Clinical resistance

to platinum

chemotherapy

in ovarian cancer

DECLARATION

I can confirm that all work presented in this thesis is my own.

DEDICATIONS

I would like to dedicate this thesis to my husband James, who has been very supportive throughout.

ABSTRACT

Platinum drugs are the most active agents in ovarian cancer. Their cytotoxicty results from DNA crosslinking. High tumour response rates are seen, but 80 % of patients relapse. Major mechanisms of platinum resistance in patients remain to be established. We have studied DNA interstrand crosslinking and its repair in response to *ex vivo* treatment with cisplatin in forty patients with ovarian cancer using the single cell gel electrophoresis (comet) assay. Tumour cells from resected tumours or tumour and mesothelial cells from ascites were obtained from chemonaïve patients and those relapsing after platinum-based therapy. The average percent decrease in tail moment at the peak of crosslinking was 61.1%±9.25 in 34 pre-chemotherapy patient samples following treatment with 100μM cisplatin. In 14 post-chemotherapy patient samples it was 58.1%±9.94. The average percentage repair at 24 hours was 3.6%±18.89 in pre-chemotherapy patients and 44.6%±43.4 for post-chemotherapy patients ($p<0.001$).

In 6 paired samples, before and after chemotherapy the average percentage repair at 24 hours was 7.2%±12.64 increasing to 69.5%±23.42 after chemotherapy. Differences in cell cycling, and cell signalling gene expression levels using microarray analysis was found, between pre- and post-chemotherapy patients. Real time PCR was also used to investigate the levels of ERCC1 (excision-related cross complementation group 1) in 3 of these paired patient samples, which was found to be increased by an average of 14.4% +/-0.8% in 3 post-chemotherapy samples.

In ten pre-chemotherapy and seven post-chemotherapy patient tumours incubated *ex vivo* with 50μM melphalan, the percent decrease in tail moment at the peak of

crosslinking was 41.4±11.2, and 44.6 ±7.6, respectively. 24 hours later the percentage repair was 3.1±.25.6 for untreated and 2.8±26.3 for treated tumours. In conclusion, repair of DNA interstrand crosslinks appears to be an important mechanism of clinical platinum resistance in ovarian cancer. Repair of melphalan crosslinks is unaffected.

CONTENTS

1. INTRODUCTION

1.1 Ovarian cancer

Ovarian cancer is the leading cause of death from gynaecological malignancies. It is the fourth most common cancer among women in the UK (Cancer Research UK, 2004), and the lifetime risk of a woman developing ovarian cancer is 1 in 70 (Ozols *et al*., 2001). The cause of ovarian cancer is unknown however there are risk factors, such as increased age. Half of all ovarian cancers occur in women after the age of 65 (Cancer Research UK, 2004). Women who have been pregnant have a 50% decreased risk of developing ovarian cancer compared to nuliparous women (Garcia *et al*., 2004), and further pregnancies confer additional protective effect, while the oral contraceptive pill decreases the risk. These risk factors support the theory that the risk of ovarian cancer is related to ovulation, and that suppressing ovulation plays a protective role (Garcia *et al.,* 2004). In addition, a prior history of breast cancer increases a woman's risk of ovarian cancer (Garcia *et al*., 2004).

Another important risk factor is family history. The lifetime risk for developing ovarian cancer is 1.6% in the general population. This risk increases to 5% when one first-degree relative has had ovarian cancer, rising to 7% when two first-degree relatives have been affected (Garcia *et al*., 2004). Less than 5% of all ovarian cancers are inherited, and so far two syndromes have been identified: breast/ovarian cancer syndrome, and Lynch II syndrome (also called hereditary nonpolyposis colorectal cancer) (Garcia *et al.*, 2004). Breast/ovarian cancer syndrome is

associated with early onset of either or both of the diseases. There are often several family members affected. The inheritance follows an autosomal dominant transmission, and can be inherited from either parent. Most cases are associated with *BRCA1* and *BRCA2* gene mutations (Garcia *et al.,* 2004). Families that have the Lynch II syndrome are at high risk of developing colorectal, endometrial, stomach, small bowel, breast, pancreas, and ovarian cancers, which is caused by mutations in the mismatch repair genes (Garcia *et al*., 2004).

About 70% of women diagnosed with ovarian cancer are stage III or IV (Rustin *et al.,* 2004). They usually present at an advanced stage due to the vagueness of the symptoms, the difficulty of diagnosis, and the lack of a screening test. Symptoms include malaise, weight loss, pressure symptoms (e.g from ascites or an enlarging tumour), pelvic pain, vaginal bleeding, abdominal distension, and change in bowel habit. Physical findings are uncommon in patients with early disease, while patients with advanced disease may have an ovarian pelvic mass, ascites, pleural effusion, abdominal mass or bowel obstruction, or a combination of these. Investigations include an ultrasound scan of the pelvis, a blood test for a tumour marker called Cancer antigen –125 (Ca-125), an MRI scan of the abdomen and pelvis, and a Chest X-ray, as well as routine blood tests, such as a full blood count, and urea and electrolytes. Other investigations may be appropriate depending on the patients' symptoms e.g a colonoscopy if they have bleeding from the rectum.

The standard care for ovarian cancer includes a primary staging and cytoreductive or debulking laparotomy (Baird *et al.,* 2001). Peritoneal washings are usually taken

immediately on entering for cytology. A thorough exploration of the abdomen and pelvis is performed to assess the extent of spread. The surface of the liver, diaphragm, and spleen are examined, as well as the small and large bowel, omentum, para-aortic and pelvic lymph nodes. A total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, para-aortic and pelvic lymph node sampling, peritoneal biopsies, and peritoneal cytology (peritoneal washings) are usually performed if ovarian cancer is confirmed. In advanced disease cytoreduction or debulking surgery (where as much tumour tissue as possible is removed) is performed, which will then offer the best response to subsequent chemotherapy. It is recommended to achieve a residual disease of less than 1cm (Stuart *et al*., 2001). When doubt exists about the diagnosis of ovarian cancer, a frozen section can be performed. The ovaries are removed and placed in a dry container for a pathologist to look at them immediately. They convey their findings whilst the patient is still on the operating table, which helps the surgeon to decide what other tissues need to be taken (if any).

1.1.1 Staging of Ovarian Cancer

The FIGO (Federation International of Gynaecology and Obstetrics) staging for ovarian cancer is as follows:

1. Stage I – Growth limited to the ovaries
    - Stage 1a – Growth limited to one ovary, no ascites, no tumour on external surface, capsule intact
    - Stage 1b – Growth limited to both ovaries, no ascties, no tumour on external surface, capsule intact

- Stage 1c – Tumour either stage 1a or 1b but with tumour on surface of one or both ovaries, ruptured capsule, ascites with malignant cells or positive peritoneal washings

2. Stage II – Growth involving one or both ovaries, with pelvic extension
    - Stage IIa – Extension and/or metastases to the uterus or tubes
    - Stage IIb – Extension to other pelvic tissues
    - Stage IIc – Stage IIa or IIb but with tumour on surface of one or both ovaries, ruptured capsule, ascites with malignant cells or positive peritoneal washings.
3. Stage III – Tumour involving one or both ovaries, with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes; superficial liver metastases equals stage III.
    - Stage IIIa – Tumour grossly limited to pelvis, negative lymph nodes but histological proof of microscopic disease on abdominal peritoneal surfaces
    - Stage IIIb – Confirmed implants outside of pelvis in the abdominal peritoneal surface; no implant exceeds 2cm in diameter and lymph nodes are negative
    - Stage IIIc – Abdominal implants larger than 2cm in diameter and/or positive lymph nodes.

4. Stage IV – Distant metastases; pleural effusion must have a positive cytology to be classed as stage IV; parenchymal liver metastases equals stage IV.

The survival rates for ovarian cancer depend on the stage at diagnosis:

- Stage 1 – 90% 5 year survival
- Stage 2 – 70% 5 year survival
- Stage 3 – 21% 5 year survival
- Stage 4 – 6% 5 year survival (Cancer Research UK, 2004)

1.1.2 Histopathology of Ovarian Tumours

All tissues removed are sent to the hisopathologist who examines the material and helps in assessing the stage (see above), grade and type of cancer. The grade of the ovarian cancer refers to the cells themselves. The most important features in assessment of grade are mitotic activity, nuclear size and pleomorphism, and differentiation from the original cells (Underwood, 2000). Grading is not entirely objective, and different pathologists may disagree on the grade of a particular ovarian cancer, as there are different systems for grading ovarian cancers (Dolson, 2004). Pathologists usually use a combination of *the pattern system* and *Broder's grading system*.

There are three different grades; grade 1 (well differentiated), grade 2 (moderately differentiated), and grade 3 (poorly differentiated) (Dolson, 2004). Tumours are often heterogenous, and the grading should be performed on what appears to be the least differentiated area (Dolson, 2004). Overall low-grade (grade 1) tumours grow more slowly, and have a better prognosis than high-grade (grade 3) tumours (Underwood, 2000). However, determining prognosis is multi-factorial (Dolson,

2004). Ovarian tumours may be divided into five broad categories (Underwood, 2000) according to the World Health Organisation :

- Epithelial
- Germ-cell
- Sex-cord stromal
- Metastatic
- Miscellaneous

1.2 Chemotherapy

1.2.1 Chemotherapy agents used in ovarian cancer

There are many chemotherapeutic agents used to treat ovarian cancer, depending on the type, grade, stage of the disease, and the health of the patient. Metaanalyses of randomized clinical trials have shown that platinum compounds (cisplatin/carboplatin), anthracyclines (doxorubicin/epirubicin), alkylating agents (cyclophosphamide/melphalan), spindle poisons (paclitaxel/docetaxel), antimetabolites such as gemcitabine, and the topoisomerase I inhibitor topotecan are the most active agents in ovarian cancer (Berkenblit *et al.,* 2005).

1.2.1.1 Spindle Poisons

Spindle poisons include taxanes such as paclitaxel and docetaxel, and promote the assembly of microtubules by binding to β-tubulin, which inhibits depolymerisation

(Yoshida *et al*., 2001). This action disturbs mitosis in normal and malignant cells (Schiff *et al*., 1979). The taxanes were originally derived from the bark of the Pacific Yew tree, *Taxus brevifolia*, and paclitaxel was identified as the active constituent in 1971 (McGuire *et al*., 2003). Docetaxel is a semi-synthetic taxoid derived from the needles of *T. baccata* (Lister-Sharp *et al*., 2000) and has been shown to be less neurotoxic than paclitaxel (Vasey *et al*., 2002). The introduction of paclitaxel in the 1990's to treat ovarian cancer increased the survival rates of women with the disease (Rustin *et al*., 2004).

1.2.1.2 Anthracyclines

Anthracyclines such as doxorubicin and epirubicin, have several cytotoxic actions. Their main mechanism of action appears to be mediated by their effect on topoisomerase II, whose activity is markedly increased in dividing cells (Rang *et al*., 2005). Topoisomerase II binds to the double stranded DNA, cleaves both strands of duplex DNA and passes a second duplex through this transient cleavage, involving hydrolysis of ATP, and enzyme recycling (Swift *et al*., 2006). The intermediate form is termed the 'cleavable complex' (Swift *et al*., 2006). As a result of its double-stranded DNA passage mechanism, topoisomerase II is able to remove negative or positive superhelical twists (i.e., under- or overwinds) from the genetic material and resolve intramolecular DNA knots as well as intermolecular tangles (Wang *et al*., 1996)

Doxorubicin poisons the cleavable complex, inhibiting religation of the cleaved complex, resulting in double strand breaks of the DNA. Doxorubicin intercalates into the DNA and becomes trapped (Epstein *et al*., 1998). Apoptosis results if the cell is unable to repair these DNA double-strand breaks. However, these drugs have additional modes of action such as production of oxygen free radicals, and formation of covalent adducts which may contribute to its toxicity (Minotti *et al*., 2004).

1.2.1.3 Topoisomerase inhibitors

Topoisomerase I inserts a nick into one strand of the DNA to relax supercoiled DNA that occurs when DNA replicates itself. It also re-ligates the cleaved DNA. Irinotecan and topotecan (9-dimethylaminoethyl-10-hydroxycamtothecin) are potent inhibitors of topoisomerase I (Xu *et al*., 2002).

In patients with platinum-resistant ovarian cancer, there was a low probability of responding to single agent Topotecan (Main *et al*., 2006). Unfortunately Topotecan in combination with gemcitabine in the treatment of platinum refractory ovarian cancer in a phase I/II trial and has shown disappointing results (Goff *et al.,* 2008).

1.2.1.4 Antimetabolites

Antimetabolites such as gemcitabine, interfere with nucleic acid production, either by mimicking nucleotide structure, enabling them to be incorporated into nucleic

acids, thus terminating their production, or by preventing synthesis of nucleotides directly.

Gemcitabine (2', 2'-difluorodeoxycytidine dFdC) is a fluorinated deoxycytidine derivative, which can be used in a variety of solid tumours including ovarian cancer (usually in combination with carboplatin), non-small cell lung cancer, head and neck cancer, and genitourinary cancer (Fruscella *et al*., 2003). Gemcitabine enters the cell through a membrane nucleoside transporter (Mackey *et* al., 1998), and it is activated by phosphorylation to gemcitabine monophosphate, which is subsequently phosphorylated to the 5'-diphosphate (dFdCDP) and triphosphate (dFdCTP), which represent the active forms of gemcitabine (Fruscella *et al*., 2003). dFdCTP is incorporated into DNA instead of cytosine, followed by one or more deoxynucleotides, thus blocking DNA synthesis and preventing repair by 3'-5'-exonuclease activity (Fruscella *et al*., 2003). Gemcitabinetriphosphate is also incorporated into RNA, thus terminating RNA synthesis (Fruscella *et al*., 2003). Only a proportion of gemcitabine is converted into the di- or triphosphate forms. The majority of gemcitabine is rapidly inactivated in the blood, liver and kidneys by deamination into 2', 2'-diflourodeoxyuridine in a reaction catalysed by deoxycytidine deaminase (CDD) (Fruscella *et al*., 2003).

1.2.1.5 Platinum compounds cisplatin and carboplatin

Cisplatin (*cis*-dichlorodiammine platinum(II)) was discovered serendipitously by Rosenberg in the 1950's while investigating inhibition of bacterial growth by

electric currents (Rosenberg 1985). The resulting inhibitory complexes were produced by the reaction of platinum electrodes with growth media. Cisplatin (a neutral complex) was the most active of these, and was tested in clinical trials (Higby *et al.,* 1973).

Cisplatin and Carboplatin

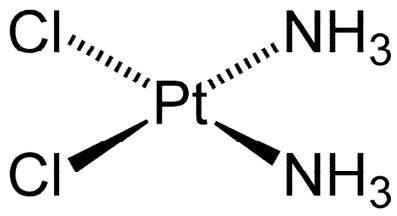
It is thought that cisplatin enters the cell by both passive diffusion and facilitated transport, possibly by a gated channel (Gateley *et al*., 1993). Some of the uptake is dependent on energy because pharmacological agents such as ouabain (a Na+, K+-ATPase inhibitor) reduce it (Judson I *et al*., 2002).

Cisplatin is a water-soluble complex, which has a central platinum atom surrounded by two chlorine atoms and two ammonia groups (Rang *et al*., 1995) (figure 1). When cisplatin enters the cell, the chloride ions dissociate (due to the low concentration of intracellular chloride ions) leaving a reactive diamine-platinum complex, which reacts with water and then, can interact with DNA (Rang *et al*., 1995). Cisplatin binds preferentially to the N7 atom of guanine and adenine residues (Eastman *et al*., 1987), although there are numerous potential reaction sites in all four bases (Singer *et al*., 1977).

Cisplatin is widely used not only for the treatment of ovarian cancer, but also for many other solid tumours such as those arising from the testes, bladder, lung, and head and neck. Cisplatin was first introduced in 1973 for treatment of ovarian cancer, which improved disease-free survival and response rates when compared to

the then-standard treatments (Rustin *et al*., 2004). Cisplatin was either used alone or in combination with cyclophosphamide (Rustin *et al*., 2004). Cisplatin causes very severe nausea and vomiting, and is highly nephrotoxic (Rang *et al*., 1995).

(a)



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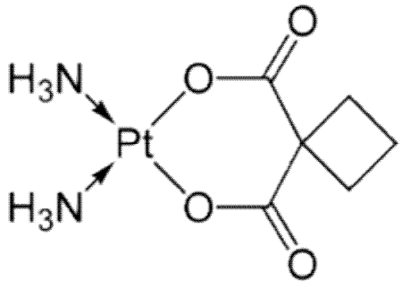

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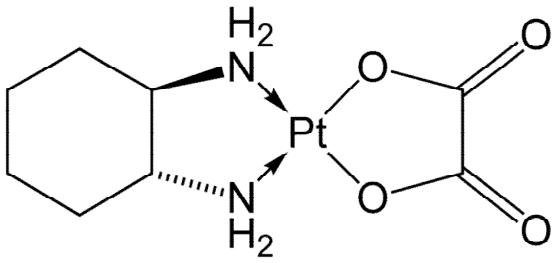
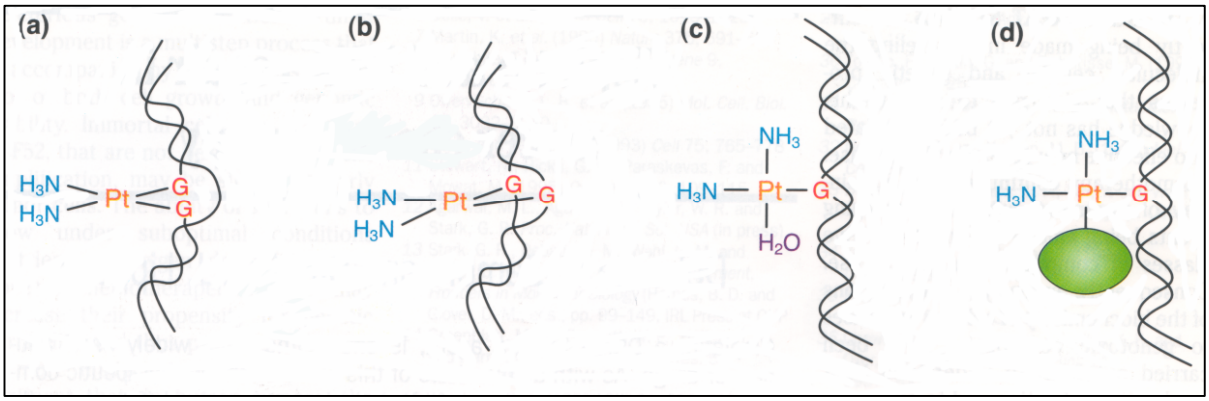


Figure 1: Structure of (a) cisplatin (b) carboplatin (c) oxaliplatin

In the early 1980's carboplatin, which is a derivative of cisplatin, was introduced (figure 1). Carboplatin causes less nephrotoxicity, neurotoxicity, and ototoxicity, and less severe nausea and vomiting than cisplatin, but it is more myelotoxic (Rang *et al*., 1995). Tinnitus and hearing loss in the high frequency ranges may occur, as may peripheral neuropathies, hyperuricaemia, myelotoxicity and nephrotoxicity.

The cytotoxicity of cisplatin and carboplatin are believed to result from the formation of platinum-DNA adducts. These include mononadducts, intrastrand crosslinks, interstrand crosslinks (ICLs), and DNA-protein crosslinks (Fichtinger-Schepman *et al*., 1995) (figure 2). There has been much debate about which adduct is responsible for the cytotoxicity of cisplatin. Fischtinger-Schepman *et al* concluded that 1,2 intrastrand crosslinks produced by cisplatin were responsible for its cytotoxicity because the peak of formation of these lesions co-incided with the peak of cytotoxicity in Chinese hamster ovary cells (CHO) (Fischtinger-Schepman *et al*., 1995). Zamble *et al.,* demonstrated that the 1,2-cisplatin adducts are poorly recognised, adding support to the argument that they are a critical cytotoxic lesion (Zamble *et al*., 1996). In contrast Zwelling L.A *et al* found a correlation between cytotoxicity and ICLs in L1210 cells *in vitro* using alkaline elution (Zwelling *et al.*, 1979). Meyn *et al* demonstrated that an ERCC1 mutant CHO cell line, UV20, was extremely sensitive to cisplatin and defective in the uncoupling of ICLs, which was taken to demonstrate a direct relationship between sensitivity and ICL repair (Meyn *et al*., 1982). In the nitrogen mustard class, there is a clear correlation between the extent of interstrand crosslinks (ICL) and cytotoxicity (O'Connor *et al*., 1990).

However, the platinum drugs produce a high proportion of intrastrand crosslinks that clearly contribute to their activity (McHugh *et al.*, 2000).



95% Intrastrand crosslinks	2% Interstrand crosslinks	2% Monoadducts	1% DNA-protein crosslinks

Figure 2: The different types of DNA adducts produced by cisplatin, and frequencies of occurrence.

Carboplatin has a much slower rate of aquation and ICL formation compared to cisplatin (Knox *et al.*, 1986), and therefore the time to reach the peak of ICL formation is much longer with carboplatin.

Oxaliplatin

Oxaliplatin is an analogue of cisplatin that was developed to try and overcome drug resistance (figure 1). It has a different spectrum of anti-tumour activity and has shown activity in treatment of advanced colorectal cancer either as a single agent or in combination with 5-fluorouracil (Becouarn *et al*., 1998). Side effects of Oxaliplatin include neurotoxicity, including facial dyaesthesia, which may be provoked by cold weather, and peripheral sensory neuropathy (Judson *et al*., 2002).

1.2.1.6 Alkylating agents - Melphalan

There are a large number of alkylating agents used in cancer chemotherapy including nitrogen mustards (e.g cyclophosphamide, melphalan, and chlorambucil), chloroethylnitrosoureas (carmustine and lomustine), the alkylalkanesuphonate busulphan, and the natural product mitomycin C. Uptake of alkylating agents into the cell is usually by passive diffusion, although melphalan is transported into the cell by a specific amino acid transport protein (Hartley, 2002).

Alkylating agents have the ability to form covalent bonds with suitable nucleophilic substances within the cell (Rang *et al*., 1995). The main step is formation of a carbonium ion – a carbon atom with only 6 electrons in its' outer shell (Rang *et al*., 1995). These ions are very reactive, and react immediately with an electron donor group such as –SH, -OH, or an amine group. The 7 nitrogen (N7) of guanine, is strongly nucleophilic, and is the main molecular target for simple alkylating agents, although N1 and N3 of adenosine, O6 of guanine and N3 of cytosine may also be affected (Rang *et al*., 1995). However, it is not just single bases that attract

alkylating agents, but groups of bases, so guanine-rich sequences will attract these agents more than a guanine base flanked by cytosines (Mattes *et al.,* 1988). Most chemotherapeutic alkylating agents have two alkylating groups (called bi-functional), and can therefore react with two groups, causing intra- and inter-strand crosslinking (Rang *et al*., 1995). Intra-strand crosslinking occurs if the two groups are on the same strand of DNA, and ICL formation occurs if these two groups are on the two opposite strands of the DNA.

Initially in the formation of crosslinks, monoadducts are formed in which only one of the alkylating groups of the drug reacts with the DNA. For many drugs (e.g melphalan) where only a fraction of monoadducts go on to form crosslinks, the second reaction is slow (Spanswick *et al*., 2000). The ICLs distort the structure of the DNA and prevent the two strands of the DNA from separating during replication and transcription. ICLs also block DNA and RNA polymerases. If not repaired, the cell undergoes apoptosis.

Melphalan

Melphalan (figure 3) was first synthesized in 1953, and is a phenylalanine derivative of nitrogen mustard (also called L-phenylalanine mustard (L-PAM)) (Rothbarth *et al.,* 2002). It has been used to treat patients with breast cancer, melanoma, and colorectal cancer, but is primarily used in multiple myeloma patients (Dollery *et al*., 1991). The most commom side effect of melphalan is bone marrow suppression, including leukopenia and thrombocytopenia (Rothbarth *et al*., 2002). However melphalan has the potential to be carcinogenic itself, causing secondary

malignancies such as acute nonlymphocytic leukaemia, and myeloproliferative syndrome (Dollery *et al*., 1991). Twenty years ago alkylating agents such as melphalan, cyclophosphamide, and chlorambucil were used as monotherapy to treat ovarian cancer (Mcguire *et al*., 2003). These drugs were associated with overall objective response rates ranging between 33% and 65%, with complete clinical responses seen in 20% of patients (Young *et al*., 1979).

Melphalan can be given intravenously as well as orally, and enters the cell by diffusion and by active transport via two distinct amino acid transport systems (Pu *et al*., 2000). Melphalan is completely hydrolysed after 8 hours in water at 37ºC to monohydroxymelphalan and dihydroxymelphalan – the chlorine atoms are replaced in a nucleophilic attack by hydroxyl groups. The hydrolysis rate is dependent on pH, temperature, protein concentration, and chloride ion concentration. Hydrolysis decreases with increasing concentration of plasma proteins and in an acidic environment (Bolton *et al.*, 1993).

Melphalan exerts its cytotoxic effect by formation of interstrand and intrastrand crosslinks as well as DNA-protein crosslinks by alkylation via the two chloroethyl groups of the molecule (Kohn *et al*., 1981). Common sites of alkylation in DNA include the N-7 position of guanine, the N-1, N-3, and N-7 positions of adenine, the N-3 position of cytosine and the O-4 position of thymidine (Rothbarth *et al*., 2002).

As cell killing is observed throughout the cell cycle (although there are variations within it), melphalan is a cell cycle phase non-specific agent (Tannock *et al., 1986*),

and although rapidly dividing cells are killed preferentially, slower dividing cells are also killed.

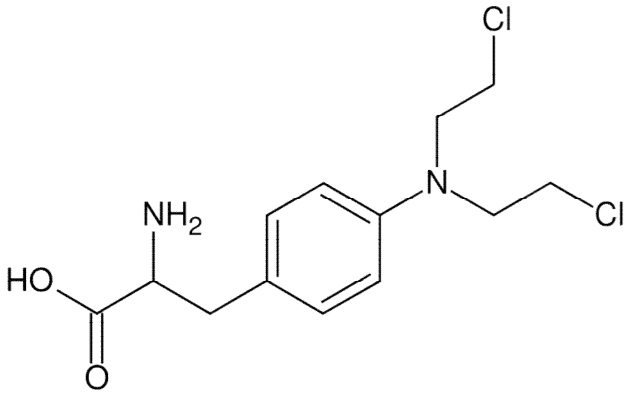


Figure 3: Structure of melphalan

1.2.2 Chemotherapy protocols in Ovarian Cancer

The usual first-line treatment for ovarian cancer is cytoreductive surgery, followed by paclitaxel-carboplatin chemotherapy for stage III –IV disease which has been tested (Baird *et al*., 2001) by the Gynaecologic Oncology Group (GOG) 111 trial (McGuire *et al*., 1996) and the National Cancer Institute of Canada (NCIC) OV10 trial (Piccart *et al*., 2000). The GOG trial was a phase III randomised trial comparing cisplatin and cyclophosphamide, with cisplatin and paclitaxel in patients with suboptimal stage III/IV disease. Three hundred eighty-six patients with advanced ovarian cancer entered the trial. Progression-free survival was significantly longer (P <0.001) with cisplatin/paclitaxel (median, 12.9 v 17.9 months). Overall survival was also significantly longer (P <0.001) with cisplatin/paclitaxel (median, 24.4 v 37.5 months) (McGuire *et al*., 2003). However, there has been some doubt about the clinical value of platinum/taxane combinations after the publication of the large

International Collaberative Ovarian Neoplasm Group 3 (ICON3), which looked at 2074 patients with ovarian cancer. The data from this trial suggest that there was no benefit, in terms of either progression-free or overall survival, from the use of paclitaxel/carboplatin compared with carboplatin alone or of cyclophosphamide/doxorubicin/cisplatin (ICON 2002). Furthermore, the incidences of alopecia, fever, and sensory neuropathy were significantly higher in the taxane treatment arm compared with carboplatin alone (ICON 2002) This has lead to many European women being offered single-agent carboplatin.

A recent randomised phase III study by Arbeitsgemeinschaft Gynaekologische Onkologie-Group d'Investigateurs Nationaux pour L'Etude des Cancers Ovariens (GINECO), involving 1200 patients with advanced ovarian cancer looked at the addition of epirubicin to standard carboplatin-paclitaxel 1st line regimens. The results showed no statisticall significant difference in overall survival between the standard carboplatin-paclitaxel arm versus the carboplatin-epirubicin-paclitaxel arm. Furthermore, the addition of epirubicin increased the number of side effects such as nausea and vomiting, and grade 3 or 4 haematological toxicities (Du Bois *et al.*, 2006). As a result epirubicin cannot be recommended for clinical use in this population of patients.

Another recent randomised controlled phase III study by the AGO-GINECO group examined whether sequential administration of topotecan could improve the efficacy of carboplatin and paclitaxel in first-line treatment of advanced epithelial ovarian cancer. 1300 previously untreated patients with advanced ovarian cancer (FIGO

stages III-IV) were randomized to receive the standard carboplatin-paclitaxel regimen followed by four cycles of topotecan, or surveillance. The sequential addition of topotecan to carboplatin-paclitaxel did not result in superior overall response or progression-free or overall survival. Therefore, this regimen cannot be recommended as standard of care treatment for ovarian cancer (Pfisterer *et al*., 2006).

The Gynaecologic Oncology Group (GOG), have shown that intraperitoneal chemotherapy regimen represents a new standard of care for patients with optimally resected stage III ovarian cancer, but should be offered on an individualized basis (Runowiscz *et al*., 2006) (Armstrong *et al.*, 2006*).* In this study, 400 patients with stage III ovarian cancer with residual disease of less than 1cm after debulking laparotomy were included. Participants were randomly assigned to receive six cycles of treatment with intravenous paclitaxel (135 mg/m^2) on day 1 followed by intravenous cisplatin (75 mg/m^2) on day 2 (intravenous therapy), or six cycles of intravenous paclitaxel (135 mg/m^2) on day 1 followed by intraperitoneal cisplatin (100 mg/m^2) on day 2 and intraperitoneal paclitaxel (60 mg/m^2) on day 8 (intraperitoneal therapy). The patients in the intraperitoneal chemotherapy arm had statistically significant increased survival compared to the intravenous arm (Armstrong *et al*., 2006). However, the quality of life up to 6 weeks after treatment was much worse in the intraperitoneal chemotherapy arm due to side effects such as haematological, gastrointestinal, neurologic toxicities, and catheter complications. However, 1 year after treatment, there was no difference in quality of life between the two groups (Armstrong *et al*., 2006). As a result of this trial, The National

Cancer Institute (NCI) issued a bulletin suggesting that, in women with stage III epithelial ovarian cancer, consideration should be given to the administration of intraperitoneal cisplatin and a taxane (National Institute of Health, 2006).

Pegylated liposomal doxorubicin (PLD) has been used as consolidation chemotherapy in patients with advanced ovarian cancer who have had a complete response to standard carboplatin-paclitaxel regimens. In two studies, a total of 41 patients were given PLD at a dose of 40 mg/m^2 every 28 days for four cycles (Rocconi *et al*., 2006) (Disilvestro *et al*., 2006). These studies concluded that PLD was well tolerated, and the main side effect was palmar-plantar erythrodysesthesia. Overall survival results were promising, but more work needs to be done on optimal dosage and schedule for these patients (Rocconi *et al*., 2006) (Disilvestro *et al*., 2006).

In the recurrent disease setting, there are a number of second-line single chemotherapeutic regimens. In a phase II Gynaecologic Oncology Group study Rose *et al* (1998) reported a 27% response rate in platinum-resistant and 34% in platinum sensitive patients given oral etoposide at 50 mg/m^2/day for 21 days out of a 28-day cycle. Bookman et al used topotecan 1.5 mg/m^2 for 5 days on a 21 day schedule in a phase II trial, and reported response rates of 12.4% in platinum-resistant patients, and 19.2% response rate in platinum-sensitive patients (Bookman *et al*., 1998). There are also combination chemotherapeutic regimens for treating relapsed disease. Gemcitabine combined with either carboplatin or cisplatin has demonstrated an

increase in progression-free survival in patients with clinically resistant ovarian cancer compared to platinum chemotherapy alone (Villella *et al*., 2004).

Carboplatin is much less toxic than cisplatin, and has been used instead of cisplatin since publication of a meta-analysis of 37 trials in over 5000 patients that showed equivalent efficacy of cisplatin and carboplatin (Aabo *et al*., 1998). It also showed the superiority of platinum- over non-platinum-based treatment (Aabo *et al*., 1998).

There have been very few clinical trials to assess the effectiveness and tolerability of melphalan in patients with ovarian cancer which is resistant to platinum chemotherapy. One small study investigated double intermediate-dose melphalan ($100mg/m^2$) supported by autologous stem cells in 14 patients with refractory ovarian cancer and poor performance status (Magagnoli *et al*., 2004). This regimen was well tolerated, and converted one patient from partial remission (PR) to complete remission (CR). Another four patients had disease stabilisation with this melphalan regimen. Nine patients with progressive disease (PD) showed a partial response. Interestingly all patients had a marked improvement in their performance status (Magagnoli *et al*., 2004).

In another study the effectiveness of 10mg of oral melphalan given once a day for 5 days every 6 weeks for 6 cycles was assessed in patients with platinum-resistant relapsed ovarian cancer (Hasan *et al*., 2003). Melphalan was well tolerated, but there were no responses in these 22 patients. Melphalan had no impact on progression-free survival or overall survival (Hasan *et al.,* 2003). Given these two small trials it

is still unclear whether melphalan has a place in the clinical setting in patients with platinum-resistant relapsed ovarian cancer.

Aggressive treatment that involves debulking surgery followed by carboplatin and paclitaxel chemotherapy results in complete response rates of 70-80% (Ferry *et al*., 2000). Unfortunately, 80% of complete responders eventually relapse at a median of 18-28 months (Ozols *et al*., 1999) with disease that is resistant not only to platinum compounds, but also to a wide range of other chemotherapeutic agents (Rustin *et al*., 2004). The prognosis for these patients remains poor, with a 5-year survival of 20% (National Institues of Health, 2006). It is therefore important to overcome resistance to chemotherapy to improve patient survival.

1.3 Cellular pathways

When DNA damage occurs, the cell activates a complex series of pathways, which are incompletely understood that involve DNA repair, cell cycle arrest and/or cell death. There is a critical balance between cell cycle arrest (promoting DNA repair and survival), and cell death following chemotherapy (Longley *et al*., 2005). If the cell is unable to repair its damaged DNA following cell cycle arrest, then apoptosis occurs.

BRCA 1 is involved in many of these cellular pathways. The gene encodes a 220kDa nuclear protein (Kennedy *et al*., 2004) and was discovered in 1994 by genetic linkage studies, which localised it to chromosome 17q (Miki *et al*., 1994). Patients with loss-of-function mutations in BRCA1 gene have an 82% risk of developing breast cancer and up to 54% risk of developing ovarian cancer by 80 years of age (King *et al*., 2003). However, these mutations only account for about 5% of all ovarian cancers (Garcia, 2004). Approximately 70% of ovarian cancers and 30% of breast cancers have reduced BRCA1 expression that is partly caused by methylation of the BRCA1 promoter (Wang *et al*., 2004).

1.3.1 The Cell cycle

Cellular proliferation needs to be tightly controlled as loss of this control can lead to cell death or deregulated proliferation characteristic of cancers. Dividing cells enter the cell cycle in order to grow, replicate their DNA and divide in an organised and

controlled manner. There are 4 distinct phases in a well defined order, each of which should be completed successfully before the next begins. Between each step of the cell cycle there are regulatory checkpoints.

The cell cycle controls cellular proliferation through the integration of negative signals called cell cycle checkpoints, and positive signals (Lowndes *et al*., 2000). Serine-threonine protein kinases called cyclin-dependent kinases (CDK1, 2, 4, 6) and their essential activating coenzymes (cyclins A, B, D, E), as well as CDK-inhibitory proteins (CDKIs) control cell cycle transitions (Funk *et al*., 1999).

The cell cycle starts with G1 in which the cells grow. The next step is S phase where initiation of DNA synthesis takes place. Promotion of the transition from G1 to S phase is regulated by cyclin D-cdk4, cyclin D-cdk6, cyclin E–cdk2 and cyclin A-cdk2 (Lee *et al*., 2002). G2 is the next phase of the cell cycle followed by M phase (mitosis). Cyclin A and B are involved in G2/M transition (Viallard *et al*., 2001).

1.3.1.1 The effect of DNA damage on the cell cycle

When DNA damage such as double-strand breaks (DSBs) occur in G1 or S phases of the cell cycle, entry into S phase or progression through S phase is prevented or slowed, respectively. When DSBs arise in G2, entry into mitosis is prevented (Jackson *et al*., 2002)

It has been proposed that ICLs do not activate the G1 or G2 cell cycle checkpoint, suggesting that they are tolerated by the cell until a DNA replication fork is

encountered (Akkari *et al*., 2000). The S-phase checkpoint could have a major role as co-ordinator of the cellular response to ICLs (Akkari *et al*., 2000).

The combined mechanisms of BRCA1 promote DNA repair and prevent replication of damaged DNA by activating cell cycle checkpoints, although these processes have different mechanisms. This is illustrated by mutation of the serine 988 residue of BRCA1, (this residue is phosphorylated by CHK2 in order to activate BRCA1) which results in defective DNA repair, but not activation of cell cycle checkpoints (Zhang et al., 2004). Once BRCA1 is phosphorylated, it is thought that it stimulates the transcription of p21 (cyclin-independent kinase inhibitor 1A), and p27 (cyclin-dependent kinase inhibitor 1B) (Williamson *et al*., 2002), which arrests cells at the G_1/S boundary and S phase, respectively, by inhibiting cyclin-dependent kinase (Williamson *et al*., 2002). BRCA1 also arrests cells at the G_2/M–phase checkpoint, through the reduction in cyclin B-cdc2 levels (Mullan *et al*., 2001), by a number of mechanisms. These include, inhibiting its expression (Mullan *et al*., 2001), and inducing other proteins such as GADD45 (Growth Arrest and DNA Damage-inducible protein 45) (Mullan *et al*., 2001), Wee 1 kinase and 14-3-3σ. 14-3-3σ binds to cdc25C, which is then prevented from activating the cyclin B-cdc2 complex (Yarden *et al*., 2002). Wee1 kinase inhibits cdc2 by phosphorylation, which means it cannot bind to cyclin B (Yarden *et al*., 2002). GADD45 binds to cyclin B-cdc2 preventing its localisation in the nucleus (Mullan *et al*., 2001).

1.3.2 DNA Repair Pathways

There are many different DNA repair pathways that occur in the cell. The type of lesion present in the DNA determines activation of a particular pathway.

1.3.2.1 Mismatch repair (MMR)

MMR plays a critical role in the maintenance of genomic stability in prokarytes, simple eukaryotes and humans (Kolodner *et al*., 1999). DNA MMR deficiency produces the microsatellite instability phenotype, which is detected as variations in lengths of DNA repeat sequences present in the genome (Fischel *et al*., 1995). MMR is an evolutionary conserved process that corrects mismatches generated during DNA replication which escape proofreading (Kunkel *et al*., 2005). MMR is also responsible for correcting base substitution mismatches and insertion-deletion mismatches (IDLs) generated during DNA replication in organisms from bacteria to mammals (Kunkel *et al*., 2005) Studies have indicated that excision is mismatch dependent, is initiated at a nick or a gap, and has bidirectional capacity (Genschel *et al*., 2002). MMR also preferentially proceeds along the shortest path to the mismatch, and terminates about 150 nucleotides beyond the mismatch (Genschel *et al*., 2002).

MMR starts with recognition of the lesion. The mammalian *E.coli* MutS homologs (MSH proteins) are thought to directly contact double-stranded DNA, and slide along it like a 'sliding clamp' until they encounter a base pair containing a mismatch (Acharya *et al*., 2003). The MSH proteins then interact with a host of other proteins including the mammalian *E.coli* MutL homologs (MLH), and yeast post-meiotic

segregation (PMS) homolog proteins, as well as RPS, EXO1, and RFC (Kunkel *et al*., 2005)**.** Specific MSH proteins identify and repair different sizes of lesions, for example, MSH2-MSH6 (MutSα) heterodimer is thought to primarily repair single base substitutions and 1 base pair insertion-deletion mutations, while MSH2-MSH3 (MutSβ) heterodimer is thought to primarily repair larger 1-4 base pair insertion-deletion mutations (Kunkel *et al*., 2005). MLH-PMS2 is the primary MutL complex that interacts with both MSH2/6 and MSH2/3 complexes to help catalyse their different functions (Kunkel *et al*., 2005).

There is evidence that PCNA (proliferating cell nuclear antigen) interacts with both replication and MMR proteins (Gu *et al*., 1998), and is required at both early and late stages of MMR (Gu *et al*., 1998), suggesting that it could be involved with the initiation of MMR. RFC (replication factor C) is the factor required to load PCNA onto the DNA, and may also be involved in MMR (Xie Y *et al*., 1999). PCNA is also a processivity factor for replicative polymerases, and it interacts with and stimulates the activity of proteins involved in processing Okazaki fragments. Early evidence suggests that PCNA is required for MMR prior to DNA repair synthesis and that PCNA interacts with MSH2 and MLH1 (Umar *et al*., 1996), leading to the suggestion that replication and MMR may be physically coupled and that primers at the replication fork may provide the strand discrimination signal.

In addition to undamaged mismatches, the MMR machinery also recognizes certain DNA lesions generated by normal intracellular metabolism (e.g oxidative stress) (Russo *et al*., 2004), and by physical and chemical insults from the external

environment. MMR proteins activate cell cycle checkpoints and signal apoptosis (Stojic *et al*., 2004). Loss of these functions decreases apoptosis, increases cell survival, and results in resistance to chemotherapy (Stojic *et al*., 2004).

1.3.2.2 Base excision repair

Base excision repair corrects small alterations to bases, including oxidation and alkylation, and is distinct from nucleotide excision repair which repairs bulky adducts and helix distorting lesions. In base excision repair a lesion-specific glycosylase removes the base and the resulting apurinic/apyrimidinic site is converted to a break. A small gap, one or two nucleotides long is filled in by DNA polymerase plus ligase (Evans *et al*., 2000). BER is also a key pathway in the repair of DNA single-strand breaks (Wooster *et al*., 2003), and involves the enzyme poly (ADP-ribose) polymerase (PARP) (Wooster *et al*., 2003). PARP inhibition leads to persistent single-strand breaks in the DNA (Boulton *et al*., 1999). When single-strand breaks are encountered by a replication fork, they become double-strand breaks, and arrest of DNA replication occurs (Haber *et al*., 1999).

Tumours in carriers of BRCA1 and BRCA2 mutations lack wild-type BRCA1 and BRCA2, but normal tissues retain a single copy of the relevant wild-type gene (Bryant *et al*., 2005). This difference could be exploited to treat these patients using PARP inhibitors, as the tumours would be unable to repair the persistent single-strand breaks in the DNA effectively, leading to apoptosis (Bryant *et al*., 2005). This principle could also be applied to other sporadic cancers that have impairments in

the homologous recombination pathways, and thus has wider implications (Bryant *et al*., 2005).

It is interesting that no diseases that are defective in BER have been reported. This is probably because defects in this repair process include defective repair of oxidation DNA damage caused by oxygen free radicals. Due to the abundance of oxygen free radicals, many mutations would occur and thus would be lethal *in embryo* (Collins *et al*., 2002).

1.3.2.3 Nucleotide excision repair (NER)

This is the main pathway used to repair bulky, helix-distorting DNA adducts including those produced by platinum-based chemotherapy (McHugh *et al*., 2000), such as intrastrand crosslinks, and ICLs. NER removes oligonucleotides of approximately 28 bases long, and repairs the correspondingly long repair patch (Collins *et al*., 2002). The importance of NER is highlighted, by the finding that defects in this pathway result in hypersensitivity to cisplatin, and restoration of NER activity reduces sensitivity to more normal levels (Furuta *et al*., 2002).

NER can be further subdivided according to how the lesion is initially recognised into transcription-coupled repair, and global genomic repair pathways (Le Page *et al*., 2000). Once recognition of the lesion is completed, both transcription-coupled NER and global genomic NER have a common pathway involving TFIIH complex, XPA, XPG, ERCC1 and XPF (Mitchell *et al*., 2003).

Transcription-coupled nucleotide excision repair is initiated by the arrest of RNA polymerase II at a lesion on the transcribed strand during transcription. TFIIH, XPG, CSA and CSB are then recruited to the site of blocked transcription (Mitchell *et al*., 2003), where they all participate in the removal of the stalled RNA polymerase (Mitchell *et al*., 2003). Therefore DNA that is transcriptionally active is repaired preferentially. MSH2 (mutS homolog 2) and MSH6 (mutS homolog 6) are also involved. BRCA1 forms a complex with both MSH2 and MSH6 (Wang *et al*., 2000), and is therefore indirectly linked to this pathway, which may explain the increased cisplatin sensitivity shown in BRCA1-deficient cells (Quinn *et al*., 2003).

The first step of global genomic repair is recognition of the lesion. There is supporting evidence for the involvement of the XPC (xeroderma pigmentosum group C) and hHR23B (the human homologue of yeast RAD23) proteins in humans (Batty *et al*., 2000). It is thought that XPC is one of the first proteins to be localised to sites of helical distortions within the DNA (Sugasawa *et al*., 2000). Breast-Cancer susceptibility gene 1 (BRCA1) can modulate the transcription of XPC, DDB2 (damaged DNA binding protein), and GADD45 (growth arrest and DNA damage response protein 45), and has been implicated in global genomic repair (Hartmann *et al*., 2002).

The zinc-finger protein XPA (xeroderma pigmentosum group A) and the heterotrimeric replication protein RPA (replication protein A) are also involved in recognising the damaged DNA (Ferry *et al*., 2000). The XPA-RPA complex is

believed to recruit a large complex called TFIIH (transcription factor IIH), (Ferry *et al*., 2000) which contains helicases (XPB and XPD) (McHugh *et al*., 2000). The helicases unwind the DNA in the vicinity of the DNA lesion in an ATP-dependent manner (Evans *et al*., 1997). The unwinding of the DNA produces a 'bubble' surrounding the lesion, which acts as a structural landmark for asymmetric dual incisions that release the damage in the form of a lesion-containing nucleotide (McHugh *et al*., 2000) (figure 4).

ERCC1 (excision repair cross-complementation group 1) is a single stranded DNA endonuclease (Park *et al*., 1995) that forms a tight complex with its heterodimeric partner XPF (xeroderma pigmentosum group F), and this complex is believed to interact with both XPA (xeroderma pigmentosum group A) and RPA (replication protein A) (Saijo *et al*., 1996). This suggests that ERCC1-XPF plays a role in DNA damage recognition. The XPF-ERCC1 complex makes the initial cut in the DNA strand 5' (Sancar *et al*., 1996) (where XPF is thought to act as the nuclease (McHugh *et al*., 2000) and the XPG (xeroderma pigmentosum group G) protein makes the incision in the 3'to the DNA lesion. The oligonucleotide 24-32 nucleotides long, containing the damage is released (Sancar *et al*., 1996). DNA synthesis occurs across the gap, followed by ligation, and the DNA is restored.The rate-limiting step within this process is DNA damage recognition and excision (Mu *et al*., 1995), particularly involving ERCC1.

The inherent sensitivity of testicular cancer to platinum has led to its curability, which may be due to a defect in the NER pathway. They are defective in NER

because they have very low amounts of XPA and XPF-ERCC1 proteins (Koberle *et al*., 1999). In confirmation of this, the absence of ERCC1 is associated with the most severe DNA repair defect yet discovered (Weeda *et al*., 1997).

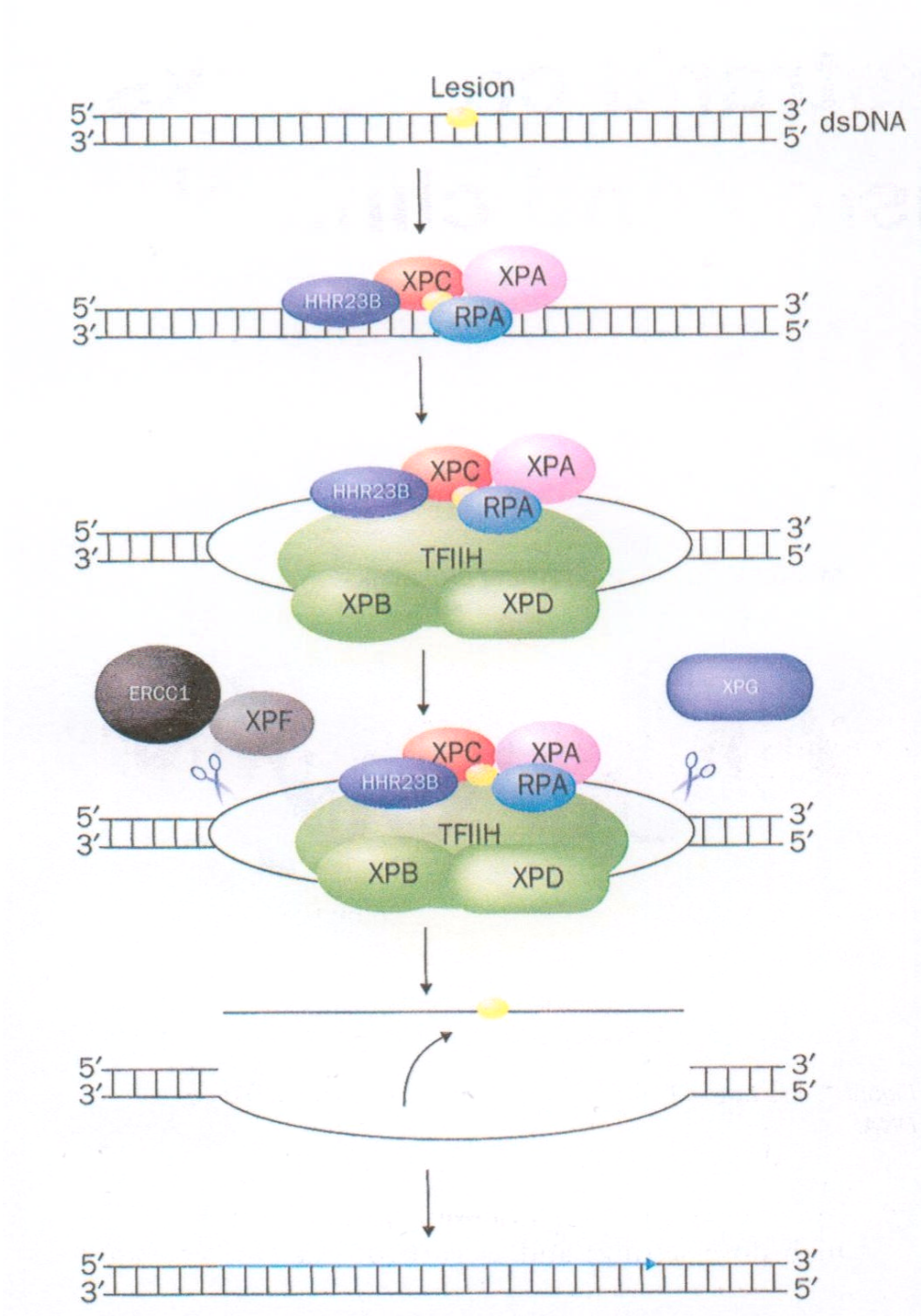


Figure 4: Pictorial representation of the key steps involved in mammalian nucleotide excision repair (McHugh *et al*., 2001).

The organisation of chromatin is crucial for the regulation of gene expression. The nucleosome core within the cell contains DNA wrapped around a central histone octamer comprising two molecules of each of the core histones. The four core histones (H2A, H2B, H3, and H4) are subject to a wide variety of enzyme-catalysed post-translational modifications, thereby modulating the function of the chromatin such as acetylation (Yoshida *et al*., 2001). The primary sites of histone acetylation are specific lysine residues in the positively charged N-termini tails that protrude from the octamer, resulting in neutralisation of these residues (Yoshida *et al*., 2001). Histone acetylation by histone acetyltransferases (HAT's) loosens histone-DNA contacts, and as such is an important step in transcription, and allows access by DNA repair proteins to DNA (Kouzarides *et al*., 1999).

The architecture of the nucleosme, with the helical DNA strands wrapped almost twice around a tight octameric histone core, presents an obvious physical barrier to any repair proteins that need to interact with damaged DNA. The histones H2A and H2B are known to be reversibly post-translationally modified by ubiquitination. The globular ubiquitin molecule is added to specific lysine residues located on the flexible histone C-termini, which is capable of relaxing chromatin (Wolffe *et al*., 1999). This would be expected to make sites of DNA damage more accessible to proteins of the NER pathway. Lending support to this hypothesis is the transient unfolding of nucleosomes during NER in both normal and XPC human cells (Baxter *et al*., 1998). It is also intriguing that the carboxy termini of the human hHR23A and hHR23B RAD23 homologs contain a 50-amino-acid domain highly homologous to E2 ubiquitin-conjugating enzymes (Masutani *et al*., 1997). The hHR23A and

HHR23B enzymes form complexes with the XPC DNA damage recognition protein (Masutani *et al*., 1997).

Proteasome inhibition stabilizes polyubiquitinated proteins, depletes cytosolic unconjugated ubiquitin, and thereby promotes the deubiquitination of nucleosomal histones in chromatin, resulting in the condensation of chromatin. The balance of histone ubiquitination/deubiquitination is rapidly tilted in favour of the enzymatic cleavage of ubiquitin from the histones because the nuclear pool of unconjugated ubiquitin is normally low (Mimnaugh *et al*., 1997, Chen *et al*., 1998).

1.3.2.4 Homologous Recombination

Homologous recombination is a template-dependent DNA repair pathway that is most efficient in late S and G2 phases of the cell cycle found in all organisms studied (Heyer *et al*., 2006). It is critical for the repair of DNA damage, and the recovery of stalled and broken replication forks which preserves genomic stability (Heyer *et al*., 2006)**.** Homologous recombination involves the exchange of DNA between sequences of perfect or near perfect homology over several hundreds of base pairs. In contrast, nonhomologous recombination occurs between sequences with little or no sequence homology (Symington *et al*., 2002).

Homologous recombination can be divided into three stages, pre-synapsis, synapsis, post-synapsis. In pre-synapsis formation of a protein complex consisting of BRCA1, and RAD50-MRE11-Xrs2/ Nbs1 (neijman breakage syndrome) (Zhong *et al*., 1999),

nicks the DNA using its 5'-3' exonuclease activity to expose the 3' ends on either side of the DSB (Hoeijmakers *et al*., 2001). Exo1 is also involved in this process (Heyer *et al*., 2006). Phosphorylation of the serine 1423 residue of BRCA1 activates it, which is important for its function in DNA repair (Cortez *et al*., 1999). RPA then binds to the ssDNA produced at the break site, and is able to recruit and activate ATR (Zou *et al*., 2003).

The mediator proteins Rad52, Rad54, Brca2 (Chen *et al*., 1998), and the Rad 51 paralogs (Rad51B, Rad51C, Xrcc2, Xrcc3), orchestrate the formation of the pre-synaptic Rad51 filament on RPA-coated ssDNA. In support of this, BRCA2-deficient cells have increased sensitivity to ionising radiation, which indicates a defect in DSB repair (Moynahan *et al*., 2001). BRCA2 regulates the intracellular location and function of RAD51 (Davies *et al*., 2001). RAD51 directly binds 6 of the 8 BRC repeats on the BRCA2 protein (Davies *et al*., 2001).

The homology driven pathway then enters synapsis in which single strand annealing, or strand invasion pathways occur. During synapsis a physical connection (d-loop) is generated between the recombinogenic substrate and an intact homologous duplex DNA template leading to the formation of a heteroduplex (or hybrid) DNA (Heyer *et al*., 2006).

The third and final part of homologous recombination is post-synapsis in which a double-holiday junction intermediate is formed. This is resolved by crossover or

non-crossover outcomes involving Rad51 and Rad54, in which the DNA strands are restored (Heyer *et al*., 2006).

In strand invasion RAD51 coated single stranded DNA nucleoprotein filament invades and pairs with a homologous region in the sister chromatid, (Baumann *et al*., 2006) leading to crossing over of genetic material. This usually happens in G_2 and S phases of the cell cycle (Kennedy *et al*., 2004).

Single strand annealing (SSA) forms part of homologous recombination, and occurs when a DSB is flanked either side by homologous regions. This is likely to occur frequently in the mammalian genome because there is a high proportion of repetitive DNA (e.g Alu repeats) (McHugh *et al*., 2000). The DSB is resected as above, and the complimentary 3' ends anneal in the regions of homology, while the overhanging ends are clipped off. Ligation completes the repair (McHugh *et al*., 2000). Again a member of the RAD52 family is involved. The 3' ends of the DNA are removed by the XPF-ERCC1 heterodimer (Sargent *et al*., 2000). SSA is non-conservative and an error prone mechanism of DSB repair.

1.3.2.5 Non-homolgous end-joining (NHEJ)

NHEJ is active throughout the cell cycle (Chu *et al*., 1997), but it is especially important in G1. It appears to be the predominant pathway for repairing DSBs in mammalian cells such as those caused by ionising radiation (McHugh *et al*., 2000). In NHEJ the two ends of the DSB are directly joined together, requiring very little

homology (McHugh *et al*., 2000) and because no template is used, it is less accurate, and therefore is error prone.

NHEJ requires the removal of 1-10 nucleotides to uncover microhomology sequences to allow proper base pairing and ligation of the broken ends (Ting *et al*., 2004). It has been proposed that the MRN complex (Mre/Rad50/Nbs1) is involved in processing the ends. *In vitro* Mre11 has 3'-5' endonucleolytic activity which is enhanced by the presence of Rad50 and Nbs1 (Paull *et al*., 1999). Brca1 inhibits the nucleolytic activity of Mre11, suggesting that Brca1 regulates the function of the MRN complex to prevent extensive DNA end processing (and hence loss of genetic material) (Paull *et al*., 2001).

Once the ends have been processed, a heterodimeric complex of the Ku70 and Ku80 proteins stabilise the ends, by recruiting DNA protein kinase, and facilitates rejoining by DNA ligase IV and ERCC4 proteins (McHugh *et al*., 2001) (figure 5). Under electron microscopy a region of Brca1 (452-1079) is seen to form protein-DNA complexes cooperatively between multiple DNA strands suggesting that Brca1 may act like DNA-PKcs to bridge adjacent DNA molecules (Paull *et al*., 2001).

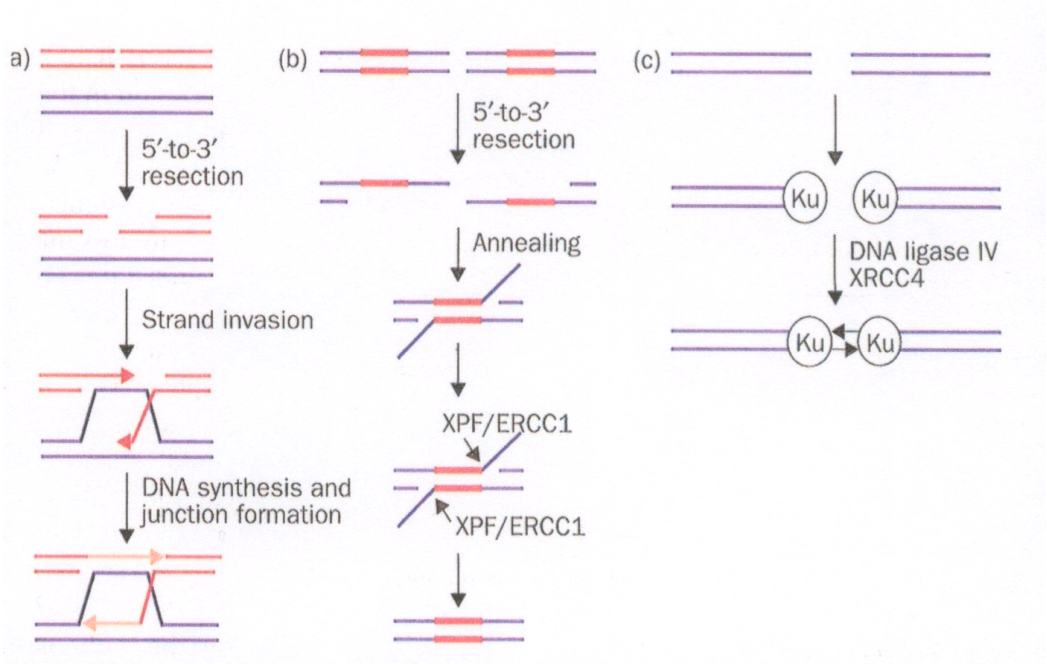


Figure 5: The main pathways for the repair of DSBs in eukaryotic cells. (a) classic model for DSB repair based on experimental data from *Saccharomyces cerevisiae*. (b) single-strand annealing (c) Non-homologous end-joining (McHugh *et al*., 2001).

In BRCA1- and BRCA2- deficient cells, RAD51-dependent homologous recombination cannot occur, and the DSB is repaired by other error-prone mechanisms such as non-homologous end-joining (NHEJ) (Lomonosov *et al*., 2003). This results in large numbers of aberrations and chromatid breaks, leading to loss of viability (Lomonosov *et al*., 2003).

1.3.3 Repair of different DNA lesions

1.3.3.1 Repair of single strand breaks

Single strand breaks can occur during DNA replication or repair, after UV irradiation, or from intermediates of type 1 topoisomerases (Pacques *et al*., 1999).

PARP-1 (poly(ADP-ribose)) binds to single-strand DNA breaks, and initiates the recruitment of the DNA repair machinery (Leppard *et al*., 2003). There is evidence that the DNA ligase IIIa-XRCC1 complex is recruited to DNA single-strand breaks by virtue of the preferential binding of both subunits of the complex to automodified PARP-1 (Leppard *et al*., 2003).

1.3.3.2 Repair of double-strand breaks

There are many ways in which Double strand breaks (DSB) may occur. They can occur naturally, for example in meiosis where rearrangement of gene segments (VDJ joining) during immune-cell development, are important developmentally regulated processes involving DSB intermediates. They can also result from normal metabolic processes that generate reactive oxygen species within the cell that attack DNA (Sankaranarayanan *et al*., 2005). Ionising radiation (McHugh *et al*., 2000) mechanical stress, endonucleases, or replication of a single-stranded nicked chromosome can also produce DSBs (Pacques *et al*., 1999). DSBs can also arise indirectly when a DNA replication fork collides with an un-repaired single-strand break (SSB), giving rise to a collapsed replication fork (Haber *et al*., 1999).

DSBs are the sole instigators of recombination in meiotic cells and are a major factor in recombination in mitotic cells (Pacques *et al*., 1999). DSB are lethal if left unrepaired, and if mis-repaired can lead to translocations and other potentially carcinogenic-causing chromosomal abnormalities (McHugh *et al*., 2000).

DSBs are repaired by mechanisms such as, homologous recombination, and non-homologous end joining (NHEJ), but there is co-operation between them (Richardson *et al*., 2000). Single strand annealing, which is a variant of HR, is also involved in the repair of DSBs. This takes place when direct repeat sequences flank the two DNA ends (Jackson *et al*., 2002). Brca 1 is linked to HR and NHEJ, and is likely to participate in establishing the mechanism of repair of the DSB (Ting *et al*., 2004). In support of this notion, cells display either Rad50 or Rad51 IRIF (ionising radiation-induced foci), and Brca1 co-localises with either Rad50 or Rad51, but not with both at the same time (Zhong *et al*., 1999). Since the MRN complex may be involved in both HR and NHEJ, Brca1 likely executes this decision through coordinating the activities of the MRN complex (Ting *et al*., 2004).

BRCA1 is activated by ataxia telangiectasia mutated protein (ATM) and checkpoint kinase 2 (CHK2) in the response to double strand breaks of the DNA (Tutt *et al*., 2002). ATM and/or ATR become activated in response to DSB, and then phosphorylate histone H2AX (Burma *et al*., 2001) at serine 139, which enables it to recruit a number of proteins: Brca1, MRN (Mre11/Rad50/Nbs1) complex, DNA-PKcs, Chk2 kinases, Mdc1/Nfbd1, 53BP1, and Rad51 all co-localise to histone H2AX following ionising radiation and DSB production (Paull *et al*., 2000). The phosphorylation of histone H2AX produces a type of nuclear foci (or ionising radiation induced foci (IRIF)), and is rapidly detected within 3 minutes of ionising radiation (Rogako *et al*., 1998). There is evidence that Mdc1/Nfbd1 and 53BP1 may play redundant roles in initially associating with histone H2AX, which in turn recruit Brca1 and MRN to histone H2AX (Goldberg *et al*., 2003) (Wang *et al*.,

2002). In response to ICL formation, Fancd2 (one of the proteins involved in the cancer susceptibility syndrome Fanconi anaemia (FA)) becomes mono-ubiquinated and colocalises with Brca1 at IRIF (Garcia-Higuera *et al*., 2001).

1.3.3.3 Repair of DNA interstrand crosslinks (ICLs)

As the ICLs involve both strands, they are critical lesions since they block transcription and replication (McHugh *et al*., 2001). The repair of ICLs in mammalian cells involves components of different repair pathways such as NER, and homologous recombination (McHugh *et al*., 2001). However, the full mechanistic details of mammalian ICL repair have not been fully elucidated (McHugh *et al*., 2001). It has been established that NER and homologous recombination are important for the removal of cisplatin DNA adducts in *Eschierichia coli* (Zdraveski *et al*., 2000) and yeast (McHugh *et al*., 2000). De Silva *et al* demonstrated that the high cisplatin sensitivity of ERCC1- and XPF-mutant cells likely results from a defect other than in excision repair (De Silva *et al*., 2002). It has also been shown in human tissues from patients with ovarian cancer, a possible molecular basis for co-ordinate mRNA expression of genes involved in NER, suggesting that NER is important in human cancers (Zhong *et al*., 2000).

Defects in the homologous recombination repair pathways for DSBs result in increased sensitivity to ICLs in human cells, suggesting that these pathways are

involved in ICL repair (Moynahan *et al*., 2001). In support of this Brca1 (which is involved in HR), deficient cells are sensitive to mitomycin C, which induces ICLs (Dronkert *et al*., 2001). The observation that XRCC2 and XRCC3 mutants are extremely sensitive to cisplatin suggests that homology-driven recombination plays a major role in the repair of cisplatin DNA damage (Caldecott *et al*., 1991). XRCC2 and XRCC3 mutants are also unable to uncouple cisplatin ICLs, which raises the possibility that homologous recombination is initiated prior to the incisions which uncouple the cisplatin cross-link, and that the incision reaction depend upon an early recombination intermediate being formed (De Silva *et al*., 2002)

1.3.4 Apoptosis

Apoptosis plays a major role in chemotherapy-induced tumour cell killing. There is a careful balance between cell cycle arrest to enable DNA repair to occur, and apoptosis. Apoptosis is carried out by the activation of caspases. There are two main pathways to activate caspases: the intrinsic pathway regulated by Bcl-2 proteins, and the extrinsic pathway regulated by tumour necrosis factor (TNF) receptor superfamily (Reviewed in Hengartner *et al*., 2000).

In the intrinsic pathway, the pro-apoptotic Bcl-2 family proteins (such as bax, bad, bak) promote the release of cytochrome c from mitochondria, which forms a complex called the apoptosome with caspase 9 and APAF-1 in the cytosol

(Reviewed in Hengartner *et al*., 2000). Caspase 9 is activated by the apoptosome, leading to cleavage and activation of effector caspases such as caspase 3 and 7. These executioner caspases bring about the morphological and biochemical changes that characterise apoptosis, including, chromatin condensation and nuclear fragmentation, membrane blebbing, and cell shrinkage (Reviewed in Hengartner *et al*., 2000). The cell then breaks into membrane bound fragments called apoptotic bodies that are cleared by phagocytosis without causing an inflammatory response (Longley *et al*., 2005). Other Bcl-2 family members are anti-apoptotic such as Bcl-2 itself, Bcl-X_L and Mcl-1. They bind to the pro-apoptotic members of the Bcl-2 family, and prevent them from becoming activated.

The extrinsic apoptotic pathway is regulated by cell surface 'death' receptors of the TNF-receptor family, such as Fas (CD95/APO-1), DR4 (TNF-related apoptosis-inducing ligand recptor 1, TRAIL-R1), and DR5 (TRAIL-R2). When Fas ligand (FasL), binds to Fas, caspase 8 is recruited via an adaptor molecule FADD (Fas-associated death domain) to form a death-inducing signalling complex (DISC) (Nagata *et al*., 1999), which in turn activates the effector caspases 3 and 7 (Nagata *et al*., 1999). FLIPP (FADD-like interleukin-1β-converting enzyme-inhibitory protein) binds to the DISC and inhibits capase 8 activation (Krueger *et al*., 2001).

Apoptosis is also inhibited by members of the IAP (inhibitors of apotosis) family, which include c-IAP1, c-IAP2, X-IAP, and surviving (Salvesen *et al*., 2002). IAPs can bind directly to caspases, such as caspase 3, 7, and 9, which inhibit their activity (Salvesen *et al*., 2002). IAPs themselves are inhibited by Smac/DIABLO, which is

released from mitochondria along with cytochrome c in response to pro-apoptotic stimuli (Salvesen *et al*., 2002).

MEK is a MAP kinase kinase apoptosis inhibitor that plays a role in signal transduction from growth factors in a receptor tyrosine kinase –RAS-RAF-MEK-ERK cascade (Garrett *et al.,*1999). It has been shown that MEK may stimulate antiapoptotic Bcl-2, Bcl-XL, and MCL-1 proteins (Boucher *et al.,* 2000), as well as inactivate proapoptotic protein BAD (Scheid *et al., 1998*). The role of MEK kinase in response of cancer cell lines to cisplatin-based chemotherapy is unclear: some report MEK inhibition of TP53-dependent apoptosis after cisplatin administration (Hong *et al.*, 1999), and others report an activation (Wang *et al.,* 2000), although in the latter case apoptosis was blocked by MEK inhibitors (Wang *et al.,* 2000). In a study by Kupryja *et al,* 2003, high MEK expression was associated with the highly aggressive endometriod and clear cell carcinomas (p=0.049).

P53 is a tumour suppressor protein encoded by the TP53 gene, and has a central role in the regulation of the cell cycle, apoptosis, survival, DNA repair, transcription, differentiation, senescence as well as glucose metabolism oxidative stress and angiogenesis (Vogelstein *et al*., 2000). Normal cells have low levels of P53 as it has a short half life. Mutations in P53 often encode proteins that are resistant to degradation, and mutant P53 often accumulates in the nucleus of cancer cells.

P53 protein downregulates Bcl-2 (an apoptosis inhibitor), and upregulates BAX expression (an apoptosis promoter), thus promoting apoptosis (Miyashita *et al.*,

1994). It has also been shown that cisplatin-induced apoptosis is associated with wild-type TP53/BAX complex formation (Raffo *et al*., 2000). Cell line studies show that TP53-regulated protein levels may differ before or after cisplatin administration depending on the TP53 status (Jones *et al*, 1998), therefore evaluating functional and dysfunctional TP53 protein, may possibly mask the biological significance of proteins regulated or interacting with wild-type TP53, but not with mutant TP53. BAX expression was a strong prognostic indicator of disease free survival in TP53 (+) ovarian carcinomas, but high levels of BAX expression negatively influenced complete remission in all patient groups, which is in contrast to its pro-apoptotic function (Kupryja *et al*., 2003)

Cisplatin induces apoptosis, which in the majority of ovarian carcinoma cell lines is TP53-dependent (Jones *et al*., 1998). Impaired TP53 protein function resulting from TP53 gene mutation contributes to cisplatin resistance in ovarian cancer cell lines (Jones *et al.,* 1998). TP53 is the most frequently mutated gene in human cancers, with up to 50% of tumours carrying a mutation (Levine *et al*., 1997) and has a central role in determining the response of tumour cells to chemotherapy following DNA damage. This lack of functional p53 may contribute to drug resistance through the inability of cells to undergo apoptosis in response to DNA damage (Ferreira *et al*., 1999).

DNA damage results in the activation of upstream kinases such as ATM (ataxia-telangiectasia-mutated), ATR (ATM and Rad-3 related), and DNA-PK (DNA-dependent protein kinase), which directly or indirectly activate p53 (Ljungman *et*

al., 2000). ATR (ATM-related kinase) also phosphorylates and activates BRCA1 (Zou *et al*., 2003).

Once activated, p53 acts as a transcription factor by up-regulating genes such as GADD45 and p21, which induce cell cycle arrest in response to DNA damage (Dotto *et al*., 2000). However, p53 can also trigger apoptosis by up-regulating pro-apoptotic genes such as Bax, NOXA, TRAIL-R2 (DR5), and Fas (Schuler *et al*., 2001). These processes act to maintain genomic integrity and prevent damaged DNA from being passed on to daughter cells.

In breast cancer cell lines, BRCA1 induces apoptosis, mediated by caspases 9 and 3, in response to spindle damage through activation of the c-jun N-terminal kinase pathway (JNK) (Harkin *et al*., 1999). This pathway also involves activation of the kinases H-Ras, MEKK4, Fas ligand and its receptor (Thangaraju *et al*., 2000), and is specifically activated after treatment with spindle poisons such as paclitaxel (Mingo-Sion *et al*., 2004).

However, there have been conflicting results in ovarian cancer cell lines. BRCA1 expression decreased paclitaxel sensitivity in BRCA1-mutant SNU human ovarian cancer cell lines (Zhou *et al*., 2003), and expression of a dominant negative BRCA in the ID-8 murine ovarian cancer cell line increased the sensitivity of the cells to paclitaxel (Sylvain *et al*., 2002).

1.4 Drug Resistance in Ovarian Cancer

Chemotherapy resistance is clinically defined as the progression of disease during therapy, absence of regression during therapy, or recurrence within 6 months after completed treatment. Progression of disease during therapy is defined by the RECIST (response evaluation criteria in solid tumours) guidelines as a 20% increase in the sum of the longest diameter of the target (i.e ovary) or non-target lesions (Therasse *et al*., 2000). Absence of regression during therapy is defined by the RECIST guidelines as neither disappearance of all target lesions (complete response), >30% decrease in the sum of the longest diameter of target lesions (partial response), or progressive disease (defined above) (Therasse *et al*., 2000). Recurrence of disease is confirmed as a rise of serum Ca-125 levels to more than twice the upper limit of normal (Rustin *et al*., 1996), and in patients with persistently elevated Ca 125 serum levels, recurrence of disease is a doubling of Ca 125 above the nadir (Rustin *et al*., 2001). Recurrence of disease can also be confirmed by CT scans.

Tumours are considered to be sensitive if they exhibit complete clinical response to therapy, or if relapse occurs after remission and treatment has not been administered for more than 6 months (Ringborg *et al*., 1998). Resistance to cisplatin can be intrinsic or acquired. Intrinsic is present at the time of diagnosis, and patients fail to respond to first line chemotherapy. Laboratory studies have shown that cisplatin

resistance is multifactorial, consisting of mechanisms such as: (1) decreased drug accumulation (2) increased drug inactivation (3) evasion of apoptosis (4) enhanced ability to repair DNA damage. However, because cancer cells are heterogenous, more than one mechanism of drug resistance may be present at one time.

1.4.1 Decreased drug accumulation

This can be due to an insufficient dose, low bioavailiblity, poor drug distribution, increased metabolism (e.g extensive first-pass metabolism) and excretion, impaired diffusion of drug to tumour cells, high plasma protein binding, low tissue binding and increased drug efflux. In some human ovarian cancer cell lines, decreased cellular accumulation of platinum compounds has been found to be partly responsible for the resistant phenotype (Jekunen *et al*., 1994). The adenosine binding cassette (ABC) superfamily can confer drug resistance *in vitro* (Taniguchi *et al*., 1996) , and are an important part of enhancing drug efflux. In other non-ovarian cancer cell lines, two cell membrane proteins that may be involved in platinum compound uptake and efflux have been indentified that may account for the cisplatin phenotype (Bernal *et al*., 2005). However due to practical limitations, the importance of decreased drug accumulation as a mechanism of platinum resistance in the clinical setting remains to be established.

A major problem in the treatment of cancer patients with chemotherapy is the development of multi-drug resistance (MDR). This is the resistance of tumour cells

to structurally and functionally unrelated drugs such as the anthracyclines, Vinca Alkaloids, and the epipodophyllotoxins. The multidrug resistance-associated protein gene (MRP1) encodes a protein that is part of the ABC transporters, and is very important in the development of chemotherapy resistance. cMOAT(MRP2), MRP3, MRP4, and MRP5, are all homologues of MRP1. Two proteins, *MDR1* gene encoded-P-glcoprotein (P-gp), and MRP1 (MDR associated protein), are involved in drug transport and are well known for causing MDR, usually by drug efflux. They are both members of the ATP binding cassette transporter superfamily, that transport diverse compounds such as metal ions, phospholipids, and nucleosides (de Jong *et al*., 2001). Despite their common involvement in MDR, there are clear differences in their function and substrate specificity of P-gp, and MRP1. Pgp binds and transports neutral or positively charged, hydrophobic compounds, whereas MRP1 transports conjugated organic anions such as the leukotriene C_4 and GSH S-conjugates of prostaglandin A_2 and aflatoxin B_1 (de Jong *et al*., 2001). GSH is required for the transportation of anthracyclines by MRP1, but GSH is not necessarily conjugated to the drug, instead GSH may be co-transported (Loe *et al*., 1998).

Increased drug efflux can be due to enhanced expression of drug transporter proteins such as P-glycoprotein, and MRP (Gottesman *et al*., 1993). The reduction of expression of MDR1 by RNAi has been found to sensitise cells to paclitaxel (Duan *et al*., 2004), and the reduction of expression of MRP2 with ribozymes confers sensitivity to cisplatin (Materna *et al*., 2005). This suggests that the ABC family of transporters are involved with transporting cisplatin and paclitaxel out of the cancer cells, which confers resistance. Other inhibitors of these drug efflux pumps include

verapamil and cyclosporin A, and have been developed to reverse multidrug resistance (Kool *et al*., 1999). However, P-gp and MRP have also been expressed in normal tissues, so the inhibition of these pumps has distinct adverse effects and is difficult to use these inhibitors in clinical therapy (Kool *et al*., 1999).

Furthermore, in ovarian cancer up to two thirds of tumour specimens have been found to overexpress P-glycoprotein on immunohistochemistry. This overexpression has been shown in some cases to correlate with poor overall survival, (Baekelandt *et al*., 2000) and poor response to standard chemotherapy (Yokoyama *et al*., 1999). As a result of this, inhibitors of P-glycoprotein (P-gp) have been studied in trials. One such phase III trial compared carboplatin and paclitaxel with or without PSC-833 (Valspodar, which is an inhibitor of P-gp) for the treatment of 762 patients with advanced ovarian cancer. The results showed that the addition of PSC-833 led to a reduction in response rates and no difference in overall survival (Baird *et al*., 2001). However it has since been shown that valspodar has unpredictable pharmacokinetic interactions and in addition targets other transport proteins (Thomas *et al*., 2003), which may contribute to the disappointing clinical results. There are other novel p-gylcoprotein inhibitors such as tariquidar and zosuquidar that are highly specific for p-glycoprotein (Starling *et al*., 1997). They are currently being tested in phase III clinical trials to determine if they can reduce drug resistance (Starling *et al*., 1997).

ABCC2 is a human ABC transporter also called multidrug resistance-associated protein (MRP2), or canalicular multiple organic anion transporter (cMOAT), and is expressed in the apical membranes of canalicular cells in the liver (Kool *et al*., 1997)

where it functions as the major exporter of organic anions from the liver into the bile (Wada *et al*., 1998). *In vitro* experiments showed that overexpression of ABCC2 could confer resistance to platinum-based chemotherapy in ovarian cancer cell lines by increasing cisplatin efflux (Taniguchi *et al*., 1996). Although xpression of ABCC2 could be detected in clinical specimens of ovarian carcinoma, an immunohistochemical study using frozen tissue sections of tumours could not show any prognostic value of ABCC2 assessment for response to chemotherapy or progression-free survival (Arts *et al*., 1999).

1.4.2 Increased drug inactivation

Gluthathione is a powerful antioxidant, which inhibits oxidative stress that can damage DNA and RNA (Rothbarth *et al*., 2002). It is a tripeptide thiol which has an important role in cellular detoxification of various toxins (Van der Zee *et al*., 1995). Binding of drugs such as cisplatin and carboplatin by glutathione, catalysed by glutathione-S-transferase (GST-π) (Gottesman *et al*., 1993), inactivates them. The resulting complex is a substrate for ABC transporter proteins in leukaemia cells, which transport it out of the cell (Ishikawa et al., 1993), contributing to drug resistance further.

High levels of glutathione have been found in tumour cell lines resistant to platinum chemotherapy (Kelland *et al*., 1993) and melphalan (Schroder *et al*., 1996). In a study by Bratasz *et al.* 2008, it was shown that NCX-4040, a nitric oxide-releasing

derivative of aspirin, could resensitize drug-resistant ovarian cancer cells (ovarian cell lines and xenograft tumours) to cisplatin, possibly by depletion of cellular thiols (Bratasz *et al*., 2008)

A relationship has also been reported between high levels of GST-π, worse prognosis, and resistance to platinum chemotherapy in ovarian cancer (Green *et al*., 1993). Another study has also shown high levels of GST-π in platinum resistant ovarian cancer cells (Sakamoto *et al*., 2001) and tumours (Green *et al*., 1993). However, other studies did not support these findings and could not find any relationship between GST-π expression and survival or response to chemotherapy in ovarian cancer (Van der Zee *et al*., 1995). In a large recent study by Nagle *et al*, 2007, the common glutathione-S-tansferase polymorphisms (GSTM1, GSTP2, GSTT1) of 239 Australian women with primary epithelial ovarian cancer were analysed to see if there was a relationship to survival. Reduced GST function was associated with better survival outcomes (Nagle *et al.*, 2007).

A platinum analogue called ZD0473 avoids binding to cytoplasmic thiols, thereby overcoming thiol-mediated detoxification. This was tested in phase II clinical trials in 94 patients with ovarian cancer that had been previously treated with carboplatin. The results were disappointing and showed that ZD0473 had much poorer response rates, median survival, and median time to progression than carboplatin (Gore *et al*., 2002). This pathway therefore does not appear to be a major factor in the clinical resistance to platinum compounds.

1.4.3 Evasion of apoptosis

P53 is a tumour suppressor protein encoded by the TP53 gene, and has a central role in the regulation of the cell cycle and apoptosis (Vogelstein *et al*., 2000). The TP53 gene is often mutated in ovarian cancer, and a number of studies have shown that these patients have worse clinical outcomes following platinum-based chemotherapy, (Marx *et al*., 1998). However, it is difficult to know if the p53 status predicts poor response to chemotherapy, or an inherently aggressive tumour type. The Gynecologic Oncology Group (GOG) performed a detailed analysis of p53 overexpression in previously-untreated women with early invasive or advanced stage epithelial ovarian cancer (EOC). Women included had participated in either the GOG-157, a randomized phae III trial of three vs six cycles of paclitaxel + carboplatin in high risk, early stage EOC, or GOG-111, a randomized phase III trial of cyclophosphomide + cisplatin, vs paclitaxel + cisplatin in sub-optimally resected, advanced stage EOC (Darcy *et al*., 2008). P53 overexpression was assessed by DO-7 immunostaining, and was found to be overexpressed in 51% (73/143) and 66% (90/136) of cases on the GOG-157 and GOG-111 cohorts, respectively. P53 overexpression was not associated with any clinical characteristics or overall survival (OS) but was associated with worse progression free survival (PFS) (logrank test: p=0.013; unadjusted Cox modelling: p=0.015). In the GOG-111 cohort, p53 overexpression was associated with GOG performance status (p=0.018) and grade (p=0.003), but not with age, stage, cell type, or tumour response and disease status after primary chemotherapy, PFS, or OS. Adjusted Cox regression

modelling demonstrated that P53 overexpression was not an independent prognostic factor in either cohort (Darcy *et al*., 2008).

Experimental and clinical data seem to show that paclitaxel enhances apoptosis through a p53 –independent pathway that probably involves the BAX gene. Whereas patients with wild type p53 tumours have a good chance to respond to platinum, patients with mutant p53 tumours may have a clinical need to add paclitaxel to platinum-based chemotherapy (Gadducci *et al*., 2002). Therefore determining p53 status can be useful in predicting therapeutic response to specific drugs. Other studies are being performed to assess whether wild-type p53 can be reintroduced into tumour cells using adenovirus ADP53 to restore chemosensitivity in these cells (Wolf *et al*., 2000). A large scale randomised trial is underway to assess intraperitoneal ADP53 in combination with platinum-based chemotherapy (Vasey *et al*., 2003).

Lactacystin (LC) and *N*-acetyl-leucyl-leucyl-norleucinal (ALLnL), which are both proteasome inhibitors potently increase p53 levels in cell lysates and stimulated the binding of p53 to chromatin (Mimnaugh *et al*., 2000). Bcl-2 and Bcl-x_L inhibit apoptosis by regulating the release of cytochrome *c* from the mitochondrial membrane. Expression of Bcl-x_L is associated with a shorter disease-free survival period in patients receiving platinum-based chemotherapy for ovarian cancer (Williams *et al*., 2005). In confirmation of this, ectopic expression of Bcl-x_L renders the A2780 ovarian cancer cell line more resistant to cytotoxic agents both in vitro and in tumour models *in vivo* (Williams *et al*., 2005). Conversely, down–regulation

of Bcl-2 (in this case in combination with antisense directed to MDR1) leads to a significant sensitisation to doxorubicin (Pakunlu *et al*., 2003).

Several cytotoxic agents have been shown to induce the ligand for Fas, which contributes to drug-induced apoptosis, since the Fas receptor is part of a death-induced signalling complex (Pakunlu *et al*., 2003). FLIP (FLICE-like inhibitory protein) inhibits apoptosis by preventing the assembly of the Fas receptor with its signalling complex. Ectopic expression of FLIP inhibits cisplatin-induced apoptosis and RNAi directed to FLIP sensitises cells to cisplatin (Abedini *et al*., 2004). Similarly TRAIL, another ligand, which induces apoptosis, is able, in some cells to potentiate apoptosis induced by cytotoxic agents, including carboplatin and paclitaxel (Cuello *et al*., 2001). Decoy receptor 3 (DcR3) is a soluble tumour necrosis factor receptor found in ascites of patients with ovarian cancer. Ascites from 44 patients inhibited fas-ligand mediated apoptosis, and higher levels of DcR3 were associated with stage 4 disease, and higher incidence of platinum resistance (Connor *et al*., 2008).

Another molecule that has been implicated in drug resistance through its anti-apoptotic mechanism is the caspase inhibitor XIAP. In cell lines that are sensitive to cisplatin, XIAP is down regulated by the drug, which leads to a corresponding activation of the pro-apoptotic caspase 3. In contrast, drug-resistant cell lines have shown no decrease in XIAP after exposure to an equimolar concentration of cisplatin (Yang *et al*., 2004). Ectopic expression of XIAP can render cells more resistant to cisplatin (Asselin *et al*., 2003) and this appears to depend on activation

of the PI 3-kinase/Akt signalling pathway (Asselin *et al*., 2003). Conversely anti-sense oligonucleotides directed to XIAP can sensitise resistant cells to cisplatin (Yang *et al*., 2004). These studies suggest that by preventing apoptotis, XIAP can potentially contribute to drug resistance.

The phosphatidylinositol 3-kinases (PI3Ks) are widely expressed lipid kinases that phosphorylate phosphoinositides at the D-3 position of the inositol ring. These enzymes function as signal transducers downstream of cell-surface receptors (Reviewed in Garcia-Echeverria *et al.,* 2008). The products of PI3K-catalzed reactions are second messengers and have central roles in a number of cellular processes including cell growth, differentiation and survival. In the PI3K/Akt pathway, PI3K recruitment to tyrosine kinase receptors leads to a transient rise in phosphatdylinositol 2,4,5-triphosphate (PIP3), which recruits PH-domain containing proteins such as Akt (Bellacosa *et al.*,1998). After translocation to the plasma membrane, Akt is activated by phosphorylation, which then activates downstream cytosolic and nuclear effectors. In tumour cells, Akt is constitutively activated which leads to dysregulated proliferation, increased angiogenesis, cell growth and survival (Bellacosa *et al.,* 1998).

PIK3CA gene encodes a catalytic subunit of PI3K. In a study by Kolasa *et al*, molecular analysis on 117 ovarian carcinomas revealed PIK3CA mutations occurred in 5/117 (4.3%) carcinomas, exclusively in the endometrioid and clear cell sub-types (p=0.0002). PIK3CA amplification occurred in 28/117 (24%) of ovarian tumours and strongly diminished odds of complete remission (OR=0.25, p=0.033), and

platinum sensitive response (PS, OR=0.12, p=0.004) in the taxane-platinum patients, suggesting that PIK3CA amplification may be a marker predicting ovarian cancer response (Kolasa *et al.*, 2009).

The PI 3-kinase-Akt pathway has been identified as a potential contributor to drug resistance by preventing chemotherapeutic drugs from inducing apoptosis (Pommier *et al.*, 2004). The tumour suppressor PTEN catalyses the degradation of the 3-phosphoinositides that are generated by the PI 3-kinase pathway, and thus antagonising this pathway. Although the gene encoding Akt is amplified in only 12% of ovarian cancers (Bellacosa *et al.*, 1995) it is reported to be hyperphosphorylated and activated inbetween 36 and 68% of clinical samples (Altomare *et al.*, 2000). Activation of this survival pathway by ectopic expression of PI 3-kinase (Lee *et al.*, 2005), constitutively active Akt (Yuan *et al.*, 2003) or by RNAi directed to PTEN (Lee *et al.*, 2002) appears to increase resistance of ovarian cancer cells. Similarly, inhibiting this pathway by inhibition of PI 3-kinase (Hu *et al.*, 2002) or expression of a dominant-negative interfering variant of Akt (Yuan *et al.*, 2003) can increase sensitivity to cytotoxic drugs.

1.4.4 Enhanced ability to repair DNA damage

Increased platinum-DNA adduct removal has been shown by several DNA repair assays to be associated with cisplatin resistance in an A2780 cisplatin resistant human ovarian cancer cell line (A2780cisR) (Ferry *et al*., 2000). Up-regulation of ERCC1 mRNA in response to cisplatin exposure correlates with the development by tumour cells of an acquired resistance to this chemotherapeutic agent (Parker *et al*., 1991). The importance of this enzyme is demonstrated by the fact that cisplatin-DNA adducts cannot be repaired without functional ERCC1 enzyme (Lee *et al*., 1993), and expression of antisense ERCC1 leads to sensitisation of cells to cisplatin both *in vitro* and in xenograft tumour models (Selvakumaran *et al*., 2003). Proteasome inhibitors (such as LC and ALLnL) prevent the increase in ERCC1 mRNA expression that occurs in cells exposed to cisplatin (Mimnaugh *et al*., 2000).

Clinically in ovarian cancer, there is a direct association between mRNA levels of ERCC1 gene and clinical resistance to platinum-based chemotherapy (Dabholkar *et al*., 1994). Activator protein 1 (AP-1) is an important transcription factor for ERCC1 (Li *et al*., 1998), and pharmacological modulation of AP1 levels appear to impact strongly on ERCC1 mRNA and protein expression (Li *et al*., 1998). AP1 transcription factor consists of either, heterodimers formed between Jun and Fos family members of proto-oncoproteins, or homodimers of Jun proteins (Gottlicher *et al*., 1997).

There is evidence that cisplatin induces expression of proto-oncogenes *c-fos/c-jun* and activates c-Jun NH_2 –terminal Kinase / stress-activated protein kinase (JNK/SAPK) in ovarian cancer cells and other tumour cells (Liu *et al*., 1996). JNK/SAPK is a subfamily of MAP kinases in the Ras pathway, which is responsible for the phosphorylation of Jun protein (Li *et al*., 1998). Phosphorylation of the transcription factor c-Jun at serine residues 63 and 73 in its NH_2-terminal domain greatly enhances the transcriptional activity of the AP1 binding sites (Pulverer *et al*., 1991) and AP1 regulated genes (Derijard *et al*., 1994). In addition, cell lines resistant to cisplatin exhibited higher levels of c-jun and c-Fos, and down-regulation of c-jun and c-Fos expression sensitizes the cells to cisplatin (Moorehead *et al*., 2000). In support of this, a dominant-negative c-jun reduces the repair of cisplatin adducts (Gjerset *et al*., 1997). Therefore it is possible that the effect of cisplatin on ERCC1 could be through AP1 induction or c-Jun phosphorylation.

The JNK/SAPK pathway has been reported to protect against cisplatin-induced DNA damage and that this response is required for DNA repair and survival following cisplatin treatment (Potapova *et al*., 1997). This suggests that the Ras/JNK pathway may mediate a physiological response to DNA damage such as the induction of one or more DNA repair enzymes. Youn *et al* have shown in NIH3T3 mouse embryo cell lines that activation of oncogenic H-Ras increases ERCC1 promoter activity through Ap1-binding sites. This is an essential role for oncogenic H-Ras mediated cell survival against platinum-based agents, and is important in the development of resistance to chemotherapy and ionising radiation in tumour cells (Youn *et al*., 2004).

To try and inhibit the increase in DNA repair, ways of creating a physical barrier to the components of the NER pathway have been tried. Proteasome inhibitors promote deubiquitination of nucleosomal histones in chromatin, resulting in the condensation of chromatin. When cells were pretreated with proteasome inhibitors (such as lactacystin) and then treated with cisplatin, chromatin condensation, increased amounts of cisplatin-DNA adducts, diminished NER-dependent repair of cisplatin-DNA lesions were all observed, compared to control cells which were just treated with cisplatin alone (Mimnaugh *et al*., 2000). Another component of the NER pathway, XPA, is overexpressed in cells that are cisplatin resistant (Dabholkar *et al*., 1994).

Ovarian cancer cell lines over-expressing BRCA1 have been shown to be cisplatin resistant, which has been attributed to BRCA1-dependent DNA repair (Husain *et al*., 1998). Also, in support of this, inhibition of BRCA1 by antisense RNAs increased cisplatin sensitivity, and overexpression of BRCA1 in murine ovarian cancer cells increased resistant to cisplatin (Sylvain *et al*., 2002). Experiments in mouse embryonic stem cells with a mutant BRCA1 showed increased sensitivity to alkylating agents such as mitomycin C, and cisplatin, compared with cells expressing wild type BRCA1 (Moynahan *et al*., 2001). This is probably because BRCA1 mediated DNA repair does not happen and the cell undergoes apoptosis. In the clinical setting, it has been shown in a retrospective trial that patients with mutations in BRCA1 or BRCA2 respond better to platinum-based chemotherapy,

and have an overall better prognosis than those patients with sporadic disease (Cass *et al*., 2003).

Cell lines deficient in the mismatch repair genes hMLH1 or hMSH2 are resistant to cisplatin *in vitro* (Brown *et al*., 1997). A common reason for their deficiency in these genes is hypermethylation of the hMLh1 promoter (Plumb *et al*., 2000). In support of this, a clinical study found that residual ovarian tumours exhibited microsatelite instability after platinum-based chemotherapy, which was linked to a loss of expression of hMLH1 (Watanabe *et al*., 2001). In response to this 2'-deoxy-5-azacytidine (DAC), which inhibits DNA methyltransferase activity, can be used to re-sensitize cells to chemotherapy drugs including cisplatin (Plumb *et al*., 2000) by reducing the hypermethylation of the hMLH1 promoter. DAC is now in clinical trials in combination with carboplatin in ovarian cancer (Longley *et al*., 2005). However, newer platinum-based drugs such as oxaloplatin, do not show the same resistance caused by loss of expression of hMLH1 (Raymond *et al*., 2002). This is because the MMR machinery does not recognise the DNA adducts introduced by oxaloplatin (Raymond *et al*., 2002).

1.5 Aims

The comet assay has made it possible to study levels of ICLs in individual cells. It can also be used in the clinical setting as the number of cells it requires can easily be obtained from patients.

The human paired A2780 and A2780cisR ovarian cancer cell line will be used as an *in vitro* model to establish peak of ICL formation and repair, after treatment with cisplatin. These time points will then be used in clinical samples. Little is known about clinical resistance, especially ICL formation and repair and their contribution to platinum chemotherapy resistance. Ovarian cancer cells obtained from patients with ovarian cancer that are clinically resistant and sensitive to platinum chemotherapy will be compared to determine the importance of ICL formation and repair in clinical platinum resistance. It is unclear if upstream mechanisms such as drug efflux, drug detoxification mechanisms, or decreased drug influx play an important role in platinum resistance in the clinical setting. By measuring ICL formation it will become apparent if upstream mechanisms significantly contribute to platinum resistance.

Melphalan used to be used in the treatment of ovarian cancer, and also causes interstrand crosslinking. Results from our laboratory suggest that multiple myeloma patients resistant to melphalan may be sensitive to cisplatin, and it was hypothesised that ovarian cancer patients resistant to platinum chemotherapy, may be sensitive to melphalan. Again, the human paired A2780 and A2780cisR ovarian cancer cell line

will be used as an *in vitro* model to establish peak of ICL formation and repair, after treatment with melphalan. These time points will be used in clinical samples.

Microarrays have enabled as many as 30,000 genes to be analysed in a single sample. This has enabled great advances in the understanding of gene expressions in different cell populations. Gene expression will be investigated in platinum-resistant and platinum-sensitive patients with ovarian cancer to look for any possible differences in ICL formation and repair found in patient samples.

2. MATERIALS AND METHODS

2.1 Patient Recruitment

Ethics Approval for this study was granted by the joint UCL/UCLH committees on the ethics of Human research. Ethics reference number 04/Q0505/77 (Appendix 1 – study protocol).

All patients being treated at University College Hospital between October 2004 and January 2006 with known epithelial ovarian cancer were included in the study. Patients with suspected or known epithelial ovarian cancer were approached in clinics, and on the wards. They were given the patient information leaflet to read (Appendix 2 – Patient information sheet), and the study was explained to them. If patients wanted to enter the study, written consent was obtained using the patient consent form (Appendix 3 – Patient consent form). There were two parts of the consent form, the first signature was required for the patient to take part in the study, and the second signature was required to store samples received from the patient for future research. All patients needed to sign the first part to be included in the study, but not all patients signed the second part so their samples could not be used for future research. Only patients with proven epithelial ovarian cancer by cytology and/or histopathology were included. There were no other exclusion criteria.

2.2 Measurement of cisplatin cytotoxicity on human ovarian cancer cell lines

First the cells were prepared, counted, and plated up for the experiment. The SRB (sulphorodamine Blue) assay was used.

2.2.1 Preparation of A2780 and A2780cisR human ovarian cancer cell lines

A2780 and A2780 cisplatin resistant cell lines were used. All cell lines tested negative for mycoplasma. An aliquot of each cell line containing $1x10^6$ cells was taken from a stock in liquid nitrogen. The vials were quickly thawed at room temperature, and 9mls of RPMI media containg 10% FCS and 1% glutamine was added to each sample and pipetted into a 20ml conical tube. The tubes were spun in a Jouan CT422 centrifuge (Thermoelectron, Basingstoke, UK) at 270g for 5 minutes. The supernatant was poured off and the pellet was re-suspended in 12mls of RPMI media containg 10% FCS and 1% glutamine and the whole sample was pipetted into an $80cm^2$ flask. The flasks were put into an incubator (37°C, 5% CO_2) overnight for the cells to attach.

The cells were looked at under the microscope to assess confluency, which is seen as cells touching each other with very little space between them. Once the cells were reaching confluency, they were split to enable their growth and multiplication to continue. The RPMI media containing 10% FCS and 1% glutamine was poured off, and 3mls of trypsin (Autogen Bioclear Uk ltd. Wiltshire, UK) was pipetted into the flask to wash the cells, and then poured off. A further 5mls of trypsin was pipetted into the flask and left in the incubator for 5 minutes, at 37˚C and 5%CO_2. After 5 minutes the trypsin was pipetted off and added to 10mls of RPMI media containing

10% FCS and 1% glutamine in a 20ml conical tube, and spun in a centrifuge at 270g for 5 minutes. The supernatant was poured off and the cell pellet was re-suspended in 12mls of RPMI media containing 10%FCS and 1% glutamine. A variable amount of the suspension, according to the size of the cell pellet, was pipetted into sterile 80cm^2 flasks. If the cell pellet was approx 0.2mls then 10mls of the RPMI media containg 10% FCS and 1% glutamine with the cells suspended in it was used. If the cell pellet was approx 1.0mls, then only 2mls of the RPMI media containg 10% FCS and 1% glutamine containing the cells was used.

Once the cells were reaching confluency, as viewed down an inverted microscope, they were then split again. This was repeated, until the cells were ready to use for the experiments below. Cells were only used if they were at the point of reaching confluency, as this meant they were in the exponential phase of growth.

2.2.2 Determining the concentration and amount of cells present in media

1ml of RPMI media (containing 10% FCS and 1% glutamine) containing the single cell suspension of tumour cells, was pipetted onto a haemocytometer which already had a cover slip pushed on the slide to create a vacuum. The haemocytometer had a grid of 9 large squares printed on it which was clearly seen under the microscope. The cells in each large corner square were counted (4 in total) and the number averaged to give the number of cells present. This was the number of cells present x10^4 in 1ml of sample (i.e the concentration of the sample). The solution was diluted

as appropriate to obtain the right concentration of cells to be used in each experiment.

2.2.3 Measurement of cisplatin cytotoxicity using the SRB assay

Four 96 well plates were plated up with 100µl of $0.5x10^4$/ml of cells in each well. Two plates had A2780 and the other two had A2780cisR cells. They were left to adhere overnight. They were all treated with a range of cisplatin concentration: 0µM, 0.03µM, 0.1µM, 0.3µM, 1µM, 3µM, 10µM, 30µM, 100µM. The cisplatin was added to RPMI media containing only 1% glutamine and no FCS, to obtain these concentrations, and 100µl was pipetted into each well. The cisplatin was left on for 1 hour and then carefully pipetted off. 100µl of RPMI media with 1% glutamine and 10% FCS was pipetted into each well. The plates were then left for 4 days in the incubator (37º C, 5%CO_2).

After this time, the media was removed by inverting and flicking the plates. 100µl of 30% acetic acid was added to each well to fix the cells for 20 minutes at 4°C in the fridge. The acetic acid was removed by flicking the plates. The plates were then washed with tap water 4 times. 100µl of a protein stain called sulphorodamine blue (SRB) (sigma, Poole, Dorset, UK) was then pipetted into each well, and left at room temperature for 20 minutes. The SRB was removed by flicking the plates. Excess SRB was removed by washing with 1% acetic acid 5 times. The plates were left to dry overnight. 100µl of 10mM trizma base (Sigma, Poole, Dorset, UK) was added to each well to solubilise, and left for 20 minutes at room temperature. The plates were read at 540nm.

2.3 Measurement of ICL formation and repair in human ovarian cancer cell lines

2.3.1 Preparation of A2780 and A2780cisR human ovarian cancer cell lines

These cell lines were prepared as above (see section 2.2.1)

2.3.2 Determining the concentration and amount of cells present in media

The cells were counted to ensure the correct concentrations of cells to be used in the experiments (see section 2.2.2).

2.3.3 Drug Treatment

Cells were treated *in vitro* with varying concentrations of cisplatin to measure the level of ICL formation in the cells at the peak of crosslinking (9 hours for cisplatin). Other cells were treated *in vitro* with either 100μM cisplatin or 50μM melphalan, and the level of ICL formation and repair measured over time using the comet assay.

2.3.3.1 Treatment *in vitro* with different concentrations of cisplatin

Two 6 well plates were plated up with 2mls of $5x10^4$/ml of cells in each well. One plate had A2780 cells and the other A2780cisR. They were left to adhere overnight. Each well was treated with a different concentration of cisplatin: 0μM, 25μM, 50μM, 100μM, 150μM, 200μM. The cisplatin was added to RPMI media containing only 1% glutamine and no FCS, to obtain these concentrations, and 1ml was added to each well. It was then incubated with the cells for 1 hour in an incubator (37ºC, 5% CO_2).

The cisplatin was pipetted off and discarded. This was done by adding sodium hydroxide to neutralise the drug, before pouring down the sink with plenty of water. 2mls of RPMI media containing 10% FCS and 1% glutamine was pipetted into each well. After a further 8 hours, the cells were harvested using trypsin: The RPMI media containing 10% FCS and 1% glutamine was pipettd off, and 1ml of trypsin was pipetted into the appropriate well and left in the incubator at 37ºC, 5% CO_2 for 5 minutes. After 5 minutes the trypsin was pipetted off and added to 5mls of RPMI media containing 10% FCS and 1% glutamine in a 10ml conical tube, and spun in a centrifuge at 270g for 5 minutes. The supernatant was poured off. Into each tube 1ml of DMSO (VWR International, Leicester, UK) with 10% FCS was pipetted, and the sample stored at -80ºC ready for the comet assay (see section 2.3). This was repeated 4 times.

2.3.3.2 Treatment *in vitro* with 100μM cisplatin

Ten wells of two 6 well plates were plated up by pipetting 2mls of $5x10^4$/ml of A2780 cells into each well, and another ten wells of two 6 well plates were plated up with 2mls of $5x10^4$/ml of A2780cisR cells. They were left to adhere overnight. Cisplatin was added to RPMI media containing only 1% glutamine and no FCS, to obtain a concentration of 100μM, and 1ml was pipetted into each well. Cisplatin was incubated with the cells in an incubator at 37ºC, and 5%CO_2 for 1 hour. After 1 hour the cisplatin was pipetted off and replaced with 2mls of RPMI media containing 10% FCS and 1% glutamine (except the ‘0 hour’ and ‘0 hour control’ wells).

The “0 hour” well and “0 hour control” on each plate were harvested using trypsin: The RPMI media containing 10% FCS and 1% glutamine was pipetted off. 1ml of trypsin was pipetted into the appropriate well incubated at 37ºC, 5% CO_2 for 5 minutes. After 5 minutes the trypsin was pipetted off and added to 5mls of RPMI media containing 10% FCS and 1% glutamine in a 10ml conical tube. It was spun in a centrifuge at 270g for 5 minutes and the supernatant was poured off. The cell pellet was resuspended with 1ml of DMSO (VWR International, Leicester, UK) with 10% FCS, and the sample stored at -80ºC ready for the comet assay (see section 2.3). After a further 3, 6, 9, 24, 24 control, 32, 48 control and 48 hours the appropriate well on each plate was harvested, and frozen as above. The comet assay was performed on the samples, and the results plotted. The experiment was repeated 4 times.

2.3.3.3 Treatment *in vitro* with 50μM Melphalan

Seventeen wells of three 6 well plates were plated up with 2mls of $5x10^4$/ml of A2780 cells in each well, and another seventeen wells of three 6 well plates were plated up with 2mls of $5x10^4$/ml of A2780cisR cells in each well. They were left to adhere overnight. The melphalan was added to RPMI media containing only 1% glutamine and no FCS to obtain 50μM, and 1ml was added to each well. It was then incubated with the cells for 1 hour in an incubator at 37ºC, and 5%CO_2.

The melphalan was then pipetted off, and replaced with 2mls of RPMI media containing 10% FCS and 1% glutamine. The “0 hour” well and “0 hour control” on each plate were harvested using trypsin: The RPMI media containing 10% FCS and

1% glutamine was pipettd off, and 1ml of trypsin was pipetted into the appropriate well and incubated at 37ºC, 5% CO_2 for 5 minutes. Then, the trypsin was pipetted off and added to 5mls of RPMI media containing 10% FCS and 1% glutamine in a 10ml conical tube. It was spun in a centrifuge at 270g for 5 minutes. The supernatant was poured off. Into each tube 1ml of DMSO (VWR International, Leicester, UK) with 10% FCS was pipetted and the cell pellet was resuspended. The sample was stored at -80ºC ready for the comet assay (see section 2.3). After a further 3, 6, 8, 11, 16, 18, 20, 24, 30, 40 and 48 hours the appropriate well on each plate was harvested, and frozen as above. The comet assay was performed on the samples, and the results plotted.

2.3.4 Comet assay

2.3.4.1 Background

Techniques such as flurometric alkaline elution, which measure ICL formation and repair (Rudd *et al*., 1995), have been hindered in clinical practice due to the high levels of cells required. The single cell gel electrophoresis (comet) assay uses small numbers of cells, and is therefore a very useful tool in the clinical setting, where it is often difficult to obtain large samples. The comet assay has been modified to sensitively detect and quantify the levels of ICLs in patient lymphocytes and other cells (Hartley *et al*., 1999). It can also measure unhooking of the ICLs, which is part of the DNA repair pathway.

The comet assay was used to process samples received from patients, and on human paired ovarian cancer cell lines, to detect levels of ICL formation and repair.

2.3.4.2 Preparation of slides

Single-frosted glass microscope slides 25 x 75mm, 1mm thick (VWR International Ltd, Leicester, UK) were pre-coated by pipetting 0.8ml of molten 1% type 1A agarose in water, onto the centre of the slide. They were allowed to dry overnight at room temperature.

2.3.4.3 Preparation of samples with $10x10^4$ cells present

All procedures were carried out on ice. Samples were thawed on ice and the cell suspension diluted with RPMI (with 10% FCS and 1% glutamine) to $2.5x10^4$cells/ml. There was at least 4 ml in each sample. Each sample was divided into two appropriately labelled tubes. Duplicate agarose precoated slides were labelled and placed in a tray on ice. One of the duplicate samples was irradiated with 12Gy using an X-ray source running at 212.5Kv and 12.5mA (2.35Gy/min).

Into a 24 well multidish, 0.5ml of the appropriate sample was pipetted. 1ml of 1% LGT agarose (sigma, Poole, Dorset, UK) was also added and mixed with the sample by pipetting up and down. 1ml of this mixture was then pipetted onto the centre of the appropriate slide and a coverslip was placed on top of each gel. After the gel had set, the coverslip was removed. This was repeated for all the slides.

2.3.4.4 Preparation of samples with $1x10^4$ cells present

For the samples that only had $1x10^4$ cells/ml in each well of the 6 well plates, the comet assay was adjusted to make the gels smaller. Samples were thawed on ice and the cell suspension diluted with RPMI (with 10%FCS and 1% glutamine), to wash the cells, and spun in a centrifuge at 270g for 5 minutes. The supernatant was discarded, and the cell pellet was diluted with 500µl of RPMI media containing 10% FCS and 1% glutamine to give a concentration of $2x10^4$cells/ml. Each sample was divided into two appropriately labelled tubes. Duplicate agarose precoated slides were labelled and placed in a tray on ice. One of the duplicate samples was irradiated with 12Gy using an X-ray source running at 212.5Kv and 12.5mA (2.35Gy/min).

A 96 well plate was used, into which 100µl of the appropriate sample was pippetted. 200µl of 1% LGT agarose was also added and mixed with the sample by pipetting up and down. 300µl of this mixture was then pipetted onto the centre of the appropriate slide and a small round coverslip was placed on top of each gel. After the gel had set, the coverslip was removed. This was repeated for all the slides.

These slides resulted in fewer cells present in the gel overall, and the gel was a lot smaller. However, the concentration enabled adequate reading of the required 25 cells per slide.

2.3.4.5 Lysis treatment

After all the gels had been prepared, they were then treated exactly the same, regardless of the method used. Lysis buffer (100mM disodium EDTA, 2.5M sodium chloride, 10mMtris-Hcl, pH to 10.5 with sodium hydroxide) containing 1% triton X-100 (Sigma, Poole, Dorset, UK) was added, ensuring all the slides were sufficiently covered. They were left for 1 hour on ice in the dark.

The lysis buffer (containing 1% triton X-100) was carefully removed using a vacuum pump, and ice-cold double distilled water was added which completely covered the slides. The gels were left for 15 minutes on ice in the dark, and then the water was carefully removed using the vacuum pump. Ice-cold double distilled water was added a further three times, and each time left on for 15 minutes on ice in the dark.

2.3.4.6 Alkali treatment and electrophoresis

After the four washes, the slides were transferred to an electrophoresis tank so that all the slides were laid lengthways in the same direction. Two litres of ice-cold alkali buffer (50mM sodium hydroxide (Sigma, Poole, Dorset, UK), 1mM disodium EDTA, pH 12.5) were poured into the tank completely immersing the slides, and left for 45 minutes in the dark. The slides were then electrophoresed in the dark for 25 minutes at 18V (0.6V/cm), 250mA.

The slides were then removed from the tank and placed on a horizontal slide rack. Each slide was flooded with 1ml neutralisation buffer (0.5M Tris-HCl, pH 7.5) and

left for 10 minutes. The slides were then rinsed twice with 1ml phosphate buffered saline for 10 minutes each rinse. The slides were then allowed to dry at room temperature overnight.

2.3.4.7 Preparation of slides for analysis

The slides were rehydrated with double distilled water and left for 30 minutes. Each slide was flooded with 1ml of 2.5μg/ml propidium iodide solution which stains DNA, and incubated for 15-20 minutes at room temperature in the dark. Propidium iodide is toxic, and so precautions should be used when handling it. Gloves and laboratory coats were worn at all times, hair was tied back, and eye protection worn.

The propidium iodide was rinsed off with double distilled water into the tray and then left for 20-30 minutes. The water containing propidium iodide was disposed of into plastic containers specific for this toxin. Once these containers were full, they were disposed of in the correct manner. Under no circumstances was propidium iodide flushed down the sink. The slides were then dried in the oven at 37°C for 1-2 hours and stored in a slide box until image analysis.

2.3.4.8 Analysis of comets

Visualisation and analysis of comets could be done at any time after preparation of the slides because they keep indefinitely.

A few drops of distilled water was placed onto each slide and covered with a coverslip. A NIKON inverted epifluorescent microscope (consisting of a high power

mercury vapour light source, a 580nm dichromic mirror, 510 to 560nm excitation filter and 590nm barrier filter) at X20 magnification was used to visualise the comets, which was relayed to a computer screen. The entire slide was visualied to determine 25 representative cells on the slide. These cells were analysed using Komet analysis software (Kinetic imaging, Liverpool, UK), by activating the computer software programme to read each cell selected.

The tail moment for each cell was defined as the product of the percentage in the comet tail, and the distance between the means of the head and the tail distributions. This was described by Olive et al (1990) and recently summarized by Spanswick et al (1999). Crosslinking was calculated as the percentage decrease in tail moment and compares the drug treated and irradiated control to the non-drug treated irradiated control. From both samples the comet tails from the non drug-treated unirradiated control sample is subtracted as this represents background damage to these cells. The background damage was always negligible in these experiments. Percent decrease in tail moment is calculated using the formula:

$$\% \text{ decrease in tail moment} = 1 - \frac{TMdi - TMpreu}{TMprei - TMpreu} \times 100$$

Where;

TMdi = tail moment of drug treated irradiated sample

TMpreu = tail moment of predose unirradiated control

TMprei = tail moment of predose irradiated control

2.4 Measurement of ICL formation and repair in ovarian cancer cells obtained from patient ascites treated *ex vivo* with 100μM cisplatin or 50μM melphalan

2.4.1 Preparation of ascites

Ascites was obtained from patients consented for the study. Samples were collected from theatre and hospital wards. Ascitic fluid was collected from patients in theatre via suction into a sterile bag once the abdominal cavity had been opened. Ascitic fluid was also collected from patients on the ward who had had ascitic drains placed under aseptic conditions. Ascitic fluid was collected from the sterile bags into which the fluid was drained on the wards. A minimum of 50mls of ascitic fluid was needed to enable enough ovarian cancer cells to be extracted for experiments. As much ascites as possible was collected from the patients, up to 3 litres in some cases. Any ovarian cancer cells that were not needed for experiments, were stored (see below for protocol) for future research if the patient gave permission by signing part 2 of the patient consent form (Appendix 3 – Patient consent form).

The ascites obtained was aliquoted into 50ml sterile tubes and spun in a centrifuge (Megafuge 1.0, Heraeus Instruments, Thermoelectron, Basingstoke,UK) at 249g for 5 minutes. The supernatant was poured off, the cell pellet was examined, and the cells re-suspended in 25mls RPMI media containing 10% FCS and 1% glutamine. The cell suspension was then pipetted into one or a varying number of 175cm^2 flasks according to the size of the cell pellet. The smallest cell pellet was 0.2mls, and in this case all of the 25mls was pipetted into one flask. The largest cell pellet was

approximately 1ml, and in this case 5mls was pipetted into 5 flasks. This was to ensure a good concentration of cells present in each flask.

The flasks were left for 1 hour in the incubator (37°C, 5%CO_2) for the mesothelial cells to attach. After 1 hour, the RPMI media containing the remaining cells (mostly tumour cells) was pipetted off and added to another sterile 175cm^2 flask and left overnight for the tumour cells to attach. 25 mls of RPMI media containing 10% FCS and 1% glutamine was then added to the first flask containing predominantly mesothelial cells and left in the incubator (37°C, 5%CO_2) to grow. This resulted in one flask containing predominantly tumour cells, and the other one containing predominantly mesothelial cells. However, further separation was often necessary (see section 2.4.2 below).

2.4.2 Separation of cell types from ascites

The morphology of mesothelial and tumour cells are different. Mesothelial cells look like spindles and are much thinner than tumour cells. They spread out and have thin projections from the cell. Tumour cells are much rounder than mesothelial cells and cluster together. The morphology of the two cell types was observed down the microscope and the levels of contamination by each type of cell, was estimated.

Mesothelial cells and tumour cells both come from ascites. Mesothelial cells become detached quicker than tumour cells, when exposed to trypsin. Mesothelial cells also attach to the flasks quicker than ovarian tumour cells when re-suspended in media

and left in an incubator at 37°C, and 5%CO_2. These differences were utilised to enable the separation of mesothelial cells from ovarian cancer cells.

2.4.2.1 Method to obtain mesothelial cells from ascites

The RPMI media containing 10% FCS and 1% glutamine was poured off, and 5mls of trypsin was added to wash the cells, and then poured off. A further 7mls of trypsin was added to the 175cm^3 flask, and left in the incubator for 5 minutes. After 5 minutes the trypsin was pipetted off, and added to 10mls of RPMI media containing 10% FCS and 1% glutamine and spun in a centrifuge at 249g for 5 minutes. The supernatant was poured off and the cell pellet was examined, and then re-suspended in 25mls of RPMI media containing 10% FCS and 1% glutamine. The smallest cell pellet was 0.2mls, and in this case all of the 25mls was pipetted into one flask. The largest cell pellet was approximately 1ml, and in this case 5mls was pipetted into 5 flasks. This was to ensure a good concentration of cells present in each flask. The flasks were then left for 1 hour in an incubator (37°C, 5%CO_2) for the mesothelial cells to attach.

After 1 hour the media containing mostly tumour cells was pipetted off and discarded. This results in a higher percentage of mesothelial cells in the first flask. This was repeated if necessary.

2.4.2.2 Method to obtain ovarian cancer cells from ascites

The RPMI media containing 10% FCS and 1% glutamine was poured off, and 5mls of trypsin was pipetted to cover the cells, and then poured off. A further 7mls of trypsin was added and left in the incubator at 37°C, and 5%CO_2, for 12 minutes. After 12 minutes the trypsin was poured off and added to 20mls of RPMI media containing 10% FCS and 1% glutamine and spun in a centrifuge at 249g for 5 minutes. The supernatant was poured off and the cell pellet was re-suspended in 25 mls of RPMI media containing 10% FCS and 1% glutamine. Cell pellets ranged in size from 0.2-1ml. If the cell pellet was 0.2mls, then all of the 25mls was pipetted into one flask. If the cell pellet was approximately 1ml, then 5mls was pipetted into 5 flasks. This was to ensure a good concentration of cells present in each flask. The flasks were then left for 1 hour in an incubator (37°C, 5%CO_2) for the mesothelial cells to attach.

After 1 hour the media containing mostly tumour cells was pipetted off and pipetted into another sterile 175cm^2 flask. This resulted in a higher percentage of tumour cells in the second flask. This was repeated if necessary.

2.4.3 Confirming presence of ovarian cancer cells using immunohistochemistry

In order to be sure of the cell type before experiments were commenced, the cells were stained using antibody markers.

2.4.3.1 Preparation of samples

First the cells were harvested using trypsin. The RPMI media containing 10% FCS and 1% glutamine was poured off, and 5mls of trypsin was pipetted into the flask to coat the cells, and then poured off. A further 7mls of trypsin was added and left in the incubator at 37°C, and 5%CO_2, for 10 minutes. After 10 minutes the trypsin was poured off and added to 20mls of RPMI media containing 10% FCS and 1% glutamine and spun in a centrifuge at 249g for 5 minutes. The supernatant was poured off, and the cell pellet was resuspended in 10mls of RPMI media containing 10% FCS and 1% glutamine.

The cells were counted to determine their concentration (see section 2.2b), and diluted to obtain a concentration of 1x 10^4 cells/ml. Four drops were placed into a cytofunnel (Shandon, Cheshire, UK) mounted onto filter paper with a hole in it, which was on a slide (Vision Biosystems, Newcastle-upon-Tyne, UK). This was then put into a Shandon Cytospin II (Shandon, Cheshire, UK) and spun at 650 rpm for 5 minutes. The centrifugal force pushes the cells onto the slide in the gap in the filter paper. The filter paper absorbs the remaining media. The slides were then removed from the funnel and filter paper and left to air dry for 1 hour. They were then placed in 100% ethanol to fix the cells.

For each sample two slides were made. One was stained with CK5 antibody (stains mesothelial, but not epithelial cells) and the other was stained with CK7 antibody (stains epithelial i.e ovarian cancer cells). This was done to estimate the percentage of each cell type in the sample. Calretinin antibody (stains mesothelial, but not

epithelial cells) was sometimes used to stain for mesothelial cells instead of CK5. The slides were stained by the Immunocytochemistry Department at University College London, using the staining protocol below (see section 2.4.3.2)

2.4.3.2 Immunohistochemistry protocol

The slides were transferred to an incubation tray and rinsed in 0.05% Tween® 20 (Polyoxyethylene sorbitan mono-laurate) in tris-buffered saline (TBS). The circular area on the slide that contained cells was marked with a hydrophobic pen. The primary antibodies (CK5, or CK7) were applied at optimal dilution for 60 minutes. The primary antibody was rinsed off with 0.05% Tween® 20 in tris- buffered saline (TBS) for 2-3 minutes, and Dako ChemMate peroxidase block was applied for 10 minutes. The peroxidase block was rinsed off with 0.05% Tween® 20 in tris-buffered saline (TBS) for 2-3 minutes, and the biotinylated secondary antibody (AB2) was applied for 30 minutes. This was rinsed off with 0.05% Tween® 20 in tris-buffered saline (TBS) for 2-3 minutes, and peroxidase- labelled streptavidin (HRP) was applied for 30 minutes. This was rinsed off with 0.05% Tween® 20 in tris-buffered saline (TBS) for 2-3 minutes, and DAB solution (100µl DAB + 5mls substrate buffer) was applied for 7 minutes. The DAB was rinsed off with 0.05% Tween® 20 in tris-buffered saline (TBS) for 2-3 minutes, and washed in running tap water. The slide was stained with Dako ChemMate Haematoxylin for 10 seconds, and blued in running water. It was differentiated in 1% acid alcohol and blued again in running water. The slide was dehydrated, and mounted.

The slides were looked at under an inverted microscope and the percentage of each cell type was estimated. This was done by counting the total number of cells stained with each antibody in a particular field. Four different fields were counted, and the result averaged.

2.4.4 Determining the concentration and amount of cells present in media

See section 2.2.2. The cells were diluted to obtain a concentration of $5x10^4$cells/ml, and a minimum volume of 28mls (14mls each for the cisplatin and melphalan experiments). If this was not possible the cells were diluted to obtain a concentration of $1x10^4$cells/ml with a minimum volume of 14mls (7mls each for the cisplatin and melphalan experiments). If this was not possible, the drug treatment experiments were not performed.

2.4.5 Drug Treatment

Cells were either treated with 100μM cisplatin or 50μM melphalan. If there were limited numbers of cells, and only one drug treatment experiment was possible, then 100μM cisplatin was used.

2.4.5.1 Treatment *ex vivo* with 100μM Cisplatin

Seven wells in two 6 well plates were plated up with 2mls of $5x10^4$/ml of cells in each well. They were left to adhere overnight.

For some samples, there were not enough cells to use. Only 1 ml of $1x10^4$/ml of cells was plated into seven wells in two 6 well plates (same number of wells as above). They were left to adhere overnight also, and then treated the same as the higher concentration of cells (see below).

The plates were treated with 100μM cisplatin. The cisplatin was added to RPMI media containing only 1% glutamine and no FCS to obtain a concentration of 100μM, and 1ml was added to each well. It was then incubated with the cells for 1 hour. After 1 hour the cisplatin was then removed and replaced with 2mls of RPMI media containing 10% FCS and 1% glutamine, and left for the appropriate amount of time.

The "0 hour" well and "0 hour control" on each plate were harvested using trypsin (see section 2.2.2), and frozen at -80°C in 1ml of DMSO with 10% FCS. After a further 9, 24, 24 control, 48 control and 48 hours the appropriate well on each plate was harvested, and frozen as above. The comet assay was performed on the samples, and the results plotted.

2.4.5.2 Treatment *ex vivo* with 50μM Melphalan

2mls of $5x10^4$/ml of cells was pipetted into each of 4 wells in a 6 well plate. The cells were left to adhere overnight.

For some samples, there were not enough cells to use. Only 1 ml of $1x10^4$/ml of cells was pipetted into each well. They were left to adhere overnight also, and then treated the same as the higher concentration of cells (see below).

The plates were treated with 50μM melphalan. The melphalan was added to RPMI media containing only 1% glutamine and no FCS to obtain this concentration, and 1ml was added to each well. It was then incubated with the cells for 1 hour at 37°C, and 5%CO_2. After 1 hour the melphalan was removed and replaced with 2mls of RPMI media containing 10% FCS and 1% glutamine and left for a further 16 hours.

The "16 hour" well and "16 hour control" on each plate were harvested using trypsin (see section 2.2.2), and frozen at -80°C in 1ml of DMSO with 10% FCS. After 40 hours following drug removal, the remaining wells (40 hours, 40 control) on the plate were harvested, and frozen as above. The comet assay was performed on the samples, and the results plotted.

2.4.6 Comet Assay

This was performed as previously described (see section 2.3.4).

2.5 Measurement of ICL formation and repair in ovarian cancer cells obtained from patient solid tissue

2.5.1 Preparation of tumour cells from ovarian solid tumour

Solid tumour was obtained from patients consented for the study. Samples were collected from theatre. Solid ovarian tumour tissue was removed from the patient by the surgeon. At this point the tissue was cut under asceptic conditions into 3mm cubes, using a scalpel. Areas of obvious cancer were chosen. At least two pieces were needed for experiments. As much tissue as possible, was obtained -up to 6 pieces in some cases. Any pieces of solid tumour tissue that were not needed for experiments were stored for future research if the patient gave permission by signing part 2 of the patient consent form (Appendix 3 – Patient Consent form). Some pieces were put into cryotube vials (Nunc, Kamstupvej, Denmark), which immediately went into enough liquid nitrogen to cover the tubes (within 5-10 minutes). Others were put into tubes containing 10mls of RPMI media (Autogen Bioclear Uk ltd. Wiltshire, UK), which contained 10% FCS (Autogen Bioclear Uk ltd. Wiltshire, UK) and 1% glutamine (Autogen Bioclear Uk ltd. Wiltshire, UK). The samples put into media were immediately processed.

The solid ovarian tumour tissue was removed from the tubes containing 10mls of RPMI media, and chopped repeatedly (approximately 100 times) in the same 10mls of RPMI media (containing 10% FCS and 1% glutamine) on a sterile petri dish using sterile scalpels to create a ‘slurry’ of material. The petri dish was carefully tilted to 45 degrees, and the media was carefully pipetted off into a sterile 20ml

conical tube. By tilting the petri dish, the large particulate tumour material was avoided, whilst still obtaining a single cell suspension of tumour cells. 10mls of RPMI media (containing 10% FCS and 1% glutamine) was added to the tumour 'slurry' and the process was repeated. The resulting 20mls of media containing the cells was slowly (to ensure cells were not fragmented) pipetted approximately 20 times through a sterile 1ml sized pipette to ensure the cells were in single cell suspension. 1ml of this 20ml sample was pipetted onto a glass slide, a cover slip placed on top of the slide, and the slide looked at under the microscope. This was done to ensure the sample was a single cell suspension. If not, the sample was repeatedly pipetted approximately 20 times through a sterile 1ml sized pipette, and rechecked by looking under the microscope. Only samples with a single cell suspension were used in the comet assay experiments.

2.5.2 Confirming presence of ovarian cancer cells using immunohistochemistry

2.5.2.1 Preparation of samples

The cells were counted to determine their concentration (see section 2.2b), and diluted to obtain a concentration of 1x 10^4/ml. Four drops of each sample were placed into a cytofunnel (Shandon, Cheshire, UK) mounted onto filter paper with a hole in it, which was on a slide (Vision Biosystems, Newcastle-upon-Tyne, UK). This was then put into a Shandon Cytospin II (Shandon, Cheshire, UK) and spun at 650 rpm for 5 minutes. The slides were removed and left to air dry for 1 hour. They were then placed in 100% ethanol to fix the cells for 30 minutes.

2.5.2.2 Immunocytochemistry protocol

See section 2.4.3.2

2.5.3 Determining the concentration and amount of cells present in media

See section 2.2.2

2.5.4 Drug Treatment

As for the cells obtained from ascites, tumour cells from solid tissue were either treated with 100μM cisplatin or 50μM melphalan. If there were limited numbers of cells, and only one drug treatment experiment was possible, then 100μM cisplatin was used.

2.5.4.1 Treatment *ex vivo* with 100μM Cisplatin

The solid ovarian tumour tissue was non-adherent, so a suspension cell protocol was used. The single cell suspension of solid ovarian tumour tissue was counted (see section 2.2), and 2mls of a concentration of $5x10^4$/ml was added to each of 7 conical shaped tubes. If there were some extra cells, the experiment was run in duplicate (14 tubes in total) or even triplicate (21 tubes in total).

The tubes were spun in a centrifuge at 249g for 5 minutes, and the supernatant was carefully discarded. Cisplatin was added to RPMI media containing only 1% glutamine and no FCS to obtain a concentration of 100μM. 1ml was added to each tube, and pipetted up and down to mix with the cell pellet. All of the conical tubes

were placed in the incubator for 1 hour at 37°C, and 5%CO_2. After 1 hour the cisplatin was removed by spinning the tubes in the centrifuge at 249g for 5 minutes and carefully pouring off the supernatant. 2mls of RPMI containing 10% FCS and 1% glutamine was added to each tube (except the '0 hour' and '0 hour control' tubes), and pipetted up and down to mix with the cell pellet.

The '0 hour' and '0 hour control' tubes were frozen at -80°C in 1ml of DMSO with 10% FCS, which was pipetted up and down to resuspend the cell pellet. After a further 9, 24, 24 control, 48 control and 48 hours the appropriate tube was centrifuged at 249g for 5 minutes. The supernatant was poured off and 1ml of DMSO with 10% FCS was added, and pipetted up and down to mix with the cell pellet, and then frozen at -80°C. The comet assay was performed on the samples, and the results plotted.

2.5.4.2 Treatment *ex vivo* with 50µM Melphalan

The solid ovarian tumour tissue was not adherent, so a suspension cell protocol was used (the same as for treatment *ex vivo* with 100µM cisplatin – see section 2.5b(i)). The single cell suspension of solid ovarian tumour tissue was counted (see section 2.2b), and 2mls of a concentration of $5x10^4$/ml was added to each of 4 conical shaped tubes.

The tubes were spun in a centrifuge at 249g for 5 minutes, and the supernatant was carefully discarded. Melphalan was added to RPMI media containing only 1% glutamine and no FCS to obtain a concentration of 50μM. 1ml was added to each tube, and pippetted up and down to mix with the cell pellet. It was then incubated with the cells for 1 hour. After 1 hour the melphalan was then removed by spinning the tubes in the centrifuge at 249g for 5 minutes and carefully pouring off the supernatant. The cells were then resuspended with 2mls of RPMI media containing 10% FCS and 1% glutamine and left for 16 hours.

After 16 hours the “16 hour” and “16 hour control” tubes were centrifuged at 249g for 5 minutes. The supernatant was poured off and 1ml of DMSO with 10% FCS was added and frozen at -80°C.

After a further 24 hours, the remaining tubes (40 control and 40hours) were harvested as above. The comet assay was performed on the samples, and the results plotted.

2.5.5 Comet Assay

This was performed as previously described (see section 2.3.4).

2.6 Comparison of gene expression between A2780 and A2780cisR human ovarian cancer cell lines using microarrays

2.6.1 Preparation of RNA

2.6.1.1 Isolation of total RNA from A2780 and A2780cisR human ovarian cancer cell lines

The cells were grown as previously described (see section 2.2.1), and the sample was harvested using trypsin just before the cells reached confluency. The RPMI media containing 10% FCS and 1% glutamine was poured off, and 3mls of trypsin was pipetted into the flask to cover the cells. The trypsin was then poured off, and a further 5mls of trypsin was added to the flask, and left in the incubator at 37ºC, 5% CO_2 for 5 minutes. After 5 minutes the trypsin was pipetted off and added to 5mls of RPMI media containing 10% FCS and 1% glutamine in a 10ml conical tube, and spun in a centrifuge at 270g for 5 minutes. The supernatant was poured off. 10mls of RPMI media containing 10% FCS and 1% glutmaine was added to the sample, and pipetted up and down to resuspend the cell pellet.

The cells were counted (see section 2.2.2). $4x10^6$ cells were used for total RNA extraction, and so the correct volume of media containing this number of cells was pipetted into a 20ml conical tube. The QIAGEN RNeasy mini kit was used and the instructions followed in the manufacturers' handbook:

The sample was centrifuged at 249g at 20°C for 5 minutes and the supernatant poured off. The pellet was loosened by flicking the tube, and 600µl of RLT buffer

(from the RNeasy mini kit QIAGEN) was added, and vortexed to mix. The lysate was passed at least 5 times through a 20-gauge needle (0.9mm diameter) fitted to a 1ml RNase-free syringe, to homogenise the sample. 600µl of 70% ethanol (VWR International, Leicester, UK) was added to the homogenised lysate and mixed well by pipetting.

Up to 700µl of the sample was added to an RNeasy mini column, which was placed in a 2 ml collection tube. The tube was closed gently and centrifuged in an Eppendorf 5145D centrifuge for 15 seconds at 9,300g. The flow through was discarded. Aliquots of the sample were successively loaded onto the RNeasy mini column and centrifuged as above. The flow-through was discarded each time.

700µl of Buffer RW1 was added to the RNeasy mini column, the tube closed gently, and centrifuged for 15 seconds at 9,300g to wash the column. The flow-through and collection tube was discarded.

The RNeasy column was transferred to a new 2ml collection tube, and 500µl of RPE buffer was pipetted into the column. The tube was centrifuged again for 15 seconds at 9,300g to wash the column. The flow-through was discarded. Another 500µl of RPE buffer was pipetted into the RNeasy column and centrifuged, this time for 2 minutes at 9,300g, to dry the RNeasy silica-gel membrane.

To elute, the RNeasy column was transferred to a new 1.5ml collection tube, and 30-50µl of RNase-free water was pipetted directly onto the RNeasy silica-gel membrane. The tube was centrifuged for 1 minute at 9,300g to elute.

2.6.1.2 Determining quantity of RNA in sample

To determine the quantity of RNA extracted, the absorbance at 260nm (A260) was measured in a spectrophotometer. Sometimes it was necessary to dilute the RNA sample, and this was done using water, and in an RNase-free cuvette. The following equation was used to calculate the concentration of RNA (where an absorbance of 1 unit at 260nm corresponds to 40µg of RNA per ml):

Concentration of RNA sample = 40 x A_{260} x dilution factor

Another method for determining the quantity and purity of total RNA in the sample, involved a nanodrop machine. Only 1.2µl of sample was loaded onto the machine, which then calculated the concentration, and purity (see below- section 2.6a(iii)) of the sample using the formula already described.

2.6.1.3 Determining purity of RNA in sample

To determine the purity of the RNA, the ratio of the readings at 260nm and 280nm (A_{260}/A_{280}) were calculated. The absorbance of the RNA was measured in dH_2O. Pure RNA has an A_{260}/A_{280} ratio of 1.9-2.1 in dH_2O, and samples in this range only were used. Others not in this range were discarded.

2.6.1.4 Determining quality of RNA in sample

To determine the quality of the total RNA, the sample was checked by gel electrophoresis.

Tris-acetic ethylenediaminetetraacetic acid (TAE) stock buffer (x50) was initially made, by dissolving 18.6g of ethylenediaminetetraacetic acid disodium salt dihydrate in 500ml of distilled water. 121g of Trizma base was then added, followed by 28.55ml of acetic acid (VWR international, Leicester, UK). 20ml of 50x TAE buffer was added to 980ml of distilled water, to make 1xTAE buffer, and from this 100ml was taken to make the gel.

A 1% gel was made by adding 1g of electrophoresis agarose to 100ml of the 1x TAE buffer, and heating it until the agarose had dissolved completely. Once the solution was cooled to be touch hot, 5µl of ethidium bromide was added, mixed well, and poured into a gel holder with combe in place. The gel was allowed to solidify, before the combe was removed and the gel placed into the gel electrophoresis tank. The remaining 1x TAE buffer was poured into the tank, up to the fill line.

The required amount of the sample (usually 2µg) was added to 3µl of formamide dye and mixed in a 50µl Eppendorf tube. The whole mixture was then pipetted into the wells in the gel. 5µL of the RNA ladder (Invitrogen life technologies, Paisley, UK) was added to 3µl of the dye and also loaded into the gel. The tank was then turned on to a voltage of 130mV and left for one hour. The gel was read under UV

light (to see the bands of RNA) by a computer program called Scion Pro, and the results analysed.

2.6.2 Preparation of microarray sample

2.6.2.1 Synthesis of double-stranded cDNA from total RNA

Invitrogen Life Technologies' protocol using the SuperScript choice system was used. It was supplemented with instructions from Affymetrix gene chip systems:

The minimum amount of total RNA per reaction was 10μg, which was the amount used in each experiment. Each experiment produced different amounts and concentrations of total RNA, and so the amounts of the other reagents altered also (figure 6).

Step	Reagents in reaction	Volume	Final concentration/amount in reaction
1	DEPC-H_20 (variable) T7-(dT)24 primer (100pmol/µl) RNA	For final reaction volume of 20µl 1µl variable amount (µl)	 100pmol 10µg
2	5X First strand cDNA buffer 0.1M DTT 10mM dNTP mix	4µl 2µl 1µl	1X 10mM DTT 500µM each
3	SuperScript II RT (variable)(200U/µl)	2µl	200 U to 1000U
Total volume		**20 µl**	

Figure 6: Shows the steps, and amounts of reagents used to make cDNA

Step 1 was primer hybridisation. All the reagents listed (DEPC-H_20, T7-(dT)24 primer (MWG biotech, London, UK) and RNA) were added together and incubated at 70°C for 10 minutes. They were then quickly spun and put on ice. Step 2 was temperature adjustment. The reagents (Invitrogen life technologies, Paisley, UK) were added to the reagents from step 1, vortexed and incubated at 42°C for 2

minutes. Step 3 was first strand synthesis. The Superscript II RT was added to the tube, vortexed, incubated at 42°C for 1 hour, and then put on ice.

The first strand reaction tube was briefly centrifuged to bring down condensation on the sides of the tube, and the following reagents for the second strand reaction were added (figure 7).

Component	Volume	Final concentration /amount in reaction
DEPC- treated water	91µl	
5X second strand reaction buffer	30µl	1X
10mM dNTP mix	3µl	200µM each
10U/µl E.*coli* DNA ligase	1µl	10U
10U/µl E.*coli* DNA Polymerase	4µl	40U
2U/µl E.*coli* Rnase H	1µl	2U
Final volume	**150µl**	

Figure 7: Volumes of the components of the reaction

The tube was gently tapped to mix, and then briefly spun in a centrifuge to remove condensation, and incubated at 16°C for 2 hours in a cooling water bath. 2µl (10U) T4 DNA polymerase (Invitrogen life technologies, Paisley, UK) was added and returned to 16°C for 5 minutes. 10µl of 0.5M EDTA (93g EDTA, and 10g sodium

hydroxide in 500mls dH_2O, pH 8) was then added and the sample stored at -20°C or the cleanup procedure for cDNA was immediately done.

2.6.2.2 Cleanup of Double-Stranded cDNA

Phase Lock Gels (PLG) (Eppendorf AG, Hamburg, Germany), were used in the cleanup of cDNA. They form an inert, sealed barrier between the aqueous and organic phases of phenol-chloroform extractions. The solid barrier allows more complete recovery of the sample (aqueous phase) and minimizes interface contamination of the sample.

The PLG was pelleted in a microcentrifuge at 16,100g for 20-30 seconds. 162µl of (25:24:1) phenol:chloroform:isoamylalcohol (saturated with 10mM Tris-Hcl pH 8.0, 1mM EDTA) was added to the final cDNA synthesis preparation (final volume 324µl) and vortexed briefly. The entire cDNA-phenol/chloroform mixture was added to the PLG tube (it was NOT vortexed), and was centrifuged at 16,100g for 2 minutes. The aqueous upper phase was added to a fresh 1.5ml tube.

0.5 volumes (of the sample) of 7.5M Ammonium acetate (Sigma, Poole, Dorset, UK), and 2.5 volumes of absolute ethanol (VWR international, Leicester, UK), were added, vortexed and immediately microcentrifuged at 16,100g at room temperature for 20 minutes. The supernatant was removed, the pellet washed with 0.5ml of 80% ethanol, and centrifuged again at 16,100g at room temperature for 5 minutes. The 80% ethanol was removed carefully, and the 80% ethanol wash was repeated once more. The pellet was air dried and re-suspended in a 12µl of RNase-free water.

2.6.2.3 Synthesis of *in vitro* transcription products

Starting Material (total RNA)	Volume of cDNA to use in IVT
10 to 100ng	All (12μl)
1.0 to 8.0μg	All (12μl)
8.1 to 15.0μg	6μl

Figure 8: Relationship between starting material and volume of cDNA to use *In Vitro* Transcription (IVT)

The correct amount of template cDNA was transferred to RNase-free microcentrifuge tubes and the following reagents were added:

RNase-free water (to give final reaction volume of 40μl), 10X IVT labelling buffer (4μl) (Affymetrix, Bucks, UK), IVT labelling NTP mix (12μl) (Affymetrix, Bucks, UK), IVT labelling enzyme mix (4μl) (Affymetrix, Bucks, UK).

All the reagents were carefully mixed together and the mixture collected at the bottom of the tube by brief (5 seconds) microcentrifugation. The tube was incubated at 37°C (in an oven incubator) for 16 hours. The labelled cRNA was stored at -20°C, or -70°C if not quantifying and cleaning up cRNA immediately.

2.6.2.4 Cleaning up and quantifying *in vitro* transcription products

This protocol comes from the Affymetrix technical manual (manufacturers' manual). The sample was aliquoted into two halves. One half was purified first, using the manufacturers instructions (see QIAGEN RNeasy mini handbook – protocol for RNA cleanup):

The sample was adjusted to a volume of 100µl with RNase-free water, 350µl of RLT buffer was added, and mixed thoroughly. 250µl of 96-100% ethanol was added and mixed thoroughly by pipetting. 700µl of the sample was added to an RNeasy mini column placed in a 2 ml collection tube, and centrifuged for 15 seconds at 10,000rpm. The flow-through was discarded.

The RNeasy column was transferred to a new 2ml collection tube and 500µl of RPE buffer was pipetted onto the column. The column and tube were centrifuged at 10,000rpm for 15 seconds, to wash it. The flow-through was discarded again.
Another 500µl of RPE buffer was added to the RNeasy mini column and centrifuged for 2 minutes at 10,000rpm to dry the silica-gel membrane.

The RNeasy mini column was transferred to a new 1.5ml collection tube and 30-50µl RNase-free water was pipetted onto the column. The column was centrifuged for 1 minute at 10,000rpm to elute.

Another method for cleaning up *in vitro* transcription products was used instead of the protocol above, once it became available. This protocol is also from Affymetrix and has been updated from the previous protocol above. It uses the Affymetrix genechip sample cleanup module:

600µl of cDNA binding buffer was added to the double-stranded cDNA (volume 162µl) and mixed by vortexing for 3 seconds. The colour of the mixture was checked to ensure that it was yellow.

500µl of the sample was applied to the cDNA cleanup spin column, which sits in a 2ml collection tube. It was centrifuged at 8,000g for 1 minute, and the flow-through was discarded. The spin column was reloaded with the remaining sample (262µl), and centrifuged at 8,000g for 1 minute. The flow-through and collection tube were discarded and the spin column was transferred to a new 2ml collection tube. 750µl of cDNA wash buffer was pipetted onto the spin column, and centrifuged at 8,000g for 5 minutes, and the flow-through was discarded again.The cap of the spin columN was opened and centrifuged at 16,100g for 5 minutes to allow complete drying of the membrane.

The spin column was transferred to a new 1.5ml collection tube, and 14µl of cDNA elution buffer was pippetted directly onto the membrane of the spin column. It was incubated for 1 minute at room temperature, and then centrifuged at 16,100g for 1 minute to elute.

Sometimes, the cRNA was concentrated by ethanol precipitation if it was too dilute (minimum concentration 0.6μg/μl). The method for ethanol precipitation is as follows:

0.5 volumes of 7.5M ammonium acetate and 2.5 volumes of absolute ethanol was added to the sample and vortexed. The sample was left to precipitate at -20°C for 1 hour to overnight. The sample was then centrifuged at 15,000rpm at 4°C for 30 minutes. The pellet was washed twice with 0.5ml of 80% ethanol and air dried. The pellet was resuspended in 10 to 20μl of RNase-free water.

2.6.2.5 Quantifying cRNA

To quantify the cRNA (IVT product) a spectrophotometer or a nanodrop was used. The absorbance of the sample was measured at 260nm and the equation below used:

1 absorbance unit (at 260nm) = 40μg/ml RNA

However, an adjustment needed to be made to reflect carryover of unlabeled total RNA as this was used as the starting material. The following equation was used:

Adjusted cRNA yield = RNA_m – (total RNA_i)(y)

RNA_m = amount of cRNA measured after IVT (μg)

Total RNA_i = starting amount of total RNA (μg)

(y) = fraction of cDNA reaction used in IVT

The absorbance of the sample was measured at 260nm and 280nm and the ratio calculated to determine the concentration and purity. A ratio of between 1.9 and 2.1 are acceptable.

2.6.2.6 Fragmenting the cRNA for target preparation

The cRNA was at a minimum concentration of 0.6μg/μl. If it was not, it was precipitated with ethanol (see section 2.6.2.4).

2μl of 5X fragmentation buffer for every 8μl of RNA plus H_2O was added. The fragmentation buffer has been optimised to break down full length cRNA to 35-200 bases fragments by metal-induced hydrolysis.

The reaction mixture was incubated at 94°C for 35 minutes, and then put on ice. An aliquot of at least 1 μg was saved for gel analysis. The undiluted, fragmented sample of RNA was stored at -20°C until ready to perform hybridisation.

2.6.2.7 Checking the cRNA

The unfragmented, and fragmented samples were checked by gel electrophoresis to estimate the yield and size distribution of labelled transcripts. 1% of each sample was analysed by gel electrophoresis on a 1% agarose gel (see previous protocol for gel electrophoresis). The century marker and RNA ladder were used as the ladder for the fragmanted cRNA and unfragmanted cRNA respectively.

2.6.3 Processing the Microarray sample

2.6.3.1 Hybrdisation of the probe array

The genechip arrays used were the Human genome U133 plus 2.0 from Affymetrix. The protocols for these procedures can be found online at www.affymetrix.com under expression analysis technical manual, and were used by the Wolfson Institue of Biomedical Sciences at University College London to process the samples.

A summary of the protocol is as follows: The genechip eukaryotic hybridisation control cocktail (see figure 9 for components) was heated to 65°C for 5 minutes to completely re-suspend the cRNA before aliquotting.

Component	Hybridisation cocktail	Final Concentration
Fragmented cRNA (adjusted amount)	15µg	0,05µg/µl
Control Oligonucleotide B2 (3nM)	5µl	50pM
20X Eukaryotic Hybridisation controls (bioB, bioC, bioD, cre)	15µl	1.5, 5, 25 and 100pM respectively
Herring Sperm DNA (10mg/ml)	3µl	0.1mg/ml
Acetylated BSA (50mg/ml)	3µl	0.5mg/ml
2x Hybridisation buffer	150µl	1x
dH_2O	To final volume 300µl	
Final Volume	**300µl**	

Figure 9: contents of the hybridisation cocktail

The human U133 plus 2.0 probe array was equilibrated at room temperature before use, while the hybridisation cocktail was heated to 99°C for 5 minutes in a heat block.

The probe array was made wet by filling it through one of the septa, with the appropriate volume of 1x hybridisation buffer. To make 100mls of 1x hybridisation buffer the following was added; 8.3ml of 12X MES stock, 17.7ml of 5M NaCl, 4.0 ml of 0.5M EDTA, 0.1ml of 10% Tween^{20}, 69.9ml of water. The probe array filled with 1x hybridisation buffer was incubated at 45°C for 10 minutes.

The hybridisation cocktail was transferred to a 45°C heat block for 5 minutes after it had been heated to 99°C. It was then spun in a microcentrifuge for 5 minutes to remove any insoluble material.

The buffer solution was removed from the probe array, and replaced with 200µl of the hybridisation cocktail. It was then placed in a rotisserie box in a 45°C oven, rotating at 60rpm for 16 hours, to hybridise.

2.6.3.2 Washing, staining and scanning the probe array

After 16 hours, the probe array was washed and stained (see figure 10 below for the steps involved).

Post hybridisation wash 1	10 cycles of 2 mixes/cycle with non-stringent wash Buffer (see below) at 25°C
Post hybridisation wash 2	4 cycles of 15 mixes/cycle with stringent wash Buffer (see below) at 50°C
Stain	Stain the probe array for 10 minutes in SAPE solution (see below) at 25°C
Post wash stain	10 cycles of 4 mixes/cycle with wash buffer A at 25°C
2nd stain	Stain the probe array for 10 minutes in antibody solution (see below) at 25°C
3rd stain	Stain the probe array for 10 minutes in SAPE solution at 25°C
Final wash	15 cycles of 4 mixes/cycle with non-stringent wash buffer at 30°C. The holding temperature is 25°C

Figure 10: Steps involved in the washing and staining of the microarray chip

See figure 11 below for the composition of the different buffers and stains involved in the process above:

Solution	**Composition**
12x MES stock buffer	70.4g MES free acid monohydrate 193.3g MES sodium salt 800ml of Molecular biology grade water pH 6.5-6.7 filter through 0.2μm filter
2x MES stain buffer	41.7ml of 12x MES stock buffer 92.5ml of 5M sodium chloride 2.5ml of 10% Tween ^{20} 112.8ml of water filter through 0.2μm filter store at 2-8°C and shield from light
Non-stringent wash buffer	300ml of 20x SSPE 1.0ml of 10% Tween ^{20} 698ml water filter through 0.2μm filter
Stringent wash buffer	83.3ml 12x MES stock buffer 5.2ml of 5M sodium chloride 1.0ml of 10% Tween ^{20} 910.5ml water filter through 0.2μm filter store at 2-8°C and shield from light
SAPE solution	600μl of 2x MES stain buffer 48μl of 50mg/ml acetylated BSA 12μl of 1mg/ml Streptavidin-Phycoerythrin (SAPE) 540μl dH_2O
Antibody solution mix	300μl of 2x MES stain buffer 24μl of 50mg/ml acetylated BSA 6.0μl of 10mg/ml of normal Goat IgG 3.6μl of 0.5ml/ml of biotinylated antibody 266.4μl of dH_2O

Figure 11: Shows the components of each of the buffers

The probe array was scanned using Affymetrix microarray suite computer software.

The pixel value was 3μm and the wavelength was 570nm.

2.6.3.3 Analysis of microarray results

The results were analysed by Dr Stephen Henderson, of the department of Viral Oncology, Wolfson Institute for Biomedical Research. University College London, UK using the following protocol:

All microarrays were quantile-quantile normalised to the array with the median average signal (see mean variance pairs (MVA-pairs). Differential expression was analysed using the limma package, part of the Bioconductor suite of bioinformatic packages (http://www.bioconductor.org/), for the R statistical programming language (http://cran.r-project.org/). Briefly the limma package is a variant of simple linear models but using a moderated empirical Bayesian estimate of the standard deviation to calculate p-values. The empirical estimate comes from the strong mean variance relationship of microarrays, each observed variance being adjusted towards the population variance of genes with a similar mean expression. This is appropriate for studies with relatively few replicates in which the standard deviation of a population of similar genes may be a better estimate than the observed standard deviation of a single gene (Smyth *et al.*, 2004).

The significance of contrasts is further moderated by the use of a "false discovery rate" (FDR) algorithm that compensates for the massive multiple testing error inherent in microarray experiments, adjusting p-values upwards (Benjamini *et al.*, 1995).

2.7 Comparison of gene expression between newly diagnosed and treated patients using microarrays

2.7.1 Preparation of RNA

2.7.1.1 Isolation of total RNA from ovarian tumour cells obtained from patient ascites

For the isolation of total RNA from patient ascites tumour cells the TRIzol (Invitrogen Life technology, Paisley, UK) protocol was used. This reagent is a mono-phasic solution of phenol and guanidine isothiocyanate.

The sample was harvested using trypsin once the cells had reached the exponential phase of growth as viewed down a microscope. The RPMI media containing 10% FCS and 1% glutamine was poured off, and 3mls of trypsin was pipetted into the flask to cover the cells. The trypsin was then poured off, and a further 5mls of trypsin was added to the flask, and left in the incubator at 37ºC, 5% CO_2 for 5 minutes. After 5 minutes the trypsin was pipetted off and added to 5mls of RPMI media containing 10% FCS and 1% glutamine in a 10ml conical tube, and spun in a centrifuge at 270g for 5 minutes. The supernatant was poured off. 10mls of RPMI media containing 10% FCS and 1% glutmaine was added to the sample, and pipetted up and down to resuspend the cell pellet.

The cells were counted (see section 2.2b). $4x10^6$ cells were used for total RNA extraction, and the appropriate volume of RPMI media containing 10% FCS and 1% glutamine, and the cells was pipetted into a 20ml conical shaped tube. The sample

was spun in a centrifuge at 249g for 5 minutes, and the supernatant poured off. 1ml of TRIzol was added to the sample on dry ice, and the sample left overnight at -80°C.

0.2ml of chlorofom-phenol solution (Sigma, Pool, Dorset,UK) was added, the caps secured and the tube shaken vigorously by hand for 15 seconds. The sample was then incubated at room temperature for 2-3 minutes before being centrifuged at 15,000g for 15 minutes at 2-8°C. Following this, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase.

The aqueous phase was carefully pippetted into a fresh eppendorf (capacity1.5ml), and 0.5ml of isopropyl alcohol (VXR International, Leicester, UK) was added and left at room temperature for 10 minutes to precipitate the RNA. The sample was then centrifuged at 15,000g for 10 minutes at 2-8°C. The RNA was often seen as a gel-like pellet at the bottom of the tube. The supernatant was removed by pipetting and 1ml of 75% ethanol was added and centrifuged at 7,500g for 5 minutes, at 2-8°C, to wash the sample. The supernatant was carefully removed, taking care not to dislodge the RNA pellet, and the sample was air-dried. The pellet was re-suspended in 40μl of RNase-free water.

2.7.1.2 Isolation of total RNA from ovarian solid tumour tissue from patients

The sample was removed from liquid nitrogen and placed on dry ice. 1 ml of TRIzol was pipetted into the eppendorf, and the tissue homogeniser was used for several

minutes to break up the tissue. The sample was then left at -80°C overnight. The tissue homogeniser was cleaned meticulously between samples. This was done by using the homogeniser in a beaker of 100% ethanol, followed by a beaker of DEPC water, followed by spraying it with a solution to decontaminate the homogeniser from RNAases. The protocol for extraction of total RNA using TRIzol was then followed (see section 2.7.1).

2.7.2 Preparation of microarray sample

See section 2.6.2

2.7.3 Processing the microarray sample

See section 2.6.3

2.8 Real-time polymerase chain reaction (PCR)

Real time PCR was done using a selection of primers of genes that were significantly up or down regulated to confirm the microarray results obtained. The genes chosen were Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (used as a control), and excision repair cross-complementation group 1 (ERCC1) gene. Only cDNA from the paired samples were used for this experiment, which included patient 17 asc.tu, pt 17B asc.tu, pt 33 asc.tu, pt 33B asc.tu, pt 41 asc.tu, and pt 41B asc.tu..

cDNA was made using the protocol previously described for microarrays (see section 2.6b(i)). Only 6µl of the 12µl sample of cDNA was used for microarrays, and the remaining 6µl sample was diluted with 73.97µl DEPC water to produce a concentration of cDNA of 0.0625µg/µl.

First experiments were done to assess the relative efficiencies of the primers used. The relative primer efficiencies (comparing each primer to GAPDH – the control primer) had to be less than 0.1 to ensure that one primer was not binding more efficiently than the other, which would bias the results.

Serial dilutions of 1:2, 1:5, 1:10, 1:20, 1:50, and 1:100 of the cDNA were made using DEPC water. A mastermix containing 250µl of Taqman universal PCR mastermix (Applied biosystems, Warrington, UK), 25µl of the appropriate primer/probe mix (Applied biosystems, Warrington, UK), and 222.5µl of DEPC water, was made and 17.5µl was added to each of the 21 wells (which does not include the wells containing only water) on the 96 well PCR detection plate (ABgene, Surrey, UK). 2.5µl of the appropriate concentration of cDNA was added to each well. This was repeated for each primer/probe set that was being investigated, so on one 96 well PCR detection plate 2 genes (and their respective primers) were investigated for 1 of the patient samples. Different plates were used for all of the 6 different samples of patient cDNA.

The wells were checked to make sure all of the reagents were at the bottom, and a PCR seal (Applied biosystems, Warrington, UK) was placed over the top of the plate before being put into an ABI prism 7000 (Applied biosystems, Warrington, UK). Initially the machine heated the plate to 50°C for 2 minutes, and then 95°C for 10 minutes before 40 cycles were performed as follows:

95°C for 15 seconds

60°C for 1 minute

Once the relative primer efficiencies were shown to be less than 0.1, the main experiment to determine differences in gene expression was then set up in triplicate. The RNA concentration of each patient sample that had been diluted by 50% was used (serial dilution ½). For each patient sample 2.5µl of this ½ concentration of RNA was added to each of three wells on the 96 well plate, followed by 17.5µl of mastermix (see above for making the mastermix). The same cycling conditions as the standard curve experiment were used (see above). This meant there were a total of 18 wells used – three for each patient 17, patient 17B, patient 33, patient 33B, patient 41, patient 41B.

3. RESULTS

3.1 Introduction

A2780 and A2780cisR human ovarian cancer cell lines were used to establish cisplatin cytotoxicity using the SRB assay. These same cell lines were used to measure the peak of ICL formation and repair after exposure to cisplatin to identify differences that could be further investigated in the clinical setting.

Human ovarian cancer cells were obtained from patients before and after chemotherapy and the peak of ICL formation and repair were measured after exposure to cisplatin and melphalan *ex vivo* to establish any differences which may relate to clinically acquired resistance. Microarray technology was used to determine if these differences could be explained at the gene level.

3.2 Human ovarian cancer cell lines treated *in vitro* with cisplatin

3.2.1 Measurement of cisplatin cytotoxicity in paired cisplatin sensitive (A2780) and resistant (A2780cisR) human ovarian cancer cell lines

The A2780 and A2780cisR cell lines are a paired cell line produced from human ovarian cancer cells, and were used as an *in vitro* model. The A2780 cisplatin resistant (A2780cisR) cell line used in these experiments was produced by chronic

exposure of the parent cisplatin-sensitive A2780 cell line to increasing concentrations of cisplatin (Behrens *et al*., 1987). The resulting cell line was originally called 2780CP8 where CP8 refers to this cell line's growth in medium containing 8μM cisplatin (Behrens *et al*., 1987). Once the A2780cisR cell line had been produced it was stable, and therefore remained resistant to cisplatin without the need for continually growing it in the drug.

A2780cisR resistance to cisplatin chemotherapy was demonstrated using the Sulphur-Rhodamine Blue (SRB) assay. The IC_{50} is the concentration of the drug that inhibits growth by 50%. SRB assays to determine the cisplatin IC_{50} for the A2780 and A2780cisR cell lines following 1 hour exposure to cisplatin were performed (figure 12).

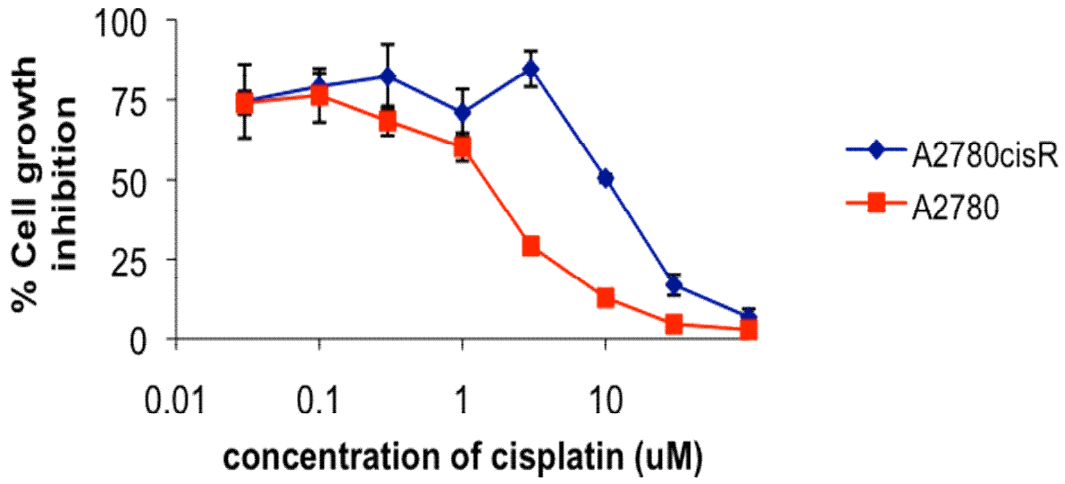


Figure 12: Inhibition of growth of A2780 and A2780cisR cell lines after treatment with cisplatin, as measured by the SRB assay (A2780 n=2, A2780cisR n=3, standard error bars are shown)

The cisplatin IC_{50} for A2780 cell line was 1.6μM, and for the A2780cisR cell line was 9.9μM, which resulted in a 6.2-fold difference between their sensitivities to cisplatin.

3.2.2 Measurement of ICL formation and repair in human ovarian cancer cell lines treated with cisplatin using the comet assay

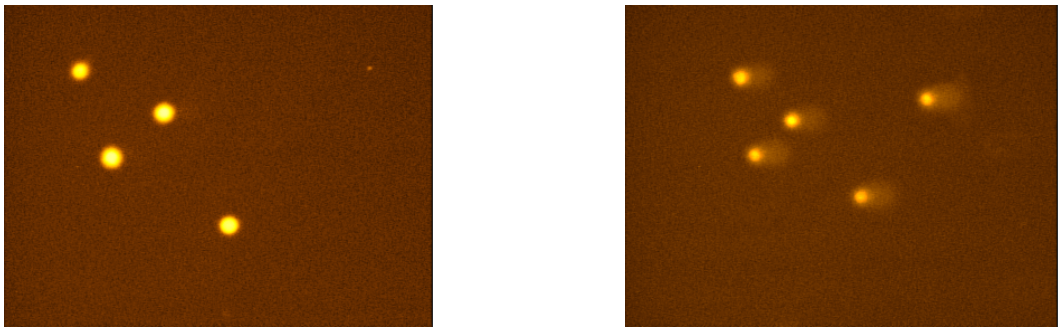
The single cell gel electrophoresis (comet) assay has enabled the study of strand breaks and DNA interstrand crosslinking in clinical material due to the low number of cells needed from patients, its sensitivity (compared with techniques such as alkaline elution), and the reproducibility of results (Hartley *et al.*, 1999). It has

become an important tool in the investigation of ICL formation and repair in the clinical setting.

The comet assay was originally developed to measure strand breaks, and has been adapted to measure interstrand crosslinking (Hartley *et al*., 1999). Cells are irradiated to produce a fixed level of random single strand breaks (which will include some double strand breaks if two single strand breaks occur at the same point). The shortening of the comet tails in the crosslinking drug treated and then irradiated sample, compared to the irradiated control is a measure of the level of ICLs present (Hartley *et al*., 1999). Shortening of the comet tails is due to the decrease in electrophoretic mobility caused by the presence of interstrand crosslinks within the DNA.

The figures below represent the DNA of individual cells, and are typical examples of the images that are produced by the comet assay. The percent decrease in tail moment is a measure of the amount of interstrand crosslink formation present in the cells at a given time, and is calculated using the formula shown in methods section 2.3d(viii). Figures 13(a)-13(d) are typical images from A2780 cells of the four components used in the formula.

(a) (b)



(c) (d)

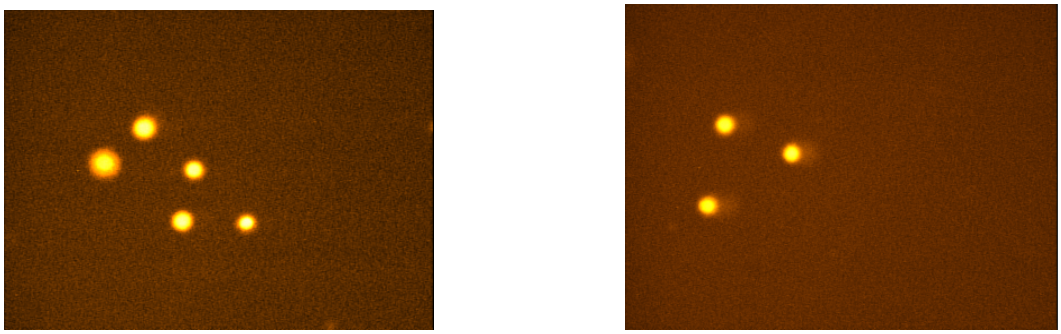


Figure 13: (a): non-irradiated non-drug treated cells, (b): irradiated non-drug treated cells, (c): non-irradiated drug-treated cells, (d): irradiated drug-treated cells

In Figure 13(a), the cells have almost no comet tails. This is because their DNA is not damaged and is of high molecular weight and therefore has low electrophoretic mobility. This sample is a control to establish the amount of background DNA damage present within these cells, before any drug is added. In this sample there is very little background DNA damage.

In figure 13(b) the cells have very large comet tails. This results from the controlled dose of irradiation introducing a fixed number of random DNA strand breaks. As the DNA is broken into smaller fragments its electrophoretic mobility increases resulting in large comet tails. The amount of DNA damage in this sample is a baseline to which other irradiated drug-treated samples are compared.

In Figure 13(c), the cells have almost no comet tails after drug treatment with cisplatin. This is because cisplatin does not produce strand breaks and therefore the DNA has a high molecular weight, and low electrophoretic mobility, similar to figure 13(a). This sample is a control to show very few single or double strand breaks are produced by cisplatin, and therefore it is the irradiation alone that produces fixed levels of strand break DNA damage.

The cells in Figure 13(d) have received the same amount of irradiation as those in figure 13(b), but they have smaller comet tails due to the presence of ICLs produced by the drug. The interstrand crosslinks that are present in this sample decreases the electrophoretic mobility in the gel due to the increased size of the DNA fragments, compared to fragments produced by irradiation alone. This difference (percent decrease in tail moment) is a measure of the amount of DNA interstrand crosslinks present in this sample.

In figure 14, media containing different concentrations of cisplatin were incubated with the cells for 1 hour and then removed. The cells were incubated for a further 9 hours in drug-free media. 9 hours was chosen because it has previously been shown

to be the peak of ICL formation in these cells (Wynne *et al*., 2007). As can be seen from figure 14, the relationship between concentrations of cisplatin and the percent decrease in tail moment is hyperbolic. Above 100µM the amount of ICL formation present plateaus as the concentration of cisplatin increases.

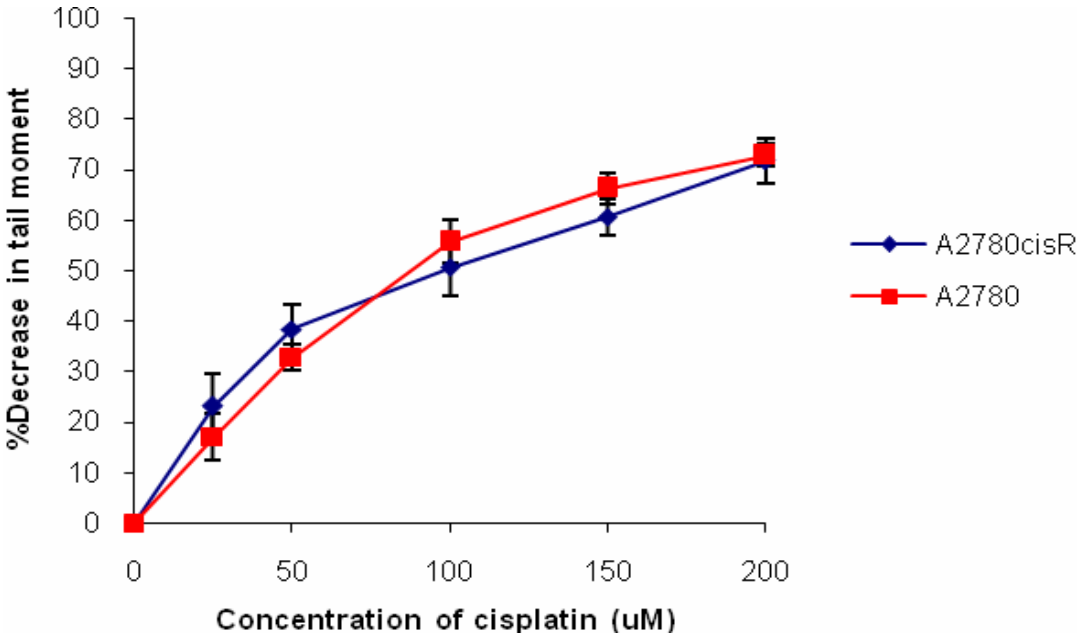


Figure 14: Relationship between the percent decrease in tail moment and concentration of cisplatin (data used from three individual experiments, standard error bars are shown)

In figure 14, 100µM cisplatin produces approximately 50% decrease in tail moment in both A2780 and A2780cisR cell lines, and this concentration of cisplatin was used to investigate formation and repair of ICLs in the A2780 and A2780cisR cell

lines. Students paired t-test was used to see if there was any statistical difference between the % decrease in tail moment of A2780 and A2780cisR cells when treated with 100μM cisplatin. Statistical significance was achieved if $p \leq 0.05$. This test was used as these cell lines are paired, and only differ in their resistance to cisplatin. Their results do not significantly differ ($p < 0.40$). However, it is noted that 100μM cisplatin is a far higher concentration that the cells are exposed to, than in the clinical setting. The dose of carboplatin that the patients are exposed to decreases over time, but a concentration of between 5 to 7mg/ml min^{-1} which is the area under the concentration curve (AUC) gives a maximal response rate (Duffull *et al*., 1997), which is in the order of 10 fold lower than in these experiments. 100μM cisplatin was also used to investigate formation and repair of ICLs in clinical samples taken from newly diagnosed and treated patients. As 100μM cisplatin produces approximately 50% of interstrand crosslink formation, this concentration was arbitarly chosen as it provided a level from which formation and repair could be measured over time. Therefore small changes in either ICL formation or repair could be detected.

In figure 15, 100μM cisplatin was incubated with A2780 and A2780cisR human ovarian cancer cell lines for 1 hour and then removed. The cells were then incubated in drug-free media for 0, 3, 6, 9, 24, 30 and 48 hours, and then harvested, and the level of ICL formation present at each time point was measured using the comet assay.

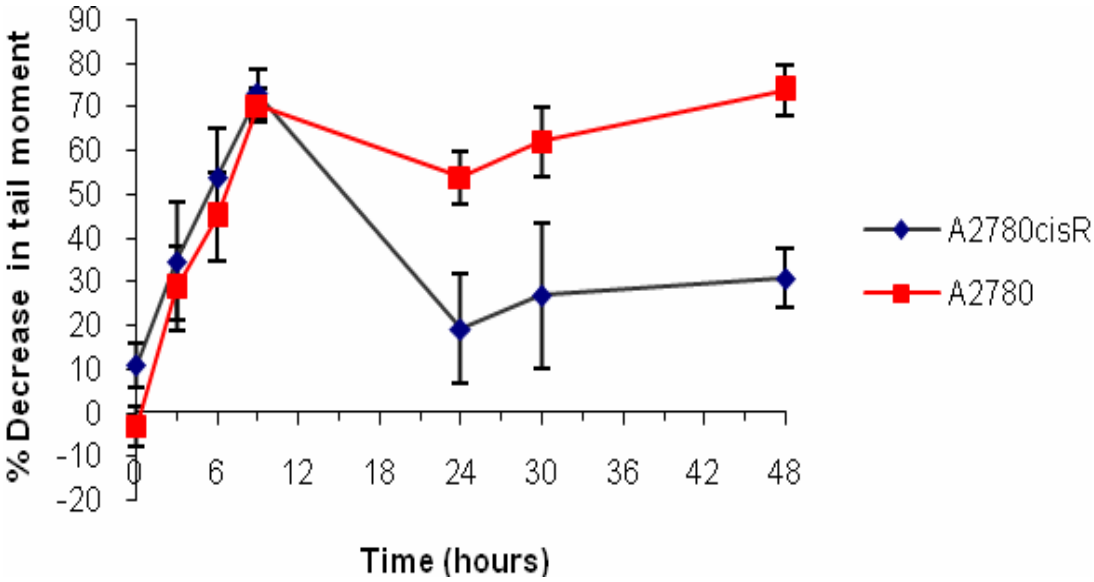


Figure 15: Time course over 48 hours to show the relationship between the percent decrease in tail moment in A2780 and A2780cisR cell lines when incubated *in vitro* with 100μM cisplatin for 1 hour (data used from three individual experiments, standard error bars are shown).

The percent decrease in tail moment after treatment with 100μM cisplatin at 9 hours for the A2780 and A2780cisR cell lines are similar at 70%±6 and 73%±10 respectively (figure 15). Using students paired t-test there is no significant difference in the peak of crosslinking ($p<0.40$). This is the maximum decrease in tail moment over a 48-hour period, corresponding to the peak of ICL formation.

The levels of crosslinks remain at a high level over a 48 hour period in the A2780 cell line. In contrast there is a rapid decrease in the % decrease in tail moment by 24 hours in the A2780cisR cell line as a result of repair (unhooking) of the crosslinks. At 24 hours there is a significant difference of the %decrease in tail moment between the A2780 and A2780cisR ($p<0.025$, using students paired t-test). This significant difference in % decrease in tail moment continues at 48hours ($p<0.05$, using students paired t-test). This repair of ICLs is likely to be an important mechanism in the resistance to cisplatin seen in the A2780cisR cell line when compared to the parent A2780 cell line.

3.2.3 Discussion

The results from the SRB assay found the A2780cisR human cancer cell line had a 6.2-fold increase in resistance to cisplatin compared to the A2780 human cancer cell line. Behrens et al (1987) found that the A2780cisR (or 2780CP8) resistant cell line was 7.3-fold resistant to cisplatin compared to the A2780 cell line (Behrens *et al*., 1987). Another group found this A2780cisR cell line was 7.7-fold resistant to cisplatin (Schmidt *et al*., 1993). Therefore, the results obtained are of a similar magnitude to those found in the literature.

As the concentration of cisplatin increases, so too does the formation of ICLs, in a hyperbolic relationship up to 100μM cisplatin, demonstrating similar amounts of ICLs are present in both A2780 and A2780cisR human ovarian cancer cell lines.

This is supported by Masuda H *et al* (1990) who showed that binding of platinum to DNA as measured by flameless atomic absorption spectrophotometry, of either A2780 or A2780cisR cell line was a linear function of concentration ranging from 20-80μM cisplatin (Masuda *et al*., 1990). This implies that similar concentrations of cisplatin interact within the DNA of these cells to produce ICLs, suggesting that mechanisms upstream of DNA damage such as increased efflux, or reduced influx of cisplatin, or drug detoxifying mechanisms are not different between the two cell lines. However, in contrast, Schmidt *et al* (1993) have shown decreased accumulation of cisplatin in the A2780cisR cell line as measured by atomic absorption spectrometry, resulting in less formation of platinum-DNA adducts, including ICLs, compared to the parent cell line, which they suggested contributes to cisplatin resistance (Schmidt *et al*., 1993).

Importantly, other cells such as lymphocytes show similar properties when treated with other crosslinking agents. Hartley et al (1999) demonstrated a linear relationship between decrease in tail moment as measured by the comet assay and the level of ICL formation in lymphocytes treated *ex vivo* with increasing concentrations of chlorambucil (Hartley *et al*., 1999).

The increase in repair of ICLs found in the A2780cisR cell line as compared to the A2780 cell line is supported by Masuda H *et al* (1990). They suggested that the mechanism of resistance in the A2780cisR (or A2780/CP8) cell line was due to their increased ability to remove platinum-DNA adducts (mostly intrastrand crosslinks were measured), and not to a difference in initial binding of cisplatin, as measured

by flameless absorption atomic spectophotometry (Masuda *et al*., 1990). There is further evidence using a 3H-thymidine incorporation assay that showed a two-fold increase in the repair ability of the A2780cisR cell line (Masuda *et al*., 1988). Further studies have also showed increase in repair of the A2780cisR cell line as a mechanism of cisplatin resistance (Scmidt *et al*., 1993) (Zhen *et al*., 1992).

Parker *et al*. 1991, and Masuda *et al*. 1990 found the A2780/CP8 cell line to have similar levels of DNA damage, including ICL formation when treated with 100μM cisplatin compared to the parental A2780 cell line. Masuda *et al*. also found the A2780/CP8 resistant cell line to be two-fold more efficient at repairing DNA damage, including ICLs and intrastrand crosslinks, compared to the parent A2780 cell line (Masuda *et al*., 1990). This shows that these resistant cell lines behave very similarly to the ones used in the experiments, and supports the data from our experiments.

3.3 Clinical ovarian cancer tissue treated *ex vivo* with cisplatin

3.3.1 Obtaining ovarian cancer cells from patient samples

A total of 48 patients were included in this study, who were all suspected to have epithelial ovarian cancer. Informed consent was obtained from all of them. From six of these patients a sample was collected before they received any chemotherapy, and also, after they had received platinum-based chemotherapy. These are the six 'paired' patients. Three of the 48 patients did not have ovarian cancer when the

histopathology was examined, and so were excluded from the study. There were cell culture problems with a further five patients, which meant they were not included in the study as experiments were unable to be carried out. Overall cells from 40 patients with epithelial ovarian cancer were analysed in the study, including the six paired patient samples. Patients who had not yet received chemotherapy and are newly diagnosed are called ‘pre-chemotherapy’ patients, and those that had received platinum-based chemotherapy are called ‘post-chemotherapy’ patients.

3.3.2 Patient characteristics

Table 1 and 2 show the individual patient characteristics in both newly diagnosed (pre-chemotherapy) and treated (post-chemotherapy) patient groups. The average age in the pre-chemotherapy group is 66 ± 11, and in the post-chemotherapy group is 66 ± 10.

The Platinum-free interval (PFI) is the time taken from when the patient had their last dose of chemotherapy (before the sample was taken), until they had evidence of disease relapse (around the time the sample was taken), such as a doubling of their Ca-125, or evidence on CT scan. Therefore, only post-chemotherapy patients have a PFI. The PFI is used to guide future chemotherapy treatment, as it describes the likelihood of responding to future platinum-based chemotherapy. A PFI of 6 months or less suggests the patient is clinically resistant to platinum, and their next chemotherapy should be changed to second-line chemotherapy. A PFI of 7 months

or more suggests the patient may still respond to platinum based chemotherapy, and therefore their next course of chemotherapy should include platinum.

The progression-free survival (PFS) is the time from the patients first dose of chemotherapy (usually around the time the sample was taken) until the patient clinically relapses. Doubling of Ca-125 levels from the upper limit of normal has been shown to predict progression (Rustin *et al*., 2001). Persistently elevated levels, or doubling of Ca-125 from its nadir level has now also been shown to accurately define progression (Rustin *et al*., 2001) with a false positive rate of less than 2% (Rustin *et al*., 2003). Clinical relapse is also defined as evidence of disease on CT scan (Rustin *et al*., 2003).

Interval debulking surgery (IDS) patients are in the post-chemotherapy group of patients as they have already received three doses of chemotherapy. They then undergo an operation to remove as much of the tumour as possible. However, these patients will not have received a full course of platinum-based chemotherapy as three doses are half the six doses required for a full course.

Patient number	Age	Treatment	FIGO stage	PFS	Outcome	Sample and hitological type
1	63	Carboplatin/ paclitaxel	4	11	Ca125 36 to 67, with symptoms	ascites/solid tumour, serous papillary adenocarcinoma
2	68	Carboplatin/ paclitaxel	2c	>13	NED(no evidence of disease)	solid tumour, endometrioid adenocarcinoma
3	91	none	Not known		No treatment given	ascites/solid tumour, endometrioid adenocarcinoma
4	72	Carboplatin/ paclitaxel	3c	>10	NED	solid tumour, serous papillary adenocarcinoma
5	65	Carboplatin/ paclitaxel	3c	>4	NED	solid tumour, peritoneal serous adenocarcinoma
8	74	Carboplatin/ paclitaxel	3c	10	Doubled Ca125	ascites/solid tumour, serous papillary adenocarcinoma
10	51	Carboplatin/ paclitaxel	3c	>8	NED	ascites/solid tumour, serous papillary adenocarcinoma
12	63	Carboplatin/ paclitaxel	3c	7	Doubled Ca125	ascites/solid tumour, serous papillary adenocarcinoma
15	50	Carboplatin/ paclitaxel	2c	>4	NED	solid tumour, serous papillary adenocarcinoma
16	63	Carboplatin/ paclitaxel	3c	>4	NED	solid tumour, not known
17	54	Carboplatin/ paclitaxel	3c	0	Progressive disease on chemo	Ascites, serous papillary adenocarcinoma
18	62	Carboplatin	1c	>6	NED	ascites/solid tumour, serous papillary adenocarcinoma
19	45	Carboplatin/ paclitaxel	3a	>8	NED	solid tumour
22	77	Carboplatin	2b	>6	NED	solid tumour
24	62	Carboplatin	2b	>7	NED	solid tumour
26	73	Carboplatin/ paclitaxel	3c	>7	NED	Ascites, serous papillary adenocarcinoma
27	70	Carboplatin/ paclitaxel	3c	9	Doubled Ca125, R inguinal lump	Ascites, serous papillary adenocarcinoma
29	61	Carboplatin	1a	>4	NED	solid tumour
30	54	None (too unwell)	3c	N/A	Died Aug 05	ascites
32	64	Carboplatin/ paclitaxel	3	3	Doubled Ca125,CT-abdo disease	ascites
33	58	Carboplatin/ paclitaxel	4	0	Disease progression on chemo	ascites
34	63	Carboplatin/ paclitaxel	3c	5	CT-progressive disease, symptoms	ascites
36	87	Carboplatin	3c	>6	NED	solid tumour
38	80	Carboplatin	3c	>6	NED	ascites
39	76	Carboplatin	3c	>4	NED	ascites/solid tumour
40	78	None (too unwell)	3c	N/A	Died	ascites
41	77	Carboplatin (intraperitoneal)	3c	0	Disease progression on chemo	ascites
43	66	Carboplatin/paclitaxel	4	>3	NED	ascites
45	56	Info not available	3c		Info not available	ascites
46	66	Carboplatin/paclitaxel	3c	>4	NED	ascites
48	63	Carboplatin	3c	19	NED	ascites

NED is no evidence of disease, PFS Progression free survival

Table 1: Newly diagnosed patient characteristics

Patient number	Age	Treatment	FIGO stage	category	PFI	PFS	outcome	Sample and histological type
1B	63	Carboplatin/paclitaxel	4	IDS	N/A	11	Ca125 36 to 67, with symptoms	solid tumour, serous papillary adenocarcinoma
27B	70	Carboplatin/paclitaxel	3c	IDS	N/A	9	Doubled Ca125, R inguinal lump	Ascites, serous papillary adenocarcinoma
28	53	Etoposide	4	IDS	N/A	11	Doubled Ca125, CT-lymph node	solid tumour, serous papillary adenocarcinoma
37	69	Cisplatin/etoposide	3c	IDS	N/A	0	Disease progression, died jan 06	Ascites/solid tumour, Clear cell carcinoma
38B	80	Carboplatin	3c	IDS	N/A	>6	NED	ascites
44	82	Carboplatin	3c	IDS	N/A	>8	NED	solid tumour
6	69	Carboplatin	4	<6months	0	0	Died, unable to assess	Ascites, serous papillary adenocarcinoma
7	66	None	3c	<6months	3	0	Died, unable to assess	Ascites, serous papillary adenocarcinoma
17B	54	None	3c	<6months	0	0	Disease progression	Ascites, serous papillary adenocarcinoma
21	49	Carboplatin/paclitaxel	3c	<6months	4	0	Died	Ascites, Clear cell carcinoma
33B	58	Cisplatin/etoposide	4	<6months	0	>6	Responding to treatment	ascites
35	63	Carboplatin	4	<6months	4	0	Doubled Ca125, symptoms. Died Dec 05	ascites
41B	77	none	3c	<6months	0	N/A	Unwell with severe COPD	ascites
47	68	Gemcitabine/ Carboplatin	3c	>6months	16	>6	NED	ascites

NED is no evidence of disease
PFI Platinum free interval
PFS progression free survival

Table 2: Post-chemotherapy patient characteristics

Ovarian tumour cells were obtained from ascites by separating them from the mesothelial cells using cell culture techniques (see methods section 2.4c(i)). The samples were observed under the microscope (see figure 16), and stained to confirm presence of specific cell types.

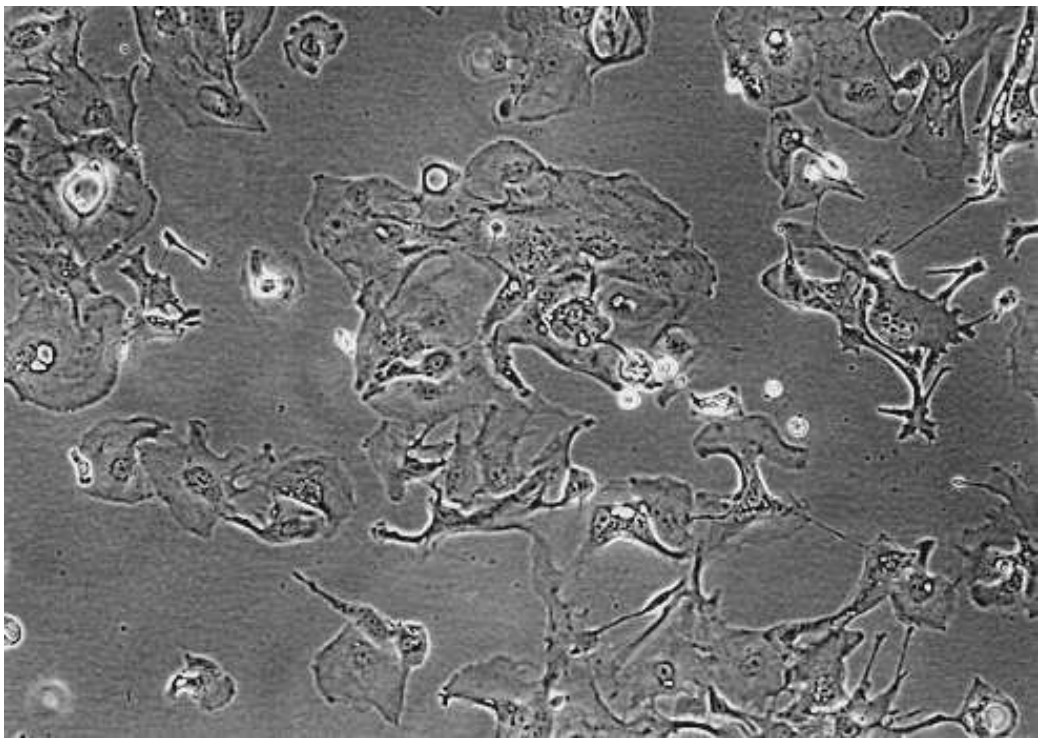


Figure 16: Phase contrast images taken down a microscope of primary culture of ovarian tumour cells isolated from ascites (Wynne, 2006).

Figure 16 illustrates the morphology characteristic of ovarian tumour cells. They are more rounded, tend to cluster together, and have abnormal nuclei. This is in contrast to mesothelial cells (figure 17).

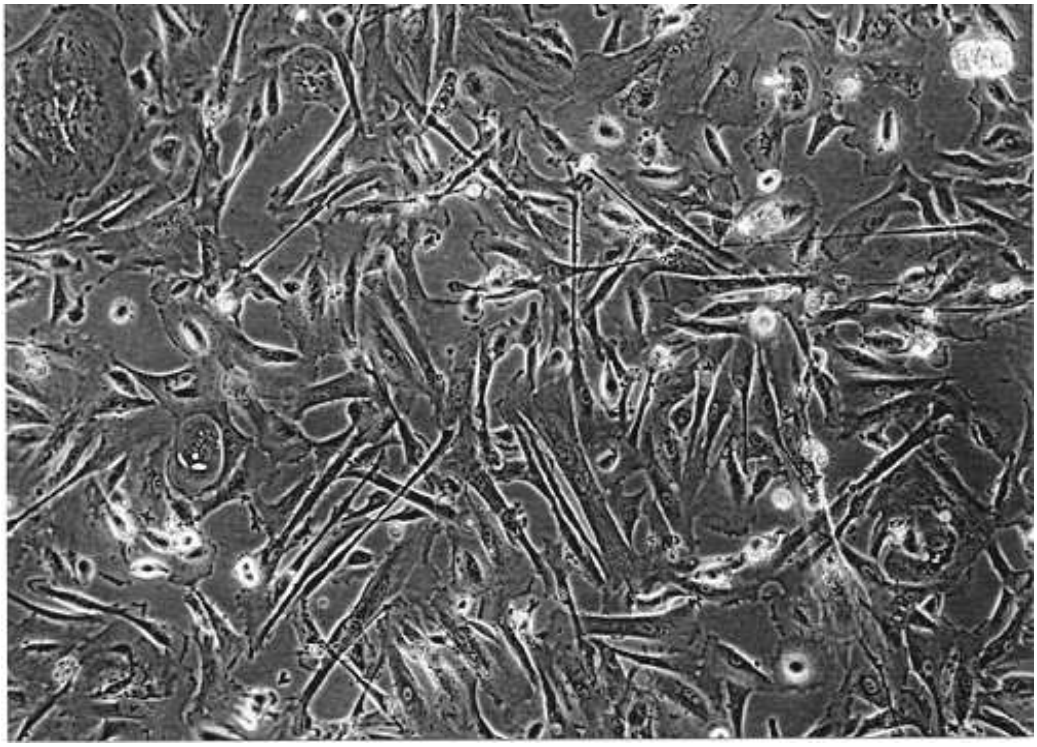


Figure 17: Phase contrast images taken down a microscope of primary culture of mesothelial cells isolated from ascites (Wynne, 2006).

Figure 17 illustrates the morphology characteristic of mesothelial cells. They are more spindle-shaped compared to ovarian tumour cells. Once the sample had been separated into mesothelial cells and ovarian tumour cells, the samples were stained to confirm the percentage of mesothelial and ovarian tumour cells.

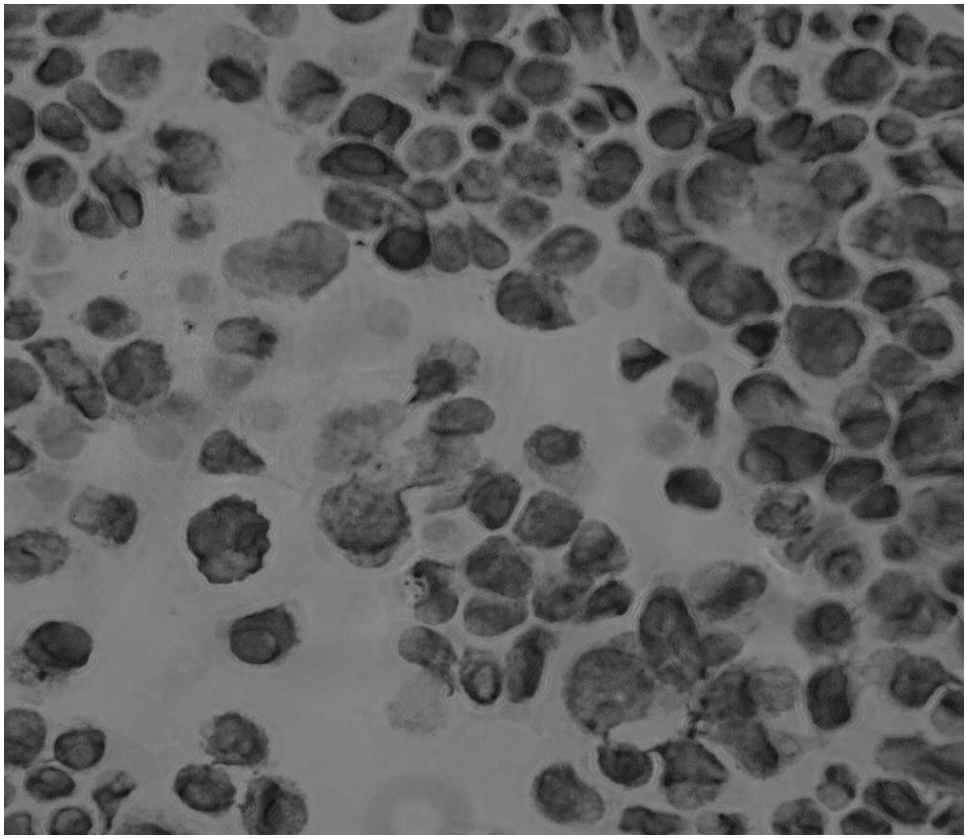


Figure 18: Ovarian tumour cells from ascites stained using CK7 from a typical patient sample

Cytokeratin 7 (CK7) stains epithelial cells, and therefore stains ovarian tumour cells as they are of epithelial origin. CK7 does not stain mesothelial cells (Plaza *et al*., 2004). In the tumour cell sample shown in figure 18, there is almost 95% positive staining for CK7, indicating the presence of epithelial cells. This is representative of all the separated tumour cell samples that were stained.

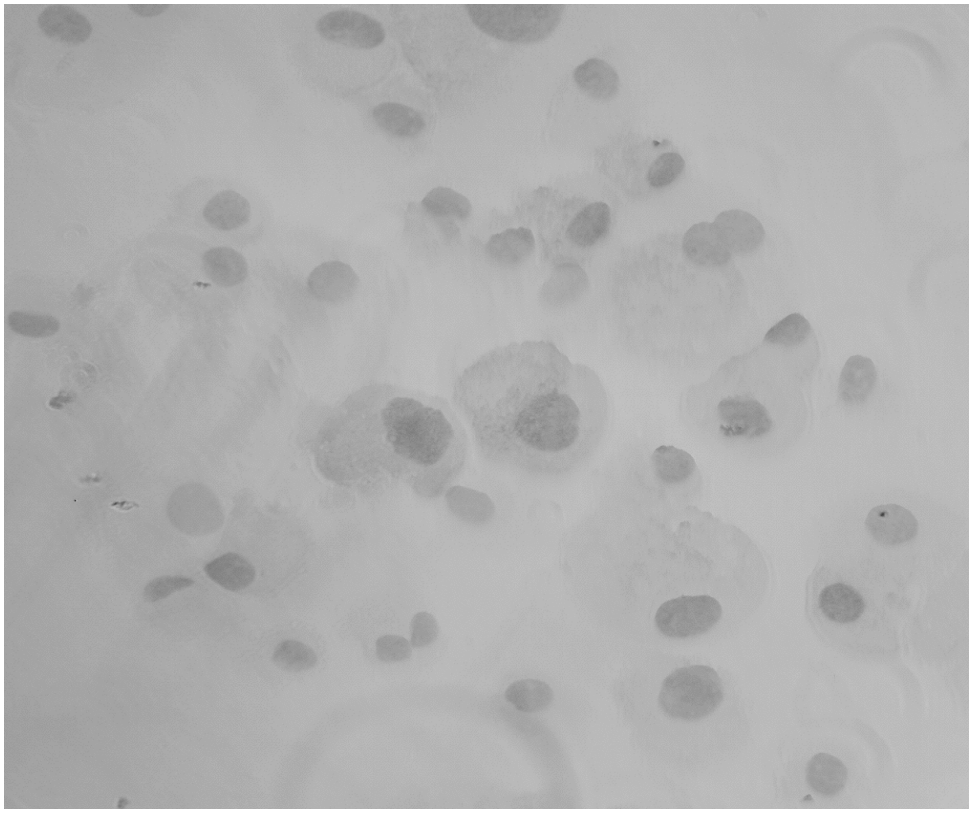


Figure 19: Ovarian tumour cells from ascites stained with CK5 from a typical patient sample

Cytokeratin 5 (CK5) stains mesothelial cells (Plaza *et al*., 2004), and not epithelial cells. In figure 19, the same tumour cell sample as in figure 18 was used. There is almost no staining present, as there are very few mesothelial cells present. Figure 19 and figure 18 confirms the presence of approximately 95% epithelial cells present in this sample. As ascites is mainly a mixture of ovarian tumour cells and mesothelial cells, the epithelial cells confirmed in this sample are ovarian tumour cells.

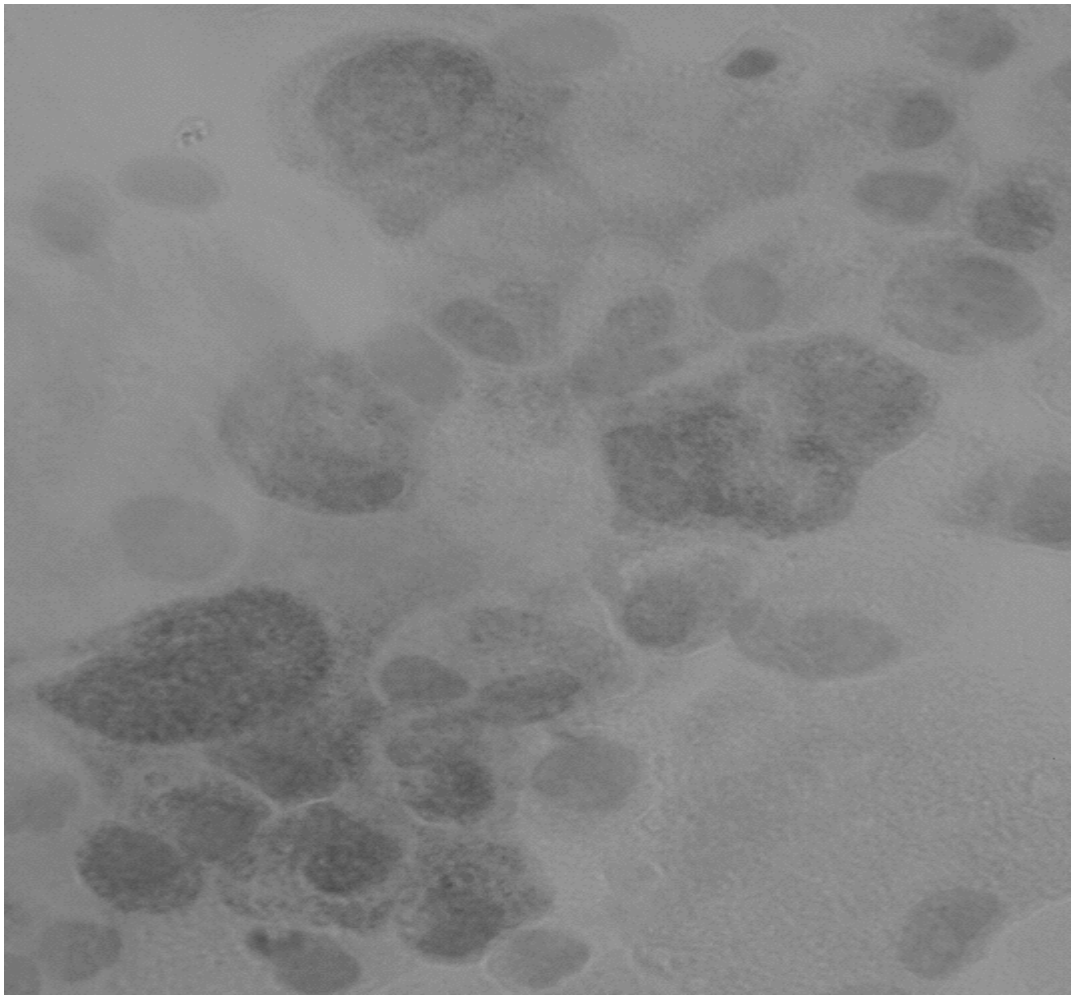


Figure 20: Mesothelial cells from ascites stained with CK5 from a typical patient sample.

Figure 20 shows the staining with CK5 of a typical mesothelial cell sample isolated from ascites. There is positive staining for mesothelial cells using the CK5 antibody marker, confirming the presence of mesothelial cells in this sample.

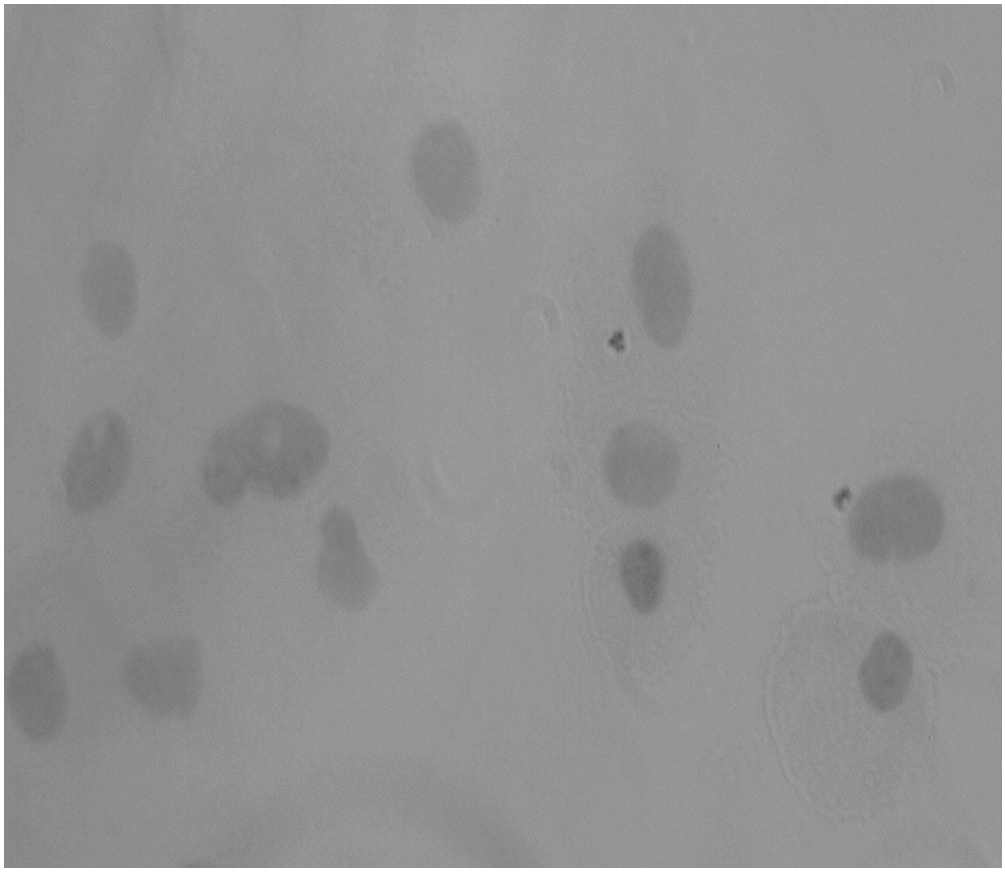


Figure 21: Mesothelial cells stained with CK7 from a typical patient sample.

Figure 21 shows the same sample as figure 20, but stained with CK7. There is no staining, which means there are no epithelial cells present. These two figures confirm the presence of approximately 80% mesothelial cells in this sample. Only samples with >80% ovarian tumour cells or mesothelial cells were used in further experiments. The majority of ovarian tumour cell samples stained >95% positive with the CK7 marker. Other techniques such as fluorescent active cell sorting (FACS) or magnetic bead sorting could have been used to further purify the cells, but because the vast majority of cells were 95% pure, it was deemed an unnecessary expense.

Solid tumour cell samples obtained from patients with ovarian cancer were also stained to confirm the presence of ovarian cancer cells. They were stained with CK7 and were all shown to be strongly positive (>95% ovarian cancer cells).

3.3.3 Measurement of ICL formation and repair in ovarian cancer cells obtained from patient ascites and solid tumour when treated with cisplatin

ICL formation and repair were measured in ovarian cancer cells obtained from patients. 100μM cisplatin was used as this has been shown to produce a significant level of ICL formation in the A2780 and A2780cisR cell lines.

Figure 22 shows an individual crosslink response curve for patient 37. This patient had not received any chemotherapy before a sample was taken. The peak of ICL formation at 9 hours is maintained over 48 hours in these cells. No repair or unhooking of ICLs has occurred in this patient. It is thought that the inability to repair platinum ICLs confers sensitivity to the drug and a clinical response. However, this patient had progressive disease and died soon after diagnosis. Histopathology showed clear cell ovarian carcinoma which is known to be a very aggressive form of the disease. This may have contributed to the progressive nature of her disease despite chemotherapy.

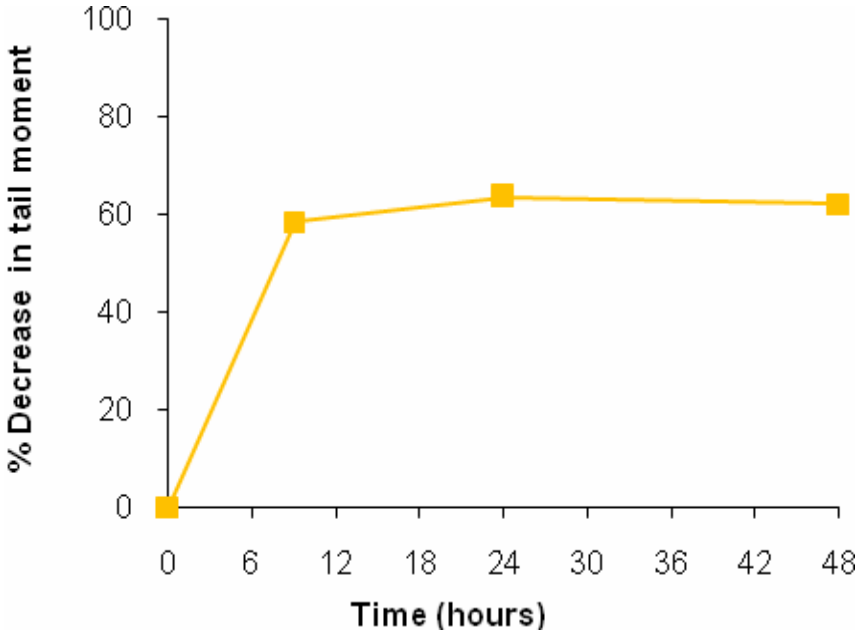


Figure 22: Time course over 48 hours illustrating ICL formation and persistence in ovarian cancer cells isolated from ascites in patient number 37 before chemotherapy.

There were limited cells obtained from some patient samples, and so only two time points were chosen to measure ICL formation and repair. These were 9 hours, as this is the peak of ICL formation (as already demonstrated), and 24 hours.

In a number of patients it was possible to repeat the comet assay two or three times depending on the amount of cells obtained. Patient numbers 1, 1B, 3, 4 and 8 had duplicate comet assay results, patient numbers 2, 5, 6 and 10 had triplicate comet assay results, and the other patients had one result from the comet assay. Where

duplicate or triplicate samples were processed successfully, error bars are present on the relevant figures.

Some of the values of percent repair at 24 hours are ‘negative’. This means that the percent decrease in tail moment, and therefore the level of crosslinks, increased from 9 hours to 24 hours. All the patient data for the peak of ICL formation at 9 hours are shown in figure 23.

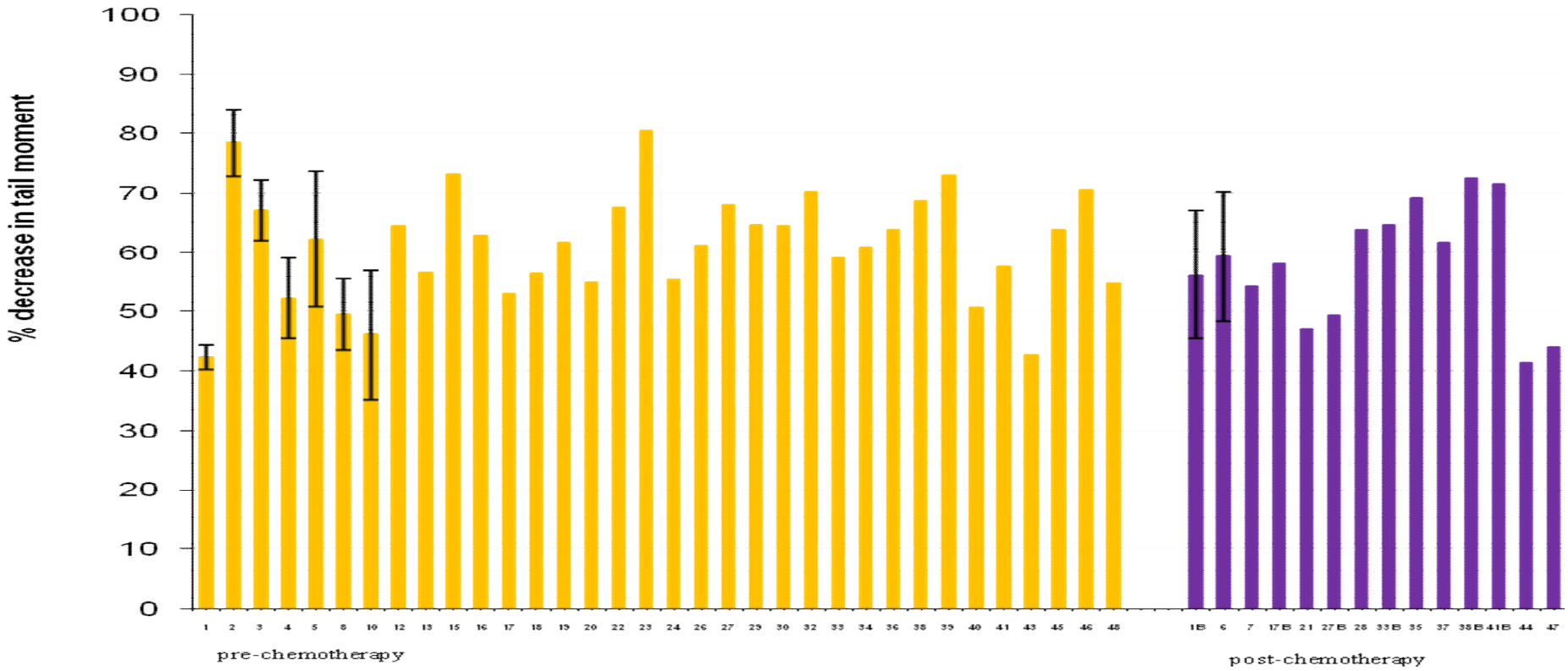


Figure 23: Peak of ICL formation in patients before and after chemotherapy (some standard error bars are shown on this figure for the patients that had duplicate or triplicate results)

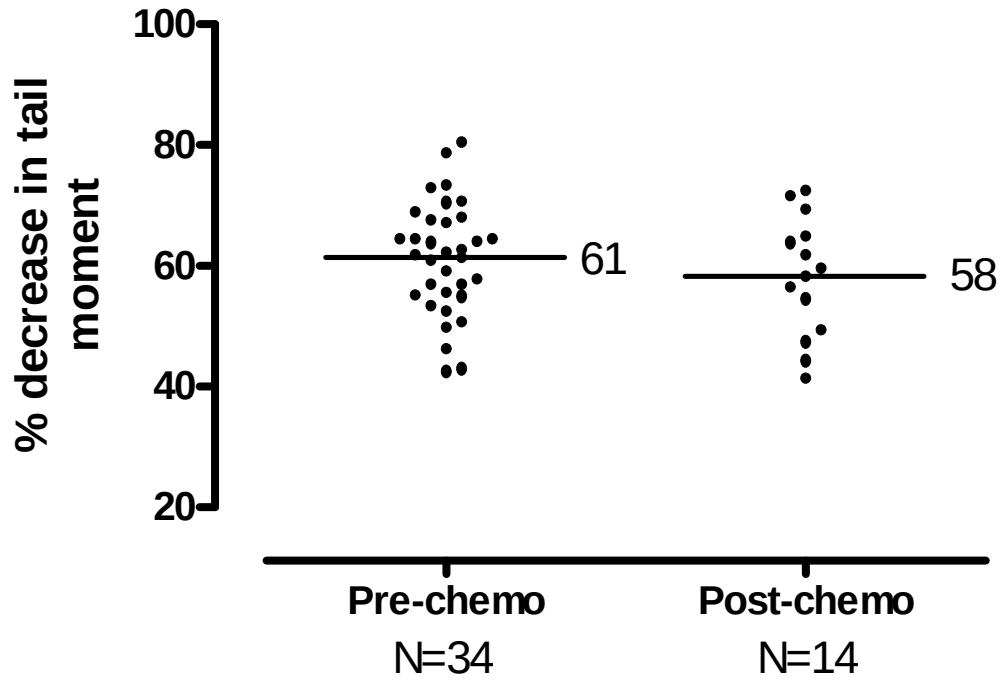


Figure 24: Peak of ICL formation in pre and post-chemotherapy samples. Horizontal line shows mean of each group. Dots show distribution of results.

Figures 23 and 24 both show data for the peak of ICL formation in pre- and post-chemotherapy samples. The results are clearly similar for the two groups of patients. There is no statistically significant difference between the two groups, using students paired t-test ($p<0.75$), and this is especially noticeable in the scatter plot of figure 24 which shows the distribution of the data clustered together. Figure 23 illustrates the results obtained for each patient. Students paired t-test was used as the two groups of patients are paired as some samples are taken before chemotherapy, and others are taken after chemotherapy. Statistical significance was achieved if $p \leq 0.05$.

In total there are 34 pre-chemotherapy (newly diagnosed) patient samples, and 14 post-chemotherapy (treated) patient samples. Overall, the level of ICL formation as measured by the % decrease in tail moment ranged from 41-80 (see figure 24). The mean level of crosslinking in all samples was 60% ±10, while it was 61% ±9 in the pre-chemotherapy group of patients (newly diagnosed), and 58% ±10 in the post-chemotherapy patients (treated patients). There was no significant difference in these values using students paired t-test ($p<0.40$). This suggests that similar amounts of cisplatin interact with the DNA of both groups of patient cells, to cause similar amounts of ICL formation within the DNA. These results also suggest that upstream mechanisms such as drug efflux, and detoxification mechanisms are not contributing to reducing the levels of cisplatin entering and reaching the DNA of these cells.

In five patients it was possible to obtain mesothelial and ovarian cancer cells from ascites in pre- and post-chemotherapy patients. The peak of ICL formation was measured in these two cell populations to determine if their cellular metabolism of cisplatin was similar. These cells were treated with 100μM cisplatin for 1 hour, and then the drug was removed. The cells were left for 9 hours and the level of ICL formation at 9 hours was measured using the comet assay (figure 25).

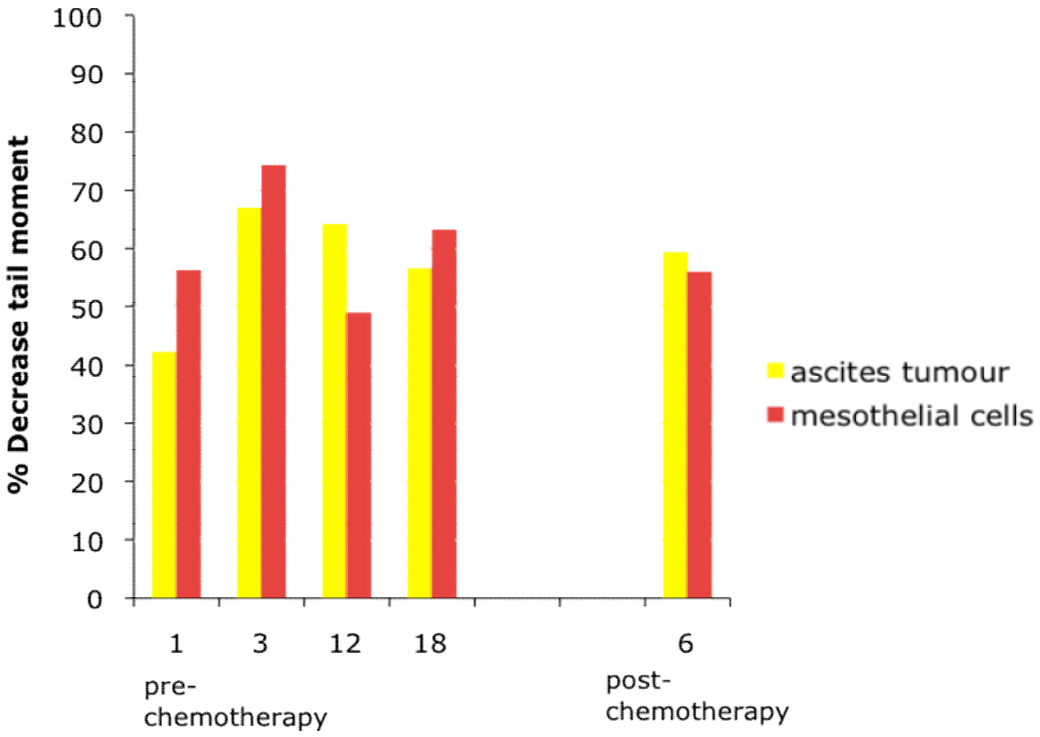


Figure 25: Percent decrease in tail moment at 9 hours in ascites and mesothelial cells from the same patient before and after chemotherapy.

Figure 25 shows similar levels of ICL formation in both tumour (i.e ovarian cancer), and non-tumour (i.e mesothelial) cells. The average level of ICL formation as measured by percent decrease in tail moment at 9 hours in the mesothelial cells from pre-chemotherapy patients was 58±11, and for the corresponding ovarian cancer cells was 61±11 (figure 25). There was no significant difference in these values using students paired t-test ($p<0.40$). The percent decrease in tail moment at 9 hours

in the mesothelial cells from one post-chemotherapy patient was 56, and for the corresponding ovarian tumour cells it was 60.

Ovarian cancer cells obtained from ascites and solid tumour samples were investigated to determine if these cells had similar levels of ICL formation at 9 hours, or if ovarian tumour cells obtained from different tissues behaved differently.

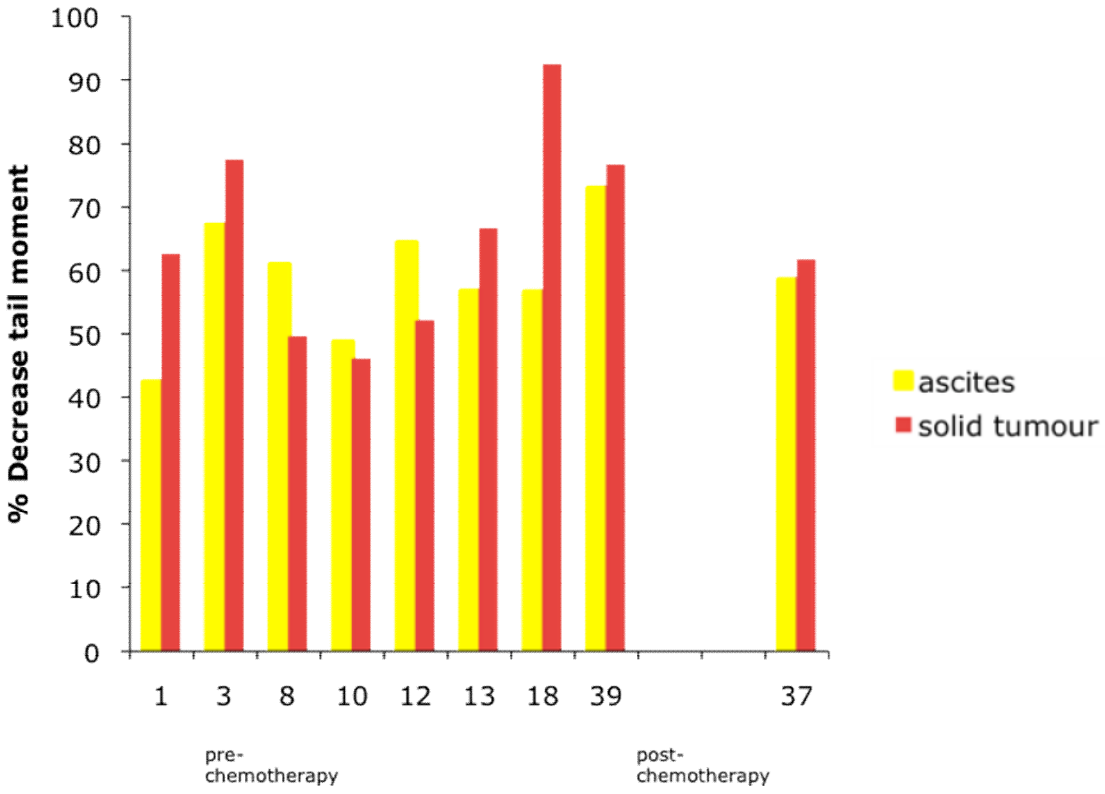


Figure 26: percent decrease in tail moment at 9 hours in ascites and solid tumour patient samples taken before and after chemotherapy.

In eight patients it was possible to obtain both ascites and solid tumour samples. Figure 26 shows similar levels of ICL formation in both ascites and solid tumour samples taken from the same patient, suggesting these cell types do not differ significantly in their cellular uptake or metabolism of cisplatin which has enabled similar levels of DNA damage to occur. The level of ICL formation as measured by the average % decrease in tail moment at 9 hours was 59±10and 65±16 for ascites and solid tumour cells, respectively, taken before chemotherapy. There was no significant difference in these values using students paired t-test ($p<0.40$) The one patient sample collected after chemotherapy showed comparable levels of percent decrease in tail moment at 9 hours for ascites (59), and solid tumour (62). Patient 13 has benign ovarian disease, and was included to illustrate the similarity of ICL formation present after treatment with cisplatin, in mesothelial cells and benign ovarian cells before receiving any chemotherapy in the clinical setting.

Levels of ICL formation were measured at 9 hours and 24 hours after the samples were incubated *ex vivo* with 100μM cisplatin, and then the percent repair at 24 hours calculated. The percent repair (at 24 hours) is the difference between the peak of ICL formation at 9 hours and 24 hours. The data are shown in figure 27 for the individual patients and figure 28 as a scatter plot to show the distribution of the data.

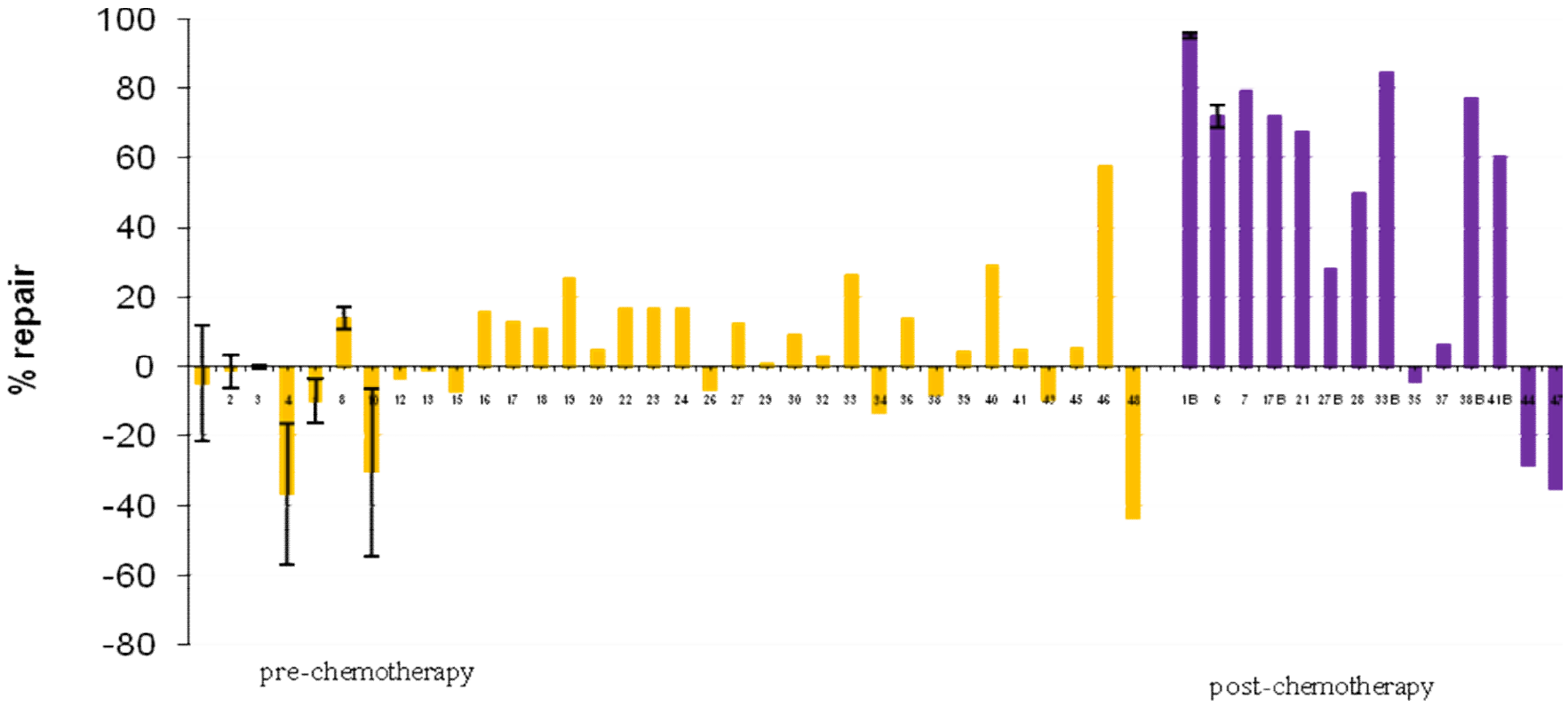


Figure 27: Percent repair at 24 hours in patients before and after chemotherapy (standard error bars are shown on the patient samples that have been done in duplicate or triplicate).

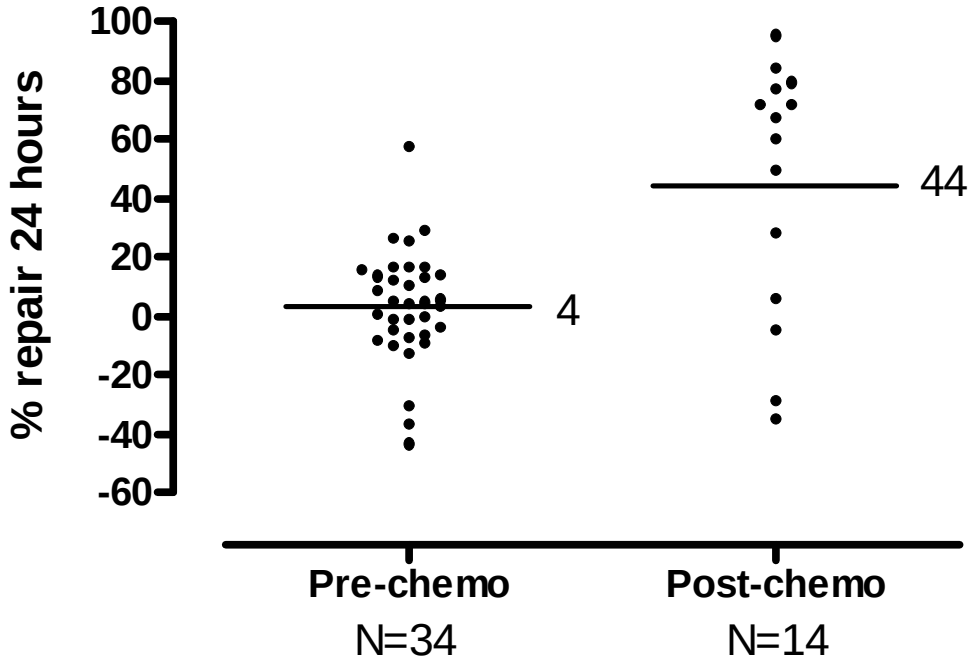


Figure 28: Percent repair in patient samples taken before and after chemotherapy. Horizontal lines indicate the mean of each group.

From figures 27 and 28, there appears to be a much more heterogeneous response between the patient samples, with values for repair at 24 hours ranging from no repair to almost 100% repair. Some patients demonstrated 'negative' repair, in which the levels of ICLs present actually increased between 9 and 24 hours. There is a clear difference between the patient groups. In the pre-chemotherapy patient group, the average percent repair after 24 hours was 4%±19, but it was 45%±43 in the post-chemotherapy group (figure 28). This was statistically significant using students paired t-test ($p<0.001$). In the pre-chemotherapy patient population only four patients had >20% ICL repair, while the remaining 30 patients had <20% repair. In contrast only four patients had <20% ICL repair in the post-chemotherapy group, while 1 patient had 28% repair, and the remaining nine patients had >50% repair.

In six patients a sample was collected before the patient had received any chemotherapy, and again after the same patient had received platinum-based chemotherapy. Each sample was incubated with 100μM cisplatin for 1 hour, and then analysed after 9 hours and 24 hours. The levels of ICL formation and repair were measured using the comet assay (figure 29).

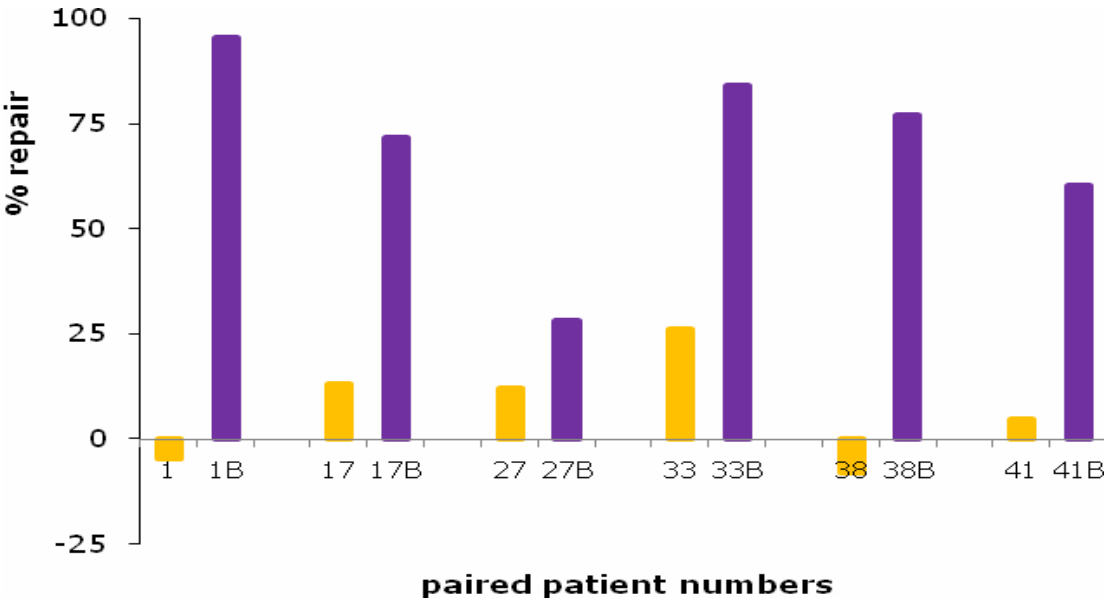


Figure 29: percent repair at 24 hours in six patients treated with cisplatin before and after they had received platinum-based chemotherapy. B denotes the sample taken after chemotherapy.

Although similar levels of ICL formation are present in the two patient populations, the percent repair after 24 hours was very different. The ovarian tumour cells taken before chemotherapy show very little unhooking of crosslinks ranging from -8% to 26%, but in contrast the ovarian tumour cells taken after chemotherapy show extensive unhooking with % repair ranging from 28% - 95%. Average % ICL repair in the pre-chemotherapy patient samples was low at 7%±13 but was much higher at 70%±23 in the post-chemotherapy samples from the same patient, respectively (figure 29). This was statistically significant using students paired t-test ($p<0.002$). Patients 1B, 27B, and 38B all had samples taken at interval debulking surgery (IDS), which means they had only had 3 doses of carboplatin before the sample was taken. These data clearly demonstrate that increase in ICL repair at 24 hours plays a role in resistance to platinum chemotherapy after as little as three doses of carboplatin. What is also clear is that nearly all patients who are treated with platinum chemotherapy will show some level of repair of ICLs, but not all of them will relapse. Therefore not all ICL repair will be clinically relevant.

In six patients it was possible to obtain mesothelial and ovarian cancer cells from ascites in pre- and post-chemotherapy patients. The repair of ICLs was measured in these two cell populations to determine if their response to treatment with cisplatin were similar.

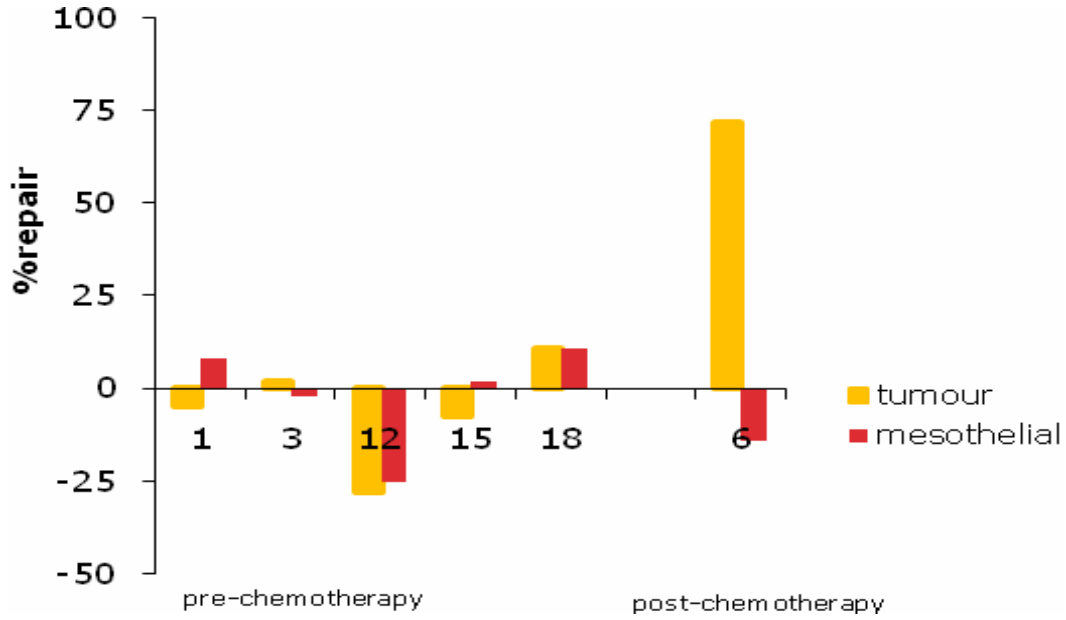


Figure 30: Percent repair (or unhooking) of ICLs in ovarian cancer cells and mesothelial cells before and after chemotherapy

In the ovarian cancer and mesothelial cells obtained from patients before chemotherapy, there does not appear to be any difference in the levels of ICL repair at 24 hours, as they all have <10% repair. There was no significant difference in these values using students paired t-test ($p<0.40$). This suggests that these cells have similar metabolic pathways when incubated with cisplatin *ex vivo*, and that the ovarian cancer cells have not yet acquired the resistance mechanism of ICL repair

.

In the post-chemotherapy patient there is an obvious difference between the percent repair (at 24 hours) of ICLs in the ovarian cancer cells and the lack of repair at 24 hours in the mesothelial cells (72 and -14%, repectively). Although these data are from only one treated

patient, it does seem that the ovarian cancer cells obtained from the ascites of patient 6 have acquired this mechanism of increased ICL repair. The mesothelial cells are also obtained from ascites, but do not seem to have acquired this same mechanism of resistance, suggesting that this acquired resistance is unique to the cancer cells. Cancer cells are genetically unstable, and are therefore more likely to acquire mutations within the DNA. However, the time interval is much too short for this to occur. A more likely mechanism is that ovarian cancer cells that repair ICLs have been selected out by treatment with carboplatin.

3.3.4 Discussion

The similarity in levels of ICL formation at 9 hours between pre-chemotherapy, and post-chemotherapy patients resembles the results from the A2780 platinum sensitive and A2780 cisplatin resistant human ovarian cancer cell lines (figure 15). Data from ICL formation and repair in clinical ovarian cancer samples has not been published before now.

The initial levels of ICL formation in ovarian cancer cells obtained before and after chemotherapy are very similar (figure 23). Many studies have shown that the level of platinum-DNA adducts correlates with patient response to platinum-based chemotherapy (Altaha *et al*., 2004), which implies that drug influx and efflux mechanisms that influence the level of drug available to reach the DNA play an important role in response to platinum chemotherapy.

The clinical response of patients to platinum chemotherapy was investigated to see if data from the level of ICL formation at 9 hours obtained from the laboratory could be used to predict which patients would not respond to platinum-based chemotherapy.

Patient 17, 33, and 41 had intrinsic (or primary) platinum resistance as their disease actually progressed whilst on carboplatin chemotherapy. As the levels of ICL formation in these patients are similar to the average for the newly diagnosed patients, it seems that drug influx/efflux or other upstream mechanisms such as detoxification do not play an important role in inherent (or primary) platinum resistance in these patients. This is also true for the other patients that developed acquired resistance to platinum chemotherapy (patients 37, 6, 7, 17B, 21, 33B, 35, 41B) as they have similar levels of ICL formation at 9 hours. Since ICLs are critical cytotoxic lesions, similar levels of cell death within these cancer cells might be expected to occur. The similar level of ICL formation is also striking in the six paired patient samples taken before and after each patient had received platinum chemotherapy. From these data it seems that the level of ICL formation at 9 hours in ovarian cancer cells obtained from newly diagnosed patients (pre-chemotherapy), and treated patients (post-chemotherapy) cannot be used to predict their response to platinum based chemotherapy.

There are a number of patients that have very different values of percent repair at 24 hours compared to the mean. In the pre-chemotherapy group, patient 19, 33, 40, and 46 have percent repairs at 24 hours of 25%, 26%, 29%, and 57%, respectively. This is in comparison to the mean for this pre-chemotherapy group of 4%. The clinical data for some of these patients was compared to see if the data for repair of ICLs can be supported with clinical evidence of resistance.

Looking at their clinical data patient 33 has a PFS of 0, and patient 40 was not given any chemotherapy, as she was not fit enough, therefore PFS cannot be calculated (she died soon after diagnosis). A PFS of 0 means that not only is patient 33 clinically resistant, but

this patient actually progressed whilst on carboplatin chemotherapy. Progression whilst on chemotherapy is very significant and is strong clinical evidence of an intrinsic resistance mechanism within the tumour cells. This intrinsic quality may be due to the higher than average levels of ICL repair. However, resistance is multi-factorial and it is always difficult to assign clinical outcome to only one resistance mechanism.

Clinical details of newly diagnosed patients, was compared to the levels of ICL repair to see if there are any correlations. Patient 17 and 41 have a PFS of 0, patient 32 has a PFS of 3, and patient 35 has a PFS of 5. This means these patients had disease recurrences soon after starting platinum chemotherapy. For further support of the theory that high levels of ICL repair correlate with *inherent* clinical resistance to carboplatin chemotherapy, we would expect these patients to have high levels of ICL repair. Patient 17 had 13% repair, patient 32 had 3% repair, patient 34 had -13% repair and patient 41 had 4.7% repair, so overall these patients had low levels of ICL repair. There is little correlation with clinical outcome and levels of ICL repair. This does not support the theory that high levels of ICL repair are a major mechanism of *inherent* clinical resistance to platinum chemotherapy. However, ICL repair could still play a role in *acquired* resistance to platinum.

The other newly diagnosed patients that have not previously been discussed have low levels of ICL repair suggesting platinum sensitivity, which seems to be consistent with their PFS. As can be seen from table 1, most patients do not have a specific number for PFS. This is because these patients have not yet had a recurrence of their disease, and so the exact PFS cannot be calculated. Most of the patients with no exact PFS have only 1 or 2 months more before they are considered to be platinum sensitive, and is this is such a short time interval, it is unlikely they will relapse within this time. However, it must be

pointed out that there are 6 patients with a PFS of >3, or >4, and so they have 5-6 months in which to relapse to be considered platinum resistant. So this means no firm conclusions can be drawn from these 6 patients. However, overall it seems as though there is a good correlation between low levels of ICL repair and sensitivity to platinum based chemotherapy in newly diagnosed patients with ovarian cancer.

Another important practical aspect of deciding which patient is put into either platinum sensitive or resistant groups, is the timing of particular blood tests and scans e.g. if a patient has been free of recurrent disease for 6 months since starting chemotherapy (PFS>6), and they are reviewed in clinic in a further 3 months at which time recurrence is detected, their PFS is 9. This means they are in the platinum sensitive group (only just), but their disease recurrence may have been apparent before this if the patient came to the clinic sooner and reported symptoms, and/or blood tests had been done sooner, which would have put them into the platinum resistant group.

It may be that if a newly diagnosed patient has high levels of ICL repair (above 20%) this could be used to predict inherent resistance to carboplatin chemotherapy. However there would still be a few patients that would not be identified as platinum resistant using this method. Measuring ICL repair could be used in conjunction with other methods currently being developed to determine outcome to platinum chemotherapy e.g. microarray profiling. If outcome to platinum chemotherapy can be pre-determined, it would prevent unnecessary treatment and serious side effects for some patients.

In the post-chemotherapy group patient 35, 37, 44, and 47 have low percent repairs at 24 hours of -4%, 6%, -28%, and -35% respectively. This is in comparison to the mean for this

group of 45%. The clinical data for these patients will now be compared to see if there are any correlations. Patient 44 has a PFS >8 which means that this patient has no evidence of disease recurrence at this time, and is therefore likely to fit into the platinum sensitive group. This is consistent with this patients' low levels of ICL repair. Patient 47 has a PFI of 16 months, and a PFS >6 months which means that this patient is in the platinum sensitive group (their PFI is 16 and therefore >6 months). This is also consistent with low levels of ICL repair in the ovarian cancer cells.

Of the remaining ten treated patients, the majority of them have a high level of repair which is consistent with their PFI of <6, as they are clinically resistant to platinum chemotherapy. There are four patients (1B, 27B, 28, 38B), who all have high PFS, which correlates with clinical sensitivity to platinum chemotherapy, but have all got high levels of ICL repair. However, on closer inspection, two of these patients (patients 27B and 28) have levels of ICL repair less than 50%. This could mean that the cut-off for the prediction of resistance to platinum chemotherapy could be an ICL repair level above 50% at 24 hours.

Overall what is striking is the marked difference in ICL repair in the six paired patient samples taken before and after chemotherapy (figure 29). Initially all six patients have very little capacity for repair of ICLs, but after undergoing chemotherapy the cells were able to repair ICLs. Some of these samples (patients 1, 27, and 38) taken after chemotherapy were after as little as three doses as they were taken at interval debulking surgery. One possible explanation is that these tumour cells aquired this ability within a short time, but a more plausible explanation is that platinm-based chemotherapy killed the sensitive cells, and so those that were left behind were resistant. When ovarian cancer cells were taken at interval

debulking surgery these resistant populations of cells were taken and shown to be resistant at the cellular level by repair of ICLs. There are probably other mechanisms present within these cells that also confer resistance as resistance is likely to be multifactorial.

3.4 Human ovarian cancer cell lines treated *in vitro* with melphalan

3.4.1 Measurement of ICL formation and repair in paired human ovarian cancer cell lines treated with melphalan using the comet assay

Having established the formation and repair of ICLs produced by cisplatin chemotherapy in resistant patients with ovarian cancer, other crosslinking agents were investigated for cross-resistance, to see if increased repair of ICLs was relevant to other crosslinking agents, such as melphalan. The A2780 and A2780cisR paired human ovarian cancer cell line was used initially as the *in vitro* model.

A2780cisR resistance to melphalan chemotherapy was demonstrated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The IC_{50} is the concentration of the drug that causes 50% cell death. MTT assays to determine the cisplatin IC_{50} for the A2780 and A2780cisR cell lines following 1 hour exposure to melphalan were performed (figure 30a).

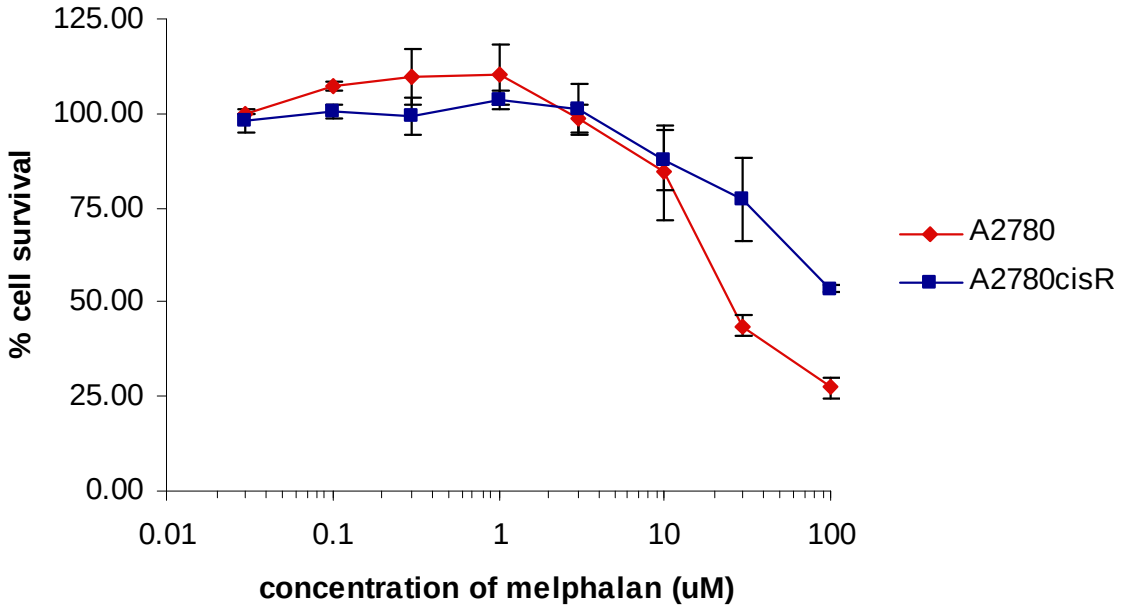


Figure 30a: Percentage cell survival of A2780 and A2780cisR cell lines after treatment with melphalan, as measured by the MTT assay (A2780 n=2, A2780cisR n=3, standard deviation bars are shown) (unpublished data H.Lowe 2006).

The melphalan IC_{50} for A2780 cell line was 26μM, and for the A2780cisR cell line was 100μM, which resulted in a 4-fold difference between their sensitivities to melphalan.

Melphalan is another chemotherapy agent that causes formation of ICLs. There is very little known about the formation and repair of melphalan induced crosslinks in ovarian cancer cells obtained from patients.

A2780 and A2780cisR ovarian cancer cells were incubated with melphalan for 1 hour (similar to cisplatin), and the cells analysed after varying amounts of time to establish the peak of ICL formation. The peak of ICL formation was found to be at 16 hours post melphalan incubation. Therefore the time points used in this experiment were 16 hours,

and 40 hours which is 24 hours after the peak of ICL formation to investigate the level of ICL repair.

The concentration of melphalan that gave 50% decrease in tail moment at the peak of ICL formation was also investigated. Too high a concentration would kill the cells, and too low a concentration of melphalan would result in not enough ICL formatio to enable measurement. The optimum concentration of melphalan was found to be 50µM.

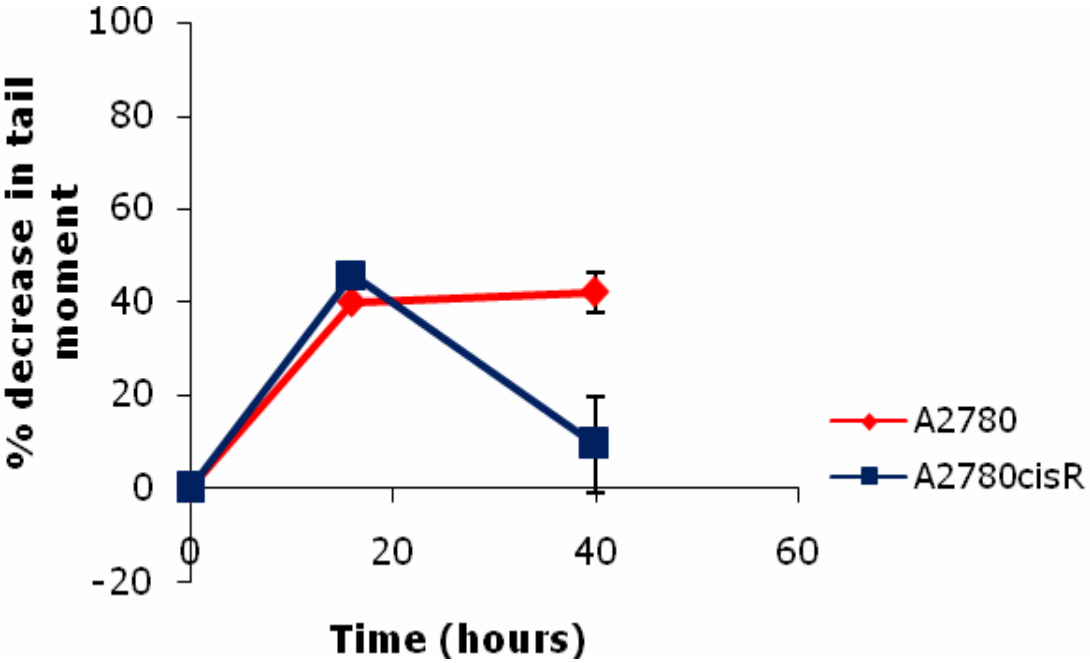


Figure 31: A time course over 40 hours to show the change in percent decrease in tail moment over time in A2780 and A2780cisR cell lines after treatment with melphalan (n=3, standard error bars are shown)

The level of ICL formation as measured by the percent decrease in tail moment at 16 hours for the A2780 and A2780cisR cell lines are similar at 40%±1 and 46%±0, respectively (figure 31). There was no significant difference in these values using students paired t-test ($p<0.40$). The peak of ICL formation is very similar in both cell lines, suggesting similar amounts of melphalan reaches the DNA in both cell lines, to produce similar levels of DNA damage in the form of ICLs.

After 24 hours the percent decrease in tail moment in the A2780cisR cell line decreases to 10%±18, while the A2780 cell line maintains a level of 42%±7. This was not significantly different using students paired t-test $p<0.10$, because significance is usually taken if $p<0.05$. The lack of statistical significance was probably because the sample size was too small. This illustrates the persistence of ICLs in the A2780 cell line, and the unhooking (or repair) of ICLs in the A2780cisR cell line over 40 hours.

3.5 Clinical ovarian cancer tissue treated *ex vivo* with melphalan

3.5.1 Measurement of ICL formation and repair in ovarian cancer cells obtained from patient ascites and solid tumour when treated with melphalan *ex vivo.*

As the peak of ICL formation was confirmed to be 16 hours in A2780 and A2780cisR cell lines, this time point was used in the clinical samples. ICL formation and repair were measured in clinical samples at 16 hours and 40 hours following a 1 hour treatment of 50μM of melphalan.

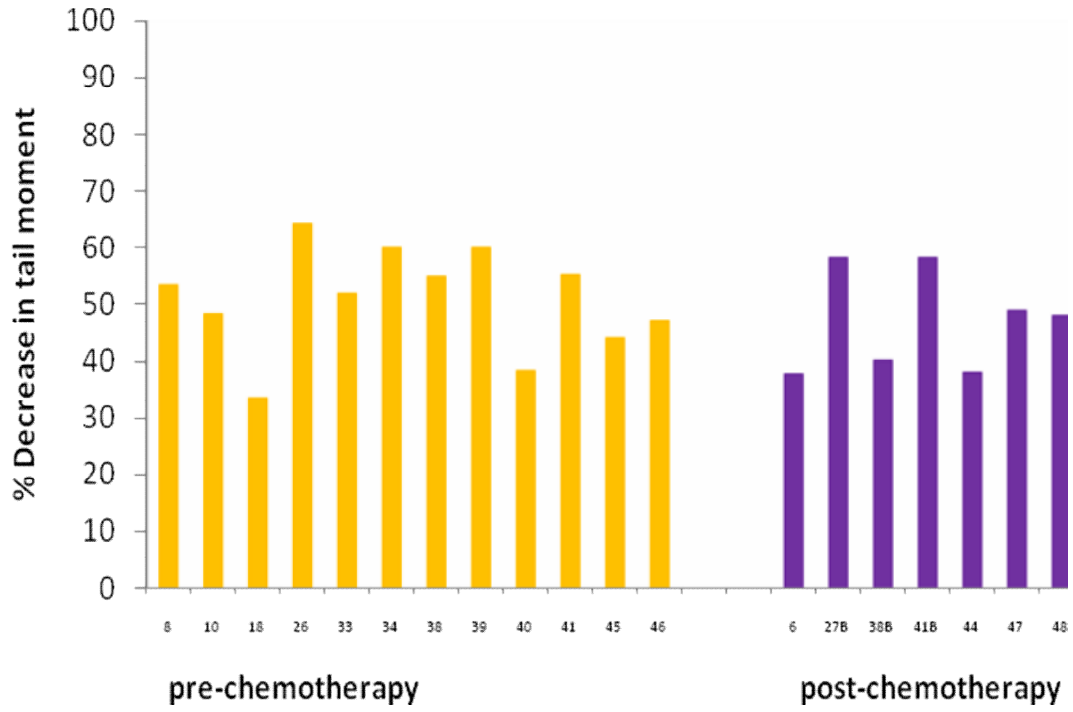


Figure 32: Percent decrease in tail moment at 16 hours in pre- and post-chemotherapy patients treated *ex vivo* with 50μM melphalan.

In 12 pre-chemotherapy patients the average percent decrease in tail moment at 16 hours was 49%± 12, and for 7 post-chemotherapy patients was 45%± 8 (figure 32 and figure 33). These values are statistically similar, using students paired t-test ($p<0.65$), which suggests there is no difference in the level of ICL formation at 16 hours in these two groups of cells.

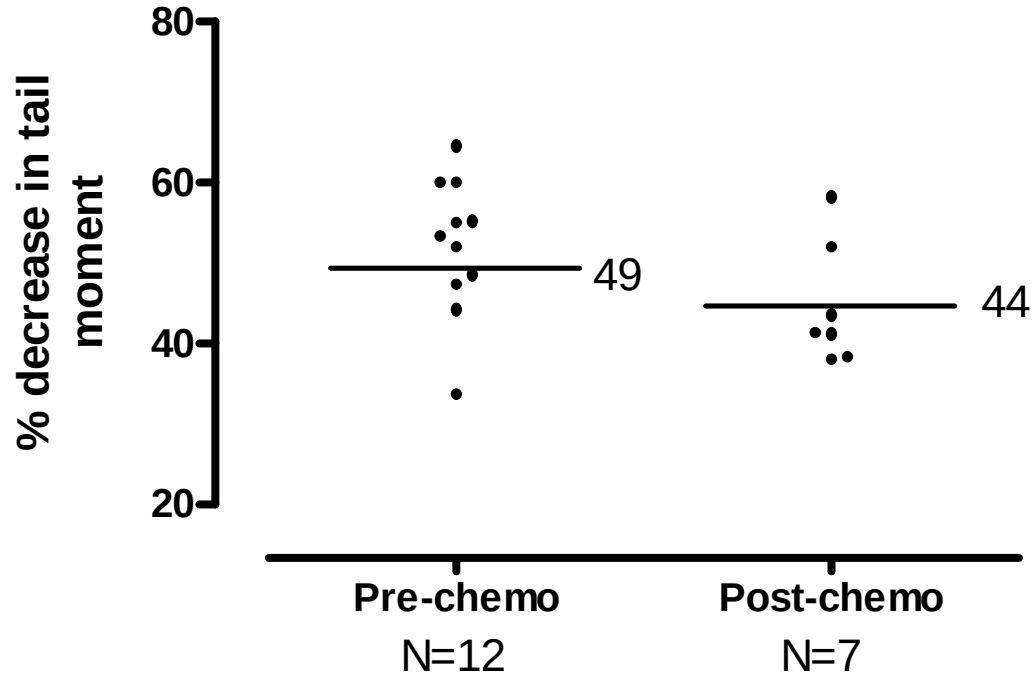


Figure 33: Percent decrease in tail moment at 16 hours in pre- and post-chemotherapy patients treated *ex vivo* with 50μM melphalan. Horizontal bars indicate mean of each group.

In the pre-chemotherapy patient group, samples ranged from 30-64% decrease in tail moment, with the majority between 44-64% decrease in tail moment. The post chemotherapy group of patients had a similarly narrow range of results between 38-58% decrease in tail moment (figure 33). There was no significant difference in these values using students paired t-test ($p<0.40$). The similar levels of ICL formation present in both these patient groups illustrates that similar amounts of melphalan reaches the DNA of the cells to produce similar levels of ICLs.

After the peak of ICL formation had been established, the percent repair was measured at 40 hours (figure 34 and 35).

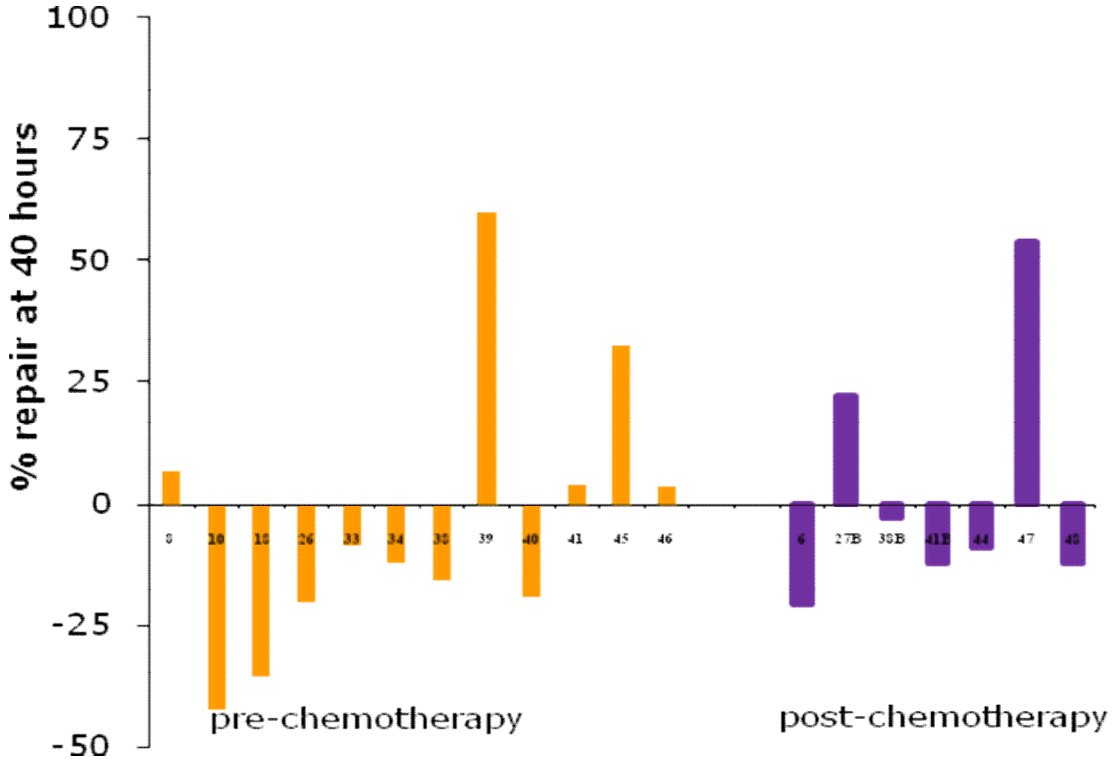


Figure 34: Percent repair at 40 hours in pre- and post-chemotherapy patients treated with melphalan.

Figure 34 and 35 show the percent repair at 40 hours in both patient populations. In general, both pre- and post-chemotherapy patients have low levels of melphalan ICL repair. The mean was 4%± 28 and 3%± 26 for the pre- and post-chemotherapy patients respectively, which is very similar. There was no significant difference in these values using students paired t-test ($p<0.40$)

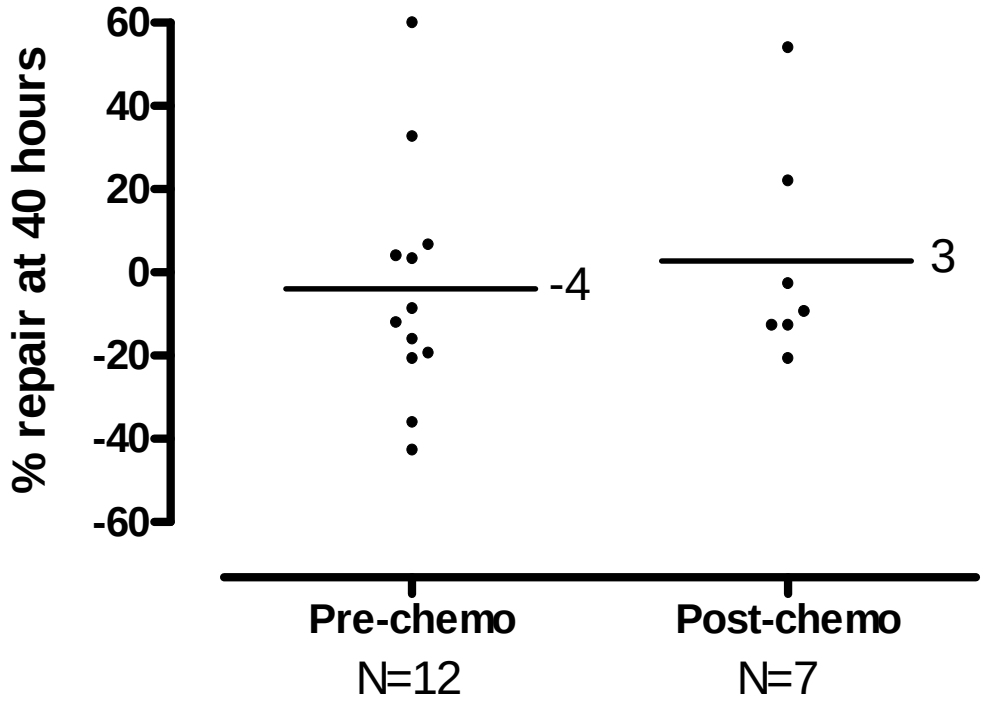


Figure 35: percent repair at 40 hours in pre- and post-chemotherapy patients treated with melphalan. Horizontal lines indicate mean of each group.

In some pre-chemotherapy patients the number of ICLs present actually increased at 40 hours, which is why the percent repair figure is negative. This occurred in twelve patient samples, both before and after chemotherapy. Overall both groups of patients show very little repair of the ICLs at 40 hours. There are, however exceptions to this in both groups, which can be seen from the distribution in figure 35. Patient 39, and 45, who are from the pre-chemotherapy group, repair 60% and 33% of the ICLs that are present, respectively, whilst the mean for this group is -4%. Patients 27B and 47 from the post-chemotherapy group, repair 22% and 54% of their ICLs respectively, whilst the mean for this group is 3%.

Interestingly, patients 39 and 45 have high levels of melphalan ICL repair – 60% and 33%, respectively (figure 34), but they have very low levels of cisplatin ICL repair – 4% and 6%, respectively (figure 27). This supports the theory that differect mechanisms of ICL repair exist.

There are two patients in which, a sample was obtained before and after they had received platinum-based chemotherapy from the data set in fugure 23 and 24. These patients are numbers 38 (and 38B) and 41 (and 41B). Their percent decrease in tail moment at 16 hours is 55% and 64% for patients 38 and 41, respectively, and their percent repair at 40 hours is -16% and 4%, respectively. Both these samples from pateints post-chemotherapy show very little repair of the ICLs after 40 hours.

Melphalan is not currently given to patients with ovarian cancer. As melphalan and cisplatin both cause ICLs (which is their main cytotoxic lesion), it was hypothesised that patients that are clinically sensitive to platinum chemotherapy (and therefore do not repair cisplatin induced ICLs), would also be clinically sensitive to melphalan if the drug was given to them, and therefore not repair the ICLs induced by melphalan. It is also hypothesised that the reverse would also be true, i.e. those patients that are clinically resistant to platinum chemotherapy, would also be clinically resistant to melphalan, and therefore repair ICLs induced by either cisplatin or melphalan. However, our data suggest that this hypothesis is wrong (figure 34 and 35).

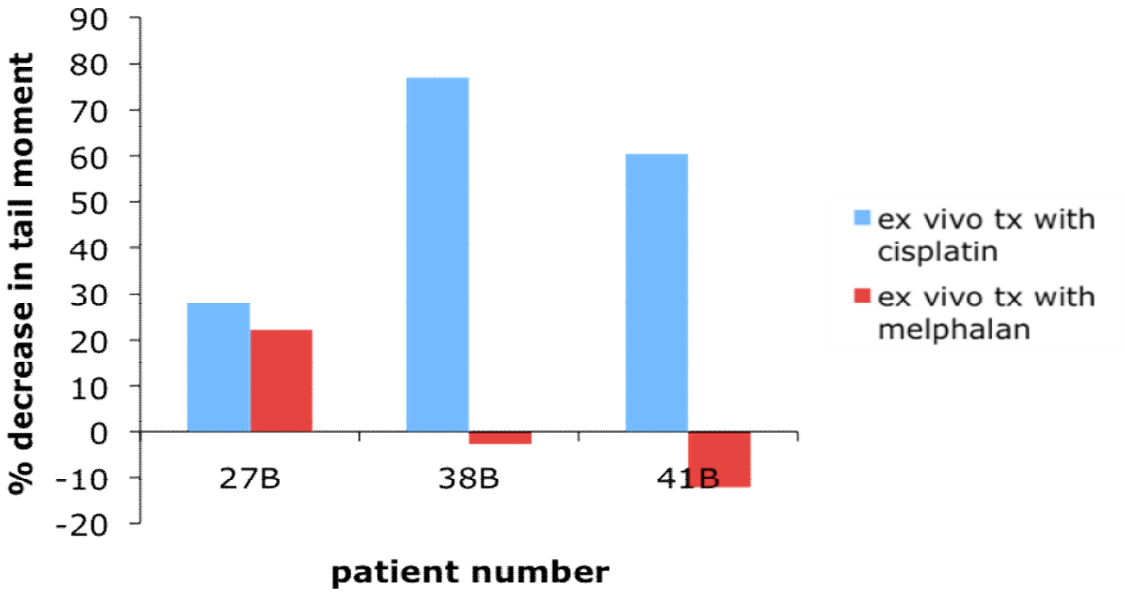


Figure 36: Percent repair in post-chemotherapy patients after treatment *ex vivo* with either cisplatin or melphalan

Post-chemotherapy patient ascites samples have been treated *ex vivo* with either 100μM cisplatin, or 50μM melphalan, and the percent repair has been measured at 24 hours for cisplatin, and 40 hours for melphalan (figure 36). There are high levels of repair in the cells treated *ex vivo* with cisplatin ranging from 28% to 77% repair with a mean of 55%±25. However, ICL repair in the same cells after treatment *ex vivo* with melphalan is much lower (mean 2± 18). This is statistically significant using students paired t-test ($p<0.05$). This means that not only is the ICL repair mechanism different in cells treated *ex vivo* with cisplatin or melphalan, but that these cells may be sensitive to melphalan chemotherapy.

This has implications for the clinical setting and suggests that melphalan could be used to treat these patients.

3.5.2 Discussion

The results from the MTT assay found the A2780cisR human cancer cell line had a 4-fold increase in resistance to melphalan compared to the A2780 human cancer cell line (figure 30a). This demonstrates that there is cross resistance of the A2780cisR cell line to melphalan as well as cisplatin.

In figure 31 the A2780 and A2780cisR cell lines have a very similar percent decrease in tail moment at 16 hours after incubation with melphalan, indicating they have similar levels of ICL formation. However at 40 hours (24 hours after the peak of ICL formation), the A2780 cell lines show little evidence of repair, while the A2780cisR cell lines repair (unhook) the ICLs, indicating resistance to melphalan. This has not been shown before.

Roy *et al* (2000) used A2780 and A2780 (100) human ovarian cancer cells that were selected by exposure to high levels of the nitrogen mustard chlorambucil. They demonstrated a 5 to 10-fold increase in resistance to nitrogen mustards with A2780(100) compared to A2780 ovarian cancer cell lines. However Roy *et al* showed that there was no difference in the level of DNA repair proteins found in these cells, but instead, the increased resistance of the A2780(100) cell line was due to the 4 to 8-fold increase of anti-apoptotic proteins such as Bcl-XL and Mcl-1 (Roy *et al*., 2000). This mechanism of resistance appears to be different to the increase in ICL repair which was demonstrated in the A2780cisR cell line in figure 31. However, the cell lines that were used in their

experiments were different as the resistant lines had been selected using chlorambucil instead of cisplatin.

The results found in the A2780 and A2780cisR cell lines after incubation with melphalan, are very similar to that obtained when the same cell lines were incubated with cisplatin. It appears the A2780cisR cell line repairs the crosslinks produced by both cisplatin and melphalan, suggesting there is cross-resistance between the two drugs. However, resistant mechanisms to melphalan *in vitro* can differ from cisplatin e.g drug transport, drug efflux, or detoxification mechanisms using glutathione (Ozols *et al.*, 1992).

Ovarian cancer cells obtained before and after chemotherapy, and then incubated with melphalan show similar levels of ICL formation (figure 32). This implies that cellular metabolism of melphalan, influx/efflux, and other 'upstream' mechanisms do not play important roles in the potential resistance to melphalan in the clinical setting. This suggests that the same concentration of melphalan interacts with the DNA as the same levels of ICL formation are produced. It also shows that melphalan, like cisplatin, can cause ICL formation in these cells.

Overall, newly diagnosed, and treated patients show very little repair of melphalan induced ICLs (figure 34). This implies that these cells are not resistant to melphalan through an increase in ICL repair. The levels of ICLs that are produced by melphalan appear to be maintained in the cells over 40 hours, and as these are the main cytotoxic lesions, it follows that these cells should be sensitive to melphalan. These data also imply that most of the ovarian cancer cells obtained from newly diagnosed patients are not intrinsically resistant to melphalan chemotherapy.

Ovarian cancer cells from post-chemotherapy patients had very different levels of ICL repair following melphalan or cisplatin exposure. Those cells incubated *ex vivo* with melphalan demonstrated very little repair, while those incubated *ex vivo* with cisplatin had high levels of repair. This suggests that ovarian cancer cells obtained from post-chemotherapy patients should be resistant to cisplatin, but sensitive to melphalan chemotherapy, which could have implications in clinical practice.

There are a few patients that do not fit the overall trend of low levels of ICL repair after treatment *ex vivo* with melphalan, as they actually show high levels of ICL repair at 40 hours. These are newly diagnosed patients 39 and 45, and treated patients 27B and 47.

Patients 27B and 47 both have high levels of melphalan induced ICL repair, but have low levels of repair of cisplatin-induced ICLs (compared to their treated patient group) which supports the possibility of two distinct mechanisms to repair (unhook) cisplatin and melphalan induced ICLs. As for patient 39, patient 45 can repair ICLs induced by melphalan, but not ICLs induced by cisplatin. Both these patients again support the hypothesis that the repair (or unhooking) of ICLs differs depending on the drug that caused their formation. However there are lots of other possible resistance mechanisms, and the comet assay just looks at one aspect, which is repair of ICLs. So it is still possible these patients could be resistant to melphalan, but via a different mechanism(s). These mechanisms (if they exist) are unlikely to include drug influx/efflux or other drug detoxification mechanisms because there are the same levels of ICLs present at 16 hours in newly diagnosed and treated patients, suggesting that the same concentration of melphalan encounters the DNA to produce ICLs.

There are also conflicting reports of patients' response to melphalan in the clinical trial setting. There have been two trials, one by Magagnoli *et al*., (2004), and the other by Hasan et al., (2003) that have given melphalan to platinum resistant patients with advanced disease (FIGO stages III-IV). Magagnoli *et al*., (2004) gave double intermediate-dose melphalan (100mg/m^2) supported by autologous stem cells in 14 patients with refractory ovarian cancer and poor performance status, and Hasan et al., (2003) gave 10mg of oral melphalan once a day for 5 days every 6 weeks for 6 cycles in patients with platinum-resistant relapsed ovarian cancer. These studies report conflicting data. The regimen by Magagnoli *et al*., (2004) was well tolerated, and converted one patient from partial remission (PR) to complete remission (CR). Another four patients had disease stabilisation, while nine patients with progressive disease (PD) showed a partial response. Interestingly all patients had a marked improvement in their performance status. The retrospective study by Hasan *et al* (2003) showed melphalan was well tolerated, but there were no responses in these 22 patients. Melphalan had no impact on progression-free survival or overall survival.

In the analysis undertaken by Hasan *et al* (2003), it is unclear why nearly all of the patients did not continue the treatment. This may have a major impact on the poor responses that were seen. Also, each trial had a different dosage regimen of melphalan, which could have a major impact on patient response. It appears from the trial by Magagnoli *et al* (2004), that high dose melphalan could be used in the clinical setting once it has undergone further evaluation. This is supported by data in figure 36 in which clinically platinum resistant patients that repair their ICLs after treatment *ex vivo* with cisplatin, do not appear to repair the ICLs caused by treating ovarian cancer cells from the same patient with *ex vivo* melphalan.

3.6 Gene expression Studies

3.6.1 Comparison of gene expression between newly diagnosed and treated patients, and between A2780 and A2780cisR human ovarian cancer cell lines, using microarrays

It has been demonstrated that most ovarian cancer cells obtained from post-chemotherapy patients repaired cisplatin-induced ICLs, more efficiently than the ovarian cancer cells from pre-chemotherapy patients. The gene expression profiles for each patient population were investigated to determine if specific differences in gene expression could account for this difference in their phenotypes. It is also evident that A2780cisR cells repair cisplatin-induced ICLs which may play an important role in their resistance to platinum chemotherapy, whereas A2780 cells do not repair cisplatin-induced ICLs. Gene expression profiles were also investigated in the A2780 and A2780cisR paired human ovarian cancer cell line to determine if differences in gene expression could account for their difference in ICL repair.

The Microarray technology used oligonucleotide arrays to analyse 30,000 genes (from the human genome) from a single sample, and was used to compare gene expression between pre- and post-chemotherapy patients. A single microarray chip contains thousands of single-stranded oligonucleotide sequences that are complementary to target sequences, which are bound onto a glass support about the size of a microscope slide. Fluorescent dyes were used to label the mRNAs from the cell sample to be analysed, and hybridised with the oligonucleotide array. The array was then washed to remove any non-specific

hybrids. A laser then excites the attached fluorescent dyes to produce light which is detected by a (confocal) scanner which generates a digital image. The digital image is further processed by specialized software to transform the image of each spot to a numerical reading. This process is performed, first, finding the specific location and shape of each spot, followed by the integration (summation) of intensities inside the defined spot, and, finally, estimating the surrounding background noise. Background noise generally is subtracted from the integrated signal. This final reading is an integer value assumed to be proportional to the concentration of the target sequence in the sample to which the probe in the spot is directed (Trevino *et al.*, 2007). A statistical test is used to assess each gene to determine whether the expression is statistically different between the two groups of samples.

The microarray data was analysed by Stephen Henderson at the Wolfson Institute for biomedical research, University College London using the following protocol:

Data analysis was carried out using the R statistical environment and programming language (Venables *et al*., 2002) and packages from the Bioconductor open source bioinformatics project (Gentleman *et al*., 2004). The affymetrix package and specifically the 'rma' algorithm for pre-processing (background correction), normalisation and calculation of expression values were used (Irizarry *et al*., 2003). To determine differential expression between groups of genes in pre-chemotherapy and post-chemotherapy patient samples, the ‘limma’ package was used. This method is a simple extension of standard linear models that uses a moderated estimate of the standard deviation modeled on the overall mean-variance characteristics of the data (Smyth *et al*., 2004). In essence a moderated t-test for paired data was used.

The first step to produce a sample for microarray analysis is to extract good quality total RNA from ovarian cancer cells from patient samples. It was very difficult to establish an effective protocol to extract good quality total RNA in the clinical setting. It was found that ovarian solid tumour tissue needed to be frozen in liquid nitrogen within 5 minutes of being removed from the patient otherwise all the RNA would degrade and become unusable. This meant that a flask of liquid nitrogen was taken into the theatre at the start of the operation, in readiness for the surgeon to remove the ovarian tissue. However, this meant that patients 1, 1B, and 2 had no usable total RNA extracted from the solid tumour samples, as it was unknown at this stage how important it was to freeze the samples in liquid nitrogen within 5 minutes. It was easier to extract total RNA from ovarian cancer cells from ascites, as it was less likely the RNA would degrade, because the cells were cultured and grown in the laboratory.

Altogether 17 patients had good quality total RNA extracted from samples as determined by gel electrophoresis (figure 37), and by analysis of nucleic acid content using the nanodrop machine where only samples with a ratio of 1.8-2.05 were used. Of these 17 patient samples, 4 patients had paired samples (taken before and after chemotherapy), and 1 patient had both ascites and solid tumour microarray samples.

Pt 33 pt 37 pt 41

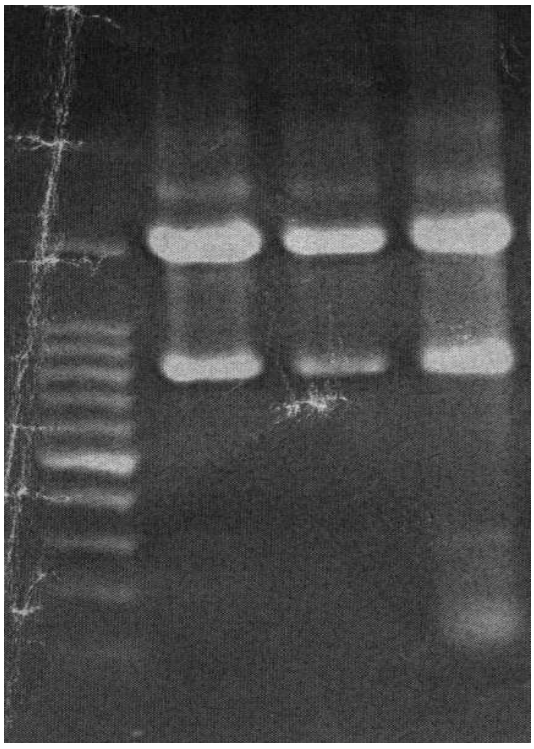


Figure 37: A typical gel produced after gel electrophoresis on total RNA extracted from 3 patients. All these samples are extracted from ascites.

Microarray experiments were conducted using patients numbered 21, 24, 25, and 26 but the quality of these results were very poor, because the fragmented cRNA had degraded. Therefore these results are not included. In total there are 12 samples from patients before chemotherapy, 6 from patients after chemotherapy. Of these samples 4 patients had a sample taken before and after chemotherapy (paired samples).

Heat map 1 (figure 38) shows the difference in gene expression in pre- and post-chemotherapy samples taken from the same patient, in 4 patients. The gene expression in samples from post-chemotherapy patients, are used as a baseline to which gene expression from pre-chemotherapy patients are compared. This subset of patients is shown separately,

as within this group of patients are statistically significant different levels of gene expression. The red colour denotes an increase in gene expression and blue denotes a decrease in gene expression. White means there is not a decrease or an increase in gene expression. The letter B indicates a post-chemotherapy sample, and 1 or 2 denotes repeated microarray experiment using the same sample, but processed independently. Figure 39 and 40 show the names of the differentially expressed genes found, and the statistical analysis.

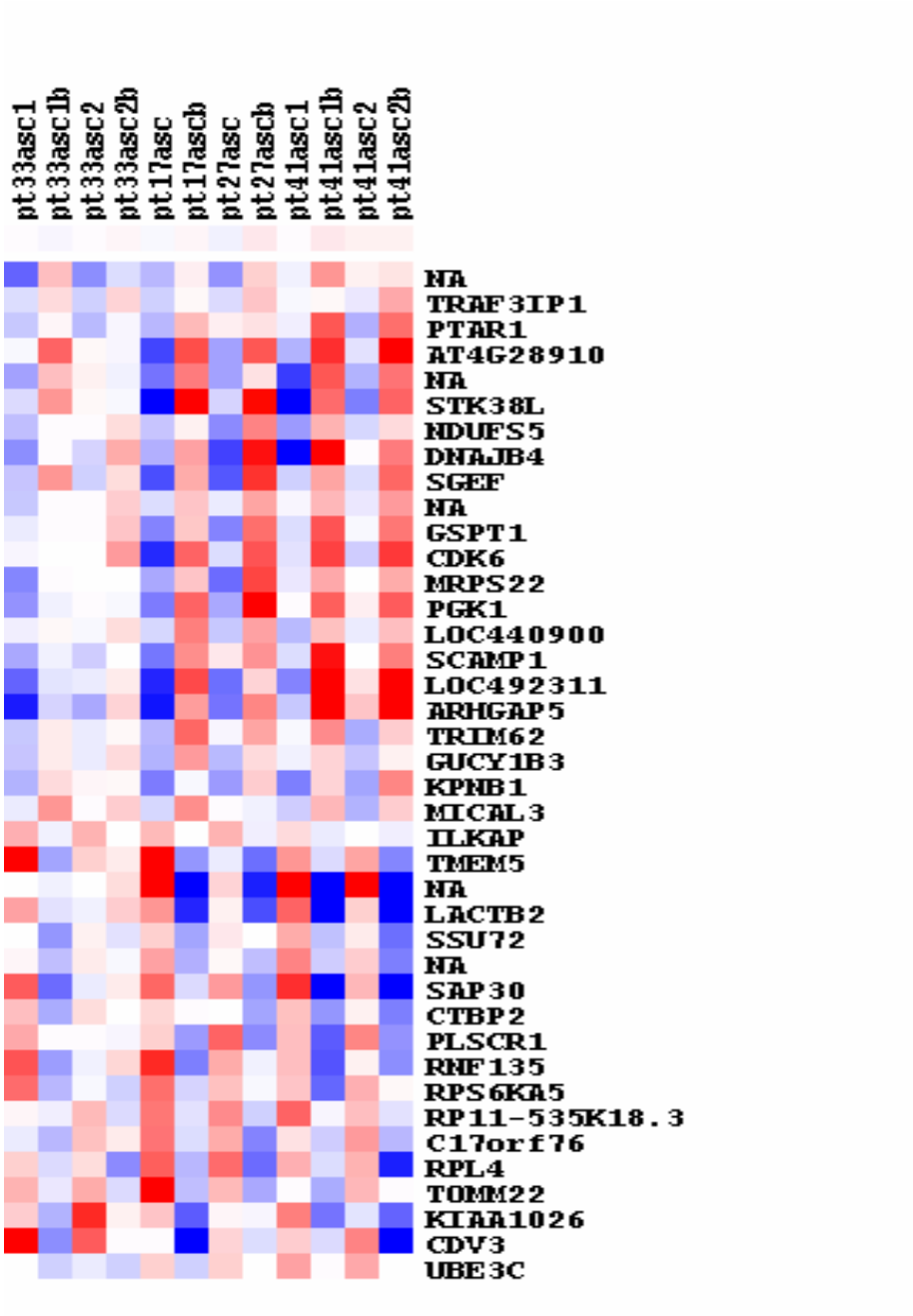


Figure 38: Heat map 1 shows the difference in gene expression in 4 paired patient ascites samples. Gene abbreviations are shown down the right hand side, and patient sample numbers are across the top (asc. is ascites, pt is patient)

Genes upregulated in post-chemotherapy samples:

symbol	Gene name	M value	A value	t value	p value
TRAF3IP1	TNF receptor-associated factor 3 interacting protein 1	-0.3219	3.7635	-4.0572	0.0017491
PTAR1	Protein prenyltransferase alpha subunit repeat containing 1	-0.4798	3.4530	-4.2153	0.0013298
AT4G28910	N/A	-0.9991	3.6493	-4.4545	0.0008833
STK38L	Serine/threonine kinase like	-1.0745	6.6846	-4.6433	0.0006430
NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5	-0.4683	11.0169	-3.9615	0.0020678
DNAJB4	DnaJ (heat shock protein 4) homolog, subfamily B, member 4	-1.0322	6.4489	-4.8552	0.0004529
SGEF	Src homology 3 domain-containing guanine nucleotide exchange factor	-0.8252	3.903	-5.966	0.0000798
GSPT1	G1 to S phase transition 1	-0.5990	3.0582	-4.3073	0.0011351
CDK6	Cyclin dependent kinase 6	-0.7719	8.3511	-4.7224	0.0005636
MRPS22	Mitochondrial ribosomal protein s22	-0.5480	4.8587	-3.8699	0.0024293
PGK1	Phosphoglycerate kinase	-0.6810	3.4497	-4.4319	0.0009180
LOC440900	N/A	-0.4331	3.4623	-4.3742	0.0010126
SCAMP1	Secretory carrier membrane protein 1	-0.5792	6.6094	-4.7199	0.0005661
LOC492311	N/A	-1.0677	4.7226	-4.1283	0.0015457
ARHGAP5	Rho GTPase activating protein 5	-0.9813	4.521	-5.976	0.0000787
TRIM62	Tripartite motif-containing 62	0.4888	5.5970	-4.2370	0.0012810
GUCY1B3	Guanylate cyclase 1, soluble, beta 3	-0.3951	3.2064	-4.3878	0.0009893
KPNB1	Karyopherin (importin) beta 1	-0.5415	7.7464	-4.3336	0.0010853
MICAL3	Microtubule associated monoxygenase, calponin and LIM domain containing 3	-0.4041	3.8238	-4.0189	0.0018701

Figure 39: Statistical analysis of the genes shown in heat map 1 (figure 38), that are upregulated in post-chemotherapy samples. M is the log difference between the two groups. A is the average expression. A t value an estimation of a coefficient, the t-statistic for that coefficient is the ratio of the coefficient to its standard error. That can be tested

against a t distribution to determine how probable it is that the true value of the coefficient is really zero.

Genes down-regulated in post-chemotherapy samples:

symbol	Gene name	M value	A value	t value	p value
ILKAP	Integrin –linked kinase-associated serine-threonine phosphatase 2C	0.2912	2.7734	3.9893	0.0019694
TMEM5	Transmembrane protein 5	0.8255	9.1113	4.3747	0.0010118
LACTB2	Lactamase beta 2	0.9287	7.2117	4.0665	0.0017212
SSU72	RNA polymerase II CTD phosphatase homolog (yeast)	0.4407	4.4793	3.8891	0.0023485
SAP30	Sin-3-associated polypeptide, 30kDa	1.1544	6.9173	4.2875	0.0011745
CTBP2	C-terminal binding protein 2	0.4470	9.4976	4.4742	0.0008544
PLSCR1	Phospholipid scramblase 1	0.6761	8.1481	4.6702	0.0006149
RNF135	Ring finger protein 135	0.6891	7.9558	3.9057	0.0022807
RPS6KA5	Ribosomal protein s6 kinase	0.5550	3.7897	3.9339	0.0021704
RP11	Retinitis pigmentosa 11	0.5101	6.8815	5.161	0.0002758
C17orf76	Chromosome 17 open reading frame 45	0.5128	3.7123	3.8603	0.0024709
RPL4	Mitogen-activated protein kinase 13/ ribosomal protein 4	0.8080	9.127	5.638	0.0001308
TOMM22	Translocase of outer mitochondrial membrane 22 homolog (yeast)	0.6196	6.3192	3.9544	0.0020937
KIAA1026	Kazrin	0.6559	7.7342	3.9396	0.0021490
CDV3	CDV3 homolog (mouse)	0.8663	9.2605	4.1380	0.0015200
UBE3C	Ubiquitin protein ligase E3C	0.2892	2.6860	4.0613	0.0017368

Figure 40: Statistical analysis of the genes shown in heat map 1 (figure 38), that are downregulated in post-chemotherapy samples. M is the log difference between the two groups. A is the average expression. T-value is an estimation of a coefficient, the t-statistic for that coefficient is the ratio of the coefficient to its standard error. That can be tested against a t distribution to determine how probable it is that the true value of the coefficient is really zero.

Heat map 1 is divided into two with the top half indicating genes that are overexpressed in post-chemotherapy samples, and the bottom half indicates genes that are underexpressed in post-chemotherapy samples.

Genes that are most upregulated in the post-chemotherapy samples (i.e show a more negative M value in figure 39, and show a darker red colur in the heat map) include STK38L (Serine/threonine kinase like), DNAJB4 (DnaJ (heat shock protein 4) homolog, subfamily B, member 4), SGEF (Src homology 3 domain-containing guanine nucleotide exchange factor), CDK6 (cyclin-dependent kinase 6), and ARHGAP5 (Rho GTPase activating protein 5). The probe sites for these genes are blue/white for low expression in the pre-chemotherapy samples and red for over expression in the post chemotherapy samples (the darker the colours, the greater the difference in gene expression). The colours alternate from blue/white to red as the heat map is read from left to right as the pre-chemotherapy sample is next to its corresponding post-chemotherapy sample taken from the same patient. These genes are clearly consistently expressed at a higher level in the post-chemotherapy samples as the colours on the heatmap demonstrate.

Genes that are most down regulated in post-chemotherapy samples (i.e show a more +ve M value in figure 40, and a darker blue colour on the heatmap) include LACTB2 (Lactamase beta 2), SAP30 (Sin-3-associated polypeptide, 30kDa), and RNF135 (Ring finger protein 135). The probe sites for these genes are red/white for high expression in the pre-chemotherapy samples, and blue for low expression in the post-chemotherapy samples. These genes are clearly consistently expressed at a lower level in the post-chemotherapy samples as the colours on the heatmap demonstrate. However, it should be noted that

patient 33 ascites tumour (pt 33 asc.tu), and its corresponding post-chemotherapy sample (pt 33 asc.tu b) have overall lighter colours on heatmap 1. This means the genes are not as highly or lowly expressed compared to other patient samples e,g patient 41. However, the results still fit into the overall pattern of gene expression.

Comparing gene expression levels between all pre- and post-chemotherapy patient samples yielded results that were not statistically significant, and could have occurred by chance. Instead, the panel of differentially expressed genes that were identified in the paired patient data set were looked at, to see if the same genes were up- or down regulated in these samples also. The gene expression profiles in ovarian cancer cells from post-chemotherapy patients were used as a baseline (samples; pt 06 asc. tu., pt 37 asc. tu., pt 33B asc. tu, pt 17B asc. tu., pt 27B asc.tu, pt 41B asc. tu.) to which the gene expression of pre-chemotherapy patients, were compared (figure 41). The red colour denotes an increase in gene expression and blue denotes a decrease in gene expression. White means there is not a decrease or an increase in gene expression.

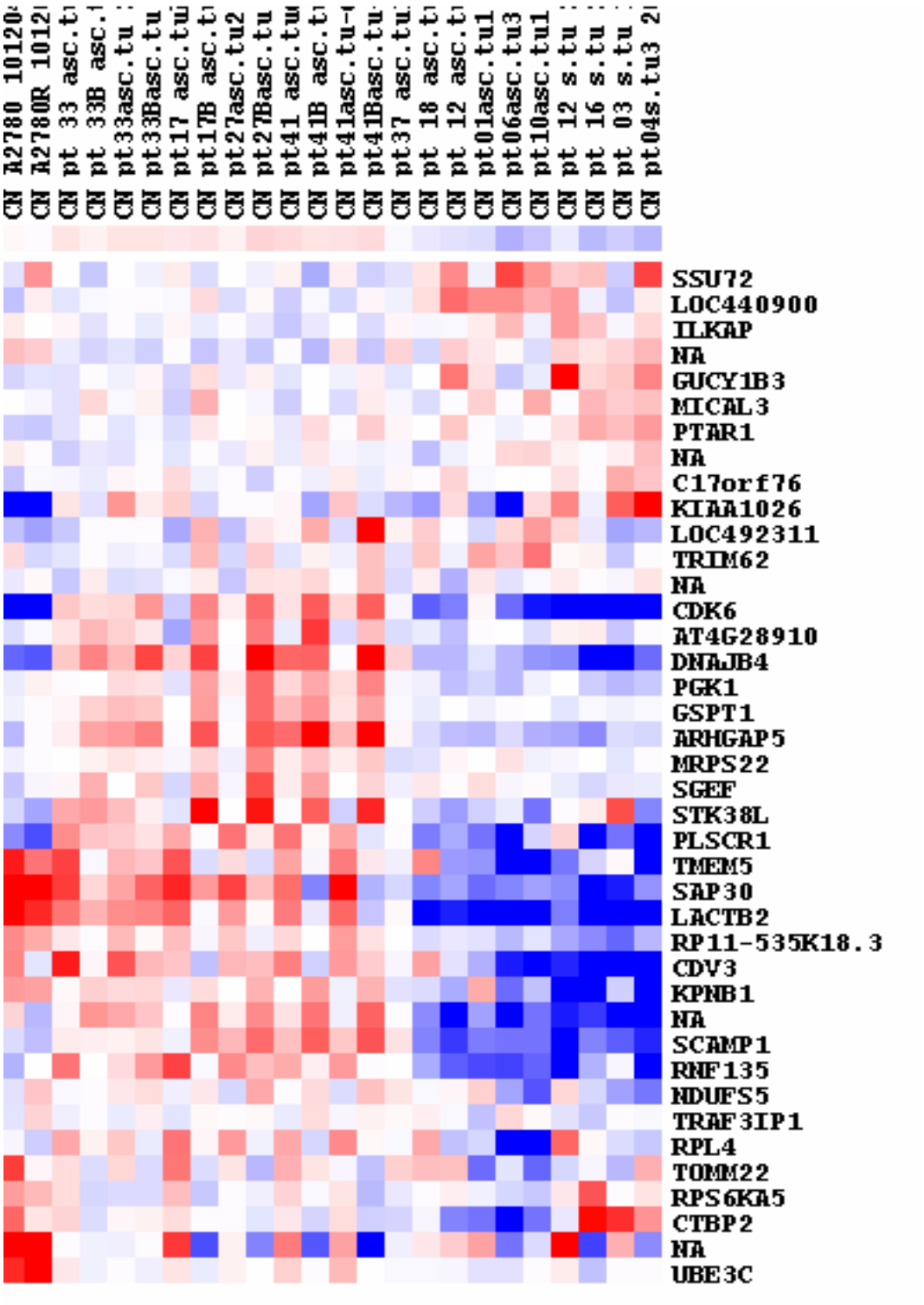


Figure 41: heat map 2 shows the gene expression profiles of cell lines, and all patient samples. Gene abbreviations are shown down the right hand side, and patient sample numbers are across the top (asc.tu is a sample from ascites, s.tu is a sample from solid tumour)

Heat map 2 (figure 41) shows the difference in gene expression in 14 patient samples. There are 6 patients that have post-chemotherapy samples, and they are numbers 6 and 37, as well as patients 17, 27, 33, and 41 that have paired samples (taken before and after chemotherapy). All the others are pre-chemotherapy samples (patient numbers 1, 3, 4, 10, 12, 16, 18 as well as patients 17, 27, 33 and 41 that have paired samples taken before and after chemotherapy). Most these samples are from ascites, but patient 3, 4, 12, and 16 are all from pre-chemotherapy solid ovarian tumour.

In heatmap 2 there are only 2 post-chemotherapy samples (patient 6 ascites tumour, and patient 37 ascites tumour), apart from the paired patient samples. This makes it difficult to make comparisons between the two groups of samples. Also some of the samples contain solid tumour as well as ascites, which can have an impact on the levels of gene expression because variations occur between tissue types.

The top part of heatmap 2 from gene SSU72 down to TRIM62 demonstrate lower gene expression levels of these genes in the post-chemotherapy paired patient samples (on the left hand side). This is demonstrated by the darker blue colour in these probe sites. It would be expected, therefore that pre-chemotherapy patient samples would have slightly higher levels of these genes expressed. The right hand side contain mainly pre-chemotherapy samples, and these probe sites are mainly red indicating that genes SSU72 to TRIM62 are indeed expressed at a higher level in these samples.

The bottom part of heatmap 2 from gene CDK6 downwards demonstrate higher gene expression levels of these genes in the post-chemotherapy paired patient samples (on the left hand side). This is demonstrated by the darker red colour in these probe sites. It would

be expected, therefore that pre-chemotherapy patient samples would have slightly lower levels of expression of these genes. This is demonstrated by the mainly blue colours of the pre-chemotherapy sample probe sites on the right hand side.

The two post-chemotherapy samples (pt 6 asc.tu, and pt 37 asc.tu) that are within the pre-chemotherapy samples on the right hand side do not appear to stand out according to their levels of gene expression. Patient 37 overall has very low levels of gene expression, and only a few genes from the gene expression profile of patient 6 appear to fit the pattern. These include genes GUCY1B3, and KIAA1026 which are down regulated, and gene MRPS22 (Mitochondrial ribosomal protein s22) which is up-regulated in the post-chemotherapy patient 6 sample, and appears to fit with the pattern. However, although the trend of gene expression seems consistent in these data set compared to the paired patient data set, the results are not statistically significant.

3.6.2 Relative levels of gene expression in ovarian cancer cells from newly diagnosed and treated patients using real-time polymerase chain reaction

ERCC1 and GAPDH (internal control) were investigated using real time PCR. ERCC1 was used because this gene is known to be involved in repair of cisplatin damage and in ICL repair. An increase in ERCC1 gene expression in post chemotherapy patients could therefore contribute to their increase in ICL repair. The primers for ERCC1 had already been optimised, but further experiments were carried out to ensure the primers had very similar relative binding efficiencies compared to the internal control primer (GAPDH). This meant that both primers bound their respective sequences of DNA at the same rate.

Therefore if differences in gene expression were detected, it would not be due to a higer efficiciency of one primer set.

The experiment was set up according to the template for the standard curve (see method). This meant that RT-PCR with each primer set was performed on serial dilutions of cDNA. Once the experiment was complete, the cycle threshold (Ct) values were arbitrarily set by hand in the region of exponential flouresence on the amplification plot (for both GAPDH and ERCC1), and the results were analysed at this given Ct value. The Ct values for the primer (ERCC1) being investigated had to be the same as GAPDH to make it a direct comparison. Flouresence values for GAPDH and ERCC1 were read at the given Ct value and the results subtracted from one another. Results were then plotted and the slope of the figure was calculated to give the relative primer efficiencies over a given cDNA starting amount.

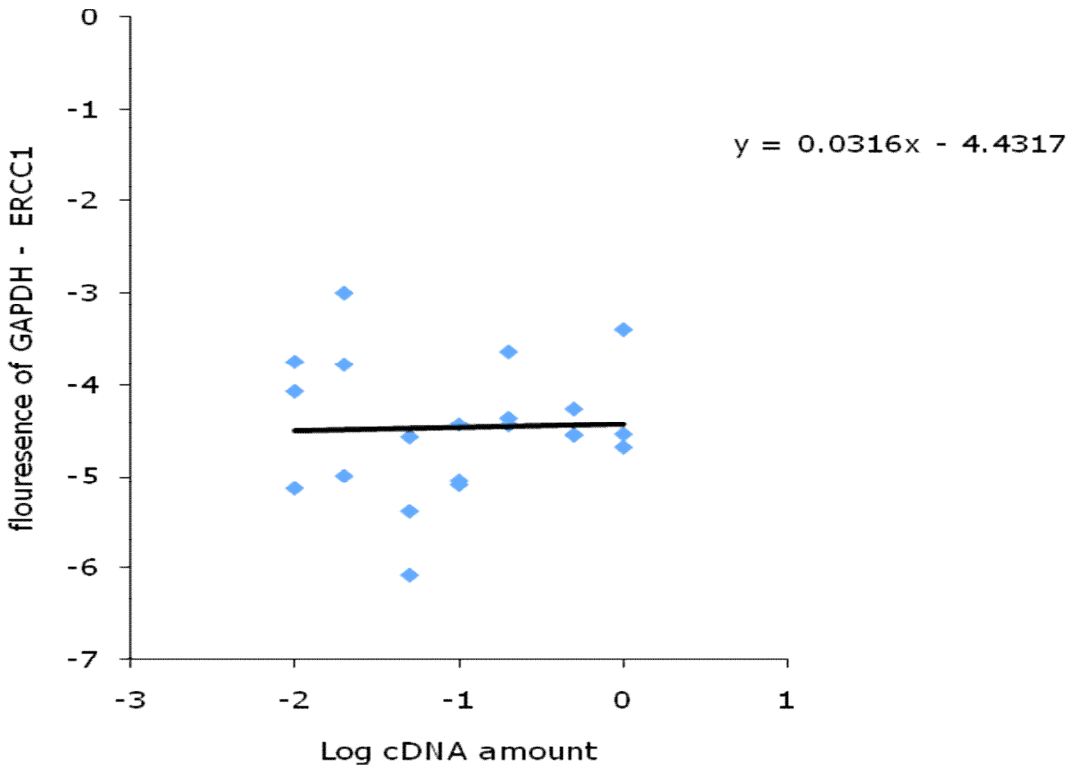


Figure 42: shows the relative efficiency for ERCC1 primer compared to GAPDH.

The relative primer efficiency for ERCC1 compared to GAPDH is 0.0316, which is the slope of the line in figure 42. This means this primer is optimised as the relative efficiency is less than 0.1.

The Ct value for ERCC1 and GAPDH was 0.597. The experiment was then set up in triplicate using the same cycling conditions as the standard curve experiment. The same Ct value was used to analyse the data. The fluorescence of GAPDH was taken away from the ERCC1 value and the results averaged for each patient sample.

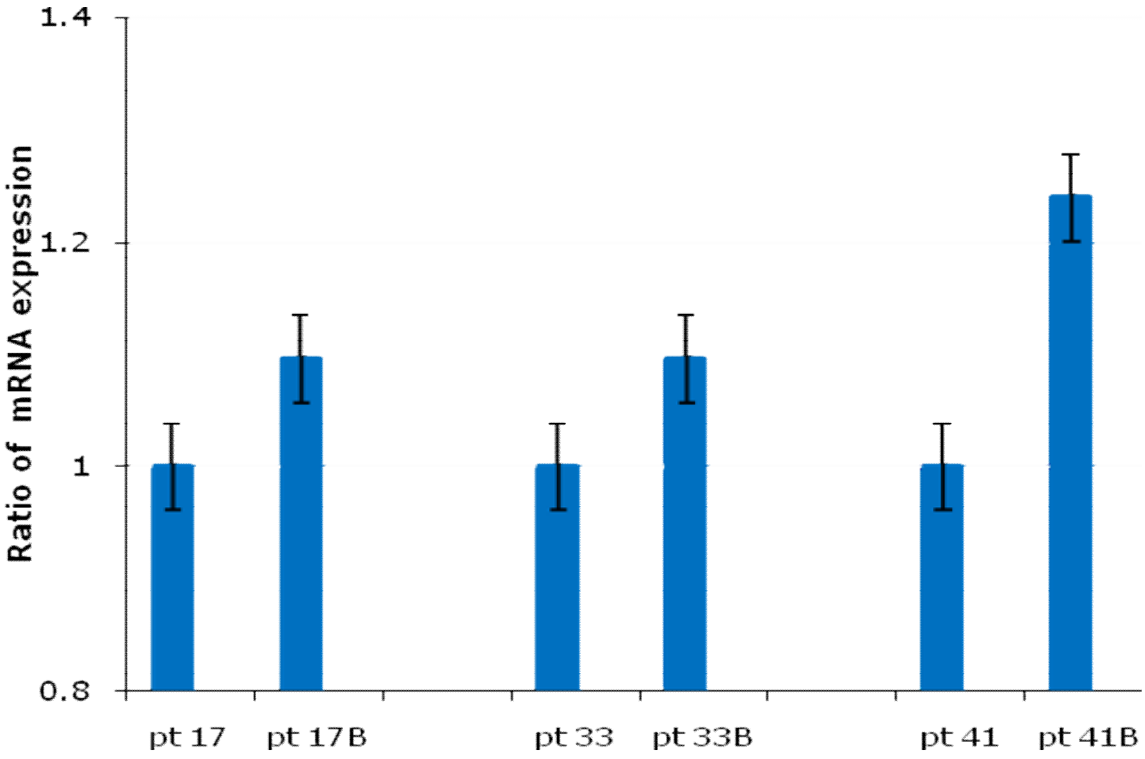


Figure 43: Relative gene expression levels of ERCC1 in pre- and post-chemotherapy patient samples, using the pre-chemotherapy samples normalised to 1.

As can be seen from figure 43 there is an increase in ERCC1 gene expression in the post-chemotherapy samples. The average increase is 14.4% +/-0.8%. This apparent difference in gene expression may contribute to the increase in ICL repair seen in the post-chemotherapy patient samples. Results obtained from microarray samples also showed a small increase in ERCC1 expression in post-chemotherapy samples, but this was not statistically significant, using students paired t-test ($p<0.40$).

3.6.3 Discussion

The samples used to investigate gene expression using microarray analysis were taken straight from the patient and either processed immediately (such as solid tumour samples), or cultured in the lab (such as ovarian cancer cells extracted from ascites). The gene expression levels measured are basal levels. The discussion relates to the different basal levels of gene expression found between the two groups. In the comet assay, the data are derived following cisplatin treatment. No attempt to measure changes in gene expression following cisplatin treatment was made in this study.

Interestingly, none of the differentially expressed genes identified included apoptotic regulators, ATP binding cassette transporters, metallothionein, and DNA repair components, which have all been implicated in cisplatin resistance in cell model systems. This was similar to a study by Hartmann *et al* (2002) where microarray analysis was performed on solid ovarian cancers obtained from patients to predict early relapse after treatment with carboplatin-paclitaxel. In a different study Hartmann *et al* showed genes involved in inhibiting BRCA1, and other genes involved in activating nuclear factor κB were up-regulated (Hartmann *et al*., 2005). But there were other genes identified in our experiments that were up or down regulated in pre-chemotherapy patients when compared to post-chemotherapy patients.

Genes that are most upregulated in the post-chemotherapy samples (i.e show a –ve M value) include unknown genes such as LOC492311, and AT4G28910. Other genes highly upregulated in post-chemotherapy samples encode proteins involved in cell-cycling such as ARHGAP5 (Rho GTPase activating protein 5), CDK6 (cyclin-dependent kinase 6), and

GSTP1 (G1 to S phase transition 1). GUCY1B3 (Guanylate cyclase 1, soluble, beta 3) is involved in cell signalling, and is also upregulated.

Rho GTPase activating protein 5 is part of the family of Rho GAPs (Rho GTPases activating protein) that are key protein regulators of the various and numerous small GTPases. Rho GAPs mediate a variety of receptor-transduced signals, and appear to play an essential role in growth factor dependent GTPase regulation (Bernards *et al.*, 2005). Rho GAPs are also involved in cell-cycle progression (Settleman *et al*., 1992), but there are no known link to DNA repair, and it is unclear whether its role in cell-cycle progression could have an effect on resistance to chemotherapy as shown in cells from post-chemotherapy patients.

CDK4/6 on binding to cyclin D begins the phosphorylation of retinoblastoma protein (pRb) complexed to E2F/DP (transcription factor E2F with dimerisation partner). Following pRb phosphorylation, cyclin E activates CDK2 (cyclin-dependent kinase 2) to effect further phosphorylation of pRb, thereby enabling the cells to cross the G_1 restriction point (Sridhar *et al*., 2006). CDK6 is important in promoting cell gowth and division, and therefore has oncogenic properties. Its upregulation in post-chemotherapy samples indicates it may have a role in the cells resistance to chemotherapy by overcoming the cell cycle checkpoints, and by promoting cell growth and division. This is a different mechanism of resistnace to the increase in ICL repair seen in these cells. However, cellular chemotherapy resistance is likely to be multifactorial.

Guanylyl cyclases are a family of enzymes that catalyze the conversion of GTP to cGMP. Accumulation of cGMP regulates complex signaling cascades through immediate

downstream effectors, including cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases, and cyclic nucleotide-gated ion channels (Lucas *et al.,* 2000). Guanylyl cyclases are regulated by peptide hormones, bacterial toxins, and free radicals, as well as intracellular molecules, such as calcium and adenine nucleotides. It is unclear why Guanylate cyclase 1 is upregulated in post-chemotherapy samples, and what relevance this has to increase in ICL repair shown in these cells. However their involvement in diverse signalling pathways could play a role in cellular communication of yet unknown relevance.

Genes that are most down-regulated in post-chemotherapy samples (i.e show a +ve value) include ring finger protein 135, and SAP30 (Sin-3-associated polypeptide, 30kDa). SAP30 interacts with parts of the Sin3A corepressor complex which recruits histone deacetylases and in many cases represses transcription (Viiri *et al.,* 2006). The down-regulation of this gene repressor could potentially result in the over-expression of other genes, such as those involved in promoting chemotherapy resistance.

The protein encoded by Ring finger protein 135 (RNF 135) contains a RING finger domain that is known to be involved in protein-protein, and protein-DNA interactions. This gene is located in a chromosomal region known to be frequently deleted in patients with neurofibromatosis (Douglas *et al.,* 2007). It is thought that mutations within RNF135 can promote human growth, as well as give rise to dysmorphic features, and learning disability which is characteristic of this syndrome (Douglas *et al.,* 2007). It is unclear what involvement in the promotion of growth this protein has, and future developements are awaited.

This type of microarray analysis is used to determine levels of gene expression, by measurement of mRNA levels. However post-transcriptional and post-translational modifications of mRNA and proteins are not accounted for and therefore different techniques need to be used to investigate these modifications. These modifications can have a profound impact on the functions of the cell, as a small increase in gene expression may have a greatly amplified affect caused by post-transcriptional and post-translational modifications. The reverse is also true that a large increase in gene expression may have only limited affects on the cell due to these modifications to mRNA and proteins. This could contribute to why there are no clear differences in DNA repair gene expression in the post-chemotherapy patients. It is therefore appropriate to investigate differences in gene expression at the protein level.

It is also unclear what impact treatment of patient samples with cisplatin *ex vivo* would have on the relative levels of gene expression in pre and post-chemotherapy patients. This may have had a profound impact on the results obtained from microarray analysis, and will be the subject of future research.

4. DISCUSSION

The results presented demonstrate that ICL repair may play an important role in the resistance of both the A2780cisR cell lines and in 14 treated patients to cisplatin chemotherapy. Interestingly this was demonstrated in both primary ovarian solid tumour, and ovarian cancer cells obtained from ascites, suggesting they have similar cellular pathways that process cisplatin and its adducts.

However, it should be noted that measurement of % decrease in tail moment is really a measurement of 'unhooking' of one arm of the ICL that releases the linkage of the two DNA strands. Unhooking is the first step in the repair of ICLs, and is also thought to be the rate limiting step of this complex process (McHugh *et al*., 2001). The comet assay measures unhooking, but does not provide information on the completion of repair and the resulting restoration of integrity of both DNA strands of the double helix.

Although ovarian cancer cells from treated patients (post-chemotherapy) are not necessarily the same as resistant patients, they behave similarly to the A2780cisR ovarian cancer cells as they both repair ICLs after treatment with cisplatin. However, this does not appear to be true when the cells are treated with melphalan, another crosslinking agent. The A2780cisR cell line appears to repair the crosslinks produced by melphalan, but the post-chemotherapy patients do not appear to repair the ICLs after exposure to melphalan *ex vivo.* This means caution should be used when examining the responses of this cell line as it may not correspond completely to the clinical situation. However, cell lines are still a valuable tool, because obtaining good quality ovarian cancer cells from patients is both

time consuming and difficult, and cell lines may help to guide experimentation in the clinical setting.

4.1 Cisplatin-induced ICL repair in human ovarian cancer cell lines

A possible mechanism of enhanced ability to repair ICLs by the A2780cisR cell line involves ERCC1 (excision repair cross-complementation group 1), part of the nucleotide excision repair (NER) pathway (Yan *et al*., 2006) which is one of the major repair pathways and also part of the mechanism of repair of ICLs. ERCC1-XPF plays a role in DNA damage recognition and makes the initial cut in the DNA strand 5' (Sancar *et al*., 1996), which is termed unhooking of the ICL, and is thought to be the rate limiting step (Mu et al., 1995). In support of this, cells defective in ERCC1 are very sensitive to ICL agents, such as nitrogen mustards (De Silva *et al.,* 2000), and cisplatin (De Silva *et al.*, 2002). It is thought that NER, as well as homologous rembination (HR) are involved in the repair of cisplatin ICLs, although the role of ERCC1 is thought to be mainly unhooking of ICLs and not part of the recombinational components of cross-link repair (De Silva *et al.*, 2000).

The importance of ERCC1 is demonstrated by Li *et al* who demonstrated a six-fold increase in the mRNA levels of ERCC1 in A2780/CP70 cells after treatment with cisplatin. The A2780cisR cell lines that were used in our experiments were A2780/CP8, and therefore are different to the A2780/CP70 cell lines mentioned here. The A280/CP70 cell lines were made resistant to cisplatin by exposure to higher concentrations of cisplatin. However, there are still similarities in their behaviour. In *in vitro* studies, over-expression

of ERCC1 is associated with a platinum-resistant phenotype in ovarian cancer cells (Parker *et al*., 1991). Cisplatin hypersensitive, repair deficient cells have 30- to 50-fold lower levels of ERCC1 compared to inherently resistant cells (Lee *et al*., 1993). Furthermore, Interleukin (IL)-1α has been shown to increase cisplatin cytotoxicity *in vitro* in the A2780/CP70 cell line by reduction of ERCC1 mRNA levels (Li *et al*., 1998). This has led to a reduction in repair of ICLs, and further supports the role of ERCC1 in resistance of A2780/CP70 cells to cisplatin (Li *et al*., 1998). XPA (xeroderma pigmentosum A) is also important in NER and down-regulation of gene expression by antisense RNA transfection can reduce DNA repair, and therefore reduce resistance of tumour cells to cisplatin (Wu *et al*., 2003).

The MCAS cell line and the A2780/CP70 cell line are equally resistant to cisplatin, although the MCAS cell line differs by an ERCC1 polymorphism at codon 118, which markedly reduces induction of ERCC1 mRNA levels compared to A2780/CP70 cells (Yu *et al*., 2000). The A2780/CP70 cell line repaired 50% more cisplatin-DNA adducts present compared to the MCAS cell line, although these cells have less cisplatin-DNA adduct formation present due to their increased cytosolic inactivation of the drug (Yu *et al*., 2000). This mechanism may be a compensatory cellular response to the decreased ability to repair the ICLs (Yu *et al*., 2000). Antisense ERCC1 RNA was used to sensitize the resistant OVCAR10 human ovarian cancer cells to cisplatin in an ovarian cancer xenograft model (Selvakumaran *et al*., 2003). These results suggest the importance of ERCC1 in cisplatin-DNA adduct repair in A2780 cell lines as well as other human ovarian cancer cell lines.

ERCC1 appears to be regulated by genes such as AP-1 (activator protein 1) and MZF1 (myeloid zinc finger gene 1) (Li *et al*., 1998) located approx 410bp upstream from the

ERCC1 gene (Yan *et al*., 2006). AP-1 is bound by c-fos and c-jun transcription factors, which have a 4-fold increase in mRNA levels, and a 14-fold increase in c-jun phosphorylation, in response to a 1-hour incubation with cisplatin in A2780/CP70 cells (Li *et al*., 1998). This corresponds to increased binding of the AP-1 site (Li *et al*., 1998).

MZF1 encodes a transcription factor that functions as a transcription repressor of ERCC1, as well as a repressor of the promoter of ERCC1 (AP-1) (Yan *et al*., 2006). Studies have shown that cisplatin reverses this repression of ERCC1 by MZF1 in A2780/CP70 cells, which could explain the mechanism of cisplatin resistance in these cells (Yan *et al*., 2006). Inhibition of DNA repair by reduction of ERCC1 mRNA levels could potentially be used in the clinical setting to combat resistance to cisplatin, if a suitable inhibitor was found. Lactacystin is a selective inhibitor of the proteasome that can inhibit the ubiquitin pathway, and was used at concentrations that do not appear harmful, to increase cisplatin cytotoxicity in three resistant human ovarian carcinoma cell lines via inhibition of ERCC1 transcription (Li *et al*., 2001). Lactacystin also significantly enhanced DNA platination (Li *et al*., 2001).

Ets-1 is a transcription factor which has been found to be important in resistance of a human ovarian cancer cell line called C13 to cisplatin (Bergoglio *et al*., 2001). Ets-1 was up-regulated in cisplatin-resistant C13 cells as compared with the sensitive 2008 cell line and over-expression of this protein in 2008 cells led to a seven fold increase in resistance (Wilson *et al*., 2004). There is also evidence that Ets-1 transcriptionally activates genes whose products are well described in cisplatin resistance such as DNA repair enzymes (Wilson *et al*., 2004). The association of Ets-1 to cisplatin resistance may have therapeutic significance, but this remains to be established.

Other possible mechanisms to up-regulate NER in mammalian cells have been found. Wang et al have shown that selective post-translational modification of histones on platinated nucleosomes may provide a general strategy for recruiting NER factors more efficiently and thus help to overcome the nucleosome barrier to excision repair (Wang *et al*., 2003). This may lead to cellular resistance to platinum compounds. The comet assay measures unhooking of interstrand crosslinks, and it is clear from the results (figure 15) that there is increased repair in the A2780 cisplatin resistant cell line, which could partly be due to histone modifications.

It has also been shown that binding of the MMR complex to DNA adducts appears to increase the cytotoxicity of cisplatin in tumour cells (Papouli *et al*., 2004). This may occur by activating downstream signalling pathways that lead to apoptosis or by causing 'futile cycling' during translesion synthesis past DNA adducts of cisplatin (Vaisman *et al*., 1998). Both hMSH2 and MutS proteins (Fourrier *et al*., 2003), as components of the MMR complex have been shown to bind to cisplatin adducts. Defects in MMR in human kidney cells may result in resistance or tolerance of cancer cells to cisplatin (Papouli *et al*., 2004). This may contribute to the resistance found in the A2780cisR cell line to cisplatin.

P53 is a tumour suppressor involved in DNA repair, cell cycle, and apoptosis, and its levels increase upon exposure to genotoxic compounds due to several post-transcriptional mechanisms (Brabec *et al*., 2005). Interestingly, cisplatin-induced stabilisation of p53 protein in A2780 cell line (platinum sensitive) is markedly less pronounced then in the A2780cisR cell line (Yazlovitskava *et al*., 2001). This is consistent with the observation that inactivation of wild-type p53 function in A2780 cells by transfection with HPV-16 E6

(a protein that binds p53 and targets it for degradation) increases cisplatin sensitivity and decreases cisplatin-DNA adduct repair (Pestell *et al.*, 2000).

There is clearly evidence that A2780cisR resistance to platinum based chemotherapy is characterised by up-regulation of DNA repair mechanisms, and increase in ICL unhooking in cell lines. The literature suggests this could include up-regulation of ERCC1. Little is known about the occurrence of this in the clinical setting. The comet assay has not been used before to measure ICL unhooking in ovarian cancer cells direct from patients. This was investigated to determine if increased ICL repair was involved in the development of platinum resistance in the clinical setting.

4.2 Cisplatin-induced ICL repair in ovarian cancer cells obtained from patient samples

There is evidence that there is a difference in the levels of DNA repair in cells obtained from patients at different ages. This was clearly demonstrated in a study by Rudd *et al* (1995) where peripheral blood mononuclear cells were taken from patients of varying age. It was found that peripheral blood mononuclear cells from older patients treated with cisplatin, had markedly reduced levels of ICL repair as measured by alkaline elution, compared to peripheral blood mononuclear cells obtained from younger patients (Rudd *et al*., 1995). This is also thought to be true in other cell types. In this study there were no significant differences in the ages of newly diagnosed and treated patients (mean age newly diagnosed patients 66 +/- 11, and mean age treated patients 66 +/- 10). This means

that this variable should have equal affect on each patient group, and therefore should not bias the results.

In figure 27 (results chapter) there is a clear increase in ICL repair in ovarian cancer cells from patients after they have received platinum chemotherapy. This suggests that the increase in ICL repair is not likely to be intrinsic to these ovarian cancer cells, but they acquire this mechanis. It could also be that by treating these cells with chemotherapy, the cells that survive are selected for for their ability to repair DNA. It is possible that this increase in ICL repair is mediated by an increase in the levels of ERCC1, which is an important protein involved in the rate limiting step of the ICL repair process. AP-1 is an important transcription factor of ERCC1, and can induce the levels of ERCC1 mRNA and protein expression in A2780 human ovarian cancer cell lines when they are incubated *in vitro* with cisplatin (Li *et al*., 1998). There is some evidence, that cisplatin can induce expression of proto-oncogenes *c-fos/c-jun* and activate c-Jun NH_2 –terminal Kinase / stress-activated protein kinase (JNK/SAPK – a subfamily of the MAP kinases involved in the Ras pathway) in ovarian cancer cells and other tumour cells (Liu *et al*., 1996). JNK/SAPK phosphorylates transcription factor c-jun protein which greatly increases the transcriptional activity of AP-1, and AP-1 related genes (Derijard *et al*., 1994). These observations of induction of ERCC1 expression have been seen in cancer cell lines, rather than in clinical samples obtained from patients. However, it is still possible that cisplatin can induce ERCC1 protein expression, through activation of the transcription factor AP-1, that accounts for the acquired mechanism of increased ICL repair in these patients.

To support this theory, high levels of ERCC1 mRNA and protein in patient samples have been associated with clinical resistance to platinum chemotherapy (Altaha *et al*., 2004).

Furthermore, drugs that decrease expression of ERCC1 cause the reversal of platinum resistance when given to cancer cell lines *in vitro* prior to platinum-based chemotherapy (Altaha *et al*., 2004). Also, in patients with completely resected non-small-cell lung cancers that are ERCC1 negative benefit from adjuvant cisplatin chemotherapy, whereas those that are ERCC1 positive do not benefit (Olaussen *et al*., 2006). Interestingly, cells defective in ERCC1 are much more sensitive to cisplatin than cells defective in other components of the NER pathway (e.g XPB, XPD, XPG mutants), even though all these cells are equally defective in the unhooking step of ICL repair (De Silva *et al*., 2002). This suggests that ERCC1 is involved in other repair processes as well as NER that are important in ICL repair such as homologous recombination. In support of this, XRCC2 and XRCC3 mutants are extremely sensitive to cisplatin and, as these proteins are involved in HR, this suggests that HR is necessary for the repair of cisplatin adducts (Caldecott *et al*., 1991). It has also been shown in human tissues from patients with ovarian cancer, a possible molecular basis for co-ordinate mRNA expression of genes involved in NER, suggesting that NER is important in human cancers (Zhong et al., 2000).

Results from qPCR indicate that there is a small up-regulation of basal ERCC1 mRNA levels in the post-chemotherapy samples from patients 17B, 33B, and 41B, as compared to their pre-chemotherapy samples (figure 43). However, these results are not statistically significant. This is probably because there are only three samples, and so further experiments should be undertaken. An increase in ERCC1 mRNA levels may be more following cisplatin treatment. This was not studied, but could be in the future.

These results are supported by microarray analysis, as the trend is towards a small upregulation of ERCC1 in post-chemotherapy patients (results not shown). However, these

results are not statistically significant either. This could be because there has to be a large fold increase in gene expression to be able to be detected using microarray analysis. As was previously discussed, only small increases in mRNA may be necessary to have an effect due to post-transcriptional, and post-translational modifications. Results from both microarray analysis, and qPCR are supported by the literature as discussed above.

Other methods to validate qRT-PCR and microarray experiments at the protein level could have been used, such as western blots that use fluoresecent antibodies to measure protein levels. Other types of functional assays, other than those used, could also have been employed, as well as candidate-based approaches to identify genes involved in chemotherapy resistance.

4.3 Melphalan-induced ICL repair in A2780 and A2780cisR human ovarian cancer cell lines, and patient samples

It is also apparent that treated patients resistant to cisplatin chemotherapy, repair cisplatin-induced ICLs, but do not appear to repair melphalan-induced ICLs. Interestingly, in the A2780, and A2780cisR paired human ovarian cancer cell line the resistant cells repair both cisplatin-induced ICLs and melphalan-induced ICLs, which is in contrast to the clinical samples. This suggests that the ovarian cancer cells obtained from treated patients behave differently to the A2780cisR ovarian cancer cell line model, and if so, the cell line would be of limited use to study melphalan induced ICL repair *in vitro*.

The difference between melphalan-induced ICL repair and cisplatin-induced ICL repair in treated patients with ovarian cancer suggests the possibility that melphalan could be used to treat these patients. Also these results suggest that melphalan-induced ICLs and cisplatin-induced ICLs are repaired differently. Previous clinical trials using melphalan to treat platinum-resistant patients with ovarian cancer as already discussed have been very small, and therefore larger trials are needed.

Interestingly, Spanswick *et al* demonstrated that multiple myeloma patients that were clinically resistant to melphalan chemotherapy had between 42-100% ICL repair, even though they had similar levels of ICL formation at the peak of crosslinking compared to melphalan-sensitive patients with multiple myeloma who did not repair the ICLs (Spanswick et al., 2000). Work carried out in our laboratory suggests that patients with multiple myeloma that are resistant to melphalan chemotherapy may be sensitive to cisplatin chemotherapy. Lymphocytes from patients with multiple myeloma demonstrated low levels of cisplatin-induced ICL repair compared to high levels of melphalan-induced ICL repair (unpublished data H. Lowe). Not only does this suggest that cisplatin chemotherapy could be used to treat these patients, but also is more evidence that melphalan-induced ICLs and cisplatin-induced ICLs are repaired differently.

4.4 Comparison of gene expression between A2780 and A2780cisR human ovarian cancer cell lines, and between newly diagnosed and treated patients using microarrays

The gene expression profiles obtained from patients before and after chemotherapy did not show any statistical difference in expression of known genes involved in DNA repair, even though there was a clear phenotypical difference between these cells. However there were other genes involved in cell cycle and cell signalling that showed up- or down-regulation in ovarian cancer cells from post-chemotherapy patient samples, but the significance, and their involvement in DNA repair is as yet unknown. Cells used to extract total RNA for use in microarray experiments were not treated *ex vivo* with cisplatin or melphalan, although cells used in the comet assay were treated *ex vivo* with these drugs. It may be that treatment *ex vivo* with either cisplatin or melphalan is necessary to induce differential changes of gene expression within these cells. In support of this, it has been shown that there is an increase in c-fos and c-jun transcription factors that bind Ap-1 (a gene that regulates ERCC1) in A2780/CP70 cells after a 1 hour exposure to cisplatin (Li *et al.*, 1998), although it is unclear if the same changes are seen in ovarian cancer cells obtained from clinical samples.

Microarray is a relatively new and powerful technique, but does have limitations that may have influenced the difficulties of obtaining statistically significant results for differences in gene expression of DNA repair genes between pre- and post-chemotherapy patients. According to the Goldie-Coldman hypothesis, acquired chemotherapy resistance is driven

by clonal selection of cells that have acquired a genetic mutation that has conferred an advantage (Goldie *et al*., 1979). This means that in primary tumours only a small percentage of cells will possess a chemo resistant phenotype with the associated molecular change, resulting in a 'dilution' of any changes in gene expression in this population (Goldie *et al*., 1979). This may mean that there are other genes associated with chemotherapy resistance (e.g DNA repair genes) that are not apparent from this kind of microarray analysis.

Within solid tumours there are blood vessels and connective tissue as well as ovarian cancer cells. This mixture of cells influences the gene expression profiles for each patient sample, and also adds to the dilution of any changes in gene expression. To minimise this problem, all samples were stained with antibodies to assess the percentage of ovarian cancer cells present, and only those with >80% of ovarian cancer cells present were used.

The 'dilution' effect can be problematic because microarray experiments accurately detect differences in gene expression of 2 fold or more. This means that subtle differences in gene expression will not be detected. This was evident in the RT-PCR experiments which showed an increase in ERCC1 gene expression levels by 15% in ovarian cancer cells obtained from patients that had received platinum-based chemotherapy. However, differences in ERCC1 gene expression levels were not detected using microarray techniques on all samples of ovarian cancer cells from the same patient. Some patients did show a small increase in ERCC1 expression in post-chemotherapy samples using microarray analysis. Analysis of microarrays may also add to this dilutional affect. The primary expression data are in the form of logarithmic intensity ratios. Secondary data such as average gene expression levels of genes within a group, are derived by calculating the

geometric rather than the arithmetic means of logarithmic intensity ratios, resulting in smaller values, and smaller apparent differences (Jazaeri *et al*., 2005).

It is important to point out that the microarray technology used only determines levels of mRNA of specific genes, but does not provide any information about the post-translational and post-trancriptional modifications of mRNA. These modifications may have dramatic effects on the overall function of the proteins that are produced by these genes. Many genes involved in chemotherapy resistance have been identified through microarray analysis, and as is often the case there is no genetic overlap between results obtained from different research groups. Differences in gene expression signatures between two groups of investigators may be due to the end point of interest (eg, chemoresistance *v* natural history such as tumour growth rate and metastatic potential), the algorithms used to define gene expression thresholds, the bioinformatics used to define expression clusters, as well as more subtle differences between the patient populations under study. Many different gene expression signatures may be identified over the next few years, each containing different genes, but each converging on an equally powerful and valid prognostic end point (Spentzos *et al*., 2005).

4.5 Clinical Relevance and Future Work

It may be possible to use the comet assay to predict which patients will respond to platinum based chemotherapy. This may be used along with microarray technology, that use gene profiling to try and predict a patient's response to chemotherapy. These techniques should be evaluated in a clinical trial, because if they are successful, it would

prevent patients suffering from side effects of chemotherapy drugs with very little benefit in terms of disease response.

The comet assay may also be used to determine which patients may respond to melphalan chemotherapy. Although as the two clinical trials already discussed from Magagnoli *et al* (2004) and Hasan *et al* (2003) demonstrate, more clinical trials need to be done in this area to establish whether melphalan chemotherapy is effective (Magnoli *et al*., 2004), (Hassan *et al*., 2003), and also to clarify the dosing regimen. The clinical trial by Maganoli *et al* (2004) and data obtained from our experiments support the possibilty of melphalan usage in some patients with clinically platinum resistant ovarian cancer (Maganoli *et al*., 2004).

With the emergence of microarray technology, it is hoped that, along with other techniques, it can be used to tailor chemotherapy to each patient. Although this has wide cost implications, it has the potential to improve patient response, maintain disease stabilisation, and perhaps improve the cure rates for ovarian cancer.

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5. REFERENCES

Aabo, K., Adams, M., Adnitt, P., Alberts, D.S., Athanazziou, A., Barley, V., Bell, D.R., Bianchi, U., Bolis, G., Brady, M.F., Brodovsky, H.S., Bruckner, H., Buyse, M., Canetta, R., Chylak, V., Cohen, C.J., Colombo, N., Conte, P.F., Crowther, D., Edmonson, J.H., Gennatas, C., Gilbey, E., Gore, M., Guthrie, D. and Yeap, B.Y. **1998** Chemotherapy in advanced ovarian cancer: four systematic meta-analysis of individual patient data from 37 randomised trials. Advanced Ovarian Cancer Trialists Group. *Br J Cancer*, **78**, 1479-1487.

Abedini, M.R, Qiu, Q., Yan, X. and Tsang, B.K. **2004** Possible role of FLICE-like inhibitory protein (FLIP) in chemoresistant ovarian cancer cells in vitro. *Oncogene* **23**, 6997-7004.

Acharya, S., Foste,r P.L., Brooks, P., and Fischel, R. **2003** The coordinated functions of the E.coli MutS and MutL proteins in mismatch repair. *Mol. Cell*. **12**, 233-246.

Advanced Ovarian Cancer Trialists Group. **1991** Chemotherapy in advanced ovarian cancer: an overview of randomised clinical trials. *Br Med J* **303**, 884-893.

Akkari, Y.M, Bateman, R.L, Reifsteck, C.A, Olson, S.B and Grompe, M. **2000** DNA replication is required to elicit cellular responses to psoralen-induced DNA interstrand crosslinks. *Mol Cell Biol* **20**, 8283-8289.

Altaha, R., Liang, X, Yu, J.J., and Reed, E. **2004** Excision repair cross complementing group-1: gene expression and platinum resistance. *Int J Mol Med* **14**(6), 959-70.

Altomare, DA., Wang, H.Q., Skele, K.L., De Rienzo, A., Klein-Szanto, A.J., Godwin, A.K., and Testa, J.R. **2000** Akt and mTOR phosphorylation is frequently detected in ovarian cancer and can be targeted to disrupt ovarian tumour growth. *Oncogene* **23**, 5853-5857.

Armstrong, D.K., Bundy, B., Wenzel, L., Huang, H.Q., Baergen, R., Lele, S., Copeland, L.J., Walker, J.L. and Burger, R.A. **2006** Gynaecologic Oncology Group. Intraperitoneal cisplatin and paclitaxel in ovarian cancer. *N Eng J Med* **354**(1), 34-43.

Arts, H.J., Katsaros D., de Vries E.G. **1999** Drug resistance-associated markers P-glycoprotein, multidrug resistance-associated protein 1, multidrug resistance-associated protein 2, and lung resistance protein as prognostic factors in ovarian carcinoma. *Clin Cancer Res* **5**, 2798-805.

Asselin, E., Mills, G.B. and Tsang, B.K. **2003** XIAP regulates Akt activity and caspase-3-dependent cleavage during cispplatin-induced apoptotsis in human ovarian epithelial cancer cells. *Cancer Res* **3**, 502-516.

Atlas of Genetics and Cytogenetics in Oncology and Haematology. http://www.infobiogen.fr/services/chromcancer/Tumors/OvarianTumOverviewID5231.html Accessed December 18th, 2004

Baekelandt, M.M., Holm, R., Nesland, J.M., Trope, C.G. and Kristensen, G.B. **2000** P-glycoprotein expression is a marker for chemotherapy resistance and prognosis in advanced ovarian cancer. *Anticancer Res* **20**, 1061-1067.

Baird, R.D. and Kaye, S.B. **2001** Drug resistance reversal –are we getting closer? *Eur J Cancer* **39**, 2450-2461.

Batty, D.P. and Wood, R.D. **2000** Damage recognition in nucleotide excision repair of DNA. *Gene* **241**, 193-204.

Baumann, P., Benson, F.E. and West, S.C. **1996** Human RAD51 protein promotes ATP-dependent homologous pairing and strand transfer reaction *in vitro*. *Cell* **87**, 757-66.

Baxter, B.K. and Smerdon, M.J. **1998** Nuclesosome unfolding during DNA repair in normal and xeroderma pigmentosum (group C) human cells. *J Biol Chem* **273**, 17517-17524.

Becouarn, Y., Ychou, M., Ducreux, M., Borel, C., Bertheault-Cvitkovic, F., Seitz, J.F., Nasca, S., Nguyen, T.D., Paillot, B., Raoul, J.L., Duffour, J., Fandi, A., Dupont-André, G. and Rougier, P. **1998** Phase II trial of oxaliplatin as first-line chemotherapy in metastatic colorectal cancer patients. Digestive Group of French Federation of Cancer Centers. *J Clin Oncol* **16**, 2739-44.

Behrens, B.C., Hamilton, T.C., Masuda, H, Grotzinger, K.R., Whang-Peng, J., Louie, K.G., Knutsen, T., Mckoy, W.M., Young, R.C. and Ozols, R.F. **1987** Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res* **47**(2), 414-8.

Bellacosa, A., Defeo, D., Godwin, A.K., Bell, D.W., Cheng, J.Q., Altomare, D.A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V., Ferrandina, G., Benedetti Panici P., Mancuso, S., Neri, G. and Testa, J.R. **1995** Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int. J. Cancer* **64**, 280-285.

Bellacosa, A., Chan, T.O., Ahmed, N.N., Datta, K., Malstrom, S., Stokoe, D. **1998**Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene* **17,** 313-325.

Benjamini, Y., and Hochberg, Y. **1997** Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society* Series_B **57**(1), 289-300.

Bergoglio, V., Canitrot, Y., Hogarth, L., Minto,L., Howell, S.B., Cazaux, C. et al. **2001** Enhanced expression and activity of DNA polymerase beta in human ovarian tumour cells:impact on sensitivity towards antitumour agents. *Oncogene* **20**, 6181-6187.

Berkenblit, A., and Cannistra, S.A. **2005** Advances in the management of epithelial ovarian cancer. *J Reprod Med* **50**(6), 426-38.

Bernal, S., Speak, J., Boeheim, K., Dreyfuss, A., Wright, J., Teicher, B..A., Rowsowsky, A., Tsao, S.W. and Wong, Y.C. **2005** Reduced membrane protein associated with

resistance of human squamous carcinoma cells to methotrexate and cisplatin. Mol Cell Biochem **95**, 61-70.

Bernards, A. and Settleman, J. **2005** GAPs in growth factor signalling. *Growth Factors* **23**(2), 143-9.

Bolton, M.G., Hilton,J., Robertson, K.D., Streeper, R.T., Colvin, O.M. and Noe, D.A. **1993** Kinetic analysis of the reaction of melphalan with water, phosphate and glutathione. *Drug Metab. Dispos.* **21**, 986-996.

Bookman, M.A., Malmstrom, H., Bolis, G., Gordon, A., Lissoni, A., Krebs, J.B. et al. **1998** Topotecan for the treatment of advanced epithelial ovarian cancer: an open label phase II study in patients treated after prior chemotherapy that contained cisplatin or carboplatin and paclitaxel. *J Clin Oncol* **16**, 3345-3352.

Boucher, M.J, Morisset, J., Vachon, P.H., Reed, J.C., Laine, J., Rivard, N. **2000** MEK/ERK signalling pathway regulates the expression of bcl-2, bcl –XL, and Mcl-1 and promotes survival of human pancreatic cancer cells. *J Cell Biochem* **79**, 355-369.

Boulton, S., Kyle, S. And Durkacz, B.W. **1999** Interactive effects of inhibitors of poly(ADP-ribose) polymerase and DNA-dependent protein kinase on cellular to DNA damage. *Carcinogenesis* **20**, 199-203.

Brabec, V. and Kasparkova, J. **2005** Modifications of DNA by platinum complexes: Relation to resistance of tumours to platinum antitumour drugs. *Drug resistance updates* **8**(3), 131-146.

Bratasz, A., Selvendiran, K., Wasowicz, T., Bobko, A., Khramtsov, V.V, Ignarro, L.J. and Kuppusamy, P. **2008** NCX-4040, a nitric oxide-releasing aspirin, sensitizes drug-resistant human ovarian xenograft tumours to cisplatin by depletion of cellular thiols. *J Transl Med* 6-9.

Brown, R., Hirst, G.L., Gallagher, W.M., McIlwrath, A.J., Margison, G.P., Van der Zee, A.G. and Anthoney D.A. **1997** hMLH1 expression and cellular responses of ovarian tumour cells to treatment with cytotoxic anticancer agents. *Oncogene* **15**, 45-52.

Bryant, H.E., Schultz, N., Thomas, H.D., Parker, K.M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N.J. and Helleday, T. **2005** Specific killing of BRCA2 deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* **434**(7035), 913-7.

Burma, S., Chen, B.P., Murphy, M., Kurimasa, A. and Chen, D.J. **2001** ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem* **276**, 42462-42467.

Caldecott, K. and Jeggo, P. **1991** Cross-sensitivity of gamma-ray-sensitive hamster mutants to cross-linking agents. *Mutat Res*, **255** 111-121

Cancer Research UK. www.cancerresearchuk.org/aboutcancer/specificcancers/ovariancancer Accessed December 18th, **2004**

Cancer Research UK. Ovarian Cancer. Statistics and Prognosis. www.cancerhelp.org.uk Accessed July 27, **2004**

Cass, I., Baldwin, R.L., Varkey, T., Moslehi, R., Narod, S.A. and Karlan, B.Y. **2003** Improved survival in women with BRCA-associated ovarian carcinoma. *Cancer* **97**, 2187-95.

Chen, J., Silver, D.P., Walpita, D., Cantor, S.B., Gazdar, A.F. and Tomlinson, G. **1998** Stable interaction between the products of the BRCA1 and BRCA2 tumour suppressor genes in mitotic and meiotic cells. *Mol Cell* **2**, 317-28.

Chu G. **1997** Double strand break repair. *J Biol Chem* **272**, 24097-100.

Collins, A. and Harrington, V. **2002** Repair of oxidative damage: assessing its contribution to cancer prevention.*Mutagenesis* **17**(6), 489-493.

Connor, J.P. and Felder, M. **2008** Ascites from epithelial ovarian cacner contain high levels of functional decoy receptor-3 (DcR-3) and is associated with platinum resistance. *Gynaecologic Oncology* **111**(2), 330-335.

Cortez, D., Wang, Y., Qin, J. and Elledge, S.J. **1999** Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science* **286**, 1162-1166.

Cuello, M., Ettenberg, S.A., Nau, M.M. and Lipkowitz S. **2001** Synergistic induction of apoptosis by the combination of trail and and chemotherapy in chemoresistant ovarian cancer cells.*Gynaecol Oncol* **81**, 380-390.

Dabholkar, M., Vionnet, J.A., Bostick-Bruton, F., Yu, J.J. and Reed, E. **1994** mRNA levels of XPAC and ERCC1 in ovarian tumour tissue correlates with response to platinum containing chemotherapy. *J Clin Invest* **94**, 703-708.

Darcy, K.M., Brady, W.E., McBroom J.W, Bell, J.G., Young, R.C., McGuire, W.P., Linolla, R.I., Hendricks, D., Bonome, T. And Farley, J.H. **2008** Associations between p53 overexpression and multiple measures of clinical outcome in high-risk, early stage or suboptimally resected, advanced stage epithelial ovarian cancers: A Gynecologic Oncology Group study. *Gynecologic Oncology* **111**(3), 487-495.

Davies, A.A., Masson, J.Y., Mcilwraith, M.J., Stasiak, A. Z., Staisak, A., Venkitaraman, A.R. and West, S.C. **2001** Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. *Mol Cell* **7**, 273-82.

De Silva, I.U., McHugh, P.J., Clingen, P.H., and Hartley, J.A. **2002** Defects in interstrand cross-link uncoupling do not account for the extreme sensitivity of ERCC1 and XPF cells to cisplatin. *Nucleic Acids Res*, **30**(17), 3848-3856.

Derijard, B., Hibi, M.and Wu, I.H. **1994** JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**, 1025-1037.

Disilvestro, P.A., Fisher, M., Pearl, M.L., Buhl, A., Chalas, E. and Valea, F.A. **2006** Pilot Phase 2 Trial of 4 Months of Maintenance Pegylated Liposomal Doxorubicin in Patients with Advanced Ovarian Cancer after Complete Response to Platinum and Paclitaxel-Based Chemotherapy. *Gynaecol Obstet Invest* **63**(1), 1-6.

Dollery C (Editor). **1991** Melphalan, Therapeutic drugs 2, *Churchill Livingstone, London uk* 48-52.

Dolson L. Cell Grading and Differentiation www.baymoon.com/~gyncancer/library/weekly Accessed December 18th, **2004**

Dotto G.P. **2000** p21(WAF1/CIp1): more than a break to the cell cycle? *Biochem Biophys Acta* 1471:M43-56.

Douglas, J., Cilliers, D., Coleman, K., Tatton-Brown, K., Barker, K., Bernhard, B., Burn, J., Huson, S., Josifova, D., Lacombe, D., Malik, M., Mansour, S., Reid, E., Cormier-Daire, V., Cole, T.; Childhood Overgrowth Collaboration, Rahman, N. **2007** Mutations in RNF135, a gene within the NF1 microdeletion region, cause phenotypic abnormalities including overgrowth. *Nat Genet* **39**(8), 963-5.

Dronkert, M.L. and Kanaar R. **2001** Repair of DNA double-strand breaks. *Mutat Res* **486**, 217-247

Duan, K.A., Brakore, K.A., and Seiden, M.V. **2004** Inhibition of ABCB1 (MDR1) and ABCB4 (MDR3) expression by small interfering RNA and reversal of paclitaxel resistance in human ovarian cancer cells. *Mol. Cancer. Ther* **3**, 833-838.

Du Bois, A., Weber, B., Rochon, J., Meier, W., Goupil, A., Olbricht, S., Barats, J.C., Kuhn, W., Orfeuvre, H., Wagner, U., Richter, B., Lueck, H.J., Pfisterer, J., Costa, S., Schroeder, W., Kimmig, R. and Pujade-Lauraine E. (GINECO). **2006** Addition of epirubicin as a third drug to carboplatin-paclitaxel in first-line treatment of advanced ovarian cancer: a prospectively randomized gynecologic cancer intergroup trial by the Arbeitsgemeinschaft Gynaekologische Onkologie Ovarian Cancer Study Group and the Groupe d'Investigateurs Nationaux pour l'Etude des Cancers Ovariens. *J Clin Oncol* **24**(7), 1127-35.

Duffull, S.B. and Robinson, B.A **1997** Clinical pharmacokinetics and dose optimization of carboplatin. *Clin Pharmacokinet* **33**(3), 161-83.

Eastman, A. **1987** The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. *Pharmacol Ther* **34**, 155-166.

Epstein, RJ. **1988** Topoisomerases in human disease. *Lancet* **1**, 521-524.

Evans, A.R., Limp-Foster, M., Kelly, M.R. **2000** Going APE over ref-1. *Mutat Res* **461**, 83-108.

Evans, E., Moggs, J.G., Hwang, J.R., Egly, J.M. and Wood, R.D. **1997** Mechanism of open complex and dual incision formation by human nucleotide excision repair factors. *EMBO J* **16**, 6559-73.

Ferreira, C.G., Tolis, C. and Giaccone, G. **1999** p53 and chemosensitivity. *Ann Oncol* **10**, 1011-1021.

Ferry, K.V., Hamilton, T.C., and Johnson, S.W. **2000** Increased nucleotide excision repair in cisplatin-resistant ovarian cancer cells. Role of ercc1-xpf. *Biochemical Pharmacological* **60**, 1305-1313.

Fishel, R. and Kolodner, R.D. **1995** Identification of mismatch repair genes and thei role in the developement of cancer. *Curr Opin Genet Dev* **5**, 382-395.

Fichtinger-Schepman, A.M., Van der Veer, J.L., den Hartog, J.H., Lohman, P.H., and Reedijk, J. **1985** Adducts of the antitumour drug *cis* –diamminedichloroplatinum(II) with DNA: Formation, identification and quantitation. *Biochemistry* **24**, 707-713.

Fischtinger-Schepman, A.M.**,** Van Dijk-Knijnenburg, H.C., van der Velde-Visser, S.D., Berends, F. and Baan, R.A. **1995** Cisplatin- and carboplatin-DNA adducts: is PT-AG the cytotoxic lesion? *Carcinogenesis* **16**(10), 2447-53.

Fourier, K.B., Brooks, P. and Malinge, J.M. **2003** Binding discrimination of MutS to a set of lesions and compound lesions (base damage and mismatch) reveals its potential role as a cisplatin-damaged DNA sensing protein. *J Biol Chem Biol* **278**, 21267-21275.

Fruscella, E., Gallo, D., Ferrandina, G., D'Agostino, G. and Scambia, G. **2003** Gemcitabine: current role and future options in the treatment of ovarian cancer. *Clin Rev Oncol/Haematol* **48**(1), 81-88.

Funk, J.O. **1999** Cancer cell cycle control. *Anticancer Res* **19**, 4772-80.

Furuta, T., Ueda, T., Aunne, G., Sarasin, A., Kraemer, K.H. and Pommier, Y. **2002** Transcription-coupled nucleotide excision repair as a determinant of cispplatin sensitivity of human cells. *Cancer Res* **62**, 4899-4902.

Gaduuci, A., Cosio, S., Muraca, S. and Genazzani, A.R. **2002** Molecular mechanisms of apoptosis and chemosensitivity to platinum and paclitaxel in ovarian cancer: biological data and clinical implications. *Eur J Gynae Oncol* **23**(5), 390-6.

Garcia AA. Ovarian Cancer. www.emedicine.com/med updated October 2004. Accessed December 18th, **2004**

Garcia-Echeverria, C. and Sellers, W.R. Drug discovery approaches targeting the PI3K/Akt pathway in cancer. *Oncogene* **27**, 5511-5526, **2008**

Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M.S., Timmers, C., Hejna, J., Grompe, M. and D'Andrea, A.D. **2001** Interaction of the fanconi anaemia proteins and BRCA1 in a common pathway. *Mol cell* **7**, 249-262.

Garrett, M.D., Workman, P. **1999** Discovering novel chemotherapeutic regimens for the third millennium. *Eur J Cancer **35,*** 2010-2030

Gateley, D.P. and Howell, S.B. **1993** Cellular accumulation of the anticancer agent cisplatin: a review. *Br J of Cancer.* **67**, 1171-5.

Genschel, J., Bazemore, L.R., Modrich, P. **2002** Human exonuclease I is required for 5' and 3' mismatch repair. *J Biol Chem* **277**, 13302-11.

Gentleman, R.C., Carey, V.J., Bates D.M., Bolstad B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry J., Hornik, K., Hothorn, T., Huber W., Iacus S., Irizarry R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smythe, G., Tierney, L., Yang J.Y. and Zhang, J **2004** Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80.

Gershenson, DM. **1993** Update on malignant ovarian germ cell tumours. *Cancer* **71**(suppl 4), 1581-90.

Gjerset, R.A., Lebedeva, S., Haghighi, A., Turla, S.T., Mercola, D. **1999** Inhibition of the jun kinase pathway blocks DNA repair, enhances p53-mediated apoptosis and promotes gene amplification. *Cell Growth Differ* **10**, 545-54.

Goff, B.A., Holmberg, L.A., Veljovich, D., Kurland, B.F. **2008** Treatment of recurrent or persistent platinum-refractory ovarian, fallopian tube or primary peritoneal cancer with gemcitabine and topotecan A phase II trial of the Puget Sound Oncology Consortium. *Gynecol Oncol (epub ahead of print).*

Goldberg, M., Stucki, M., Falck, J., D'Amours, D., Rahman, D., Pappin, D., Bartek, J. and Jackson, S.P. **2003** MCD1 is required for the intra-S-phase DNA damage checkpoint. *Nature* **421**, 952-956.

Goldie, G.H. and Coldman, A.J. **1979** A mathematical model for relating the drug sensitivity of tumours to their spontaneous mutation rate. *Cancer Treat Rep* **63**, 1727-33.

Gordon, K. and Smyth. **2004** Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology*: **3**(1), Article 3.

Gore, M.E., Atkinson, R.J., Thomas, H., Cure, H., Rischin, D., Beale, P., Bougnoux, P., Dirix, L. and Smit, WM. **2002** A phase II trial of ZD0473 in platinum-pretreated ovarian cancer. *Eur J Cancer* **38**, 2416-2420.

Gottesman, M.M., Fojo, T. and Bates, S.E. **2002** Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* **2**, 48-58.

Gottesman, M.M. and Pastan, I. **1993** Biochemistry of mutidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62**, 385-427.

Gottlicher, M., Rahmsdorf, H.J. and Herrlich, P. **1997** Transcription Factors in Eukaryotes **1**, 67-93.

Green, J.A., Robertson, L.J. and Clark, A.H. **1993** Glutathione S-transferase expression in benign and malignant ovarian tumours. *Br J Cancer* **68**, 235-239.

Gu, L., Hong, Y., McCulloch, S., Watanabe, H. and Li, G.M. **1998** ATP-dependent interaction of human mismatch repair proteins and dual role of PCNA in mismatch repair. *Nucleic Acids Res* **26**, 1173-8.

Haber J.E. **1999** DNA recombination: the replication connection. *Trends Biochem Sci* **24**, 271-275.

Harkin, D.P., Bean, J.M., Miklos, D., Song, Y.H., Truong, V.B. and Englert, C. et al. **1999** Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. *Cell* **97**, 575-86.

Hartley, J.A. **2002** Alkylating agents from Souhami R.L, Tannock I, Hohenberger and Horiot J-C. Oxford textbook of oncology, second edition.

Hartley, J.M., Spanswick, V., Gander, M., Giacomini, G., Whelan, J., Souhami, R.L. and Hartley, J.A. **1999** Measurement of DNA cross-linking in patients on ifosfomide therapy using the single cell gel electrophoresis (comet) assay. *Clin Can Res* **5**, 507-512.

Hartmann, A.R. and Ford, J.M. **2002** BRCA1 induces DNA damage recognition factors and enhances nucleotide excision repair. *Nat Genet* **32**, 180-4.

Hartmann, L.C., Lu, K.H., Linette, G.P., Cliby, W.A., Kalli, K.R., Gershenson, D., Bast, R.C., Stec, J., Iartchouk, N., Smith, N.I., Ross, J.S., Hoersch, S., Shridhar, V., Lillie, R., Kaufmann, S.H., Clark, E.A. and Damokosh, A.I. **2005** Gene Expression Profiles Predict Early Relapse in Ovarian Cancer after Platinum-Paclitaxel Chemotherapy. *Clin Cancer Res* **11**, 2149-2155.

Hasan, J. and Jayson, G.C. **2003** Oral melphalan as a treatment for platinum-resistant ovarian cancer. *Br J Cancer* **88**, 1828-1830.

Hengartner, M.O. **2000** The biochemistry of apoptosis. *Nature* **407**, 770-776.

Heyer, W-D., Li, X., Rolfsmeier, M. and Zhang, X-P. **2006** Rad54: the swiss army knife of homologous recombination. *Nucleic Acids Res* **34**(15), 4115-4125.

Higby, D.J., Wallace, H.J Jr and Holland, J.F. **1973** *Cis*-dichlorodiammineplatinum (NSC-119875): a phase I study. *Cancer Chemotherapy Reports* **57**, 459-63.

Hoeijmakers, J.H. **2001** genome maintenance mechanisms for preventing cancer. *Nature* **411**, 366-74.

Hong, M., Lai, M.D., Lin, Y.S., Lai, M.Z. **1999** Antagonism of p53-dependent apoptosis by mitogen signals. *Cancer Res* **59** 2847-2852

Hu, L., Hofman, J., Lu, Y., Mills, G.B., and Jaffe, R.B. **2002** Inhibition of phosphatidyl 3'-kinase increases efficacy of paclitaxel in in vitro and in vivo ovarian cancer cell models. *Cancer Res.* **62**, 1087-1092.

Husain, A., He, G., Venkataraman, E.S. and Spriggs, D.R. **1998** BRCA1 up-regulation is associated with repair-mediated resistance to cis-diamminedichloroplatinum(II). *Cancer Res* **58**, 1120-3.

The International Collaberative Ovarian Neoplasm Group. **2002** Paclitaxel plus carboplatin versus standard chemotherapy with either single agent carboplatin or cyclophosphamide, doxorubicin, and cisplatin in women with ovarian cancer: the ICON3 randomised trial. *Lancet* **360**, 505-515.

Irizarry, R.A., Hobbs, B., Collin F., Beazer-Barclay, Y.D., Antonellis K.J., Scherf, U., Speed, T.P **2003** Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249-264.

Ishikawa, T. and Ali-Osman, F. **1993** Glutathione-associated *cis*-diammine-dichloroplatinum(II) metabolism and ATP-dependent efflux from leukaemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. *J Biol Chem* **268**, 20116-20125.

Jackson S.P. **2002** Sensing and repairing DNA double-strand breaks. Commentary. *Carcinogenesis* **23**, 687-696.

Jekunen, A.P., Homm, D.K., Alcaraz, J.E., Eastman, A. and Howell, S.B. **1994** Cellular pharmacology of dichloro(thylenediamine)platinum(II) in cispaltin sensitive and resistant human ovarian carcinoma cells. *Cancer Res* **54**, 2680-2687.

Jones, N.A., Turner, J., McIlwrath A.J., Brown R., Dive C. **1998** Cisplatin- and paclitaxel-induced apoptosis of ovarian carcinoma cells and the relationship between bax and bak up-regulation and the functional status of p53. *Mol Pharmacol* **53**, 819-826.

De Jong, M.C., Slootstra, J.W., Scheffer, G.L., Schroeijers, A.B., Puijk, W.C., Dinkelberg, R., Kool, M., Broxterman, H.J., Meloen, R.H. and Scheper, R.J **2001** Peptide transport by the multidrug resistance protein MRP1. *Cancer Res* **61**, 2552-2557.

Judson, I. and Kelland, L.R. Cisplatin and analogues from Souhami R.L, Tannock I, Hohenberger and Horiot J-C. Oxford textbook of oncology, second edition, **2002**

Kelland, L.R. **1993** New platinum antitumour complexes. *Crit Rev Oncol Haematol* **15**, 191-219.

Kennedy, R.D., Quinn, J.E., Mullan, P.B., Johnston, P.G. and Harkin, P.D. **2004** The role of BRCA1 in the cellular response to chemotherapy. *J Nat Cancer Institute*. **96** (22), 1659-1668.

King, M.C., Marks, J.H. and Mandell, J.B. **2003** Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. *Science* **302**, 643-6.

Knox, R.J., Friedlos, F., Lydall, D.A. and Roberts, J.J. **1986** Mechanism of cytotoxicity of anticancer platinum drugs: evidence that *cis* -diamminedichloroplatinum (II) and *cis*-diammine-(1,1 cyclobutanedicarboxylatoplatinum (II) differ only in the kinetics of their interaction with DNA. *Cancer Research* **46**, 1972-9.

Koberle, B., Masters, J.R., Hartley, J.A. and Wood, R.D. **1999** Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours. *Curr Biol* **9**, 273-76.

Kohn, K.W., Sartorelli, A.C., Lazo, J.S. and Bertino, J.R. **1981** Molecular mechanisms of crosslinking of alkylating agents and platinum complexes. Molecular actions and targets for cancer chemotherapeutic agents Academic, San Diego 3-16.

Kolasa, I.K., Rembiszewska, A., Felisiak A., Ziolkowska-Seta, I., Murawska M., Moes J., Timorek, A., Dansonka-Mieszkowska, A., Kupryjancyk, J. **2009** PIK3CA amplification associates with resistance to chemotherapy in ovarian cancer patients. *Cancer Biol Ther*. **17**, 8(1) Epub ahead of print.

Kolodner, R.D. and Marsischky, G.T. **1999** Eukaryotic DNA mismatch repair. *Cur.r Opin. Genet. Dev.* **9**, 89-96.

Kool, M., de Haas, M., Scheffer G.L. **1997** Analysis of expression of cMOAT(MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* **57**, 3537-47.

Kouzarides T. **1999** Histone acetylases and deacetylases in cell proliferation. *Curr Opin Genet Dev* **9**, 40.

Krueger, A., Baumann, S., Krammer, P.H. and Kirchoff, S. **2001** FLICE-inhibitory proteins:regulators of death receptor-mediated apoptosis. *Mol Cell Biol* **21**, 8247-8254.

Kunkel, T.A. and Erie, D.A. **2005** DNA mismatch repair. *Annu. Rev. Biochem*. **74**, 681-710.

Kupryja, J., Szymanska, T., Dry, R.M., Timorek, A., Stelmachow, J., Karpinska, G., Rembiszewska, A., Ziokowska, I., Kraszewska, E., Debniak, J., Emerich, J., Ulanska, M., Pluzanska, A., Jedryka, M., Goluda, M., Chudecka-Glaz, A., Rzepka-Gorska, I., Klimek, M., Urbanski, K., Breborowicz, J., Zielinski, J. And Markowska J. **2003** Evaluation of clinical significance of TP53, BCL-2, BAX and MEK1 expression in 229 ovarian carcinomas treated with platinum-based regimen. *Br J Cancer* **88**, 848-854.

Lafarg, S., Sylvain, V., Ferrara, M. and Bignon, Y.J. **2001** Inhibition of BRCA1 leads to increased chemoresistance to microtubule-interfering agents, an effect that involves the JNK pathway. *Oncogene* **20**, 6597-606.

Lee, K.B., Parker, R.J., Bohr, V., Cornelison, T. and Reed, E. **1993** Cisplatin sensitivity/resistance in UV repair-deficient Chinese hamster ovary cells of complementation groups 1 and 3. *Carcinogenesis* **14**, 2177-2180.

Lee, K.Y. and Bae, S-C. **2002** TGFβ-dependent cell growth arrest and apoptosis. *J Biochem and Mol Biol* **35**(1), 47-53.

Lee, S., Choi, E.J., Jin, C. and Kim, D.H. **2005** Activation of PI3K/Akt pathway by PTEN reduction and PI3KCA mRNA amplification contributes to cisplatin resistance in ovarian cell line. *Gynaecol. Oncol* **97**, 26-34.

Le Page, F., Randrianarison, V., Marot, D., Cabannes, J., Perricaudet, M., Feunteun, J. And Sarasin, A. **2000** BRCA1 and BRCA2 are necessary for the transcription-coupled repair of the oxidative 8-oxoguanine lesion in human cells. *Cancer Res* **60**, 5548-52.

Leppard, J.B., Dong, Z., Mackey, Z.B., and Tomkinson, A.E. **2003** Physical and functional interaction between DNA ligase IIIalpha and poly(ADP-Ribose) polymerase 1 in DNA single-strand break repair. *Mol Cell Biol*, **23**, 5919.

Levine, A.J. **1997** p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-331.

Li, Q., Bostick-bruton, F., and Reed, E. **1998** Effect of interleukin-1 alpha and tumour necrosis factor-alpha on cisplatin-induced ERCC1 mRNA expression in a human ovarian carcinoma cell line. *Anticancer Res* **18**(4a), 2283-7.

Li, Q., Gardner, K., Zhang, L., Tsang, B., Bostick-Bruton, F. and Reed, E. **1998** Cisplatin induction of ERCC1 mRNA expression in A2780/CP70 human ovarian cancer cells. *J Biol Chem* **273**, 23419-23425.

Li, Q., Yunmbam, M.K., Zhng, X., Yu, J.J., Mimnaugh, E.G., Neckers, L. and Reed, E. **2001** Lactacystin enhances cisplatin sensitivity in resistant human ovarian cancer cell lines via inhibition of DNA repair and ERCC1 expression. *Cell Mol Biol (Noisy-le-grand)* **47** online Pub, OL61-72.

Lister-Sharp, D., McDonagh, M.S., Khan, K.S. and Kleijen, J. **2000** A rapid and systematic review of the effectiveness and cost-effectiveness of the taxanes used in the treatment of advanced breast and ovarian cancer. *Health Technol Assess* **4**, 1-113.

Liu, Z.G., Baskaran, R. and Lea-Chou, E.T. **1996** Three distinct signalling responses by murine fibroblasts to genotoxic stress. *Nature* **384**, 273-276.

Ljungman, M. **2000** Dial 9-1-1 for p53:mechanisms of p53 activation by cellular stress. *Neoplasia* **2**, 208-225.

Loe, D.W., Deeley, R.G. and Cole, S.P. **1998** Characterization of vincristine transport by the M_r 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. *Cancer Res* **58**, 5130-5136.

Lomonosov, M., Anand, S., Sangrithi, M., Davies, R. and Venkitaraman, A.R. **2003** Stabilization of stalled DNA replication forks by the BRCA2 breast cancer susceptibility protein. *Genes Dev* **17**, 3017-3022.

Longley, D.B. and Johnston, P.G. **2005** Molecular mechanisms of drug resistance. *J of Pathology* **205**, 275-292.

Lowndes, N.F. and Murguia, J.R. **2000** Sensing and responding to DNA damage. *Curr Opin Genetics and development* **10**(1), 17-25.

Lucas, K.A., Pitari, G.M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K.P. and Waldman ,S.A. **2000** Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* **52**(3), 375-414.

Mackey, J.R., Mani, R.S., Selner, M., Mowles D., Young, J.D., Belt J.A., Crawford C.R. and Cass C.E **1998** Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. *Cancer Res* **58**(19), 4349-4357.

Magagnoli, M., Masci, G., Castagna, L., Rimassa, Bramanti, S. and Santoro, A. **2004** Intermediate-dose melphalan with stem cell support in platinum refractory ovarian cancer. *Bone Marrow Transplantation* **33**(12), 1261-1262.

Main, C., Bojke, L., Griffin, S., Norman, G., Barbieri, M., Mather, L., Stark, D., Palmer, S. and Riemsa, R. Topotecan. **2006** pegylated liposomal doxorubicin hydrochloride and paclitaxel for second-line subsequent treatment of advanced ovarian cacner: a systematic review and economic evaluation. *Health Technol Assess* **10**(9), 1-148.

Marx, D., Meden, H., Ziemek, T., Lenthe, T., Kuhn, W. and Schauer, A. **1998** Expression of the p53 tumour suppressor gene as a prognostic marker in platinum-treated patients with ovarian cancer. *Eur J Cancer* **34**, 845-850.

Masuda, H., Ozols, R.F., Lai, G.M., Fojo, A., Rothenberg, M. and Hamilton, T.C. **1988** Increased DNA repair as a mechanism of acquired resistance to cis-diamminedichloroplatinum(II) in human ovarian cancer cell lines. *Cancer Res* **48**(20), 5713-6.

Masuda, H., Tanaka, T., Matsuda, H., Kusaba, I. **1990** Increased removal of DNA-bound platinum in a human ovarian cancer cell line resistant to cis-diamminedichloroplatinum(II). *Cancer Res* **50**(6), 1863-6.

Masutani, C., Araki, M., Sugasawa, K., Van der Spek, P.J. and Yamada, A. **1997** Identification and characterisation of XPA-binding domain of hHR23B. *Mol Cell Biol* **17**, 6924-6931.

Materna, V., Liedert, B., Thornale, J. and Lage, H. **2005** protection of platinum-DNA adduct formation and reversal of cisplatin resistance by anti-MRP2 hammerhead ribozymes in human cancer cells. *Int. J. Cancer* **115**, 393-402.

Mattes, W.B., Hartley, J.A., Kohn, K.W. and Matheson, D.W. **1988** GC-rich regions in genomes as targets for DNA alkylation. *Carcinogenesis* **9**, 2065-72.

Mcguire, W.P., Hoskins, W.J., Brady, M.F., Kucera, R.R., Partridge, E.E., Look, K.Y., Clarke-Pearson, D.L. and Davidson M. **1996** Cyclophosphamide and cisplatin versus paclitaxel and cisplatin: a phase III randomised trial in patients with suboptimal stage III/IV ovarian cancer (from the Gynaecologic Oncology Group). *Semin Oncol* **23**(suppl 12), 40-47.

Mcguire, W.P. III. and Markman, M. **2003** Primary ovarian cacner chemotherapy: current standards of care. *Br J Cancer* **89**(suppl 3), S3-S8.

McHugh, P.J., Sones, W.R., and Hartley, J.A. **2000** Repair of intermediate structures produced at DNA interstrand crosslinks in *Saccharomyces cerevisiae*. *Mol Cell Biol*, **20**, 3425-3433.

McHugh, P.J., Spanswick, V.J., Hartley, J. A. **2001** Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. *Lancet* **2**, 483-490.

McLachlin CM. Diagnostic and Systemic Pathology; Ovarian Neoplasms. http://www.uwo.ca/pathol/MedsII/Reproduction/REPRO-Ovarian.pdf Revised July 2003. Accessed December 19th **2004**

Meyn, R.E., Jenkins, S.F. and Thompson, L.H. **1982** Defective removal of DNA cross-links in a repair deficient mutant of Chinese hamster cells. *Cancer Res* **42**, 3106-3110.
Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K. and Tavtigian, S. et al. **1994** A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* **266**, 66-71.

Mimnaugh, E.G., Chen, H.Y., Davie, J.R., Celis, J.E. and Neckers, L. **1997** Rapid deubiquitination of nucleosomal histones in human tumour cells caused by proteasome inhibitors and stress response inducers: Effects on replication, transcription, translation and the cellular stress response. *Biochemistry* **36**, 14418-14429.

Mimnaugh, E.G., Yunmbam, M.K., Li, Q., Bonvini, P., Hwang, S.G., Trepel, J., Reed E. and Neckers, L. **2000** Prevention of cisplatin-DNA adduct repair and potentiation of cisplatin-induced apoptosis in ovarian carcinoma cells by proteasome inhibitors. *Biochem Pharmacol* **60**(9), 1343-1354.

Mingo-Sion, A.M., Marietta, P.M, Koller, E., Wolf, D.M., Van Den Berg, C.L. **2004** Inhibition of JNK reduces G2/M transit independent p53, leading to endoreduplication, decreased proliferation, and apoptosis in breast cancer cells. *Oncogene* **23**, 596-604.

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G. and Gianni, L. **2004** Anthracyclines: molecular advances and pharmacologic evelopments in antitumour activity and cardiotoxicity. *Pharmacol Rev.* **56**, 185-229.

Mitchell, J.R., Hoeijmakers Jan, H.J., and Niedernhofer, L.J. **2003** Divide and conquer:nucleotide excision repair battles cancer and ageing *Curr Opin Cell Biol* **15**, 232-240.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B., Reed, J.C. **1994** Tumour suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. *Oncogene* **9,** 1799-1805.

Moorehead, R.A. and Singh, G. **2000** Influence of the proto-oncogene c-fos on cisplatin sensitivity. *Biochen Pharmacol* **59**, 337-45.

Moynahan, M.E., Cui, T.Y. and Jasin, M. **2001** Homolgy-directed DNA repair, mitomycin-c resistance, and chromosome stability is restored with correction of the BRCA1 mutation. *Cancer Res* **61**, 4842-50.

Moynahan, M.E., Pierce, A.J. and Jasin, M. **2001** BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol Cell.* **7**, 263-72.

Mu, D., Hsu, D.S. and Sancar, A. **1995** Reaction mechanism of human DNA repair excision nuclease. *J Biol Chem* **270**, 2415-2418.

Mullan, P.B., Quinn, J.E., Gilmore, P.M., Mcwilliams, S., Andrews, H., Gervin, C. et al. **2001** BRCA1 and GADD45 mediated G2/M cell cycle arrest in response to antimicrotubule agents. *Oncogene* **20**, 6123-31.

Nagle, C.M., Chenevix-Trench, G., Spurdle, A.B. and Webb, P.M. **2007** The role of glutathione-S-transferase polymorphisma in ovarian cancer survival. *Eur J Cancer* **43 (2)**, 283-90.

Nagata, S. **1999** Fas ligand-induced apoptosis. *Annu Rev Genet* **33**, 29-55.

National Institutes of Health (online January 2006) NCI issues clinical announcement for preferred method of treatment for advanced ovarian cancer [http://www.nih.gov/news/pr/jan2006/nci-04.htm] (accessed 21 April **2006**)

National Cancer Institute. www.imsdd.meb.uni-bonn.de/cancer.gov/ Accessed December 18th, **2004**

National Institutes of Health. **1994** Ovarian cancer: Screening, treatment and follow up. *NIH Consensus statement.* **12**(3), 1-30.

O'Connor, P.M. and Kohn, K.W. **1990** Comparative pharmacokinetics of DNA lesion formation and removal following treatment of L1210 cells with nitrogen mustards. *Cancer commun* **2**, 387-94.

Olaussen, K.A., Dunnant, A., Fouret, P., Brambilla, E., Andre, F., Haddad, V., Taranchon, E., Filipits, M., Pirker, R., Popper, H.H., Stahel, R., Sabatier, L., Pignon, J.P., Le Chavalier, T. and Soria, J.C: IALT Bioinvestigators. **2006** DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Eng J Med* **355**(10), 983-91.

Ozols, R.F., Schwartz, P.E. and Eifel, P.J. **2001** Ovarian cancer, Fallopian tube Carcinoma and Peritoneal Carcinoma: Cancer principles and Practice of Oncology, 6th edn. Philadelphia: Lippincott Williams and Wilkin.

Ozols, R.F. **1992** Role of chemotherapy in the future treatment of ovarian cancer. *Acta Obstet Gynaecol Scand Suppl.* **155**, 55-60.

Pacques, F. and Haber, J. **1999** Multiple pathways of recombination induced by double-strand breaks in *saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **63**(2), 349-404.

Pakunlu, R.I., Cook, T.J. and Minko, T. **2003** Simultaneous modulation of multidrug resistance and antiapoptotic cellular defence by MDR1 and Bcl-2 targeted antisense oligonucleotides enhance the anticancer efficacy of doxorubicin. *Pharm Res* **20**, 351-359.

Papouli, E., Cejka, P. and Jiricny, J. **2004** Dependence of the cytotoxicity of DNA-damaging agents on the mismatch repair status of human cells. *Cancer Res* **64**, 3391-3394.

Park, C.H., Bessho, T., Matsunaga, T. and Sancar, A. **1995** Purification and characterization of the XPF-ERCC1 complex of human DNA repair excision nuclease. *J Biol Chem* **270**, 22657-22660.

Parker, R.J.,Eastman, A., Bostick-Bruton, F. and Reed, E. **1991** Acquired cispaltin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation. *J Clin Invest* **87**, 772-777.

Paull, T.T., Cortez, D., Bowers, B. and Elledge, S.J. Gellert M. **2001** Direct DNA binding by Brca1. *Proc. Natl. Acad. Sci U.S.A* **98**, 6086-6091.

Paull, T.T. and Gellert, M. **1999** Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11-Rad50 complex. *Genes Dev* **13**, 1276-1288.

Paull, T.T., Rogakou, E.P., Yamazaki, V., Kirchgessner, C.U., Gellert, M. and Bonner, W.M. **2000** A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* **10**, 886-895.

Pestell, K.E., Hobbs, S.M., Titley, J.C., Kelland, L.R. and Walton, M.I. **2000** Effect of p53 status on sensitivity to platinum complexes in a human ovarian cacner cell line. *Mol Pharmacol* **57**, 503-511.

Pfisterer, J., Weber, B., Reuss, A., Kimmig, R., du Bois, A., Wagner, U., Bourgeois, H., Meier, W., Costa, S., Blohmer, J.U., Lortholary, A., Olbricht, S., Stahle, A., Jackisch, C., Hardy-Bessard, A.C., Mobus, V., Quaas, J., Richter, B., Schroder, W., Geay, J.F., Luck, H.J., Kuhn, W., Meden, H., Nitz, U., Pujade-Lauraine, E; AGO-AVCAR; GINECO. **2006** Randomized phase III trial of topotecan following carboplatin and paclitaxel in first-line treatment of advanced ovarian cancer: a gynecologic cancer intergroup trial of the AGO-OVAR and GINECO. *J Natl Cancer Inst* **98**(15), 1024-6.

Piccart, M.J., Bertelson, K., James, K., Cassidy, J., Mangioni, C., Simonsen, E., Stuart, G., Kaye, S., Vergote, I., Blom, R., Grimshaw, R., Atkinson, R.J., Swenerton, K.D., Trope, C., Nardi, M., Kaern, J., Tumolo, S., Timmers, P., Roy, J.A., Lhoas, F., Lindvall, B., Bacon, M., Birt, A., Anderson, J.E., Zee, B., Paul, J., Baron, B. and Pecorelli, S. **2000** Randomized intergroup trial of cisplatin-paclitaxel versus cisplatin-cyclophosphamide in women with advanced epithelial ovarian cancer: three year results. *J Natl Cancer Inst* **92**, 699-708.

Plaza, J.A., Dominguez, F. and Suster, S. **2004** Cystic adenomatoid tumour of the mediastinum. *Am J Surg Pathol* **28**(1), 132-8.

Plumb, J.A., Strathdee, G., Sluddeen, J., Kaye, S.B. and Brown, R. **2000** Reversal of drug resistance in human tumour xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. *Cancer Res* **60**, 6039-6044.

Pommier, Y., Sordet, O., Antony, S., Hayward, R.L. and Kohn, K.W. **2004** Apoptosis defects and chemotherapy resistance: molecular interactions and networks. *Oncogene* **23**, 2934-2949.

Potapova, O., Haghighi, A., Bost, F., Liu, C., Birrer, M.J., Gjerset, R. and Mercola, D. **1997** The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. *J Biol Chem* **272**, 14041-14044.

Pu, Q., Bianchi, P. and Bezwoda, W.R. **2000** Alkylator resistance in human B lymphoid cell lines: melphalan accumulation, cytotoxicity, interstrand DNA-crosslinks, cell cycle analysis, and glutathione content in the melphalan-sensitive B-lymphocytic cell line (WL2) and in the resistant B-CLL cell line (WSU-CLL). *Anticancer Res* **20**, 2561-2568.

Pulverer, B., Kyriakis, J.M., Avruch, J., Nikolakakie, E. and Woodgett, J. **1991** Phosphorylation of c-jun mediated by MAP kinases. *Nature* **353**, 670-674.

Quinn, J.E., Kennedy, R.D., Mullan, P.B., Gilmore, P.M., Carty, M., Johnston, P.G., et al. **2003** BRCA1 functions as a differential modulator of chemotherapy-induced apoptosis. *Cancer Res* **63**, 6221-8.

Rang, H.P., Dale, M.M. and Ritter, J.M. **1995** Pharmacology 3rd edn. Churchill Livingstone.

Raffo, A.J., Kim, A.L. and Fine, R.L. **2000** Formation of nuclear bax/p53 complexes is associated with chemotherapy induced apoptosis. *Oncogene* **19**, 6216-6228.

Raymond, E., Faivre, S., Chaney, S., Woynarowski, J. and Cvitkovic, E. **2002** Cellular and molecular pharmacology of oxaloplatin *Mol Cancer Ther* **1**, 227-235.

Richardson, C. and Jasin, M. **2000** Coupled homologous and nonhomologous repair of double-strand breaks preserves genomic integrity in mammalian cells. *Mol Cell Biol* **23**, 9068-75.

Ringborg, U., Henriksson, R. and Friberg, S. **1998** Ovarian cancer. *Onkologi*, Liber, Stockholm 457-479.

Rocconi, R.P., Straughn, J.M., Leath, C.A. 3rd, Kilgore, L.C., Huh, W.K., Barnes, M.N. 3rd, Partridge, E.E. and Alvarez, R.D. **2006** Pegylated liposomal doxorubicin consolidation therapy after platinum/paclitaxel-based chemotherapy for suboptimally debulked, advanced-stage epithelial ovarian cancer patients. *Oncologist* **11**(4), 336-41.

Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S. and Bonner, W.M. **1998** DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* **273**, 5858-5868.

Rose, P.G., Blessing, J.A., Mayer, A.R.and Homesley, H.D. **1998** Prolonged oral etoposide as second-line therapy for platinum-resistant and platinum-sensitive ovarian carcinoma: a Gynaecologic Oncology Group study. *J Clin Oncol* **2**, 405-410.

Rosenberg, B. **1985** Fundamental studies with cisplatin. *Cancer* **55**, 2303-16.

Rothbarth, J., Vahrmeijer, A.L. and Mulder, G.J. **2002** Modulation of cytostatic efficacy of melphalan by glutathione:mechanisms and efficacy. *Chemico-Biological Interactions* **140**(2), 93-107.

Roy, G., Horton, J.K., Roy, R., Denning, T., Mitra, S., and Boldogh, I. **2000** Acquired alkylating drug resistance of a human ovarian carcinoma cell line is unaffected by altered levels of pro- and anti-apoptotic proteins. *Oncogene* **19**(1), 141-150.

Rudd, G.N., Hartley, J.A. and Souhami, R.L. **1995** Persistence of cisplatin-induced DNA interstrand crosslinking in peripheral blood mononuclear cells from elderly and young individuals. *Cancer Chemother Pharmacol* **35**(4), 323-6.

Runowiscz, C. **2006** Should patients with ovarian cancer receive intraperitoneal chemotherapy following initial cytoreductive surgery? *Nature Clin Practice Oncol* **3**, 416-7.

Russo, M.T., Blasi, M.F., Chiera, F., Fortini, P., Degan, P., Macpherson, P., Furuichi, M., Nakabeppu, Y., Karran, P., Aquilina, G. and Bignami, M. **2004** The oxidized deoxynucleoside triphosphate pool is a significant contributor to genetic instability in mismatch repair-deficient cells. *Mol Cell Biol* **24**, 465-74.

Rustin, G.J. **2003** Use of Ca-125 to assess response to new agents in ovarian cancer trials. *J Clin Oncol* **21**(10), 187-193.

Rustin, G.J., Bast Jr, R.C., Kelloff, G.J., Barrett, J.C., Carter, S.K., Nisen, P.D., Sigman, C.C., Parkinson, D.R. and Ruddon, R.W. **2004** Use of Ca-125 in clinical trial evaluation of new therapeutic drugs for ovarian cancer. *Clinical Cancer Res.* **10**, 3919-3926.

Rustin, G.J., Marples, M., Nelstrop, A.E., Mahmoudi, M. and Meyer, T. **2001** Use of Ca-125 to define progression of ovarian cancer in patients with persistently elevated levels. *J clin Oncol.* **19**(20), 4054-7.

Rustin, G.J., Nelstrop, A.E., Tuxen, M.K. and Lambert, H.E. **1996** Defining progression of ovarian carcinoma during follow-up according to Ca 125: a North Thames Ovary Group Study. *Ann Oncol* **7**(4), 361-4.

Saijo, M., Kuraoka, I., Masutani, C., Hanaoka, F. and Tanaka, K. **1996**
Sequentioal binding of DNA repair proteins RPA and ERCC1 to XPA *in vitro*. *Nuceil Acids Res* **24**, 4719-4724.

Sakamoto, M., Kondo, A., Kawasaki, K., Goto, T., Sakamoto, H., Miyake, K., Koyamatsu, Y., Akiya, T., Iwabuchi, H., Muroya., Ociai, K., Tanaka, T., Kikuchi Y. And Tenjin, Y. **2001** Analysis of gene expression profiles associated with cisplatin resistance in human ovarian cancer cell lines and tissues using cDNA microarray. *Hum Cell* **14**, 305-315.

Salvesen, G.S. and Duckett, C.S. **2002** IAP proteins: blocking the road to death's door. *Nature Rev Mol cell Biol* **3**, 401-410.

Sancar A. **1996** DNA excision repair. *Annu Rev Biochem* **65**, 43-81.

Sankaranarayanan, K. and Wassom, J.S. **2005** Ionising radiation and genetic risks XIV. Potential research directions in the post-genome era based on knowledge of repair of radiation-induced DNA double-strand breaks in mammalian somatic cells and the origin of deletions associated with human genomic disorders. *Mutation Res* **578**, 333-370.

Sargent, R.G., Meservy, J.L., Perkins, B.D., Kilburn, A.E., Intody, Z., Adair G.M., Nairn, R.S. and Wilson, J.H. **2000** Role of nucleotide escision repair gene *ERCC1* in formation of recombination-dependent rearrangemtns in mammalian cells. *Nucleic Acids Res* **28**, 3771-78.

Scheid, M.P., Duronio, V. **1998** Dissociation of cytokine-induced phosphorylation of bad and activation of PKB/akt: involvement of MEK upstream of bad phosphorylation. *Pro natl Acad Sci USA* **95**: 7439-7444.

Schiff, P.B., Fabt, J., Horwtiz, S.B. **1979** Promotion of microtubule assembly *in vitro* by Taxol. *Nature* **227**, 665-667.

Schmidt, W. and Chaney, S.G. **1993** Role of carrier ligand in platinum resistance of human carcinoma cell lines. *Cancer Res* **53**(4), 799-805.

Schroder, C.P., Godwin, A.K., O'Dwyer, P.J., Tew, K.D., Hamilton, T.C. and Ozols, R.F. **1996** Glutathione and drug resistance. *Cancer Invest* **14**, 158-168.

Schuler, M. and Green, D.R. **2001** Mechanisms of p53-dependent apoptosis. *Biochem Soc Trans* **29**, 684-688.

Selvakumaran, M., Pisarcik, D.A., Bao, R., Yeung, A.T. and Hamilton, T.C. **2003** Enhanced cisplatin cytotoxicity by disturbing the nucleotide excision repair pathway in human ovarian cancer cell lines. *Cancer Res* **63**(6), 1311-6.

Serroy, S.F., Scully, R.E. and Robin, I.H. **1973** International Histological classification of Tumours: No. 9. Histological Typing of Ovarian Tumours. Geneva World Health Organisation.

Settleman, J., Albright, C.F., Foster, L.C. and Weinberg, R.A. **1992** Association between GTPase activators for Rho and Ras families. *Nature* **359**(6391), 153-4.

Singer, B. **1977** Sites in nucleic acids reacting with alkylating agents of differing carcinogenicity or mutagenicity. *J of Toxicology and Environmental Health* **2**, 1279-95.

Smyth, G.K. **2004** Linear models and empirical bayes method for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* **3**(1), Article 3.

Spanswick, V.J., Craddock, C., Sekhar M., Mahendra, P., Shankaranarayana, P., Hughes, R.G., Hochhauser, D., Hartley, J.A. **2002** Repair of DNA interstrand crosslinks as a mechanism of clinical resistance to melphalan in multiple myeloma. *Blood* (1):224-9.

Spentzos, D., Levine, D.A., Kolia, S., Out, H., Boyd, J., Libermann, T.A., Cannistra, S.A. **2005** Unique Gene Expression Profile Based on Pathologic Response in Epithelial Ovarian Cancer. *J Clin Oncol* **23**(31), 7911-7918.

Sridhar, J., Akula, N. and Pattabiraman, N. **2006** Selectivity and potency of cyclin-dependent kinase inhibitors. *The aaps journal* **8**(1), E204-21.

Trevino, T., Falciani, F., and Barrera-Saldaña, H.A. **2007** DNA Microarrays: a Powerful Genomic Tool for Biomedical and Clinical Research. *Mol. Med* **13**(9-10), 527–541.

Starling, J.J., Shepard, R.L., Cao, J., Law, K.L., Norman, B.H., Kroin, J.S. et al. **1997** Pharmacological characterization of LY335979: a potent cyclopropyldibenzosuberane modulator of p-glycoprotein. *Adv Enzyme Regul* **37**, 335-347.

Stojic, L., Brun, R. and Jiricny, J. **2004** Mismatch repair and DNA damage signalling. *DNA Repair* **3**, 1091-101.

Stuart, G.C.E. **2003** First Line treatment regimens and the role of consolidation therapy in advanced ovarian cancer. *Gynaecologic Oncology* **90**(3) suppl 1, S8-S15.

Sugasawa, K., Ng, J.M., Matsutani, C., Iwai, S., Van der Spek, P.J., Eker, A.P., Hanaoka, F., Bootsma, D. and Hoeijmakers, J.H.J. **2000** Xeroderma pigmentosum group C protein complex is the initiator of global genomic nucleotide excision repair. *Mol Cell* **5**, 737-744.

Swift, L.P., Raphaeli, A., Nudelman, A., Philips, D.R. and Cutts, S.M. **2006** Doxorubicin-DNA adducts induce a form of non-topoisomerase II- mediated form of cell death. *Cancer Res* **66**(9), 4863-4871.

Sylvain, V., Lafarge, S. and Bignon, Y.J. **2002** Dominant-negative activity of a BRCA1 truncation mutant:effects on proliferation, tumorigenicity in vivo, and chemosensitivity in a mouse ovarian cancer cell line. *Int J Oncol* 845-53.

Symington, L. **2002** Role of *RAD52* Epistasis Group Genes in Homologous Recombination and Double-Strand Break Repair.*Microbiol Mol Biol Rev* **66**(4), 630–670.

Taniguchi, K., Wada, M., Kohno, K. **1996** A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. *Cancer Res* **56**, 4124-9.

Tannock, I.F. **1986** Experimental chemotherapy and concepts related to the cell cycle. *Int J of Radiation Biol* **49**, 335-55.

Thangaraju, M., Kaufmann, S.H. and Crouch, F.J. **2000** BRCA1 facilitates stree-induced apoptosis in breast and ovarian caner cell lines. *J Biol Chem* **275**, 33487-96.

Therasse, P., Arbuck, S.G., Eisenhauer, E.A., Wanders, J., Kaplan, R.S., Rubinstein, L., Verweijj, J., Van Glabbeke, M., Van Oosterom, A.T., Christian, M.C., Gwyther, S.G. **2000** New guidelines to evaluate the response to treatment in solid tumours. *J Natl Cancer Institute* **92**(3), 205-216.

Thomas, H. and Coley, H.M. **2003** Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting p-glycoprotein. *Cancer Control* **10**, 159-165.

Ting, N.S.Y. and Lee, W-H. **2004** The DNA bouble-strand break response pathway:becoming more BRCAish than ever. *DNA repair* **8(**9), 935-944.

Tutt, A. and Ashworth, A. **2002** The relationship between the roles of BRCA genes in DNA repair and cancer predisposition. *Trends Mol Med* **8**, 571-6.

Umar, A., Buermeyer, A.B., Simon, J.A., Thomas, D.C. and Clark, A.B. **1996** Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. *Cell* **87**:65-73.

Underwood, J.C.E. General and Systematic Pathology, 3rd edn. Churchill and Livingstone **2000**.

Vaisaman, A., Varenchenko, M., Umar, A., Kunkel, T.A., Risinger, J.I., Barrett, J.C., Hamilton, J.C. and Chaney S.G. **1998** The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and oxaliplatin resistance:correlation with replicative bypass of platinum-DNA adducts. *Cancer Res* **58,** 3579-3585.

Van der Zee, A.G., Hollema, H., de Brujin, H.W., Willemse, P.H., Boonstra, H., Mulder, N.H., Aalders, J.G. and de Vries, E.G. **1995** Cell Biological marker of drug resistance in ovarian carcinoma. *Gynaecol Oncol* **58**, 165-178.

Van der Zee, A.G., Hollema, H., Suurmeijer, A.J.H., Krans, M., Sluiter, W.J., Willemse, P.H., Aalders, J.G. and de Vries, E.G.. **1995** Value of P-glycoprotein, glutathion S-transferase pi, c-erbB-2, and p53 as prognostic factors in ovarian carcinomas. *J Clin Oncol* **13**, 70-78.

Vasey, P.A. **2003** Resistance to chemotherapy in advanced ovarian cancer: mechanisms and current strategies. *British J Cancer* **89**(suppl 3), S23-S28.

Vasey, P.A. **2002** Survival and longer-term toxicity results of the SCOTROC study: docetaxel-carboplatin (DC) vs paclitaxel-carboplatin (PC) in epithelial ovarian cancer (EOC). *Proc Am Soc Clin Oncol* **21**, 202a.

Venables, W.N. and Ripley, B.D. **2002** *Modern Applied Statistics with S* (Springer-Verlag, New York).

Venkitaraman, A.R. **2001** Functions of BRCA1 and BRCA2 in the biological response to DNA damage. *J Cell Sci* **114**, 3591-8.

Viallard, J.F., Lacombe, F., Belloc, F., Pellegrin, J.L. and Reiffers, J. **2001** Molecular mechanisms controlling the cell cycle: fundamental aspects and implicatins for oncology. *Cancer Radiother* **5**(2), 109-29.

Villella, J., Marchetti, D., Odunsi, K., Rodabaugh, K., Driscoll, D.L., and Lele, S. **2004** Response of combination platinum and gemcitabine chemotherapy for recurrent epithelial ovarian carcinoma.*Gynecology Oncology* **95**(3), 539-545.

Viiri, K.M., Korkeamaki, H., Kukkonen, M.K., Nieminen, L.K., Lindfors, K., Peterson, P., Maki, M., Kainulainen, H., Lohi, O. **2006** SAP30L interacts with members of the Sin3A corepressor complex and targets Sin3A to the nucleolus. **34**(11), 3288-98.

Vogelstein, B., Lane, D. and Levine, A.J. **2000** Surfing the p53 network. *Nature* **408**, 307-310.

Wada, M., Toh, S., Taniguchi, K. **1998** Mutations in the canalicular multispecific organic anion transporter (cMOAT) gene, a novel ABC transporter, in patients with hyperbilirubinaemia II/Dubin-Johnson syndrome. *Hum Mol Genet* **7,** 203-7.

Wang, B., Matsuoka, S., Carpenter, P.B. and Elledge, S.J. **2002** 53BPI, a mediator of the DNA damage checkpoint. *Science* **298**, 1435-1438.

Wang, C., Horiuchi, A., Imai, T., Ohira, S., Itoh, K., Nikaido, T., Katsuyama, Y. And Konishi, I. **2004** Expression of BRCA1 protein in benign, borderline, and malignant epithelial ovarian neoplasms and its relationship to methylation and allelic loss of the BRCA1 gene. *J Pathol* **202**, 215-23.

Wang, D., Hara, R., Singh, G., Sancar, A., Lippard, S.J. **2003** Nucleotide excision repair from site-specifically platinum-modified nucleosomes. *Biochem* **42**, 6747-6753.

Wang, J.C. **1996** DNA topoisomerases. *Annu Rev Biochem* **65**, 635-692.

Wang, Y., Cortez, Yazdi, P., Neff, N., Elledge, S.J. and Qin, J. **2000** BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev* **14**, 927-39.

Watanabe, Y., Koi, M., Hemmi, H., Hoshai, H. and Noda, K. **2001** A change in microsatelite instability caused by cisplatin-based chemotherapy of ovarian cancer. *Br J Cancer* **85**, 1064-1069.

Weeda, J., Donker, I., deWit, J., Morreau, R., Janssens, R., Vissers, C.J., Nigg, A., van Steeg, H., Bootsma, D. And Hoeijmakers, J.H. **1997** Disruption of mouse *ERCC1* results in novel repair syndrome with growth failure, nuclear abnormalities and senescence. *Curr Biol* **7**, 427-439.

Williams, J., Lucas, P.C., Griffith, K.A., Choi, M., Fogoros, S., Hu,Y.Y. and Liu, J.R. **2005** Expression of Bcl-x_L in ovarian carcinoma is associated with chemoresistance and recurrent disease. *Gynaecol Oncol* **96**, 287-295.

Williamson, E.A., Dadmanesh, F. and Koeffler, H.P. **2002** BRCA1 transactivates the cyclin-dependent kinase inhibitor p27 (kip1). *Oncogene* **21**, 3199-206.

Wilson, L.A., Yamamoto, H., Singh, G. **2004** Role of the transcription factor Ets-1 in cisplatin resistance. *Mol Cancer Ther* **3**, 823-832.

Wolf, J.K., Bodurka-Bevers, D., Gano, J., Deavers, M.T., Levenback, C. **2000** A phase 1 trial of ADP53 for ovarian cancer patients:correlation with p53 and anti-adenovirus ab status. *Proc Am Soc Clin Oncol* **19**, 1510.

Wolffe, A.P. and Hayes, J.J. **1999** chromatin disruption and modification. *Nucleic acids Res* **27**, 711-720.

Wooster, R. and Weber, B. **2003** Breast and ovarian cancer. *N Engl J Med* **348**, 2339-2347.

Wu, X.M., Fan, W., Xu, S.W. and Zhou, Y.K. **2003** Sensitization to the cytotoxicity of cisplatin by transfection with nucleotide excision repair gene xeroderma pigmentosum group a antisense RNA in human lung adenocarcinoma cells. *Clin Cancer Res* **9**, 5874-5879.

Wynne, P., Newton, C., Ledermann, J.A., Olaitan, A., Mould, T., Hartley, J.R. **2007** Enhanced repair of DNA interstrand crosslinking in ovarian cancer cells from patients following treatment with platinum-based chemotherapy. *Br J Cancer* **8;97**(7), 927-33.

Wynne, P. Enhanced repair of DNA interstrand crosslinking in ovarian cancer cells from patients following treatment with platinum-based chemotherapy. **2006** PhD thesis.

Xie, Y., Counter, C. and Alani, E. **1999** Characterisation of the repeat-tract instability and mutator phenotypes conferred by a Tn3 insertion in RFC1, the large subunit of the yeast clamploader. *Genetics* **151**(2), 499-509.

Xu, Y. and Villalona-Calero, M.A. **2002** Irinotecan: mechanisms of tumour resistance and novel strategies for modulating its activity. *Ann Oncol* **13**, 1841-1851.

Yan, Q-W., Reed,E., Zhong, X-S., Thornton, K., Guo, Y., Yu, J.J. **2006** MZF1 possesses a repressively regulatory function in ERCC1 expression. *Biochem Pharmacol* **71**(6), 761-771.

Yang, X., Xing, H., Gao, Q., Chen, G., Lu, Y., Wang, S., Zhou, J., Hu, W. and Ma, D. **2004** Resistance to chemotherapy-induced apoptosis via decreased caspase-3 activity and overexpression of antiapoptotic proteins in ovarian cancer. *J. Cancer Res. Clin. Oncol.* **130**, 423-428.

Yarden, R.I., Pardo-Reoyo, S., Sgagias, M., Cowan, K.H. and Brody, L.C. **2002** BRCA1 regulates the G_2/M checkpoint by activating Chk1 kinase upon DNA damage. *Nat Genet* **30**, 285-9.

Yazlovitskava, E.M., DeHaan, R.D. and Persons, D.L. **2001** Prolonged wild-type p53 protein accumulation and cisplatin resistance. *Biochen. Biophys. Res. Commun* **283**, 732-737.

Yokoyama, Y., Sato, S., Fukushi, Y., Sakamoto, T., Futagami, M. and Saito, Y. **1999** Significance of multi-drug-resistant proteins in predicting chemotherapy response and prognosis in epithelial ovarian cancer. *J Obstet Gynaecol Res* **25**, 387-394.

Yoshida, K. and Yoshio, M. **2004** Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci* **95**, 866-871.

Yoshida, M., Furumai, R., Nishiyama, M., Komatsu, Y., Nishino, N., Horinouchi, S. **2001** Histone deacetylase as a new target for cancer chemotherapy. *Cancer Chemother Pharmacol*, **48**(suppl 1), S20-S26.

Youn, C-K., Kim, M-H., Cho, H-J., Kim, H-B., Chang, I.Y., Chung, M.H. and You, H.J. **2004** Oncogenic H-Ras up-regulates expression of ERCC1 to protect cells from platinum-based anticancer agents. *Cancer Research* **64**, 4849-4857.

Young, R.C., Von Hoff, D.D., Gormley, P., Makuch, R., Cassidy, J., Howser, D., Bull, J.M. **1979** Cis-dichlorodiammineplatinum(II) for the treatment of advanced ovarian cancer. *Cancer Treat Rep* **63**, 1539–1544.

Yuan, Z.Q., Feldman, R.I., Sussman, D., Coppola, D., Nicosia, S.V. and Cheng, J.Q. **2003** AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: implication of AKT2 in chemoresistance. *J. Biol. Chem* **278**, 23432-23440.

Zamble, D.B., Mu, D., Reardon, J.T., Sancar, A., and Lippard, S.J. **1996** Repair of cisplatin-DNA adducts by the mammalian excision nuclease. *Biochemistry* **35**, 10004-10013.

Zdraveski, Z.Z., Mello, J.A., Marnus, M.G., and Essigmann, J.M. **2000** Multiple pathways of recombination define cellular responses to cisplatin. *Chem Biol* **7**, 39-50.

Zhang, J., Willers, H., Feng, Z., Ghosh, J.C., Kim, S., Weaver, D.T., Chung, J.H., Powell, S.N. and Xia, F. **2004** Chk2 phosphorylation of BRCA1 regulates DNA double-strand break repair. *Mol Cell Biol* **24**, 708-18.

Zhen, W., Link, C.J. Jnr., O'Connor, P.M., Reed, E., Parker, R., Howell, S.B. and Bohr, V.A. **1992** Increased gene-specific repair of cisplatin interstrand crosslinks in cisplatin-resistant human ovarian cancer cell lines. *Mol Cell Biol* **12**(9), 3689-98.

Zhong, Q., Chen, C.F., Li, S., Chen, Y., Wang, C.C., Xiao, J., Chen, P.L., Sharp, Z.D. and Lee, W.H. **1999** Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. *Science* **285**, 747-50.

Zhong, X., Thornton, K. and Reed, E. **2000** Computer based analyses of the 5'-flanking regions of selected genes involved in the nucleotide excision repair complex. *Int J Oncol* **17**(2), 375-80.

Zhou, C., Smith, J.L. and Liu, J. **2003** Role of BRCA1 in cellular resistance to paclitaxel and ionizing radiation in an ovarian cancer cell line carrying a defective BRCA1. *Oncogene* **22**, 2396-404.

Zou, L. and Elledge, S.J. **2003** Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* **300**, 1542-8.

Zwelling, L.A., Anderson, T. and Kohn, K.W. **1979** DNA-protein and DNA interstrand cross-linking by cis- and trans-platinum(II) diamminedichloride in L1210 mouse leukemia cells and relation to cytotoxicity. *Cancer Res* **39**(2 pt 1), 365-9.

APPENDIX 1 – Study protocol

University College London Hospitals NHS
NHS Foundation Trust

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Telephone: 020 7387 9300

STUDY PROTOCOL

Version: 2
October 7th 2004
Ref no: 04/Q0505/77

TITLE : The Mechanism of Resistance to Chemotherapy Drugs in Ovarian Cancer

Aims:
It is thought that patients who are resistant to chemotherapy agents (particularly platinum-based), repair the DNA interstrand crosslinking that is caused by these agents.
The objective of the study is to look at the gene expression in the chemotherapy resistant cells that repair their DNA, and the chemotherapy sensitive cells, to see if there are any differences in gene expression.
This will be done by splitting patients into two groups based on clinical findings. The amount of DNA interstrand crosslinking will be looked at in each patient, to confirm or refute their resistance to chemotherapy. Then gene expression will be measured.

Selection of patients
Patients with ovarian cancer are included in the study, there are no exclusions. Patients will be approached in clinics, and on wards. They will be given study information sheets and told about the study. They will be given time to think about whether or not they want to be included. Written signed consent will be taken from the patients wanting to participate. Their samples will be held up to 5 years if they have given consent specifically for this, and then destroyed. If they have not consented for storage for 5 years, the samples will be destroyed after this study.

1. Collection of tumour, and mesothelial cells
Tumour and mesothelial cells will be separated from ascitic, or pleural effusion samples, received from patients at the time of drainage, or operation. Separation will involve splitting them by trypsinisation. Mesothelial cells attach and detach from the flasks quicker than tumour cells. Therefore timing of removing cells still suspended in media is essential.
Gross phenotypical differences between the two cell types exist, which will make it easy to visibly gauge the level of contamination between the two cell types. Immunocytochemistry will be performed to confirm the predominance of each cell type.

2. Collection of solid tumour cells
Primary tumour tissue will be finely chopped in sterile conditions, and suspended in media.

3. Collection of blood samples
Lymphocytes will be isolated from patients' blood as a source of germ line DNA.

4. Ovarian cell lines
Ovarian Cell lines will also be used in this study.

5. Experiments involving Ovarian cell lines
Initially these cell lines will be used to ascertain the cisplatin IC50. This is the dose of cisplatin that will cause 50% of the cells to die. The ovarian cell lines will be incubated with cisplatin for 1 hour, and the cells incubated in media for 6 days following this. The SRB (sulphorhodamine B) assay will be used in this experiment
Different doses of cisplatin will be incubated for 1 hour in these cell lines. The amount of DNA interstrand crosslinking present in the two cell lines will be investigated using the Comet Assay.
The IC50 dose will be used in a timed drug response experiment. The two Ovarian cell lines will be incubated with cisplatin for 1 hour, and the level of DNA interstrand crosslinking measured (using the Comet Assay) at different times e.g 0,6,9,24,30,48,72 hours.
DNA microarrays will be used on these cell lines to investigate the up-regulation, or down-regulation of genes involved in DNA repair mechanisms.

6. Experiments involving Tumour, Mesothelial, and solid Tumour cells
The timed drug response experiment using cisplatin, and possibly other chemotherapeutic agents will be repeated in these cells.
DNA microarrays will also be repeated in the same cells.

APPENDIX 2 – Patient information sheet

University College London Hospitals NHS
NHS Foundation Trust

Department Of Oncology
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Telephone: 020 7387 9300

PATIENT INFORMATION SHEET

Version: 2
October 7th 2004
Project I.D: 04/Q0505/77

Study Title : Mechanism of Resistance to Chemotherapy Drugs in Ovarian Cancer

Invitation Paragraph

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

Some patients with Ovarian Cancer become resistant to some forms of chemotherapy. Chemotherapy drugs cause damage to the tumour cell which kills it. It is thought that the tumour cells acquire mechanisms to repair this damage, thus making them resistant. The purpose of this study is to investigate the tumour cell repair mechanisms involved in this process. This will involve looking at expression of different proteins and genes involved in these repair mechanisms, within the cells.

It is hoped that this research will help the design and development of newer drugs for treating Ovarian Cancer. Patients involvement will only be brief, as all we require are small samples of tissue taken at the same time as any necessary procedures.

Why have I been Chosen?

All patients with ovarian cancer are eligible for this study. We will be treating samples of patients tissue, which have been removed, with chemotherapy agents and monitoring them. This means it does not matter if you have had chemotherapy or not.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect your future medical care.

What will happen to me if I take part?
As part of your treatment for ovarian cancer, you may undergo surgery, or other procedures such as draining some fluid from your abdomen or chest. At this time, samples of tumour or fluid will be taken as part of your treatment. They will be examined, and then thrown away. You are invited to allow these samples to be used in this study. They will still be examined in the proper way. No extra tissue or fluid will be taken from these procedures.
During your treatment you will have several blood tests to monitor your general condition. We would like 20mls of your blood (1 tube) which would normally be taken at the same time as your other blood tests.
This means that you are only in the study for less than one day, while we collect the samples during your procedures.
If you were to return for additional procedures, then we would also collect tissue or fluid samples from these, unless you wanted to withdraw your participation from the study.
Tissue samples taken may be retained for future research. We will hold your samples for a maximum of 5 years, and then they will be destroyed. Your name and personal details will be separate from your samples. Any new research will be reviewed and passed by a research ethics committee before the research starts. However, we will not contact you in the future to obtain consent.
Some insurance companies ask whether you have had gene studies to see if you suffer from a hereditary disease. This research does not involve looking at genes which cause ovarian cancer, we are investigating genes which could become involved in treating ovarian cancer. There will be no link between your DNA and your name or your notes.
Please note that volunteers give up their legal title to their samples when they sign the consent form.

What do I have to do?
No extra procedures are needed for this study apart from a single blood test.

What are known risks of the study or the side effects of any treatment received?
There are no added risks or side effects if you take part in this study. There are also no disadvantages.

What are the possible benefits of taking part?
The study will not affect your treatment directly. However, if the study is successful it may lead to further studies to develop new treatments for cancer that could benefit patients in the future.

Will my taking part in the study be kept confidential?
Access to your medical records is necessary to find out details about your treatment and your general condition. Personal details including initials of first and last name, date of birth and hospital number will be taken and stored securely on a computer at UCL. Only the local investigator will have access to this information. However, sometimes, representatives from the ethics board at UCLH may be allowed to see your records to check on the study. These details will be kept for a limited period of time and then destroyed.
Your GP will not be informed of your participation because it will not affect your treatment in any way. It will also not affect your participation in any other studies, should you wish to join.

Your clinical record will be kept separate from the laboratory result. No individual identities will be used in any reports or publications resulting from the study.
All data will be used and stored according to the Data Protection Act.

What will happen to the results of the research study?
Your clinical record will be kept separate from the laboratory result. No individual identities will be used in any reports or publications resulting from the study.
The results are likely to be published in approximately 2 years. Any patients wanting to obtain a copy of the results can write to us at the above address.

Who is organising and funding the research?
The trustees of the Elizabeth Garrett Anderson Hospital are funding the research, as well as Cancer research UK, and the department of Oncology, UCL.

Who has reviewed the study?
All proposals for research using human subjects are reviewed by an ethics committee before they can proceed. This proposal was reviewed by the joint UCL/UCLH Committees on the ethics of human research.

Contact for further information
If you have any questions after reading this information, please ask Dr Claire Newton (0207 679 9319), Dr Jonathan Ledermann (020 7679 8040), or the Gynaecological Cancer Research Nurse, Elga Atkins (020 7387 9300 x3133) for further information.

Thank you for taking time to read this information and for considering participation in this research study. You will be given a copy of this information to keep, and a copy of the consent form if you decide to join this study.

APPENDIX 3 – Patient consent form

University College London Hospitals NHS
NHS Foundation Trust

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Telephone: 020 7387 9300

UCLH project number: 04/Q0505/77
Form version and date: version 2, 7th October
Patient identification number for this study:
Name of principal investigator: Dr. Ledermann

CONSENT FORM

TITLE : The Mechanism of Resistance to Chemotherapy Drugs in Ovarian Cancer

Please initial box

1. I confirm that I have read and understood the information sheet dated 7th October, version 2 for the above study and have had the opportunity to ask questions ☐
2. I confirm that I have had sufficient time to consider whether or not I want to be included in the study ☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason, without my medical care or legal rights being affected ☐
4. I understand that sections of any of my medical notes may be looked at by responsible individuals from UCLH or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records ☐
5. I agree to take part in the above study ☐

1 form for patient
1 to be kept as part of study documentation
1 to be kept with hospital notes

UCLH project number: 04/Q0505/77
Form version and date: version 2, 7thOctober
Patient identification number for this study:
Name of principal investigator: Dr. Ledermann

CONSENT FORM

TITLE : The Mechanism of Resistance to Chemotherapy Drugs in Ovarian Cancer

_____ _____ _____

Name of patient Date Signature

_____ _____ _____

Name of Person taking consent Date Signature

Part B: initial box

I agree to my samples retained for up to 5 years, and then destroyed**.** I understand that my consent will not be sought if they are used for future research. I understand that any future research must be approved by the

UCL/UCLH ethics committee on human research. ☐

_____ _____ _____

Name of patient Date Signature

_____ _____
_____

Name of Person taking consent Date Signature

Researcher (to be contacted if any problems): Dr Claire Newton, tel. 020 7679 9319

Version. 2 7th October 2004
PI: Dr Ledermann

Appendix 4 - NAMES AND ADDRESSES OF SUPPLIERS

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