# Combatting Chemoresistance: An Analysis of DNA Repair Proteins ERCC1 and XPF and their Chemotherapeutic Implications in Ovarian Cancer

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## Certificate of Originality and Compliance

### CERTIFICATE OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this thesis, that the original work is my own, except as specified in the acknowledgements and in references, and that neither the thesis nor the original work contained therein has been previously submitted to any institution for a degree.

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**Abstract**

Platinum-based drugs form a key aspect of multimodal treatment to various cancers. Despite this, resistance to platinum therapy is a colossal threat encircling the treatment of ovarian cancers. It is well documented that an upregulation in DNA repair pathways is an advantageous characteristic for cancer cells and results in the emergence of chemoresistance. Nucleotide excision repair (NER) is essential in the DNA damage response (DDR) and removes bulky DNA adducts caused primarily by UV light. This pathway utilises Xeroderma Pigmentosum F (XPF) and Excision Repair Cross-Complementation Group 1 (ERCC1) endonucleases in the final cleavage of the 5' DNA lesion. An intact nucleotide excision repair (NER) pathway is required for the correction of platinum-induced crosslinks; cells lacking functional NER show exquisite sensitivity to platinum agents.

A targeted study of both ERCC1 and XPF was carried out to determine their significance in association with chemoresistance and synthetic lethality in ovarian cancer. Preclinical analysis by the siRNA depletion of ERCC1 and XPF were tested against platinum sensitivity and inhibition by PARPi in a panel of platinum sensitive and platinum resistant ovarian cancer cell lines (A2780 and A2780cis). Furthermore, the clinical significance and clinicopathological correlations of ERCC1 and XPF was established in a cohort of 334 ovarian tumours.

Functional analysis revealed that ERCC1 knockdown increased platinum sensitivity activating apoptosis, inducing S phase arrest and resulting in higher double strand breaks (DSBs). PARP inhibition of ERCC1 and XPF depleted A2780cis cells show increased levels of DSBs, and a G2/M arrest. Clinicopathological analysis revealed increased ERCC1 nuclear expression was linked with poor progression free survival ($p=0.048$), higher histological grade ($p=0.047$) and higher histological stage ($p=0.049$). No significance data was seen between XPF and prognostic factors upon clinicopathological analysis. ERCC1 was shown to be an independently associated prognostic indicator for ovarian cancer, with high ERCC1 expression significantly correlating to poor progression free survival and overall survival.

Data from this study allows us to conclude that ERCC1 is a key predictor of platinum resistance in a large clinical cohort of ovarian cancers. Pre-clinically, ERCC1 or XPF depletion not only increased cisplatin sensitivity but also increased cytotoxicity to Olaparib (PARP1 inhibitor) therapy. Together, the data suggests that ERCC1 deficiency is not only a predictor of platinum sensitivity but may also be utilized to personalise Olaparib therapy in ovarian cancer patients.

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**Abbreviations**

XPF – *xeroderma Pigmentosum* F
XPG - *xeroderma Pigmentosum G*
ERCC1 - excision repair cross-complementation group 1,
DSB – double strand breaks
PFS – progression free survival
OS – overall survival
RPA – replication protein A
PARP - poly ADP ribose polymerase
AGCT - Adult-type granulosa cell tumour
SCCOHT – small cell carcinoma of the ovary hypercalcaemic type
ROS – reactive oxygen species
CPD – Cyclobutane pyrimidine dimers
IR – ionising radiation
NHEJ – non-homologous end joining
BER – base excision repair
DDR – DNA damage response
HR – homologous recombination
NER – nucleotide excision repair
MMR – mis-match repair
ICLS – interstrand crosslinks
TC NER – transcription coupled nucleotide excision repair
GG NER – global genome nucleotide excision repair
XPC - *xeroderma Pigmentosum C*
TFIIH - transcription Factor II H
CSA – cockayne syndrome A
CSB – cockayne syndrome B
CEN2 – centrin 2

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# 1 Chapter 1: Introduction

## 1.1 Ovarian Cancer

The ovary is a specialised female organ that functions predominantly in menstrual regulation and hormone balance, fertilisation and bone remodelling. In women that are premenopausal, the ovaries serve as the main source of progesterone and oestrogen and in the maintenance of reproductive health. Ovarian cancer is deemed one of the most prevalent malignancies in adult women, accounting for around 2.5% of cancer diagnoses but 5% of all cancer deaths, relative to its poor survival rates (Furuya, 2012). This is a direct result of the generally late staging at diagnosis whereby the cancer has already metastasised into the abdominal cavity. In 2018 there is expected to be 22,240 diagnoses of ovarian cancer and 14,070 ovarian cancer caused deaths across the United States. The median age of ovarian cancer diagnosis is 63 years of age; however, the age distribution differs per tumour subtype and partially through ethnicity (Torre *et al*., 2018). The recognised average life time risk for ovarian cancer is 1.3% with the highest risk being amongst older women and non-Hispanic Caucasian women, however compared to other cancers, the racial variation is significantly lower. Reproductive factors such as the use of contraceptives – particularly long-term use and the number of children (births) acts as a large risk factors in ovarian cancer. Unfortunately, due to the low survival rate, the mortality trends in ovarian cancer tend to mimic incidence (Hedemann *et al*., 2018).

When compared with malignancies of other organs, the composition of ovarian tumours presents with more heterogenous histologic types amongst each different subtype, particularly epithelial cell tumours. The monolayer of epithelial cells covering the surface of the ovary are bombarded with different sources of mechanical stresses predominantly caused by ovulation (Mackenzie *et al*., 2015). Regardless of the histological subtype, the morphology of these cancers presents as multi-cystic

however the pathogenesis between them differs greatly, partly a cause of genetic backgrounds and pre-cancerous conditions.

Risk factors for developing ovarian cancers include familial history, genetic predispositions, child birth/pregnancy, hormonal/surgical birth control, diet/weight, height and fertility drugs. Familial history plays a large role in the likelihood of cancer diagnosis, whereby individuals who have a first degree relative who has suffered from the disease have a two-fold increase in predisposition than those with second degree relatives that have received a cancer diagnosis. Similarly, the likelihood of developing ovarian cancer is around 70% for those who have a first degree relative that has had a breast cancer diagnosis. Almost 40% of patients with a history of ovarian cancer in the family are due to mutations in the BRCA1 and BRCA2 cancer susceptibility genes (Minion *et al*., 2015). As a consequence, genetic predisposition forms a huge risk factor, as around 20% of all ovarian cancer diagnoses are suggested to be a result of mutations in the BRCA1 and BRCA2 genes that confer a significantly increased risk – particularly with developing high grade tumours. Amongst the general public with BRAC1 and BRCA2 mutations (1%) the estimated likelihood of them developing ovarian cancer by the age of 80 years is 44% and 17% respectively. The lifetime risk of an individual carrying BRCA1 and BRCA2 mutations of developing ovarian cancer is 45–60% and 11–35% respectively (Paul., 2014).

Medical abnormalities such as the rare hereditary condition, hereditary nonpolyposis colorectal cancer (Lynch syndrome) is linked to a significant increase in risk for a number of cancers, namely colorectal and ovarian (Webb and Jordan., 2017). Women who have this condition are subjected to an 8% risk of developing ovarian cancer by 80. It is also noted that women who have suffered from breast cancer previously have a 30% increase in ovarian cancer likelihood, increasing to 5x more likely if breast cancer diagnosis is before the age of 40 years. Breast cancer patients

have a 29% risk of developing ovarian cancer pre-50 years and a 44% risk before age 70 (Mehrgou and Akouchekian 2016).

Ovarian cancer research has fully established that women carrying to full term pregnancy have a reduced cancer risk than those who have not carried full term. Further reduction of risk is linked to multiple pregnancies, with risk decreasing by 40% after the first birth and then a further 12% after each following birth (Webb and Jordan, 2017). Similarly, the use of oral contraceptives is noted to have a reductive effect on risk, with longer duration of prescriptions being of higher benefit. Women who take oral contraceptives for 5-9 years have a decreased risk of around 35%. Surgical methods of birth control such as fallopian tube ligation decrease risk by 30% with complete removal of the fallopian tubes having a reductive effect of 60%.

## 1.2 Molecular Subtypes of Ovarian Cancer

### *1.2.1* Epithelial Cancers

Around 90% of current ovarian diagnoses are found in the epithelium layer covering the ovaries; predominantly in post-menopausal women. Age standardised risk ranges from 7–17 per 100,000 population per year. Five-year survival is <45% and the proportion of women who die from their disease has not improved substantially over time (Webb and Jordan, 2017).

Research suggests that epithelial ovarian tumours may develop along two distinct molecular pathways which result in different clinical characteristics, treatments and prognosis. Tumours can be classified into type I and type II. Type I tumours are inclusions of the Mullerian system germ cell layers and epithelium giving rise to clear cell, endometrial, well differentiated serous and mucinous tumours. These tumour types present as more indolent, with tumorigenesis suggested to be due to the ‘multi hit hypothesis’ (Kirman and Shih, 2011).

Type II serous tumours present as the highest-grade carcinomas and involve both ovaries, they exhibit aggressive behaviour – a characteristic of their late diagnosis and reduced overall survival rate. Evidence suggests that the primary origin of these tumours may be the fallopian tubular epithelium, where microscopic preliminary lesions migrate to the ovaries. This gives rise to poorly differentiated tumour type (Wu *et al*., 2013). Often believed to be a consequence of p53 mutations, type II tumours are responsible for a large proportion of metastatic cancer and present a more aggressive clinical phenotype. Metastasis of epithelial ovarian cancer is usually through exfoliation of the primary organ to the peritoneal cavity or lymphatic spread. Due to this chief method of spread and often a distinct lack of symptoms in early stage, metastasis often results in malignancy of multiple organs (Koshiyama, Matsumura and Konishi, 2018).

The standard procedure for the optimal treatment of metastatic epithelial ovarian cancer often includes surgery to de-bulk any mass disease present. The uterus and pelvic peritoneum are often involved in this kind of surgical intervention, along with portions of affected intraperitoneal organs. This is usually shadowed with adjuvant chemotherapy, principally with a platinum compound i.e. cisplatin and a taxane. As previously stated, the peak incidence of ovarian cancers comes within the fifth or sixth decade of life, and as such the preservation of fertility is not a consideration, therefore complete removal of ovaries, fallopian tubes and uterus is deemed necessary as part of surgical procedures. Incidence in women 40 years and younger is only between 3 and 17% with prognosis following a similar treatment regime, however there is an emphasis placed on the balance between the preservation of fertility and curative measures in women of childbearing age (Joseph *et al*., 2014).

### *1.2.2* Germ Cell Carcinomas

Comprising of only around 5% of all ovarian tumours, germ cell carcinomas are ovarian neoplasms derived from the primitive egg forming germ cells of the

embryonic gonad. These cells may undergo germinomatous or embryonic differentiation. Diagnosis can be confirmed at any age but is particularly prevalent in women in their early twenties. This molecular subtype can be divided into seven further subtypes characterising different ovarian cancer types: teratomas, dysgerminomas and endodermal sinus tumour are the most common of the seven (Table 1) (Lee-Jones, 2013).

Table 1 Subtypes and characteristics of ovarian germ cell tumours (data derived from Rice, 1999 and John Hopkins Pathology, 2001) (Lee-Jones, 2013).

| **Subtype** | **Percentage Prevalence** | **Malignancy** |
|---|---|---|
| Dysgerminoma | 35-50% | Malignant |
| Endodermal Sinus Tumour | 20% | Malignant |
| Embryonal Carcinoma | Rare | Malignant |
| Polyembryoma | Rare | Malignant |
| Choriocarcinoma | Very Rare | Malignant |
| Teratoma | Immature count for 20% malignant GCT | Benign or Malignant |
| Mixed Germ Cell Tumour | 10-15% | Dependant on Cell Type |

Currently, no aetiological factors have been associated with increase prevalence of germ cell carcinomas, apart from a noted increase in incidence with dysgenetic gonads. Cytogenic abnormalities involving either the whole of or sections of the Y chromosome such as gonadal dysgenesis (45, X), mixed gonadal dysgenesis (46, XY) and testicular feminisation (46, XY) are apparent in around 5% of all patients diagnosed with dysgerminomas. Despite this, the remaining 95% of dysgerminoma patients have normal cytogenetics (Lee-Jones, 2013).

Germ cell tumours of the ovary chiefly affect young females; however, they have been observed in infants, accounting for 60% of ovarian cancers in this demographic. Of this 60% one third of the neoplasms are deemed malignant. Worldwide the frequencies of ovarian germ cell carcinomas are invariable, showing no racial predisposition. Ovarian germ cell cancer incidence shows an increase between 8-9 years with a peak at 18 years, with the mean age of diagnosis currently sitting at 19 years old. Clinical data shows that patients normally present early, at stage I with symptoms of adnexal torsion and abdominal pain, however some patients are asymptomatic through early stages of germ cell carcinomas. The developing tumour mass may result in acute pain in the local area and in potentially rupture or haemorrhage, intermittent vaginal bleeding may also occur. Teratomas diagnosis typically occurs in premenopausal women with a lack of any presenting symptoms, however a mature cystic teratoma can incur complications such as rupture and haemolytic anaemia (Lee-Jones 2013).

Despite their high levels of radio sensitivity, chemotherapy is the preferable treatment approach, particularly in young patients where there is an emphasis on preserving fertility. In the same way, surgery is conservative. If fertility is not of concern, then a total hysterectomy can be performed alongside a bilateral salpingo-oophorectomy in order to remove the highest amount of tumour mass possible. This is a particularly common option in stage II, III, IV dysgerminatous and non-dysgerminatous germ cell tumours (Kraggerud *et al.,* 2013). All patients with the exception of immature teratomas will receive will receive chemotherapy post-surgery. As a result of their significantly high recurrence rate, adjuvant chemotherapy is given to those patients with completely resected staged I, II or III endodermal sinus tumours, choriocarcinomas, immature teratomas and embryonal carcinomas. Combination cisplatin therapy with vinblastine and bleomycin (VBP) or with cisplatin and etoposide is the mainstay in chemotherapy treatment for these tumours (Mangili *et al*., 2016).

Dysgerminomas recur in around 15-20% of patients, however the survival rates for early presenting tumours are 95% and 80% for advanced tumours. Endodermal sinus tumours presenting at stage I and II have a survival rate of 60-100% whereas later stage disease (stage III or IV) is decreased to 50-75%.

### *1.2.3* Stromal Carcinomas

Stromal carcinomas of the ovary are a rare class of tumours that derive from the surrounding connective tissue that holds the ovary together. Of this molecular type the most common tumours to arise are granulose-theca tumours and Sertoli-Leydig cell tumours. These rare tumours are considered low grade, with 70% presenting at an early stage disease (Stage I) in these cases the malignancy is limited to the ovaries with no metastasis to lymphatic system or other organs (Horta and Cunha, 2015).

Adult granulose cell tumours (AGCT) are a class of sex cord stromal cell tumours that comprise 2-5% of all ovarian malignancies, and account for 70% of malignant stromal cell carcinomas. AGCTs present at early stage, tumours are typically indolent and have a good prognosis despite the fact that late recurrences can occur up to 37 years post the diagnosis. Typical symptomology includes abnormal bleeding (uterine), pain and pressure in the abdomen resulting from tumour mass and virilisation. AGCT tumours present with an oestrogen excess, expressing aromatase activity and thus promoting the synthesis of oestrogen (Mancari, Portuesi and Colombo, 2014).

The noted rarity of this tumour type means that the risk factors of the disease have not been well determined, however on the contrary to other ovarian tumours, fertility agents and oral contraceptives may increase risk in AGCTs but is yet to be proven. Genetic analysis of ATGCs has revealed that >95% of these tumours have a somatic

point mutation in the FOXL2 gene – missense 402C>G. FOXL2 is a transcription factor that codes for the maintenance of ovarian function and granulosa cell differentiation. This mutation results in a AGCT cells having a partial loss of apoptotic function due to a reduction in GnRH receptors, resulting in a cellular resistance to GnRH-induce apoptosis (Kommoss *et al*., 2013).

Treatment of early stage AGCTs follows surgery as the mainstay, with the aim of removing the tumour, leaving no gross residual disease. Surgeries suggested include: bilateral salpingo-oophorectomy and total abdominal hysterectomy, in the case of stage IA nodal dissection is not recommended as these tumours have low incidences of metastasis to the lymph nodes (Horta and Cunha, 2015). In terms of chemotherapy, the efficacy of adjuvant therapy is unclear and remains only an option when tumour is unresectable or is at an advanced stage. Studies have shown that when treat with neoadjuvant chemotherapy, patients experience recurrence two times earlier than patients who had immediate surgical resection. Several regimes may be used for the cytotoxic treatment of AGCTs including: cisplatin, doxorubicin, cyclophosphamide and taxes (Hodeib *et al*., 2017).

Late stage disease presents further issues with the end goal of cytoreduction aiming to achieve no residual disease. A multi-modal approach is utilised in this situation and where resection cannot happen a combination of radiation, hormonal and chemotherapies is used.

### *1.2.4* Small Cell Carcinoma of the Ovary

Small cell carcinoma of the ovary (SCCOHT) is a very rare (representing <1% of all cases), malignant ovarian tumour that is primarily present in young women, with the average diagnosis at 24years.  It is the most common undifferentiated carcinoma of

the ovary found in young women, and as such is essential to be included in differential diagnoses (Stewart *et al*., 2016).

The histology of these tumours is widely unknown as the tumour cells cannot be subtyped into germ cell, neuroendocrine or epithelial cells. SCCOHT is a highly undifferentiated malignancy that can be distinguished from other tumour types by characteristic early onset, brisk mitotic activity and the dominance of hyperchromatic cells presenting scant cytoplasm. Symptomology includes abdominal pain, cramping, nausea, irregular menstrual cycles and infertility and patients tend to present at early stage disease (Ghazi *et al*., 2017). Amongst these patients roughly two thirds have hypercalcaemia however, only a minority of these patients present associated symptoms i.e. hypertension, lumbar and abdominal pain, fatigue and polydipsia. The presence of hypercalcaemia helps distinguish this cell type from the neuroendocrine type which presents similar morphology (Callegaro-Filho *et al*., 2016)

Familial studies of small cell carcinoma of the ovary demonstrate autosomal dominant transmission, potentially suggesting that a single gene mutation may underlie these cases. Whole exome sequencing of individuals from families with presumed familial SCCOHT identified that SMARCA4 (the chromatin remodelling gene) was the only gene showing mutation in all of the families tested. In condition one analysis of the mother's tumour a somatic mutation was detected, and the tumour from the daughter showed a distinguished lack of heterozygosity. In the second family the mother and daughter carried an identical mutation. Following families showed missense mutations and base pair shifts. However fresh samples weren't available, so all analysis was carried out on formalin fixed paraffin embedded tissues (Stewart *et al*., 2016).

Overall, small cell carcinoma of the ovary has a poor prognosis with staging at time of diagnosis being the most important factor. Upwards of 50% of stage IA patients die within two years of initial diagnosis, patients with a tumour size of <10cm have an

improved prognosis compared to larger tumours (Kasack *et al*., 2016). Recurrence is found in 65% of patients after the primary treatment and any long-term survival is capped at 33% of patients. Currently there have been no reliable treatment options that significantly improve disease outcome, however a combined chemotherapy approach is thought to extend survival.

## 1.3 DNA Damage Repair in Ovarian Cancer

It is commonly known that a defective DNA damage response is a key player in many cancers, so it is no surprise that there are multiple studies defining malfunctioning DDR as an imperative hallmark of ovarian cancers. However, DNA damage repair is a multifaceted multi pathway process, utilising a vast number of specialist proteins, giving rise to a number of potential targets.

Data reveals that an alteration on one of the genes in the NER pathway is present in 8% of high grade serous ovarian cancers, with several of the individual NER proteins being implicated in predisposition to cancers, namely ERCC1. In vitro studies of ERCC1 in cell lines, revealed that its protein expression directly correlates to platinum resistance as a result of NER upregulation. Paradoxically, XPA, a protein involved with binding and stabilising damaged DNA is associated with improved chemotherapy response and overall survival in females who had metastatic ovarian cancers (Gee *et al*., 2018).

An extensive TCGA study revealed that NER gene dysfunctions in epithelial ovarian cancers (high grade) could be related to positive therapy out comes, and a high association with increased progression free survival and over all survival when looked at in comparison with matched tumours that did not have NER gene alterations. Most strikingly, the ERCC6-Q524 and ERCC4-A583T mutations held a functional association which potentiated in vitro sensitivity to platinum therapy (Ceccaldi *et al*., 2015).

Various reported polymorphic variants and alternations in the NER protein expression levels are implicated in breast and gynaecological cancers, particularly ovarian cancers with dysregulation of NER speculated to be responsible for the accumulation of mutations. Mapping of these polymorphisms has clearly identified variants of proteins that are significantly associated with ovarian cancers. When aberrantly expressed, NER proteins are linked to being a causal element in development of cancer (Shen *et al*., 2014).

## 1.4 DNA Damage Repair

Cellular DNA is relentlessly assaulted daily with thousands of exogenous and endogenous attacks on each of the body's cells. This continual bombarding of the DNA causes instability, eventually leading to DNA lesions and an inability for effective cellular replication. If not repaired effectively and efficiently the cell is left at risk of mutations and wide scale genome aberrations. Both of which seriously threaten the cells viability and are significantly linked to an increased disease bearing potential, particularly cancers (Ali et al., 2017). Because of this, each cell has evolved a group of multifaceted DNA repair mechanisms that work in co-ordination to preferentially repair lesions in the DNA. Such mechanisms must remain at an equilibrium, thus keeping a balance between genetic diversity in germ cells and reducing harmful mutations to a minimal level. Repair of damaged DNA is essential in multiple human processes. For example, biological processes and immune development rely on functional DNA replication mechanisms, and any lesions or mutations will result in fundamentally flawed pathways. Consequences of DNA repair defects or mutations in DNA repair genes have monumental impact on human health,

with their presence leading to premature aging and neurodegeneration, significantly increased cancer risk and immune-deficiency disorders.

### 1.4.1 Types of DNA Damage

#### 1.4.1.1 Endogenous DNA Damage

Generally speaking, a large majority of modifications or damage to DNA come in the form of endogenous attacks – intrinsic mechanisms which result in DNA damaging products. Spontaneous acid catalysed hydrolysis of the N-glycosidic bond between the deoxyribose DNA base is one of the simplest forms of endogenous damage. Hydrolytic base loss is estimated to happen around 10,000 times a day and I the main mechanism behind abasic site products. These abasic sites are known to be 'chemically liable' and are subject to β-elimination resulting in DNA scission (Dexheimer, 2013). Similarly, DNA base deamination lesions involving the formation of transformation of uracil from cytosine are frequent forms of intrinsic damaging agents. Less commonly, the respective spontaneous deamination of adenine and guanine to hypoxanthine and xanthine can occur.

Additionally, endogenous lesions by chemical modification are often an accidental by product of cellular respiration. Reactive oxygen species (ROS) such as O2-, H2O2 and •OH are produced by normal mitochondrial respiration and readily attack cellular DNA. They have the ability to generate >100 oxidative lesions such as DNA adducts caused by lesions in sugar bases, breaks in single strands and crosslinks between DNA and proteins (Rowe, Degtyareva and Doetsch, 2008).

DNA damage in the form of endogenous genomic malfunctions causes unavoidable errors in DNA genome wide. Normal physiological processes can leave DNA vulnerable to various methods of damage, such as DNA polymerases mis-incorporating bases, deletion of bases and insertions of bases. All of which have significant effect on the genome and have the ability to completely alter the

functionality of genes or completely prevent DNA replication all together. Errors in DNA may occur if specific check points are deactivated allowing damaged DNA to go unidentified, particularly if specific DNA enzymes are dysregulated. An overwhelming damage load can also prevent restorative capacity on DNA repair pathways, thereby hindering DNA replication and giving rise to further damage. It was initially though that exogenous agents were the primary source of major mutations; however, advances now demonstrate that endogenous damage holds significant impact of DNA viability (Tubbs and Nussenzweig, 2017).

#### 1.4.1.2 Exogenous DNA Damage

Exogenous DNA damaging agents are extrinsic and can come from various environmental sources. UV light from the sun is one of the most common physical agents of exogenous damage and it causes two distinct types of lesions (Tubbs and Nussenzweig, 2018). Cyclobutane pyrimidine dimers (CPD) are formed when coupling of the C=C double bond in pyrimidines cause a four-membered ring to develop, disrupting DNA base pairing. Secondly, 6-4 pyrimidone photoproducts are the less common of the UV bulky helix distorting lesions but are increasingly more mutagenic than CPDs. Both types of lesions induced by UV light are repaired primarily by nucleotide excision repair and are detected by UV-DDB (Osakabe *et al*., 2015).

Ionising radiation (IR) also plays a significant role in external sources of physical DNA damage. Derived from both nature and artificial sources, ionising radiation is able to induce a wide variety of damage onto DNA. The most significant of these is double strand breaks. Direct action of IR occurs when alpha particles, beta particles or x-rays produce ions that are able to physically break the bases or sugar phosphate backbones of the DNA (Vingared *et al.,* 2013). Various DRR signalling cascades are elicited by IR induced DNA damage, these cascades mediate both cell cycle arrest and DNA repair. The most lethal form of IR damage comes in the form of DSBs,

which are repaired by one of two pathways: nonhomologous end-joining (NHEJ) or homologous recombination (HR )(Santivasi and Xia, 2014).

Not only does cellular DNA have to contend with physical attacks from the environment, it also has to fend off a variant of chemical agents. Clinical drugs, particularly chemotherapeutic agents function in specifically targeting cellular DNA in the attempt to cause damage sufficient enough to induce apoptosis. Alkylating agents and platinum compounds are two of the most popular drug categories that utilise DNA damage to treat cancer. These agents induce inter and intra-strand crosslinks and bulky DNA adducts, whereas topoisomerase inhibitors are effective inducing DSBs. Various other well noted chemicals that damage DNA are cigarette smoke and exhaust emissions due to the presence or polycyclic aromatic hydrocarbons (Dexheimer, 2013). These compounds form bulky helix distorting adducts by binding to DNA bases. Studies also suggest that long term exposure to cigarette smoke increases the level of ROS present, therefore producing an endogenous attack as well (Chen *et al*., 2015).

### 1.4.2 DNA Repair Pathways

All eukaryotes possess the ability to detect and repair DNA damage, with each process preferentially repairing a particular type of DNA lesion. Lesions take the form of double strand breaks (DSBs), inter-strand crosslinks, intra-strand cross links, single strand breaks, bulky adducts, modified bases, abasic sites and base mismatches (figure 1).

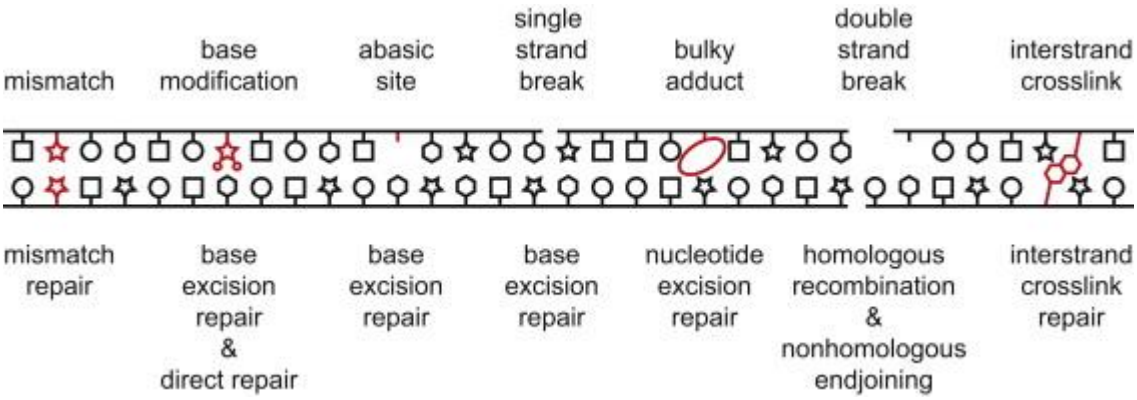



Figure 1 Schematic demonstrating types of DNA damage and the respective repair mechanisms (Burkhalter, Rudolph and Sperka, 2015)

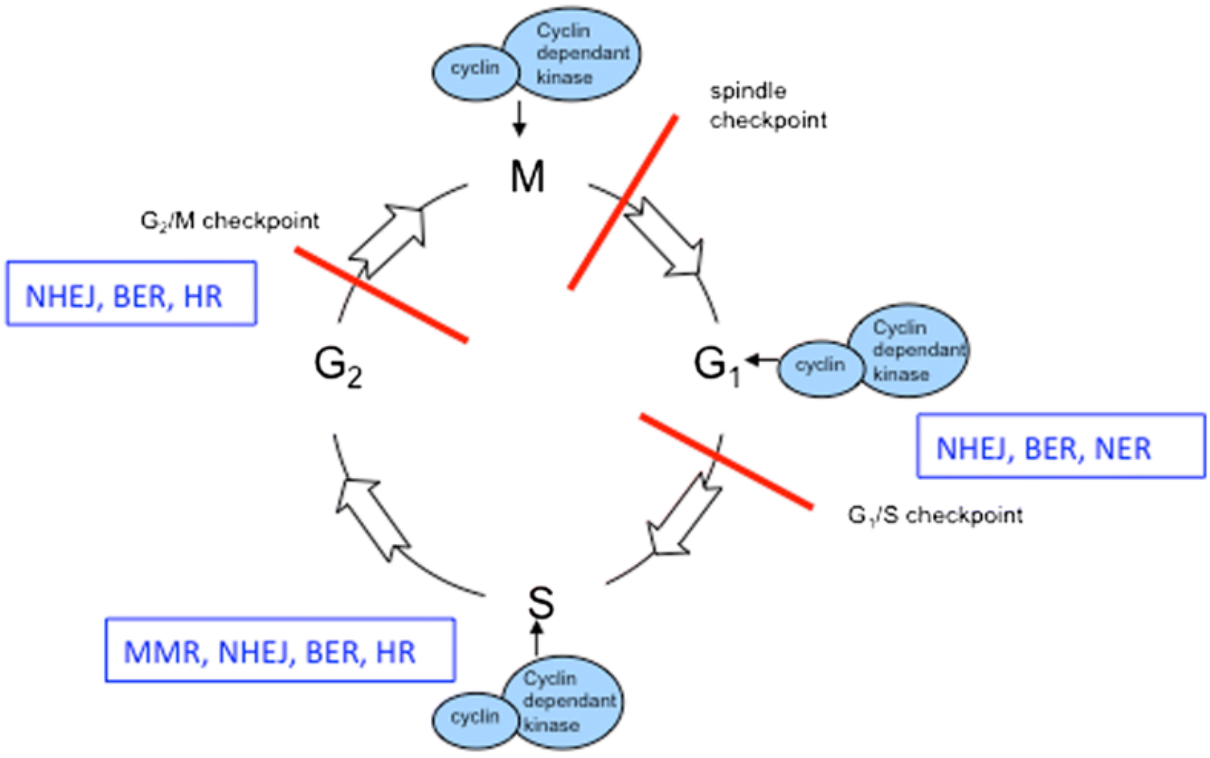



Figure 2 Schematic representing DNA repair mechanisms relevant to cell cycle check points (Gee *et al*., 2018)

These pathways include: base excision repair (BER) that functions specifically in the excision of damaged DNA bases, mainly arising from cellular metabolism events i.e. deamination, oxidative damage and alkylation. BER is a highly co-ordinated pathway with consecutive enzyme reaction, however there are several sub-pathways

associated with BER that are determined on the damage recognised. Around 12 structurally diverse glycosylases are associated with BER and they all act on small overlapping sections of the lesion. Initiated by lesion specific glycosylases, BER relies on the production of an abasic site via the removal of damaged bases by the removal of N-glycosidic bond that links the base to deoxyribose back bone (Burkhalter, Rudolph and Sperka, 2015). APE-1 then immediately creates a single strand break by hydrolysing the backbone 5' to the abasic site. Following this where a DNA polymerase fills the gap and a DNA ligase seals the nick. The synthesis/ligation step is then divided into two sub-pathways, short-patch and long patch BER. Which pathway is used is determined by whether a single or several nucleotides are incorporated at the DNA break (Wallace, 2014).

Miss-match repair (MMR) process is highly conserved from e. coli to humans and works to repair of base-pairing mismatches and/or insertion–deletion loops which are usually a result of cellular metabolism. These mismatches, if left unrepaired can be highly mutagenic and lead to carcinogenesis (Burkhalter, Rudolph and Sperka, 2015). Cells deficient in MMR display a mutator phenotype, characterised by microsatellite instability and an elevated mutation frequency and germline mutations in MMR genes are predisposed to a variety of cancers. The MMR pathway is carried out by two primary proteins, MutS (responsible for recognition) and MutL (responsible for coupling recognition complexes to MMR events, leading to error strand removal). Degradation of the error containing strand is dependent on the assembly of the ATP-dependant MutS-MutL-DNA complex, whereby degradation is performed by Exo1 with 5'-3' exonucleolytic activity. DNA polymerase δ, PCNA and RPA then fill the remaining gap and DNA ligase I seals the nick (Dexheimer, 2013).

Homologous recombination (HR) is most commonly utilised in the repair of double strand breaks (DSBs) and inter-strand crosslinks (ICLs) by method of exchanging nucleotide sequences between DNA molecules. It also provides significant support in

DNA replication, holding a fundamental role in recovery of stalled replication forks. Homologous recombination can be divided into three separate phases, the first being the pre-synapsis phase. During HR, DNA ends of the DSBs are process through 5'-3' resection, producing DNA molecules containing 3 single stranded tails, initiated by the MRN complex and CtIP. RPA then binds the ss-tails in the aid of removing any secondary structures that may prevent binding of further repair proteins. Rad51 then initiates the central reaction of HR (the search for DNA sequence homology). Once located, strand invasion of the DNA duplex is mediated by Rad51 and DNA polymerase η synthesises DNA from the 3' end of the invading strand. Ligation by DNA ligase I follows. The intermediate of the recombination reaction is either resolved by asymmetric cleavage or dissolution (Samadder *et al*., 2016).

Non-homologous end-joining (NHEJ) is similar in its role as it also repairs DSBs, however it is unique in that break ends are directly ligated without a homologous template, in contrast to homologous repair which requires a homologous template to guide the repair. Depending on the extent of the DNA DSB, modification may be warranted throughout the NJEH process, for example DNA overhangs may be made ligatable via resection. NHEJ is one of the most fundamental mechanisms for sustaining genome stability and first evolved in bacteria (Bartlett *et al*., 2016). NHEJ is mediated by the sequential assembly of factors recruited at DSB sites. Binding and recognition of Ku70-Ku80 heterodimer defines the initial recognition step at the exposed DSB terminal. Ku encircles the DNA duplex and recruits DNA-dependant protein kinase (catalytic subunit). Binding of this translocates Ku, allowing DNA-PKcs to make contact with the DNA termini, and also promoting synapsis of the two molecules of DNA. DNA-PKcs is the auto-phosphorylated allowing access to the DNA terminals. Pol μ and Pol λ then resynthesise the missing DNA nucleotides. Ligation is then carried out by DNA ligase IV and XRCC4 (Dexheimer, 2013).

Nucleotide excision repair (NER) functions specifically in the universal excision of a wide variety of bulky DNA lesions/adducts by excising the damage site and filling in the gap using a DNA polymerase. The pathway consists of two distinct sub-pathways which differ only in the initial recognition step. NER has implication in many biological pathways and disease progression such as in the case of *Xeroderma Pigmentosum* (Spivak, 2015).

### 1.4.3 Nucleotide Excision Repair (NER) Pathway

Fundamentally NER works on the basis of two individual pathways: global genome NER (GGNER) and transcription-coupled repair (TCR); of the two, GGNER is better understood and holds central roles in removing lesions from all damage sites across the genome. It operates by sequential assembly of the proteins that are involved in DNA damage repair. TCNER, on the other hand is the preferential repair of lesions formed in damaged transcribed strands of active genes (Figure 3) Various NER proteins are able to diffuse freely around cells, and when recruited travel in a defined order to the locations of lesions caused by DNA damage. Once the pathway is complete, and the lesion repaired, the individual proteins are released from the NER complex. Current research points towards the initial recognition factor of DNA damage being a subcomplex of XPC, HR23B and Centrin2. XPC then follows this with the recruitment of TFIIH to the various sites of UV instigated lesions, along with two helicase subunits of TFIIH (XPD and XPB). This sequence initiates the opening of the DNA around the lesion site; consequently, recruiting XPA, RPA and XPG to join the forming complex and stabilise it (Spivak, 2015).

The most significant helix distorting lesions that are found are pyrimidine dimers such as 6-4 photoproducts produced in the present of UV light. Mechanistically, NER and BER hold many similarities, however NER is unique in the complexity of its pathway – requiring around 30 specific proteins to carry out functions to excise and fill DNA

lesions. Mediated by the sequential assembly of repair proteins at the damage site, NER relies specifically on the formation of complexes to initiate and carry out damage repair. The steps of NER include: recognition of the DNA lesion, opening of the DNA around the lesion area, excision of the damaged DNA segment (spanning no more than the lesion), sequential strand repair and ligation (Williams *et al*., 2016).

NER holds monumental biological importance, a fact supported by the number of genetic disorders that arise as a result of NER defects. Such disorders include: *Xeroderma Pigmentosum* and Cockayne syndrome both of which exhibit overlapping symptoms with not only certain skin cancers but also in neurodegeneration and premature aging. In placental mammals, NER is the only DNA repair pathway that can remove UV-photoproducts.

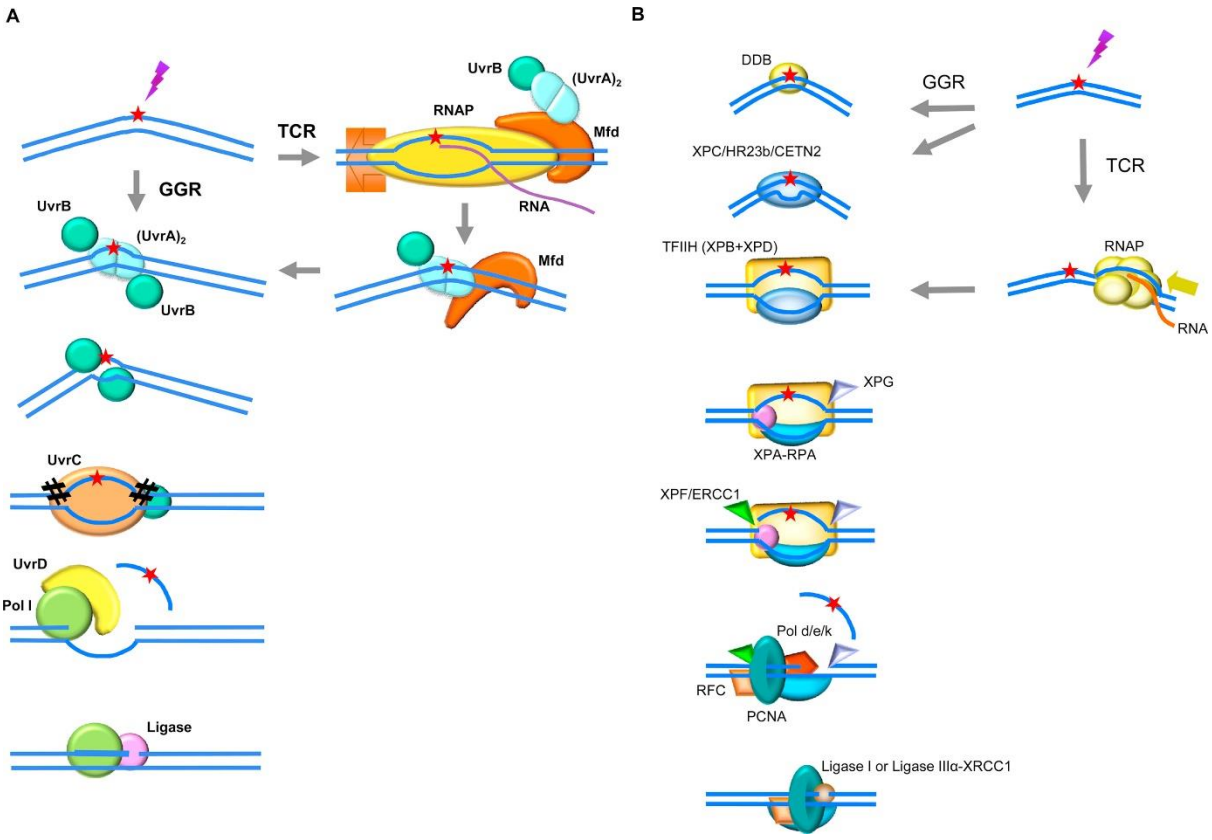



Figure 3 Schematic demonstrating nucleotide excision repair, including the two distinct pathways: TC-NER and GG-NER (Spivak, 2015)

#### 1.4.3.1 Transcription Coupled NER

Transcription coupled NER (TC-NER) preferentially repairs lesions that are found on the coding strand of active genes. It is a highly conserved pathway that can be observed in humans, yeast and some bacteria and it is noted that UV-photoproducts are more readily removed from actively transcribed genes than non-transcribed genes. In TC-NER damage recognition begins when RNA polymerase II arrests upon encountering a DNA damage site, where CSA and CSB displace the RNA polymerase II to allow NER to carry out (Ranes *et al*., 2016). Succeeding initial recognition, both TC-NER and GG-NER then ensue onto the fundamental NER pathway and recruit the ten sub-unit complex to the damage site along with transcription factor TFIIH. Following this, the two TFIIH-associated Xeroderma Pigmentosum complementation group B (XPB) and Xeroderma Pigmentosum complementation group D (XPD) helicases coordinate the unwinding of the DNA helix, forming a nucleotide bubble at the flank of the lesion. It is this initial unwinding that allows XPA access to the lesion activating the secondary level of damage recognition which aids to prevent undamaged DNA being excised. Following this, replication protein A (RPA) binds, stabilising the now forming pre-incision complex and allowing for complete extension (Pani and Nudler, 2016). The penultimate step includes the structure specific endonucleases excision repair cross complementing 1-Xeroderma Pigmentosum complementation group F heterodimer (ERCC1-XPF ) and Xeroderma Pigmentosum complementation group G (XPG) orchestrate the cleavage of the DNA 3’ and 5’ to the lesion respectively. Finally, using the undamaged strands as a template to resynthesise, DNA polymerase δ or ε fills the gap. DNA ligase then seals the nick found in the repaired strand, finalising the NER pathway (Shuck., *et al* 2008).

#### 1.4.3.2 Global Genome NER

The initial damage recognition in GG-NER is carried out by a complex formed of Xeroderma Pigmentosum complementation group C (XPC)/Rad23 homolog B(HR23B)/Centrin 2(CEN2), where HR23B and CEN2 act as accessory proteins to increase binding affinity and specificity of XPC to DNA damage. XPC has a low affinity to minor lesions and as a result, the level of binding of XPC directly correlates with the level of helix distortion present. In the case of minor lesions, a secondary DNA damage recognition complex formed of DDB1 and XPE initially detects the damage, such as CPDs induced by UV light. Binding of this complex distorts the helix further by inducing DNA bending, consequently facilitating the employment of XPC onto the site (Nouspikel, 2008).

### 1.4.4 NER Deficiency Related Disorders

The significant clinical consequences of a defect in one of the NER associated proteins are evident in several recessive genetic syndromes which are characterised by increased photosensitivity. *Xeroderma Pigmentosum* (XP), Cockayne Syndrome (CS) and the photosensitive form of trichothiodystrophy (TTD) are all the products of a defect leading to a deficiency in particular NER protein, resulting in reduced DNA repair (Ribezzo,, Shiloh,  and Schumacher, 2016). There are seven different complementation groups found within the group of XP individuals that are NER deficient ranging alphabetically from XPA-XPG, three of which are combined with CS symptoms (XPB, XPD and XPG). CS patients are found within the two comple mentation groups CSA and CSB, whereas TTD is found in XPB, XPD and TTDA, with each group representing a defect in a specific gene. Importantly, these different complementation groups give rise to different clinical outcomes, ranging in severity from mild to severe. It is important to note that conditions of UV-light (sun) sensitivity

are major risk factors in predisposing an individual to cancer, particularly in the case of XP (de Boer and Hoeijmakers, 2000).

#### 1.4.4.1 Xeroderma Pigmentosum

Xeroderma Pigmentosum is a rare autosomal recessive genetic disorder that is characterised by a deficiency in the NER pathway, resulting in an extreme sensitivity to UV light. Mean onset of symptoms is around 2 years old with the media age of death being 37 years (Singh *et al*., 2015). Prominent hallmarks of this condition are the presence of freckles and parchment skin and the eventual carcinogenesis of skin tissues. The affected areas are specifically contained to light exposed regions, such as the neck, head and face. Progressive degeneration of the skin and the eyes occurs as a result of continual sun exposure of XP patients. Degradation includes: ocular involvement as a direct consequence of sunlight exposure (photophobia, keratitis, atrophy of the skin of the lids) and severe increase in the risk of developing cutaneous neoplasms that are UV induced such as melanomas and more often basal cell carcinomas (Clever, Lam and Revet, 2009). Additionally, around 25% of XP patients have neurological symptoms including absent or diminished deep tendon stretch reflexes, hearing loss and progressive cognitive impairment. It is thought that the basis for the neurological symptoms is neuronal loss. Individuals that fall into the partial NER defect category (XP-F and XP-C) and experience ‘mild’ symptoms generally do not develop neurological symptoms, and if symptoms do develop they have a much later onset. Potential reasoning for neurological abnormalities is deficient or defective repair of oxidative lesions on nerve cells inducing death of neurones. The median age of death of XP patients who also experience neurodegeneration is 29 years, lower than the median of those without neurological involvement (Mirecka *et al*., 2014).

XP is associated with dramatic increase in skin cancer risk (>1000-fold) with the majority of these malignancies being confined to head and neck areas. Basal cell carcinomas are amongst the most common malignancy to occur along with squamous cell carcinomas. Less frequently melanomas can occur. The mean onset of skin cancer amongst XP patients is 8 years old compared to 56 years in the normal population, and it is the primary cause of death reducing life span by around 30 years (Kraemer and DiGiovanna, 2015)..

Genetic heterogeneity characterises XP, consequently demonstrating heterogeneity in the severity of observed symptoms (neuronal degeneration and UV sensitivity). In particular; XPA, XPB, XPD and XPG individuals exhibit a severe NER deficiency resulting in most severe symptoms. Whereas XPC, XPE and XPF exhibit symptoms on the milder end of the spectrum. Research suggests that the moderate UV sensitivity found in XPF individual could be a consequence of the dual function of the ERCC1-XPF heterodimer in the repair of inter-strand crosslinks in NER (Kraemer and DiGiovanna, 2015). Deep phenotyping analysis on a large cohort of patients demonstrated the heterogeneity of clinical features between the different complementation groups. Development of skin cancer was found to be the most common in the previously considered moderate to mild XPC and XPE groups. It is thought that this is due to late diagnosis as patients experience normal sunburn reactions and less likely to keep to UV protection. Compared to other groups, XPG and XPF were found to be much less susceptible to noted skin cancers (Fassihi *et al*., 2016). These studies provide indication of the essential role of DNA repair in protection from skin cancer and neurodegeneration.

#### 1.4.4.2 Cockayne syndrome

Cockayne syndrome (CS), also known as Neill-Dingwall syndrome is a rare and fatal autosomal recessive neurodegenerative disorder that characterised by a defect in the TC-NER pathway. Thus, resulting in cutaneous photosensitivity particularly to UV light and physical and mental retardation (Ranes *et al*, 2016). Correspondingly, skeletal abnormalities such as dental caries and spinal cord kyphosis are observed, with osteoporosis present in older patients. Severe progressive neurodegeneration and microcephaly are present in the majority of patients over the age of 2 years old. Unlike XP patients, those who suffer with CS are not predisposed to developing skin cancers. The mean age of death of CS patients is 12.5 years old, primarily through respiratory infections and pneumonias. CS is classified into two different complementation groups: (1) CSA as a result of a mutation on ERCC8 (chromosome 5q12-q31), and (2) CSB, due to a mutation on ERCC6 (chromosome 10q11), accounting for 70–75% of cases. Type 1 usually presents in early childhood, around 12months to 24 months, whereas type 2 presents a more severe phenotype and presents from birth (Karikkineth *et al*., 2017)

Comparably, the clinical symptoms are difficult to explain in terms of partial NER defect due to the fact that XPA patients are completely deficient in NER and do not exhibit the same symptoms. Thus, it is suggested that it is the transcriptional engagement of CSA and CSB that contributes to these symptoms and that DNA damage induce transcriptional deficiency is the main cause. Studies have also suggested that endogenous damage by oxidation may be associated in CS developmental defects. Knock out studies of CSB in mice showed partial symptoms, but double mutant mice with CSB/XPA repair deficiency suffered severe growth failure and died before weaning. Similarly, TC-NER defects in oxidative damage

repair were observed in CS cell, but not XPA, therefore rendering CS cells more sensitive to IR that wild type (de Boer and Hoeijmakers, 2000).

In NER, the presence of defective DNA repair stalls RNA polymerase transcription. The CSB is thought to be responsible in aiding RNA polymerases in these situations and helps deal with any DNA damage induced transcription blocks. CSB then recruits NER proteins to remove the damage (Aamann et al., 2013).

#### 1.4.4.3 Deactivation of DNA Damage Repair in Cancer

As source of genetic diversity, mutations are beneficial and essential in evolution allowing for advantageous phenotypes to arise. However, widespread mutations causing genomic instability can hold catastrophic consequences, leaving individuals susceptible to an array of diseases. Said mutations are a consequence of countless sources, but defects in DNA repair pathways hold significant jurisdiction over disease causing mutations, particularly those associated with age. General cellular processes such as transcription and replication expose the cell to genotoxic stresses which can overwhelm normal functioning DNA repair mechanisms, promoting genetic instability and eventually carcinogenesis (Ali *et al*., 2017).

The mutator hypothesis (Loeb *et al*., 2016) proposes that a deficiency or loss in genes associated with DNA repair leads to instability within the genome. This consequently amplifies rates of mutations, transforming cells to mutagenic states. In line with this, increased mutation load paired with faulty DNA repair forms the basis of many hereditary cancers. Many breast and ovarian cancers have an overwhelming association with mutations in the BRCA1 and BRCA2 gene, which function specifically in the repair of double strand breaks via homologous recombination (Tubbs and Nussenzweig, 2017). BRCA1 is a pleiotropic DDR protein, holding roles in DNA repair and checkpoint activation. BRCA2 mediates the central mechanism that runs HR. One mutation of either copy of these tumour supressing genes results in hereditary breast and ovarian cancer (HBOC) syndrome, leading to early onset

breast carcinogenesis. Its direct role in HR – the process most essential in maintaining and protecting the integrity of the genome – suggest that it is defects in this pathway in particular that are responsible in development of breast cancer. Similarly, there are other factors involved in HR pathway (PALB2 and BRIP1) that act as facilitators and, when mutated, are predisposing for HBOC. Thus, suggesting that the BRCA1/BRCA2 pathway in HR is a suppressor of tumorigenesis, and when defective, leads to breast carcinogenesis (Roy, Chun and Powell, 2012).

Next generation sequencing has allowed for huge advances to develop in detecting germline and somatic mutations and has reveal much about DNA repair deficiencies in cancer development. Men with high-risk prostate cancer are >5 times more likely to have mutations (either inherited or somatic) in repair genes that mediate HR, particularly BRCA2 and ATM genes, and in MMR genes MLH1 and MLH2 (Pritchard *et al*., 2016). These strategies will hold monumental impact in the development of targeted therapies. However, little understanding is had over why these defects in universal repair genes are responsible for organ specific cancer, even though they are present and function in all cell types and expressed ubiquitously. Tissue variations along with extrinsic factors may result in specific vulnerabilities that aid the process of tumorigenesis.

### 1.4.5 Consequences of Increased NER

In the normal cell, average and high levels of DNA repair pathways and genes act as an advantage in the efficient detection and repair of DNA damage and allow for the prevention of mutations by maintenance of genomic integrity. Contrastingly, efficient repair of DNA damage in cancer cells is an imperative participant in the development of resistance to chemotherapy drugs that function in promoting cell death by inducing DNA lesions. Platinum compounds are particularly subjected to this mechanism of resistance. Patients receiving platinum therapy usually produce promising results

with treatment, but with relapse and secondary platinum treatment show high levels of drug resistance (Du *et al.*, 2016).

Increased levels of DNA repair proteins, particularly those involved in the NER pathway, are highly associated with drug resistance. This correlation was first noted in ovarian cancer samples, where ERCC1 was observed at higher levels in the cancer tissue compared to normal tissues. Similarly, even higher mRNA levels of ERCC1 were found in patients known to have particularly resistant cancer and lower levels were observed in patient who were sensitive to platinum treatment. A linear correlation of ERCC1 expression and relative sensitivity to cisplatin is noted (Amable, 2016).

## 1.5 ERCC1-XPF: The Heterodimer

Around 20 genes are involved in the NER pathway and they all function in sync to provide genomic protection against damages to DNA. The XP genes are particularly of note and are responsible for the rate limiting of the process. The ERCC1 protein forms a tight heterodimer with XPF – forming the ERCC1-XPF complex (figure 4). It has the functional purpose of being a structure specific endonuclease, with the key role in catalysing the 5' incision at the site of DNA damage (Pei *et al.,* 2014). The functional heterodimer forms three attractive binding sites for other proteins involved in the NER pathway; the XPA binding site that is fundamental in the formation of the NER complex, the ERCC1-XPF heterodimerisation domain, integral in upholding the heterodimers stability and the XPF endonuclease domain which is required for all of the known roles of XPF (McNeil *et al*., 2012).

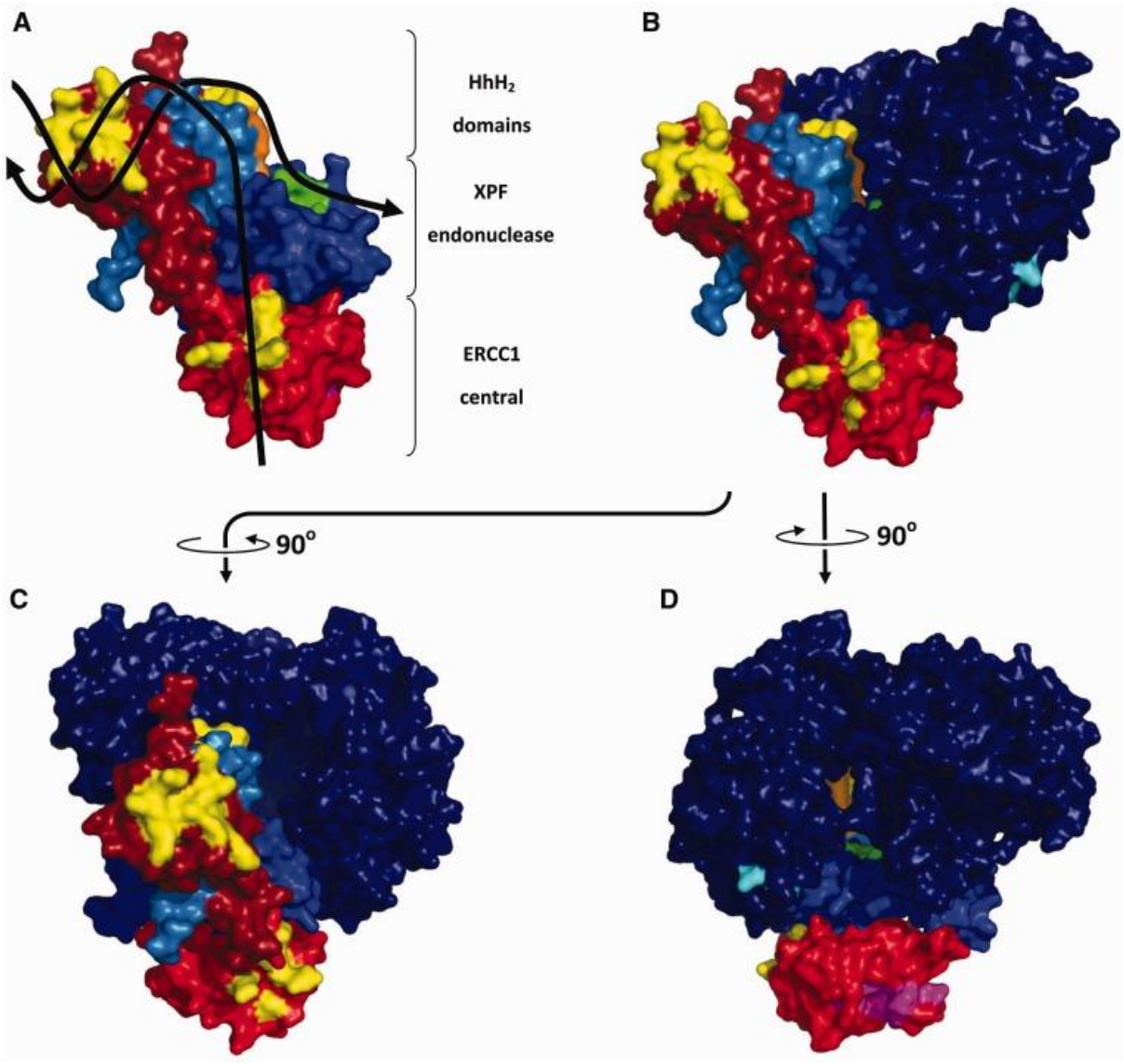



Figure 4 ERCC1-XPF heterodimer showing the HhH2 domain

ERCC1-XPF is demonstrated as critical in NER and variations in the genes are likely to hold significant value in the influence of genomic stability which consequently plays a role in carcinogenesis. As such, it is likely that different polymorphisms of the ERCC1 gene can have a bearing on NER pathways and tumour sensitivities to chemotherapeutic drugs, namely Cisplatin (Amable, 2016).

### 1.5.1 ERCC1-XPF

#### 1.5.1.1 ERCC1-XPF Structure

ERCC1 is a 297 amino acid protein located on chromosome 19q13.32 and consists of 10 exons. The functional gene encodes a protein that serves in rectifying DNA damages as part of the NER pathway. ERCC1 is formed of a helix-hairpin-helix domain (HhH2) (figure 5) that is essential in the formation of the heterodimer with XPF (McNeil, 2012).

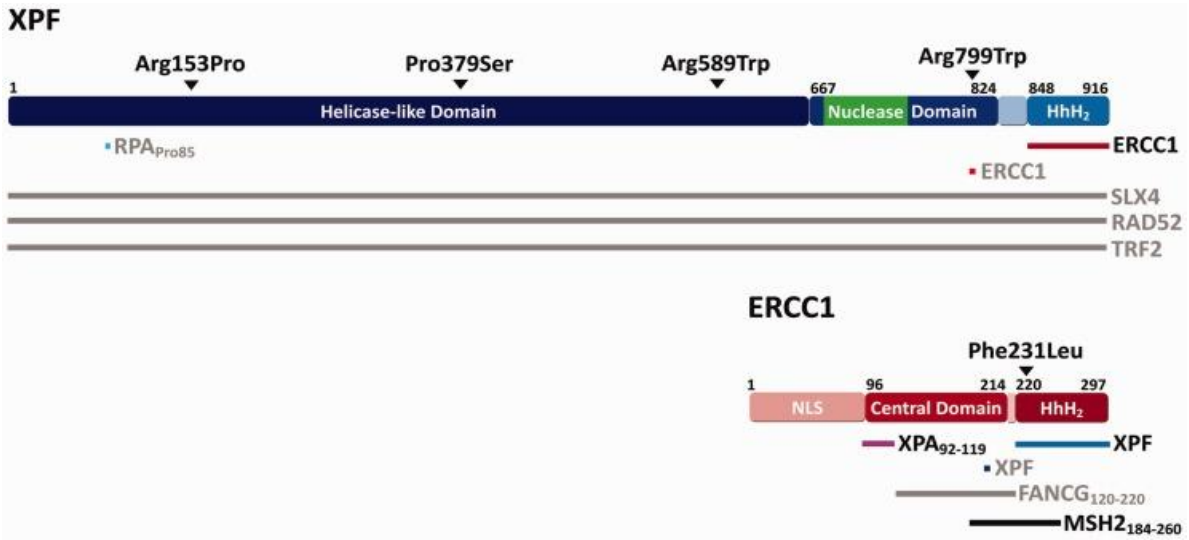



Figure 5 Schematic demonstrating the structure of XPF and ERCC1 (McNeil et al., 2014)

The XPF gene is located on chromosome 16p13.12, containing 11 exons and spanning 28.2kb. Its protein product (FANCQ protein) also holds a key role in NER, with it being imperative in the 5' incision of the lesion. The non-specific PK-C inhibitor UNC-01 interacts with the Tyr residues within the XPA interaction site on the ERCC1 protein, and it is this interaction that promotes the recruitment of ERCC1 to the DNA damage site (Barakat *et al*., 2009).

XPF is highly evolutionarily conserved and is a member of the Fen1 family of structurally related endonucleases. This family includes Rad1 (the yeast homolog) and the Mus81p nuclease, which along with XPF contain the highly conserved

$GDX_nERKX_3D$ region. This region is imperative in maintaining that catalytic activity of the endonuclease. Along with other members of the XP family, XPF houses a $Mg^{2+}$ metal binding site and $Mn^{2+}$ ions, preferentially binding $Mn^{2+}$ whereby the residues synchronise to coordinate binding. Mutations in any of the XPF residues lead to diminished binding to ssDNA and dsDNA.

RPA is essential in the employment of XPF to sites of UV damage this recruitment is mediated by the Pro85 region of XPF. XPF has been investigated in both biophysical and biochemical assays which reveal the main interaction sites of the protein. Primary interaction of the DNA phosphate backbone occurs via the Lys850, Ser854, His877, Arg853 and His858residues and with the DNA nucleotides via Ser875, Leu877, Ile876, Gly878 and Asn879 residues (Das *et al*., 2012).

Analysis of the XPF HhH2 motif (residues 863-905) present in the C terminal of XPF reveals several residues specifically required for the ERCC1-XPF interaction, that are imperative in heterodimer formation. ERCC1 only has the ability to accurately fold when complexed with XPF, however homologous XPF derived from *Aeropyrum pernix* has the ability to bind both ssDNA and dsDNA in the complete absence of other NER proteins (Feltes and Bonatto, 2015).

#### 1.5.1.2 ERCC1-XPF Function

The ERCC1-XPF complex is required for DNA damage repair upstream from the site of the lesion. It performs functionally as a structure specific endonuclease, incising the 5’ end of the DNA. Structure specific endonucleases are normally the first enzymes within a DNA repair pathway that cleave the DNA backbone, altering the primary DNA structure and consequently performing the first irreversible step in the named pathway (Schärer, 2017).

ERCC1-XPF demonstrates functional roles outside of NER, with ERCC1 being indicated in mitotic progression. In ERCC1 knockdown studies in hepatocellular carcinoma cell cycle delay and multinucleation was observed. Similarly, when XPF was knocked down, abnormal mitoses and morphology was observed. The heterodimer has also been linked to telomere maintenance and shows interactions with TRF2 (McNeil *et al*., 2012). The heterodimer itself is observed to have many individual functions, donating much of its time to repair damaged DNA. Its role is not only in NER, but has functional roles in inter-strand crosslink repair, single stranded annealing in HR and potentially acts as a backup nuclease in the repair of oxidative damage.

In terms of secondary roles, post translational modifications of XPF have not yet been demonstrated. There has been widespread interest into the individual role of XPF, however the results still remain elusive (Scharer, 2017).

### 1.5.2 ERCC1-XPF in Cancer

There are three noted ERCC1 variants that have been associated with increased cancer risk: rs321986, rs321961 and rs11615 (Zhang *et al*., 2012). Conversely, studies on ovarian, non-small cell lung cancer and squamous cell carcinomas demonstrate that high levels of ERCC1 expression are linked to poor responses to chemotherapy ((Bai *et al*, 2012) (Bauman *et al* 2013)). Comparably, research observing low levels of ERCC1 found that patients with testicular cancer received effective treatment with cisplatin without relapse when ERCC1 levels were below the average value (Usanova *et al*., 2010). On the same note, in knock-down studies of ERCC1-XPF, siRNA depletion of the endonuclease heterodimer increased the cancer cells sensitivity to Cisplatin in ovarian and breast cancers.

Despite the lack of conclusive data suggesting link between ERCC1-XPF polymorphisms and breast cancer susceptibility, research carried out on a Chinese population demonstrated that the rs11615 and re3212968 polymorphisms did in fact show an increased risk (Pei *et al*., 2014). Inconsistencies in results may be a result of various ethnic background and sample sizes being used, particularly when studies use all samples from one hospital. Research into a Chinese population demonstrated that the number of women getting breast cancer is increasing, however, the frequency of breast cancer related deaths is on the decline. Multiple factors including familial history, increased breast density, decreased physical activity and menstruation, reproductive history (Ma *et al*., 2016).

The ERCC1 variant ERCC1rs11615GA polymorphism was found to be associated with an increased risk in breast cancer susceptibility (3.53 folds higher than the healthy population) (Pongsavee *et al*., 2017). Similarly, the rs11615AA variant was reported to have a 1.62 fold increase in Chinese patients.

Owning to their multiple functions, mutations in either of the heterodimer proteins are linked to various human syndromes. XPF has significant links to XP, CS and a syndrome of premature ageing named XFE. XP being a significant risk factor in the development of multiple skin malignancies. Mutations in ERCC1 are responsible for cerebro-oculo-facio-skeletal syndrome.

## 1.6 XPG: The Structural Endonuclease

Found on the xeroderma pigmentosa complementation group G, XPG is a protein domain that is involved in many key factors within molecular biology. XPG (Xeroderma Pigmentosum G) is a structure specific endonuclease that is essential in the role of nucleotide excision repair. Its specific role is to repair DNA damage that is caused as a result of exposure to UV light and specifically functions in the NER

pathway. Defects within this protein domain lead to dysfunctional DNA repair mechanisms, and as such are associated with various disease factors including a predisposition to cancer (namely skin cancers).

### 1.6.1 Structure

The XPG protein domain belongs to the Fen1 family of proteins and shares it homology with various mammalian and yeast species. For example, RAD2 from S*accharomyces cerevisiae* and the mouse and human protein Fen1, a structure specific endonuclease and DNA repair protein that is heavily implicated in base excision repair (BER) pathways. When analysed via sequence alignment, similarities between the members of the Fen1 family are largely contained to two specific regions (nuclease domains). The first likeness is located at the N terminal region and the second at the I (internal) region that is located towards the C terminal region and expands 140 residues (Figure 6). An area containing 27 highly conserved amino acids and a pentapeptide (E-A-[DE]-A-[QS]) is thought to be the region responsible for the highly conserved catalytic mechanism of NER. As a contrast, the amino acids that link the N and I regions are not highly conserved (Barros *et al*., 2016), (Xu *et al*., 2014).

Regardless of the similarities in the highly conserved functional domains between XPG and the remainder of the Fen1 family of proteins (particularly the Fen1 flap endonuclease), the XPG protein has remarkably unique functional domains. These can be indicated in the specific function of NER mediation, giving XPG a defined role in this DNA damage pathway. The highly acidic spacer region doesn't contain any functional sequence motifs, however various parts have been known to interact with other proteins. A particular interaction of importance is noted between the spacer region of the N terminus and TFIIH, holding fundamental capabilities within NER. Conversely interactions with RPA have been suggested but have yet have their significance determined (Scharer 2008).

At the level of primary sequence analysis, it is shown that XPG contains two conserved domains including the highly conserved acidic Glu77 and Glu791 domains. Further analysis of these sequences reveals mediation properties – due to a mutation, alanine abolishes its catalytic activity. A 300 amino acid motif at the C terminal end of the protein is also indicated in protein-protein interactions. Containing two recognised localisation signals, it also holds Specific interaction sites with both a PCNA mediator (PIP box motif) and TFIIH and CSB. The intracellular behaviour of XPG points towards the protein having mobility that is consistent with it being present in monomeric form (Fagbemi, Orelli and Schärer, 2011).

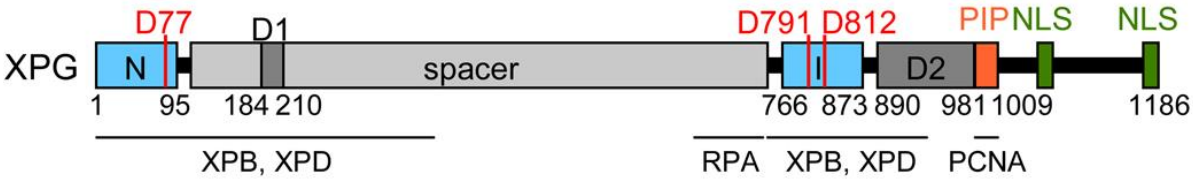



Figure 6. Functional domains of the XPG protein. The N and I domain (blue) comprise the active site of XPG. The 600 amino acid spacer region separates these two domains. Both the D1 and D2 areas are conserved in higher eukaryotes (Scharer et al, 2008).

The protein domain of XPG refers to the N terminus. It is an Mg2+ dependant single stranded DNA endonuclease that operates via making endonucleolytic incisions that are structure specific, within a duplex containing DNA substrate. These incisions cleave one strand of the duplex at the border of the single stranded region. The XPG protein can be corrected by the acidic 133 kDa nuclear protein XPGC that confers usual UV resistance in cells. Functionally, the internal domain of XPG contains numerous glutamate and cysteine amino acid residues that are found within the amino acid sites of DNA nucleases. The N terminal along with the I (internal) domain forms the catalytic domain of the protein and forms the active site. It functions both in the precision and catalytic complexes making the 3’ incision in the damaged DNA site.

### 1.6.2 Function

XPG's role in GGNER is both structural and catalytic, once it arrives at the damage site it is able to then complex with TFIIH, RPA and XPA, forming a very stable complex. When in this form the XPG protein holds little nuclease activity; with its full potential only being revealed when ERCC1-XPF arrives with its catalytic activity. ERCC1-XPF efficiently carries out the 5' incision in the presence of the catalytically inactive XPG protein. This mechanism in the pathway suggests that the 3' incision (via XPG) is triggered by a conformational change in the NER complex that is generated by the catalytic activity of the ERCC1-XPF incision of the 5' strand. Correspondingly, research has suggested that XPG proteins show distinctive requirements for its action in both binding and the cleavage of its substrates. Diverse protein-protein interactions are indispensable in the modulation of the NER pathway; for example, XPG interacts closely with TFIIH resulting in recruitment to DNA damage sites (Laine *et al*., 2006).

On the contrary to the role of XPG in GGNER, its role in TCR is less well understood. It is noted that TCR is initiated by and RNA polymerase that becomes stalled at the site of a DNA damage associated lesion. The mechanisms of TCR involve all of the factors associated with GGNER, however XPC is exempt and an additional requirement for CSA, CSB and XAB2. The recruitment of core NER factors is dependent on CSB found at stalled RNA polymerases, it also acts in enabling the dual incision reaction. XPG – one of the core NER factors – plays a similar role as it does in GGNER, interacting directly with RNA polymerase II, therefore it is suggestive of XPG holding a functionality in an earlier role in the NER pathway (Fousteri *et al*., 2006).

Recently published works suggest a direct link with the WRN protein as a 3'-5' ReCQ like DNA helicase, both proteins are implicated in similar subnuclear redistribution

during the S phase of the cell cycle where they eventually colocalise in nuclear foci. (Aracil *et al*, 2013).

During DNA repair the XPG protein acts fundamentally in cleaving the 5'overhanging flap structure which is produced as a result of DNA polymerase encountering a 5' end of and okazaki fragment. Cleavage of the junction between double and single strand fragments of DNA result in the removal of the strand. Functionally, the XPG protein domain has both 5' endo and exonuclease activity as well as 5'pseudo Y endonuclease activities (Feltes and Bonatto, 2015). The endonuclease binds 2 mg2+ ions per subunit and it has been suggested that after substrate binding that it may bind to an additional third mg2+ ion. XPG is able to cleave a variety of SS/DS DNA junction containing substrates that include both bubbles substrates (with 5' single strand overhang) and splayed arms.

A secondary function for XPG has been suggested, whereby it has a stimulatory effect on the HsNTH protein, which in turn leads to the stimulation of thymine glycol DNA glycosylase (the repair enzyme). This has a functional role within the base excision repair (BER) pathway therefore suggesting a fundamental role of XPG within multiple repair pathways, signifying its molecular importance in both biological pathways and the predisposition to cancers. XPG has the ability to enhance the binding of the BER protein DNA glycosylase (HsNTH protein) to its specific substrate thymine glycol (Samadder *et al*., 2016) The XPG protein functions as a cofactor for DNA glycosylase – removing oxidised pyrimidines from DNA damage sites and has been indicated in transcription coupled repair and transcription of RNA polymerase II.

Outside of its central roles in NER, XPG is implicated in other biological functions. The severe phenotype that is observed in XP/CS could provide evidence for this; as the CS phenotype results not only in a TCNER defect, but also in a mild transcription defect (Mirecka *et al*., 2014). Despite the role of XPG within transcription not yet being noted in mammals, it has been suggested in studies on *s. cerevisiae* and the

role of Rad2 (a homolog of XPG). Nuclease activity deficient mouse models still maintain their full transcriptional capabilities. However, as a contrast, when the Rad2 is truncated and the 200 amino acid C terminal nuclease motif is lost there was not supporting evidence to suggest a role in transcription (Scharer, 2008).

### 1.6.3 XPG in Cancer

Various polymorphisms in XPG can lead to alterations in the expression of its protein, therefore variations in its ability to perform correct DNA repair, thus having an influence on susceptibility to cancers (Xu *et al*., 2014).

When subjected to point mutations, ERCC5 (the gene that codes for XPG) causes the cancer prone disorder XP. Polymorphisms and alterations in mRNA expression are also linked to an increased risk. There are 130 genes that are implicated in the DNA repair pathway, with the main role of XPG being in extreme UV sensitivity and leads to an increases genetic predisposition to sunlight caused cancers. Previous studies assessing the relative relation between XPG mutations and cancers suggest a strong link with head and neck, colorectal, digestive, laryngeal and prostate cancers (Yu *et al*., 2015).

A 2011 metanalysis identified a total of 17 studies including 5,235 cases and 5,685 controls for the XPG variant Asp1104His and 3,910 cases and 3,985 controls for XPF variant Arg415Gln. Data from this revealed that there was not significant association between the XPG Asp1104His polymorphism and increased breast cancer risk. The XPF Arg415Gln was demonstrated as potential low penetrant risk factor in the Caucasian population (Ding, He and Zhang 2011).

A further recent study on XPG polymorphisms suggested that the specific polymorphism: rs873601 had a significant association to cancer risk. There was a noteworthy and statistically significant correlation between rs873601 and risk of

developing cancer in homozygous models (GG vs AA: OR=1.16, 95% CI=1.07–1.26, *P*=.001). Moreover, under the 'allele contrast model' a significant connection between rs873601 and breast cancer was detected. The rs751402 and rs2296147 polymorphism held moderate effects on cancer susceptibility, data which might be highly dependent on various conditions such as the ethnicity of the patients and the type of cancer studied (Han *et al*., 2017).

Mutations of the XPG protein domain result in a condition called Xeroderma Pigmentosum – a rare autosomal recessive disorder in which the ability to repair DNA lesions cause by UV light is significantly deficient. Due to these defective repair mechanisms, multiple basal cell carcinomas occur frequently in patients, especially in those who are young. The most common cause of death in these patients are metastatic malignant melanomas providing a direct link between XPG protein domains and cancer fatalities. Similarly, if truncation mutations occur, Cockayne's syndrome is observed providing the XP/CS complex. However, the molecular pathway for this particular XP phenotype has yet to have been distinguished. Phenotypically, XPG is the most heterogenous form of Xeroderma Pigmentosum and is the rarest subtype, demonstrating a wide range of symptoms from mild to extreme dysfunction. Defects in the incision step of NER are responsible for the eventual symptoms of XPG. XPG was initially established by Jim Cleaver providing evidence for all of the XP genes working in the same biochemical pathway, suggesting linearity between each phenotypic subtype (Fassihi *et al*., 2016).

## 1.7 Platinum compounds in the Treatment of Ovarian Cancer

In the modern world of cancer chemotherapy, platinum compounds have emerged as an effective treatment for a vast majority of cancers, these include small cell and non-small cell lung cancer, gynaecological and aerodigestive cancers and has proven to be significant in the treatment of patients with ovarian cancer. Despite novel molecular targets emerging, and new therapies being developed, the use of cytotoxic drugs is liable to continue to be the mainstay in cancer therapy (McNeil *et al*., 2015).

### 1.7.1 Cisplatin

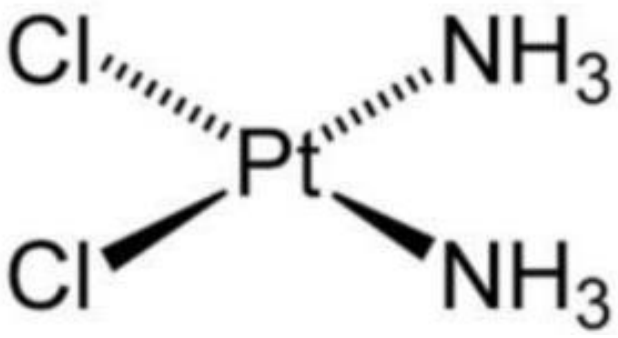



Figure 7 Schematic depicting the molecular formula for Cisplatin [Pt(NH3)2Cl2] (Dasari and Tchounwou, 2015)

The square planar metallic platinum compound (figure 7), cisdiamminedichloroplatinum, also known as cisplatin/cisplatinum is a cytotoxic chemotherapeutic agent widely utilised in the treatment of many cancers. It presents as a white or deep yellow powder at room temperature and has a molecular weight of 301.1g/mol and a melting point of 270°C. It is slightly soluble in water and is soluble in both dimethlyprimadine and N, N-dimethylformamide. Stable at room temperature, this metallic compound has the ability to slowly transform to its trans-isomer.

Cisplatin was first synthesised in 1844, but scientific investigations did not gain propulsion until the 1960s. Its anticancer properties were first noted when observations revealed that electrolysis products (of the mesh platinum electrodes) held the ability to inhibit cell division in *e*. coli. This breakthrough then lead to the interest in various platinum compounds as cancer agents. Cisplatin is now widely used in cancer treatments and demonstrates a good level of anticancer properties in head, neck, ovarian, testicular and breast cancers and is the first ever FDA approved platinum therapy (Dasari and Bernard Tchounwou, 2014).

Cisplatin enters the cell via passive diffusion along a concentration gradient, in yeast and mammals, uptake is mediated by the copper membrane transporter Ctr1 and it becomes activated upon entering the cell. (Dasari, and Tchounwou, 2014). In the cytoplasm the chloride groups on the cisplatin molecule are displaced by water molecules, producing a hydrolysed product with potent electrophilic properties. This electrophile can react with any neutrophile, particularly those found on proteins and nitrogen donor atoms on nucleic acids. Cellular DNA forms the primary target of Cisplatin, to which is binds reversibly and causes DNA adducts such as intra-strand crosslinks and inter-strand crosslinks. These lesions act in blocking DNA replication, resulting eventually in cell death. Despite cisplatin being very targeted, it can also bind to other unspecific macromolecules such as RNA and proteins. Cisplatin had been found to be largely inactivate essential RNAs i.e. spliceosomal, ribosomal and telomeric, with the precise site of binding in RNA unknown, therefore lack sufficient study as a primary target (Melnikov *et al*., 2016).

Around 80-85% of cisplatin caused adducts are a result of intra-strand cross links, for example via its binding to the N(7) reactive centre position of purines (Figure 8), resulting in 1,20d(GpG) intra-strand adducts. Conversely, inter-strand crosslinks are much less common (around 5%) but do contribute significantly to its toxicity. Highly toxic covalent bonds form between the two strands of DNA, rendering the helix

distorted and unfunctional, inhibiting replication and RNA transcription. If said lesions are not repaired cell death can occur as a result of unwarranted chromosomal rearrangements and DNA breaks. The repair of these particular lesions is yet to be specifically defined, however it is thought to involve a wide range of pathways including NER (Gatti *et al*., 2015).

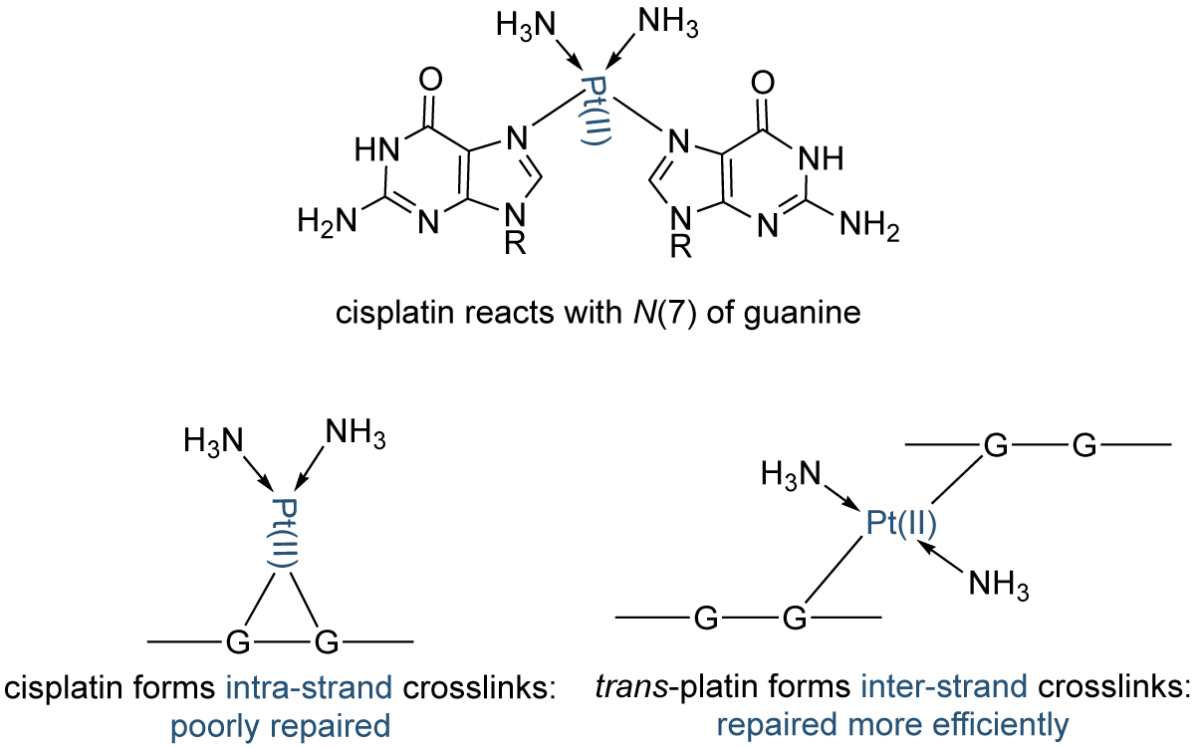



Figure 8 Formation of cisplatin induced inter and intra-strand crosslinks

Cisplatin has been approved for both individual and dual treatments for bladder, testicular, cervical, non-small cell lung cancer, ovarian cancer and head and neck cancers wherein randomized trials its efficacy in combination therapy significantly increases survival rates. Similarly, Cisplatin and its derivative carboplatin are amongst the most common chemotherapeutics used in the treatment of ovarian cancer. Research has shown increased levels of therapeutic benefit when combining cisplatin with various other cancer agents, with particular interest into gold nanoparticles. Cisplatin is utilised in the clinical setting frequently as a radiosensitiser; in spite of this, its relative toxicity limits the available therapeutic

window. Gold nanoparticles (AuNPs) and cisplatin have been explored in concomitant chemoradiotherapy, wherein they elicit their effects by distinct and overlapping mechanisms. Research has concluded that the combination of cisplatin and gold nanoparticles provides a new and promising area in increasing the efficacy of cisplatin treatment (Cui *et al*., 2017). Combination of cisplatin with mitoses inhibitors, such as paclitaxel, and anthracyclines such as doxorubicin are quickly becoming the mainstay for ovarian cancer therapies. Cisplatin is administered in cycles via continuous intravenous infusions or sequentially with other drugs. Local administration may aid in decreasing side effects of the therapy. Despite the relative success of cisplatin, its effectiveness as a treatment option is limited to the specific cancer type and the duration of the treatment. Resistance to therapy is highly associated with cisplatin treatment, with first time treatment yielding positive results, but this diminishing in relapsed patients. There are many mechanisms by which cisplatin resistance can occur, but most often high levels of DNA repair contribute significantly to resistance.

### 1.7.2 Mechanisms of Cisplatin Resistance

Resistance is a prominent issue that causes many clinical limitations with a vast majority of chemotherapeutic agents, thus proving it to be essential to understand the mechanisms underlying resistance mediation. Basic cellular mechanisms include diminished accumulation of the drug, detoxification via metallothionein and glutathione and the improved repair of DNA lesions caused by drug damage, therefore reducing cellular death. Increased cellular survival without the presence of DNA damage through defected signalling pathways is also an area of interest in increased resistance. (Shen *et al*., 2012).

It is clearly understood that the cytotoxicity of cisplatin is reliant on the establishment of DNA adducts that result in cellular death. Decreased levels of cisplatin

accumulations by either decreased uptake, or greater levels of efflux can both have significant effect on reducing the amount of cisplatin available to form said lesions. When studied, cells with high levels of resistance to cisplatin showed decreased accumulation through active transported, suggesting a strong link between accumulation of cisplatin and its efficacy.

Cisplatin resistance can be found in four different stages. The first stage is pre-target, usually caused by decrease uptake of the drug by various reasons. Secondly, on target resistance, namely increased DNA repair mechanisms repairing lesions caused by the platinum compounds. Thirdly, post target resistance, often caused by inactivation of TP53 resulting in no cell death. Off target mechanisms of resistance are usually a consequence of activation pro survival pathways (Ghor *et al*., 2017) (figure 9).

The mediator of cisplatin uptake (CTR1) has been indicated to have a significant role in the development of cisplatin resistance. The deletion or mutation of the gene causes noteworthy effect on the uptake of cisplatin into the cell, with cisplatin resistant cell lines demonstrating reduced expression of the gene. In cell lines that over-expressed CTR1, there was an increased uptake of cisplatin and consequently increases cellular sensitivity. In vivo, when CTR1 was genetically knocked out a resistance of cisplatin was observed. Furthermore, cells that held increased expression of CTR1 display amplified uptake of platinum compounds and in the majority of cases, an increased sensitivity to cisplatin (Shen *et al*., 2012). Another protein implicated in cisplatin resistance is of the multidrug resistance (MRP) – associated family – MRP2 or cMOAT. MRP2 is consistently increased in cisplatin resistant cells thereby suggesting an association.

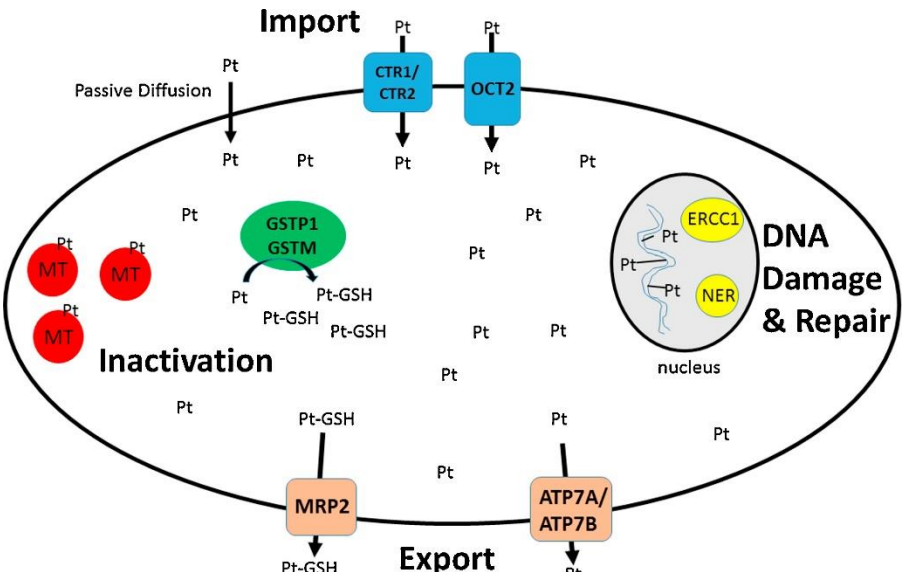


Figure 9 The cellular fate of cisplatin (Amable, 2016)

## 1.8 Inhibitors of DNA Damage Repair

A recent study on in silico drug screens demonstrated that when at very high concentrations, inhibitors of the heterodimeric interaction domain of ERCC1-XPF could disrupt the stability of the complex in cell lysates. Despite this, analysis suggests that the potency and stability of these particular inhibitors requires much work and amendment (Jordheim *et al.,* 2013). Similarly, when bound by an inhibitor of ERCC1-XPF, reduced level of ERCC1-XPF complex was observed. This consequently inhibited NER in melanoma cells, in turn increasing the level of cisplatin sensitivity (McNeil *et al*., 2015).

A recent study demonstrated that the natural compounds ellagic acid and resveratrol could prevent the development of cisplatin resistance in A2780 ovarian cancer cell lines and a intermediate shift into cisplatin sensitivity to the cisplatin in A2780CisR, the resistant clone of A2780, therefore demonstrating that the use of natural phenolic compounds may be a promising step in the prevention of cisplatin resistance in ovarian cancers (Engelke *et al*., 2016) This effect of this may be in part due to the prevention of ErbB2 and ErbB3 activation in long term cisplatin treatment.

IGF-1R activation has also been demonstrated to be important in the development of resistance to cisplatin. When A2780 cells were treated in 26 weekly cycles with

cisplatin, a significant rise in in IGF-1R that corelated with cisplatin resistance was observed, therefore proving it a promising target for cisplatin resistance (Eckstein *et al*., 2009). To further support this, a 1988 study demonstrated a link between blood levels of IGF-1 in pre-menopausal women and breast cancer. Binding to IGF1R promotes the initiation of the downstream network of signalling pathways via p13k-Akt-mTor and RAF-MAPk, both of which are fundamental in apoptosis and cell survival. It wasn't the high expression, but the high levels of phosphorylation of IGF-1R that was predictive of poor prognosis in breast cancer patients.

When carried forward for phase II clinical trials unpromising results were found for IGF-1R targeted therapies, this could be due to compensatory signalling mechanisms through growth hormone receptors. As a result; combination therapies have undergone research to tackle these unpromising results, in vitro studies have found synergistic effects with the small molecule inhibitors gefitinib (Beckwith and Yee, 2015).

Receptor tyrosine kinase phosphorylation analysis exposed the activation of both IGF1R and EGFR as a direct result of resistance to cisplatin. The respective inhibitors of these, (NVP-AEW541 and lapatinib) elevated the potency of lapatinib in dual cisplatin treatment in the triple negative breast cancer cell line HCC38CisR, however it didn't increase cisplatin sensitivity. Alternatively, another small molecule inhibitor, NVP-BEZ235 fully restored cisplatin sensitivity. To further this point, when tested with the highly resistant breast cancer cell line MDAMB231CisR, dual treatment with lapatinib and NVP-AEW541 enhanced the potency of cisplatin by 4-8x. Similarly, this combination of drugs was shown to increase levels of apoptosis compared to when used individually, with a reduction of the fraction of cells seen in G2/M phase of the cell cycle from 25.7% to 14.2%. Cells in the G1 phase were elevated from 62.8% to 77.7% (Gohr *et al*., 2017).

Increasing the sensitivity of patients to platinum-based therapies would be monumentally beneficial, not only in increasing the efficacy of the first line chemotherapeutics, but also in the ability to induce further remissions amongst patients with relapsed disease.

# 2 Chapter 2: Aims

The aim of this study is to analyse the existing data surrounding the NER pathway and its associated proteins ERCC1, XPF and XPG. It aims to determine whether the inhibition of the NER pathway via depletion of ERCC1-XPF can increase sensitivity to cisplatin treatment as a means of combatting resistance in ovarian cancers.

# 3 Chapter 3: Methods and Materials

## 3.1 Sub-culturing of Cell Lines

### 3.1.1 Cell selection

Cell lines were selected according to phenotype, including: derivation, primary tumour, tumour characteristics, organ of origin and adherence. Six breast cell lines and six ovarian cell lines were selected from previously maintained cultured cells purchased from ATTC (UK). These cell lines were used for initial screening to progress on to further experimentation, reference list of cell lines and selected growth media and tumour properties can be found in table 2.

Table 2 Panel of breast and ovarian cell lines used for initial testing, including growth medium, tumour/cell type and source of purchase.

| **Cell Line** | **Growth Media** | **Primary Tumour** | **Cell Type** | **Properties** | **Purchased** |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| MDA-MB-468 | **dMEM** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Metastatic Carcinoma | Breast cancer | TNBC (cisplatin sensitive) | American Type Culture Collection (ATCC, Manassas, USA) |
| SKBR3 | **McCoys** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Invasive Ductal Carcinoma | Breast Cancer | HER2 + | American Type Culture Collection (ATCC, Manassas, USA) |
| MCF10a | **DMEMf12** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | N/A | Breast, Normal | N/A | American Type Culture Collection (ATCC, Manassas, USA) |
| MDA-MB-231 | **dMEM** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Invasive Ductal Carcinoma | Breast Cancer | TNBC (cisplatin resistant) | American Type Culture Collection (ATCC, Manassas, USA) |

| | | | | | |
|---|---|---|---|---|---|
| T47D | **dMEM High Glucose** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Invasive Ductal Carcinoma | Breast Cancer | ER+ | American Type Culture Collection (ATCC, Manassas, USA) |
| MCF7 | **RPMI phenol red** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Invasive Ductal Carcinoma | Breast Cancer | ER+ | American Type Culture Collection (ATCC, Manassas, USA) |
| PEO1 | **RPMI** (Thermofisher Scientific) -10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Adenocarcinoma | Ovarian Cancer | BRCA2 def (cisplatin sensitive) | American Type Culture Collection (ATCC, Manassas, USA) |
| PE04 | **RPMI** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Adenocarcinoma | Ovarian Cancer | BRCA2 def (cisplatin resistant) | American Type Culture Collection (ATCC, Manassas, USA) |

| | | | | | |
|---|---|---|---|---|---|
| A2780 | **RPMI** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Carcinoma | Ovarian Cancer | Cisplatin Sensitive | Sigma Aldrich (Gillingham, UK) |
| A2780cis | **RPMI** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Carcinoma | Ovarian Cancer | Cisplatin Resistant | Sigma Aldrich (Gillingham, UK) |
| OVCAR3 | **RPMI** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Adenocarcinoma | Ovarian Cancer | Triple + (Cisplatin sensitive) | American Type Culture Collection (ATCC, Manassas, USA) |
| OVCAR4 | **RPMI** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Adenocarcinoma | Ovarian Cancer | Triple + (Cisplatin resistant) | American Type Culture Collection (ATCC, Manassas, USA) |

### 3.1.2 Initial Culturing

Cell lines were cultured in humidified incubators at a constant 37°c at 5% CO2 atmosphere. Culture environment was maintained to be completely sterile and each cell line were handled individually within a biohazard hood with laminar air flow to prevent contamination. Each cell line was grown in specifically selected media (table 1) in a culture flask (without the presence of special matrixes) to encourage optimal growth conditions. Cells were checked microscopically daily; attached cells should be present at the bottom of the flask.

To split or collect the cells, media was pipetted off and discarded in aid to remove any non-adherent (dead cells) leaving the remaining (live) cells adhering to the flask. The cell monolayer was then washed in ~2ml (depending flask size and trypsin sensitivity of cell lines) of phosphate buffered solution (Thermofisher Scientific) without $Mg^{2+}$ or $Ca^{2+}$. Adherent cells must be trypsinised to detach them from the bottom of the flask, therefore 2ml of 0.5mg/ml trypsin-EDTA (Sigma-PAA, UK) was added in order to disrupt the monolayer and incubated at 37°c for ~3minutes. The detachment was then visually analysed microscopically to observe free cells. Following this, selected media was added to the flask to inhibit the activity of the trypsin. The suspension of cells was collected and then and centrifuged at 1000 rpm (800xg) to collect a cell pellet. Supernatant was discarded, and the pellet was resuspended in 2.5-5ml of media. According to this, the suspension was transferred to a new flask along with media. This was then incubated in the above conditions and cells left to grow until around 60-80% confluency (60-80% of the bottom of the flask covered by the cell mono-layer) where they are at a suitable level for experimentation. Cell lysates were collected and stored a -20°c until use.

### 3.1.3 Cell Counting

Certain experiments require a number of cells in a particular range, to ensure this cell counting techniques are used. To count cells, 10µl of the cell suspension is aliquoted

onto a haemocytometer chamber and suspended with 10µl of trypan blue . All of the cells within the 5x5 division grids are counted and the mean number of cells calculated. The following equation is then used to determine cell number:

**number of cells/ml=dilution factor (10) x mean cell count x 10**

## 3.2 Western Blot Analysis

### 3.2.1 BCA Protein Assay

Prior to western blotting, protein levels of the lysates, previously collected as described in3.1.2, need to be analysed. The Pierce™ BCA Protein Assay Kit (Thermofisher LTD) is a two-component kit used to measure total protein concentration compared to a protein standard. Preparation of a dilution series as according to the protocol was carried out to form a set of protein standards to be used as the standard curve to determine cell lysate protein concentration.

The following equation was used to determine the volume of working reagent (WR) needed:

**(#standards + #unknowns) × (#replicates) × (volume of WR per sample) = total vol WR required**

The working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). Following the ‘microplate procedure’ the sample: working reagent (1:8) of each standard or unknown sample replicate was pipetted into a microplate well (working range = 20-2000µg/mL). 200µL of the working reagent was added to each well and plate. The plate was covered and incubated at 37°C for 30 minutes. Following this plate was cooled at room

temperature and absorbance was measured at 540nm. To analyse data the following equation was used:

**(Individual standard and unknown replicates) – (Av. 540nm reading of blanks)-Blank corrected 540nm reading**

From this a standard curve was prepared by plotting the average blank corrected 540nm measurement vs. its concentration µg/mL. The standard curve was prepared by plotting the average Blank-corrected 540nm measurement for each BCA standard vs. its concentration in µg/mL. The standard curve was then used to determine the concentration of each sample.

### 3.2.2 Western blotting

To begin initial screening of cells lines, the western blotting technique was employed to determine levels of protein expression within the selected cell lines. This was carried out in order to determine the two cell lines with the highest and lowest expression of the selected genes: XPF, ERCC1 and XPG in both the breast cancer cell lines and control ovarian cancer cell lines.

Cells were harvested and lysed in RIPA buffer (R0278, Sigma) with the addition of protease cocktail inhibitor (P8348, Sigma), phosphatase inhibitor cocktail 2 (P5726, Sigma) and phosphatase inhibitor cocktail 3 (P0044, Sigma) and stored at -20°C. Protein was quantified using BCA Protein Assay kit (23225, Thermofisher)

From this the working volumes of sample, reducing agent, sample buffer and water required for western blot analysis were deduced i.e. values used for breast and ovarian samples in condition 3 (table 2) volume of sample buffer is worked out at a 1:4 ratio to total volume of the sample, and reducing agent is worked out as a 1:10 ratio.

After samples were prepared, NuPAGE reducing agent buffer (Invitrogen™ NP0009, Fisher Scientific), sample buffer (Invitrogen™ NP0007, Fisher Scientific) and water (as stated in example table 3) they were denatured on a hot plate at 100°c for 5 minutes then allowed to cool. Samples were briefly centrifuged to ensure mixing of suspension for 2 minutes at 13,000rmp. Following this, the samples were loaded into precast 4-12% SDS gels, with the sample then running in a running buffer constituting of propanesulfonic acid sodium dodecyl sulphate (MOPS-SDS) at a 1:20 ratio to water in the blot module (Invitrogen). This was run at a constant voltage of between 90v to 125v for 60-90 minutes. Following this the gel is transferred to a nitrocellulose membrane in Bolt™ Transfer Buffer (20X) (Invitrogen™, Thermofisher) of 20% methanol, where by a constant current was applied for 60 minutes at 10v.

After transfer the membrane was removed and blocked in a blocking solution made of 5% milk solution in PBST (PBS, 0.1% Tween-20) (Thermo Scientific™) for one hour at room temperature on a roller. Following this the membrane was washed 3x5minutes in PBST and incubated overnight with the primary antibody diluted in 1%BSA, PBST in a constant temperature cold room on a roller. Full list of antibodies and concentrations used can be found in table 3.

Following primary antibody incubation, the membrane was washed 3x5minutes in PBST. To allow for normalisation of results, the membrane was then incubated with a house keeping antibody (diluted in 1% BSA-PBST) appropriated to the lysate and cell fraction, for 1 hour at room temperature. 3x5minute washes in PBST then followed. Finally, the membranes were then incubated with fluorescent secondary antibodies

(anti-mouse or anti-rabbit) for 1hour at room temperature, noting the light sensitivity of these antibodies, they were kept under cover constantly. Finally, the membranes were washed 2x5minutes in PBST and imaged using Licor Oddessy imaging system to detect protein bands.

Table 3 List of antibodies used for western blotting, including: concentration, species and company.

| **Antibody** | **Species** | **Company/Clone** | **Concentration Used** | **Application** |
|---|---|---|---|---|
| XPG | Monoclonal Mouse | Thermo Fisher (8H7) | 1:1000 | Western Blot – Primary Antibody |
| XPF | Polyclonal Rabbit | Novus Biologicals – NB100-60689 | 1:2000 | Western Blot – Primary Antibody |
| ERCC1 | Monoclonal Mouse | Dako – Clone 49F M3648 | 1:1000 | Western Blot – Primary Antibody |
| PARP1 | Monoclonal Mouse | BD Pharmigen - 516639GR | 1:1000 | Western Blot – Primary Antibody |
| ß-III Tubulin | Monoclonal Rabbit | AbCam - AB6046 | 1:1000 | Western Blot – House Keeping Antibody |
| YY1 | Monoclonal Rabbit | AbCam - AB109228 | 1:1000 | Western Blot – House |

| | | | | |
|---|---|---|---|---|
| | | | | Keeping Antibody |
| GADPH | Polyclonal Rabbit | AbCam - AB9485 | 1:1000 | Western Blot – House Keeping Antibody |
| Anti-Rabbit | Donkey IgG | LiCor - IRDye 800CW | 1:5000 | Western Blot – Secondary Antibody |
| Anti-Mouse | Donkey IgG | LiCor - IRDye 680CW Anti-Mouse | 1:5000 | Western Blot – Secondary Antibody |

## 3.3 Nuclear and Cytoplasmic Extraction

Cells were cultured, and lysates taken as per 1.1.2 wherein the pellet was resuspended and 10µl of the lysate was stained with 10µl of trypan blue and cell number was counted using an automated cell counter. The final cell lysate was re-centrifuged at 1000rpm (800xg) for 5 minutes to form a secondary pellet. Based on the number of cells counted the following volumes of each reagent were used whereby $2x10^6$ cells was considered equivalent to 20 µl of packed cell volume (table 4). All reagents and samples were kept on ice to ensure viability.

Table 4 Reagent volumes for cytoplasmic and nuclear extraction

| **Packed Cell Volume (µl)** | **CER (µl)** | **CER II (µl)** | **NER (µl)** |
|---|---|---|---|
| 10 | 100 | 5.5 | 50 |

| | | | |
|---|---|---|---|
| 20 | 200 | 11 | 100 |
| 50 | 500 | 27.5 | 250 |
| 100 | 1,000 | 55 | 500 |

The volume ratio of the reagents must be scaled to number of cells present and remain in the ratio CER I: CER II: NER at 200:11:100µl. Any supernatant was carefully removed to ensure a completely dry pellet and ice cold CER I was added to the pellet (volume as according to table 4). The tube was the vigorously vortexed on the highest setting for 15 seconds in order to fully disrupt the pellet. The sample was then incubated on ice for 10 minutes. Following this, ice cold CER II was added to the suspension and vortexed at the highest speed for 5 seconds. Samples were then centrifuged at 13,000 rpm (>16,000g) for 5 minutes. After this the supernatant (fraction of sample containing the cytoplasmic extract) formed was immediately transferred to a pre-chilled Eppendorf and stored at -80°C. The remaining pellet (which contains the nuclei) was the resuspended in the appropriate amount of NER reagent and vortexed on the highest setting for 15 seconds. Vortexing was repeated every 10 minutes for 40 minutes, with samples being incubated on ice between vortexing. Samples were then centrifuged at 13,000 rpm (>16,000g) for 10 minutes and the supernatant now containing the nuclear extract was immediately transferred to a pre-chilled eppendorf and stored at -80°C.

## 3.4 Real-time Polymerase Chain Reaction (rtPCR)

### 3.4.1 mRNA Extraction

For the isolation and extraction of cellular mRNA, the RNeasy® Mini Kit (Quiagen, UK) was used. Cell lysates were collected as according to 1.1.2 whereby the final cell suspension was re-centrifuged at 1000rpm (800xg) for 5 minutes to form a secondary

pellet. In order to disrupt the pellet, it was then resuspended in 350µl of RLT lysis buffer provided by the kit and transferred to a 1.5ml Eppendorf (350µl of RLT buffer for < 5 x$10^6$ cells). The suspension was mixed by pipetting and then centrifuged at 4°C for 5 minutes at >8000g and up to 350µl of the supernatant was transferred to an Eppendorf. Following this, equal volume to RLT buffer (350µl) of 70% ethanol was added to the supernatant and mixed by pipetting. Immediately after up to 700µl of the suspension was transferred to an RNeasy spin column placed in a 2ml collection tube and centrifuged for 15 s at ≥8000 x g. The flow through was discarded. This process was repeated twice. 700 µl buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Flow through discarded. Next, 500µl of Buffer RPE was added to the RNeasy spin column and centrifuged for 15 s at ≥8000. A further 500µl of Buffer RPE was added and centrifuged for 2minutes at >8000g. Finally, the RNeasy spin column was placed in a new 1.5 ml collection tube (supplied). 30–50 µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA. Nano drop analysis then occurred to determine the amount of RNA present in each 1µl sample.

Polymerase Chain Reaction: cDNA synthesis was performed using the $RT^2$ First Strand Kit (330404, Qiagen). Real time PCR was carried out on an Applied Biosystems 75000 FAST cycler.

Table 5 List of primers and concentrations used for PCR reactions

| **Gene** | **Company** | **Sample Concentration** |
|---|---|---|
| ERCC1 | Qiagen- QuantiTect® QT00059374 | 1:50 |
| ERCC4 (XPF) | Qiagen- QuantiTect® QT00063091 | 1:80 |

| | | |
|---|---|---|
| ERCC5 (XPG) | Qiagen- QuantiTect®<br>QT00029246 | 1:80 |
| GADPH | Qiagen- QuantiTect®<br>QT00079247 | 1:80 |

Table 6 PCR Master mix

| **Master Mix/Well 8µl** | |
|---|---|
| **Sybr Green** | 5µl |
| **Primer** | 1µl |
| **Water** | 2µl |
| **Sample** | 2µl |

## 3.5 siRNA

Silencing RNA otherwise known as small interfering RNA is a popular method used to transiently knock down genes. siRNA is a class 20-25 base pairs of double stranded RNA that operate within the RNA interface pathway (RNAi). siRNA interferes with sequences of complementary nucleotides and prevents translation by degrading mRNA after transcription – thus interfering with specific genes and reversibly knocking it down.

A2780 and A2780cis Cells were transfected with 20 nM of either XPF siRNA oligonucleotide (146632 Silencer®, Ambion) or ERCC1 siRNA oligonucleotide (146877 Silencer®, Ambion) or a negative control siRNA. 24hours before transfection, the cells were seeded at a density of $8\times10^3$ cells/cm$^2$, at around 50-60%

confluency. The transfection process was as follows: Prior to transfection, Lipofectamine™ was diluted in opti-MEM growth medium and left at room temperature to incubate for 5 minutes. Following this, the siRNA (20nM) was combined with the diluted Lipofectamine™ and incubated for a further 20 minutes to enable complex formation. Media from tissue culture growth flasks was then aspirated and replaced with the Lipofectamine, Opti-MEM and siRNA mix (5ml) to the A2780 and A2780cis cell lines and incubated at 37°C in a $CO_2$ incubator. 24hours post transfection, the media was replaced with complete growth media. Cell lysates were then collected at 24 hours, 36 hours and 48 hours. Lysates were then verified via western blot analysis to confirm the knock down of the gene. A suitable window was then selected for each gene

## 3.6 Cell proliferation and Sensitivity Assays

### 3.6.1 MTS Assay

The MTS assay is a colorimetric assay designed for the quantification of viability in toxicity assays, particularly with chemotherapy drugs. This assay was used to analyse cell viability with in cisplatin-sensitive A2780 cells and cisplatin-resistant A2780cis cell, in both control and knock down conditions when treated with Cisplatin (provided by Nottingham City Hospital) and Olaparib (kindly provided by AstraZeneca Pharmaceuticals) (PARPi).

For the control condition, A2780 and A2780cis cells were seeded (3000 cells/$cm^2$) in 96-well plates and incubated for 24 hours (37°C in a 5% $CO_2$ atmosphere). In the siRNA knock down condition, cells were also seeded in the same way 24 hours post transfection. Following incubation, Cisplatin was added to samples at concentrations of 0, 2, 4, 6, 8, 10µm and Olaparib (PARPi) was added in concentrations of 0, 0.01, 0.1, 0.5, 1, 5, 10µm and incubated in the same conditions for 48hrs. It was ensured

that siRNA knock down conditions were drugged on the selected day of transient knock down (day 3). Afterwards, Cell Titer Aqueous One Solution Reagent (G3580, Promega, UK) was added into each well and left to incubate for 2 hours. The absorbance was recorded at 490nm in the 96well plate reader. Averages were recorded in biological replicates and ran in triplicate and analysed in graphpad prism.

### 3.6.2 Clonogenic Assay

Clonogenic assays serve as a particularly useful method in the analysis of the survival clonogenic survival of cancer cells when treated with chemotherapy. This assay gives insight into real-time reproductive death and the effectiveness of a drug's cytotoxicity. A2780 and A2780cis cells clonogenic survival in both control and siRNA knock down samples were analysed upon treatment of Cisplatin and Olaparib.

In the sensitivity assay 32 cells/$cm^2$ were seeded in 6-well plates and left at 37°C in a 5% CO2 atmosphere over night. Cisplatin 0, 2, 4, 6, 8, 10µm and Olaparib 0, 0.01, 0.1, 0.5, 1, 5, 10µm were then added at the indicated concentrations and the plates were left at 37°C in a 5% $CO_2$ atmosphere for 14 days or until the cells present in the control plates (0 µm) have formed colonies that are of a substantial size (50 cells per colony is the minimum for scoring. Following this the plates were washed with PBS, fixed and stained with a crystal violet, acetic acid and methanol solution and colonies were counted.

## 3.7 Functional Studies – Flow Cytometry

Functional studies by means of flow cytometry were used to analyse levels of Cisplatin and Olaparib induced double strand breaks (DSBS), apoptosis and cell cycle arrest in control A2780 and A2780cis cells and siRNA knock down cells.

### 3.7.1 Double Strand Breaks

$1.05\times10^4$ Cells/$cm^2$ were seeded in 6- well plates and left overnight at 37°C in a 5% $CO_2$ atmosphere. Next, Cisplatin or Olaparib were added to controls and siRNA

knock down A2780 and A2780cis cells and incubated for 24hrs at 37°C in a 5% $CO_2$ atmosphere. Cells were collected by trypsinization, washed with ice cold PBS and fixed in 70% ethanol for 1h at -20°C. After removal of the fixative solution by centrifugation cells were stained with 2 μg/ml of phospho-Histone (γH2AX) Ser139 (16202A, Millipore, UK) to detect DSBs. Cells were treated with 20 μg/ml RNase A (12091021, Invitrogen) and then 10 μg/ml Propidium Iodide (P4170, Sigma Aldrich) was added to determine the cell cycle distribution. The samples were analysed on a Beckman-Coulter FC500 flow cytometer using a 488nm laser for excitation and emission data for PI collected using a 620nm bandpass filter (FL3) and a 525nm bandpass filter (FL1) for FITC-anti-phospho-Histone H2A.X.

### 3.7.2 Apoptosis

For Apoptosis assay, cells were analysed using Annexin V detection kit (556547, BD Biosciences). Briefly, cells were trypsinized, washed with PBS and the cellular pellet was re-suspended in Anexin Binding Buffer (1×). Following this, 2.5 μl of FITC Anexin V and 2.5 μl of Propidium Iodide were added to the cells. After incubation 300 μl of Anexin Binding Buffer (1×) was added to each tube. All the samples were analysed on a Beckman-Coulter FC500 flow cytometer. The percentage of apoptotic cells was determined (late apoptotic = FITC-Annexin V positive, PI positive early apoptotic = FITC-Annexin V positive, PI negative; live cells = FITC-Annexin V negative, PI negative) and compared for untreated and treated samples. Graphical representation and statistical analysis was performed in GraphPad Prism 7 (GraphPad, La Jolla, USA).

## 3.8 Immunohistochemistry (IHC)

Immunohistochemistry is a widely utilised technique which holds much scope in the pathological analysis of a variety of diseased and healthy tissues. IHC analysis was used to render clinical correlations and significance of experimental results.

Immunohistochemical labelling of formalin-fixed paraffin embedded tissue micro array sections was carried out on a variety of breast and ovarian blocks, initially concentration of antibodies and incubation time was optimised, and appropriate conditions selected. Slides were laid on a hotplate at 60°C for 10minutes in order to fully melt the wax on the sections. Following this, slides were placed in a Leica auto-stainer and followed the dewaxing program: 2x5minutes Xyelene, 3x2minutes IMS, 2 minutes deionised water, 5 minutes tap water. Following this, antigen retrieval was performed: a 1% citrate solution was prepared, and pH was adjusted to 6 as appropriate. Slides were then boiled in the microwaved for 20minutes in the citrate solution. Slides were then cooled for 5minutes with tap water, and then a hydrophobic barrier was marked out on the slide to ensure maximum staining. Sections were washed 2x5minutes with TBS-Tween and then incubated with 300µl of peroxidase block (Leica Biosystems) and then washed 3x5minutes TBS-Tween. The section was then incubated with 300µl of protein block (Leica Biosystems) and washed as before. The slide was then incubated with the selected primary antibody at the optimised concentration and duration. $\beta$2 microglobulin at 1:2000 was used as a control antibody for staining, and testis were used as the control tissue. See table 7 for detailed antibody dilutions. 3x5minute washes followed and then an incubation with 300µl of post primary antibody for 30 minutes, 3x5minute washes and then a 30minute incubation with 300µl of Polymer and further wash cycle. DAB substrate/buffer was then made to a 1:20 concentration and sections were incubated in 300µl for 5 minutes, with 3 washes following. Finally, 300µl of haematoxylin was

added and incubated for 6 minutes. Slides then followed the final stage of dehydrating and clearing in the Leica Autostainer, following a program of 5 minutes wash in tap water, 3x2minute IMS and 2x5minutes in Xylene. Slides were then scored using the H-score system and verified with a trained Pathologist. This was then analysed against clinical data sets and statistical analysis was performed.

Table 7 Antibodies used for IHC analysis of ovarian TMAs

| **Antibody** | **Species** | **Company/Clone** | **Concentration Used** | **Incubation Time** |
|---|---|---|---|---|
| XPG | Monoclonal Mouse | Thermo Fisher (8H7) | 1:100 | 1hour |
| XPF | Polyclonal Rabbit | Novus Biologicals – NB100-60689 | 1:200 | Overnight – 18 hours |
| ERCC1 | Monoclonal Mouse | Dako – Clone 49F M3648 | 1:50 | 1hour |

## 3.9 Statistical Analysis

Data are displayed as mean ± SEM. Normally distributed data was statistically analysed using a two-way ANOVA and multivariate analysis unless otherwise stated. A P value of $<0.05$ was considered significant.

# 4 Chapter 4: Results

## 4.1 Screening and Selection of Cell Lines

### 4.1.1.1 ERCC1 Protein Expression

Protein analysis of ERCC1 depicted the highest levels of protein expression in SKBR3 and T47D breast cell lines, and both A2780 and A2780cis ovarian cell lines. When compared directly to its resistant clone (A2780cis), A2780 cells exhibited significantly lower expression of ERCC1 protein (figure 10).

### 4.1.2 XPF Protein Expression

Protein level analysis of XPF revealed particularly high levels of XPF expression in SKBR3, T47D (breast) and A2780cis (ovarian) cell lines. Interestingly, there was a significant difference in the expression of XPF in A2780 cells and its cisplatin-resistant clone A2780cis. Similarly, there was a highly significant difference in the expression of XPF in the ovarian PEO1 and PE04 cell lines, showing high and low levels of protein expression respectively. Both OVCAR3 and OVCAR4 demonstrated low level expression of XPF, whereas breast cell lines 231 and 468 exhibited midline expression amongst the cell cohort. When comparing overall levels of protein expression, ERCC1 expression is notably higher in all cell lines than that observed in XPF expression. Previous studies suggest that there is an overriding role of ERCC1 (McNeil, 2012) and that it plays the pivotal role in DNA excision, thus supporting the data that suggests its protein is present in higher levels (figure 10).

### 4.1.3 XPG

Following the same protocol in western blotting analysis, no protein expression of XPG was observed in any of the breast or ovarian cell lysates. Due to this several antibodies were tested from different companies, using different clones and a band failed to appear. The antibodies were all also tested on HeLa cells, which are stated to provide a clear XPG band at 187KDa (Tilllhon *et al*., 2012) however, again no

band was observed. Because of this lack of protein expression and inability to have a band appear, mRNA analysis followed to confirm any presence of XPG in these cell lines. Literature suggests that the XPG protein is present and observed via western blot in both breast and ovarian tissues (Aracil *et al*., 2013) but in particularly low levels compared to its counterpart ERCC1-XPF. XPG has also been observed in IHC analysis in a variety of tumour types, with microarrays of 28 cores showing 60% positive staining. The highest staining was found in breast tissue, with 9 out of 10 breast tumours showing XPG expression and all 5 ovarian samples staining positive for XPG (Aracil *et al*., 2013).

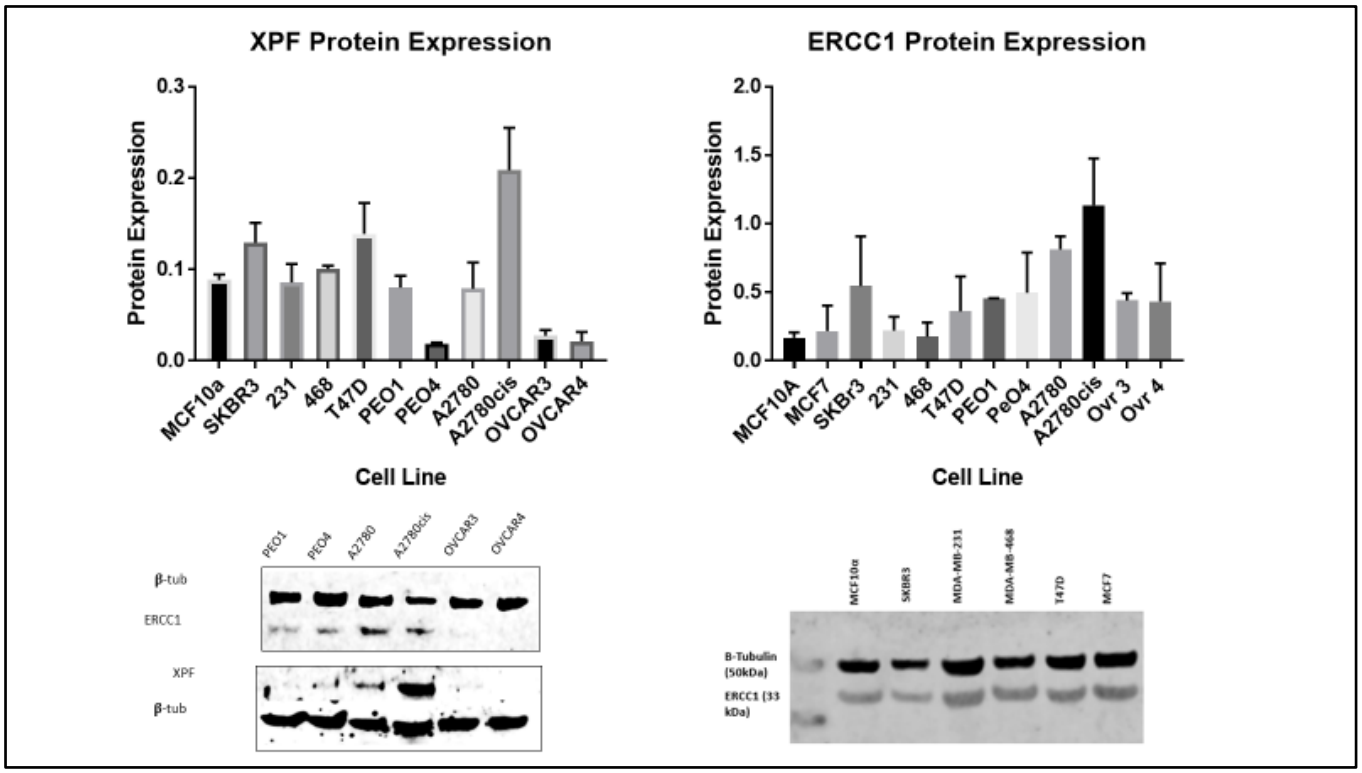



Figure 10 Western blot analysis of the protein expression of ERCC1 and XPF in a panel of breast and ovarian cancer cell lines

## 4.2 mRNA Expression

After the initial screening of cell lines via western blot, the results were analysed and T47D, SKBR3, MCF7, MCF10a, A2780 and A2780cis cell lines were selected for further analysis. This was due to the significant difference in expression of the

ovarian cell lines A2780 (exhibiting lower expression in ERCC1 and XPF when compared to its resistant counterpart) and A2780cis its cisplatin resistant clone (showing high levels of expression in both ERCC1 and XPF). The similarity in expression levels between ERCC1 and XPF is expected data as they form a heterodimer and can be expected to be present in similar levels throughout the cells. MCF10$\alpha$ cell lines were selected as a non-malignant control for the breast cell lines. Quantitative analysis by means of real time polymerase chain reaction (rtPCR) provided insight into the mRNA expression levels of the selected proteins in each cell line.

### 4.2.1 ERCC1

Expression analysis of the levels of ERCC1 mRNA of the breast and ovarian panel revealed the highest expression levels in the A2780 and A2780cis ovarian cell lines, exhibiting a significantly higher expression level than SKBR3, MCF7 and the non-cancerous MMCF10a breast line. When comparing T47D to MCF10a there was a significant increase in expression of ERCC1 at the mRNA level. Similar levels of protein expression were observed with the A2780 and A270cis cells lines, suggesting that ERCC1 mRNA expression may be indicative of protein expression (figure 11).

### 4.2.2 XPF

Unlike its counter-part ERCC1, XPF demonstrated the highest and most significant increase of mRNA expression in the MCF7 cell line when compared to the control breast cell line (MCF10a). A2780 and A2780cis revealed an overall higher expression of XPF mRNA than ERCC1 mRNA, however the expression levels remained similar, with neither the cisplatin resistant or the cisplatin sensitive line showing an increased expression. These results vary from those observed in protein

expression analysis, where there were higher levels of XPF protein expressed in the A2780cis cell line than the A2780. Overall the expression of XPF at mRNA level was higher than the expression of ERCC1 was, differing to the observed protein expression of the two genes (figure 11).

### 4.2.3 XPG

Despite protein analysis via western blotting providing no evidence of XPG being present in any of the cell lines, it was decided that the mRNA levels should be checked. Overall, the observed expression of XPG in the various cell lines was seen to be higher, on average than ERCC1. The most significant results were observed between A2780 and A2780cis cell lines, where by there was a ~4-fold increase in XPG levels in the cisplatin resistant cell line.

Despite high levels of XPG mRNA being observed, it was decided that the research into XPG would not carry forward on to functional studies or further protein analysis, as it was not feasible to continue to use a protein that could not be viewed on western blot, thus preventing data being gained. Consequently, analysis of results and consideration of further experiment revealed that A2780 and A2780cis ovarian cancers cells were the most promising cell lines to carry forward in the analysis of ERCC1 and XPF. Many studies have shown links with ERCC1 as a clinicopathological biomarker for various cancers, however there has not yet been any wide scale analysis into ERCC1 in ovarian cancers. This was particularly attractive due to fact that direct comparisons could be drawn between the platinum sensitive A2780 ovarian cells and its platinum resistant sister A2780cis cells (figure 11).

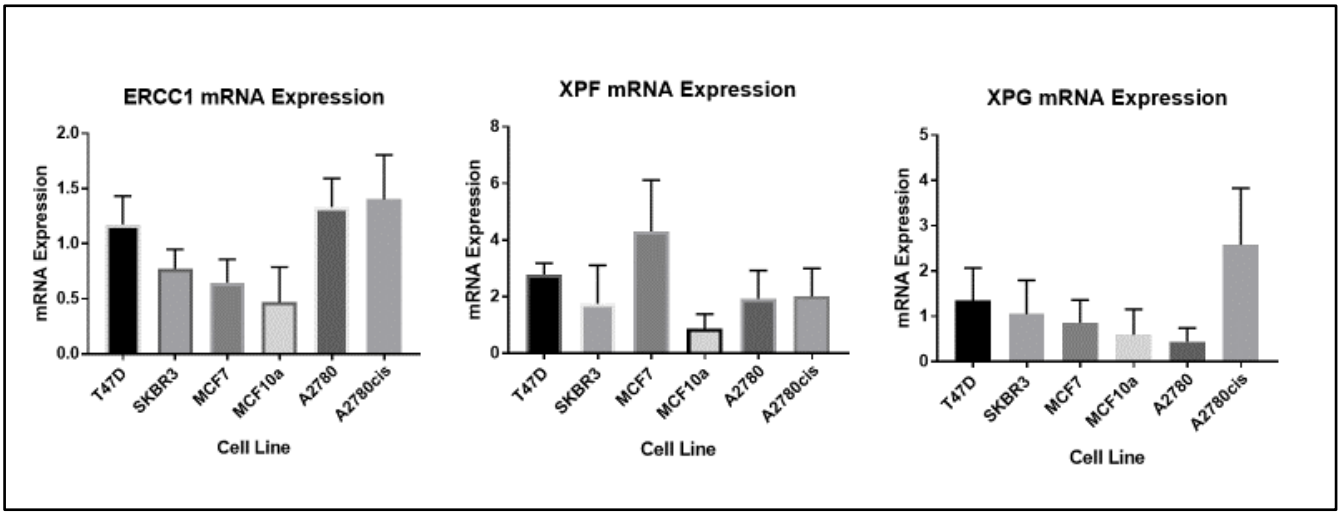



Figure 11 mRNA expression of a panel of ovarian and breast cancer cell lines. Data gained from RT-qPCR analysis of whole cells.

## 4.3 Immunohistochemistry Analysis

### 4.3.1 ERCC1 Expression in Ovarian Cancers

Ovarian TMAs were kindly provided by Nottingham City BioBank, of the samples on the block a total of 235 tumours were deemed suitable for analysis of ERCC1 expression level. Of these samples analysed, 114/235 (48.5%) tumours were graded as low in ERCC1 expression levels and 121/235 (51.5%) of the tumours were graded as high ERCC1 expression. High ERCC1 level was significantly associated with serous cystadenocarcinomas ($p = 0.000053$), higher FIGO stage at presentation ($p = 0.049$) and higher tumour grade ($p = 0.047$). It was also noted that high ERCC1 expression was associated with poor progression free survival (PFS) ($p=0.001$) and overall survival (OS) ($p=0.05$) (Appendix 1).

### 4.3.2 XPF Expression in Ovarian Cancers

Using the same criteria, tumours on the block were assessed for viability for analysis of XPF expression levels; A total of 229 tumours were suitable for analysis. Following

statistical analysis of this data it was deemed that there was no significant association between XPF and clinicopathological variables or survival (Appendix 1).

### 4.3.3 XPG Expression in Ovarian Cancer

Using the same criteria, tumours on the block were assessed for viability for analysis of XPG expression levels. Following statistical analysis of this data it was deemed that there was no significant association between XPG and clinicopathological variables such as overall survival or progression free survival (Appendix 1).

### 4.3.4 ERCC1-XPF Co-expression and Ovarian Cancer

When ERCC1 and XPF data was combined together (206 samples), ERCC1+/XPF- were more likely to be serous cystadenocarcinomas (p = 0.005)). Patients with ERCC1-/XPF- tumours had a favourable PFS and OS compared to ERCC1+/XPF- tumours (Appendix 1).

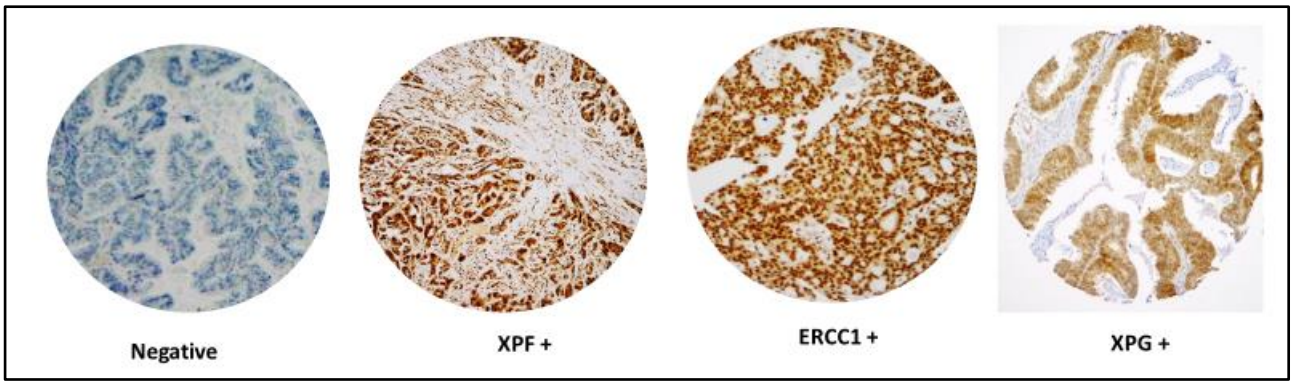



Figure 12 ERCC1, XPF and XPG positive staining of ovarian TMA cores, kindly provided by Nottingham BioBank and scored by Dr Michael Toss.

## 4.4 Pre-Clinical Studies

Following the initial screening of the cell lines and the promising data gained from clinical analysis of ovarian cancer TMAs; experimentation proceeded forward to pre-clinical analysis of the two selected cell lines. In particular, the protein and mRNA evidence suggest that ERCC1 is a clear biomarker for ovarian cancers (Bogush,

2015), predicting various features such as progression free survival and overall survival of patients. More specifically, its deficiency is a strong indicator of platinum sensitivity in ovarian cancers.

Previous research into patients with platinum sensitive ovarian cancers has linked the use of Olaparib therapy to the improvement of progression free survival. Therefore, using this logic it was speculated that ERCC1 deficiency may not only be predictive of platinum therapy response, but also to Olaparib therapy.

### 4.4.1 Protein Expression in Cisplatin and Olaparib Treated Whole Cell Lysates

Before progressing on to functional studies, the protein levels of ERCC1 and XPF were assessed in whole cell lysates of A2780 and A2780cis cells. This was used to give a general idea of the protein distribution in the cell lines upon 24hours of treatment with either Cisplatin 5µM or Olaparib 10µM. Drug concentrations were selected from data sheets and previous analysis.

Cisplatin treatment (5µM) was used to assess the protein levels of the two selected genes in both cisplatin resistant (A2780cis) and cisplatin sensitive (A2780) ovarian cancer cell lines. The A2780 cisplatin treated cells revealed a noted increase in ERCC1 expression, whereas in the resistant cell line A2780cis there was an overall similar level of ERCC1 expression observed. When looking at XPF expression after cisplatin treatment there was no change in the expression levels in A2780 cells, however there was an observed decrease in XPF expression in the A2780cis cell 24 hours post cisplatin treatment (figure 13).

When treated with 10µM of olaparib there was an increase in ERCC1 expression in the A2780 cells, where the A2780cis cells demonstrated a decrease expression of ERCC1. Furthermore, similar to the effect of cisplatin treatment, XPF levels remained

similar in the A2780 cells post olaparib treatment and there was a noted decrease in XPF expression in A2780cis cells (figure 13).

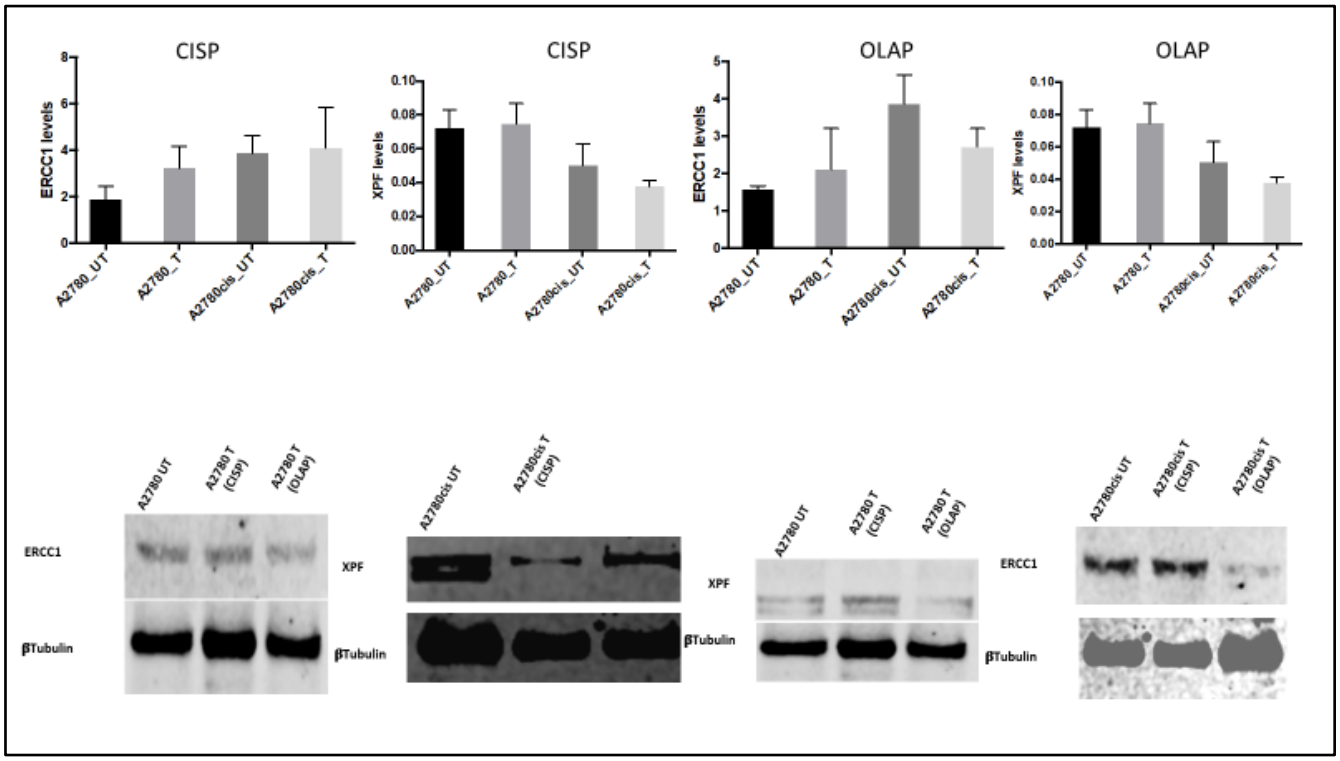



Figure 13 Protein analysis via western blot of the expression of ERCC1 and XPF in A2780 and A2780cis whole cell lysates after treatment with 10uM olaparib and 5uM cisplatin.

### 4.4.2 Cisplatin and Olaparib treatment Enhance ERCC1 and XPF nuclear subcellular localization.

After the whole cell lysate analysis, instead of following a whole cell lysis protocol, the following results come from the extraction of both the nuclear and cytoplasmic fractions of the cells, in treated and untreated samples at time intervals of 24 hours and 48 hours. This process allows the distribution of the proteins around the cell to be analysed under different conditions. As ERCC1 and XPF function fundamentally in DNA repair, it is expected that they are nuclear associated proteins. Theory would suggest that treatment by a platinum compound i.e. Cisplatin and its induction of DNA damage would increase the subcellular localisation of these DNA repair

proteins into the nuclear fraction of the cell. Results of this process were confirmed by western blot analysis of protein levels.

#### 4.4.2.1 Olaparib Treatment

Olaparib treatment was used to analyse the effect of PARP inhibition on the sub cellular localisation of ERCC1 and XPF, the different fractions of the cell – cytoplasmic and nuclear – were assessed individually for their protein expression at time points of 24 and 48 hours.

The A2780cis cells treated with olaparib revealed a significant increase in ERCC1 expression in the nuclear fraction compared to the cytoplasmic fraction at 48hours post treatment ($p = <0.05$), with a slight increase in protein expression observe 24 hours post treatment. In turn, the cytoplasmic fraction of the A2780cis cells reflected at decrease in ERCC1 expression at both 24 hour and 48 hours post treatment, reflecting nuclear localisation of the two DNA repair proteins in the resistant ovarian cancer cell lines (figure 15). Further to this, there was an observed increase in nuclear expression of XPF 48 hours post olaparib treatment accompanied by a cytoplasmic decrease in XPF in A2780cis cells (figure 16).

Upon analysis of A2780 cells under olaparib treatment, there was an overall reduction in ERCC1 nuclear protein expression in the 24hour treated samples compared to the non-olaparib treated controls. In the cytoplasmic fraction of the A2780 cells there was a decrease in cytoplasmic expression of ERCC1 at 24 hours post treatment, however at 48hours there was an increase in the cytoplasmic expression of ERCC1 (figure 14). The nuclear expression of XPF in A2780 cells reflected an overall decrease associated with an increase in cytoplasmic XPF expression at 48hours post treatment, suggesting cytoplasmic localisation of XPF upon olaparib treatment in a platinum sensitive ovarian cancer (figure 16).

PARP1 localisation provided insight into the effect of PARP1 inhibitors and the distribution of PARP within the two ovarian cancer cell lines. Despite no statistically significant data, there were very high levels of PARP expressed in the nuclear fraction of A2780 cells (40x that of the cytoplasmic fraction) (figure 14). Despite this, upon treatment (48 hours) with olaparib there was a significant increase in PARP1 expression in the nuclear fraction of A2780cis cells ($p = <0.05$) (figure 15).

#### 4.4.2.2 Cisplatin Treatment

Cisplatin treatment of A2780 and A2780cis cells reveals information on the expression of the two NER proteins, XPF and ERCC1 in the cisplatin sensitive and resistant cell lines. Cisplatin induces DNA lesions that are repaired by the nucleotide excision repair pathway, a resistance to cisplatin suggest an increase in DNA repair of the lesions via NER proteins (ERCC1 and XPF), thus the localisation of these proteins upon cisplatin treatment is suggestive of the resistance status of the cell.

A2780cis – the cisplatin resistant ovarian cancer cells exhibited low levels of ERCC1 expression when treated with cisplatin, with a noted decrease in expression at 48 hours compared to the untreated samples. Following suite, there was a significant increase observed at 24 hours post cisplatin treatment of ERCC1 expression in the nuclear fraction ($p = <0.05$) and at 48hours treatment ($p = <0.01$) (figure 18). Similarly, an overall decrease in cytoplasmic XPF expression was observed at 24 hours and 48hour post cisplatin treatment, with an increase in nuclear localisation of XPF exhibited at 48 hours (figure 19). This data revealed that in the cisplatin resistant cells there is a cisplatin association subcellular localisation of the ERCC1 and XPF protein into the nucleus after 24- and 48-hours treatment.

Further to this, A2780 cells demonstrated a slight increase of ERCC1 expression in the cytoplasmic fraction, however at 48hours post treatment there was a noted

decrease in cytoplasmic expression. However, in the nuclear fraction the expression of ERCC1 remained the same in the treated and untreated samples at 24 hours, however a noted increase was observed 48hours post cisplatin treatment (figure 17). XPF expression in A2780 cells presented no characteristic increase or decrease in either the cytoplasmic or nuclear fractions post cisplatin treatment, with overall levels remaining similar (figure 19).

Further to these results, it was decided to analyse the localisation of the PARP1 protein in the resistant and sensitive cell lines under both treatment methods. Upon cisplatin treatment there was no significant association found between PARP1 localisation in either of the cell lines. However, there were very high levels of cytoplasmic PARP1 being expressed at 24hours in both treated and treated A2780 cells (figure 17).

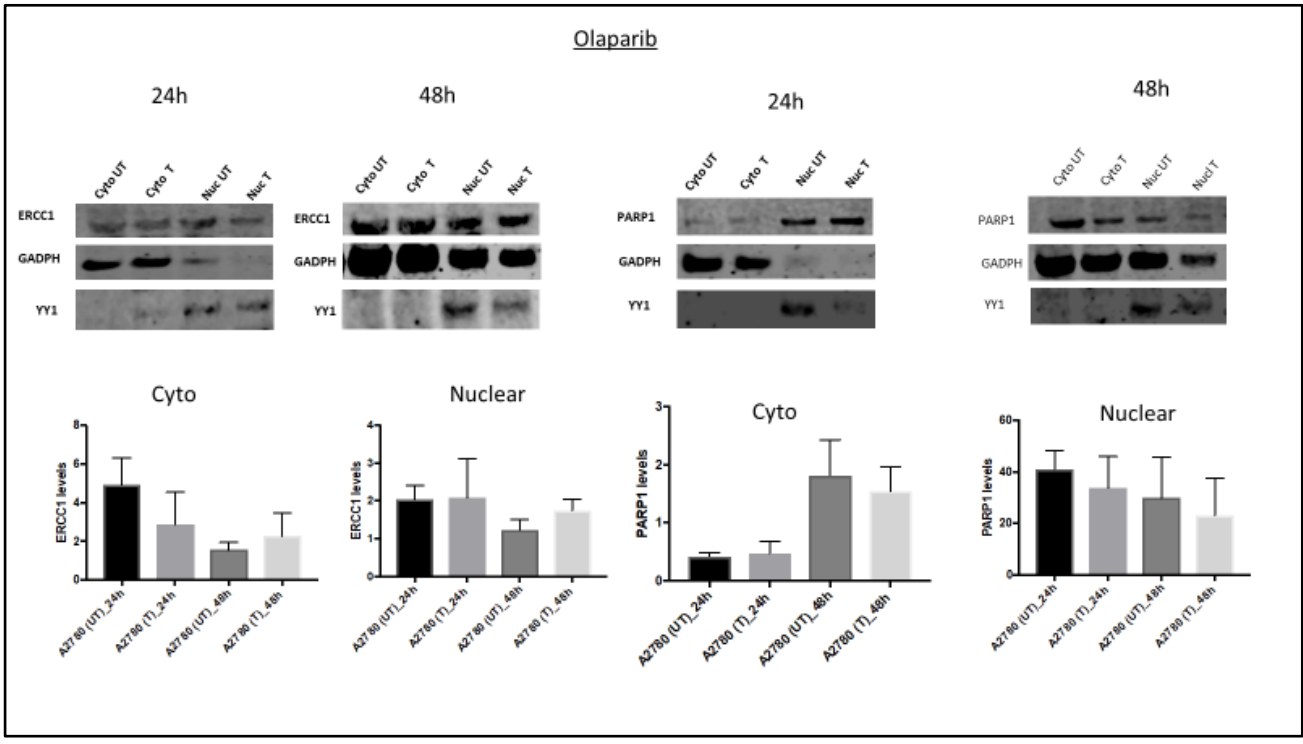



Figure 14 Western blot analysis of the subcellular localisation of ERCC1 and PARP1 in A2780 cells treated with 10uM olaparib at 24 and 48 hours.

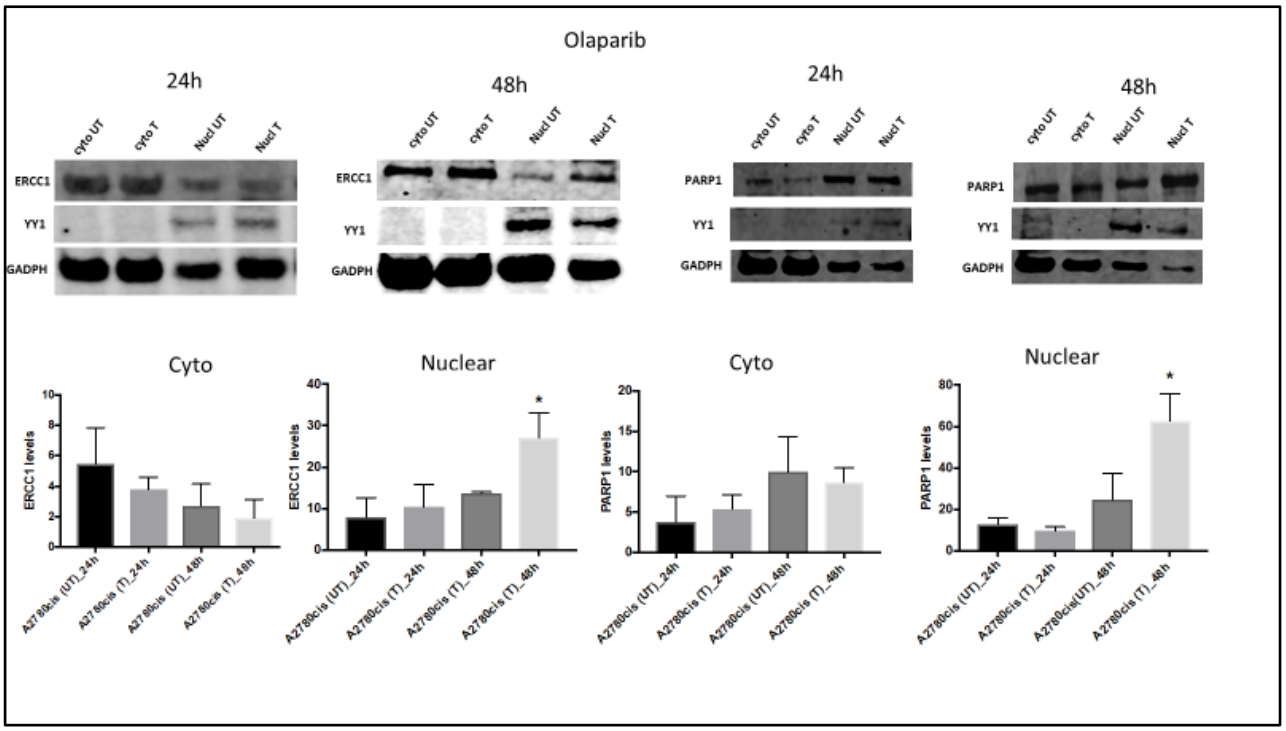



Figure 15 Western blot analysis of the subcellular localisation of ERCC1 and PARP1 in A2780cis cells treated with 10uM olaparib at 24 and 48 hours. Many thanks to Dr Katia Almeida Mesquita for compiling western blot images.

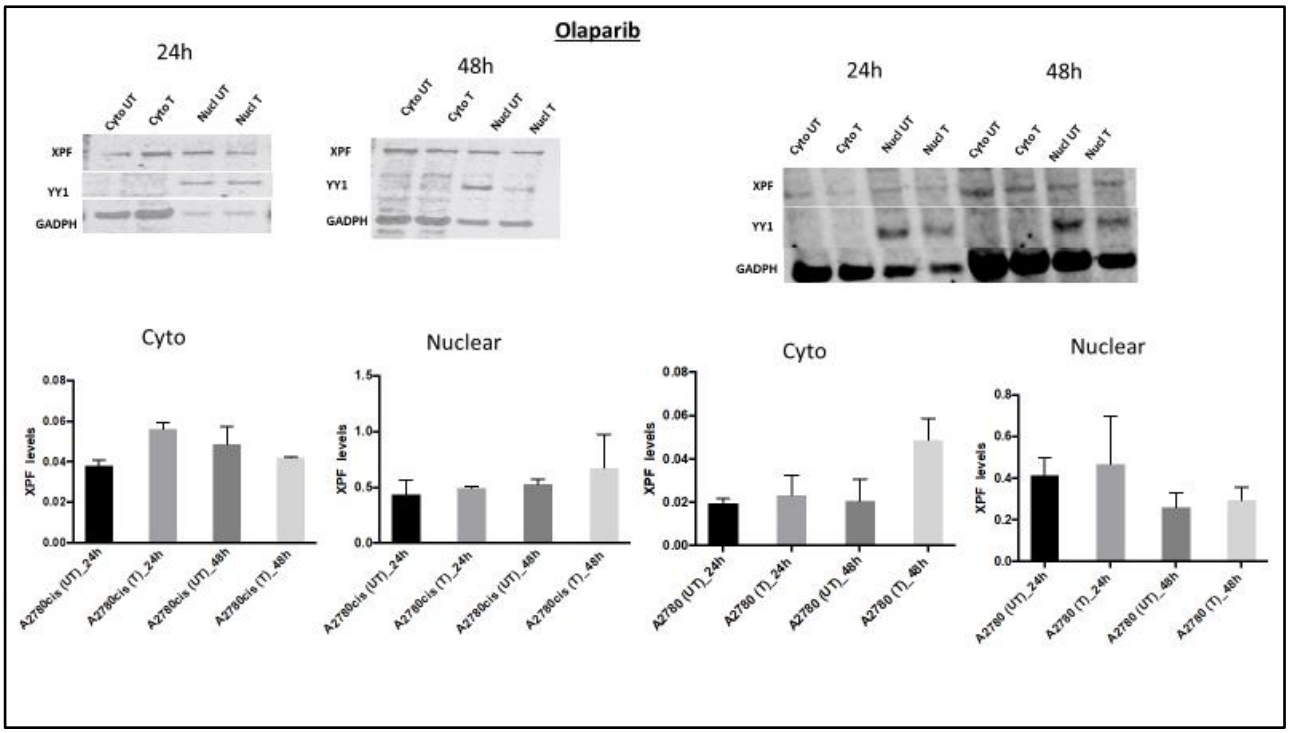



Figure 16 Western blot analysis of the subcellular localisation of XPF in A2780cis and A2780 cells treated with 10uM olaparib at 24 and 48 hours.

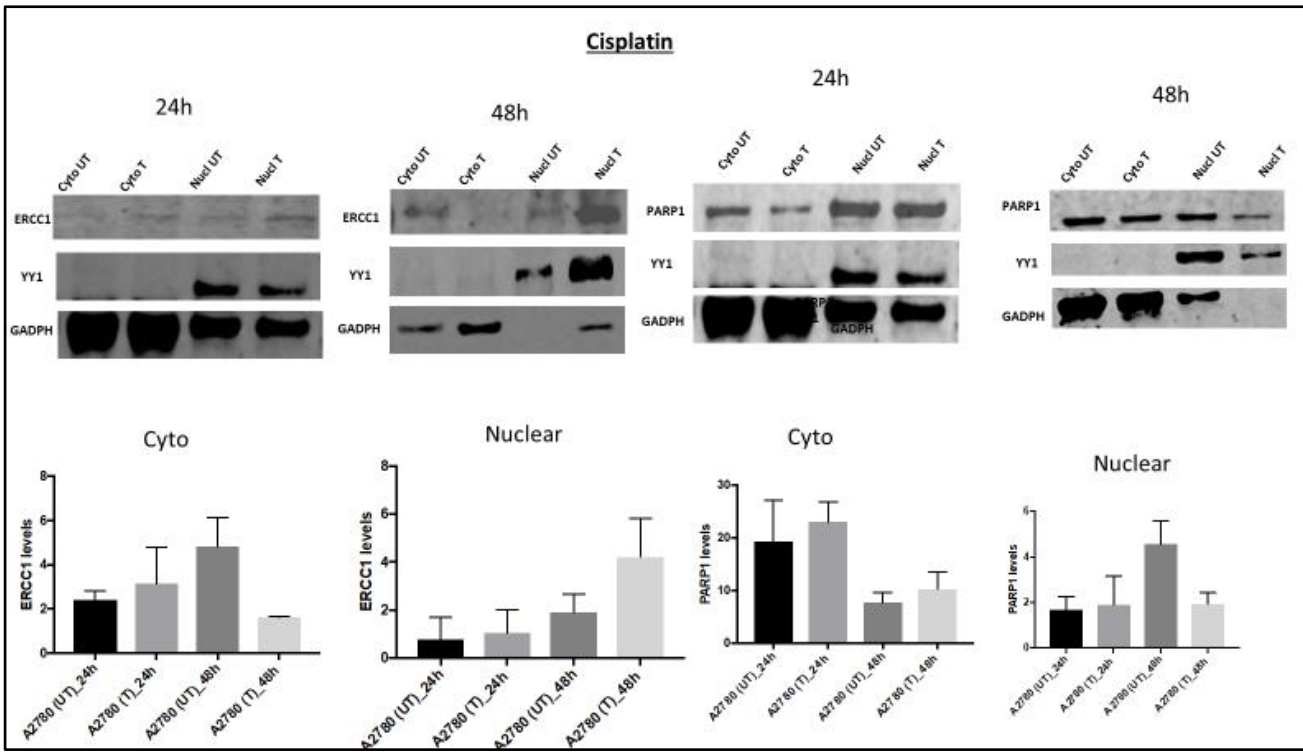



Figure 17 Western blot analysis of the subcellular localisation of ERCC1 and PARP1 in A2780 cells treated with 5uM cisplatin at 24 and 48 hours.

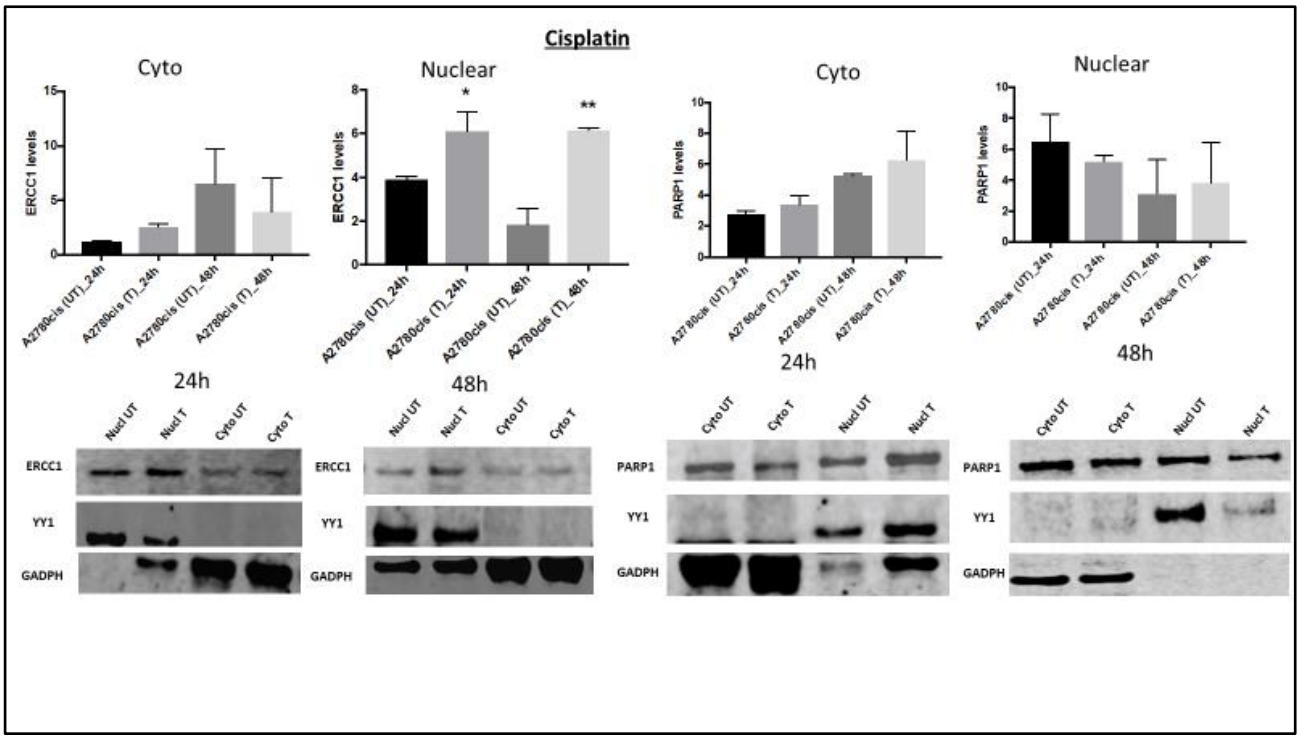



Figure 18 Western blot analysis of the subcellular localisation of ERCC1 and PARP1 in A2780cis cells treated with 5uM cisplatin at 24 and 48 hours

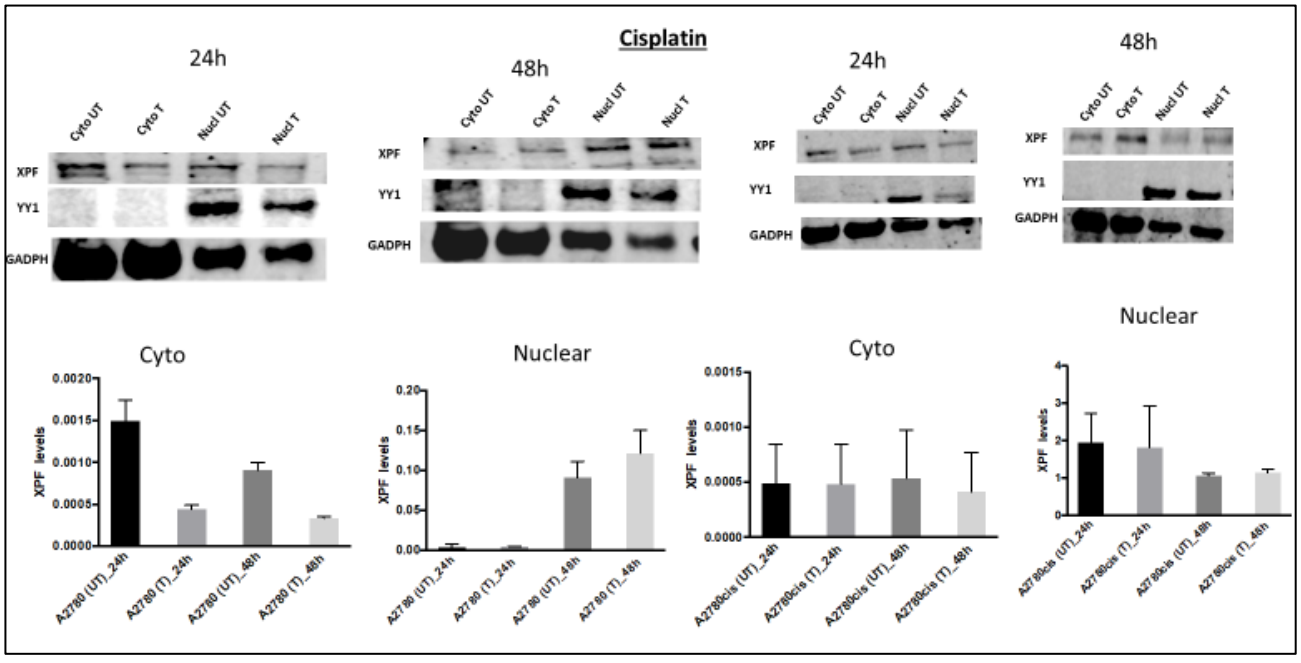



Figure 19 Western blot analysis of the subcellular localisation of XPF in A2780cis and A2780 cells treated with 5uM cisplatin at 24 and 48 hours

## 4.5 siRNA Post-transcriptional Gene Knock-down

Clinical data provided evidence that a deficiency of ERCC1 in ovarian cancers was predictive of better PFS and OS, therefore it was speculated that the transient post-transcriptional knock down of the XPF and ERCC1 genes would produce similar data. Depletion of ERCC1 and XPF protein levels, in theory should hinder the cells NER pathway, therefore the ability to repair cisplatin induced DNA lesions, leading to effective killing of the cell by chemotherapeutic agents.

### 4.5.1 ERCC1 Knock Down

Three different constructs of ERCC1 siRNA were tested and analysed via western blot for a complete knock down of the gene and subsequent protein product and a suitable construct and window of time was selected. Analysis revealed a >90% reduction in ERCC1 protein at 72 hours. This was then selected as the experimental window (figure 20).

### 4.5.2 XPF Knock Down

Similarly, 2 constructs of XPF siRNA were analysed, with a subsequent protein reduction of >85% of XPF at 72 hours (figure 20).

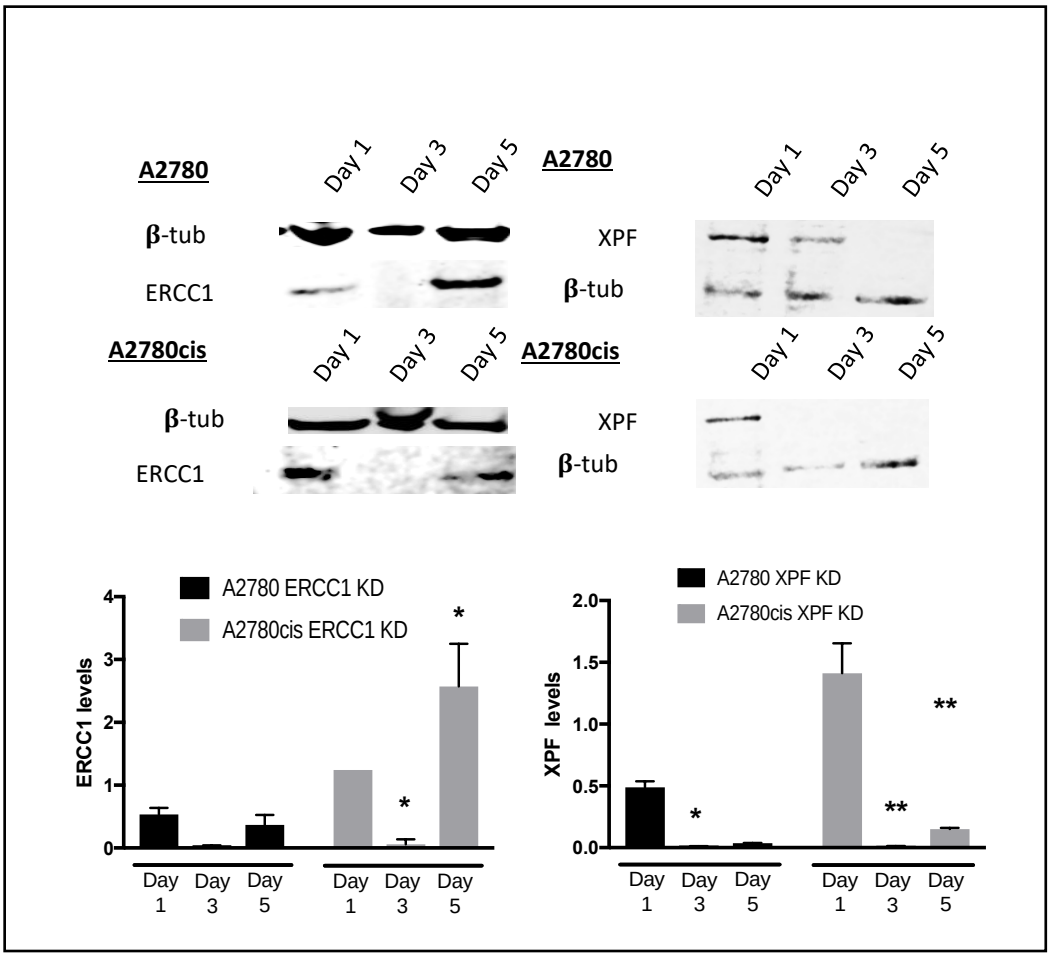



Figure 20 siRNA post-transcriptional knock down of XPF and ERCC1 in A2780 and A2780cis cell lines

## 4.6 Cell Viability Assays – ERCC1 depletion increased platinum sensitivity in ovarian cancer cells

### 4.6.1 MTS Cell Proliferation Assay

To assess the viability of cells after XPF and ERCC1 genes were knocked down, MTS cell proliferation assays were completed on untreated, treated (Cisplatin and Olaparib) and both knock down and non-knock down cell lines.

Upon treatment with cisplatin as typically observed, the highest level of sensitivity of the cohort was observed in the A2780 control cell line when compared to the A2780cis control cells. The cisplatin treated ERCC1 deficient cell lines demonstrated that when ERCC1 is knocked down the A2780cis-ERCC1KD cell line become more sensitive to cisplatin treatment, with a significant reduction in the survival fraction observed at all treatment points- 2, 4, 6 and 8 μM ($p < 0.001$). Similarly, the ERCC1

deficient A2780KD cells demonstrated a significant reduction in survival at 2 μM treatment ($p < 0.001$) (figure 21). Furthermore, when XPF was knocked down in A2780 cells there was a significant reduction at 2 μM treatment ($p < 0.001$). Moreover, in the A2780cis resistant cell line, there was a significant movement into sensitivity of the cell line, with overall reduced survival at 2, 4 and 6μM treatment ($p < 0.001$) when XPF was knocked down (figure 22). This data suggests that inhibiting both ERCC1 and XPF in cisplatin resistant ovarian cancer cell lines produces an overall increase in the sensitivity of the cell lines to the drug, with a significant reduction in survival.

When treated with Olaparib, the ERCC1 deficient A2780 and A2780cis cells exhibited the lowest level of sensitivity, with no significant shift of survival fraction observed in this condition (figure 21). Furthermore, when XPF was knocked down in A2780 cells there wasn't a significant reduction in survival fraction after statistical analysis, however there is noted drop in the percentage of surviving cells at 2, 4 and 6μM olaparib. Despite this, when XPF was knocked down in A2780cis cells there was a significant reduction in the survival fraction at all treatment points 2, 4, 6, 8 and 102μM treatment ($p = < 0.001$) (figure 22).

These results are indicative that inhibition of ERCC1 has a significant effect on the sensitivity of the resistant A2780cis cells when subjected to treatment with cisplatin. Therefore, ERCC1 deficiency potentiates the cytotoxicity of cisplatin in resistant cell lines. Similarly, there was a noted movement into sensitivity with the XPF deficient A2780cis cells. However, there was no significant correlation between ERCC1 deficiency in A2780cis cells when combined with PARP inhibition.

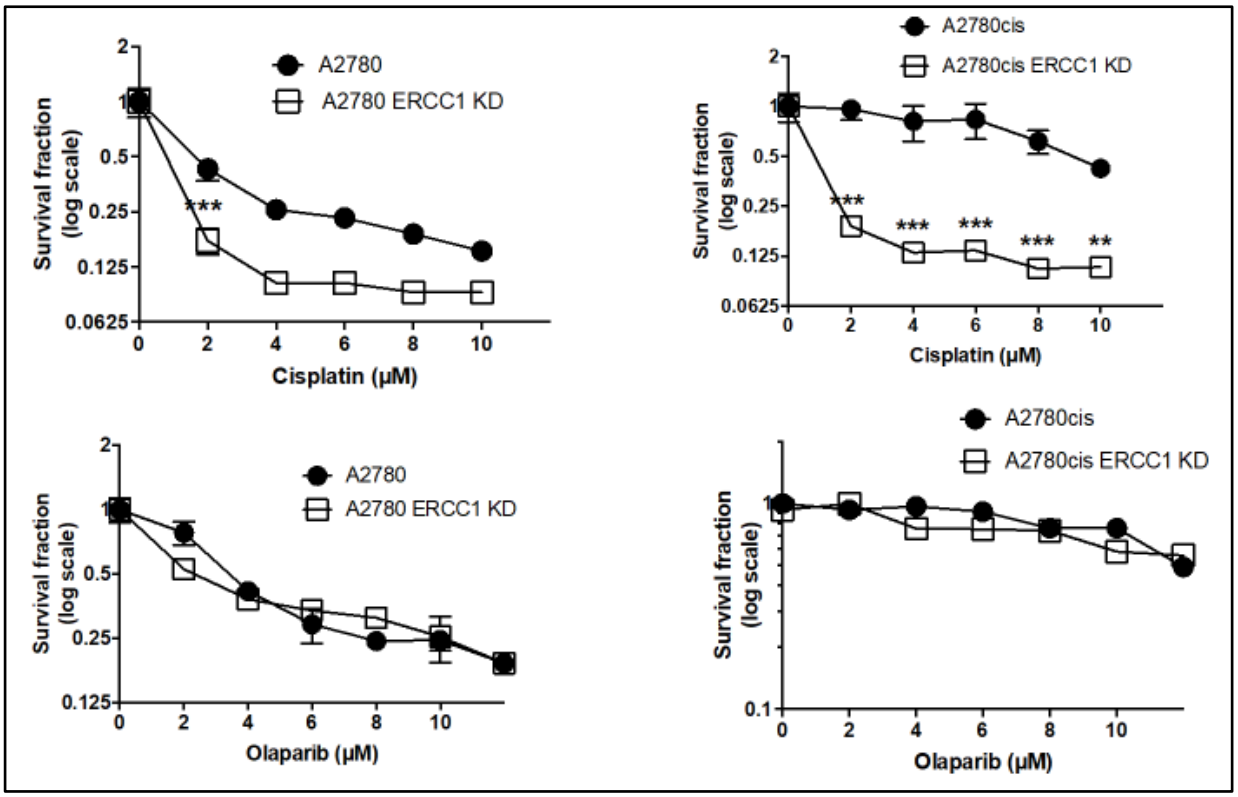



Figure 21 MTS survival assay of ERCC1 deficient A2780 and A2780cis cells treated with cisplatin 5uM or olaparib 10uM

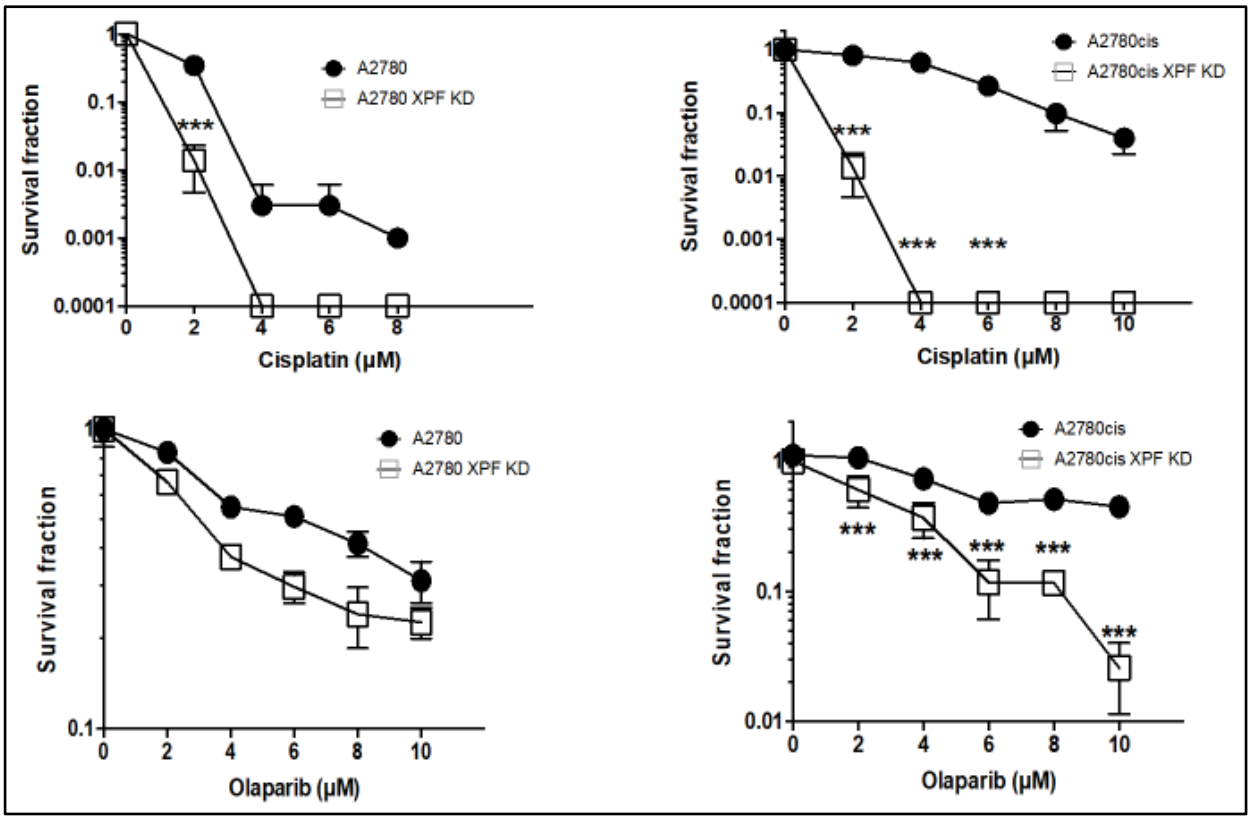



Figure 22 MTS survival assay of XPF deficient A2780 and A2780cis cells treated with cisplatin 5uM or olaparib 10uM

### 4.6.2 Clonogenics Sensitivity Assay

To assess the effect of post transcriptional knock down of both ERCC1 and XPF on the sensitivity of A2780 and A2780cis cell lines to Cisplatin and Olaparib, a clonogenic assay was performed. The viability of cells was investigated in both transfected and non-transfected cells at varying drug concentrations. This analysis allows for speculation on the long-term effects of the drug and the ability of the cells to form colonies (of 50 cells or more) after extended exposure to both Cisplatin and Olaparib, providing more realistic model for survival. The results of the clonogenic survival assays are summarised in figures 23 and 24.

Individual knock downs of ERCC1 and XPF in platinum resistant cell line A2780cis produced an overall shift towards cisplatin sensitivity, with the greatest shift

demonstrated in the ERCC1 deficient cells compared to control cells. When treated with cisplatin, the ERCC1 deficient A2780cis cells show a significant reduced survival fraction at 4μM ($p = <0.01$) and 6 and 8μM cisplatin treatment ($p = <0.001$). Upon analysis of the platinum sensitive cell line, A2780 a minor shift into sensitivity was observed in the ERCC1 deficient cell, however upon statistical analysis these results were not significant (figure 23). When the XPF was knocked down the A2780cis cells there was a significant reduction in survival at 8μM ($p = <0.01$) and in the A2780 cells there was a significant reduction at 2μM treatment ($p = <0.01$) and 8μM ($p = <0.05$) (figure 24).

Similarly, when exposed to Olaparib treatment, both the ERCC1 and XPF depleted A2780cis cells presented an overall reduction in survival fraction at all treatment points ($p = <0.01$). Moreover, ERCC1 deficient A2780 cells has significant reduction in survival fraction at all treatment points 2, 4 and 6μM ($p = <0.001$), 8μM ($p = <0.01$) and 10μM ($p = <0.05$) (figure 23). The platinum sensitive cell line again revealed reduced cellular survival with the XPF deficient cells at 4 and 8μM ($p = <0.01$) and 6μM ($p = <0.05$) (figure 24). It can be concluded from this data that ERCC1 and XPF deficiency can result in platinum resistant cell lines shifting into sensitivity with both Cisplatin and Olaparib treatment. Upon treatment with Cisplatin and Olaparib, ERCC1 deficiency is selectively toxic in ovarian cancer cells. And a synthetically lethal state is observed when NER and PARP are simultaneously inhibited.

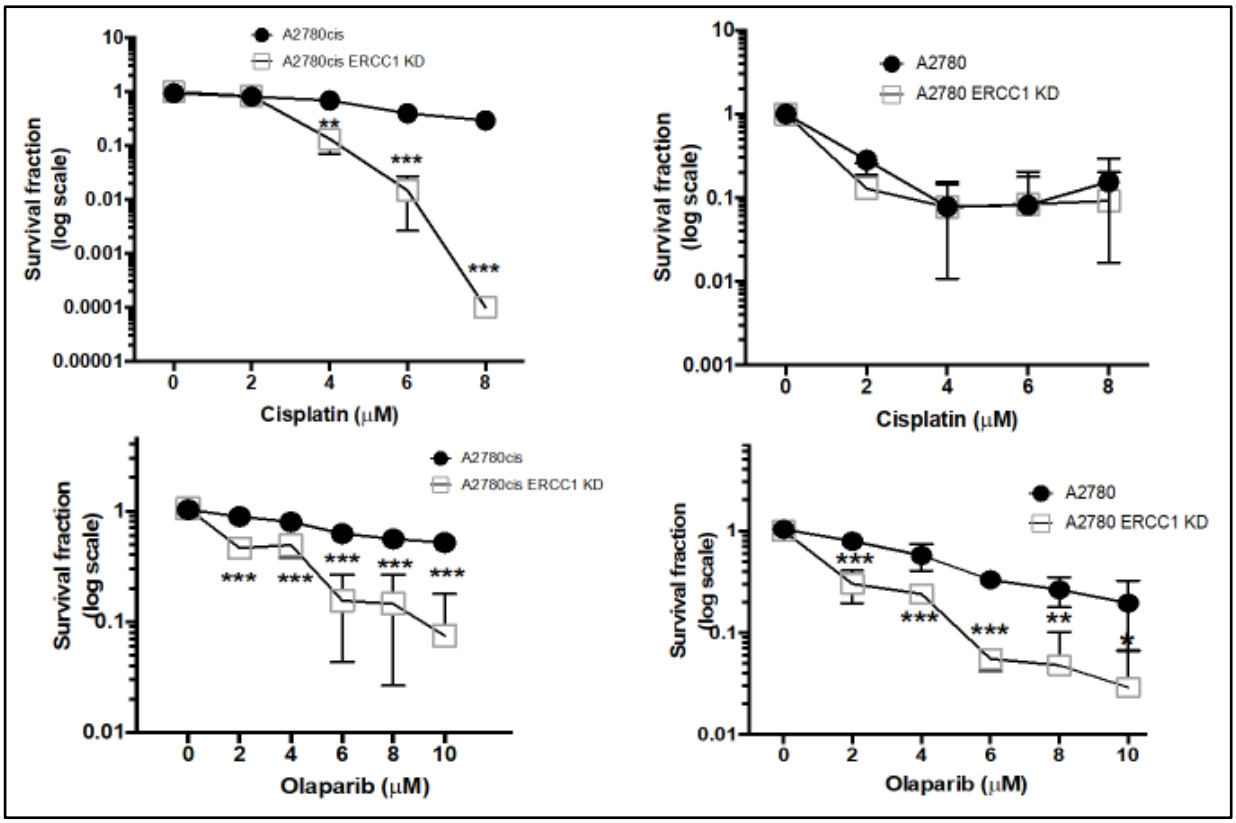



Figure 23 Clonogenic survival assay of ERCC1 deficient A2780 and A2780cis cells treated with cisplatin 5uM or olaparib 10uM

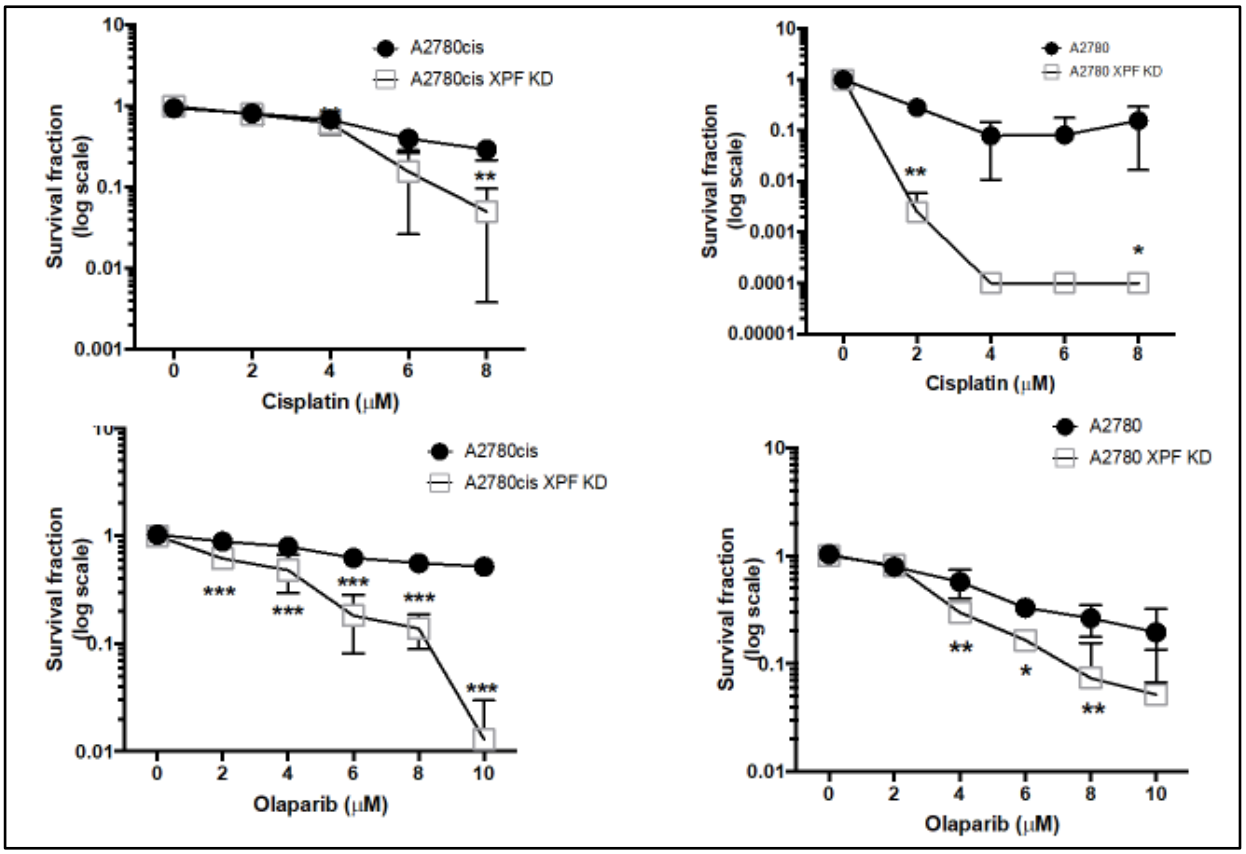



Figure 24 Clonogenic survival assay of XPF deficient A2780 and A2780cis cells treated with cisplatin 5uM or olaparib 10uM.

## 4.7 Functional studies

Following the results of the sensitivity assays and the data demonstrating that ERCC1 deficiency actively increases the sensitivity to Cisplatin, it was decided that analysis should focus on the functional effect of ERCC1 depletion at a DNA level.

### 4.7.1 Double Strand Breaks

DNA damage that induce double stranded breaks in the DNA results in the histone (H2AX) being phosphorylated. This phosphorylation occurs on the C-terminal Ser-139 of the H2AX molecule that flanks the DSB in the chromatin. This process is mediated by DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM) or ataxia telangiectasia related (ATR). The phosphorylated form, γH2AX appears in the chromatin as discrete nuclear foci, with each on indicating one double

strand break. Shortly after these DSBs occur they can be detected by immunocytochemistry. The intensity that is produced by the immunofluorescence of the γH2AX of each induvial cells' acts as a reporter of the frequency of the DSBs and therefore the extent of the DNA damage (Huang and Darzynkiewicz, 2006).

Double strand break analysis provides evidence and information about the repair capacity of the cell and how they are responding to treatment by the percentage of double strand breaks that are encountered and repaired by the cells. A lower level of double strand break present represents an efficient repair system in which cells are linked to resistance. Treatment with 5uM of cisplatin revealed no significant increase in the percentage of double stranded DNA breaks in either the resistant or sensitive cells when compared to the non-knock down cells. However, there was a significant difference observed between the resistance and sensitive cell lines ($p = <0.001$). Additionally, there was a significant difference in percentage of double strand breaks in the A2780cis cell line upon ERCC1 knock down ($p = <0.05$). At 48hours, ERCC1 knock down in both A2780 and A2780cis there was a significant increase in double strand breaks ($p = <0.05$). Similarly, there was a highly significant increase in double strand breaks in XPF deficient A2780 and A2780cis cells ($p = <0.001$ and $p = <0.01$ respectively) (figure 25).

Olaparib treatment conferred no significant increase in double stranded breaks of DNA in XPF depleted A2780 cells at 24 hours however, there was a significant increase in XPF depleted A2780cis cells ($p = <0.001$). Similarly, when ERCC1 was knocked down, there was no significant increase demonstrated in the sensitive A2780 cell at 24 hours post treatment, but there was significant increase in double strand breaks found in the resistant cell line (A2780cis) ($p = <0.05$). At 48hours post treatment there was significant increase in DSB accumulation in both A2780 and A2780cis ERCC1 deficient cells ($p = <0.001$). Similarly, this high level of DSB accumulation was seen in XPF deficient A2780 cells ($p = <0.001$) (figure 26).

Interestingly, the most significant increases were found in the cisplatin- resistant cell lines in both cisplatin and olaparib treatment. This data reflects previous studies that suggest that olaparib is a suitable choice for maintenance therapy of ovarian cancer patients with platinum resistant tumours.

### 4.7.2 Cell Cycle

Cell cycle analysis of A2780 and A2780cis cells was used to quantify the amount of DNA in each phase of the cell cycle after cisplatin and olaparib treatment, which reveals information on the damage state of the cell of ERCC1 and XPF deficient cells.

At 24hours post treatment, the control A2780 cells that were treated with cisplatin exhibited a significant $G_1/G_0$ arrest ($p = <0.01$), similarly when XPF was knocked down in these cells there was a highly significant accumulation of cells, signifying arrest at the $G_1/G_0$ stage ($p = <0.001$) and a significant S phase arrest observed ($p = <0.001$). In the XPF deficient A2780cis cell line there was an extended S phase observed, however it was not deemed to be statistically significant under the analysis. The ERCC1 deficient A2780 cells exhibited a significant cell accumulation and S phase arrest ($p = <0.05$). Similarly, the A2780cis ERCC1 knockdown cells demonstrated a significant G1/G0 accumulation and S phase arrest ($p = <0.05$). Data suggest that when ERCC1 and XPF are knock down in cells, therefore inhibiting NER the A2780cis cells undergo S phase arrest when treated with cisplatin. Furthermore, when subject to 48hours cisplatin treatment there was a significant S phase arrest observed in ERCC1 deficient A2780 cells ($p = <0.01$) and A2780cis cells ($p = <0.05$). The 48 hour treated XPF deficient A2780 cells exhibits a significant accumulation at the G1 phase ($p = <0.01$) (figure 25).

Due to the interest in synthetic lethality, it was decided that cell cycle analysis would be performed on olaparib treated A2780 and A2780cis cells, to give an insight into the behaviour of then cell when inhibiting two DNA damage repair pathways. In 24 hour control of the XPF deficient A2780 cells a significant reduction in G0/G1 phase was noted when comparing treated to non-treated cells ($p = <0.05$). This was also followed by a significantly extended S phase ($p = <0.01$). There were no significant results noted in the 24 hour olaparib treated A2780cis cells in either XPF deficient or proficient states. Further to this, analysis on ERCC1 knock down in A2780 demonstrated no significant trend or change in cell cycle upon olaparib treatment. However, in the A2780cis cells there was a significant accumulation noted at the G2 phase when ERCC1 was knocked down ($p = <0.05$) when compared to the untreated cells. Moreover, there was an observed reduction in the percentage of cells present in the G0/G1 phase in this 24hour condition ($p = <0.05$). Additionally, after 48 hours olaparib treatment there was significant $G_2$ arrest seen in ERCC1 knock down A2780cis cells ($p = <0.05$) and G1 arrest in A2780cis XPF knock down cells ($p = <0.01$) (figure 26).

### 4.7.3 Apoptosis

To further investigate the cytotoxic effect of cisplatin and olaparib on A2780 and A2780cis cells, they under-went analysis for their apoptotic properties following these treatments. Both the cisplatin and the olaparib treated cells were stained with PI and Annexin V. One of the earliest features of apoptosis in a cell is the loss of the plasma membrane asymmetry, the membrane phospholipid phosphatidylserine (PS) is translocated to the outer area of the plasma membrane. Annexin V can specifically bind to the PS found in an apoptotic cell membrane, and PI stains the DNA that is found in necrotic cells in which the membrane has disintegrated.

This assay is widely utilised as a method to assess the viability of cells after various drug treatments and conditions. In this case, it provides information regarding the

rate of cellular death of A2780 and A2780cis cells deficient in ERCC1 and XPF under treatment of cisplatin and olaparib. It allows for a direct comparison of apoptosis between the cisplatin resistant and sensitive cell lines when subject to NER inhibition. It can also be directly assessed along-side the use of synthetic lethality in the treatment of these cell lines.

Staining of cells with Annexin V and PI revealed the apoptotic capacity of the two cell lines. Upon 24 hour 5uM cisplatin treatment there was a significantly higher level of apoptosis in the XPF deficient A2780 cells when compared to the non-knock down A2780 cells ($p = <0.05$). Similarly, A278cis XPF deficient cells had a significantly higher level of apoptosis when compared to control cells ($p = <0.05$). These results were further potentiated when ERCC1 was knocked down, showing a notedly significant increase in apoptosis in both the resistant and sensitive cell lines ($p = <0.01$). Similarly, when taking into consideration the resistance status of the two cell lines, there is a considerably higher apoptotic rate in the sensitive cell lines than is observed in the A2780cis resistant cell lines. This date is in continuum with what is already developed to be a known trend and validates the use of the controls in this study. When subject to 48hours cisplatin treatment there was a significant increase in the percentage of cells entering apoptosis in the ERCC1 deficient A2780 ($p = <0.001$) and A2780cis cells ($p = <0.01$). Similarly, XPF deficiency conferred significant increase in apoptosis in A2780 cells ($p = <0.05$) (figure 25).

Upon cisplatin treatment the overall percentage of cells that had entered apoptosis was higher in the ERCC1 deficient cell lines than those with an XPF deficiency.

When the cell lines were subjected to PARP inhibition by Olaparib treatment a significant increase in the fraction of the cells undergoing apoptosis was seen in the A2780 XPF deficient cells ($p = <0.01$) at 24 hours. However, there was no noted association between the XPF depletion of the A280cis cells and treatment by olaparib. Despite this, there were significant association found at 24hours when

ERCC1 was knocked down in both cell lines, showing a significant movement in to apoptosis in in both A2780 ($p = <0.1$) and A2780cis cell lines ($p = <0.001$) when both PARP and ERCC1 are inhibited. There was a notedly more significant increase apoptotic cell fraction in the resistant cell line suggesting that inhibition of both homologous recombination and nucleotide excision repair of the resistant cell line confers a movement into sensitivity. Moreover, at 48 hours treatment there was a significant increase in apoptosis in both A2780 and A2780cis ERCC1 knock down cells ($p = <0.01$ and $p = <0.001$ respectively), and again this increase into apoptosis was observed in the XPF deficient A2780 cells at 48 hours ($p = <0.01$) (figure 26).

When comparing the results of cisplatin and olaparib treatment, they both exhibit a similar movement into sensitivity, particularly when ERCC1 is knocked down the resistant cell line.

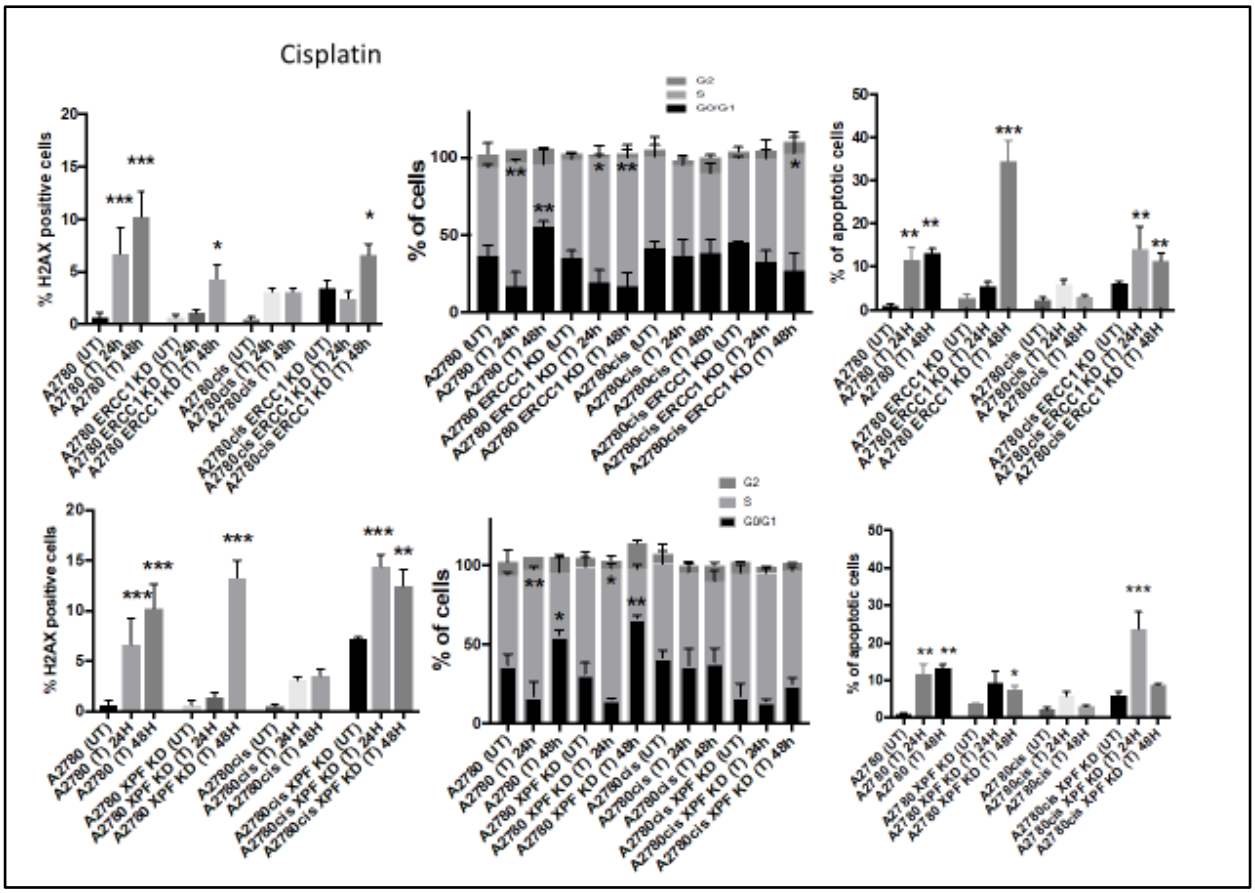



Figure 25 Functional analysis of ERCC1 and XPF knock down A2780 and A2780cis cells upon 5uM cisplatin treatment. Double strand break analysis via H2AX percentage, percentage of apoptotic cells and cell cycle analysis. Many thanks for Dr Katia Almeida Mesquita for assistance in analysis.

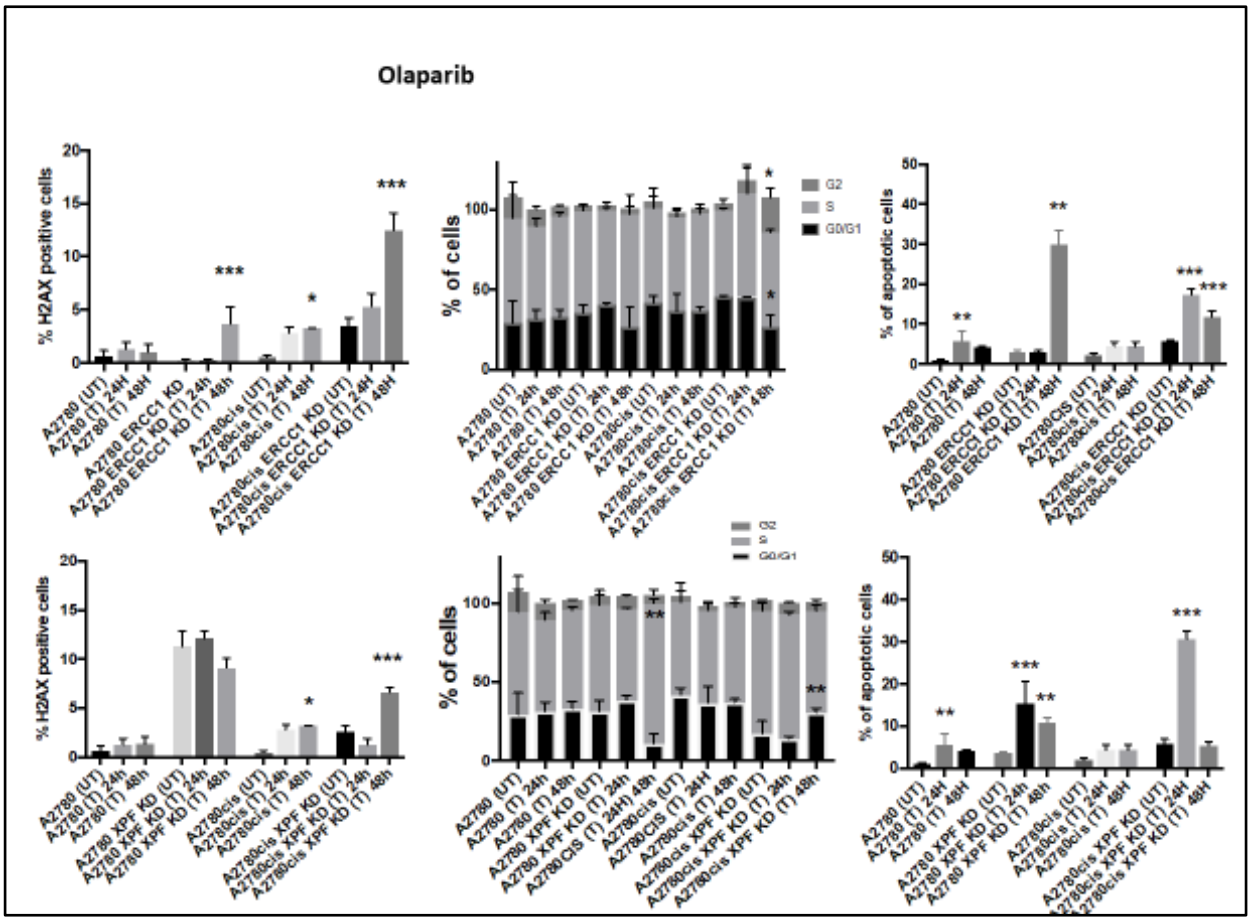



Figure 26 Functional analysis of ERCC1 and XPF knock down in A2780 and A2780cis cells upon 10uM Olaparib treatment. Double strand break analysis via H2AX percentage, percentage of apoptotic cells and cell cycle analysis. Many thanks for Dr Katia Almeida Mesquita for assistance in analysis.

# 5 Chapter 5: Discussion

## 5.1 ERCC1 is Independently Associated with Ovarian Cancer Prognosis and Cisplatin Resistance

This study aimed to further validate the concept and current data findings surrounding the usage of DNA repair inhibition in the potentiation of cisplatin-based chemotherapy. Platinum-based therapy is the first-line approach in treating ovarian cancers; unfortunately, resistance to this therapy is a major issue (Ai *et al*., 2016). If the potential sensitivity of platinum chemotherapy and associated biomarkers that link to prognosis can be determined, it will lead to individualised therapy. As such, the combination of clinical and pre-clinical analysis of ovarian cancers and certain biomarkers can protect patients from inappropriate chemotherapy and reduce the occurrence of secondary resistance. As research progressed the idea of inducing synthetic lethality became promising. Previous research has already validated the use of cisplatin and olaparib in a dual treatment, providing solid grounding for the inhibition of two repair pathways, one via PARPi and one by siRNA knock down to induce lethality in ovarian cancer cells.

The initial approach combined the screening of breast and ovarian cancer cell lines for their expression of the genes of interest to determine the most appropriate lines to work with. The selected lines, A2780 and A2780cis were deemed the most appropriate as analysis could be formed on the parent cell line (A2780) and its cisplatin resistant clone (A2780cis) to gain the most direct comparison possible. Early mRNA and protein analysis revealed markedly higher levels of the selected DNA repair endonucleases present in the A2780cis cell lines prompting interest into the mechanistic and biological factors that influence resistance in ovarian cancers. Complete transcript level analysis was performed, working alongside protein expression analysis. Literature suggests that the relative XPF and ERCC1

expression remains consistently similar throughout various cell lines. However, in this series of study it was found that the resistant cell lines had a decreased mRNA XPF level.

The human ovarian cancer cell line A2780 was established from tumour samples from a patient that received no treatment. A2780 cells are the parent cell line of the cisplatin resistant daughter cells A2780cis. Derived from an ovarian endometroid adenocarcinoma tumour (ATCC), this cell line is extremely popular in the analysis of drug toxicities in ovarian cancer. Since the patient to whom the tissue was taken from did not receive any prior treatment, it means that this cell line has not been previously exposed to any chemotherapeutics/anticancer drugs or chemicals. Therefore, it is an attractive cell line used in the analysis and observation of the potency and effects of treatments for ovarian cancer.

It was decided early on not to carry forward the research into the XPG endonuclease. This was after several failed western blot attempts where various antibodies were used and optimised, and the XPG band failed to be visualised. Despite the fact that a clear mRNA pattern and overall high mRNA expression was observed, it was decided that it was not logical to carry forward research into this endonuclease as successful protein analysis via western blot – the main confirmation method for a majority of the research, could not happen.

Immunohistochemistry analysis and consequent scoring of ovarian TMAs stained for ERCC1 and XPF revealed various significant associations between the two endonucleases and clinicopathological variables in ovarian cancers. 235 tumours were deemed suitable for analysis, presenting a larger cohort number that has previously been assessed in studies. Firstly, of the suitable cores, 48.5% stained as low in ERCC1 levels, and 51.5% stained as high in ERCC1 levels demonstrating an even split amongst the cores. Of the samples graded high in ERCC1 expression, there was a significant associated found with serous cystadenocarcinomas, a

clinically higher tumour grade and a poorer progression free survival and overall survival. Suggesting a link between increased tumour expression of ERCC1 and clinically poorer outcomes.

This clinical data gained has been validated in previous studies, finding a progression free survival of 17 and 39 months in patient samples with high and low ERCC1 expression respectively. Despite this, in that study there was no significant association with overall survival rates found within the high ERCC1 group compared to the low ERCC1 group (Du *et al*., 2016). In addition, a meta-analysis observed that when compared to patients that had a positive ERCC1 expression, patients who exhibited a negative ERCC1 expression has a significantly enhanced response to cisplatin treatment. This data is indicative that an individual's response to cisplatin chemotherapy is correlated with the status of ERCC1 expression (Fy *et al*., 2012).

A recent study on XWLC05 lung adenoma cells found that progression free survival was markedly higher in the ERCC1 negative lung cancer patients (30 months) than those who has high ERCC1 levels (11 months). Subsequently, this data identifies ERCC1 protein expression as a marker for prognosis of lung adenoma, more specifically the rate of overall survival (Wang *et al*., 2017).

In this research cox multivariate analysis of the cohort indicated that ERCC1 expression is an independent factor in the clinical variables associated with ovarian cancer. This study found ERCC1 to be independently linked to both progression free survival and cisplatin sensitivity, despite its partner XPF. Contrasting to this, A study of 92 cores identified that the expression of ERCC1 in ovarian cancer patients was not an independent or predictive factor that affects the prognosis or other clinical variables. It was found that ERCC1 expression in ovarian cancer was, in fact associated with resistance to cisplatin-based therapy, but there was no associated with overall survival with these patients (Du *et al*., 2016).

Upon analysis of XPF there was no significant association found between the gene and any clinicopathological factors in ovarian cancers. Despite this, other studies into ovarian cancers have noted that the expression of ERCC1 and XPF were closely correlated both at the protein and mRNA level, demonstrating an inverse association between mRNA levels and patient outcome (DeLoia *et al*., 2012). Another study found higher *XPF* mRNA expression could be associated with shorter PFS ($p$=0.204) and OS ($p$=0.271), but the associations were not statistically significant.

The main interest in targeting DNA repair and a means of potentiating platinum therapy in cancer chemotherapeutics initially sky rocketed upon the introduction of Olaparib – the PARP1 inhibitor. Since this, pre-clinical data has continually validated that ERCC1-XPF is an appropriate target to help modulate platinum therapy.

Data shows that ERCC1 is a key predictor of platinum resistance in a large clinical cohort of ovarian cancers.

## 5.2 Cisplatin Treatment Results in Nuclear Subcellular Localisation of ERCC1

The data gained from this study supports the hypothesised notions that ERCC1 in particular is a promising and scientifically sound target for enhancing the chemotherapeutic efficacy of cisplatin, particularly by combatting resistance in relapsed cases.

As observed by the clinical data, there was significantly higher nuclear staining of ERCC1 in a cohort of ovarian cancer tumours. Supported by other research, immunohistochemical staining of ESCC cells revealed that ERCC1 was expressed in the nuclei of 62.7% (64/102) of ESCC tissues compared with 9.8% (10/102) of normal squamous epithelial cells (Peng *et al*., 2018). This prompted the analysis of the protein expression of ERCC1 and XPF in the nuclear and cytoplasmic fractions of both A2780 and A2780cis cells when subjected to both olaparib and cisplatin

treatment. This research was carried out to determine whether treatment by cisplatin inducing DNA adducts, or inhibition of PARP by PARPi would alter the pattern of subcellular localisation of ERCC1 or XPF.

This study evaluated the ERCC1 and XPF levels in nuclear and cytoplasmic extracts from A2780 and A2780cis cells before and after 24/48 hours of cisplatin treatment. In untreated samples, nuclear levels of ERCC1 and XPF were significantly higher in A2780cis compared to A2780 cells. Whereas the cytoplasmic levels were lower in A2780cis compared to A2780 cells.

This research revealed a noted increase of ERCC1 in the nuclear fraction of the cell compared to the cytoplasmic fraction after 48hours 5μM cisplatin treatment in the cisplatin resistant cell lines. Western blot analysis of the subcellular localization of these two NER proteins revealed that there is an associated nuclear increase of them upon treatment with cisplatin. Following 24 hours of cisplatin exposure, there was a significant ($p = <0.05$) increase in the nuclear expression of ERCC1 in A2780cis cell lines, however there was no significant association between nuclear expression of ERCC1 and cisplatin treatment found in the A2780 cells. Furthermore, at 48 hours post treatment there was significantly ($p = <0.01$) increased ERCC1 expression levels in the nuclear fraction of A2780cis cells, accompanied by low cytoplasmic levels.

Upon olaparib treatment it was found that there was a significant increase of ERCC1 expression A278cis cells in the nuclear fraction, however there was no significant association found within the A2780 cells.

Whilst an increase in XPF was observed in the nuclear fraction in A2780cis cells, upon statistical analysis there was no significance found in this study.

This is indicative of the repair action of ERCC1 on the DNA adducts formed via cisplatin treatment, and thus an enhanced mechanism of repair subsequently leading to resistance.

Research into ERCC1 isoforms found that in wild type cells, ERCC1 was detectable in the nucleus, holding the ability to form nuclear foci – reflecting its role in basal repair processes of DNA damage sites. Of the four isoforms assessed, all 4 were found localised in the nucleus with nuclear foci forming abilities observed in the single-isoform clones. Isoform 203 interestingly produced a strong cytoplasmic signal. These results demonstrated that the differing isoforms of ERCC1 reflects patterns of expression that are seen to be compatible with the recognised biological functions and activities of endogenous ERCC1 that were both comparable and typical of its products (Friboulet *et al*., 2012).

Various other studies have found that XPF alone is unstable, and that in the absence of ERCC1 interaction, the NER pathway cannot carry forward. ERCC1 isoform studies have revealed a clear dependency of XPF on ERCC1 isoform 202 by western blot study of ERCC1 deficient cell lines. Despite this, ERCC1 loss had no impact on other crucial NER proteins (XPA and RPA). XPF dependency was confirmed via immunofluorescence microscopy, finding that ERCC1 was crucial for XPF to be detected in the nucleus. This study not only confirms the obligational role of ERCC1 in the recruitment of XPF to the nucleus, but also that one particular isoform is essential in doing so (Friboulet *et al*., 2012).

## 5.3 ERCC1 Depletion Leads to Cisplatin Potentiation, Altered Cell Cycle, Higher DSBs and Increased Apoptosis

Previous work from this study has provided clear evidence that low ERCC1 levels correlate with better clinical outcome, and that increased levels are associated with a poor prognosis. Using this evidence, it provided strong rationale for performing ERCC1 depletion studies alongside functional studies. XPF remains a valid point of investigation due to its formation of the heterodimer with ERCC1 during NER.

This study has shown that upon olaparib treatment there is no significant association between XPF knock down in the A2780cis resistant ovarian cancer cells and increase in apoptosis. However, there is a highly significant association when ERCC1 is knocked down in these resistant cells ($p = <0.001$) demonstrating overall increased levels of apoptosis in this condition. Therefore, this data is suggestive of ERCC1 playing a more fundamental role in NER than its heterodimeric partner XPF. It can be determined that XPF deficiency is not enough to inhibit nucleotide excision repair and confer synthetic lethality via simultaneous PARP1 inhibition by olaparib. These effects were observed in the cisplatin resistant cell line, A2780cis. Despite this, ERCC1 and PARP1 inhibition clearly suggests a role for synthetic lethality in treating ovarian cancer and potentially in the exploitation of PARPi, namely olaparib in maintenance therapy of platinum resistant ovarian cancers. It is interesting that ERCC1 has been reflected to play the essential role in NER and not its partner XPF, this claim is supported by many studies that have documented that ERCC1 is the fundamental nuclease in the process.

Further to this, cisplatin treatment revealed the typically observed pattern of apoptosis that has been observed in many other studies whereby the platinum sensitive cell line A2780 demonstrated an overall higher level of cellular death than the platinum resistant cell line. This is due to the efficient DNA repair that occurs in

A2780cis, in which the DNA adducts caused by cisplatin treatment are readily repaired by repair proteins such as ERCC1-XPF as part of the nucleotide excision repair pathway, thus leading to cellular resistance to cisplatin. Both XPF and ERCC1 knock down were significantly associated with apoptosis in both cell lines ($p = <0.05$ and $p = <0.01$ respectively) however there was a stronger correlation between the ERCC1 knock down upon cisplatin treatment. This data provides strong evidence that the inhibition of nucleotide excision repair via ERCC1 is a viable method in potentiating cisplatin toxicity and combatting chemotherapeutic resistance in ovarian cancer cells. Furthermore, this idea gives a fundamental basis for the development of ERCC1 inhibitors and to be paired alongside cisplatin chemotherapy in patients who have cisplatin resistant tumours. To select patients that would benefit from this dual treatment it would be beneficial to perform a biomarker screen for ERCC1 levels to identify those who express ERCC1 in high amounts.

Flow cytometry studies allowed for the analysis of the apoptotic rates of the sensitive and resistance cell under NER inhibition, PARP inhibition and cisplatin treatment. One of the earliest features of apoptosis in a cell is the loss of the plasma membrane asymmetry, the membrane phospholipid phosphatidylserine (PS) is translocated to the outer area of the plasma membrane. This exposure of PS to the external environment leaves it open to binding. Annexin V (the Ca2+ dependent phospholipid binding protein) has a high affinity for cellular PS and binds to the exposed PS on the surface of apoptotic cells, furthermore Annexin V can be conjugated with fluorochromes and still retain the high affinity levels for PS. These fluorochromes act as a sensitive probe that can allow cells that are undergoing apoptosis to be detected by flow cytometry.

To identify both early and late apoptotic cells, the Annexin V dye is accompanied by propidium iodide (PI). Loss of membrane integrity usually accompanies the latest stages of cellular death; therefore, Annexin V is suitable for detecting early stage

apoptosis. Viable cells, that still have intact membranes do not take up any PI, however cells that are dead or damage have membranes that are permeable to PI. If a cell is viable it will be negative for both PI and Annexin V, early apoptotic cells will stain positively to Annexin V and cells that are either dead or in the late stages of apoptosis will stain positively for both PI and Annexin V (Rieger *et al*., 2011).

Due to both staining positively for Annexin V and PI, this assay is not able to distinguish between different cellular death pathways i.e. cells that have been subject to death by apoptosis or those cells that underwent the necrotic pathway and consequently died. When apoptosis is measured over a period of time, the tracking of cells through different stages – no apoptosis, early apoptosis, late apoptosis and cell death via the staining level can be measured (Sawei and Domae, 2011).

The depletion of ERCC1 in platinum resistant A2780cis cells conferred a substantial increase in platinum sensitivity when undergoing functional analysis. This sensitization was made clear by the association with an accumulation of apoptotic cells, enhanced levels of double strand break accumulation ($p = <0.05$) and s- phase arrest in the cell cycle ($p = <0.001$). Similarly, when A2780 cells were analysed there was a moderate increase in their sensitivity to cisplatin upon siRNA depletion of ERCC1 showing a significant accumulation of cells undergoing S phase arrest ($p =< 0.05$). A significant difference in the accumulation of double strand breaks was observed between resistant and sensitive cell lines ($p = <0.001$).

XPF deficiency in A2780cis cells also conferred platinum sensitivity again rendering the cells subject to increased double strand breaks and apoptotic accumulation. Further to this, both control A2780 and XPF deficient A2780 cells were significantly associated with G1 and S phase arrest upon cisplatin treatment ($p = <0.001$).

Double strand breaks that occur in the G1 or S phase of the cell cycle will prevent or slow the S phase progression of the cycle. When the double strand breaks occur in

G2 phase, the progression onto mitosis is halted. Research has suggested that the DNA ICLs formed by cisplatin treatment do not have the ability to activate the G1/G2 cell cycle checkpoint, meaning no cell cycle arrest. Further to this, these lesions are tolerated by the cell until a replication fork is encountered, where cell cycle arrest then occurs.

However, recent research on the cisplatin treatment of HL60 cellls found that the cisplatin directly caused cell cycle arrest at the DNA synthesis stage (S phase) and the cells gradually began to accumulate at the sub G1 phase, suggesting the presence of a pre-apoptotic necrotic peak and the movement of cells into necrosis. It was also found that as the dosage of cisplatin was increased, there was a significant increase of cells moving into apoptosis. This confirms that the relationship between cisplatin treatment and levels of apoptosis are dose dependant (Velma, Dasari and Tchounway, 2016).

## 5.4 NER Deficiency Increases Sensitivity to Cisplatin and Olaparib

Clonogenic assay, also known as a colony formation assay is a routinely used technique as an in vitro cell survival assay. This assay is based on the ability of a single cell to grow into a colony, thus representing the ability to proliferate. A colony of cells is defined as a group of at least 50 cells. The assay provides information on the ability of individual cells to be able to undergo “unlimited” amounts of division – this directly correlates to the ability of a cancerous cell to be able to proliferate within the human body. This assay is the key assay of choice when examining cell reproductive death post treatment with chemotherapy. The results of the clonogenic assay were firstly used to confirm the sensitivity levels of both the A2780 and A2780cis cells to platinum treatment.

Pre-clinically, ERCC1 or XPF depletion not only increased cisplatin sensitivity but also increased cytotoxicity to olaparib (PARP1 inhibitor) therapy. The simultaneous

inhibition of the NER pathway – involved primarily in repairing bulky DNA adducts through ERCC1 and XPF depletion, and the inhibition of the homologous recombination via PARP inhibition by olaparib treatment has been noted to confer synthetic lethality in ovarian cancer cells. Significant reduction in the survival fraction was observed in the NER deficient A2780cis ($p = <0.001$) cells suggesting that there is a viable platform for the use of olaparib therapy to potentiate cytotoxicity in platinum resistance cancers.

Sensitivity studies via MTS and clonogenic assays revealed a significant association between the depletion of ERCC1 in resistance ovarian cancer cell line (A2780cis) and the potentiation of cisplatin therapy, increasing the sensitivity of the cells to the drugs. This data suggests that inhibiting both ERCC1 and XPF in cisplatin resistant ovarian cancer cell lines produces an overall increase in the sensitivity of the cell lines to the drug, with a significant reduction in survival. Together, the data suggests that ERCC1 deficiency is not only a predictor of platinum sensitivity but may also be utilised in the personalisation of targeted olaparib therapy in ovarian cancer patients.

Similarly, a study looking into cell viability assays confirmed that ERCC1 gene knock down in SKOV3 cells increased their sensitivity to cisplatin ($p<0.01$) along with functional flow cytometry analysis demonstrating that ERCC1 silencing promotes apoptosis. Upon analysis of the cell cycle, it was found that in ERCC1 deficient cells there was an extension of the G2 phase, whereas the G1 and S phase was reduced. Furthermore, the depletion of ERCC1 successfully reversed the cellular resistance to cisplatin, increasing the sensitivity of the cisplatin-resistant ovarian cancer cells (Du *et al*., 2015).

## 5.5 Olaparib Confers Synthetic Lethality in ERCC1 Deficient Cells

Previously studies on lung cancer have already confirmed that inhibition of PARP1 can confer synthetic lethality in ERCC1 deficient tumours. This, therefore initiated the rationale behind investigating the depletion of ERCC1 and XPF in ovarian cancer cells and the potential to use PARP inhibition in the sensitization of the cell to platinum therapy.

Results of this study found that there was, in fact a significant association with ERCC1 depletion and an increased level of olaparib sensitivity. This sensitivity was characterized by the increase accumulation of double strand breaks in ERCC1 deficient A2780cis cells ($p = <0.05$), with no significant association found in the A2780 ERCC1 knock down cells. Further to this, the olaparib treated ERCC1 depleted cells demonstrated a significant G2/M arrest alongside S phase arrest ($p = <0.05$). Analogous to the ERCC1 data, olaparib treatment also conferred sensitivity in the XPF deficient A2780cis cell lines producing and increase in double strand breaks ($p = <0.001$), however no significant association was observed upon analysis of XPF depletion of the platinum sensitive cell line. Likewise, significant S phase arrest was noted in A2780cis cells ($p = <0.001$).

## 5.6 Further Research

### 5.6.1 Development of Cell Culture Techniques

To further validate the results observed throughout the duration of this study, various other approaches and advancements in methodology should be utilised. This should not only include further drug assays and validation of the drugs chosen, but also additional cell culture work to introduce new conditions and parameters to provide added validation of results.

Firstly, all experiments should be repeated using 3D cell culture to mimic, as far as possible, the biological system that encapsulates and surrounds ovarian tumours so that they can be assessed in the most reliable way possible. Generally, growing cells as a monolayer (2D culture) is routinely used and is the primary choice for the analysis of cell lines and the evaluation of their behaviours when subjected to numerous conditions, namely drugs. This method usually precedes animal studies in preclinical analysis of drug development, however this method of study may not be as clinically sound as thought. In 2D culture cells are grown in supplemented growth environment, providing those cells with everything they need to become viable, this does not take into the consideration the various other factors that take on a role in a biological system. Therefore, because of this fact, results gained purely through 2D culture may not be representative of what is to be observed *in vivo* (Breslin and O' Driscoll, 2012).

3D culture, however allows for the development of models that are more analogous to those found in real tumours – giving a better idea of how these cells behave within a tumour like structure, and the innumerable interactions between cells. This method of cell culture allows for several different cell types to be co-cultured along with other cellular components that would normally be found in the natural tumour microenvironment – providing a much higher level of clinical significance. This, in the realm of anti-cancer agents, provides insight into their efficacy and effectiveness in cancer treatment in life-like models. In 2D cultures there is no signalling from cell to cell or between cells and the extracellular matrix, where as in 3D models these signals are observed to be essential for cell differentiation and various other cellular functions. Despite this, there are many parameters that need to be controlled and considered when using this method, particularly matrixes etc. A recent study using 3D cell culture models to assess EMT progression in ovarian cancers revealed that using the 3D growth conditions significantly affected not only the EMT but also the

stress responses and epigenetic processes that would not be observable in 2D monolayer culture (Paulin et al., 2017).

Similarly, a 2014 review of 3D cell cultures in drug discovery found that they are recognised as significantly better models for drug analysis particularly in cancer studies. This method holds many advantages in cancer biology, revealing information about in vivo interactions and cellular architecture (Edmondson *et al*., 2014).

### 5.6.2 Depletion of ERCC1 and XPF genes

Secondly, continuing with alternative cell culture techniques; instead of using transient knockdown of the genes by siRNA technologies, a permanent knock out of the two respective genes through the CRISPR cas 9 system should be undertaken. This will allow not only for more lab efficiency (due to cell lines continuously remaining ERCC1 and XPF deficient without the need for multiple transfections etc) but also more accurate research. It has been observed that the sequence specific off target effects seen in RNAi technologies are a growing issue. It has been found that siRNAs have the ability to induce the silencing of non-target mRNAs and the potential to supress hundreds of transcripts in a dose dependant manner (Boetcher and McManus, 2015).

As ERCC1 and XPF act as a heterodimer, there has been much research into their role both as a complex and as individuals. The research in this project focused on the way in which the proteins acted as individuals under different drug parameters. Research already tells us that ERCC1 is the key player in the ERCC1-XPF heterodimer, stabilising the complex and allowing for the nuclease activity to occur (McNeil and Melton, 2012), therefore researching the deficiency of ERCC1 can provide important insight into the destabilisation of this complex and therefore the breakdown of NER. Despite this, the role of ERCC1-XPF as a whole was not investigated. This could be achieved via a double-knock out/down (siRNA/CRISPR cas 9 technologies) of both ERCC1 and the XPF genes within one single cell line.

This dual depletion of the genes will allow for analysis of the functional role of the heterodimer as a whole.

As suggested In a PhD research thesis, when there is simultaneous knock down of both ERCC1 and XPF genes in a single cell line, that there is the greatest level of overall protein knock-down produced (Aurora *et al*., 2010). It was also found that upon analysis of DSBs in comparison to un-transfected or single transfections of just ERCC1 or XPF deficient cell lines, that the XPF–ERCC1 knockdown cells demonstrated a higher frequency of γ-H2AX foci formation. Suggesting that when both ERCC1 and XPF are depleted, the cells lines remain in a state of DNA damage which continues to be unrepaired (Aurora *et al*., 2010). Similarly, the study following on from this research project found that in MDA-MB-231 cell lines a dual knock down of the ERCC1 and XPF proteins displayed the peak level of cellular toxicity when directly compared to individual knock downs. Furthermore, the double knock down concomitantly resulted in the most reduced repair levels of Cisplatin induced DNA intra-strand adducts.

In vivo experiments suggest that there is little or no XPF present when ERCC1 is knocked down, supporting evidence that both proteins must be present for the formation of a stable complex.

### 5.6.3 Subcellular Localisation of ERCC1 and XPF

Further analysis of the subcellular localisations of each gene should be analysed after siRNA knock downs. The research in this study on non-transfected A2780 and A2780cis cell line revealed that upon treatment with cisplatin and Olaparib, that ERCC1 protein was found predominantly in the nucleus. Suggesting that the DNA damage caused by platinum adducts lead to the localisation of ERCC1 to the nucleus to perform its functional nuclease role. However, further research should progress to analyse the localisation of ERCC1 when it's heterodimeric partner XPF is knocked down (and vice versa) to assess the impact of the depletion of these proteins on the

overall functional role of the endonuclease complex. For example, a recent study focusing on subcellular localisation using CRISPR cas 9 knock out of XPF revealed that ERCC1 stays in the cytoplasm. When a fully viable cell line with complete XPF knock down was formed an increase in sensitivity to Cisplatin was observed, accompanied by no detectable ERCC1 in the nucleus. This lack of nuclear ERCC1 in XPF knock out cells indicates clearly the necessity of a fully functioning ERCC1-XPF heterodimer in the localisation of ERCC1 to the nucleus and carry out NER functions (Lehmann *et al*., 2017).

## 5.7 The Future of the Treatment of Ovarian Cancers

Chemo-resistance is a formidable problem at the forefront of the treatment of many cancers, and because of that it is imperative that we research and develop new methods to prevent this resistance occurring and increase the efficacy of known chemotherapeutic drugs. With ovarian cancer being the lead cause of death in gynaecological cancers, there is the utmost need to enhance treatments.

The resistance to chemotherapy is defined as; the progression of measurable disease during the therapeutic period, the lack of regression of disease during therapy or the recurrence of disease within a 6-month time frame of an individual finishing their initial treatment plan. Furthermore, the progression of disease during a therapeutic period is determined by a 20% increase in either non-target lesions or a 20% increase of the sum of the longest part of the diameter of the lesion i.e. the ovarian cancer. Whereas, the absence of regression during a treatment period is defined as >30% reduction in the sum of the longest diameter of the target tumour or no disappearance of target lesions (Response evaluation criteria in solid tumours, 2017).

In ovarian cancers the recurrence of disease can be measured by an increase in the serum levels of Ca-125 alongside a CT scan for confirmation. An ovarian cancer that is sensitive to platinum treatment (no recurrence of disease within 6 months of initial treatment) will exhibit a complete clinical response and thus will be eligible for platinum follow up therapy, either as a monotherapy or in combination with paclitaxel. In certain situations, an individual may be termed 'partially platinum sensitive'. This term refers to the occasion when the recurrence of disease happens 6-12 months after the last platinum treatment. Researchers have concluded that when compared to progression free interval, a platinum free interval (the time from the end of the treatment up until the time of relapse) is a more accurate method in the assessment of platinum resistance (Davis *et al*., 2014).

The median survival of a patient with a platinum resistant ovarian cancer is ~2 years (with a survival range of 3 months to 10 years). On the other hand, the median survival for patients with platinum resistant disease is significantly lower at 9-12 months and <15% of these patients will have a response to further treatment (Davis *et al*., 2014). This data confirms the dire need to broach platinum resistant in ovarian cancers, and the fundamental impact that novel cancer therapies will have on overall survival rates of patients.

The research in this study has provided evidence that not only does NER present as a very promising target for inhibition, but it has also validated the essential role of ERCC1 in the pathway and that its depletion leads to cytotoxicity in cells treated with cisplatin.

### 5.7.1 Inhibitors of Nucleotide Excision Repair

As previously discussed, targeting NER is a particularly attractive avenue in the potentiation of cisplatin therapy as there are some very clear biomarkers within this pathway – particularly ERCC1. Small molecule inhibitors of this pathway have much promise in cancer therapeutics, however, early research focused on other methods.

A 1995 study found that Anti-XPG and anti-ERCC1 polyclonal antibodies were able to inhibit the 3' and 5' incision of the damaged DNA respectively (Matsunaga *et al.,* 1995).

Interestingly, studies have found various other proteins that may hold more significant roles in the NER pathway than initially thought. Other than ERCC1, XPA has been demonstrated to have a direct effect on the response of patients to cisplatin treatment. Not unlike XPF, the interaction between ERCC1 and XPA is highly specific, proving to be fundamental in the recruitment of the ERCC1-XPF heterodimer to the site of damage in the NER pathway. However, dissimilar to ERCC1, XPA has no other known functional roles other than in the employment of the complex – offering a highly specific target in the disruption of the NER pathway (Fadda, 2016).

A proof-of concept study looked in to two different strategies in which the NER pathway could be regulated via the XPA and ERCC1 interaction. The first method utilises a short peptide to mimic the XPA binding domain on the ERCC1 protein, which possesses the ability to competitively bind with the ERCC1 over the full length XPA protein. The second approach uses a small molecule inhibitor method in which the molecules bind to the ERCC1 binding site, preventing any interaction with XPA. Both of these tactics prevent the recruitment of ERCC1 and XPF to the damage site and inhibit the progression of NER (Gentile, Tuszynski and Barakat, 2016).

Secondly, in a recent study, HTS analysis of a cohort of ~1900 small molecule inhibitors (SMIs) selected 12 inhibitors that were suitable to inhibit ERCC1-XPF. Of this subset, a further 5 SMIs were selected and validated to provided >90% inhibition of the enzymatic activity of ERCC1 and XPF at a low concentration. The most promising results were seen in NSC143099 (Hit 1) which revealed a particularly low $IC_{50}$ value against ERCC1-XPF and a secondary compound NSC16168 (Hit 2). These two small molecule inhibitors showed a potent inhibitory effect on the

enzymatic activity of ERCC1 and XPF, demonstrating 50% inhibition at ~22nM and ~420nM respectively. Furthermore, the effect of these inhibitory compounds was analysed on two non-related NER proteins; Hha1 and XPG. When treated with high concentrations of each compound there was no inhibitory effect found on Hha1 and there were very low levels of inhibition of XPG when treat with very high concentrations of Hit 2. Importantly, this lack of inhibitory effect of the two nucleases demonstrates positive results in terms of high levels of specificity. Finally, the compounds were assessed for their cytotoxicity alongside cisplatin treatment. When H460 lung cancer cells were treated with the inhibitors prior to cisplatin treatment a similar effect was shown compared to ERCC1-XPF knock down in the same cell lines, both potentiating cisplatin toxicity. Moreover, in cell lines treated with cisplatin and the SMIs, Hit 1 had 65% of cisplatin induced DNA adducts remaining at 72hours and Hit 2 had 60% in comparison to the untreated cells which presented just 15% of DNA adducts remaining at the same time point. This research validates the use of small molecule inhibitors of ERCC1 and XPF in the inhibition of DNA repair in cancer cell lines (Aurora *et al*., 2016).

Lentiviral infection of lung adenoma cell lines (XWLC05) by shRNA was used to silence the expression of ERCC1 at both the RNA and protein level. Typically, the ERCC1 expression in XWLC05 is high, contributing to its increased resistance towards cisplatin treatment. Depletion of the ERCC1 protein lead to decreased in vivo proliferation (IC50 of cisplatin 1.34 μM) and an observed increase in apoptosis post cisplatin treatment when compared to control cells (apoptotic rate of 81.2% vs. 58% respectively, $P<0.05$) (Wang *et al*., 2017).

### 5.7.2 The potentiation of cisplatin therapy via NER inhibitors

Taking into consideration the fundamental role of ERCC1 and XPF in the nucleotide excision repair pathway, and that this is also the pathway that is utilised in the repair of cisplatin induced DNA bulky adducts

The *in-silico* screen that lead to the identification of small molecule inhibitors of ERCC1-XPF interaction F06/NERI02 (NSC130813) helped to further solidify previous efforts to identify NER inhibitors. These inhibitors focus of disturbing the protein-protein interaction between ERCC1 and XPF, instead of targeting their catalytic activities individually. In vitro, F06 has been observed to show modest affinity to XPF but does exhibit the presumed biological activities of an inhibitor and is effective in reducing the cellular interaction between ERCC1 and XPF. It has also been suggested that F06 is able to induce the build-up of monomers of both ERCC1 and XPF which is presumed toxic to cells. However, this may not be the case and the single agent activity of F06 remains elusive. Despite this, the observed increase in sensitivity to cisplatin is typical of ERCC1-XPF inhibition (Jordheim *et al*., 2013). Further to this, in BRCA1 mutated breast cells, a synergistic effect was noted between F06 and olaparib. However, the exact role that ERCC1-XPF plays in mediating this synergy is not yet confirmed.

## 5.8 PARP1 and PARP2 Inhibitors

PARP1, Poly [ADP-ribose] polymerase 1 is a human enzyme encoded by the human PARP1 gene. It is implicated in many DNA repair pathways essential for all cellular processes that are involved in cell survival, predominantly the repair of DNA damage. Of the 18-member family, PARP1 was the first to be discovered and is now the centre of much research due to its functionality. PARP1 can modify itself and other proteins post transcriptionally, holding a role in a diverse array of cellular processes. It is known to be particularly essential in the stabilisation of replication forks.

PARPs act by attaching a molecule of ADP ribose either as individual units or as a polymer chair onto their target proteins via mono ADP ribosylation or by mono ADP ribosylation. Working as part of the base excision repair pathway, PARP sense any single stranded breaks in the DNA, which is either directly induced or a consequence or processing lesions. Upon binding to single strand breaks, PARP1 becomes

activated, parylating various substrates, promoting XRCC1 accumulation at the site of DNA damage. There are also roles for PARP within the alternative NHEJ pathway (which is activated in the absence of core NER (Ronson *et al*., 2018).

Due to its multifaceted roles, PARP1 very quickly became a highly regarded target for DNA repair inhibition and is now being exploited in the development of treatments for various cancers, namely DNA repair deficient ovarian cancers.

Landmark research by Bryant (Bryant *et al*., 2005) and Farmer (Farmer *et al*., 2005) into PARP1 revealed the potential therapeutic effects of its inhibition. This effect is speculated to be mediated by the inhibition of the base excision repair (BER) pathway (of which PARP1 is a key mediator), in cells that already have a defective homologous recombination (HR) pathway. Through this, a concept of synthetic lethality of BER and HR deficiency is proposed. The basis of this is established upon the fact that majority of single strand breaks to DNA are repaired by BER, with HR forming the secondary repair pathway. Small molecule inhibitors of PARP take advantage of this and exert anticancer influence via the inhibition of catalytic PARP activity, trapping the PARP at the DNA damage site (Balmaña *et al*., 2014). When PARP1, and consequently the BER pathway is inhibited by PARPi, the single stranded damage persists, thus leading to stalled replication forks, which, due to the deficiency of HR in the cells, cannot be repaired (Gee *et al*., 2018). These breakthrough findings validating the use of synthetic lethality in cancer treatments, have led to the development of many PARP1 inhibitors with several being trialled currently.

Resounding progression has been made within the development of PARPi, particularly with Olaparib trials in patients with defective or deficient HR pathways. In ovarian cancer patients with mutations in the BRCA1/2 genes Olaparib has shown high levels of efficacy and when used as a maintenance therapy, it has increased progression free survival. Niraparib, a PARPi currently undergoing phase III trials has

again exhibited efficacy in platinum sensitive relapsed patients, with the greatest effect observed in those with germline BRCA mutations (Mirza *et al*., 2016).

PARP inhibitors have gained the most advanced level of clinical application within ovarian cancers, with Olaparib receiving approval in several different settings. Olaparib was subject to rapid FDA approval following various breakthrough studies. Firstly, A phase II study of ovarian cancer patients harbouring BRCA1/2 mutations who received 400g Olaparib two times per day found an overall response rate to the drug of 31% (Audeh *et al*., 2010). Similarly, another phase II trial of 64 high grade serous ovarian cancers found a response rate of 41% in the cohort with BRCA1/2 mutations (Gelman *et al*., 2011). Approval for fourth-line (or later) treatment in BRCA1/2 mutated ovarian cancers was given in 2014.

Subsequently, many trials have investigated the potential role for Olaparib within maintenance therapy after the traditional cisplatin chemotherapy, again gaining approval from the FDA for use of Olaparib in non-BRCA mutant ovarian cancers that exhibit platinum sensitivity. Studies into patients with platinum sensitive high-grade ovarian cancers reveal that subsequent treatment with Olaparib pointedly improved progression free survival (Minchom, Aversa and Lopez, 2018).

### 5.8.1 PARPi and Platinum Sensitivity

The combination of PARPi therapy alongside cisplatin therapy has been a focal point of much research. Extensive preclinical analysis combining the use of platinum therapies i.e. cisplatin, oxaliplatin and carboplatin with PARP inhibitors has demonstrated clearly that this dual treatment can enhance the anti-cancer properties of the drug, independent of the catalytic inhibition of PARP (Murai *et a*l., 2014). A recent study looked into the patient tolerability of the two compounds when given in dual treatment, they found that continuous or intermittent olaparib in combination with cisplatin 75 mg/m2 was not considered tolerable. However, when intermittent

olaparib (at minimum dose) is combined with lower dosed cisplatin treatment, antitumour activity was noted. This effect was particularly apparent in patients with BRCA1/2 mutations. Furthermore, breast cancer patients demonstrated durable responses when olaparib monotherapy was given after cisplatin, justifying the further investigation of olaparib as a maintenance therapy in platinum therapy relapse patients.

Subsequent research has provided evidence that PARP inhibition has the ability to potentiate cisplatin agents and their chemotherapeutic effect. Not only has an increased level of DNA damage in the cells been observed in BRCA1/2 patients (Xu *et al*., 2015), but the combination of PARPi/platinum compounds has led to a statistically significant and sustained regression of the grown of breast cancer tumours in xenografts (Donawho *et al*., 2007).

Ovarian cancer patients that held BRCA1/2 mutated tumours had similar overall survival as those who had alterations in the NER genes, which is suggestive that mutations in each of the pathways contribute to the sensitivity to treatment by platinum compounds in a comparable manner. Data also suggested that unlike those with BRCA1 and BRCA2 mutations and alterations in the BER pathways; NER pathway defects were not significantly related to a sensitivity to treatment with PARPi (Ceccaldi *et al*., 2015).

Inhibition of PARP1 has been observed to improve the anticancer action of DNA damage inducing chemotherapy, not only improving efficacy but also acting to overcome known resistance to traditional therapies. When combined with cisplatin treatment, Olaparib demonstrates synergism, promoting cytotoxicity in breast cancers with a BRCA2 deficiency. Furthermore, BRCA1 mutant mouse models have validated this, showing that when compared to individual treatment, a combination therapy of cisplatin and olaparib increased the overall survival (Balmaña *et al*., 2014).

Further studies have found that upon treatment with cisplatin, cervical cancer cells reflected a significant overexpression in the PARP1 protein, demonstrating levels of hyperactivation. Following this, when in combination with cisplatin treatment, the PARP1 inhibitor olaparib has shown to have an antiproliferative effect on these cells, heightening cellular death and driving a loss of colony survival. Again, arrest in G2/M phase and an extended S phase along with delay in resolution of γH2A.X foci and increases apoptotic rate have all been observed (Prasad *et al*., 2017).

## 5.9 Targeting DDR Defects Through Synthetic Lethality

It is well documented that the induction of DNA damage represents a fundamental backbone in the treatment of cancers. Formation of DNA inter and intra-strand crosslinks by platinum compound such as cisplatin sits at the forefront of treatment. As discussed previously, cisplatin is a well-regarded treatment option for a wide spectrum of cancers, however the development of resistance via various mechanisms, particularly the up regulation of DDR proteins is a formidable issue we face. As a first line approach to combat this, various inhibitors of the DDR pathway have been developed and tested along-side treatment with platinum compounds. Unfortunately, there is a significant overlapping toxicity, stunting progression in many areas leading to a single agent approach.

This approach has been based on various experiments observing a synthetic lethality (SL) occurring between components of two or more specific DDR pathways. Synthetic lethality is defined as when deactivation of the pair or group of genes leads to cell death, however the cell can remain viable if one of the genes is active and the other is inactive. When a cancer cell has a defect in one of its DNA damage pathways, it will depend on another pathway to ensure survival. In these cases, we can exploit this knowledge by targeting the compensation pathway to ensue cellular death (Minchom, Aversa and Lopez, 2018).

Again, PARPi have been the key target in the exploitation of synthetic lethality in cancer treatments. Initially, it was suggested that the fundamental mechanism underlying synthetic lethality was due to the inhibition of PARP causing persistent single strand breaks in the DNA, which then caused collapses in DNA replication forks and progressing on to double strand breaks. However, recent research has lead to the modification of this model, with much data suggesting that the toxicity of PARPi is due to PARP trapping. Some inhibitors of PARP (namely niraparib, olaparib, and talazoparib) are known to trap the PARP1 on the DNA, preventing PARylation and interfering with its catalytic activity. It is suggested that this trapped PARP induces sufficient levels of toxicity within the cell to be lethal (Lord and Ashworth, 2017). For example, it has been demonstrated in early studies that BRCA-mutant cancer cells were up to 1000 time more sensitive to PARP inhibitors than the BRCA wild type cells (Farmer *et al*., 2005). PARP1 defective cells have been noted to be resistant to PARPi (Pettitt *et al*., 2013).

### 5.9.1 PARPi and ERCC1 Deficiency: Cancer Treatment Through Synthetic Lethality

The connection between PARPi treatment synthetic lethality in BRCA1/2 mutated tumours is already extremely well documented. However, this research has provided fundamental grounds for the utilisation of SL in ERCC1 deficient cancers. Evidence provided in this study suggests a solid platform and rationale for the use of olaparib treatment in ERCC1 defective ovarian cancer patients, particularly those that have proven to be platinum resistant.

Recently, a study assessing the individual roles of NER and HR in the process of platinum resistance found that the significance of NER and its contribution to resistance was variable when assessed *in vitro* in different cell lines. Furthermore, the expression of NER proteins showed now correlation with the activity of NER or the cell line's sensitivity to cisplatin treatment (Wang *et al*., 2011). The role of ERCC1

in the role of NER associated platinum resistance has been extensively researched, however ERCC1 also appears to play a prominent role in homologous recombination repair. Therefore, considering the association between the NER and HR pathways when developing inhibitors and modulators of repair pathways is important.

Previous studies have recorded the synthetic lethality between ERCC1 deficient NSCLC cells and PARP1 inhibition. It was found that there was a significant delay in the repair of double strand breaks and an extended $G_2$/M arrest in ERCC1 deficient cells following treatment with PARPi. Furthermore, the ERCC1 isoform (202) which is associated with the mediation of cisplatin sensitivity was also found to hold a role in PARPi sensitivity. Silencing of PARP1 was able to re-establish sensitivity in PARPi resistant ERCC1 depleted cells, however there was no effect observed in ERCC1 proficient cells. Thus, providing support for the PARP1 induced ERCC1 selectivity of PARP inhibitors (Vinay *et al*., 2013).

Further drug combination experiments have revealed that olaparib can potentiate the cytotoxicity of cisplatin but was also toxic to cells as a single agent therapy. This is clearly defined in ERCC1 deficient PC9 and HCC827 cells, but not observed in ERCC1 proficient A549 and H157 lung cancer cell lines. Additionally, the knock down of ERCC1 by siRNA technology in the two ERCC1 proficient cell lines (A549 and H157) lead to an increase in sensitivity to both olaparib and cisplatin. Syngergism was also observed between the two drugs, with combination of PARPi and cisplatin leading to sustained DNA DSB breaks, cell cycle alterations i.e. extended G2/M arrest and higher apoptotic activity in ERCC1 deficient cells (Cheng *et al*., 2012).

## 5.10 Issues with Targeting DDR Pathways

Targeting DNA damage repair pathways in an effort to combat cisplatin resistance has already proven to be a promising topic and is a frontier in the fight to improve ovarian cancer prognosis. Despite this, when trying to overcome chemotherapeutic

resistance there are several draw backs in pursuing both functional and dysfunctional pathways in DDR.

Multiple challenges arise when trying to target the DDR pathway. Primarily, in order to develop an effective and targeted therapy, clinicians need to be able to correctly identify the specific DDR pathway that is dysfunctional in a given tumour. Not only this, but an in depth understanding of the interactions of proteins within the pathway, and potential overlap with other biological processes needs to be had. To overcome this; functional assays may be utilised to distinguish key DNA repair proteins i.e. ERCC1 in the NER pathway or PARP1 in the BER pathway. This will allow assessment as to whether these key drivers network over several pathways (Gee *et al*., 2018).

Despite many attempts, determining DNA damage repair status of ovarian cancers had incurred many issued. Multiple assays have been attempted, particularly in the recognition of HR malfunctions, however the results have been poor. A distinctive gene expression is evident in those cancers that harbour a BRCA mutation, however this is not necessarily predictive.

Currently there are a vast number of small molecule inhibitors being developed and trialled as targets to DNA repair proteins, many of them showing promising results at the pre-clinical stage. However, these need to be rapidly driven through the developmental stages for clinicians and patients to reap the benefits of these therapies.

### 5.10.1 Development of Assays to Predict Defective NER and HR in Cancers

To begin to combat poor prognosis in defective DDR related cancers, and to overcome screening issues, selective assays need to be developed. The need for a rapid, quantitative and sensitive assay that can detect the activity of NER and BER in cells is colossal. Studies have revealed that Oligonucleotide Retrieval Assay (ORA),

in which human cells were transfected with oligonucleotides that contain a cyclobutane pyrimidine dimer (CPD) – UV-damage adduct- can be used for the quantification of adduct (CPD) free DNA via quantitative real time PCR methods (Shen *et al*., 2014). This methodology allows for the degree of NER activity in assorted cell types including stem-like cells to be quantified. These DNA oligonucleotide fragments can be both transfected into and retrieved from human cells efficiently.

HR proficiency has been observed in cells by studying focus formation of Rad51 in cultures from ascitic fluid of OC patients. This method has shown encouraging results as a viable method for identifying tumours with a HR deficiency, and potentially a target for PARP1 inhibition (Mukhopadhyay *et al*., 2012). Development of these assays further, for clinical use, may prove useful in selecting women for targeted therapy via identification of their DNA repair status.

Whilst developing inhibitors for DDR pathways there are several things that need to be considered. Knowing the specific protein and specific pathways that a drug is selected to target allows us to assed the specific function of that protein and it can act as an important biomarker. Assessment of the protein by means of DNA or RNA analysis, or screening of tissues via IHC analysis allows patients to be separated into groups according to likely response to the drug.

### 5.10.2 Using ERCC1 as a Biomarker for Ovarian Cancer

A study into ERCC1+ cancer tumour cells (CTS) looked into whether ERCC1+ mirrored response to chemotherapy. It was found that 17% of ovarian cancer tumour cells were detected as having exclusively high ERCC1. It was found that ERCC1+ CTCs predict post therapeutic outcome. In samples analysed post chemotherapy, the presence of ERCC1+ CTCs was found to have a significant correlation with an overall decreased progression free survival in patients (p=0.0293). Not only this, but

there was also a notedly decreased overall survival of the patients in the same condition (p= 0.0008). Furthermore, when corelating this data back to resistance to platinum-based therapy, it was found that there was a direct correspondence between the presence of ERCC1+ CTCs and platinum resistance (p=0.01). Additionally, patients who were found to be persistently positive to ERCC1+ CTCs (before and after surgery and treatment) had an extremely poor progression free survival when compared to the other groups – positive before treatment and negative after treatment, negative before treatment and positive after treatment and negative before and after treatment. Again, individuals with a newly acquired ERCC1+ status post chemotherapy were found to have a notable trend in shorter progression free survival rates (p=0.2871) and overall survival rates were also significantly compromised (p=0.0202). Interestingly, researchers noted that the singular assessment of ERCC1-transcripts on their own was sufficient enough to be able to detect any prognostically relevant CTCs that expressed ERCC1 (Chebouti *et al*., 2017).

# 6 Chapter 6: Conclusion

This study independently investigates both the expression of ERCC1 and XPF in ovarian cancer cell lines and a large cohort of ovarian cancers (courtesy of BioBank Nottingham). Through this study, evidence can be provided for the role of ERCC1 and XPF deficiency in their predictor status of sensitivity of platinum-based chemotherapies and the use of olaparib in targeted maintenance therapies in platinum resistant relapse cases. Pre-clinical analysis not only confirms data found in previous studies, but expressly marks ERCC1 as an independent marker of clinicopathological outcomes of ovarian cancers. Further to this, the role of XPF as an independent biomarker in ovarian cancer has not yet previously been

investigated, providing a unique outlook into the potentiation of cisplatin therapy in an effort to combat chemotherapeutic resistance.  Here it is confirmed that Olaparib is synthetically lethal in both ERCC1 and XPF deficient cells, this conclusion can be drawn as a direct consequence of the data observed in this study. ERCC1 and XPF knock down cells both revealed a sensitivity to treatment by olaparib in clonogenic and MTS survival assays. This data was then further verified in functional analysis whereby olaparib treatment resulted in apoptosis, the accumulation of double strand breaks and cycle arrest. Therefore, considering all the evidence presented by this study, it can be concluded that both ERCC1and XPF can both be independently identified as clear predictors of platinum sensitivity, but can also be utilised as biomarkers in personalised therapy.

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# 8 Appendix 1

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**Table 1:** Pathological features of ovarian cancer patients.

| **Characteristics** | | **Number** | **Percentages** |
|---|---|---|---|
| ***Pathology*** | | | |
| | Serous cystadenocarcinoma | 178 | 53.9% |
| | Endometrioid | 44 | 13.2% |
| | Clear cell carcinoma | 24 | 7.2% |
| | Mucinous cystadenocarcinoma | 46 | 13.8% |
| | Others | 15 | 4.5% |
| | Mixed | 17 | 5.1% |
| ***Grade*** | | | |
| | 1 | 46 | 13.8% |
| | 2 | 60 | 18% |
| | 3 | 171 | 51.5% |
| ***Residual tumour*** | | | |
| | None/Microscopic | 205 | 61.7% |
| | <1cm | 34 | 10.2% |
| | >1-2 cm | 15 | 4.5% |
| | >2cm | 40 | 12% |
| ***FIGO Stage*** | | | |
| | I | 123 | 37% |
| | II | 49 | 14.7% |
| | III | 128 | 38.5% |
| | IV | 11 | 3.3% |
| ***Chemotherapy*** | | | |
| | Carboplatin monotherapy | 81 | 24.3% |
| | Carboplatin + Paclitaxel | 86 | 25.9% |
| ***Platinum sensitivity*** | | | |
| | Sensitive | 250 | 75.3% |
| | Resistant | 26 | 7.8% |
| | Unknown | 55 | 16.5% |
| ***Relapse status*** | | | |
| | Progression-free | 169 | 50.9% |
| | Progressed/relapsed | 118 | 35.5% |
| | Unknown | 44 | 13.2% |

**Supplementary Table S2:** ERCC1 and Ovarian Cancer

| | ERCC1- N(%) | ERCC1+ N(%) | **P- value** |
|---|---|---|---|
| ***Pathological Type*** | | | **0.000053** |
| Serous cystadenocarcinoma | 54(38) | 88(62) | |
| Mucinous cystadenocarcinoma | 27(87.1) | 4(12.9) | |
| Endometrioid | 15(50) | 15(50) | |
| Clear cell carcinoma | 13(65) | 7(35) | |
| Mixed | 5(41.7) | 7(58.3) | |
| ***FIGO Stage*** | | | **0.049** |
| I | 55(57.3) | 41(42.7) | |
| II | 10(31.3) | 22(68.8) | |
| III | 46(46) | 54(54) | |
| IV | 3(33.3) | 6(66.7) | |
| ***Tumour Grade*** | | | **0.047** |
| G1 | 17(56.7) | 13(43.3) | |
| G2 | 26(59.1) | 18(40.9) | |
| G3 | 54(40.3) | 80(59.7) | |
| ***Measurable Disease pre-Chemotherapy*** | | | 0.671 |
| No measurable disease | 74(48.7) | 78(51.3) | |
| Measurable disease | 31(45.6) | 37(54.4) | |

**Supplementary Table S3:** XPF and Ovarian Cancer

| | XPF- N(%) | XPF+ N(%) | **P- value** |
|---|---|---|---|
| ***Pathological Type*** | | | 0.28 |
| Serous cystadenocarcinoma | 104(73.2) | 38(26.8) | |
| Mucinous cystadenocarcinoma | 28(93.3) | 2(6.7) | |
| Endometrioid | 23(76.7) | 7(23.3) | |
| Clear cell carcinoma | 14(82.4) | 3(17.6) | |
| Mixed | 8(80) | 2(20) | |
| ***FIGO Stage*** | | | 0.338 |
| I | 70(80.5) | 17(19.5) | |
| II | 23(65.7) | 12(34.3) | |
| III | 79(76.7) | 24(23.3) | |
| IV | 6(85.7) | 1(14.3) | |
| ***Tumour Grade*** | | | 0.184 |
| G1 | 26(86.7) | 4(13.3) | |
| G2 | 36(81.8) | 8(18.2) | |
| G3 | 94(72.9) | 35(27.1) | |
| ***Measurable Disease pre-Chemotherapy*** | | | 0.799 |
| No measurable disease | 112(75.7) | 36(24.3) | |
| Measurable disease | 48(76.2) | 15(23.8) | |

**Supplementary Table S4:** ERCC1 – XPF co-expression and Ovarian Cancer

| | **ERCC 1-/XPF-N(%)** | **ERCC1-/ XPF+ N(%)** | **ERCC1+/ XPF- N(%)** | **ERCC1+/ XPF+ N(%)** | **P- value** |
|---|---|---|---|---|---|
| ***Pathological Type*** | | | | | **0.005** |
| Serous cystadenocarcinoma | 40 (31.0) | 9 (7.0) | 53 (41.1) | 27 (20.9) | |
| Mucinous cystadenocarcinoma | 22 (84.6) | 0 (0.0) | 2 (7.7) | 2 (7.7) | |
| Endometrioid | 12 (46.2) | 3 (11.5) | 8 (30.8) | 3 (11.5) | |
| Clear cell carcinoma | 10 (62.5) | 1 (6.3) | 3 (18.8) | 2 (12.5) | |
| Mixed | 4 (44.4) | 0 (0.0) | 3 (33.3) | 2 (22.2) | |
| ***FIGO Stage*** | | | | | 0.105 |
| I | 46 (56.1) | 4 (4.9) | 19 (23.2) | 13 (15.9) | |
| II | 7 (23.3) | 3 (10.0) | 12 (40.0) | 8 (26.7) | |
| III | 33 (36.7) | 6 (6.7) | 36 (40.0) | 15 (16.7) | |
| IV | 2 (33.3) | 0 (0.0) | 3 (50.0) | 1 (16.7) | |
| ***Tumour Grade*** | | | | | 0.203 |
| G1 | 15 (57.7) | 0 (0.0) | 8 (30.8) | 3 (11.5) | |
| G2 | 20 (52.6) | 2 (5.3) | 10 (26.3) | 6 (15.8) | |
| G3 | 40 (34.2) | 7 (6.0) | 45 (38.5) | 25 (21.4) | |
| ***Measurable Disease Before ChemoTherapy*** | | | | | 0.689 |
| No measurable disease | 59 (43.7) | 9 (6.7) | 42 (31.1) | 25 (18.5) | |
| Measurable disease | 21 (36.2) | 4 (6.9) | 23 (39.7) | 10 (17.2) | |