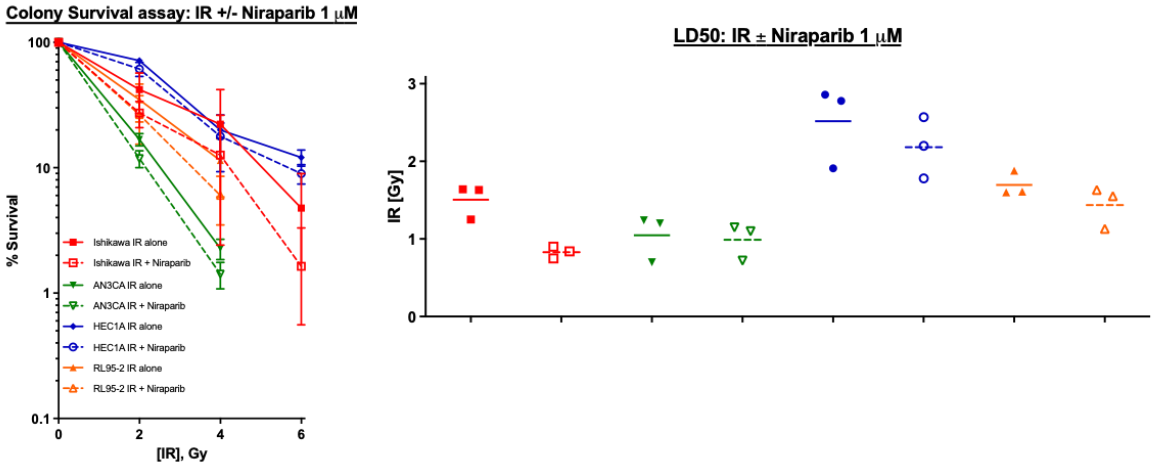
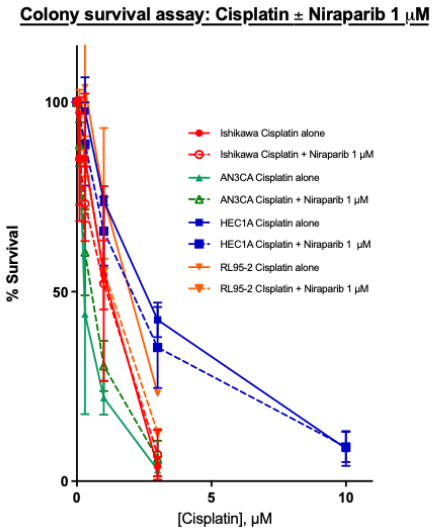


Figure 6.1. **Enhancement of IR by Niraparib**. **A**. survival of cells exposed to IR alone (solid line) or in the presence of 1μM Niraparib (dashed line) with a further 24 h incubation with Niraparib (dashed line) followed by incubation in drug free medium for colony formation. Data are normalised to unirradiated DMSO control or 1 μM Niraparib alone control as appropriate. Data are mean +/- SEM from 3 independent experiments each with triplicate measurements. **B**. LD50 values calculated by interpolation of survival curves (6.1A) following irradiation alone (solid symbols) or irradiation + 1 μM Niraparib (open symbols), colours correspond to those in figure 6.1A. Data are individual values from 3 independent experiments with mean values shown.

6.4.2 Potentiation of cisplatin with Niraparib by colony formation assay

Cisplatin sensitisation by Niraparib is shown in Figure 6.2 and summarised in Table 6.1. Chemosensitisation was very modest overall (≤1.5) at both the LC50 and at 1 μM cisplatin level.

A



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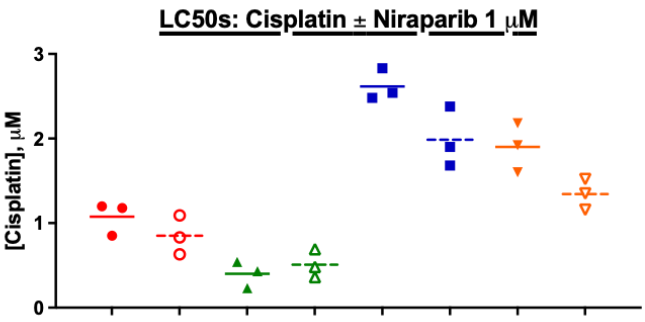


Figure 6.2. **Enhancement of cisplatin by Niraparib A**. survival of cells exposed to cisplatin alone (solid line) or in the presence of 1μM Niraparib (dashed line) with a further 24 h incubation with Niraparib (dashed line) followed by incubation in drug free medium for colony formation. Data are normalised to untreated DMSO control or 1 μM Niraparib alone control as appropriate. Data are mean +/- SEM from 3 independent experiments each with triplicate measurements. **B**. LC50 values calculated by interpolation of survival curves (6.2A) following cisplatin alone (solid symbols) or cisplatin + 1 μM Niraparib (open symbols), colours correspond to those in figure 6.2A. Data are individual values from 3 independent experiments with mean values shown.

Cell line	PF50 ± SEM Cisplatin	PF at Cisplatin 1 μM	PF50 IR ± SEM	PF at 4 Gy ± SEM
Ishikawa	1.36 ± 0.27	1.18 ± 0.28	1.83 ± 0.20	2.41 ± 0.14
AN3CA	0.77 ± 0.08	0.73 ± 0.01	1.00 ± 0.09	1.66 ± 0.25
HEC1A	1.33 ± 0.09	1.20 ± 0.16	1.31 ± 0.16	1.16 ± 0.17
RL95-2	1.44 ± 0.22	1.36 ± 0.29	1.20 ± 0.13	1.90 ± 0.08

Table 6.1. **Showing the potentiation chemo-potentiation of cisplatin (PF_{50} and 1 μM cisplatin) to Niraparib 1 μM and IR (PF_{50} and PF at 4 Gy).** Data are mean and SD of PF calculated from each of 3 independent experiments

6.4.3 Cisplatin with Niraparib 1 μM by growth inhibition with SRB assay

Growth inhibition combination SRB results are shown in figure 6.3 and table 6.2. Potentiation of cisplatin-induced growth inhibition by Niraparib was greater cytotoxicity potentiation, at least for Ishikawa and HEC1A cells but much less marked for RL95-2 cells.

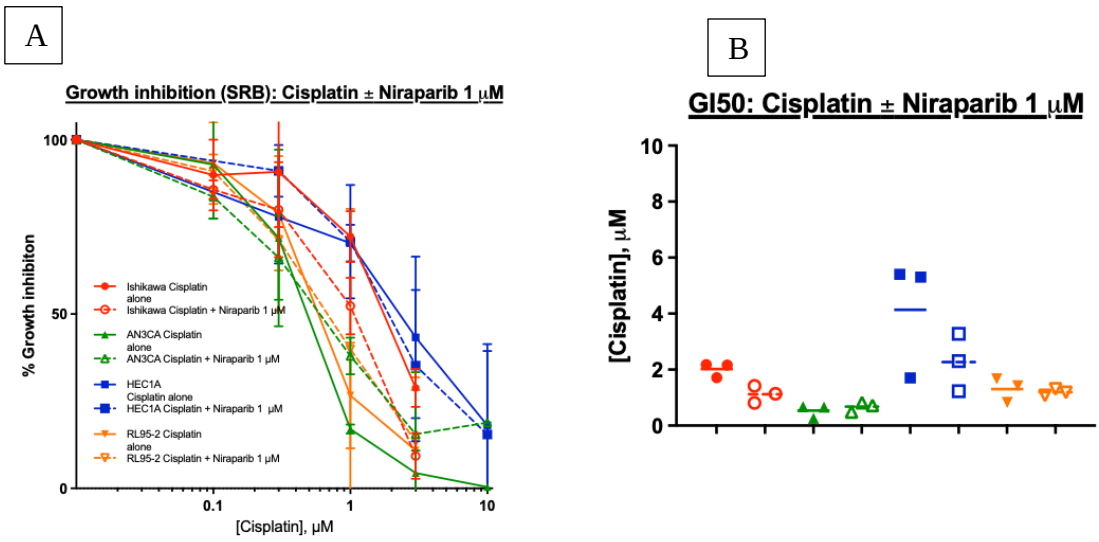


Figure 6.3. **Growth inhibition studies by SRB investigation sensitisation of four endometrial cancer cell lines to cisplatin ± Niraparib 1 μM** Exponentially growing cells in 96-well plates were exposed to increasing concentrations of cisplatin ± Niraparib 1 μM (A) for 6 days prior to fixing and staining with SRB, and cell growth compared to vehicle alone-treated cells was compared. Data are mean and SD from 3 independent experiments. The concentration required to inhibit growth by 50% (GI50) determined by interpolation

(GraphPad Prizm) of the concentration response curves is given for cisplatin alone or in combination with Niraparib 1 μM (B) where data points are from individual experiments with the mean given as a horizontal line.

Cell line	PF50 ± SEM Cisplatin	PF at Cisplatin 1 μM
AN3CA	0.87 ± 0.30	0.46 ± 0.05
HEC1A	1.78 ± 0.27	1.03 ± 0.14
Ishikawa	1.85 ± 1.67	1.39 ± 0.09
RL95-2	1.08 ± 0.16	0.94 ± 0.39

Table 6.2. **Demonstrating PF50 and potentiation at Cisplatin 1 μM when cisplatin is in combination with Niraparib 1 μM by growth inhibition**. Data are mean and SD of PF calculated from each of 3 independent experiments

6.4.4 Correlations

Correlation analysis was undertaken to determine if there were any relationships between baseline cell characteristics, HRR functional assay results, PAR studies, combination treatment and between colony survival assay and growth inhibition to determine overall effects of Niraparib in these endometrial cancer cell lines. Unfortunately, combination treatments were only undertaken in 4/6 of the endometrial cancer cell lines. Due to the small sample size with small spectrum of PFs it was not possible to accurately determine if sensitisation by colony formation was similar to sensitisation in growth inhibition, nor if chemosensitisation and radiosensitisation correlated but a trend for these to be related was observed that would be worth pursuing in additional cell lines. Chemo- and radio-sensitisation did not correlate with cell growth rate or potency of PARP inhibition.

6.4.5 Chemopotentiation in colony formation vs. chemopotentiation growth inhibition studies

Correlation studies were undertaken to compare the PF50 by growth inhibition with PF50 by colony survival (figure 6.4). Whilst there was no statistically significant correlation between the two, the numbers are small and are affected by one outlier.

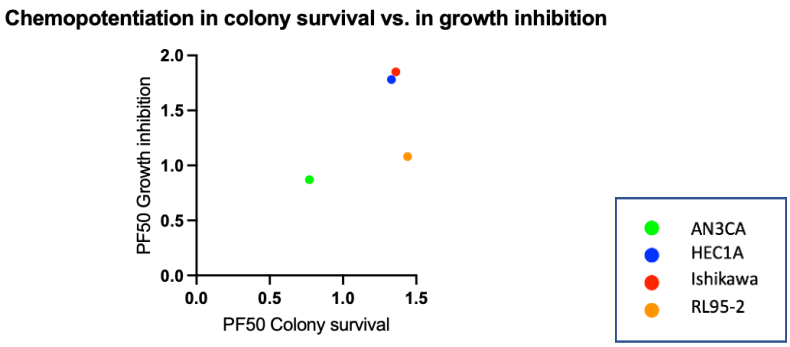


Figure 6.4 **Chemopotentiation in growth inhibition vs. chemopotentiation in colony survival.** Each point represents the mean fold potentiation seen in each cell line.

6.4.6 Correlation with chemopotentiation vs. radiopotentiation

Correlation studies were undertaken to compare the potentiation of the cytotoxicity of cisplatin and IR by Niraparib in colony survival assays (figure 6.5). Whilst there was no statistically significant correlation between the two, due to the small numbers there was a trend that suggested that both IR and cisplatin may be potentiated similarly in a cell line dependent manner.

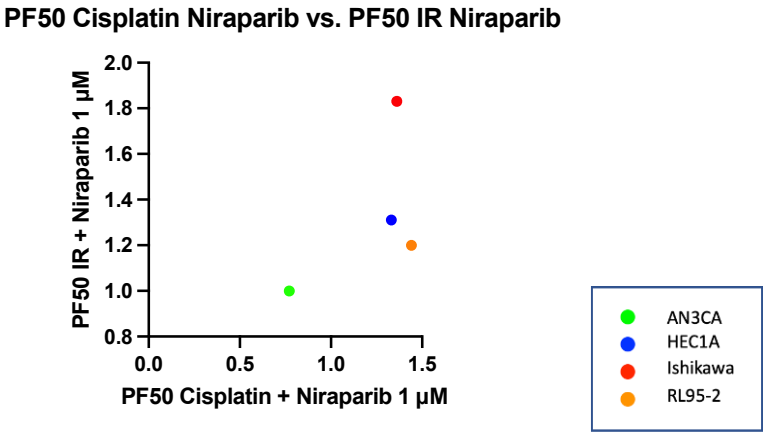


Figure 6.5. **Chemopotentiation vs. radiopotentiation by Niraparib. Each point represents the mean fold potentiation seen in each cell line.**

6.5 Discussion

The aim of this chapter was to explore whether Niraparib has a role in combination with standard of care treatments in endometrial cancer. PARPi have been found to sensitise the effects of ionising radiation and chemotherapeutic agents in many cancer types, resulting in a number of early phase clinical trials (Barcellini et al., 2021). PARPi have been used as a radiosensitiser in oesophageal, head and neck, colorectal and prostate in vivo and in vitro, where a modest radiosensitisation has been found (Tuli et al., 2014, Angel et al., 2021, Qin et al., 2022). To date, this is the first time that Niraparib has been investigated in combination as a chemo and radiosensitiser in endometrial cancer. We found a modest radio- and chemosensitation when Niraparib 1 μM was combined with increasing concentrations of IR and cisplatin. The greatest cytotoxic effect in combination was seen at high doses of IR (4 Gy). Olaparib and Telazoparib have been investigated previously in endometrial cancer cell lines showing radiosensitisation, which was found to be independent of PTEN mutation status (Wang et al., 2022, Miyasaka et al., 2014) contrary to the hypotheses of other groups that suggested that potentiation of PARPi were likely to enhance the effects of cisplatin or IR in the presence of PTEN mutation (Minami et al., 2013, Chatterjee et al., 2013). This is in keeping with the conflicting evidence of the role of PTEN in HRR. However, our cell line panel was too small to determine the role of PTEN mutation status in our sensitisation experiments. Our data shows that the extent of Niraparib radiosensitisation (up to 1.8-fold at LC50 and up to 2.5-fold at 4 Gy)) was similar to that seen with other cell types and PARPi (Jonuscheit et al., 2021, Cui et al., 2022). We did find superior radiopotentiation with Niraparib in the Ishikawa cell line (around 2-fold). Cisplatin potentiation was more modest when combined with Niraparib (by clonogenic) in line with data seen in other cancer types (1.18 - 1.44-fold) (Prasad et al., 2017, Mann et al., 2019). Notably a greater effect has been seen in HRD cancer cell lines (Evers et al., 2008). Whilst a limitation of the combination treatment with cisplatin and IR was undertaken with Niraparib alone and the impact of other PARPi in endometrial cancer combination treatment is not known, There are studies that demonstrate that the effects of PARPi are similar when co-exposed with other cytotoxic agents (Smith et al., 2022), however, with greater PARP trapping and PARP1 inhibitor potency in other PARPi, the results may be more statistically significant but may cause more off target effects in patients. Similarly, combination treatments were only studied with co-exposure, the effects of sequential exposure should

be explore to determine the effects on cytotoxicity, particularly when thinking about daily IR schedules used in endometrial cancer. A more durable PARPi such as rucaparib may show greater benefit.

There are currently no combination studies with cisplatin and PARPi in endometrial cancer cell lines which makes this study novel. Correlation of chemo- and radiopotentiation needs further investigation in more cell lines as the greatest effect seen in our data is driven largely by Ishikawa result. It is difficult to determine from this study, why the Ishikawa cell line was more sensitive to Niraparib-induced chemo and radiosensitisation. The apparent protection from cisplatin by Niraparib in the AN3CA cell line has not been seen previously in other PARPi. From our data AN3CA has similar cytotoxicity and growth inhibition effects as the Ishikawa cell line to Niraparib, but Ishikawa cells were more resistant to Niraparib-induced growth inhibition. It is tempting to speculate that in the AN3CA cell line growth arrest in G1 has protected the cell line from IR and cisplatin damage, this could be investigated further with cell cycle studies. We found a reasonable but not perfect correlation between growth inhibition and clonogenic data when used in combination and therefore this could be used as a surrogate of cytotoxicity in primary cultures that do not form colonies. Recent pre-clinical studies have proven PARPi to be effective radiosensitisers in many cancer sites (Césaire et al., 2018). Niraparib has specifically shown to have clear radiosensitising effects and has been expand into clinical trials (Wang et al., 2020). A combination of Niraparib and radiotherapy has found to be safe, well tolerated and have good efficacy in patients with recurrent glioblastoma (Jiang and Wang, 2022). Trials in other cancer sites are currently ongoing with a phase I/II studies in metastatic cervix cancer to determine its safety, tolerability, and efficacy with Niraparib and radiation combination treatment (NCT03644342), as well as in prostate cancer (Jang et al., 2020). There are several ongoing clinical trials looking to determine the role of platinum chemotherapy and PARPi combinations in breast, ovarian and lung cancers, and has been shown to be tolerated. One might expect that the overlapping myelosuppression in both drugs might limit the dose of PARPi given. There are currently no studies or clinical trials recruiting for combination treatment with Niraparib and cisplatin or radiotherapy in endometrial cancer, however, its tolerability in other cancer cell types supports its use in clinical studies, along with our data.

6.6 Conclusion

- There was minimal potentiation of cisplatin by Niraparib 1 µM in all endometrial cancer cell lines in colony survival assay or growth inhibition studies.
- There was modest radiopotentiation of IR when by Niraparib 1 µM in colony survival in the 4 endometrial cancer cell lines studied
- There appeared to be a trend toward greater radiopotentiation with the addition of Niraparib 1 µM at higher IR doses.
- There was a trend between potentiation in growth inhibition studies and those in colony survival assay.
- There was no correlation between chemo or radiopotentiation in base line cell line characteristics including cell doubling time, PARP inhibition, fold H2AX change or RAD51 in the HRR assay.
- Given the small cell line panel studied to date it was not possible to link radio- and chemopotentiation to p53, PTEN and MMR mutation status.
- The investigative work was limited by a small number of cell lines studied and available and therefore supported investigation of primary culture in endometrial cancers to determine if there were in fact in cancer that were HRD and sensitive to PARPi Niraparib.

Chapter 7. Primary culture in endometrial cancer and exploration of homologous recombination repair status

7.1 Introduction: Primary culture

Whilst cell lines provide a convenient model to investigate drug sensitivity extrapolating to the clinic has several drawbacks due to their adaptation to culture and genetic drift means that only a subset of the heterogeneous population of cancer cells grow and change with time. Given the limited cell line panel and lack of endometrial cancer primary culture data it was important to establish whether it was feasible to generate endometrial cancer primary cultures and determine functional HRR status to explore the role of PARPi. Primary culture, whilst technically challenging is an important option to explore, as the culture is more likely to represent that of the original tumour tissue and therefore more likely to be representative of a patient's condition. There have been several methods described to establish tumour primary cultures such as from ascitic fluid and solid tumour (RL et al., 2014, Kar et al., 2017, Thériault et al., 2013). Other methodologies described include the development of tissue organoids and other ex-vivo models (Suarez-Martinez et al., 2022). Ascitic fluid has been shown to be relatively easy to establish in culture, whilst solid tumour cultures are more difficult to establish due to contamination, fibroblastic overgrowth and limited cell viability (Miserocchi et al., 2017). Patients with endometrial cancer do not commonly present with ascites, unlike that of ovarian cancer. They typically present with isolated disease within the uterus or with solid spread to lymph nodes or distant tissues such as the omentum (Creasman et al., 1987). Whilst not part of FIGO staging of endometrial cancer, peritoneal washings have been found to be positive in several patients with disease that has spread outside of the uterus (Seagle et al., 2018), which affords the opportunity to study this in more detail in ex-vivo samples. Primary endometrial cancer cultures are not well described and therefore a methodology required optimisation before functional assessment of primary cultures is needed. In this chapter we explore the development of primary endometrial cancer cultures, its optimisation and refinement to include in function assessment of HRR and growth inhibition by Niraparib. The development of primary cultures is presented together in a logical sequence before presenting the final methodology at the end of the chapter.

7.2 Aims and objectives

The aims of this chapter are as follows:

- To establish endometrial primary culture from ascites, peritoneal fluid and solid tumour biopsies and determine optimum conditions to do so
- To characterise the primary cultures by morphology, growth and clonal expansion.
- To determine the HRR status of primary endometrial cancer cultures
- To determine Niraparib sensitivity in primary endometrial cancer cultures by growth inhibition studies.

This will enable to following hypotheses to be tested:

- A proportion of endometrial cancers are HRD
- HRD cell cultures are more sensitive to Niraparib

7.3 Materials and methods

7.3.1 Sample collection and primary culture

Ethical approval was obtained from the Northeast, Newcastle and North Tyneside Research Ethics Committee (REC: 12/NE/0395) as part of the DDR tissue biobank. Specimens were transferred and stored in accordance with human tissue act regulations (HTA). Written consent was taken from patients with biopsy proven endometrial cancer prior to surgery at the Northern Gynaecological Oncology Centre in Gateshead, UK. Patients were given the opportunity to decline specific tissue analysis (including genomic testing and animal testing) and were able to opt out at any time. Patients were provided with an information leaflet with contact details for the biobank and hospital research teams. Samples were collected at a variety of different time points during the procedure. Initially samples were taken at the end of procedure to ensure little disruption of the surgical case. This was taken by pipelle biopsy blindly by passing into the uterus. Where possible, additional peritoneal washings were taken during the procedure to capture any malignant endometrial cells. Peritoneal washings were collected into a 10 ml universal tube following pelvic lavage with 20mls 0.9% saline and refrigerated at -4 °C prior to transfer at the end of the surgical case. Due to the blind nature of collecting endometrial biopsy with pipelle biopsy, the protocol was modified to include the attendance of a pathologist at the end of the surgical case to bisect the uterus

to aid directed endometrial cavity visualisation for a more representative tumour biopsy. Where possible, if a patient presented with advanced or recurrent disease with ascites, ascitic fluid was taken, stored at 4 °C and transferred later that day for processing at Newcastle University.

Solid biopsies were placed into a sterile universal containing culture RF20 (RPMI 1640 medium supplemented with 20% FCS, 2 mM L-glutamine and 1% penicillin/streptomycin), prewarmed to 37 °C. Ascites was aspirated into sterile containers. All samples were transferred to the lab and processed immediately in compliance with UK category B regulations UN3373 along with appropriate written consent. Patients were link anonymised with a unique identification code, PT (solid tissue) or PA (ascites or washings) along with a consecutive number to uphold anonymity. Samples were grown and attempts were made to use cultured cells in several assays if cell number and expansion allowed. Clinicopathological data were collected, including demographics as well as overall survival (OS). Summary of all data can be found in Chapter 8 with full data in Appendix A.

7.3.2 Final primary culture protocol

Following a process of optimisation (see section 7.4.4-7.4.9), a patient tumour sample was obtained, after cleansing the cervix with surgical preparation 0.05% chlorhexidine, using a sterile pipelle endometrial biopsy sampler inserted through the cervix into the endometrial cavity. A biopsy sample was then aspirated into the pipelle sampler. Tissue was then transferred, as above, for immediate processing. Media containing the solid sample was transferred to a petri dish and tissue micro-dissected to 1 mm^3 pieces before returned to a universal container and centrifuged at 300 *g* for 5 minutes to form a pellet of endometrial tissue/cells. The supernatant was then discarded and replaced with 10 ml of 1 x digestion media (3.2mg/ml collagenase type 1:52.5 units/ml hyaluronidase). The sample was incubated for 2 hours at 37 °C in a rotating water bath with agitation every 15 minutes. The contents of the universal were poured through a 40 μM cell strainer into a falcon tube and washed with 20 ml of warmed PBS. The filtered contents of the falcon tube containing a heterogeneous mix of cells, including stromal cells such as fibroblasts, endometrial stem cells, red and white blood cells was discarded. The material remaining on the strainer which contains epithelial cells, was reverse washed onto a petri dish with RF20 medium and placed into a cell culture incubator for 1 hour to allow fibroblasts to attach. The supernatant was

then removed with a pipette and place into a T25 flask for ongoing culture and use in subsequent experiments. See sample collection and optimisation below in figure 7.1.

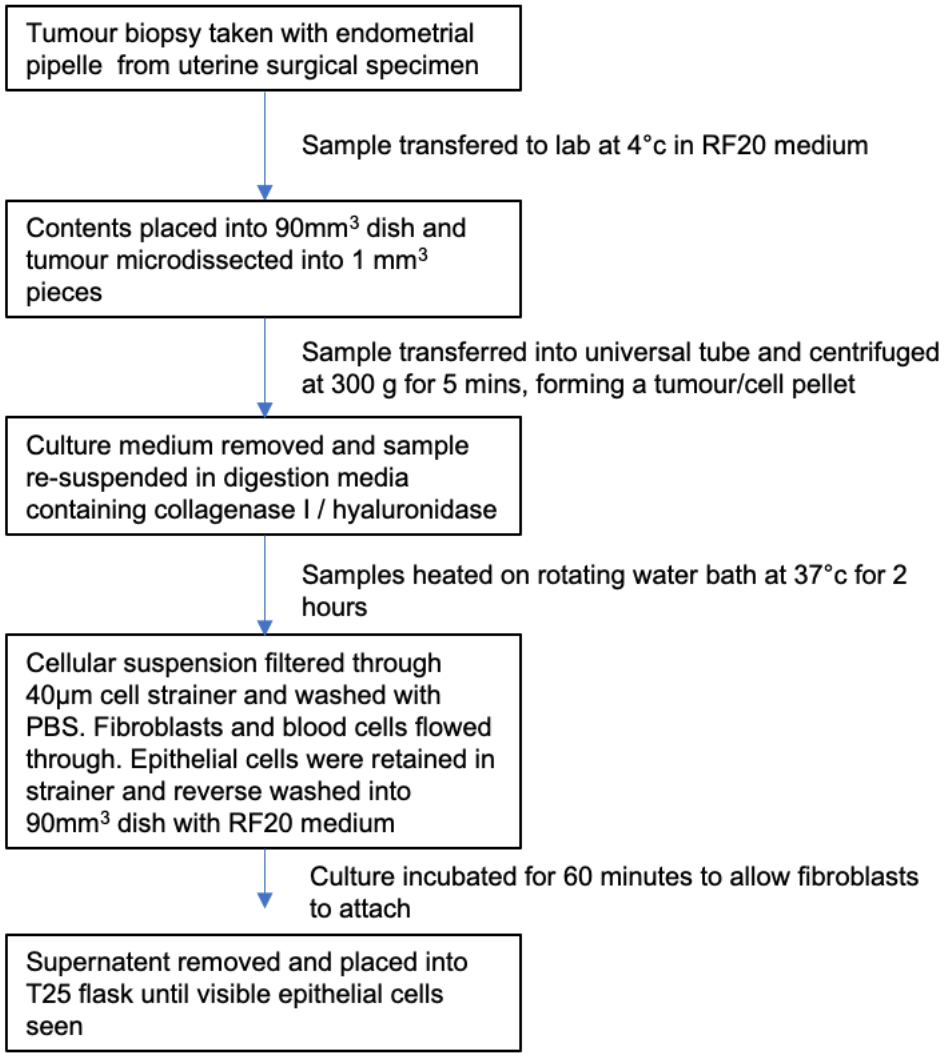


Figure 7.1 **Sample collection and protocol flow diagram**. Above is a flow diagram outlining the steps taken to collect an endometrial cancer sample through processing to culture.

7.3.3 Ascitic and washings culture

10-20 ml of ascitic fluid was added 1:1 to RF20, placed into T75 sterile cell culture flasks and incubated for 5-10 days. Medium was replaced on day 5. Old media containing non-adherent contaminants such as adipose tissue, haematological and tissue cell debris were aspirated. The adherent cellular monolayer was washed with 10 ml PBS and media replaced and allowed to continue in culture. Cultures were the observed for ongoing growth, cell

morphology, evidence of microbial contamination and confluence to allow for passage in further experimental work.

7.3.4 Formal histopathological reporting

Formal histological review of endometrial cancer samples was undertaken at the Northern Gynaecological Oncology Centre by the two lead gynaecological pathologists. Representative tumour samples were taken from the endometrial cavity prior to the main specimen being fixed with formalin. Biopsies were transferred to the lab to attempt ex-vivo culture. An FFPE block was selected for inclusion in the biobank where there were adequate blocks available. The remaining blocks were processed in the NHS lab for full diagnostic workup to present to the multidisciplinary team. Tumours were characterised for morphology on H&E, grade, presence of LVSI, depth of myometrial invasion, involvement of extra-uterine tissues. FFPE blocks were subjected to endometrial cancer IHC panel, including ER, PR, p53 and MMR proteins (post 2020). The results were ratified by the gynaecological oncology MDT with tumour stage and recommendations for further treatment. Baseline clinicopathological data was collected for inclusion in the project. A proportion of the endometrial primary cultures were reviewed by our gynaecological pathologist to observe morphology to support tumour epithelial origin as a quality control.

7.3.5 Characterisation

Primary cultures were initially made up of several different cell types, including stem cells, red and white blood cells, epithelial cells, and fibroblasts. The cultures were characterised in terms of cell growth, morphology, passage number and whether there was any contamination of the culture with other cell types. Cultures were reviewed twice weekly to note the appearance of the culture under brightfield microscope and to note any change in the above features.

7.3.6 Morphology

Images of all successful cultures were taken on a brightfield microscope to observe cell morphology. Photographs were taken a different time point during culture to observe any changes, such as pattern of culture growth, morphological changes, cell interactions, whether they formed in a uniform sheet or whether they piled on top of each other or if the cultures became overgrown with other cell types. Figure 7.2 below shows the two common

morphological types seen on primary culture, cobblestone and fibroblastic cultures. Epithelial cultures more commonly develop in cobblestone monolayers in culture flasks. They tend to be polygonal in shape, more regular in appearance and grow in discrete patches. Some can cluster and 'pile up'. Fibroblastic cultures are often bi- or multipolar, have elongated shapes and may or may not grow uniformly with or without a clear boundary. Malignant cells are often chaotic, without order, with loss of polarity, increased nuclear to cytoplasmic ratio and may have multiple prominent nucleoli. They often do not resemble their tissue of origin.

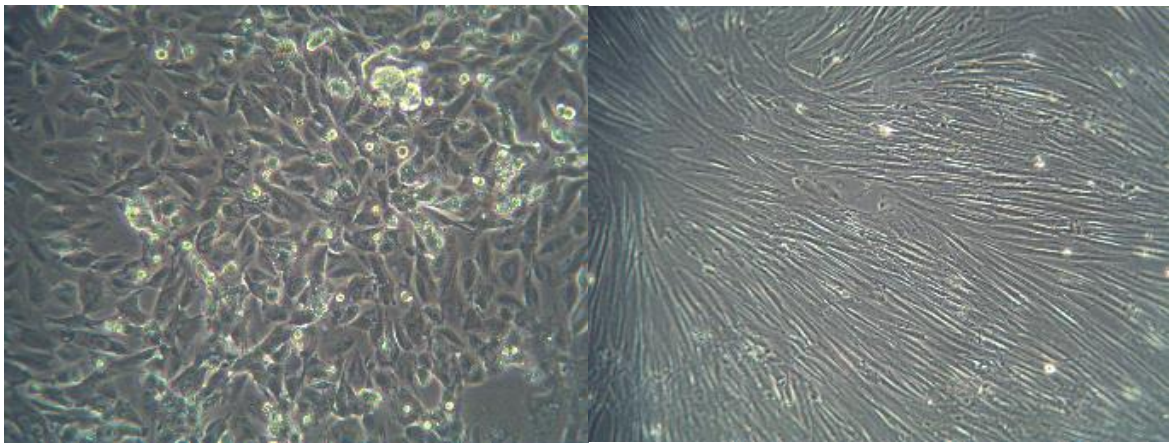


Figure 7.2. **Primary culture morphology** . The images above are taken on a brightfield microscope at x 40 magnification. The image on the left demonstrates a primary cell culture with morphology showing a cobblestone monolayer. They have a relatively large nucleus in compared to their cytoplasm. The culture is proliferating as you can see brighter more uniform cells lifting off the flasks which are replicating. This culture is a high-grade endometrial cancer cell culture. The image on the right demonstrates a more uniform culture with thin elongated appearance. The cells are smaller and appear bipolar. This culture is entirely fibroblastic.

7.3.7 Primary culture growth

A variety of methods were tested to determine cell growth, including cell counting, SRB staining. Growth was possible by SRB, but priority was given to undertaking an HRR assay and where possible growth inhibition studies. This was because cultures were delicate, particularly due to their tendency to be contaminated with microbes. Therefore, growth in this each primary culture was purely observational and regularly required assessment to ensure that experimental work was carried out in exponential growth and to ensure that the

cultures were not infected or contaminated with other non-epithelial cell types which would affect interpretation of results. Notes were taken on morphology, growth rate and appearance of culture over time at different time points to determine their growth pattern and behaviour.

7.3.8 Optimisation of EC culture

Endometrial primary cultures were optimised throughout experimental work to yield cultures that grew in culture medium, were less likely to be contaminated by bacteria and fungi, and were less prone to overgrowth with non-epithelial cell types to leave a pure epithelial culture. The steps taken to address problems during primary culture of endometrial cancer is detailed in the results section.

7.3.9 Homologous recombination repair functional assay

Primary epithelial cultures were seeded onto coverslips and examined to ensure free of bacterial or fungal contamination, 48 hours later they were incubated with primary culture medium +/- 10 μM rucaparib for 48 hours prior to processing as described in section 3.7.2.

7.3.10 Growth inhibition by SRB

Growth inhibition studies were undertaken with Niraparib where possible by SRB staining as described in chapter 3 section 5.1. To ensure that there were no clumps of tumour in the sample cells were passed through an orange needle and agitated with a cell vortex to ensure a single cell suspension then were seeded at approximately 1000 cells/well on 96-well plates, allowed to attach for 24 hours and then incubated with increasing concentrations of Niraparib. As all cell cultures differed in their observed doubling times, they were reviewed daily to ensure growth without reaching confluence in the control group prior to fixing and staining as described in section 3.5.1. Tissue/cell clumping was particularly problematic with solid tumour cultures. If large clumps of cells were noted in experimental wells, results were deemed invalid for that well and excluded. As there were 6 replicates per concentration of Niraparib used, this did not prove to be a significant problem.

7.4 Results

7.4.1 Clinical characteristics

61 patients were recruited to this study and consent with ethical approval as described above. 2 patients later withdrew their consent. Median age in the cohort was 71 (range 45-85). 2 patients had benign endometrium on biopsy and surgery was undertaken for complex atypical hyperplasia, 2 patients were diagnosed with an ovarian cancer after primary site change following review of histology from surgical specimen and 1 patient was diagnosed with a uterine adenosarcoma. This left a total of 54 with endometrial cancer following pathological review of surgical specimen. 28 (52%) patients had stage 1 disease, 5 (9%) had stage 2 disease, 12 (22%) had stage 3 disease and 9 (17%) had stage 4 disease. In this cohort 17 (32%) had serous cancer, 10 (19%) had carcinosarcoma, 10 (19%) had low grade endometrioid, 5 (9%) had grade 3 endometrioid, 4 (7%) had mixed clear cell and grade 2 endometrioid, 3 (5%) had mixed serous and clear cell, 2 (4%) had clear cell and 3 (5%) had mixed serous and endometrioid endometrial cancers. Table 7.3 shows the patient data collected for this endometrial cancer cohort.

7.4.2 Sample collection and processing to obtain single cell suspension for culture

61 samples were collected and transferred for processing, 4 were from ascites and were split 50:50 culture medium and ascites and left to grow in a T125. 57 were from solid tumour biopsies and were processed according to a previously optimised method using a combination of collagenase I (6.4 mg/ml) and hyaluronidase (125 U/ml) and stored in 5 ml aliquots at -20 °c. In summary, directed tumour biopsies were taken following surgery and sample transferred directly into 10 ml of primary culture medium and placed into 4 °c prior to transfer. Samples were transported to the university and handled in a sterile culture hood in accordance with the human tissue act. The contents were poured into a 90 mm^3 dish and dissected into small 1 mm pieces using sterile forceps and scalpel blade. Following dissection, the sample transport medium was aspirated and discarded, and media replaced with 10 ml cell culture medium containing collagenase hyaluronidase (2:1) and transferred into a sterile universal tube and placed into a water bath at 37 °c and shaken at 20 rpm. The samples were incubated for 1-2 hours depending on the yield from the directed biopsy. The solution was agitated at 30-minute intervals to ensure opportunity for the whole sample to dissociate. Following this, the sample was transferred to a centrifuge and spun down at 250g

for 3 minutes. The universal tube was then transferred into a sterile hood and the supernatant was aspirated and resuspended in media and placed into a 90 mm^3 petri dish. Samples were reviewed under high magnification and any areas with fibroblasts were removed by aspiration. The sample was then transferred into T25 flask and placed into an incubator at 37 °c until cells were seen to attach to the flask. Samples were observed daily to monitor growth and for signs of contamination with microbes or unwanted cell types such as fibroblasts. The flasks were left untouched for around 3-5 days before observations were made.

7.4.3 Primary culture success

54 patients from whom samples were collected had a final diagnosis of endometrial cancer following final histology review. Of the 54 samples collected 11 (20%) did not grow in culture, 13 (24%) became infected either following sample processing or first passage with either bacterium or fungus, leaving 34 (62%) endometrial biopsies that successfully grew in culture. Of the successful cultures 3 (10%) cell cultures did not survive past first passage. Following successful passage 4 (15%) became overgrown by fibroblasts i.e., no epithelial culture and a further 5 (19%) that did not continue in culture following seeding for HRR assay onto 6 well plates. leaving 18 cultures for functional HRR assessment and growth inhibition studies. Figure 7.3 shows a flowchart of sample outcomes from cultures.

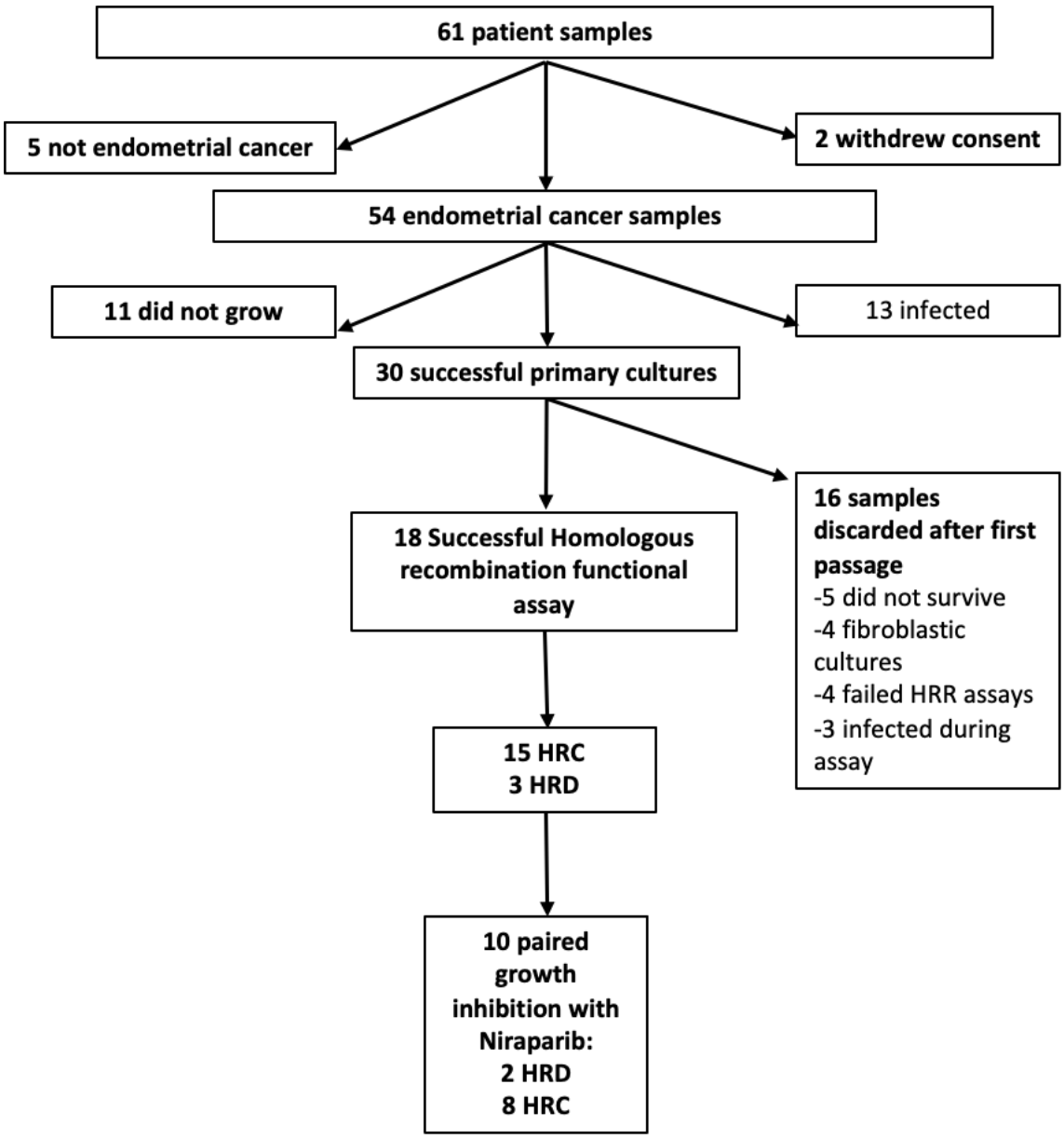


Figure 7.3. **Attrition of endometrial primary pipelle biopsy samples**. Out of 54 endometrial cancer patient biopsies, 30 successfully cultured of which 18 samples had successful functional HRR assay performed. 10 had paired HRR assay and growth inhibition studies with Niraparib by SRB.

7.4.4 Sample infection and optimisation

24% of cultures became infected with a combination of bacteria and fungi. Contaminated cultures often produce a cloudy media and often rapidly used all energy stores in the media, giving it a yellowish colour rather than a pink/purple media. However, cloudy/murky cultures may also be due to a culture that contains cellular debris. It is therefore important not to handle the culture from around 3-5 days to allow cell attachment to occur,

observations can be taken before old media is aspirated, flask washed with sterile PBS and new media replenished for ongoing cell growth. With the introduction of media containing antifungal and antibiotics, the rate of infection reduced. However, it was important to consider the lowest dose of antimicrobials to avoid impact of growth on the primary cell culture. Image D in figure 7.4 demonstrates a contaminated culture that continued to grow in culture. There were some initial concerns that cultures would be contaminated with microbes at low concentrations with antimicrobials, however, regular visible inspection took place to ensure that cultures were not containing infection, even at levels.

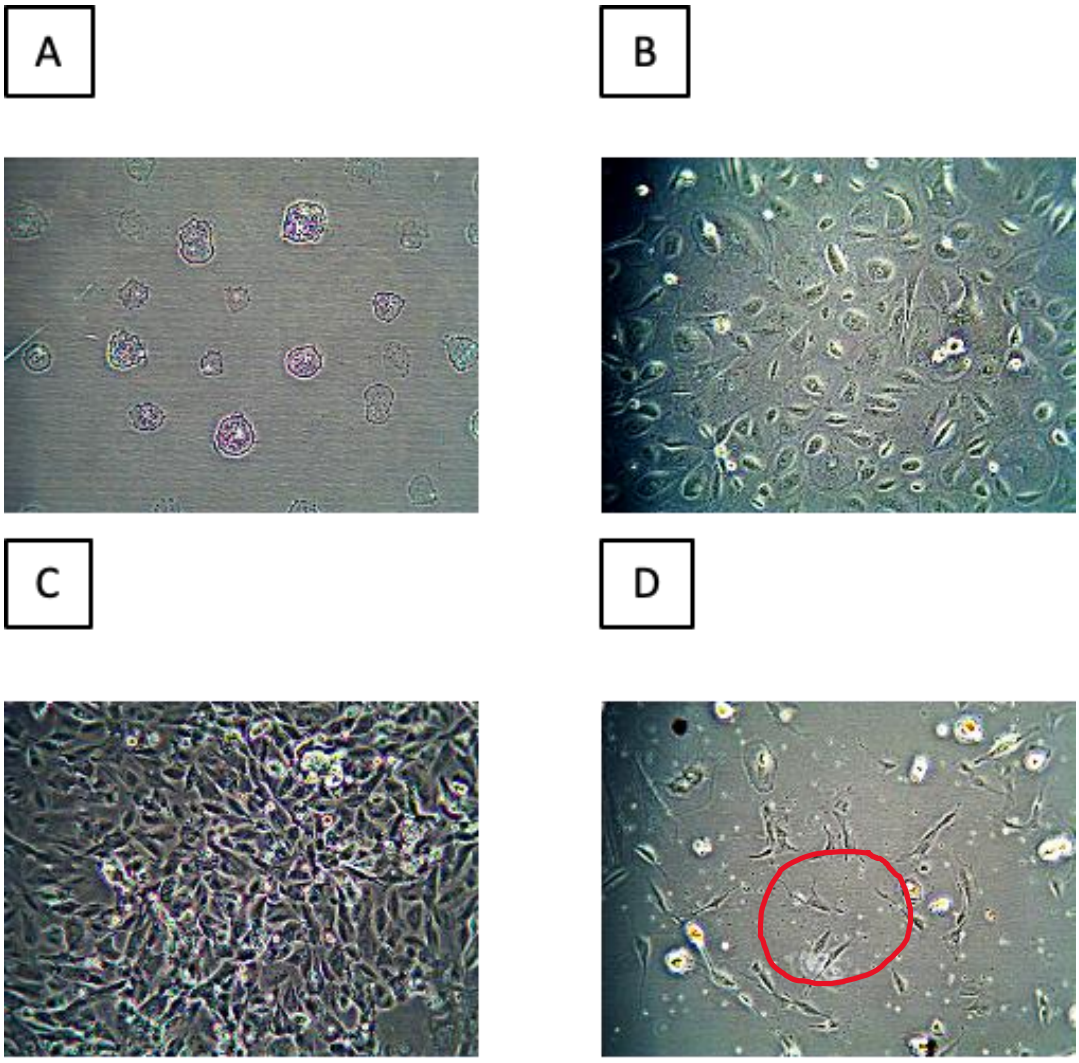


Figure 7.4. **Images of culture outcomes** . Following images were taken on a visicam brightfield microscope at x 40 magnification. A) Demonstrates a culture that has been processed and incubated for 14 days without any visible cell attachment to flask of proliferation. Cell debris can be seen floating above the surface. Cultures were discarded if no signs of cell attachment at this time point. B) Demonstrates an epithelial primary cell culture with classical cobblestone monolayer appearance. This ascitic fluid sample was taken

from a patient with advanced serous endometrial cancer. C) Solid endometrial tumour biopsy of a patient with high grade disease. A cobble stone epithelial culture is seen, however, like that seen in a proportion of the endometrial cancer cell line studies evaluated, the cells appear to crowd together and pile up, like the glandular construction of their tissue of origin. D) Shows a mixed cell culture containing red cell debris, fibroblasts and epithelial cells, however the culture was contaminated with bacterial rods (circled in red) and therefore, was discarded.

7.4.5 Histological subtype of endometrial cultures

Of the cultures that were not successful 1 were serous, 2 were clear cell, 4 were low grade endometrioid, 1 high grade endometrioid, 1 carcinosarcoma and 2 were mixed histology. Of the patients who had a successful culture, 10 were serous, 0 clear cell, 5 low grade endometrioid, 2 high grade endometrioid, 6 carcinosarcoma and 7 were mixed cell types. Table 7.1 summaries the histological subtypes of the cultures. Of the samples that did not culture, 6 failed to attach,3 initially attached but separated and 2 had mixed epithelial, fibroblast growth with contaminated red and white cell culture. Median age of successful culture 70 (range 45-75), median age of unsuccessful culture 72 (range 51-84). 3 of the successful cultures were from ascitic fluid (3/4 ascitic fluid cultures), 27 were from solid tumour biopsies (27/50 solid tumour cultures) and 0 were from peritoneal washings. The success rate of each sample method was 75% for ascitic fluid, 54% for solid directed endometrial biopsy and 0% for peritoneal washings.

Endometrial subtype	Successful culture	Unsuccessful culture	Infected
Serous	10	1	6
Clear cell	0	2	0
G1 EEC	0	1	0
G2 EEC	5	3	1
G3 EEC	2	1	2
Carcinosarcoma	6	1	3
Mixed serous and clear cell	3	0	0
Mixed serous and endometrioid	1	2	0
Mixed clear cell and endometrioid	3	0	1

Table 7.1. **Culture outcomes by endometrial cancer subtype** G1 EEC=grade 1 endometrioid endometrial cancer, G2 EEC=grade 2 endometrioid endometrial cancer, G3 EEC = grade 3 endometrioid endometrial cancer.

7.4.6 Optimisation with cell filtering to avoid mix cultures

To reduce contamination from other cell types, cell filtering was used to refine primary culture. Table 7.2 shows cell sizes. Following sample digestion with collagenase/ hyaluronidase a 40 μM cell filter was used to remove bloods cell debris and fibroblasts. Whilst the cell filter is 40 μM, endometrial cancer cells tend to clump together in a pearl necklace appearance under brightfield microscopy. It is important to look at the supernatant under brightfield microscope before undertaking the filter step to remove any obvious cell contamination and to ensure clusters of endometrial cells can be seen. Following dissociation of solid tumour tissue, the supernatant was filtered into a 50 ml falcon tube and then reverse washed with cell culture medium onto a 90 mm^3 petri dish. This media containing epithelial cells was then transferred into a T25 flask for incubation at 37 °c with daily observations recorded.

Cell origin	Size (μM)
Red blood cells	6.2-8.2
Neutrophils	8-10
Fibroblasts	10-15
Epithelial cells	20
Endometrial stem cells	5

Table 7.2**. Cell sizes** . This table Demonstrates average cell size of red blood cells, white blood cells, fibroblasts, epithelial endometrial cells, and endometrial stem cells.

7.4.7 Avoiding fibroblast overgrowth

Fibroblast contamination is a major problem in primary cultures and is particularly so with solid tumour cultures. Whilst a cell filter step was included in the development of the final protocol fibroblastic overgrowth in culture continued to be a problem. The following methods were investigated to try to eliminate fibroblasts: selective seeding, cloning rings and Matrigel. Selective seed has been used in primary culture previously. Essentially, different cell types adhere to culture flasks at different rates. Fibroblasts are most likely to attach more rapidly than epithelial cells and can therefore be used to positively select out endometrial cancer cells. Cloning rings can be placed around colonies of epithelial cells allowing their selective detachment by trypsinisation and re-seeding. Limitations of this method are that the interaction of epithelial cells in culture is important for signalling and growth, by limiting the number and volume of cells available it is likely that there will not be a significant yield. This was demonstrated in culture in our patient cohort, the two cultures attempted with this method did not grow and were discarded. Matrigels have been showed to support growth of epithelial cell cultures by providing a feeder layer. Limitation of this approach are that it is expensive and requires additional handling of delicate primary cell culture. This was attempted once, there was difficulty detaching the primary culture from the Matrigel and the resultant yield of cells in culture was small. This rapidly became infected. Therefore, it was decided that an attempt should be made to do selective seeding. Cells were transferred to T25 flasks and incubated at 37 c for around 30-60 mins. Cells were observed on brightfield microscope to see if cell attachment had started to occur. The supernatant was then aspirated and transferred to another T25 flask and incubated for daily

inspection and culture review. It was noted that fibroblast contamination considerably decreased following the introduction of this step.

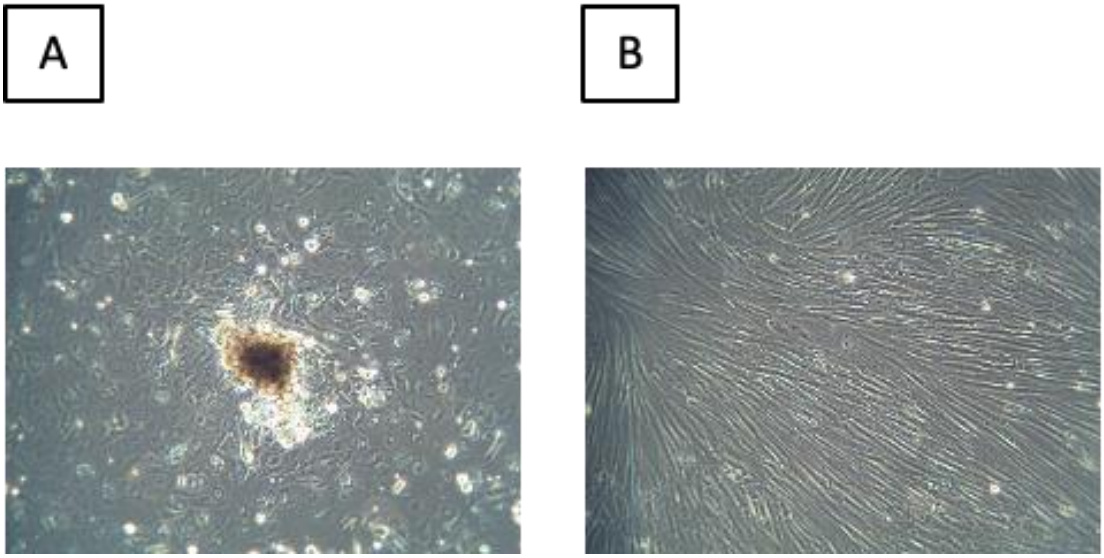


Figure 7.5. **Progression to fibroblastic overgrowth in primary culture** . Brightfield microscope images at x 40 magnification taken with a visicam at different time points in the same patient. A) Solid tumour sample, central cluster of solid endometrial tumour can be seen with cobble stone monolayer seen extending out with abnormal nuclei/cytoplasmic ratio and abnormal nuclear shape. B) Sample overtaken by fibroblasts demonstrating their classical elongated spindle shape with cytoplasm tapering off into long slender processes.

7.4.8 Morphology and longevity of cultures

As discussed previously, epithelial culture sample yield was small, and it was therefore not possible to perform growth studies with SRB as well as HRR functional assay on all samples. Each primary culture sample had notes taken which describe the growth over many observations as well as number of successful passages and is summarised in the primary culture table in appendix A. A total of 30 cultures were successful. 21 had a cobblestone morphological appearance, 1 was a mixed cell culture, 7 were fibroblastic and 1 was spindle-like. 15 cultures did not survive to first passage. 13 cultures continued past first passage, 2 survived past the second passage, 9 survived past third passage and 4 past the fourth passage. Of the 3 that did not survive to first passage, all had cobblestone morphology and were made up of the following histological subtypes; 1 serous cancer, 1 stage 3a carcinosarcoma and one stage 3C1 mixed serous and clear cell cancer. The 13 cultures that survived past the first passage were made up of 1 patient with grade 2 endometrioid

endometrial cancer (EEC), one grade 3 EEC, 5 serous endometrial cancers, 3 carcinosarcoma, 2 mixed serous and clear cell, 2 mixed serous and grade 3 EEC and one mixed clear cell and grade 2 EEC. The two cultures that survived past second passage was made up of serous endometrial cancers. Cultures that survived three passages were made up of 3 grade 2 EEC, 2 serous, 1 carcinosarcoma, 1 mixed serous and clear cell and 2 mixed clear cell and grade 2 EEC. 4 cultures survived past passage 4 and were made up of 1 serous cancer, 1 grade 2 EEC, 1 grade 3 EEC and 1 carcinosarcoma. The morphology of these cultures is summarised in table 7.3 along with age, histological subtype stage and whether a successful HRR or SRB assay was performed.

Patient number	Age at diagnosis	Stage	Tumour subtype	Culture successful	HRR assay result	HRR status	Passage	Morhology	SRB Growth inhibition	GI50 Niraparib
1	47	B	BENIGN	Y	Y	HRC	P1	C	N	
2	70	3a	CARC	Y	N	N	P0	N	N	
3	69	3c	SERCLEAR	Y	N	N	P0	C	N	
4	50	1a	CLEARG2E	Y	Y	HRC	P1	C	N	
5	82	1a	SER	Y	N	F	P0	C	N	
6	72	3C1	SER	I	N	NA	P0	N	N	
7	50	1a	CLEARG2E	I	N	NA	P0	N	N	
8	68	1a	G3E	I	N	N	P0	N	N	
9	80	1a	SER	I	N	N	P0	N	N	
10	45	1a	G3E	I	N	N	P0	N	N	
11	76	3C1	SER	I	N	N	P0	N	N	
12	71	1a	SER	I	N	N	P0	N	N	
13	64	B	BENIGN	I	N	N	P0	N	N	
14	54	4b	SER	Y	Y	HRD	P4	C	Y	1.73
15	65	2	G2E	Y	N	F	P1	S	N	
16	67	1a	SER	Y	Y	HRC	P3	F	Y	11.46
17	62	1a	SER	Y	N	F	P1	F	N	
18	68	3b	SER	I	N	N	P0	N	N	
19	71	1a	CARC	Y	N	N	P1	C	N	
20	61	1C1	Ovary	Y	N	N	P2 I	F	N	
21	56	4a	G2E	N	N	N	P0	N	N	
22	74	1a	CARC	Y	N	Failed culture	P1	F	N	
23	68	3C2	SER	Y	Y	HRC	P1	C	Y	8.82
24	81	4b	CARC	I	N	N	P0	N	N	
25	78	1a	G2E	Y	Y	HRC	P4	C	Y	7.85
26	71	1a	G3E	Y	N	Failed culture	P1	MIX	N	
27	79	3a	G2E	I	N	N	P0	N	N	
28	76	1a	CLEAR	N	N	N	P0	N	N	
29	72	2	CARC	N	N	N	P0	N	N	
30	76	1a	SER	Y	Y	HRC	P2 I	C	F	
31	68	1a	SER	Y	N	Failed culture	P1	C	F	
32	58	1a	ADENOSARC	I	N	N	P0	N	N	
33	84	2	SERG2E	N	N	N	P0	N	N	
34	83	4b	SER	N	N	Infected post passage	P1	C	N	
36	85	4b	SER	Y	N	Failed culture	P1	F	N	
37	77	1a	SERCLEAR	Y	Y	HRC	P3	C	Y	23.4
38	66	3C1	G3E	Y	Y	HRD	P4	C	F	
39	78	3C	Ovary	I	N	N	P0	N	N	
40	72	2	SERCLEAR	Y	Y	HRC	P1	C	N	
41	69	4b	CARC	Y	N	Failed culture	P1	C	N	
42	68	4b	CLEAR	N	N	N	P0	N	N	
43	70	1a	CLEARG2E	Y	N	F	P3	F	N	
45	63	4b	G2E	Y	N	F	P3	C	N	
46	59	1a	G2E	Y	Y	HRC	P3	C	Y	28.96
47	80	3C2	CARC	Y	Y	HRC	P4	F	N	
48	74	1a	CLEARG2E	Y	Y	HRC	P3	C	N	
49	83	1a	SER	N	N	N	P0	N	N	
50	62	1a	SERG2E	N	N	N	P0	N	N	
51	51	1b	G2E	N	N	N	P0	N	N	
52	69	Recurrence	G3E	N	N	N	P0	N	N	
53	66	4b	CARC	Y	Y	HRC	P3	C	Y	1.62
54	71	3C2	G2E	Y	Y	HRC	P3	C	Y	21.96
55	78	3C1	SER	Y	Y	HRD	P2	C	Y	2.03
56	79	1b	SER	Y	Y	HRC	P3	C	Y	60.2
57	77	2	SERG3E	Y	Y	HRC	P1	C	N	
58	64	2b	Ovary	N	N	N	P0	N	N	
59	74	1a	G2E	N	N	N	P0	N	N	
60	73	1a	G1E	N	N	N	P0	N	N	
61	74	1a	CARC	I	N	N	P0	N	N	

Table 7.3. **Patient-derived EC primary cultures**. Summary table of primary endometrial patient samples. Table includes patient number, age, stage, tumour subtype, culture outcome, outcome of HRR assay, morphological appearance of culture, number of culture passages and whether growth inhibition studies with Niraparib were undertaken (along with GI50 results). Y=yes, N= no C=cobblestone monolayer, F=fibroblastic culture, S=spindle-like culture, I = infected, MIX = mixed cell type culture. Tumour subtype: CARC=carcinosarcoma, SER =serous, CLEAR=clear cell, G1E = grade 1 endometrioid endometrial cancer (EEC), G2E= grade 2 EEC, G3E=grade 3 EEC, SERCLEAR= mixed serous and clear cell, SERG2E = mixed

serous and grade 2 EEC, SERG3E= mixed serous and grade 3 EEC, CLEARG2E= mixed clear cell and grade 2 EEC, OVARY=ovarian cancer, BENIGN=benign histology (no cancer).

7.4.9 Clinical characteristics and culture success

There was no significant relationship between the age of patients and ability to grow cell cultures (p=0.73).

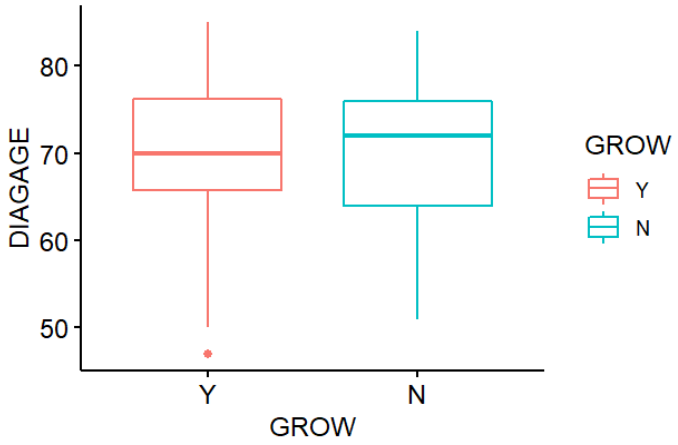


Figure 7.6**. Box and whisker plot showing summary statistics** (Mean, standard deviation, and range) for age of patients separated based on whether sampled cells could be successfully cultured (Y) or not (N). DIAGAGE = Age at diagnosis, GROW= Outcome of culture.

A Fisher's exact test was performed to test whether there was a difference in the ability to grow cell cultures (GROW) from different tumour subtypes (TUMPSPT). Table 7.4 shows the culture outcomes for each individual subgroup. There was a significant relationship (p = 0.048) suggesting that the type of cancer affects the ability to grow a cell culture. However, given the small sample size, it was not possible to determine which subtypes were the underlying cause for this. It is likely that serous cancers, carcinosarcomas and grade 2 EEC's are subtypes which are more likely to grow in culture. However, it is worth noting that our population is skewed to high grade tumours, given that the women were operated on in a cancer centre, therefore it is difficult to draw firm conclusions. The sample size is also very small so valid conclusions are difficult to conclude from this.

	GROWTH	
Subtype	N	Y
BENIGN	0	1
CARC	1	6
CLEAR	2	0
CLEARG2E	0	3
G1E	1	0
G2E	3	5
G3E	1	2
SER	2	10
SERCLEAR	0	3
SERG2E	2	0
SERG3E	0	1

Table 7.4. **Contingency table of Tumour subtype and culture outcome**. This table shows the primary culture outcomes (whether they grew or not in culture) according to the histological subtype of the endometrial cancer. There was no statistically significant correlation between a successful culture and histological cancer subtype.

A logistical regression was used to determine whether the stage of cancer was correlated with the ability to grow a cell culture. No significant relationship was detected between stage of cancer and growth of cell culture.

7.4.10 Homologous recombination repair in primary endometrial cancer culture

It was possible to perform functional HRR analysis on 18 samples, HRC was defined as ≥2-fold increase in RAD51 foci in cells showing a ≥2-fold increase in H2AX foci, HRD was defined as <2-fold increase in RAD51 foci in cells showing a ≥2-fold increase in H2AX foci and cultures in which there was <2-fold increase in H2AX were deemed invalid (Patterson et al., 2014), There were no invalid results. 15 (83%) samples were HRC and 3 (17%) were HRD. The HRC cultures were made up of 4 serous cancers, 2 carcinosarcomas, 3 grade 2 EEC, 1 mixed serous and grade 3 EEC, 2 mixed clear cell and G2E, 2 mixed serous and clear cell cancers and 1 benign histology. 8 (53%) were stage 1, 2 (13%) were stage 2, 3 (20%) were stage 3, 1 (7%) stage 4 and one ultimately had benign histology. 13 had cobblestone morphology and 2 had a fibroblastic appearing culture. Median age was 70 (47-80). Of that HRD samples, 2 were serous cancers and 1 was a grade 3 EEC (2 were stage 3 and 1 was stage 4). Further details of the culture and cell passage are included in table7.3. Figure 7.7 shows a representative HRC and a HRD image by immunofluorescence. Figure 7.8 shows a waterfall plot of all primary cultures that achieved a result from the HRR assay, it shows fold increase in increase in H2AX (red) and RAD51 foci (green).

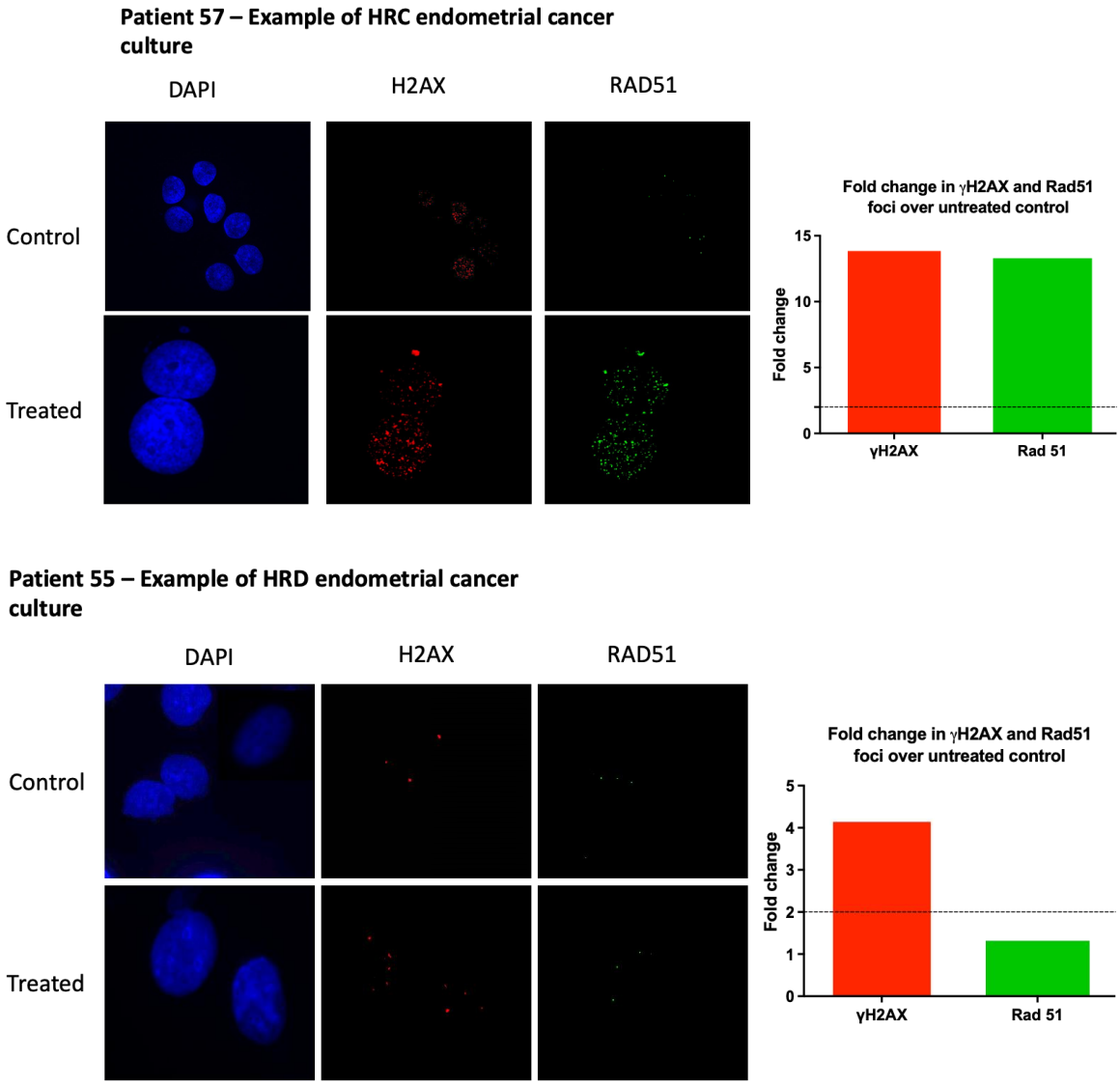


Figure 7.7. **Primary culture HRR determination**. Representative image from a HRC primary endometrial cancer culture (A) and HRD culture (C) showing nuclei from cultures exposed to 10 µM rucaparib for 48 h or not (control) stained with DAPI and H2AX and RAD51 foci identified by red and green, fluorescent antibodies. The fold increase in foci following rucaparib treatment is shown in B and D.

Data for all samples is shown in figure 7.7. Patients 14, 38 and 55, were HRD. The Median increase in RAD51 foci in the HRC group was 9 (2-24). Some primary cultures only had a 2-fold increase in RAD51 foci formation (54 and 56).

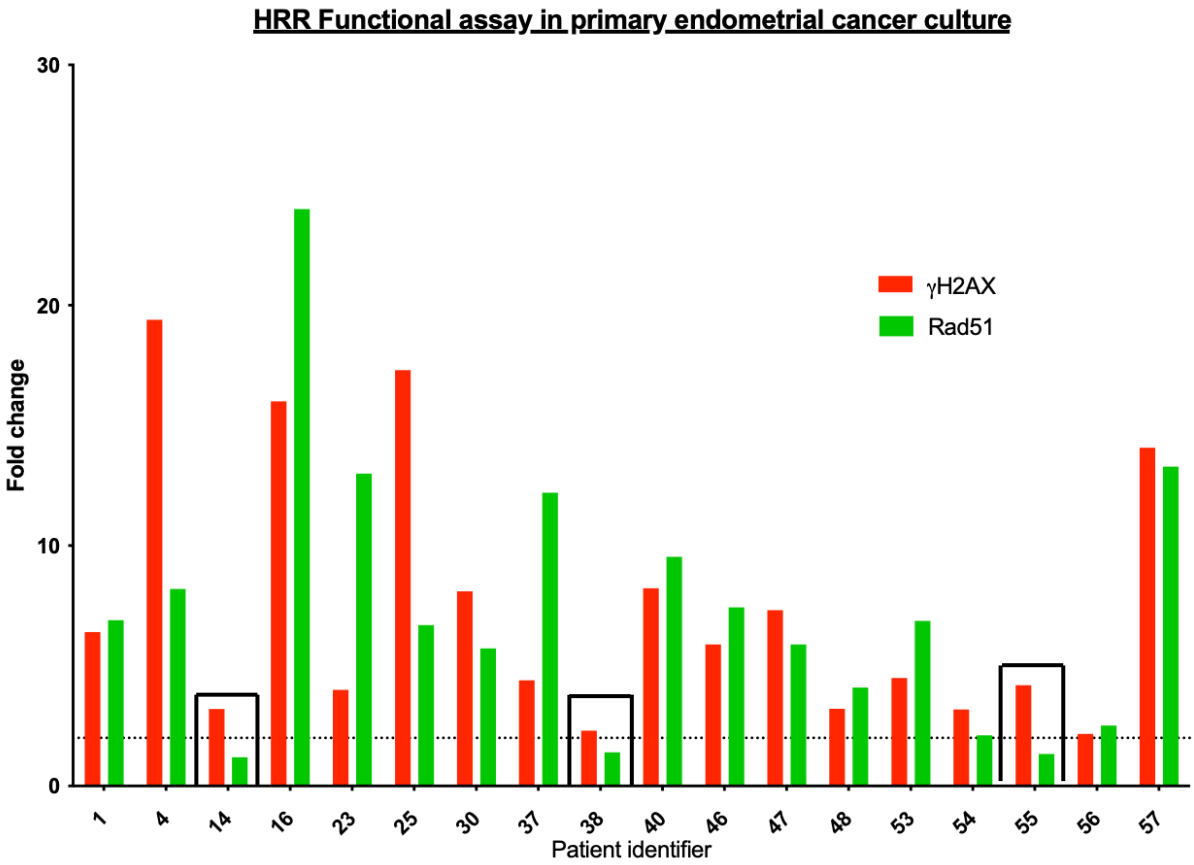


Figure 7.8. **Summary data of Functional HRR assay data in primary culture in primary**. This plot shows fold increase in H2AX foci formation (red) and RAD51 foci formation (green) (y axis) in the HRR assay for each patient (x axis). The dotted line shows a cut off at 2-fold increase to determine HRR status. A black box is drawn around the 3 HRD samples. Detailed methodology is explained in methods section 3.7.2.

7.4.11 Growth inhibition with Niraparib by SRB in primary cultures

Ten (2 HRD and 8 HRC) of 18 cultures characterised for HRR status had sufficient cell number to undergo Niraparib growth inhibition with SRB. HRC cultures had median Niraparib GI50 of 16.71 µM (1.62-60.2). The median Niraparib GI50 in the HRD cultures was 1.88 µM (1.73-2.03). HRD cultures that had successful growth inhibition measurements were serous and grade 3 EEC subtypes. Whilst HRC cultures comprised 2 serous, 3 grade 2 EEC, 1 carcinosarcoma and 1 mixed serous and clear cell subtypes. One HRC culture (patient 53 -a carcinosarcoma) was more sensitive to Niraparib than the two HRD samples. Interestingly the least sensitive HRC patient was a serous endometrial cancer (GI50 60.2 µM), however, it appears that this culture grew very slowly and has likely yielded a falsely high GI50 result.

Figure 7.9 shows a summary of growth inhibition GI50 values in the endometrial cancer primary culture samples.

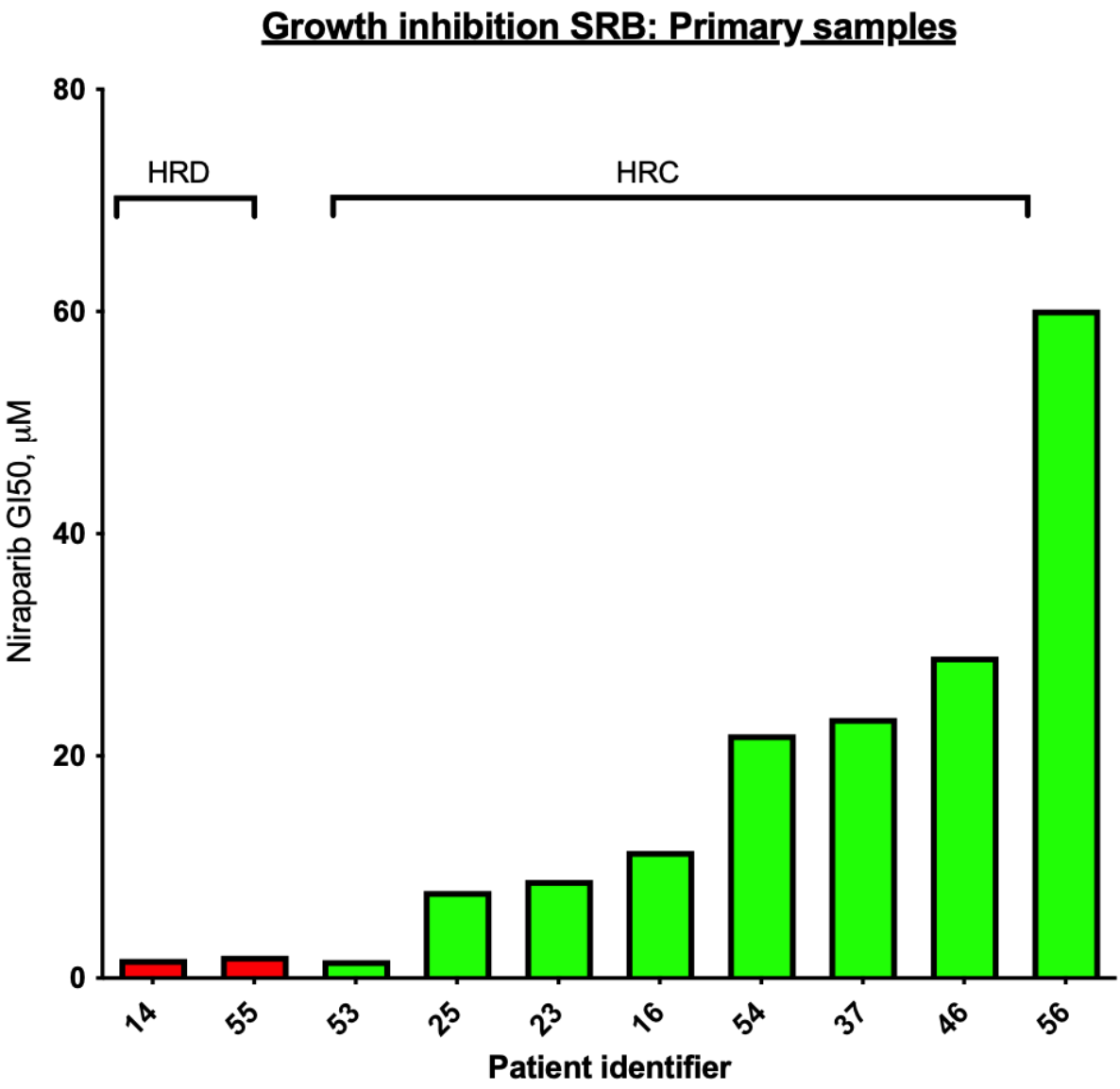


Figure 7.9 **Growth inhibition studies with single agent Niraparib from cultured primary endometrial cancer samples**. The concentration required to inhibit growth by 50% (GI50) determined by interpolation (GraphPad Prizm) of the concentration response curves is given from a single experiment. There was a range in sensitivity seen in the primary cultures to Niraparib. The patient bar charts in green are the growth inhibition GI50s in the samples which were HRC (range 1.62-60.2 µM). The red bar charts of from 2 samples that were HRD by the HRR functional assay (range 1.73-2.03 µM). All HRC patients with growth inhibition studies (apart from patient 53) were less sensitive to Niraparib. It could be hypothesised that

patient 53 is actually HRD, but the defect in HRR is downstream of RAD51 and therefore not detected by this assay.

7.4.12 Clinical characteristics and HRR status

It was important to determine if there were any clinical characteristics which are associated with homologous recombination repair status. This would serve to identify those patients who were more likely to be HRD, focus testing or serve as a biomarker. Due to the small sample size, it was not possible to associate HRR status with stage of disease or tumour subtype ($p = 0.54$ Fisher's exact test).

7.5 Discussion

In this chapter we have demonstrated that it is possible to culture endometrial cancer samples ex-vivo from ascites and solid uterine tumour biopsies. Culture success was improved in this cohort with the introduction of antimicrobial agents to reduce sample contamination with infection as well as refinement to single cell culture. As this study was investigating cancer of epithelial origin, it was important to exclude other cell types from the analysis, particularly fibroblasts. There are currently several known methodologies to achieve this, which include refinement of culture medium, selective seeding, cell filtering, cell feeder layers, as well as metallic bead filtering (Lee et al., 2018, Kelly et al., 2020, Pezzi et al., 2018, Arnold et al., 2001). In this study we optimised a technique, which included a combination of the above (excluding metallic beads) to refine the culture and increase culture yield. This was a modification of the following protocol (Chen and Roan, 2015). The success rate of culture was 62% but increased over time with method refinement. Several groups have established endometrial cancer cultures to undertake characterisation, cytotoxicity and functional studies but have seen similar culture limitations such as fibroblastic overgrowth, limited survival, and poor cell yield (Koval et al., 2015, Zhang et al., 1995). There were, however, no reported culture success rate in these groups to allow for direct comparison. Furthermore, there are limited epithelial cell specific markers for endometrial cancers to include as quality control for these cultures, to ensure that cell type included in experimental work was purely epithelial tumour and therefore relying on morphology alone was a limitation of our study. Accurate morphological assessment following passage and seeding onto coverslips is particularly tricky, further limiting quality control in experimental work, as there was no guarantee that the predominant cell type in the culture flasks did not get overgrown with fibroblasts following cell passage and seeding onto coverslips. The use of fluorescence-activated cell sorting (FACS) and magnetic cell sorting could have further refined this epithelial culture due to their ability to utilise stem cell-related antigens through antibody selection (Medema, 2013). Further experimental work with these delicate primary cultures was further limited by the small cell yield in culture. Therefore, findings within a sample was limited by only one experiment. Most of the cell volume was required to complete HRR assay and growth inhibition studies and often cultures did not survive past the first or second passage. Despite this, 18/34 (53%) of the primary culture samples underwent HRR function analysis. 17% of these were HRD and 83%

are HRC. The current estimate in other studies is that around 24% of high-grade endometrial cancers are HRD (de Jonge et al., 2018). This group were able to perform functional HRR functional analysis in endometrial cancers that were irradiated prior to fixation and embedding into paraffin but were not able to demonstrate sensitivity to PARPi in these samples. Therefore, the results from this primary culture experimental work are consistent with previous findings but augmented by growth inhibition studies with Niraparib.

Our study has demonstrated that it is possible to culture endometrial cancer cells from solid tumour biopsies taken at the end of surgery and from ascitic fluid taken from therapeutic paracentesis or intraoperative drainage. The approached used to process ascites was similar to previous methods used in our laboratory with relative ease of primary culture, however, in endometrial cancer, patients presenting with ascites is uncommon. The method of determining HRR status was the same method validated in various cancer types including (ovary, lung, breast and prostate) which also utilised a mixture of fluid and solid cultures to perform the assay. It is worth noting that this functional HRR assay was not validated previously in endometrial cancer cultures but that the methodology seemed to be reproducible across cancer and culture sample types. The primary cultures identified 3 out of 18 samples to be HRD utilising a cut off of a 2-fold increase to determine HRR status. It is interesting to note that 2 samples came very close to a < 2-fold change in RAD51 foci formation but were assigned to the HRC group (patient 54 and 56). However, both samples were significantly less sensitive to Niraparib than the HRD samples (GI50 21.96 and 60.2 µM respectively).

A significant number of the primary culture samples did not grow. Interestingly, primary cultures derived from ascites from HRD ovarian cancer are thought less likely to grow in culture, given that defects in double strand break repair are particularly lethal (Gentles et al., 2019), so the fraction of HRD in the entire sample set may be higher than the samples that were ultimately tested. A further limitation of the HRR assay is that primary cultures grow at different rates and all primary cultures undergoing RAD51 functional assay were incubated under the same experimental conditions. However, the assay controls for this in that H2AX is used as a marker of replication stress when cells with inhibited PARP enter S-phase with unrepaired SSB and/or trapped PARP. These lesions are resolved by HRR, involving RAD51 foci. Therefore, if H2AX foci increase, replication stress has occurred and HRR will be activated in cells with functional HRR. Since there were no cells in which H2AX foci did not increase in response to rucaparib all assays were deemed to be valid (Patterson et al., 2014).

The possibility that a HRR positive result is due to contaminating normal cells/fibroblasts cannot be excluded. Also, in this context it is worth noting that in 2/15 of the endometrial cancer samples with HRC status had fibroblastic morphology. Reassuringly the sample from the patient with benign histology was HRC. Interestingly the two HRD samples evaluated for Niraparib induced growth inhibition were highly sensitive compared to the HRC group. One HRC patient was sensitive to Niraparib which could have been due to a defect in HRR downstream of RAD51 foci as has recently been demonstrated for NIH-OVCAR3 cells (Bradbury et al., 2020). There was no statistically significant relationship between HRR status and patient baseline characteristics that might aid selection for PARPi therapy due to the small sample size and further work is needed to establish any relationships. Limiting primary cell culture to one cell type also limits our understanding of the complex interplay between cancer cells and associated stroma as well as understanding response to cytotoxic therapies. More recently organoid have been developed to model the tumour environment more closely, including models for genomic and functional analysis in endometrial cancer (Bonazzi et al., 2022, Wu et al., 2022, Jamaluddin et al., 2022a, Jamaluddin et al., 2022b), and it is to be anticipated that these will be more similar to the in-vivo/clinical situation. Future work should focus on further understanding our knowledge of this interplay for normal tissue and cancer, in the response to cytotoxic agents as well as biomarkers for HRD.

7.6 Conclusion

- A protocol for establishment of endometrial cancer primary cultures has been optimised
- 3/18 cultures were homologous recombination repair deficient
- Growth inhibition by Niraparib correlated with HRR status as expected
- HRR status did not correlate with stage of disease or age of patient but may correlate with tumour subtype
- Future optimisation of primary cultures is required to yield greater cell number to search for biomarkers of HRD and Niraparib sensitivity.
- Further exploration of clinicopathological data is necessary to understand if there are any markers of HRD in the next chapter.

Chapter 8. Homologous recombination repair and clinical implications in endometrial cancer

8.1 Introduction

The previous chapter demonstrates the possibility of growing primary cultures of endometrial cancer and evaluating their HRR status and sensitivity to drug-induced growth inhibition. However, these investigations are challenging, and success is not guaranteed. It is therefore important to explore if there are any markers of HRD from available clinical information and from further analysis of tumour samples. There was a correlation between tumour subtype and success of culture and trend towards a correlation between tumour subtype (serous and grade 3 EEC) and HRD. Therefore, further exploration with clinicopathological data and IHC may reveal a connection with HRR status that may be a more convenient predictive biomarker. FFPE blocks are favoured for pathological evaluation as they allow for serial sectioning of tissue samples and storage for future diagnostics and analysis. FFPE blocks can slow the decay of DNA and therefore provide an opportunity for DNA extraction later as part of research or clinical diagnostics. Following the TCGA work in endometrial cancer, the updated guidance from the European and British gynaecological cancer societies are recommends the use of additional IHC panel markers and genomic testing, to aid decision making for adjuvant therapies and opportunity for screening for Lynch syndrome. From the endometrial cancer patient cohort recruited to this biobank study, we retrieved FFPE blocks for further analysis (with patient consent) and collected clinicopathological data to determine if there were any markers correlating with HRR status. In this patient cohort IHC markers for p53 (associated with HRD in ovarian cancer), oestrogen receptor (ER), progesterone receptor (PR) and MMR, were only determined sporadically, therefore, to explore the association with these IHC markers and HRR status a TMA block was generated to undertake IHC staining of the patient samples. Around 30% of endometrial cancers are dMMR and we hypothesised that these patients may have microsatellites in regions coding for HRR proteins. In this chapter we aim to explore the patient cohort and determine factors that influence OS and to determine if any patient characteristics were associated with HRR status.

8.2 Aims and objectives

The aims to be investigated in this chapter are as follows:

- To determine p53, ER, PR, MMR and PTEN status by IHC
- To collected baseline clinicopathological data and overall survival data.

This will enable to following hypotheses to be tested:

- p53, ER, PR, MMR or PTEN status is associated with HRD in endometrial cancer
- Clinicopathological features correlate with HRR status
- p53, ER, PR, MMR or PTEN IHC marker status is associated with a difference in patients.

8.3 Materials and methods

Following processing of FFPE blocks in the NHS laboratory and clinical diagnostics. Surplus FFPE blocks were selected for each consented patient. It was important to ensure that the blocks contained areas of normal tissue and tumour. Areas of tumour were marked in each patient block and cores were taken for inclusion in TMA block (figure 8.1). IHC staining was undertaken in accordance with NHS laboratory guidelines and scoring was undertaken for each IHC stain. 4 endometrial cancer cell lines were included in the TMA block following formation of cell line thrombin clot. Methodologies for FFPE and TMA construction, IHC staining and score, and production of thrombin clot are discussed in detail in chapter 3. Sections 10 and 11. Anonymised patient clinicopathological data were collected from Northern Gynaecological Oncology Centre, Gateshead and stored securely for analysis. Overall survival (OS) was calculated from date of diagnosis to date of last follow up or death.

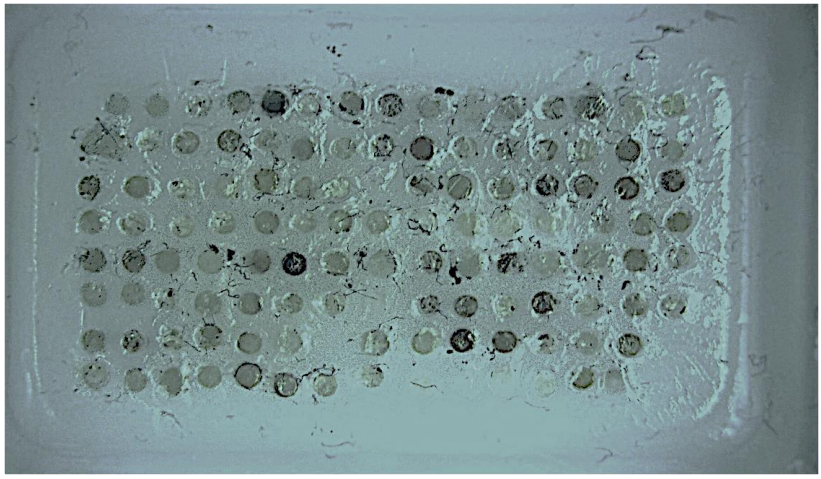


Figure 8.1 **Final TMA block image** TMA block image taken by visicam. Individual tissue cores are seen. TMA layout is shown with controls in section 3.10.

8.4 Results

8.4.1 Clinical summary

55 samples were included in the TMA block construction, this included all primary cultures samples that had HRR function characterisation. The remainder were made up of blocks from patients recruited to the study, whose samples did not grow in primary culture. Blocks used were those available prior to University laboratory shutdown during the covid pandemic. 50 endometrial patient samples were included, 4 were endometrial cancer cell line thrombin clot specimens (AN3CA, HEC1A, Ishikawa and RL95-2) and the remaining patients' final histology was an ovarian cancer. Median age 68 (53-84). Twenty-eight had stage 1 cancer, three had stage 2, 11 had stage 3 and 8 had stage 4. Four were grade 1 EEC, 6 were grade 2 EEC, 7 were grade 3 EEC, 6 were mixed histology, 16 were serous, 4 were clear cell and 7 were carcinosarcoma.

8.4.2 Lymphovascular space invasion (LVSI)

Lymphovascular space invasion or LVSI, denotes the presence of tumour cells within lymphatic channels. It highlights to potential of spread to local and distant organs through those channels and is associated with a worse prognosis. Twenty-one had LVSI negative tumours, 27 had LVSI positive tumours and 2 were unknown. Of the patients' tumours that were LVSI positive; 6 were stage 1, 2 stage 2, 11 stage 3 and 8 stage 4. 9 were serous, 5 were carcinosarcoma, 3 were mixed, 1 clear cell, 3 grade 3 EEC, 4 grade 2 EEC and 2 grade 1 EEC.

8.4.3 Serosal clearance

Serosal clearance is the distance from the greatest depth of invasion of tumour in the uterus to the outside surface of the womb. This is associated with a greater risk of metastatic spread of tumour and worse outcomes in patients. 13 had serosal clearance < 1 mm, 6 between 1-3 mm, 15 between 3-10 mm and 7 more than 10 mm. Data were missing for 9 patients. For those patients with serosal clearance < 3 mm; 5 were stage 1, 2 were stage 2, 7 were stage 3 and 5 were stage 4. Of the patients with serosal clearance greater than or equal to 3; 17 had stage 1, 1 had stage 2, 4 had stage 3 and 0 had stage 4.

8.4.4 Nodal involvement

Lymph node involvement with tumour is the result of metastatic spread of cancer cells. It increases the stage of the tumour and worsens the outcomes for patients if present. 11 patients had positive lymph nodes, 32 had negative lymph nodes and there was missing data for 7 patients. All patients with positive nodes had lymphovascular space invasion (11/11) and 12 (38%) who were node negative were LVSI positive. 7/11 (64%) patients who were node positive had a serosal clearance of < 3 mm, whereas 9/32 (28%) had with negative lymph nodes had a serosal clearance < 3 mm.

8.4.5 p53, MMR and PTEN IHC

P53 IHC is available for 39 patients' samples. 19 had mutated phenotype, 8 had null and 12 had wild-type (wt) phenotype. 11 samples were unknown. Of the p53 null or mutated phenotypes, 14 were stage 1, 2 were stage 2, 6 were stage 3 and 3 were stage 4. Of the patients will p53 wt phenotype, 6 were stage 1, 1 stage 2, 3 stage 3 and 2 stage 4. Of the patients with p53 mutated or null phenotypes; 15 were serous, 2 were carcinosarcomas, 4 were grade 2 EEC and 4 were mixed serous. Whereas there were 4 grade 2 EEC, 2 grade 1 EEC, 2 grade 3 EEC, 1 serous, 1 carcinosarcoma and one mixed cancer in the patients with p53 wt IHC. 40 of 50 patients had successful MMR status from IHC. 37 patients were pMMR and 3 were dMMR. 0 were defective in MSH2 and MSH6. 3 were PMS2 deficient, 2 of which were also deficient in MLH1 and a further was MLH1 defective only. 2 were stage 1A endometrioid cancers (grade 2 and 3) and the other was a stage 3C2 mixed low grade cancer. Unfortunately, from the 4 endometrial cancer cell lines, IHC staining was not successful from the thrombin clot samples in the TMA. 37 out of 50 had staining for PTEN. Only 3 had mutated pattern of staining, all of which were also dMMR. The remainder had a normal pattern of PTEN staining. Example positive staining is shown in figure 8.2.

8.4.6 ER and PR IHC

36 out of 50 had successful staining for ER. 7 were ER negative (3 carcinosarcoma, 2 serous, 1 clear cell and 1 G3 EEC), 3 were weakly positive (serous, carcinosarcoma and G3 EEC), 10 had moderate staining (5 serous, 2 G3 EEC, 1 clear cell and 2 mixed serous) and 16 were

strongly positive (7 low grade EEC, 2 grade 3 EEC, 2 mixed serous and low-grade EEC, 4 serous and 1 carcinosarcoma).

36 out of 50 had successful PR status. 12 were PR negative (6 serous, 3 carcinosarcoma, 2 G3 EEC and 1 clear cell). 8 were weakly positive (3 serous, 1 mixed serous and low grade, 1 grade 2 EEC, 1 clear cell and 2 carcinosarcoma), 5 were moderately positive (3 serous, 1 grade 3 EEC and 1 mixed serous and low grade) and 11 were strongly positive (6 were low grade, 1 was mixed serous and low grade, 3 grade 3 EEC and 1 serous).

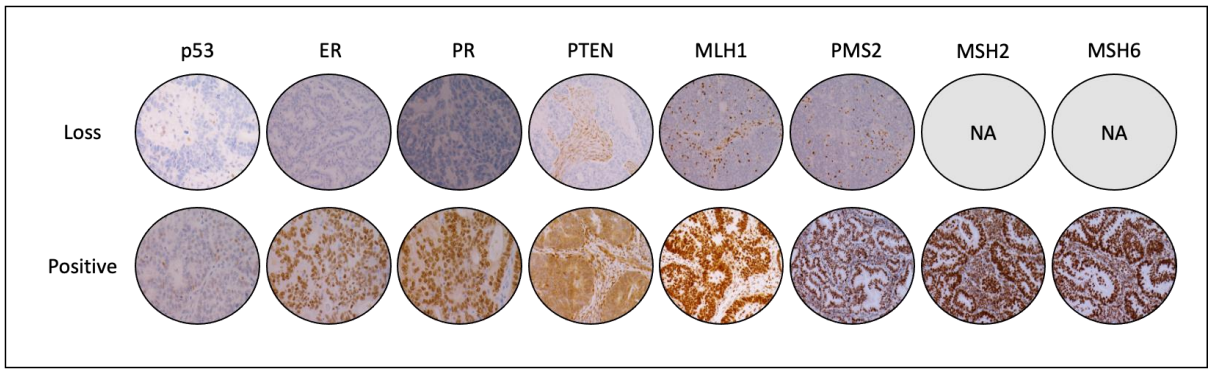


Figure 8.2 **TMA cores** Examples of positive expression and loss of protein expression in p53, ER, PR, PTEN, MLH1, PMS2, MSH2 and MSH6. There were no examples in the TMA of loss of MSH2/MSH6, therefore marked NA (not available). Images were taken at x 20 magnification with upright microscope by Dr Holly Buist, consultant gynaecological pathologist at Royal Victoria Infirmary, Newcastle.

8.4.7 Homologous recombination repair status and patient characteristics

15 patients were HRC and 3 were HRD. We defined the subtype and stage of the patient with available HRR result. Of the HRC patients, 4 had LVSI and 10 did not (1 was unavailable). In the HRD patients all patients were LVSI positive (3/3). In the HRC group, 2 were node positive and 12 were node negative. In the HRD group, 2 were node positive and the remaining was node negative. The p53 IHC status and HRR status were compared (2 were unknown). In the HRC patient group, 7 had mutated phenotype and 6 were p53wt. In the HRD group, 1 was p53mut and 2 were p53wt. MMR IHC was undertaken on the samples with HRR status, 14 were pMMR (12 HRC, 2 HRD) and 1 was dMMR (HRC). 3 samples did not have an MMR status by IHC. The IHC staining failed in 1/3 of HRD patients in the TMA. 2/3 HRD

had normal PTEN staining, 1/10 HRC were PTEN mutated. The remainder had normal pattern of staining.

In terms of ER and PR staining, 1 HRD failed for staining pattern, 1 was ER strongly positive and PR strongly positive, the other was negative. In the HRC cohort, 4 out of 11 were ER negative, 1 moderately positive and 6 strongly positive. For PR staining, 4 out 10 negative, 1 moderate and 5/10 PR strongly positive.

8.4.8 Determinants of overall survival (OS)

Survival analysis and Cox proportional hazard (PH) testing was undertaken to determine relationship between patient characteristics and survival. Stage, histological subtype and serosal clearance were all associated with reduced OS. The Cox PH regression did not find a significant relationship between age and survival (p=0.13). The log-rank test revealed a significant relationship between survival and stage of cancer ($p < 0.0001$). The Kaplan Meier plot (figure 8.3) indicated that patients with stage 4 cancer had a much lower probability of survival over time, patients with stage 1 cancer had the best survival, and patients with stages 2 and 3 cancers were in the middle. Note the sample of patients with stage 2 cancer was particularly small (n=3).

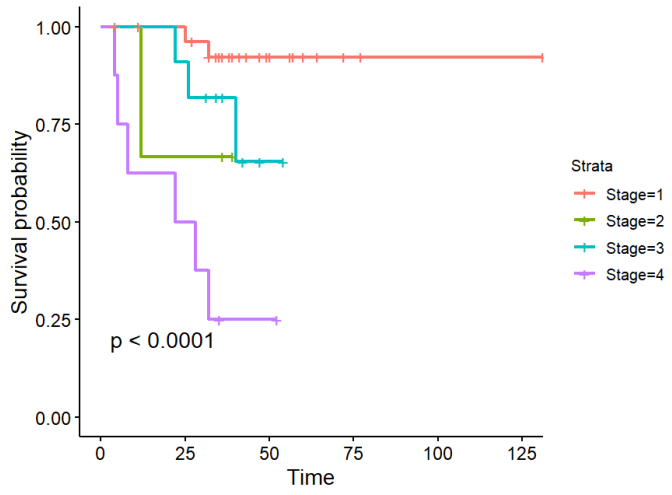


Figure 8.3. **Kaplan Meier curve; stage vs. overall survival**

The log-rank test found no significant relationship between survival and HRR status (p = 0.5). This is reflected in the Kaplan Meier curve below (figure 8.4). However, the curve is

suggestive that there may be a survival difference. However, no firm conclusions can be drawn given the small samples size.

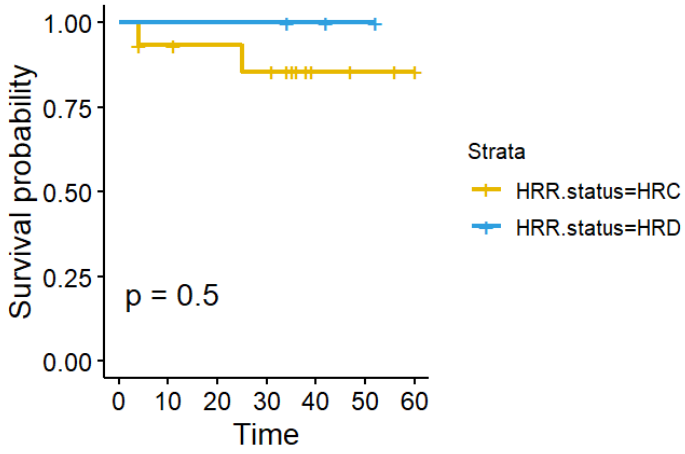


Figure 8.4. **Kaplan Meier curve; HRR status vs. survival**

A Kaplan-Meier plot (figure 8.5) indicated that there was no significant difference in survival between patients with node positive and node negative disease (p = 0.61).

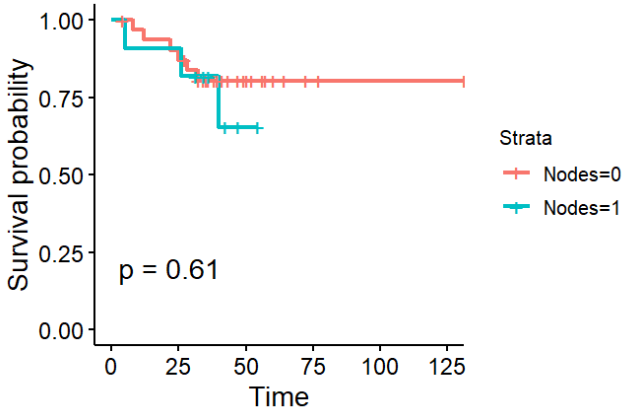


Figure 8.5**. Kaplan Meier plot; nodal status vs. survival**

A Kaplan-Meier plot (figure 8.6) indicated that patient survival varied with histological subtype. Survival probability of patients with mixed serous and G3 EEC dropped quickly compared to patients with other subtypes; however, the sample size for this subtype was very low (n=3). Patients with sub-type carcinosarcoma had the second lowest survival probability over time, followed by patients with sub-type serous. The relationship between subtype and survival was not found to be significant; however, the p-value was marginal (p=0.065).

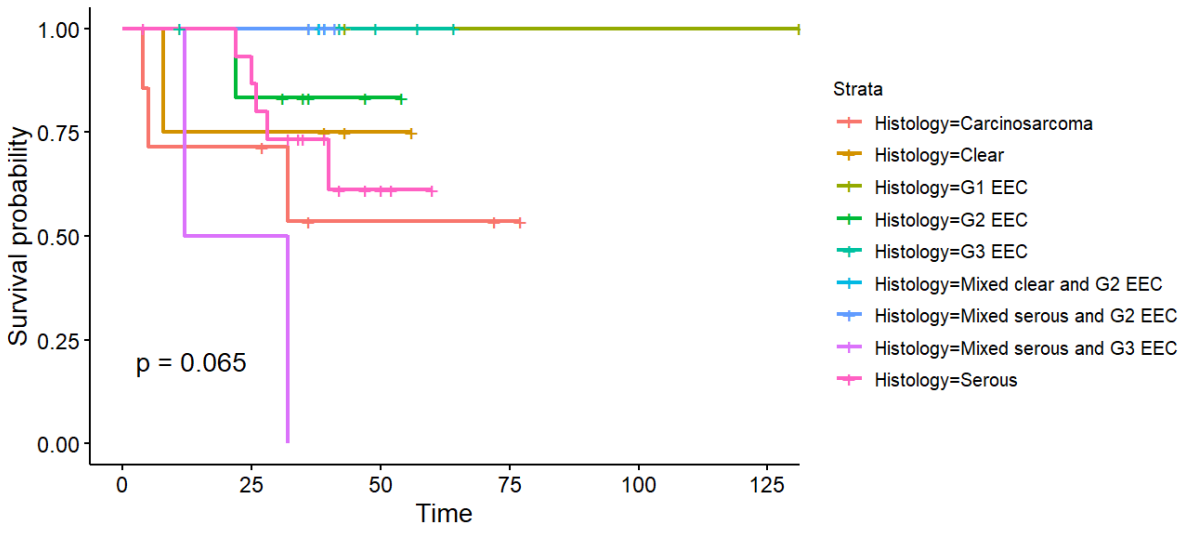


Figure 8.6. **Kaplan Meier plot; histological subtype vs. survival**

The Cox PH regression found a significant relationship between serosa clearance and survival (p=0.03), with lower serosa associated with poorer survival. (Figure 8.7); although the low and high categories created for this plot were not found to be significantly different by the log-rank test.

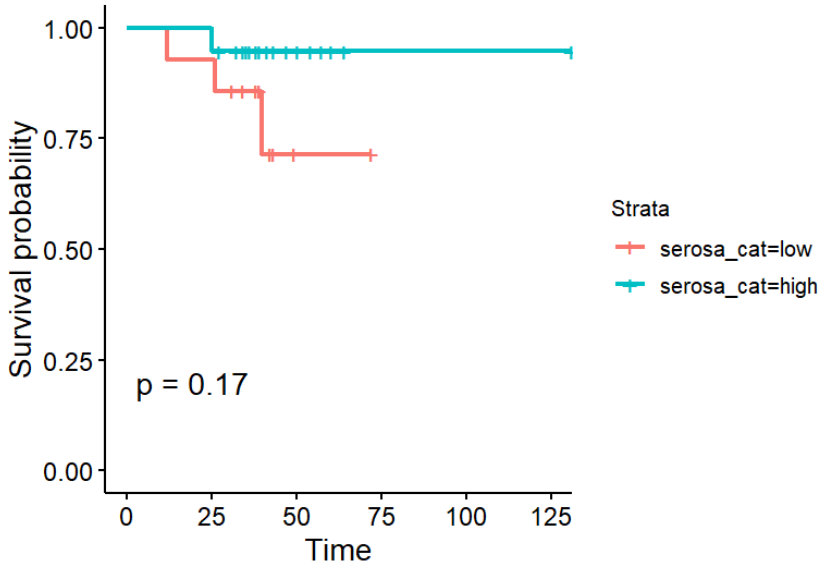


Figure 8.7. **Kaplan Meier plot; serosal clearance < 3 mm vs. survival**

8.4.9 Correlations with HRR status and IHC markers

There was no statistically significant correlation between baseline clinicopathological markers/IHC markers and HRR status. This is likely due to the small sample size in this cohort

of endometrial cancers included in this TMA. However, there is a trend towards a possible correlation between the histological subtypes and HRD, i.e., grade 3 EEC and serous cancers more likely to be HRD. There was no correlation between p53 IHC status, PTEN, MMR status and HRD, again this is likely due to the small sample size. A summary table is provided in appendix B of the clinicopathological features of the samples, along with HRR status and IHC staining pattern.

8.5 Discussion

In this chapter we have demonstrated that it is possible to compare classical clinicopathological data and current IHC markers used in stratification of risk and diagnostics in endometrial cancer with ex vivo data of HRR status. Our data was in line with the main known determinants of survival in endometrial cancer, stage and histological subtype, with poorer survival seen within the p53 mutated subgroup and more aggressive type 2 histological subtypes (Crosbie et al., 2022). Interestingly we saw that serosal clearance of tumour was also a determinant of survival which is unsurprising given is clear association with more advanced stage. This association has previously been described in our group (Chattopadhyay et al., 2012). However, we would expect to see LVSI positive tumours and those with nodal disease to have poorer overall survival (Stålberg et al., 2019, Cusano et al., 2018). This is likely due to the small sample size in our cohort and the inherent bias seen in this dataset due to samples having been taken from patients in a cancer centre which deals with more aggressive tumour types. Further work is needed to determine if our data is in line with that seen in the literature.

A tissue microarray was constructed to allow for a greater throughput of IHC staining for several patients with endometrial cancer, this allowed for direct staining during the same experiment for correlation and comparison. TMAs are already used in several cancer sites as it is a cost saving way to do multiple IHC staining on multiple sample types at the same time. TMA use in not only limited to protein expression but has been used for mutational analysis, as well as parallel in situ detection of DNA, RNA and protein targets (Behling and Schittenhelm, 2018). However, a disadvantage of using this method is that these fragile cores can ‘slip’ or be removed through the process of fixing onto slides, making it difficult to determine the current core for each sample during analysis or result in missing data (Vogel, 2014). The structure of this TMA mitigated against that by having control cores at different point to enable identification of slipped cores as well as having duplicate cores in case one of the cores fails. However, despite this, several cores were missing in the analysis reducing the number of available results making it more difficult to draw firmer conclusion with the IHC work, due to a smaller sample size.

P53 mutated IHC staining was found in 49% of patients. This is unsurprisingly given that most endometrial cancers within this group were high grade. We know that are 29% of high-grade cancers are copy number high (the group where we would expect to see HRD where a significant majority were p53 mutated (Cancer Genome Atlas Research et al., 2013)). The proportion of p53 mutated phenotype was higher in our cohort, this is likely due to an element of selection bias and targeting of serous and grade 3 EEC in the search for HRD in these endometrial cancer patients. The numbers were too small to determine a correlation between HRD and p53 IHC status and warrants further investigation.

93% of patients were pMMR and 7% were dMMR. In the dMMR patients 2/3 had combined MLH1 and PMS2 abnormality and the remaining dMMR patient was MLH1 defective only. dMMR is more commonly seen in the endometrial cancer subtypes which is reflected in our small dataset. MLH1 hypermethylation is the most common cause of dMMR in endometrial cancer and therefore was the most likely aberration likely to be found. MLH1/PMS2 loss due to epigenetic hypermethylation of the MLH1 promoter is the most common cause for dMMR in endometrial cancer (Kurpiel et al., 2022), therefore suggestive that our IHC work is likely to be valid. We would expect to see around 28% of high-grade endometrial cancers to be microsatellite unstable and 39% of endometrial cancers overall to be dMMR. In our high-grade endometrial cancer cohort, it is likely that selection bias has resulted in this discrepancy. There was no correlation between MMR status and HRR status, again the numbers of both were small and a larger sample size is needed to confirm or refute this hypothesis. Unsurprisingly there was no survival difference seen between the 2 groups, but a suggestion that there may be improved survival in the HRD group, however this is a very small sample size to draw any conclusion and warrants further work.

As discussed in the previous chapter we expected to find HRD in the serous and grade 3 EEC subtypes. Additionally, we hypothesised that MSI-high tumours may develop mutations in HRR genes which render them HRD. PTENs role in HRR in controversial as discussed in section 1.7.11 (Bian et al., 2018, Dedes et al., 2010, Shen et al., 2007). However, none of the 3 patients had MMR loss and none had PTEN loss by IHC. All 3 HRD patients were serous, or grade 3 EEC subtypes as expected by TCGA group. However, we were unable to find any biomarkers for HRD status with clinicopathological data or standard IHC work in endometrial

cancer. This was limited by missing cores, bias in the sample collection and a small sample size. Further work is needed to establish biomarkers for HRR status in endometrial cancer.

8.6 Conclusion

- Stage and tumour subtype are a determinant of survival in endometrial cancer
- It is possible to execute high throughput staining of many endometrial cancers in a TMA
- HRD was found in serous and grade 3 EEC subtypes but not association between p53, MMR and PTEN status, and HRR status was found
- Further work is needed to determine biomarkers of HRR status in endometrial cancer.

Chapter 9. Final discussion, conclusions, and future directions

The current ESMO/ESGO/ESTRO guidance (Concin et al., 2021) has identified a need for better treatment options in the relapsed setting for endometrial cancer. This is exemplified by the poor response rates to standard chemotherapy. Immunotherapy has shown promise at recurrence for dMMR tumours, however there is emerging evidence of a role for PARPi. The aim of this thesis was to extend current knowledge and understanding of the potential of PARPi, specifically Niraparib, in this disease. The investigative work in this thesis brought together for the first-time comparative work in a panel of endometrial cancer cell lines. Baseline cell line characteristics were explored, including functional DDR evaluation with PARP and HRR activity to allow for direct comparisons between sensitivity to cisplatin, IR and Niraparib, to investigate potential determinants of sensitivity. Furthermore, the ability of Niraparib to sensitise endometrial cancer cell lines to the effects of standard of care treatments IR and cisplatin were investigated in a panel of endometrial cancer cells for the first time. Similarly, this thesis optimised a protocol in primary endometrial cancer cell culture to allow investigative of HRR function and sensitivity to PARPi in a more clinically relevant situation. The aim of this thesis was to determine markers of sensitivity to common endometrial cancer agents and to determine if there was a clear role for PARPi in HRD endometrial cancers, or as sensitisers to treatment. The findings from this work could inform future studies of the relative potential of these drugs in endometrial cancer to improve the outcome in a cohort of patients with particularly poor long-term outcomes. This work with the cell line panel was somewhat limited due to size and relatively narrow spectrum of DNA repair capacity and drug/radiation sensitivity. Nevertheless, trends were seen, which may inform future clinical trials work and focus using PARPi in endometrial cancer.

To understand the effects of single agent cytotoxicity and growth inhibition more deeply on each endometrial cancer cell line, baseline cell line characteristics were investigated which included, any clinical information (pathological subtype, stage of disease), cell growth, PARP activity and HRR function. Reassuringly, the results were similar to published data. However, this is the first time that HRR status was determine by a functional assay in these cells. Unfortunately, in this small cell line panel, all were HRC, but to varying degrees, with some cell lines having greater replication stress induced by PARPi. There was no correlation between baseline cell characteristics and HRR functional assay results, which is not

surprising given all cell lines were HRC. It may have been expected that the serous endometrial cancer cell lines in this panel (ARK1 and ARK2) would be HRD, as was predicted in the TCGA dataset and follow up studies (Cancer Genome Atlas Research et al., 2013, de Jonge et al., 2018).However, it was worth noting that even in the TCGA dataset, that not all serous endometrial cancer cell lines were expected to be HRD, reflecting the heterogeneity seen within many cancers, based on the different genomics.

This thesis examined the cell survival and growth inhibition to cisplatin, IR and Niraparib. A narrow spectrum of sensitivity in all cell lines studies to these agents, two of which are used in the standard of care in endometrial cancer management. There is a trend to sensitivity with HEC1A cell line being the most resistant to IR, cisplatin and Niraparib, and AN3CA being the most sensitive. This is the first time that this panel of endometrial cell lines have had sensitivity to cisplatin and IR to allow for direct comparison of sensitivity. Other studies have looked at these individually but not together. Interestingly, HEC1A and RL95-2 were more resistant to cisplatin, compared to other cell lines. Both cell lines are known to harbour defects in MMR. This is in keeping with the notion that dMMR tumours are more resistant to platinum agents because of replication bypass (Vaisman et al., 1998). HEC1A was significantly more resistant to cisplatin than even RL95-2, this has been seen in previous cell line work and it thought to relate to cisplatin induced perturbations in the cell cycle, ROS production and autophagy, as well as the induction of cellular senescence and DNA damage (Lin et al., 2021). It should be noted that the effects of cisplatin were measured by MTT in this study and not by colony formation, limiting direct comparison with the data reported here.

It is worth noting that the LC50 concentration ranges (2.89-8.11 μM) where a profound effect on cellular toxicity was seen in the endometrial cancer cultures fall comfortably below the steady state plasma concentrations seen in pre-clinical xenograft models (65 μM) and in phase one clinical trials (4.37 μM) to warrant further exploration of single and combination treatments with Niraparib in HRD and HRC cancers (Sun et al., 2018, Bruin et al., 2022b). However, further work is needed to identify those patients most likely to see best response. We would expect to see the most profound effect in HRD cancers.

As expected, the cells ranked in a similar order of sensitivity to cisplatin and Niraparib since HRR function is a determinant of both. However, a similar trend in sensitivity to IR was also observed, which suggests sensitivity is not due to the classic determinant of PARPi sensitivity, rather that some cell lines may have more pro-survival factors that render them more resistant than others.

It might have been expected that the rank order of sensitivity to Niraparib and cisplatin to be similar because HRD is a determinant of sensitivity to both, and platinum sensitivity may be used as a surrogate for HRD. This may not have been reflected in this small cell line panel as all cells were HRC and therefore there is little spread in the sensitivity of both, but it is possible that there are degrees of impairment in HRR, e.g., if a less critical component is mutated/compromised in some way in HRR they may demonstrate different effects. A trend in the cell lines to sensitivity and resistance to these agents implies some cells may be more resilient than others.

This is the first time that Niraparib sensitivity by both cytotoxicity and growth inhibition assays has been performed in endometrial cancer cell lines. Previously, a panel of endometrial cancer cell lines had been treated with Olaparib to determine sensitivity (Miyasaka et al., 2014). Interestingly, Ishikawa, RL95-2 and AN3CA cell lines were found to be more sensitive to Olaparib compared to the more resistant HEC1A cell line in keeping with the results from this thesis. However, the rank order of Olaparib sensitivity in RL95-2, Ishikawa and AN3CA in the above published work, was different from that seen with Niraparib, this likely relates to the different effects or PARP inhibition and PARP trapping in these cell lines (Murai et al., 2012). Dedes et al. (2010) investigated the response of a panel of endometrial cancer cell lines to the PARPi KU0058948, Similarly, a narrow spectrum of sensitivity was seen with AN3CA, Ishikawa and RL95-2. The sensitivity was seen within the range acceptable for use in patients and therefore supports its use in human clinical trials. All these cell lines investigated in this thesis were found to be HRC by functional RAD51, which is supported by their relatively narrow spectrum of sensitivity, however HEC1A/HEC1B which are similar cell lines, were noted to be profoundly less sensitive to PARPi. It is therefore important to understand this relative resistance when translating this work into patients. Interestingly HEC1A has the highest PARP activity of all the cell lines and was relatively resistant to Niraparib, IR and cisplatin. It is likely that HEC1A cells are more

resistant to cell death signals/apoptosis as compared to the other cell lines, as HEC1A also appears to resistant to paclitaxel (which does not damage DNA) (Dinkic et al., 2017).

A limitation of this thesis is that NHEJ activity and protein expression (DNA-PKcs, Ku, XRCC4 and LIG4) were not measured, as defects in NHEJ are known to be a major determinant of sensitivity to IR. It would be important to understand this in the context of this research as both HRR and NHEJ are competing pathways and loss of components of NHEJ can restore HRR in BRCA mutated cells and effect sensitivity to PARPi (Patel et al., 2011).

The method of measurement of PARP activity by immunoblot was novel in endometrial cell lines. Other groups have measured PARylation by western blot. Unsurprisingly we found very similar levels of PARP inhibition to Niraparib in all cell lines, given that the activity was measured in permeabilised cells and therefore the measure was purely biochemical and does not consider the differences in transport of PARP and its cellular accumulation. It will be important to determine this in future work. Measurement of PARP inhibition by this method in the endometrial cancer cell lines allowed a concentration to be chosen for combination studies (1 μM) that inhibited PARP by ~ 90% with little impact in cell survival alone with that concentration.

There is emerging evidence to suggest that hypofractionated radiotherapy (higher radiation dose/fraction with fewer fractions as compared to conventional radiotherapy) may elicit a pronounced antitumour effect directly, as well as inducing cell death via antitumour immunity and vascular damage (Wang, 2021). Antitumour immunity is thought to be caused by the release of tumour associated antigens as well as cytokines which attract inflammatory cells to the site of tumour (Chajon et al., 2017). In clinical practice it is called stereotactic body radiotherapy (SBRT). There are now several studies in cancer which show similar or greater efficacy when compared with conventional radiotherapy without significant side effects (Koontz et al., 2015, Liu et al., 2020). The finding reported in Chap section 6.4 (table 6.1) that demonstrated greatest radiosensitisation by Niraparib in combination with higher dose IR suggests that the combination with hypofractionated radiotherapy may be of the greatest benefit. Combination treatments with radiotherapy and PARP are thought to

enhance the immune response caused by IR. The immune stimulation caused by IR can lead to what is known as the abscopal effect, resulting in tumour shrinkage elsewhere, this may be enhanced with PARPi/IR combinations (Césaire et al., 2018, Rodríguez-Ruiz et al., 2018). There are currently clinical trials ongoing using hypofractionated radiotherapy in endometrial cancer (NCT04683653, NCT05139368 and NCT04890912). The SPARTACUS phase I/II trial (Leung et al., 2021) in endometrial cancer has shown that it is feasible and well tolerated but further studies are needed to determine benefit and non-inferiority to standard treatment. Given the radiopotentiation seen with Niraparib at higher IR doses, it would be worth exploring further preclinical work in xenograft models to determine role in the future.

PARPi have been found to sensitise the effects of ionising radiation and chemotherapeutic agents in many cancer sites, resulting in a number of early phase clinical trials (Barcellini et al., 2021). This thesis was the first time that Niraparib has been used in combination as a chemo and radiosensitiser in endometrial cancer. We found a modest radio- and negligible chemosensitation when Niraparib 1 μM was combined with increasing concentrations or IR and cisplatin. The greatest cytotoxic effect in combination was seen at high doses of IR. Our data showed comparable radiopotentiation with Niraparib and IR in endometrial cancer cell lines, when compared to endometrial cancer cell line studies with other PARPi (Wang et al., 2022, Miyasaka et al., 2014, Minami et al., 2013). There are currently no combination studies with cisplatin and PARPi in endometrial cancer cell lines which makes this study novel. PARPi combinations have been taken forward in combination studies with cisplatin and radiotherapy. Recent clinical studies have proven PARPi to be effective radiosensitisers in many cancer sites (Césaire et al., 2018). Niraparib has been shown to have clear radiosensitising effects and has been expand into clinical trials (Wang et al., 2020). A combination of Niraparib and radiotherapy has found to be safe, well tolerated and have good efficacy in patients with recurrent glioblastoma (Jiang and Wang, 2022). There are currently no studies or clinical trials recruiting for combination treatment with Niraparib and cisplatin or radiotherapy, however, its tolerability in other cancer cell types supports its use in clinical studies, along with our data.

On the other hand, combinations with cisplatin and Niraparib were not as promising and available evidence suggest that any effect is compound and cell line dependent, suggesting that there is unlikely to be a promising role for this combination, unless the combination is used in HRD cell lines (Evers et al., 2008).

A primary culture method was optimised and used it to determine HRR functional status and Niraparib sensitivity by growth inhibition in a small number of samples. Nevertheless, this was at the expected frequency of HRD in endometrial cancer patients and HRD primary cultures were more sensitive to Niraparib as predicted. However, the difficulties in growing and maintaining an epithelial endometrial cancer culture enough to perform HRR and growth inhibition assays led us to search for FFPE-based predictive biomarkers but with limited success. More studies both using the HRR assay on viable tissue and available fixed histopathology tissue are needed to identify potential biomarkers.

Measurement of HRR status revealed all cell lines to be HRC but 3 out of the 18 primary cultures were HRD enabling this thesis to test the hypothesis that HRD endometrial cancers would be more sensitive to Niraparib. This proportion of HRD was expected from literature. The validity of SRB assay was found to correlate well with clonogenic survival and using this assay the 3 HRD primary cultures were more sensitive to Niraparib than the others. However, one HRC culture was also sensitive to Niraparib suggesting that HRD function as measured by RAD51 foci is not infallible. It is possible that this culture had a HRD defect downstream from RAD51 focus formation as has recently been demonstrated with OVCAR3 cells (Bradbury et al., 2020). Similarly, the GI50 concentrations in the successful primary cultures fell within the therapeutic range of Niraparib in pre-clinical models and in a phase 3 clinical trial suggesting a clear role, particularly with HRD cancers.

As expected, the HRD patients were serous and high grade endometrioid subtypes with a frequency close to what was expected in preclinical work from other groups (Cancer Genome Atlas Research et al., 2013). However, we were unable to correlate MMR and p53 status in our TMA IHC characterisation. p53 IHC status was not predictive of HRD status despite what others have suggested but with the limitation that there were only 3 HRD

cultures in our cohort (van den Heerik et al., 2021, Siedel et al., 2021, de Jonge et al., 2018). The Rainbo trial (NCT05255653) is currently recruited p53 mutated patients for randomisation to a chemoradiation followed by maintenance PARPi or placebo.

Despite the small primary culture sample size of those successful cultures, in which a successful HRR result was obtained, there appeared to be a trend towards improve survival in those patients that were HRD. Of note, two of the HRD patients were treated with combination carboplatin/paclitaxel. This improved survival was similarly found in preclinical work in ovarian cancer primary cultures with the RAD51 assay (Mukhopadhyay et al., 2012) and is reflected in those patients who are HRD treated with chemotherapy (Shao et al., 2022).

Final conclusions: Primary endometrial cancer cultures were established from patient surgical biopsy specimens in which Niraparib cytotoxicity is dependent on HRR status in primary cultures and observed clinically relevant concentrations. Niraparib may be useful as single agent in HRD, provided these can be identified. To date pathology parameters have not revealed a potential predictive biomarker of HRR status but this is due to limited number samples it was possible to characterise for HRR function. Further work is needed to determine if mutational p53 status is a predictive biomarker. Niraparib may be useful as a radiosensitiser especially with hypofractionation schedules. Combination treatment with cisplatin did not yield encouraging results. HRR may not be sole determinant of drug/IR sensitivity some may be intrinsically resilient, and more work needed to determine why. Conclusions are limited by size of study and more work needed to validate tentative hypotheses.

9.1 Plan for future work

- Expansion of endometrial cancer cell lines to find examples of HRD would allow for more intense evaluation of the implications for sensitivity to PARPi, cisplatin and IR alone and in combination.

- Genomic sequencing of FFPE material and evaluation of HRR status of corresponding primary cultures may identify more tractable IHC biomarkers of HRD

and PARPi/cisplatin efficacy. Further work to identify tumour subtypes likely to be HRD and predictive biomarkers from available clinicopathological data.

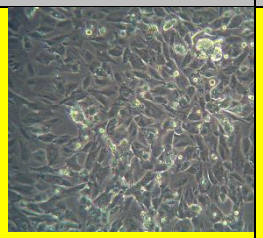
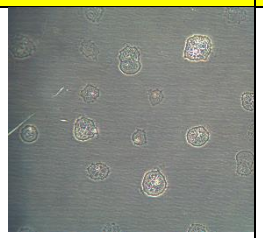
- Develop HRR assays in FFPE material e.g. RAD51 in geminin positive tumours as a way of identifying HRC as has been demonstrated in breast tumour biopsies (Graeser et al., 2010) to determine if this is a reliable and tractable indicator of HRR function that correlates with HRR function determined ex vivo in primary cultures of the corresponding tumour samples. Geminin could be used as an additional marker in the HRR assay to identify cells that are proliferating and to correlate the H2AX and RAD51 foci formation, as these are cells in which are likely to have been damaged by PARPi and then subsequently repaired.

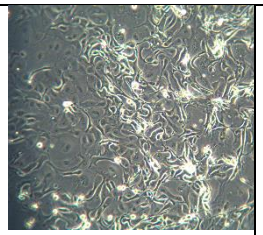
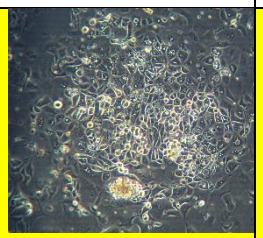
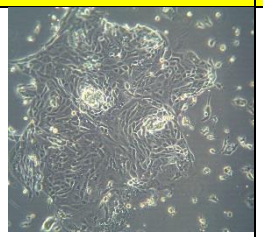
- Search for biomarkers of HRR status and other pathological markers associated likely response to PARPi

- Potential use of organoids and/or PDX to better reflect the tumour microenvironment and assess response to therapies in order to take PARPi single agent and combination into clinical trials in endometrial cancer.

Appendix A

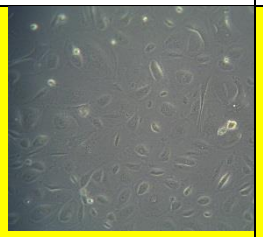
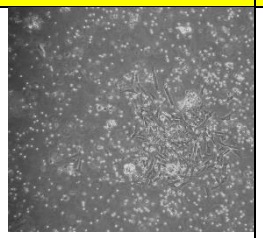
Highlight in yellow shows those cultures where HRR status was determined.

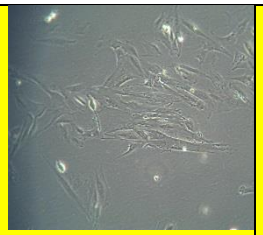
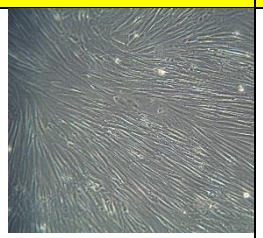
Patient ID	Age	Histological subtype	FIGO Stage	Successful Culture?	Brightfield image	Culture notes	Morphology	HRR status	Passage reached	GI50 Niraparib μM
1	47	Benign	N/A	Y		Pipelle biopsy directly transferred. Process with col/dis. Incubated for 5 days. Small colonies forming with cobblestone appearance. Media washed and changed and incubated for further 4 days. Reached 70-80% confluence. Trypsinised and placed onto coverslips. Culture remaining in flask slow to grow, cells detaching on day 4, culture not ongoing.	Cobblestone	HRC	P1	no data
2	70	Carcinosarcoma	3a	Y		Solid tumour biopsy, taken at the beginning of the procedure by pipelle, transferred directly to university for processing. Col/disp protocol. Decision to not wash and replace media on day 5, small number of colonies, however all detached on day 14 with no going culture. Media appears contaminated	senesced at P0 without monolayer growth	no data	P0	no data

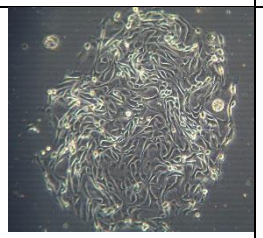
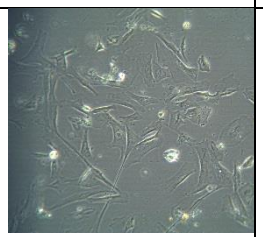
3	69	Serous and clear cell	3c	Y		Solid tumour biopsy taken at the end of the procedure, transferred immediately to lab. Col/disp protocol. Media washed and replaced day 6, colonies seen to grow slowly. Reviewed on day 10, extensive fibroblastic overgrowth suggestive of mixed culture. Culture was therefore discarded.	Cobblestone but colonies of fibroblasts seen	no data	P0	no data
4	50	G2 endo and clear cell	1a	Y		Solid tumour biopsy. Immediate transfer. col/disp protocol. Media washed and replaced day 5. Cobblestone monolayer culture forming, extending from clumps of epithelial tissue. Relatively slow growing. Media replaced day 10. 70% confluent day 12. Seeded onto coverslips for HRR culture. Small number of cell yield therefore placed back into T25. Little or no attachment seen on day 6 of passage 1. No ongoing culture at day 14, sample discarded	Cobblestone	HRC	P1	no data
5	82	Serous	1a	Y		Pipelle endometrial biopsy at beginning of procedure. Transfer at end of procedure. Col/disp. Media change day 5, fast growing culture. Cobblestone monolayer seen. Review of culture day 7 small epithelial colonies seen but	Cobblestone	Fibroblastic overgrowth	P0	no data

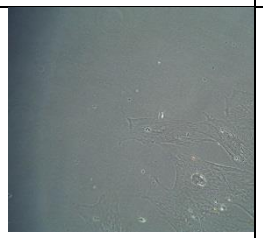
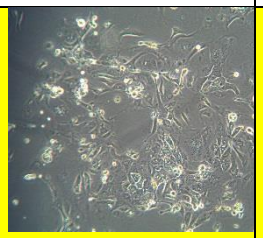
						culture becoming overgrown with fibroblasts				
6	72	Serous	3C1	infected	No image	Solid tumour biopsy taken and immediately transferred. Col/disp. Media looks murky day 5, washed and replaced with fresh media to remove debris. Day 7 media contaminated	no data	NA	P0	no data
7	50	G2 endo and clear cell	1a	infected		Solid tumour biopsy taken at the end of the procedure. Col/disp. Media washed and changed day 5. Predominantly fibroblastic culture seen. Reviewed 2 days later cloudy, offensive smelling media contaminated by microbes. Culture discarded.	no data	NA	P0	no data
8	68	G3 Endo	1a	no growth		Solid tumour biopsy taken at beginning of procedure with pipelle and stored at 4 C until transfer at end of case. Col/disp. Flask washed with PBS and media replaced day 7. No visible colonies seen. Culture observed daily but no culture at day 14 therefore sample discarded.	no data	no data	P0	no data
9	80			infected		Solid tumour biopsy taken. Col/disp. Incubated for 6 days media cloudy. Old media aspirated, flask washed with PBS and replaced with fresh media. Culture viewed and seen to be contaminated with bacteria. Samples discarded	no data	no data	P0	no data

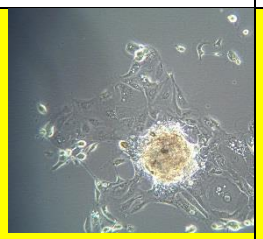
10	45	G3 Endo	1a	infected		Solid tumour biopsy taken. Col/disp. Incubated for 5 days media cloudy. Old media aspirated, flask washed with PBS and replaced with fresh media. Culture viewed and seen to be contaminated with bacteria. Samples discarded	no data	no data	P0	no data
11	76	Serous	3C1	no growth		Solid pipelle biopsy taken at start of procedure. Sample transferred at end of procedure after refrigeration at 4 C. Col/disp. Media was replaced on day 7, no visible colonies were seen. No growth at day 14 therefore sample discarded.	no data	no data	P0	no data
12	71	Serous	1a	No growth		Peritoneal washings taken intraoperatively. Washings refrigerated in normal saline and transferred to university post procedure. Sample was spun at 250g for 3 mins. Saline was aspirated and cell pellet was resuspended in 10 mls of primary culture medium. Sample was observed daily, some cellular debris was noted but no visible colonies. Media replaced day 10 and sample discarded as failed culture on day 14.	no data	no data	P0	no data
13	64	Benign	n/a	infected		Solid tumour biopsy taken. Col/disp. Incubated for 5 days media cloudy. Old media aspirated, flask washed with PBS	no data	no data	P0	no data

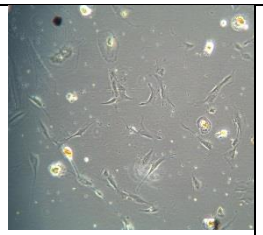
						and replaced with fresh media. Culture viewed and seen to be contaminated with bacteria. Samples discarded				
14	54	Serous Ascites	4b	Y		Ascitic fluid was taken intraoperatively from a patient with unresectable disease awaiting neoadjuvant chemotherapy. Ascites was split 1:1 well cell culture medium and transferred into a T75 flask for culture. Media was replaced on day 5. Cells grew rapidly a cobblestone monolayer and appeared similar in morphology to high grade serous ovarian cell lines. Culture continued passed 3rd passage at which point cells became more dilated and begin to detach. The culture did not survive passed P4.	no data	HRD	P4	1.73
15	65	G2 endo	2	Y		Solid directed endometrial biopsy taken post procedure. Col/disp, media containing antimicrobials. Culture medium replaced on day 6, visible colonies seen and were slow growing. Media replaced on day 10, small colonies of fibroblasts seen. Sample viewed on day 14, significant fibroblastic overgrowth with mixed culture. Therefore sample discarded.	Spindle like	Fibroblastic overgrowth	P1	no data

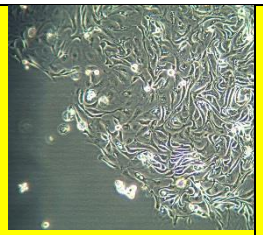
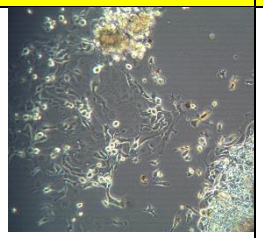
16	67	Serous	1a	Y		Solid directed endometrial biopsy taken post procedure. Col/disp, media containing antimicrobials. Culture medium replaced on day 5, visible colonies seen and were rapidly growing. Media replaced on day 10, sample discarded. Culture 70-80% confluent on day 12 therefore decision to seed onto coverslips. very limited cell count noted. HRR completed at P1, SRB completed at P2. Cells became very dilated at P2 and can be seen in the image. Culture not ongoing at P3.	Fibroblastic	HRC	P3	11.46
17	62	Serous	1a	Y		Solid directed tumour biopsy taken at the end of procedure and transferred immediately to university for processing. New primary cell culture media used for transfer and in culture containing antimicrobials. Media. Media replaced on day 5. Fibroblastic culture noted. Rapid growth and expansion. Small tumour focus on directed biopsy. Decision to passage and seed for HRR. No Growth on coverslips and no flask growth therefore sample discarded.	Fibroblastic	Fibroblastic culture	P1	no data
18	68	Serous	3b	infected	no image	Solid directed endometrial biopsy taken post procedure. Col/disp, media	no data		P0	no data

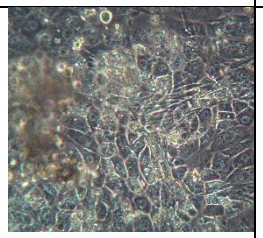
						containing antimicrobials. Culture medium replaced on day 6, visable colonies seen and were slow growing. Media replaced on day 10, small colonies of fibroblasts seen. Sample viewed on day 14, significant fibroblastic overgrowth with mixed culture. Therefore, sample discarded.				
19	71	Carcinosarcoma	1a	Y		Solid directed tumour biopsy taken at the end of procedure. Col/disp with media containing antimicrobials. Culture media changed on day 5. Visible colonies. Culture seen to form in cobblestone monolayer. Sample reached 70% confluence on day 9 and small cell yield was noted at passage. Cells were seeded onto coverslips with the remainder of culture split 1:2 with fresh media. Fibroblastic culture appeared to take over in T25 flask and coverslips failed to culture. Bacteria were noted in coverslips wells ? impacted on cell survival	cobblestone	N	P1	no data
20	61	Clear cell ovary	1C1	Y		Solid pipelle biopsy taken at start of procedure. Sample transferred at end of procedure. Col/dispase with media containing antimicrobials.	fibroblastic	N	P2 infected	no data

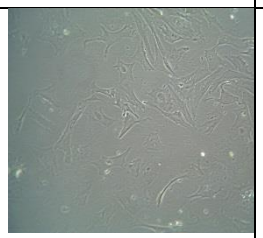
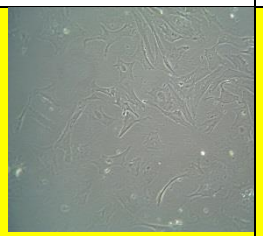
21	56	G2 endo	4a	no growth	no image	Peritoneal washings taken intraoperative in patient with overt stage 4 disease. Sample transferred immediately in normal saline. Centrifuged at 250g and pellet resuspended in media containing antimicrobials. Media was replaced on day 7. No visable colonies seen to grow. Media replaced on day 11. No visable colonies seen on day 14 therefore sample discarded.	N	N	P0	no data
22	74	Carcinosarcoma	1a	Y		Solid directed tumour biopsy taken at end of procedure. Col/disp with media containing antibiotics. Media was changed at day 5. Small number of colonises noted. Detached on day 10 and discarded.	Fibroblastic	Failed culture	P0	no data
23	68	Serous	3C2	Y		Directed solid tumour biopsy taken at the end of procedure. Transferred immediately. Col/disp with media containing antibiotics used. Media changed on day 5. Visible colonies seen which formed a cobblestone monolayer over the preceding days. Culture grew rapidly and was able to be split on day 7 at 80% confluence. Plentiful cells were available therefore samples were seeded for HRR and SRB.	cobblestone, piling up	HRC	P1	8.82

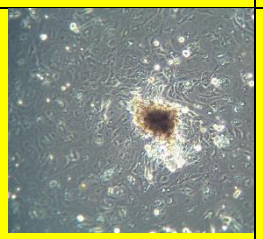
						Cell culture did not proceed passed P1. Small number of colonies formed on the flask ? split too harshly.				
24	81	Carcinosarcoma	4b	infected P0	No image	Solid tumour biopsy taken. Col/disp. Incubated for 7 days media cloudy. Old media aspirated, flask washed with PBS and replaced with fresh media. Culture viewed and seen to be contaminated with bacteria. Samples discarded	no data	no data	P0	no data
25	78	G2 endo	1a	Y		Solid directed tumour biopsy taken at end of procedure. Col/disp. Media used containing antibiotics. Media washed and replaced day 6. Visible colonies seen and appeared to be spreading out from clumps of pearl endometrial tissue. Culture continues to spread out and form a cobblestone monolayer on frank and appear to pile up ? forming glandular structures. Growth appears slow with 70% confluence reached on day 11. Coverslips seeded for HRR at P1 and SRB at P2. SRB took around 10 days to reach confluence in the control cell, again cell number was low at each passage and new flask was seeded 1:2 with fresh media. Reached P4 with fibroblastic overgrowth at this point.	cobblestone spreading out from solid tumour	HRC	P4	7.85

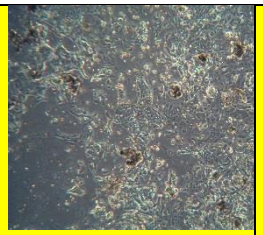
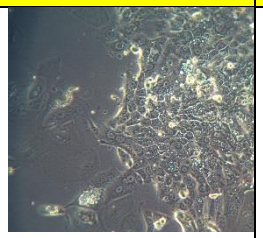
26	71	G3 endo	1a	Y			mixed epithelial and fibroblastic	Failed culture	P1	no data
27	79	G2 Endo	3a	n infected	no image	Solid tumour biopsy taken. Col/disp. Incubated for 6 days media cloudy. Old media aspirated, flask washed with PBS and replaced with fresh media. Culture viewed and seen to be contaminated with bacteria. Samples discarded.	no data	no data	P0	no data
28	76	Clear cell	1a	n no growth	no image	Peritoneal washings taken intraoperatively and stored at 4 C for the duration of the surgery. Sample was transferred and processed as per methods for washings. No growth was seen on day 5, 10, 14. Sample discarded	no data	no data	P0	no data
29	72	Carcinosarcoma	2	no growth		Solid directed endometrial biopsy taken at the beginning of the procedure and placed into refrigerator at 4 C. Col/disp. Media containing antibiotics and antifungals. Media was replaced on day 8. No visible colonies were seen. Review of culture on day 14 reviewed no active culture. Therefore, sample discarded.	No culture	no data	P0	no data

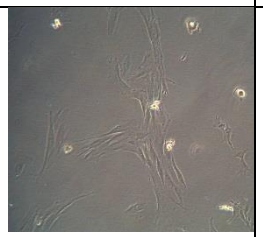
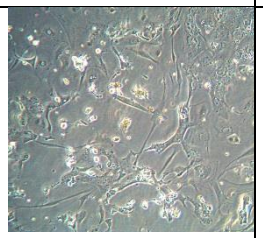
30	76	Serous	1a	Y		Direct solid tumour biopsy taken at end of procedure and transferred immediately for processing. Col/disp with cell filtering step added. Media changed on day 5 and culture observed to form colonies. Culture progressed with rapid cell growth in cobblestone monolayer fashion. Reached 70-80% confluence on day 8. HRR seeded, again, small number cell yield. Culture seemed to grow more slowly after P1	cobblestone	HRC	P2 infected	Seeded
31	68	Serous	1a	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70% confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded. Cell count was very good ? contaminated	cobblestone	Failed HRR	P1	Seeded

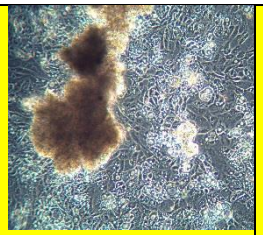
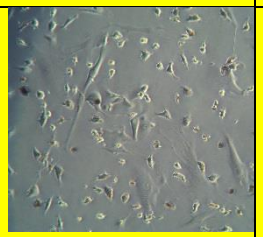
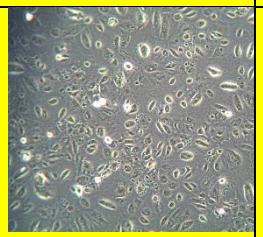
32	58	Adenosarcoma	1a	infected	No image	Solid directed tumour biopsy taken and transferred immediately. Col/disp with cell filtering and media containing antibiotics. Culture cloudy at day 5 media change. Cloudy media on day 6. Sample contaminated and discarded.	no data	no data	P0	no data
33	84	Serous and G2 endo	2	no growth	No image	Directed solid tumour biopsy taken from at the end of the procedure. Small biopsy sample as representative tumour small in specimen. Col/disp, cell filter performed. Media replenished day 7, no visible colonies, no growth on day 10 and 14, sample discarded.	no data	no data	P0	no data
34	83	Serous	4b	No growth		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70% confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension	Cobblestone	Infected post passage	P1	no data

						into T25 flask. Infected samples 2 days later, specimens discarded.				
36	85	Serous	4b	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible Fibroblastic culture. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Cells begin to swell on day 9. Media changed again on day 10, 70% confluence reached on day 13, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded.	fibroblastic	Failed HRR	P1	no data
37	77	Serous and clear cell	1a	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing. Media changed again on day 10, 70% confluence reached on day 14, culture passaged and HRR assay and SRB seeded,	cobblestone	HRC	P3	23.4

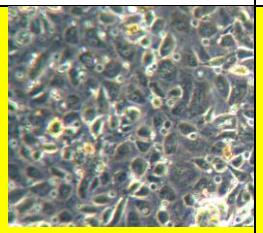
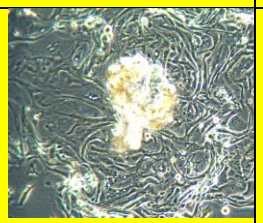
						remaining of cell suspension into T25 flask. Survived to P3, fibroblastic overgrowth at this point (see image).				
38	66	G3 endo	3C1	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears fast growing with a lot of visible cell debris even after washing with PBS and replacing media. 70% confluence reached on day 10, culture passaged and HRR assay and SRB seeded, remaining cell suspension into T25 flask. HRR effective, SRB failed as all wells confluent. Small cell yield at each passage. Survived to P4 before cells detached.	cobblestone	HRD	P4	Failed
39	78	Carcinosarcoma ovary	3C	infected	no image	Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer Cloudy offensive culture day 8, sample therefore discarded.	no data	no data	P0	no data

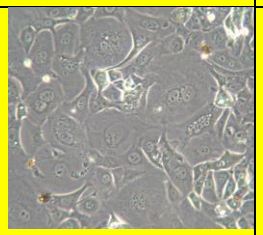
40	72	Serous and clear cell	2	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears fast growing. Media replaced day 5. 70% confluence reached at day 8. Samples passaged and HRR seeded. Fibroblastic overgrowth after P1.	cobblestone	HRC	P1	
41	69	Carcinosarcoma	4b	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70% confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded. Cell count was very good ? contaminated	cobblestone	Failed HRR	P1	no data

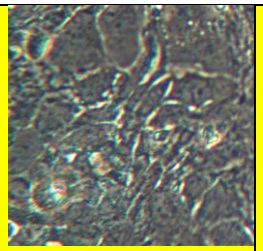
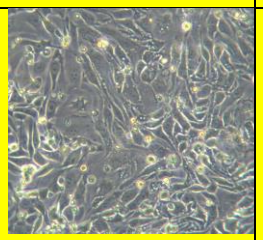
42	68	Clear cell	4b	No growth	no image	Washings taken from a patient with overt stage 4 disease. Processed as above. Media replenished day 7, no colonies at day 7, 10 and 14. Sample discarded.	no data	no data	P0	no data
43	70	Clear cell and endo	1a	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible fibroblastic culture seen appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, Cells detached day 14 ? rapid growth and cells outcompeting eachother.	fibroblastic	No growth	P0	no data
45	63	G2 endo	4b	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible fibroblastic culture appears slow growing. Media replaced day 5. Cells 80% confluent day 10 and passaged. Culture failed to grow after passage.	Fibroblastic	No growth	P1	no data

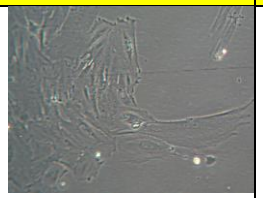
46	59	G2 endo	1a	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing media changed on day 7. 70% confluence reached on day 11, culture passaged and HRR assay. Culture continued until P3. Cells did not culture past P3.	cobblestone	HRC	P3	28.96
47	80	Carcinosarcoma	3C2	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible colonies seen at day 7, culture slow to grow initially. Culture survived to P4 but small cell yield at each passage. SRB cultured failed to grow.	fibroblastic	HRC	P4	Seeded by not successful
48	74	Clear cell and endo	1a	Y		Solid tumour biopsy taken. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer, cobblestone in appearance, cells appear quite spread out. Culture rapidly growing, reached	cobblestone	HRC	P4	Seeded but unsuccessful

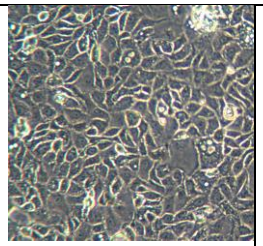
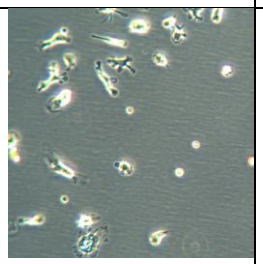
						70% confluence on day 7. Culture continued to P4 and did not continue to culture.				
49	PT282	Serous	1a	No growth	No image	Solid tumour biopsy taken. Delay in transfer until following day but refrigerated overnight at 4 C. Col/disp, cell filter, routine protocol. Media changed day 6, no visable colonies. Media replaced day 14. No colonies seen sample discarded	NA			no data
50	62	Mixed serous and endo	1b	No growth	No image	Solid tumour biopsy taken. Delay in transfer until following day but refrigerated overnight at 4 C. Col/disp, cell filter, routine protocol. Media changed day 6, no visible colonies. Media replaced day 14. No colonies seen sample discarded	NA			no data
51	PT280			No growth – delayed transfer	No image	Solid tumour biopsy taken. Delay in transfer until following day but refrigerated overnight at 4 C. Col/disp, cell filter, routine protocol. Media changed day 6, no visible colonies. Media replaced day 14. No colonies seen sample discarded	NA			no data
52	67	Recurrent grade 2 endometrioid	NA	No growth –	No image	Solid tumour biopsy taken. Delay in transfer until following day but refrigerated overnight at 4 C. Col/disp, cell filter, routine protocol. Media	NA			no data

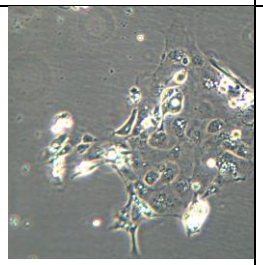
				delayed transfer		changed day 6, no visible colonies. Media replaced day 14. No colonies seen sample discarded				
53	65 xxx	Carcinosarcoma	4b	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70% confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded. Cell count was very good ? contaminated	Cobblestone	HRC	P3	1.62
54	70	G2 endo	3C1	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of		HRC	P3	21.96

						visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70% confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded. Cell count was very good ? contaminated				
55	77	Serous	3C1	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70% confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded. Cell count was very good ? contaminated	Cobblestone	HRD	P2	2.03

56	78	Serous	1b	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70% confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded. Cell count was very good ? contaminated	Cobblestone	HRC	P3	60.20
57	77	Mixed serous and endometrioid	2	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70%	Cobblestone	HRC	P1	No SRB

						confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded. Cell count was very good ? contaminated				
58	63	Serous	1a	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70% confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded. Cell count was very good ? contaminated	Fibroblastic	Failed culture	P0	no data

59	74	G2 endo	1a	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70% confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded. Cell count was very good ? contaminated	Cobblestone	Failed culture	P0	no data
60	72	G1 endo	1a	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70%	Failed attachment	Failed culture	P0	no data

						confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded. Cell count was very good ? contaminated				
61	52	High grade NOS	1a	Y		Directed solid tumour biopsy at end of procedure. Sample transferred immediately. Col/disp/cell filter and media containing antibiotics. Media replenished day 5. Visible epithelial monolayer extending out from clumps of endometrial tumour. Culture appears slow growing with a lot of visible cell debris even after washing with PBS and replacing media. Media changed again on day 10, 70% confluence reached on day 15, culture passaged and HRR assay and SRB seeded, remaining of cell suspension into T25 flask. Failed growth in all. Flasks discarded. Cell count was very good ? contaminated	Cobblestone	Infected culture bacilli seen	P0	no data

Appendix B

Block Number	Age at diagnosis	HRR status	Stage	Histology	LVSI	Cervix involved	myoinv	serosa	Nodes	Type 1/2	Death	Length FU (months) calculated	IHC	P53	ER	PR	MLH1	MSH2	MSH6	PMS2	PTEN
1	69		IA	Mixed serous and G3 EEC	0	0	0		0	2	1	32		Mutated	MOD	WEAK	PRO		PRO	PRO	PRO
2	79		IIIA	G2 EEC	1	0	1	0	0	1	1	22									
3	60		IA	Carcinosarcoma	0	0	0	5	0	2	0	27			NEG	NEG	PRO		PRO	PRO	PRO
4	66		IA	Serous	0	0	0	11	0	2	0	32		Mutated	MOD	WEAK	PRO	PRO	PRO	PRO	PRO
5	70	HRC	IIIC1	G2 EEC	1	0	1	4	1	1	0	31		wt	STRONG	WEAK	PRO	PRO	PRO	PRO	PRO
6	69		IVb	Carcinosarcoma	1	1	1			2	1	32						PRO			
7	62		IA	Serous	1	0	0	6	0	2	0	35		Null	MOD	MOD	PRO	PRO	PRO	PRO	PRO
8	77	HRD	IIIC1	Serous	1	0	0	10	1	2	0	34		Mutated	NEG	NEG	PRO	PRO	PRO	PRO	PRO
9	70	HRC	IA	Mixed clear and G2 EEC	0	0	0	10	0	2	0	38						PRO	PRO		
10	59	HRC	IA	G2 EEC	0	0	0		0	1	0	36		Null	STRONG	STRONG	PRO	PRO	PRO	PRO	PRO
11	73		IB	Carcinosarcoma	1	0	1	3	0	2	0	72		wt	WEAK	WEAK	PRO	PRO	PRO	PRO	PRO
12	59		IIIC2	Mixed serous and G2 EEC	1	0	0	13	1	2	0	36			STRONG		PRO	PRO	PRO	DEF	DEF
13	63	HRC	IVb	G2 EEC	1	1	1	0	0	1	0	35		wt	STRONG	STRONG	PRO	PRO	PRO	PRO	PRO
14	66		IVA	Clear	1				0	2	1	8									
15	78	HRC	IB	Serous	0	0	1	2.2	0	2	0	34									
16	50	HRC	IA	Clear	0	0	0		0	2	0	56							PRO		
17	72	HRC	II	Mixed serous and G2 EEC	0	1	1	2	0	2	0	39		Null	STRONG	MOD	PRO	PRO	PRO	PRO	PRO
18	73		IA	Carcinosarcoma		0	0		0	2	0	77									
19	60		IA	G1 EEC	0	0	0	7	0	1	0	131		wt	STRONG	STRONG	PRO	PRO	PRO	PRO	PRO
20	62		IB	G3 EEC	1	0	1	2	0	2	0	39		Null	STRONG	STRONG	PRO	PRO	PRO	PRO	PRO
21	70		IB	G3 EEC	1	0	1	0.7	0	2	0	49		wt	MOD	NEG	PRO	PRO	PRO	PRO	PRO
22	85		IVb	Serous	1	0	1	0	0	2	1	28		Mutated	MOD	WEAK	PRO	PRO	PRO	PRO	PRO
23	68		IIIC2	Serous	1	0	1	1	1	2	1	40		Mutated	WEAK	MOD	PRO	PRO	PRO	PRO	PRO
24	67		IA	Clear	0	0	0	5	0	2	0	43		wt	MOD	WEAK	PRO	PRO	PRO	PRO	PRO
25	65	HRC	IVb	Carcinosarcoma	1	0	1	0		2	1	4		Null	STRONG	NEG	PRO	PRO	PRO	PRO	PRO
26	76		IB	G1 EEC	0	0	1	2.5		1	0	43		wt	STRONG	STRONG	PRO	PRO	PRO	PRO	PRO
27	118	HRC	IB	G3 EEC		0	1			2	0	11		wt	NEG	NEG	PRO	PRO	PRO	PRO	PRO
28	67	HRC	IA	Serous	0	0	0		0	2	0	4			NEG		PRO		PRO		
29	78	HRC	IA	G2 EEC	0	0	1	5	0	1	0	47		Null	STRONG	STRONG	DEF	PRO	PRO	DEF	DEF
30	84		II	Mixed serous and G3 EEC	1	1	1	2.5	0	2	1	12			NEG		PRO	PRO	PRO		
31	56		IIIC2	Serous	1	1	1	0	1	2	0	47									
32	54	HRD	IVb	Serous	1	0	0		0	2	0	52		wt	STRONG	STRONG	PRO	PRO	PRO	PRO	PRO
33	53		IA	G3 EEC	0	0	0	15	0	2	0	64		Null	MOD	MOD	PRO	PRO	PRO	PRO	PRO
34	59		IVb	Carcinosarcoma	1	1	1	0	1	2	1	5		Null	NEG	NEG	PRO	PRO	PRO	PRO	PRO
35	82	HRC	IA	Serous	0	0	0	13	0	2	0	60		Mutated	MOD	NEG	PRO	PRO	PRO	PRO	PRO
36	68		IB	G3 EEC	0	0	1	2	0	2	0	38		Mutated	WEAK	STRONG	PRO	PRO	PRO	PRO	PRO
37	68		IB	G1 EEC	1	0	1	3		1	0	38									
38	61		II	G1 EEC	1	1	0	6		1	0	36									
39	66	HRD	IIIC1	G3 EEC	1	1	1	0.9	1	2	0	42									
40	75		IA	Serous	0	0	0	10	0	2	0	50		Null	NEG	NEG	PRO	PRO	PRO	PRO	PRO
41	53		IIIC2	G2 EEC	1	1	1	8	1	1	0	54		wt	STRONG	STRONG	PRO	PRO	PRO	PRO	PRO
42	75		IA	Clear	0	0	0	8	0	2	0	39		wt	NEG	NEG	PRO	PRO	PRO	PRO	PRO
43	80	HRC	IIIC2	Carcinosarcoma	1	0	1	0	1	2	0	36		wt	NEG	WEAK	PRO	PRO	PRO	PRO	PRO
44	77	HRC	IA	Serous	0	0	0	9	0	2	1	25		Mutated		NEG	PRO	PRO	PRO	PRO	PRO
45	68	HRC	IA	Serous	0	0	0	7	0	2	0	39			NEG		PRO	PRO			
46	70		IA	G3 EEC	0	0	0	6	0	2	0	57		wt	STRONG	STRONG	DEF	PRO	PRO	DEF	DEF
47	78		IIIC1	Serous	1	1	1	1	1	2	0	42		Mutated	STRONG	WEAK	PRO	PRO	PRO	PRO	PRO
48	68		IIIC1	Serous	1	0	1	0.5	1	2	1	26		Mutated	STRONG	NEG	PRO	PRO	PRO	PRO	PRO
49	0	DOM	OVARY	Serous							0	0		Mutated	STRONG	NEG	PRO	PRO	PRO	PRO	PRO
50	56		IA	Mixed serous and G2 EEC	1	0	0	9	0	2	0	41		wt	MOD	STRONG	PRO	PRO	PRO	PRO	PRO
51	80		IVb	Serous	1	1	1	0		2	1	22		Mutated	MOD	MOD	PRO	PRO	PRO	PRO	PRO

Appendix C

Presented abstracts

PARP 2019 conference: Budapest, Hungary. Poster presentation

Defective homologous recombination DNA repair in endometrial cancer confers sensitivity to Niraparib

D Blake, L Gentles, H Smith, S Johnson, S Rundle, M Adishesh, A Kucukmetin, Y Drew, RL O'Donnell and NJ Curtin

The incidence of endometrial cancer (EC) is rising. Although surgery forms the cornerstone of management it is only curative in the majority of type 1 cancers. In type 2 cancers the response rate to current chemo- and radiotherapy is <50% and there is an urgent need for better second line agents. The role of PARP inhibitors (PARPi) is established in ovarian cancers with defective homologous recombination DNA repair (HRR). A proportion of ECs display high copy number alterations, similar to ovarian cancer. We thus hypothesise that a subset of ECs are HRR defective (HRD) and sensitive to PARPi.

1) Establish incidence of HRD in EC cell lines and ex vivo patient primary cultures.
2) Explore the cytotoxic and growth inhibition effect of Niraparib (PARPi) and cisplatin as a single agents or as chemo- and radio-sensitisers.

The cytotoxicity and growth inhibition of Niraparib, cisplatin and irradiation (IR), alone and in combination, was assessed in EC cell lines (Ishikawa, HEC-1A, AN3CA, RL95-2) by colony formation and 96-well plate SRB assay. The HRR function of the cells was determined by quantification of RAD51 foci after 24-hour PARPi exposure.

Patient EC solid tumour samples (REC approval: 12/NE0395) were mechanically dissociated and cultured. Primary cultures were characterised for HRR function and related to SRB growth inhibition studies.

EC cell lines displayed a 10-fold variation in their sensitivity to cisplatin: LC50 0.22 µM (AN3CA) to 2.15 µM (HEC1A) but only a 2 to 3-fold sensitivity to Niraparib 2.08 µM (RL95-2) to 6.87 µM (HEC1A). AN3CA were the most sensitive cell line to IR (LD50 1.25 Gy) and HEC1A

were the most radio-resistant (LD50 2.20 Gy). Niraparib (1 μM) caused a modest radio-sensitisation all EC cell lines, except HEC1A. Potentiation factor (PF) at 2 Gy in AN3CA was 2.1, PF90 in RL95-2 and Ishikawa was 1.66 and 1.71 respectively. All EC cell lines were HRR competent and the EC lines most sensitive to Niraparib were type 2 endometrial cancers.
Ex vivo patient samples formed monolayer epithelial cultures with a 48% success rate. To date 2/6 cultures were HRD and were more sensitive to Niraparib with a GI50 of 1.73 μM in one HRD culture compared to a mean GI50 of 9.38 μM other HRC cultures.
The 4 EC cell lines were similarly sensitive to Niraparib despite displaying more variation in their cisplatin and IR sensitivity suggesting a potential broader role for Niraparib.
It is feasible to generate ex vivo primary EC cultures to determine HRR function. HRD is associated with increased sensitivity to PARPi in translational studies. The detection of HRD in patient cultures suggests that there may be a role for PARPi as mono-therapy or as radio-sensitiser in at least a subset of patients.

British gynaecological oncology society (BGCS) conference 2019, Cambridge, UK.

Homologous recombination DNA repair deficiency in endometrial cancer confers sensitivity to PARP inhibitor Niraparib

D Blake, L Gentles, H Smith, S Johnson, S Rundle, M Adishesh, A Kucukmetin, Y Drew, RL O'Donnell and NJ Curtin

Beyond platinum there is a need for better systemic therapies in endometrial cancers (EC). The role of PARP inhibitors (PARPi) is established in ovarian cancers with defective homologous recombination repair (HRR). A proportion of ECs display high copy number alterations and we thus hypothesised that some ECs are HRR defective (HRD) and sensitive to PARPi.
Cytotoxicity (colony formation) and growth inhibition (SRB) following Niraparib, cisplatin and irradiation, alone and in combination, was assessed in cell lines. Fresh patient tumour samples (12/NE0395) were mechanically dissociated and cultured. Primary cultures and cell lines were characterised for HRR function by quantification of RAD51 foci.
EC cell lines displayed a 10-fold variation in sensitivity to cisplatin: LC50 0.22μM to 2.15μM but only a 3-fold sensitivity to Niraparib: 2.08μM to 6.87μM, and a 2-fold sensitivity to

irradiation: LD50 1.25Gy to 2.20Gy. Niraparib caused a modest radio-sensitisation in 3/4 cell lines with a potentiation factor at 2 Gy of 2.1. All EC cell lines were HRR competent.
Ex vivo patient samples formed monolayer epithelial cultures with a 48% success rate. 2/10 cultures were HRD with greater sensitive to Niraparib: GI50 of 1.73µM compared to 9.38µM in HRC cultures.
EC cell lines were similarly sensitive to Niraparib despite displaying greater variation in cisplatin and IR sensitivity. It is feasible to generate primary EC cultures to determine HRR function. HRD is associated with greater PARPi sensitivity in translational studies.
Detection of HRD in patient cultures suggests that there may be a role for PARPi as both a radio-sensitiser and mono-therapy in this patient group.

Homologous recombination DNA repair deficiency in endometrial cancer confers sensitivity to the PARP inhibitor Niraparib

D Blake, L Gentles, H Smith, S Johnson, S Rundle, M Adishesh, A Kucukmetin, Y Drew, RL O'Donnell and NJ Curtin
Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, UK
Northern Gynaecological Oncology Centre, Gateshead, UK

Introduction

- Endometrial cancer (EC) is the 15th most common cancer globally and is rising in line with obesity. In the UK the incidence is 9000/year and mortality 2000/year [1].
- EC treatment includes surgery, radiotherapy and platinum-based chemotherapy.
- PARP inhibitors (PARPi) are known radiosensitisers, and in some circumstances also seen to sensitise to platinum therapy [2].
- Defective homologous recombination DNA repair (HRD) is predictive of sensitivity to PARPi and platinum therapy [3,4].
- The incidence of HRD in EC is thought to be 15-10% [5].

Aims

- Determine the sensitivity of a panel of EC cell lines to cisplatin, ionising radiation (IR) and Niraparib (PARPi) alone and in combination
- Establish primary cultures from patient EC material and test for HRD and drug sensitivity

Results

- All EC cell lines were HRC with >2 fold increase in RAD51 following induction of DNA DSB, Figure 1.
- EC cell lines varied 10-fold in their sensitivity to cisplatin: LC50 0.22 µM (AN3CA) to 2.15 µM (HEC1A) but only a 2 to 3-fold sensitivity to Niraparib 2.08 µM (RL95-2) to 6.87 µM (HEC1A), Figure 2.
- Niraparib caused a modest radio-sensitisation all cell lines, except HEC1A. Potentiation factor (PF) at 2 Gy in AN3CA was 2.1, PF90 in RL95-2 and Ishikawa was 1.66 and 1.71 respectively.

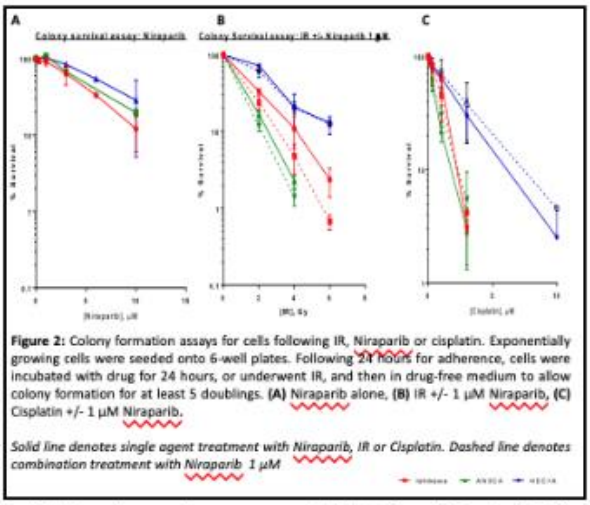


Figure 2: Colony formation assays for cells following IR, Niraparib or cisplatin. Exponentially growing cells were seeded onto 6-well plates. Following 24 hours for adherence, cells were incubated with drug for 24 hours, or underwent IR, and then in drug-free medium to allow colony formation for at least 5 doublings. **(A)** Niraparib alone, **(B)** IR +/- 1 µM Niraparib, **(C)** Cisplatin +/- 1 µM Niraparib.

Solid line denotes single agent treatment with Niraparib, IR or Cisplatin. Dashed line denotes combination treatment with Niraparib 1 µM

- Patient primary cultures were established from (18 out of 33) biopsies, forming cobblestone monolayers, Figure 3.
- Cultures reached senescence after a median of 2 passages, with culture life span ranging from 1 – 24 days.
- 2/9 cultures characterised for HRR function were HRD, Figure 4.

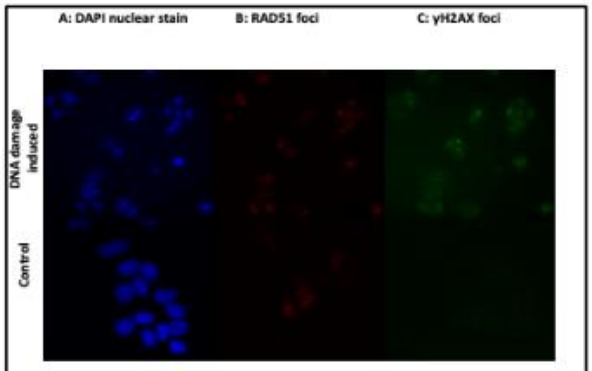


Figure1: Determination of HRR function: Cells were exposed to PARPi for 24 h to induce collapsed replication forks (γH2AX foci) **(C)** and HRR (RAD51 foci) **(B)**. A >2x induction of γH2AX but <2x induction of RAD51 indicates HRD.

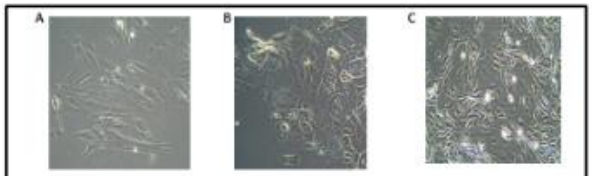


Figure 3: Ex vivo primary cultures, Patients undergoing surgery for EC were consented for intra-operative collection of endometrial tissue (REC 12/NE0395). Solid biopsies were mechanically dissociated into 1 mm pieces and incubated with collagenase (3.2mg/ml) /hyuronidase (62.5mg/ml) for 1-2 hours. An epithelial-rich cell suspension was isolated using a 40 µm filter before selective fibroblast seeding. Remaining cells were maintained in exponential growth. Bright field microscopy images of monolayer cobblestone cultures were captured using VisCam® software. **(A)** PT161, **(B)** PT218, **(C)** PT189

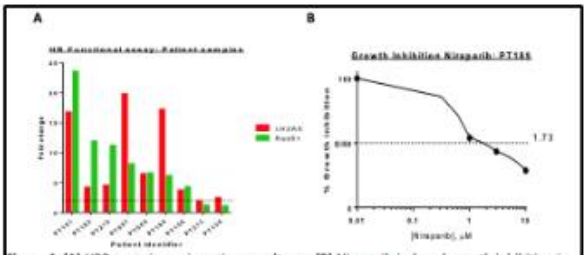


Figure 4: (A) HRR assay in ex vivo primary cultures. **(B)** Niraparib-induced growth inhibition in ex vivo primary cultures (HRD sample).

Conclusions

- The 3 EC cell lines all had functional HRR but varied in their sensitivity to cisplatin and IR
- They were similar in their sensitivity to Niraparib suggesting a potential therapeutic role for Niraparib as a single agent in EC.
- Niraparib increased the cytotoxicity of IR in 2/3 cell lines and cisplatin in 1/3 cell lines
- Primary cultures of EC patient material can be tested ex vivo for HRD status and the HRD cultures were more sensitive to Niraparib
- There may be a role for PARPi as mono-therapy or as radio-sensitiser in at least a subset of patients.

Acknowledgements and references

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