A PILOT PHARMACOGENOMIC STUDY EXAMINING THE INFLUENCE OF CYTOTOXIC AND METABOLISING GENETIC POLYMORPHISMS ON CHEMOTHERAPY TOXICITY AND OUTCOME IN OSTEOSARCOMA

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DECLARATION

‘I, Rachael Windsor confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

ABSTRACT

Background: Osteosarcoma is the most common malignant bone tumour in children and young people. Multi-agent MAP chemotherapy (**M**ethotrexate, **A**driamycin, cis**P**latin) forms the backbone of standard treatment protocols but approximately 40% patients respond poorly to chemotherapy. It was hypothesized that pharmacogenomic profiling of osteosarcoma patients may facilitate optimisation of treatment with the aim of improved outcomes and decreased burden of late effects. This pilot study aimed to investigate associations of 36 candidate genetic polymorphisms in MAP pathway genes with histological response (HR), survival and grade 3-4 chemotherapy toxicity. A secondary aim was preliminary analysis of genome-wide copy number variation in osteosarcoma.

Methods: Blood samples and retrospective chemotherapy toxicity data were obtained from 60 patients who had completed MAP chemotherapy. All patients were manually genotyped for 5 polymorphisms. The remaining 31 polymorphisms were genotyped in 50 patients using the Illumina 610-Quad microarray. Associations between candidate polymorphisms and HR, progression-free survival and toxicity were estimated using Pearson's χ^2 and Fisher's Exact tests, the Kaplan-Meier method, the log-rank test and Cox proportional hazards model. Copy number analysis was performed using PennCNV.

Results: Poor histological response was associated with ABCC2 c.24C>T (p=0.011) and GSTP1 c.313A>G p.Ile^{105}Val (p=0.009) whereas MTHFD1 c.1958G>A p.Arg^{653}Gln was protective (p=0.03). Methotrexate toxicity was associated with MTHFR c.1298A>C p.Glu^{429}Ala (p=0.038), ABCC2 c.3563T>A p.Val^{1188}Glu (p=0.028) and ABCB1 c.3435T>C Ile^{145}Ile (p=0.027). Variants of GSTP1 c.313A>G p.Ile^{105}Val were at increased risk of myelosuppression (p=0.024) and cardiac damage (p=0.008). Eight recurrent copy number variations were identified, none significant.

Conclusions: This pilot study explored the pharmacogenomics of osteosarcoma chemotherapy and although limited by small sample size secondary to retrospective recruitment and the rarity of this tumour, it remains the most comprehensive study to

date. A number of novel polymorphic associations were observed as well as confirming several previously reported findings. Cautious interpretation is required but further prospective investigation is warranted.

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List of abbreviations

A	adriamycin (doxorubicin)
ABC	ATP-Binding Cassette
ACT	anthracycline cardiotoxicity
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
ATP	adenosine triphosphate
AUC	area under the curve
BAF	B-allele frequency
BER	base excision repair
BIRC	baculoviral IAP repeat containing
CAT	catalase
CBR	carbonyl reductase
CCND1	cyclin D1
CDC5L	cell division cycle 5-like protein
CDK	cyclin dependent kinase
CGH	comparative genomic hybridisation
CHF	congestive heart failure
CI	confidence interval
COG	Children's Oncology Group
COPS3	constitutive photomorphogenic homolog subunit 3
COSS	Cooperative Osteosarcoma Study Group
CNP	copy number polymorphism
CNS	central nervous system
CNV	copy number variation
CNVR	copy number variable region
CTCAE	Common Terminology Criteria for Adverse Events
CYBA	cytochrome b-245, alpha polypeptide
DFS	disease free survival
DGV	Database of Genomic Variants
DHFR	dihydrofolate reductase
DI	dose intensity
DOX	doxorubicin
Doxol	doxorubicinol
dTMP	deoxyuridine-5-triphosphate
dUMP	deoxyuridine-5-monophosphate
EC	endometrial cancer
EF	ejection fraction
EOI	European Osteosarcoma Intergroup
EoT	end of treatment
ERCC	excision repair cross-complementation group
EURAMOS	European and American Osteosarcoma Study Group
FOLR	folate receptor
FS	fraction shortening
GFR	glomerular filtration rate
GGT	gamma glutamyl transferase
GST	glutathione S-transferase
HD-M	high-dose methotrexate
HMGB1	high-mobility group box 1
HMM	hidden Markov model
HR	hazard ratio
JNK1	c-Jun N-terminal kinase 1
JNK/SAPK	c-Jun N-terminal kinase-stress activated protein kinase
LSAMP	limbic system associated membrane protein

MAP	methotrexate, adriamycin, cisplatin
MAPK	mitogen-activated protein kinase
Mb	megabase
MMP	matrix metalloproteinase
M	methotrexate
mRNA	messenger RNA
MRP	multiresistance protein
MSH	mismatch repair gene family
MTHFD1	methylene tetrahydrofolate dehydrogenase
MTHFR	methylene tetrahydrofolate reductase
MTI	mTOR inhibitor
mTOR	mammalian target of rapamycin
MV	multivariate
NCBI	National Centre for Biotechnology Information
NCF4	neutrophil cytosolic factor 4
NCOR1	nuclear receptor compressor 1
ncRNA	non-coding RNA
NER	nucleotide excision repair
NHL	Non-Hodgkin's lymphoma
NSCLC	Non small-cell lung cancer
OR	odds ratio
OS	osteosarcoma
P	cisplatin
PE	paraffin embedded
PFS	progression free survival
PMP 22	peripheral myelin protein 22
pRB	retinoblastoma protein
RA	rheumatoid arthritis
RAC2	ras-related C3 botulinum toxin substrate 2
RB1	retinoblastoma
RFC	reduced folate carrier
ROS	reactive oxygen species
SCNA	somatic copy number alteration
SCOTROC	Scottish Randomised Trial in Ovarian Cancer
SFE	*Schisandra fructis*
shRNA	short hairpin RNA
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
SSG	Scandinavian Sarcoma Group
TOM1L2	target of myb 1-like 2 (chicken)
TOP3A	topoisomerase (DNA) III alpha
TYMS	thymidylate synthase
3R	three repeat
UGT	uridine diphosphate glucuronyltransferase
UTR	untranslated region
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VNTR	variable number tandem repeat
WT	wild type
XRCC	x-ray repair cross-complementation group

1.0 Introduction

Osteosarcoma (OS) is the most common malignant bone tumour in children and young people (1). The introduction of effective chemotherapy has dramatically improved 5-year survival from under 20% following surgery alone to 55-70% with current multimodality therapy (2-4). Cisplatin (P), adriamycin (A, referred to as doxorubicin in remainder of text) and methotrexate (M) are the most active chemotherapeutic agents against OS and as a triple drug regimen (MAP) form the backbone of most standard treatment protocols including EURAMOS 1, the current international OS Phase III trial (5). Nonetheless, approximately 40% patients will show a poor response to chemotherapy and experience subsequent inferior 5-year survival of 45-55% (6). Treatment also imposes significant toxicities with 60% of childhood bone tumour survivors experiencing severe, disabling or fatal chronic health conditions at 30 years (7). Recent technological advances now enable not only the study of genetic changes at a single-base level (polymorphisms) but also a genome-wide overview of genetic imbalance (copy number variation, (CNV)) or disease-associated changes. Using germline DNA from patients treated with MAP chemotherapy for osteosarcoma, this study aimed to: I) investigate associations between candidate polymorphisms, chemotherapy toxicity and disease outcomes and; 2) perform preliminary genome-wide analysis of copy number variation.

1.1 Candidate polymorphism study

It is well recognized that different patients respond in different ways to the same medication, with greater variation seen within populations than individuals (8). This infers that inheritance is a major determinant of drug response. Multiple sources of variation exist in the human genome including single nucleotide polymorphisms (SNP), DNA repeat sequences and insertions/deletions, SNPs being the most common. Genetic polymorphisms may be latent, silent or cause protein conformational change that enhances or inhibits enzyme function, consequently influencing drug transport, target or metabolizing properties. Use of a genome-wide approach to identify the genetic basis of interindividual differences in drug response and translation of this into individualized therapy forms the basis of pharmacogenomics.

The earliest observations of inherited differences influencing drug response appeared in the 1950's (9). Subsequent research focussed initially on elucidating the effects of single polymorphisms but the advent of high-throughput genotyping technologies has enabled the simultaneous investigation of hundreds of thousands of SNPs. Reflecting

this, several thousand studies can be found on publicly-available databases documenting the effects of genetic polymorphisms on a wide range of drugs including antivirals, anti-psychotics, anti-epileptics and anti-coagulants. The pharmacogenomics of warfarin are perhaps the most well-defined but in common with other drugs, many studies are underpowered and retrospective. This problem may be overcome by collaboration of pharmacogenomic research centres to validate findings prospectively, an example being the International Warfarin Pharmacogenetics Consortium dosing algorithm developed by collating clinical and genetic data from over 4000 patients (10).

The influence of genetic polymorphisms on chemotherapy response and toxicity has also been the subject of extensive research in haematological and solid malignancies although many studies are similarly hindered by small sample size and retrospective nature. The pharmacogenomics of breast cancer chemotherapy is well established but although the balance of evidence suggests tamoxifen metabolism and clinical outcome is adversely affected by a SNP in the CYP2D6 gene, translating retrospective data into personalised treatment guidelines is challenging (11). Pharmacogenomic data relating to rare and heterogeneous tumour groups such as sarcoma are minimal, further complicated by the complexity of multi-drug chemotherapy regimens used in treatment. The majority of patients with osteosarcoma are treated with MAP chemotherapy, a complicated regimen with significant early and late toxicities. This study proposed that pharmacogenomic profiling of these patients may facilitate optimisation of current treatment strategies with the aim of improved survival and decreased late effects burden.

Cytotoxic efficacy depends on absorption, membrane transport and interaction with intracellular metabolising and target enzymes coded for by multiple genes. Cisplatin exerts its cytotoxic effects by forming helix-distorting DNA adducts resulting in inhibition of DNA replication. Doxorubicin too binds to DNA, inhibiting replication and causing strand breaks whereas methotrexate exerts multiple inhibitory effects on the folate pathway. Although each drug has a unique pharmacological pathway, some elements such as membrane-transporters, detoxification and DNA repair are shared.

Although little is known about the pharmacogenomics of MAP chemotherapy, information gained from other malignant and non-malignant diseases provides valuable insight into potentially critical components of each pathway. The influence of genetic polymorphisms on methotrexate has been extensively studied in rheumatoid arthritis and childhood acute lymphoblastic leukaemia (ALL) with a number of polymorphisms clearly implicated in toxicity and disease outcomes (12). The ATP-Binding Cassette (ABC) superfamily of membrane efflux transporters regulate xenobiotic bioavailability,

with functional polymorphisms in several proteins influencing both chemotherapy toxicity and outcome in a number of malignancies (13-16). The glutathione-S-transferase (GST) family of isoenzymes and Nucleotide Excision Repair (NER) pathway respectively play important roles in detoxification and repair of DNA damage induced by doxorubicin and cisplatin. The impact of GST and NER polymorphisms on cancer survival has been explored in both leukaemia and solid tumours (17-23). For the current study, thirty-six candidate polymorphisms from 22 genes within MAP drug pathways were selected on the basis of previously described associations or putative functional effects. Associations between polymorphisms and histological response, survival and chemotherapy toxicity in osteosarcoma were investigated.

1.2 Genome wide analysis of CNV

Microarray technologies can now simultaneously measure millions of loci in the genome for DNA copy number changes, enabling the identification of genomic regions frequently altered in human cancers with the goal of pinpointing novel onco- and tumour suppressor genes (24). Recent analysis of the Database of Genomic Variants (DGV) showed that nearly 40% of cancer-related genes are interrupted by a CNV (25, 26).

There is currently no published literature describing genome-wide analysis of germline DNA for CNV in osteosarcoma. The USA National Cancer Institute is currently sponsoring a study investigating CNV in germline and tumour DNA from 243 patients with osteosarcoma (27).

Using microarray data available from investigating candidate polymorphisms, the current study aimed to perform genome-wide analysis of germline CNV in osteosarcoma patients.

2.0 Osteosarcoma: background

2.1 Clinical and pathological features

Osteosarcoma is the most common primary, non-haemopoietic, high-grade malignant tumour of bone and is characterised by the production of osteoid or bony matrix. There is no reported association with ethnic group but males are affected more frequently than females. Although the peak incidence of osteosarcoma is observed during adolescence, patients aged over 50 years account for almost half of all cases (28). This primarily metaphyseal tumour shows a predilection for skeletal long bones, particularly distal femur, proximal tibia and proximal humerus with only approximately 10% cases occurring in the axial skeleton. Most patients present with swelling and pain, the latter initially intermittent but eventually unrelenting. Pain is frequently attributed to a sporting injury, thus delay in diagnosis of average 3-4 months is usual (29). Pathological fractures occur in 5-10% patients and confer a poor prognosis (30). The usual radiographic appearance is of a mixed lytic/blastic lesion with associated cortical destruction and soft-tissue extension.

The histological appearance of osteosarcoma is of a highly anaplastic, pleiomorphic tumour in which tumour cells vary from spindle-shaped to polyhedral and grow in an angiocentric fashion. The tumour cells produce extracellular matrix that may contain cartilaginous, osseous or fibrous elements although the presence of osteoid is the fundamental diagnostic prerequisite (31). Historically, the main component of the extracellular matrix has subdivided conventional osteosarcoma into osteoblastic, chondroblastic and fibroblastic subtypes. However, all are high-grade tumours, treated identically with similar survival rates (31). Parental farming or residence on a farm and younger age at puberty has been consistently associated with osteosarcoma although precise mechanisms are unclear (32).

Osteosarcomas typically lack the pathognomic chromosomal rearrangements seen in Ewing's sarcoma, the second most common malignant bone tumour. However, a number of genetic conditions carry a predisposition to osteosarcoma, including Li-Fraumeni syndrome (germline p53 mutations), hereditary retinoblastoma (RB1 gene) and Werner and Rothmund-Thomson syndromes (DNA repair mutations) (33). Collectively, the study of these disorders has made a significant contribution to understanding the molecular pathogenesis of cancer *per se* and osteosarcoma in particular. Complex genetic changes are frequently seen in non-familial osteosarcoma, affecting some chromosomal regions more commonly. Indeed, germline p53 mutations

have been found in 3% osteosarcoma patients (34). Conventional cytogenetic techniques have demonstrated loss of –9, -10, -13 and –17 and gain of +1 to be the most common chromosomal abnormalities. More sensitive techniques have persistently identified amplifications in 1q21, 6p12-21, 8q23-24 and deletions at 2q, 6p, 8p, 10p and 17p13, suggesting genomic instability is an important feature of osteosarcoma (35).

Many studies have attempted to identify prognostic factors for osteosarcoma. However, poor quality or insufficient patient numbers render the findings unreliable. A recent review aimed to identify prognostic factors from literature published over the last 20 years but found only 7 of 93 studies worthy of analysis (36). Chemotherapy response was confirmed as an independent prognostic factor with a relative risk of death of 2.4 in those with <90% tumour necrosis after neoadjuvant chemotherapy (deemed poor responders). Further factors predicting a worse outcome included large tumours, inadequate resection margins, ablative surgery, age<14 yrs, male gender, high alkaline phosphatase, local recurrence, p-glycoprotein expression and absent Erb2 expression.

2.2 Current therapeutic strategy

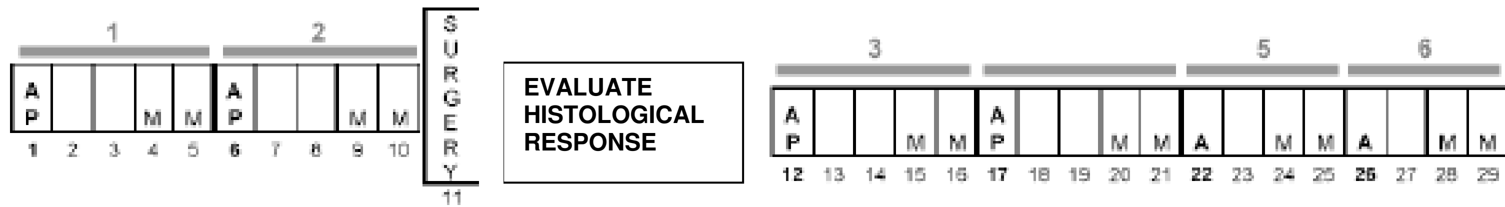
Left untreated, osteosarcoma undergoes both rapid haematogenous dissemination and local invasive tumour growth. It must be regarded as micro-metastatic at diagnosis, as the majority of patients who die despite chemotherapy, do so from pulmonary metastases. Optimal therapy for osteosarcoma is now multi-disciplinary, encompassing the expertise of surgeons, oncologists and allied health professionals to manage both local and systemic manifestations of the disease. The use of multidisciplinary therapy has resulted in disease-free survival of 60-80% while allowing the use of functional limb-sparing surgery in >80% patients (28).

A comprehensive history of osteosarcoma chemotherapy is beyond the remit of this study and has been reviewed previously (37-39). Briefly, the first report of single-agent activity against osteosarcoma was of phenylalanine mustard in 1963 (40). However, multi-agent chemotherapy was not introduced until the late 1960's when the VAC regimen (vincristine, actinomycin D, cyclophosphamide) was used. This combination had already shown efficacy in rhabdomyosarcoma and achieved a disease-free survival rate of 33% in osteosarcoma (41). Subsequently, increasingly complex multi-drug chemotherapy regimens were used, with disease-free survival rates of 76% from the USA setting a benchmark for other groups. The American, German and Italian groups followed a common theme of progressively more drugs with prolonged schedules whereas the European Osteosarcoma Intergroup (EOI) chose to focus on

shorter dose-intense regimens (38). The standard treatment strategy now consists of dose-intense neoadjuvant chemotherapy based on high-dose methotrexate, doxorubicin and cisplatin, aggressive local disease control and further adjuvant chemotherapy. The rarity of osteosarcoma and challenges of clinical trial accrual has encouraged a timely international collaboration between the North American Children's Oncology Group (COG), the German-Austrian-Swiss (COSS) Group, the EOI and the Scandinavian Sarcoma Group (SSG) to run the EURAMOS 1 Phase III clinical trial (5). This study aims to evaluate whether modification of post-operative chemotherapy can improve the outcomes of both good and poor responders. All patients receive MAP pre- and post-operatively, with post-operative randomisation dependent on the degree of tumour necrosis seen in the resection specimen. Poor responders are randomised to receive additional ifosfamide/etoposide and good responders to receive maintenance pegylated α-interferon.

The basic MAP regimen comprises six chemotherapy cycles, two pre-operatively and four post-operatively (Figure 1: MAP chemotherapy schematic). In week one of cycles 1-4, patients receive doxorubicin 75mg/m^2 plus cisplatin 120mg/m^2 followed by methotrexate 12g/m^2 at weeks four and five. In cycles 5 and 6 patients receive doxorubicin 75mg/m^2 alone at week one, followed by methotrexate12g/m^2 at weeks three and four. Cumulative maximum total doses are methotrexate 72 g/m^2, doxorubicin 450mg/m^2 and cisplatin 480mg/m^2.

Figure 1: MAP chemotherapy schematic



A = Doxorubicin 75mg/m^2/course
P = Cisplatin 120mg/m^2/course
M = Methotrexate 12g/m^2/course

The above diagram shows the standard MAP chemotherapy regimen used in the treatment of osteosarcoma. Methotrexate, cisplatin and doxorubicin are all administered in cycles 1-4, each of 5 weeks duration. Cisplatin is omitted from cycles 5 and 6, thus they are of 4 weeks duration

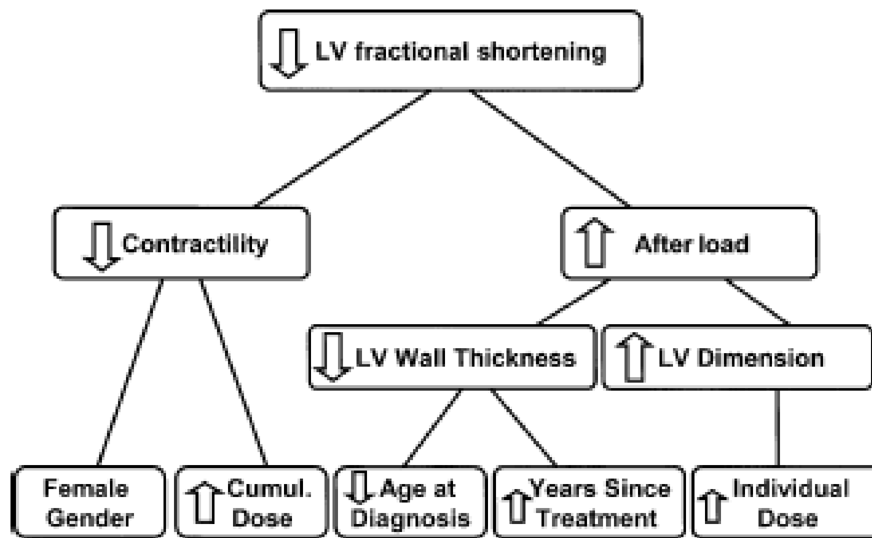
2.3 Osteosarcoma: late effects of chemotherapy

Survivors of childhood bone tumours experience severe or disabling chronic health conditions with a relative risk 38 times that of their siblings. Furthermore, they are more likely than other survivors to suffer from multiple conditions, particularly severe musculoskeletal problems, congestive heart failure, hypertension and hearing loss (7, 42). The vast majority of patients receive MAP chemotherapy for osteosarcoma with associated potential long term side-effects including cardiotoxicity, nephrotoxicity, ototoxicity, infertility, neurotoxicity, and second malignancies. The first four are discussed below, for a comprehensive review see (43).

2.3.1 Cardiotoxicity

Doxorubicin is the anthracycline responsible for late cardiac damage seen in osteosarcoma survivors. Evidence suggests anthracycline cardiotoxicity (ACT) is persistent and progressive, with childhood cancer survivors experiencing a 15-fold increase in relative risk of congestive heart failure and an 8-fold increase in standardised mortality ratio for cardiac causes (7, 44, 45). In 265 survivors of doxorubicin-treated sarcoma followed up for 3 years, 1.5% suffered from symptomatic cardiomyopathy and 16% subclinical cardiomyopathy, the relatively short follow-up suggesting the true incidence may be even higher (46). More specifically, in osteosarcoma phase 3 clinical trials, the incidence of symptomatic cardiotoxicity is reported as 0-4% (43). The cumulative dose of anthracycline is a well-recognised risk factor for cardiac damage and remains the optimal predictor of eventual cardiac dysfunction (47), with doses >550 mg/m^2 associated with a higher risk of congestive heart failure. The relationship between cardiac and non-cardiac findings in long-term survivors of childhood malignancy treated with anthracyclines is shown in Figure 2. Although superior survival rates conferred by anthracyclines ensure their enduring use, the administration of relatively high-dose doxorubicin in MAP chemotherapy (up to 450mg/m^2) has significant implications for long-term cardiac function.

Figure 2: The relationship between cardiac and non-cardiac findings in long-term survivors of childhood malignancy treated with anthracyclines.



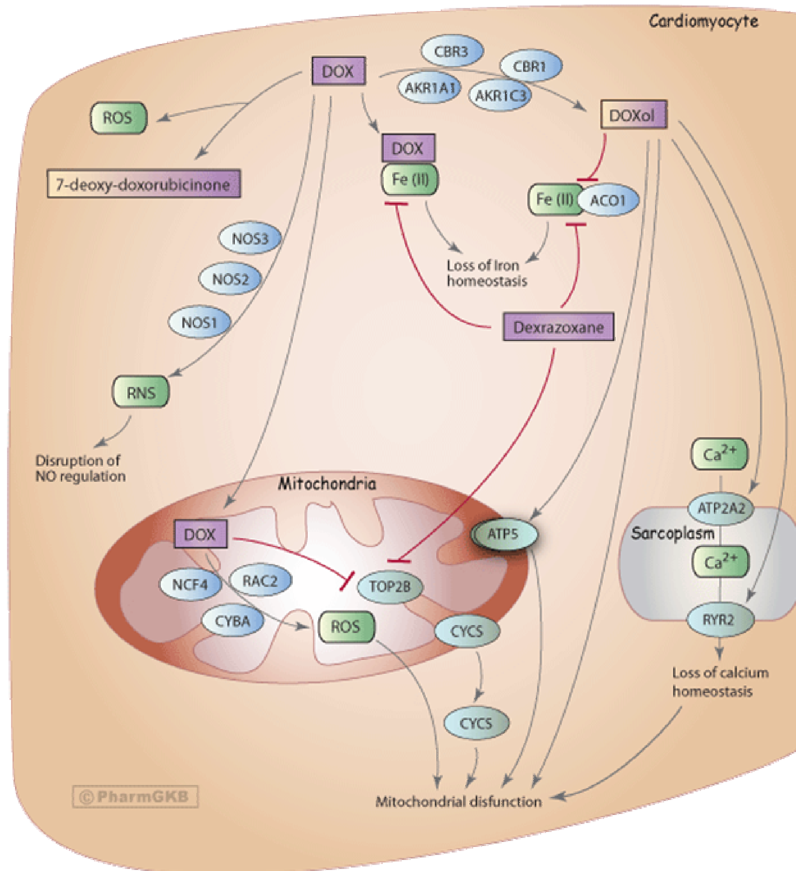
This diagram illustrates factors that contribute to anthracycline cardiotoxicity and their physiological effects on the heart. Reproduced with permission from Wouters K et al. Protecting against anthracycline-induced cardiac damage: a review of the most promising strategies. Br J Haematol 2005; 131: 561-78.

Current opinion now suggests anthracyclines themselves are directly cardiotoxic (48). Doxorubicinol, the major metabolite of doxorubicin is also significantly implicated in cardiac damage (49). The pathogenesis of doxorubicin-induced cardiomyocyte injury is thought to be secondary to reactive oxygen species (ROS) generated by redox cycling of doxorubicin on the mitochondrial electron transport chain (47). The consequences of excess ROS include lipid peroxidation, oxidation of proteins and DNA, glutathione depletion and loss of mitochondrial integrity and function (50). Cardiac myocytes are extremely susceptible to oxidative stress due to their highly oxidative metabolism and poor antioxidant defences; the activity of two important antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD) being significantly less in cardiac muscle than liver cells (51). Reactive nitrogen species are also implicated in cardiotoxicity through the disregulation of nitric oxide metabolism (52) (see Figure 3: Candidate genes involved in adverse effects of doxorubicin in a cardiomyocyte).

A large number of anti-oxidant compounds have been investigated in the search for an effective cardioprotectant, the most promising being dexrazoxane (47). Dexrazoxane prevents the generation of ROS by anthracycline-iron complexes through its chelation of intracellular iron. It has an additional anti-neoplastic effect through the inhibition of DNA topoisomerase II (51). It is the only cardioprotectant whose efficacy is proven in

clinical trials, with no convincing evidence that survival is compromised (47)[1]. In a preliminary report of a single arm study in osteosarcoma, the use of dexrazoxane did not adversely affect the proportion of patients achieving a good histological response (43).

Figure 3: Candidate genes involved in adverse effects of doxorubicin in a cardiomyocyte



This image depicts candidate genes involved in the adverse effects of doxorubicin in a cardiomyocyte (53)

DOX, doxorubicin; DOXol, doxorubicinol; CBR(1,3), carbonyl reductase (1,3); AKR1A1, aldo-keto reductase family 1, member A1; AKR1C3, aldo-keto reductase family 1, member C3; Fe, iron; ACO1, aconitase 1; NOS(1-3), nitric oxide synthase (1-3); ROS, reactive oxygen species; RNS, reactive nitrogen species; NCF4, neutrophil cytosolic factor 4; RAC2, Ras-related C3 botulinum toxin substrate 2; CYBA, cytochrome b-245, alpha polopeptide; TOP2B, topoisomerase 2 beta; CYCS, cytochrome c; RYR2, ryanodine receptor 2; ATP2A2, ATPase, Ca++ transporting, cardiac muscle, slow twitch 2. Copyright PharmGKB. Reproduced with permission from Pharm GKB and Stanford University.

[1] The European Medicines Agency completed a review of the safety and effectiveness of dexrazoxane on 23rd June 2011 (EMA/CHMP/485343/2011) and concluded it should not be used in children and adolescents. Studies have shown a 3-fold increase in the risk of new cancers such as AML and MDS plus an increased risk of severe myelosuppression (www.ema.europa.eu)

2.3.2 Nephrotoxicity

Cisplatin also has significant dose-limiting side-effects, the most important being nephrotoxicity with 20% of patients receiving cisplatin treatment experiencing severe renal dysfunction (54). The drug is directly toxic to renal tubular cells, not only activating cellular apoptotic pathways but also stimulating a brisk inflammatory response that further exacerbates tissue damage (55). In addition, cytotoxic injury to the renal vasculature leads to ischaemia and further renal damage. Nephrotoxicity clinically manifests 6-10 days after the drug, with raised creatinine, reduced glomerular filtration rate (GFR) and electrolyte disturbance (potassium and magnesium). At completion of treatment approximately 60% of children receiving a median cisplatin dose of 500-600mg/m^2 will have a decreased GFR (56). The degree of toxicity is increased by higher dose rates of cisplatin although there is no correlation with total dose (57, 58). Although the long-term consequences of cisplatin therapy are incompletely understood, there is certainly partial recovery of GFR 2 years after treatment although hypomagnesaemia persists, suggesting a long-lasting impairment (57-59). Ten-year follow-up data from 63 children treated with cisplatin has recently been published showing considerable inter-patient variability in nephrotoxicity but no significant change in the frequency or severity of renal toxicity in the follow-up period (60). However, severe nephrotoxicity was lasting, being persistent in 15% survivors ten years after completion of cisplatin treatment.

Acute renal failure is a well-documented complication of high-dose methotrexate, occurring in approximately 1.8% of patients with osteosarcoma treated on clinical trials (61). Renal damage caused by the precipitation of the drug and its metabolites in the renal tubules leads to delayed excretion, a rapid rise in plasma levels and intensification of other methotrexate toxicities such as mucositis and myelosuppression. With appropriate treatment, it appears to be reversible with a median time to recovery of renal function of 16 days (range 4-48) (62). Unfortunately there is a paucity of information relating to the long-term effects of acute methotrexate nephrotoxicity (63).

2.3.3 Ototoxicity

Ototoxicity is a further significant long-term side effect of cisplatin therapy. The probable pathogenic mechanism relates to the loss of outer hair cells of the organ of Corti secondary to oxidative stress (64). Hearing loss is variable but often permanent and symmetrical with high frequency thresholds affected first and in one study, universally in patients receiving >300mg/m^2 (65). In severe cases, hearing loss

involves the middle frequencies and is progressive on follow-up (66). The risk increases with younger age, higher cumulative and individual doses of cisplatin, co-administration of other ototoxic drugs and cranial irradiation (67). Late-onset hearing loss (>6 months after completion of therapy) is also a significant problem for children who have been treated with cisplatin, reiterating the need for prolonged follow-up (68). The incidence of educationally significant hearing loss in a small study of children treated for osteosarcoma was 4% (69). This small study is likely to represent a reliable reflection of hearing loss attributable to current therapy, as cisplatin administration was similar to current MAP guidelines.

2.3.4 Fertility

Certain malignancies, namely Hodgkin's lymphoma and testicular cancer, are associated with subfertility in males prior to the onset of treatment, suggesting this is a disease-related effect. This does not appear to be in the case in osteosarcoma, Ewing's or other soft tissue sarcomas (70, 71). It is well established that both chemo- and radiotherapy affect fertility and pregnancy outcomes with alkylating agents such as cyclo- and ifosfamide particularly associated with premature ovarian failure and male subfertility in a sex and dose-dependent fashion (72, 73).

Reported fertility outcomes following treatment for osteosarcoma are variable, possibly reflecting evolution of chemotherapy strategies and gender differences. In male long-term osteosarcoma survivors, the fertility rate was significantly lower in patients receiving dose-intense compared to moderate-dose chemotherapy (74). A different study noted normal sperm production returned in the majority of men within 2 years even when rendered azoospermic through treatment with cisplatin, doxorubicin and dacarbazine (75). Furthermore, addition of other drugs including methotrexate, bleomycin, actinomycin-D and cyclophosphamide made no significant difference. Of note however was the observation that the percentage of men achieving normal sperm counts was lower in those who had received >600mg/m^2 cisplatin. In another report of long-term follow-up after MAP, ten of 12 assessable male patients were infertile although this finding may be confounded by selection bias (76). None of the female patients in this study experienced primary or secondary amenorrhoea and the 12 patients who sought pregnancy all succeeded. A different series of 36 bone sarcoma patients reported of 15 females who attempted conception, all were successful despite cumulative chemotherapy doses of 12.8g/m^2 oral cyclophosphamide, 480mg/m^2 cisplatin and 800mg/m^2 doxorubicin (77). A further study specifically investigated residual ovarian function following osteosarcoma therapy that included high-dose

methotrexate and ifosfamide, doxorubicin and cisplatin, and found that age and alkylant dose were the most important predictive factors for early menopause (78).

3.0 Pharmacology and pharmacogenomics of MAP chemotherapy

Variations in both disease outcomes and drug toxicity may be secondary to germline mutations in genes encoding for membrane transporters, metabolising and target enzymes and signalling pathways (79). Understanding and exploiting this variation forms the basis of pharmacogenomics, with the ultimate goal of improved survival and decreased morbidity. Comprehension of the potential effects of genetic variation on drug action requires a clear understanding of individual drug pharmacological pathways.

3.1 Pharmacology of MAP chemotherapy

3.1.1 Methotrexate

Methotrexate, a reduced folate analogue, is an antimetabolite that targets endogenous cellular folate metabolism (Figure 4: Methotrexate drug pathway). The primary function of the folate pathway is the provision of single carbon donors for purine, pyrimidine and amino acid synthesis, functions all disrupted by methotrexate. Aside from activity in osteosarcoma, the drug is used to treat other malignancies including leukaemia, lymphoma and head and neck cancer and, in lower doses, as a disease-modifying agent in rheumatoid arthritis and other autoimmune disorders.

Methotrexate is transported into the cell primarily by the reduced folate carrier (RFC) protein, a saturable energy-dependent process, competitively inhibited by 5-methyl-tetrahydrofolate, the predominant circulating form of folate in mammals as well as by leucovorin (80). Passive diffusion may also play a role when high doses) are administered (>0.5g-12g/m^2) (81). Once within the cell, M binds rapidly to its target enzyme dihydrofolate reductase (DHFR), depleting the intracellular cycling of reduced folates and indirectly inhibiting the action of other enzymes including methyltetrahydrofolate reductase (MTHFR) and thymidylate synthase (TYMS) (80). TYMS plays a critical role in cellular nucleotide metabolism by methylating deoxyuridine-5-monophosphate (dUMP) to deoxyuridine-5-triphosphate (dTMP), providing the sole source of thymidylate essential for DNA replication whereas MTHFR catalyses the conversion of methylenetetrahydrofolate to methyltetrahydrofolate, the major circulating form of folate serving as a methyl donor for homocysteine remethylation to methionine (82, 83). Profound inhibition of purine and thymidylate synthesis results in failure of DNA replication and cell death. Free cellular M is

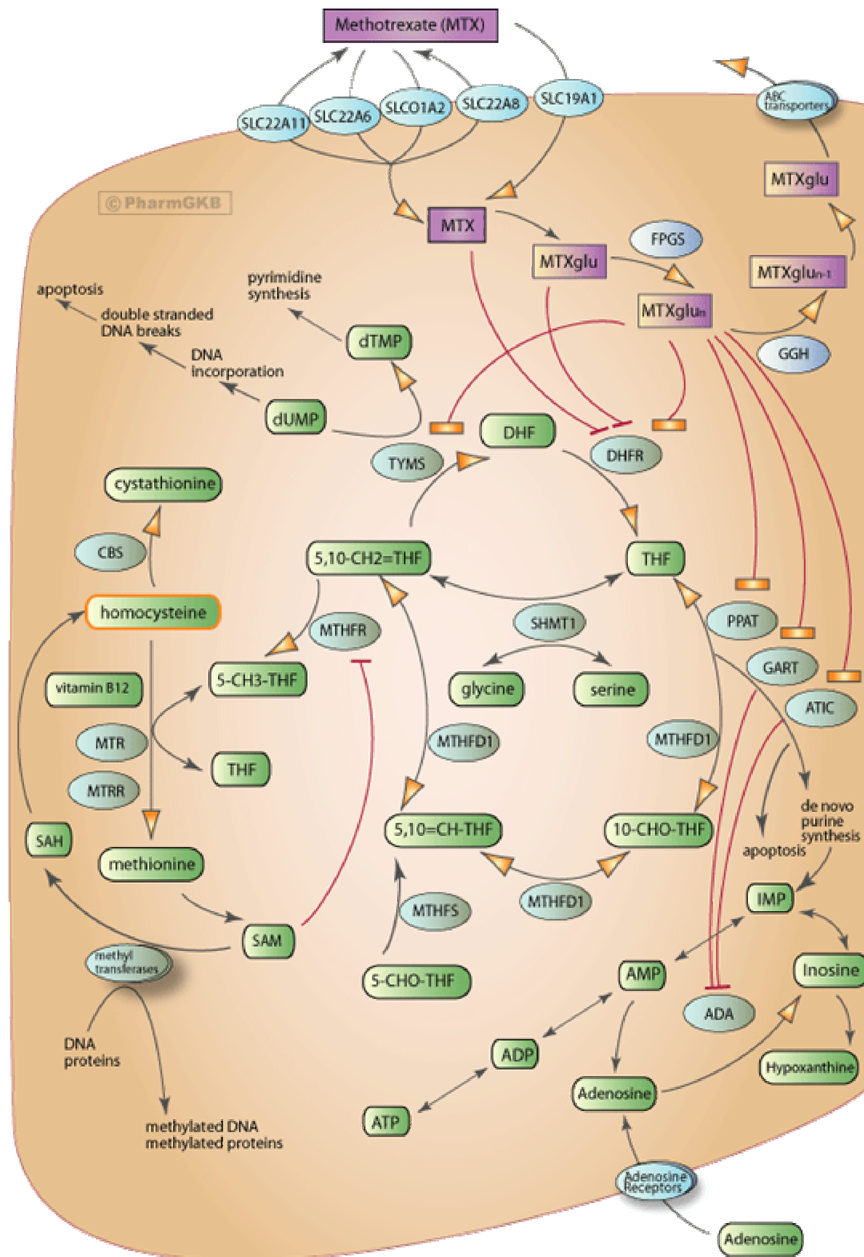
extensively metabolised to polyglutamates, biologically active forms that have two important effects; 1) reduced cellular efflux results in sustained intracellular concentrations of M and 2) sustained inhibition of DHFR and other target enzymes leads to prolonged disruption of the folate pathway and indirect inhibition of other enzymes such as MTHFR (84). The degree of polyglutamylation is influenced by peak drug concentration, exposure time and dose of leucovorin administered to "rescue" non-malignant cells (85, 86).

The concept of high-dose methotrexate (HD-M) evolved as knowledge of the cellular pharmacology of M exposed potential mechanisms of resistance. High extracellular drug concentration increases passive and active cellular uptake in resistant tumour cells whereas high intracellular concentrations overcome intracellular resistance mechanisms and exploit the dose-response relationship with respect to polyglutamylation. HD-M with leucovorin rescue is used routinely to treat not only osteosarcoma but also ALL and non-Hodgkin lymphoma (NHL) (86). Of particular importance in ALL is the ability of HD-M to increase drug concentrations in pharmacological sanctuary sites such as the CNS and testis (12).

A number of general mechanisms of M resistance have been described from both *in vivo* and *in vitro* studies and include; 1) defective membrane transport; 2) increased levels of DHFR secondary to gene amplification; 3) altered affinity of M for DHFR; 4) decreased accumulation of polyglutamates and 5) increased efflux by the multi-resistance protein (MRP) (87).

In osteosarcoma, the improved efficacy of high-dose compared to conventional-dose methotrexate suggests inherent tumour resistance (88, 89). Decreased RFC expression is frequently seen in diagnostic osteosarcoma samples suggesting impaired transport is a common mechanism. Furthermore, significantly lower RFC expression is seen in surgical resection samples showing poor response to pre-operative chemotherapy and in methotrexate-resistant osteosarcoma cell lines (90, 91). Sequence alterations in the RFC gene are associated with poor histological response after pre-operative chemotherapy (92).

Figure 4: Methotrexate drug pathway



This image depicts cellular transport and metabolism of methotrexate in addition to major sites of action of the drug and its polyglutamated derivatives (53)

MTX, methotrexate; MTXglu, MTX polyglutamates; ABC transporters, ATP-binding cassette; FPGS, folylpolyglutamyl synthetase; SLC19A1, reduced folate carrier; dUMP, deoxyuridine-5-monophosphate; DHF, dihydrofolate; THF, tetrahydrofolate; TYMS, thymidylate stnthase; DHFR, dihyhydrofolate reductase; GGH, γ-glutamyl hydrolase; MTR, methionine synthetase (vitamin B_{12} is a cofactor); MTHFD1, methylene tetrahydrofolate dehydrogenase; MTHFR, methylene tetrahydrofolate reductase; MTRR, methionine synthase reductase; SHMT1, serine hydroxymethyltransferase; ADA, adenosine deaminase; CBS, cystathionine-beta synthetase; MTHFS, 5,10-methenyltetrahydrofolate synthetase; PPAT, phosphoribosyl pyrophosphate amidotransferase; GART, phosphoribosylglycinamide formyltransferase; ATIC, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase. Copyright PharmGKB. Reproduced with permission from Pharm GKB and Stanford University.

Amplification and overexpression of the DHFR gene may be a further important mechanism of resistance in osteosarcoma. In retinoblastoma (RB1) gene-positive cell lines, the degree of methotrexate resistance was mainly associated with raised DHFR levels but in RB1-negative cells, resistance was mostly associated with decreased RFC expression (91). The RB1 gene controls cell transition from G_1 to S- phase by binding and inactivating E2F transcriptional activators (93). As a cell-cycle related gene, DHFR expression is negatively regulated by RB1, thus the latter may influence the sensitivity of the cell to drugs that target elements of the cell-cycle (94). DHFR expression also appears to be of clinical importance, a recent study showing increased protein levels in diagnostic biopsy specimens were associated with inferior event-free survival in osteosarcoma (95).

3.1.2 Doxorubicin

The anthracycline antibiotics, of which doxorubicin (DOX) is one, are naturally-occurring compounds with an undisputed role in the treatment of many haematological and solid malignancies (48). The pharmacokinetics of DOX show great inter-individual variation with implications for both anti-tumour efficacy and dose-related cardiotoxicity (96).

Intracellular movement of DOX is by free diffusion of un-ionised drug; the acidic intracellular environment protonates the drug making it unable to diffuse out. A substantial part is bound by the 20s fraction of the proteasome and actively transported to the nucleus where it dissociates due to a higher affinity to DNA (97). In humans, appproximately 50% of the dose of DOX is excreted unchanged with the remainder metabolised through three metabolic pathways; (1) two-electron reduction to its secondary alcohol metabolite doxorubicinol (DOXol); (2) one-electron reduction to DOX semiquinone radical; (3) deglycosidation to DOX aglycones (48) (Figure 5: Doxorubicin drug pathway). Metabolism to DOXol by two-electron reduction is the major pathway although performed by different enzymes depending on cell type. In the heart, it is most likely to be by aldo-keto reductase family 1 (AKR1A1), whereas in the liver it is carbonyl reductase 1 (CBR1) (98). DOXol is less potent than DOX in tumour growth inhibition but has been associated with increased cardiotoxicity in mice (99).

As a cytotoxic, DOX is active in all phases of the cell cycle but maximal in S phase. It has several modes of action including intercalation into the DNA double helix, topoisomerase II-mediated DNA damage, modification of helicase action and interference with DNA unwinding. The re-oxidation of the DOX-semiquinone radical

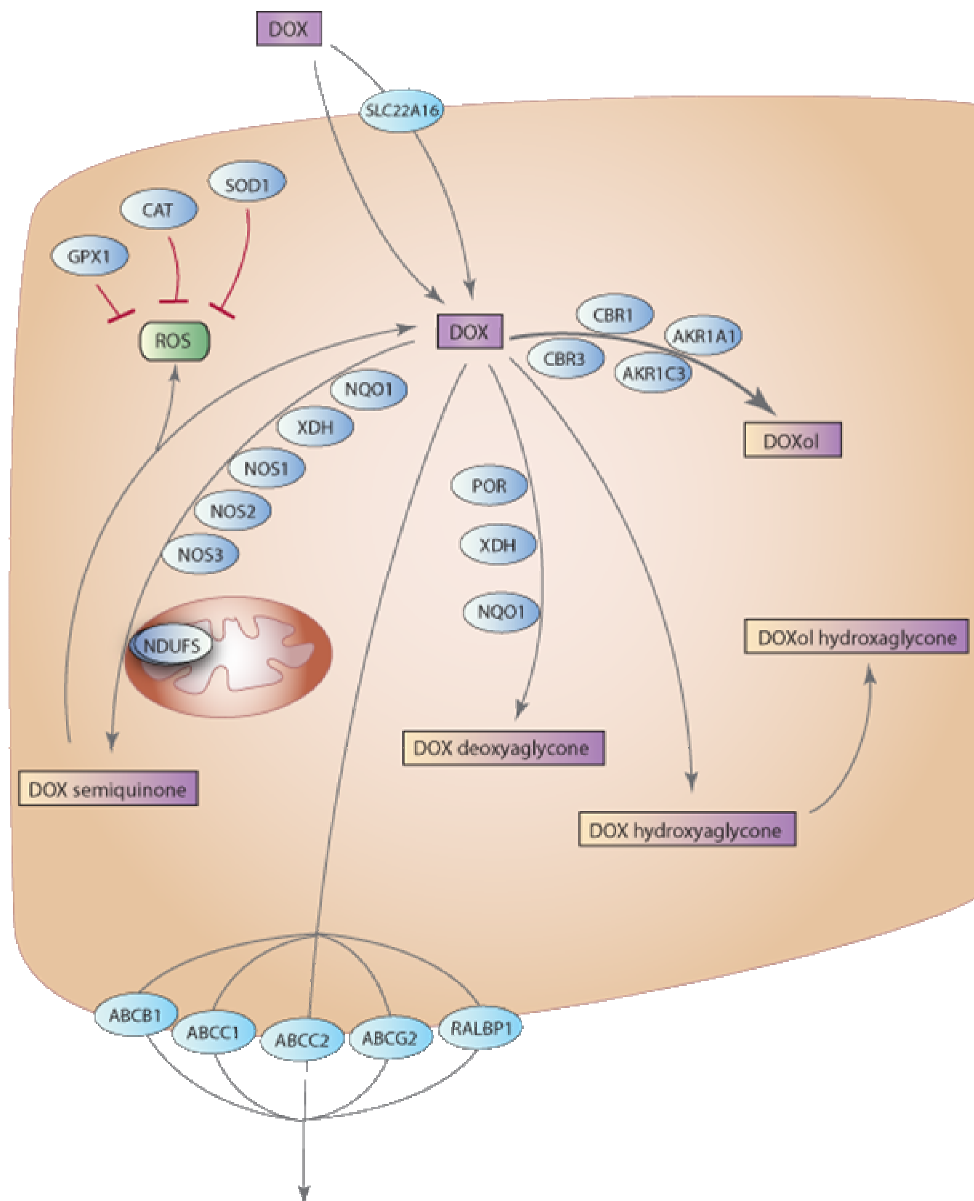
back to DOX leads to the production of ROS, accumulation of which leads to mitochondria lipid membrane damage, DNA base oxidation, disrupted calcium sequestration, energy loss and altered proliferative potential (97). Apoptotic-signalling cascades triggered by this mechanism may be responsible for cyto- and cardiotoxicity, rather than that released by one-electron reduction (53).

The highly polymorphic family of glutathione-S-transferase enzymes appear to mediate some of the cytotoxic effects of DOX, even though the drug itself is not a substrate. Overexpression of GST protects against anthracycline-induced cell-death most likely through the mediation of stress response and apoptotic pathways (100). GSTP1, as an endogenous inhibitor of c-Jun N-terminal kinase1 (JNK1), a stress response kinase, is pivotal in the regulation of the mitogen-activated protein kinase (MAPK) pathway (101). In breast cancer cell lines, DOX-induced cell-cycle withdrawal, differentiation and control of apoptosis was dependent upon Jun N-terminal kinase-stress-activated protein kinase (JNK/SAPK) signalling (102). Furthermore, decomposition of the GSTP1-JNK complex sensitizes resistant cells to doxorubicin (103).

DOX-induced DNA damage is repaired by Nucleotide Excision Repair genes including excision repair cross-complementation group 1 (ERCC1), excision repair cross-complementation group 2 (ERCC2, Xeroderma pigmentosum group D) and Base Excision Repair (BER) genes such as X-ray repair cross-complementing group 1 (XRCC1) (104). These genes detect single strand breaks and remove proteins from the DNA helix, making it more accessible to repair enzymes. Cell-cycle arrest is necessary for NER to undertake DNA damage repair, but if the damage is too extensive, apoptotic cascades are initiated.

Established mechanisms of resistance to DOX include enhanced drug efflux, altered topoisomerase II activity, altered free radical defences and altered sensitivity to apoptosis. Active drug efflux by ATP-binding cassette transporters including ABCB1, ABCC1, ABCC2 and ABCG2 has been demonstrated in a wide range of human cell lines and tumours in association with adverse outcomes (97). Intracellular distribution of DOX has been related to resistance in a number of tumours, with increased accumulation in the nucleus of drug-sensitive cells and in the cytoplasm of drug-resistant cells (105, 106). This finding has now been replicated in osteosarcoma cell lines and was found to be reversible with DOX-loaded nanoparticles but concomitant with an up-regulation of ABCB1 in the drug resistant cell lines (107).

Figure 5: Doxorubicin drug pathway



This image depicts cellular pathways involved in the transport and metabolism of doxorubicin (53)

DOX, doxorubicin; DOXol, doxorubicinol; SLC22A16, solute carrier family; CBR (1,3), carbonyl reductase (1,3); AKR1A1, aldo-keto reductase family 1, member A1; AKR1C3, aldo-keto reductase family 1, member C3; NOS (1-3), nitric oxide synthase (1-3); NDUFS, NADH dehydrogenase (ubiquinone) Fe-S protein; ROS, reactive oxygen species; NQO1, NAD(P)H dehydrogenase, quinone 1; POR, P450 (cytochrome) oxidoreductase; XDH, xanthine dehydrogenase; SOD1, superoxide dismutase 1; GPX1, glutathione peroxidase 1; ABC, ATP-binding cassette transporters; RALBP1, ralA binding protein 1. Copyright PharmGKB. Reproduced with permission from Pharm GKB and Stanford University.

A further mechanism of DOX resistance in malignant cell lines is reduced DNA cleavage and topoisomerase II activity secondary to gene mutations, decreased copy number or gene transcriptional down-regulation (97). The role of altered free radical defences in DOX cytotoxicity has been discussed previously in section 3.1.2. Finally,

modulation of cell death cascades, as above by GSTP1, or increased tolerance to DNA damage may have a significant effect on the survival of tumour cells exposed to DOX. Interestingly, recent gene expression profiling of human osteosarcoma xenografts found poorly responsive xenografts were resistant to apoptosis, with genes involving metabolic alterations and involvement of mitochondrial pathways for apoptosis and stress response more prominent for doxorubicin (108).

3.1.3 Cisplatin

Platinating agents, including cisplatin, carboplatin and oxaliplatin are widely-used in cancer chemotherapy, particularly in the treatment of head and neck, testicular, gastric, ovarian and non small-cell lung cancer (55). Cisplatin, first discovered in 1970 as an inhibitor of growth in *E.coli* (109), is the most commonly used platinating agent despite dose-limiting toxicities including nephrotoxicity, ototoxicity and peripheral neuropathy.

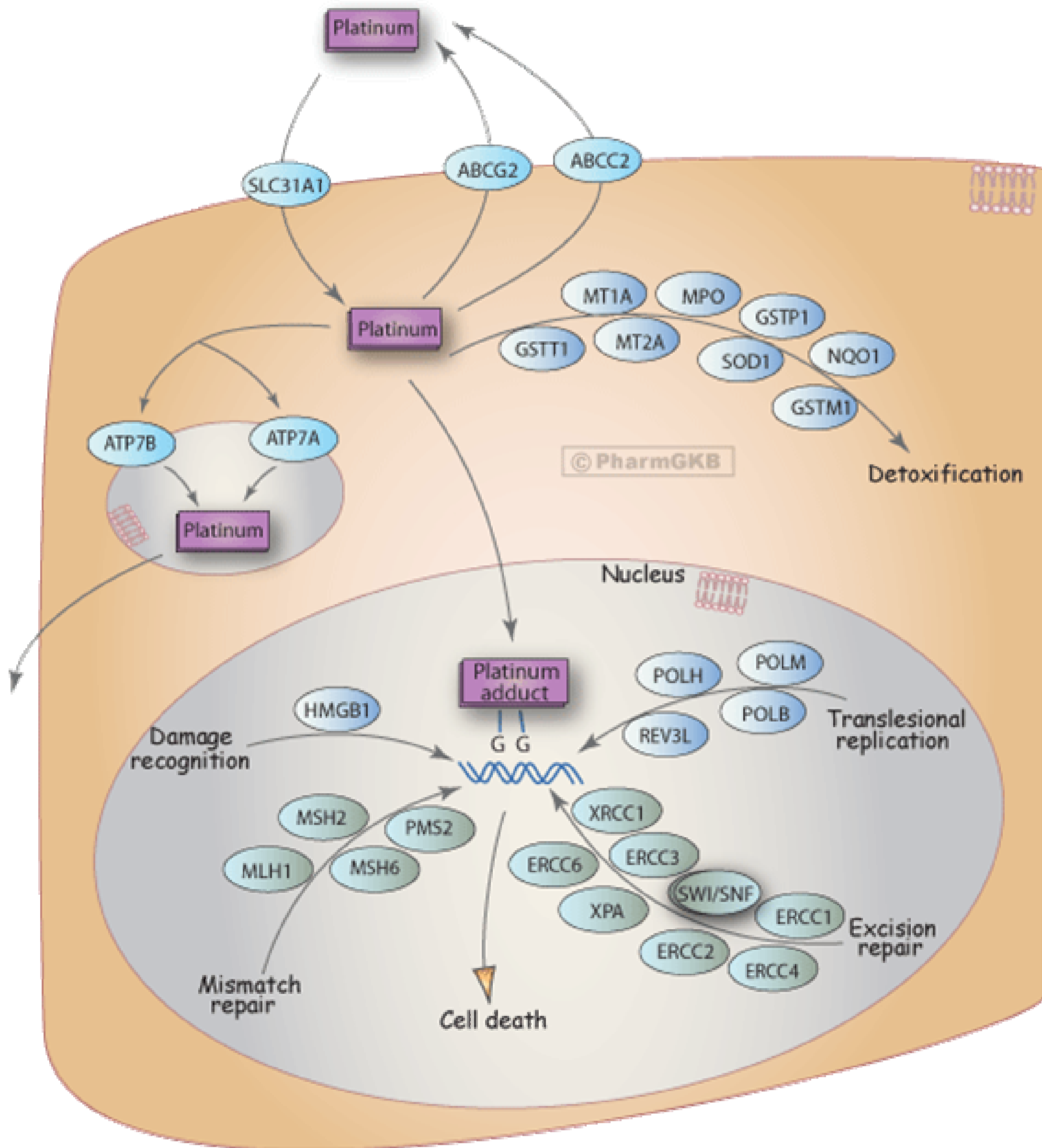
At physiological pH, cisplatin diffuses readily across the cell membrane although is actively transported by SLC31A1, (solute carrier family 31, member 1). Within the cell it becomes aquated, losing chloride or oxalate ions and gaining two water molecules. Three fates await; efflux from the cell by ATP-dependent transport proteins (ABCC2, ATP7A, ATP7B), chemical neutralisation by proteins with active sulfhydryl groups such as glutathione or metallothioneins or, interaction with nucleophilic molecules within the cell including DNA, RNA and proteins (110) (Figure 6: Platinum drug pathway). DNA is the preferential and cytotoxic target of cisplatin with three different lesions formed, monoadducts, intra- and interstrand crosslinks (111). Intrastrand adducts are probably responsible for cell death by kinking the double helix as it bends to conform to the structure of the platinum molecule, the bond angles of which are rigid compared to DNA. Recognition of DNA damage is by HMGB1 (high-mobility group box 1) with repair of bulky helix-distorting damage primarily by elements of the NER pathway and to a lesser extent, mismatch repair genes (MSH6, MLH1) (104). This occurs in two phases, initial repair of transcriptionally-active genes followed by slower repair of the quiescent parts of cellular DNA. Gene specific repair occurs in the first 6-8 hours following exposure to cisplatin and is most prominent in cisplatin resistant cells (110). If NER is overwhelmed by the degree of damage, p53, caspase and MAPK apoptotic pathways are activated (112).

Multiple mechanisms of tumour resistance to platinums have been identified of which four are well-defined; altered cellular accumulation of drug, cytosolic inactivation of drug, increased DNA repair, or an altered apoptotic process that results in increased tolerance to DNA damage.

Reduced drug accumulation is a consistent finding in cisplatin-resistant tumour cell lines and although an uptake defect appears prevalent, precise mechanisms are unclear (110). Increased cytosolic inactivation by thiol-containing molecules such as glutathione and metallothionein induce resistance to cisplatin by directly decreasing the amount of drug available to target DNA. Chronic exposure of cells to cisplatin results in increased levels of glutathione; cisplatin-glutathione interaction may be non-enzymatic or enzymatic, catalysed by GSTPi (GSTP1) (112). Overexpression of GSTP1 and γ-glutamyl transferase (GGT), a key player in glutathione homeostasis, have been found in cisplatin-resistant tumour cells whereas inhibition of glutathione synthesis leads to reversal of resistance (113-115). Protection of DNA from the effects of anti-cancer drugs seems to be a particular role of nuclear GSTP1 (116). In osteosarcoma cell lines, both increased intracellular levels and enzymatic activity of GSTP1 mediate resistance to cisplatin (117). Furthermore, overexpression in tumour samples correlates with a higher relapse rate and failure of pre-operative chemotherapy (117,118). It is likely that GSTP1 overexpression, through JNK inhibition, negatively influences the MAPK pathway, important in cisplatin-induced apoptosis (119). Evidence that the MAPK pathway is indeed an important mediator of cisplatin resistance in osteosarcoma is provided by enhanced cisplatin-induced cell death in cell lines by MPK-1 knockdown (a MAPK phosphatase that inactivates MAPK) (120).

The extent of platinum-DNA adduct formation in peripheral blood lymphocytes has been correlated with clinical response in some malignancies, suggesting that adduct formation in normal cells may parallel that in tumour tissue and may potentially be used as a predictive marker of response to chemotherapy. Children receiving cisplatin therapy show considerable inter-individual variation in adduct levels, these correlating with degree of myelosuppression (121). Furthermore, levels of circulating platinum-DNA adducts correlate with clinical outcome in non small-cell lung cancer (NSCLC) (122). Increased expression of several NER genes is associated with cisplatin resistance in solid tumours; increased intratumoural ERCC1 mRNA expression correlating with decreased survival in platinum-treated colorectal and oesophageal cancer, and transcript abundance of ERCC2, XPA and XRCC1 correlating with cisplatin resistance in NSCLC (123-125).

Figure 6: Platinum drug pathway



This image depicts the cellular pathways involved in transport and metabolism of platinum drugs (53)

ABC, ATP-binding cassette transporters; SLC31A1, solute carrier family 31, member 1; MT2A, metallothionein 2A; MPO, myeloperoxidase; GST, glutathione S-transferases; NQO1, NAD(P)H dehydrogenase, quinone 1; XRCC1, X-ray repair cross-complementing group 1; ERCC1-6, excision repair cross-complementation group; XPA, xeroderma pigmentosum, complementation group A; SWI/SNF, chromatin remodeling complex; POL, polymerases; REV3L, REV3-like, catalytic subunit of DNA polymerase zeta; HMGB1, high-mobility group box 1; MLH, mutL protein homolog 1; MSH, mutS homolog 6. Copyright PharmGKB. Reproduced with permission from Pharm GKB and Stanford University.

3.2 Pharmacogenomics of MAP chemotherapy

The advent of high-throughput microarray technology has enabled the influence of genetic polymorphisms on drug response and toxicity to be investigated not only in cancer, but many other aspects of medicine. Novel polymorphisms are continuously being added to freely available databases, although functional and clinical data is frequently lacking. Prudent selection of polymorphisms, based on putative functional effects or previous literature citations is likely to optimise the probability of revealing clinically meaningful associations. Moreover, investigation and analysis using a pathway-based approach is critical as chemotherapeutic drugs frequently share membrane transporters, detoxification enzymes and cellular targets. A lack of consensus on the clinical consequences of many polymorphisms suggests effects may be both disease and drug dependent but also reflects differing methodologies and sample size.

Currently, only three small studies have investigated the role of polymorphisms in osteosarcoma, focussing on single pathways only. Patino-Garcia et al investigated the influence of 7 folate pathway polymorphisms on histological response and toxicity of high-dose methotrexate in 96 children with osteosarcoma whereas Caronia et al genotyped 8 SNPs from 6 NER genes in 70 patients, correlating these with histological response, event-free survival and ototoxicity (19, 126). More recently, Salinas-Souza et al investigated the role of a number of GST polymorphisms in disease response (127). All are discussed further below.

Current knowledge on the pharmacogenomics of methotrexate, cisplatin and doxorubicin chemotherapy is presented with a pathway-based approach. Drugs influenced by the pathway appear in brackets by the title. For details of polymorphisms selected for the candidate polymorphism study see Table 1: Functional and clinical effects of candidate polymorphisms.

Table 1: Functional and clinical effects of candidate polymorphisms

Gene	Exon	Gene product function	rs number	Poly	Genotype	Functional effects	Clinical effects
Folate pathway							
MTHFR 1p36.22	5	Catalyses conversion of methylenetetrahydrofolate to methyltetrahydrofolate	1801133	SNP	677C>T Ala^{222}Val	T allele ↓ enzyme activity (128)	T ↑ relapse and death in ALL (129, 130), MTx toxicity in ovarian cancer (131), osteosarcoma (126) and adult ALL (133)
	8		1801131	SNP	1298A>C Glu^{429}Ala	C allele ↓ enzyme activity (82)	CC severe mucositis in NHL (133)
	8		4846051	SNP	1305C>T Phe^{435}Phe		
	12		2274976	SNP	1781G>A Arg^{594}Gln		
MTHFD1 14q24	6	Catalyses formation of substrates or purine and pyrimidine synthesis	1950902	SNP	401A>G Lys^{134}Arg		
	20		2236225	SNP	1958G>A Arg^{653}Gln	A allele ↓ enzyme activity (134)	A allele ↓ EFS in ALL (134)
RFC 21q22.3	2	Primary intracellular transporter of folates and antifolates	1051266	SNP	80G>A Arg^{27}His	AA genotype ↑ plasma levels of MTx (135), other functional studies inconclusive (136, 137)	A allele ↓ EFS in ALL (135)
DHFR 5q11.2	Intron 1	Intracellular cycling of reduced folates. Target enzyme for MTx		19bp indel		Deletion ↓ protein expression (138), dose dependent	No assoc in osteosarcoma (126). Deletion ↑ hepatotoxicity in adult ALL (132).
TS 18p11.32	5' UTR	Critical role in nucleotide metabolism by methylating dUMP to dTMP		VNTR SNP	2R or 3R alleles C/G SNP in 3R carriers	3G allele enhances transcription of TS gene (139, 83)	3R carriers adverse prognosis (140) and 3G - chemoresistance and poor OS in gastric cancer (22). 3R adverse prognosis in ALL (141)

MTHFR, methylene tetrahydrolfolate reductase; MTHFD1, methylene tetrahydrofolate dehydrogenase; RFC, reduced folate carrier; DHFR, dihydrofolate reductase; TS, thymidylate synthase; dump, deoxyuridine-5-monophosphate; dTMP, deoxyuridine-5-triphosphate; VNTR, variable number tandem repeat; SNP, single nucleotide polymorphism; 2R/3R, two or three repeat sequence; ALL acute lymphoblastic leukaemia; MTx, methotrexate; NHL, Non-Hodgkins lymphoma; EFS, event free survival; OS, overall survival;

Table 1: Functional and clinical effects of candidate polymorphisms continued

Gene	Exon	Gene product function	rs number	Poly	Genotype	Functional effects	Clinical effects
ABC efflux							
ABCB1 (MDR1) 7q21.12	13	Cellular drug efflux	1128503	SNP	1236C>T Gly^{412}Gly		T allele ↑ exposure to doxorubicin in breast cancer patients (147). No assoc. in platinum-treated ovarian cancer (143)
	26		1045642	SNP	3435C>T Ile^{145}Ile	T allele ↓ intestinal expression and drug transport activity (16), T allele ↓ expression in breast cancer (149), no change in expression (145).	CC ↑ response to platinum chemotherapy in NSCLC (13,146), CC ↑ risk of relapse in AML (14). No assoc in platinum-treated ovarian cancer (143)
ABCG2 (BCRP) 4q22.1	5		2231142	SNP	421C>A Gln^{141}Lys	A allele ↓ protein expression and drug resistance (47, 148), no change in intestinal protein expression (149)	A ↓ OS in platinum-treated lung cancer (150). No assoc in platinum-treated ovarian cancer (143)
ABCC1 (MRP1) 16p13.11			246240	SNP	A>G		G ↓ methotrexate toxicity in psoriasis (151)
			3784862	SNP	A>G		
ABCC2 10q24.2	5'UTR		717620	SNP	24C>T	T allele ↑ plasma methotrexate levels in ALL (152). T allele lower mRNA in normal kidney (153)	T ↑ response to platinum chemotherapy in NSCLC (154). No assoc in ovarian cancer (143)
	10		2273697	SNP	1249G>A Val^{417}Ile		No assoc. with response to platinum chemotherapy in NSCLC (154).
	25		17222723	SNP	3563T>A Val^{1188}Glu		A ↑ acute anthracycline cardiotoxicity, in 100% LD with Cis^{1515}Tyr (155), AT/AA poor EFS (156)
	32		8087710	SNP	4544G>A Cis^{1515}Tyr		

ABC ATP Binding Cassette transporters; SNP single nucleotide polymorphism; NSCLC non small-cell lung cancer; AML acute myeloid leukaemia; ALL acute lymphoblastic leukaemia; LD linkage disequilibrium; EFS event free survival; OS overall survival;

Table 1: Functional and clinical effects of candidate polymorphisms continued

Gene	Exon	Gene product function	rs number	Poly	Genotype	Functional effects	Clinical effects
DNA repair							
ERCC1 19q13.32	3'UTR	Recognition and repair of bulky, helix distorting DNA damage (nucleotide excision repair)	3212986	SNP	1510C>A (8092C>A)*	A allele affects mRNA stability (157), increased DNA adduct levels in AA (158) *(Now known as CD3EAP 1510C>A Gln504Lys)	A ↑ PFS and OS in cisplatin-treated cancer (164). A ↓ OS (160) and ↑ OS (161, 162) in NSCLC. A ↑ GI toxicity in NSCLC (168). No assoc in platinum-treated ovarian cancer (143)
	4		11615	SNP	354T>C Asn[118]Asn	C allele ↓ ERCC mRNA levels and DNA repair (164). T allele ↑ protein expression (165)	TT ↓ PFS in colorectal cancer (17). CC ↑ treatment response (162) and survival in NSCLC (18). No assoc in gastric (22), colorectal cancer (17) or osteosarcoma (19)
ERCC2/XPD 19q13.32	23	Required for DNA unwinding prior to lesion removal	13181	SNP	2251A>C Lys[751]Gln	C allele ↓ ERCC2 mRNA expression (166). AA genotypes suboptimal DNA repair (167)	C ↑ poor histological response and ↓ EFS in osteosarcoma (19), ↓ survival AML (168), and platinum-treated colorectal (169) and oesophageal cancer (159). No assoc. in ovarian cancer (143) or oesophagogastric cancer (170). C ↑ haem. toxicity in platinum-treated colorectal cancer (171).
ERCC4 16p13.3	8	DNA repair	1800067	SNP	1244G>A Arg[415]Glu		A allele implicated in breast cancer risk (172)
XRCC3 14q32.33	5	Base excision repair	861539	SNP	722C>T Thr[241]Met	T allele ↓ DNA repair capacity (173)	Heterozygotes ↑ DFS in childhood AML (174). TT ↑ survival in cisplatin-treated oesophagogastric cancer (170). No assoc in gastric (22) or colorectal cancer (17)
XPC 3p25	16	Nucleotide excision repair	2228001	SNP	2886A>C Lys[939]Gln	C allele ↓ DNA repair capacity (175)	No assoc with clinical outcome in AML (176) or lung cancer (161)

ERCC excision repair cross complementation; XRCC, X-ray cross complementation; XPC, Xeroderma pigmentosum, complementation group C; SNP single nucleotide polymorphism; NSCLC non small-cell lung cancer; AML acute myeloid leukaemia; EFS event free survival; PFS progression free survival; DFS disease free survival; OS overall survival;

Table 1: Functional and clinical effects of candidate polymorphisms continued

Gene	Exon	Gene product function	rs number	Poly	Genotype	Functional effects	Clinical effects
GST enzymes							
GSTP1 11q13.2	5	Glutathione conjugation and regulation of cellular response to genotoxic, metabolic and oxidative stress	1695	SNP	313A>G Ile^{105}Val	G allele ↓ enzyme activity (177)	AA ↑ chemoresistance and ↓ OS in gastric cancer (22). AA ↑ risk of relapse in ALL (178). G ↑ response to platinum chemotherapy in NSCLC (154). No assoc (143) and GG ↓ OS (179) in ovarian cancer. GG ↑ neurotoxicity in colorectal cancer (17, 180)
	6		1128272	SNP	341C>T Ala^{114}Val	T allele causes structural protein change but no catalytic change (181)	T allele ↓ CNS relapse in ALL (182). No assoc with relapse in ALL (178).
GSTT1 22q11.23		Glutathione conjugation and regulation of cellular response to genotoxic, metabolic and oxidative stress		DEL		Absent enzyme activity in null allele carriers (183)	Null genotype ↑ relapse and ↓ EFS in AML (21). Null ↓ HR for death in breast cancer (184), ↓ relapse in ALL (178), ↑ PFS in ovarian cancer (185)
GSTM1 1p13.3		Glutathione conjugation and regulation of cellular response to genotoxic, metabolic and oxidative stress		DEL		Absent enzyme activity in null allele carriers (183)	Null genotype ↓ HR for death in breast cancer (184). Null ↓ relapse in ALL (178), ↑survival in ovarian cancer (186)

GST, glutathione S-transferase; SNP, single nucleotide polymorphism; DEL, deletion; CNS, central nervous system; HR, hazard ratio; AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; NSCLC, non small-cell lung cancer; EFS, event free survival; PFS, progression free survival; DFS, disease free survival; OS, overall survival;

Table 1: Functional and clinical effects of candidate polymorphisms continued

Gene	Exon	Gene product function	rs number	Poly	Genotype	Functional effects	Clinical effects
Others							
CBR3 21q22.12	1	Metabolises doxorubicin to doxorubicinol by two-electron reduction	8133052	SNP	11G>A $Cys^{4}Tyr$	G allele ↑ conversion of doxorubicin to doxorubicinol, ↑ CBR3 mRNA (99)	A allele ↑ tumour response and haem toxicity in breast cancer (99)
	3		1056892	SNP	730G>A $Val^{244}Met$	G allele ↑ conversion of dox to doxorubicinol (187)	G allele ↑ OR for chronic ACT (188)
CCND1 11q13	4	Cell cycle regulator	9344	SNP	870A>G $Pro^{241}Pro$	A allele ↑ protein half life (189) and ↓ sensitivity to methotrexate (190)	AA ↓ toxicity (191) and ↓ EFS in ALL (192). A allele ↑ poor outcome in lung cancer (193)
NQO1 16q22.1	4	Protect myocytes against oxidative stress	1131341	SNP	415C>T $Arg^{139}Trp$	T allele ↓ enzyme activity (194)	
	6		1800566	SNP	609C>T $Pro^{187}Ser$		T allele ↓ enzyme activity and allows rapid degradation (53)
NADPH NCF4 22q13.1	5' near gene	Protect myocytes against oxidative stress	1883112	SNP	212A>G		AA genotype assoc with chronic ACT (155). G ↓ haem, infectious and cardiac toxicity in DLBL (156)
CYBA p22phox 16q24	4	Protect myocytes against oxidative stress	4673	SNP	242C>T $His^{72}Tyr$	Functional consequences unclear (155)	T allele carriers associated with acute and chronic ACT (155). TT ↓ EFS in DLBL (156)

CBR3, carbonyl reductase 3; CCND1, cyclin D1; NQO1, NAD(P)H dehydrogenase, quinone 1; OR, odds ratio; ACT, anthracycline cardiotoxicity; EFS, event-free survival; ALL, acute lymphoblastic leukaemia; DLBL, diffuse large B-cell lymphoma

3.2.1 The folate pathway (MTX)

Extensive studies in childhood ALL and rheumatoid arthritis (RA) have helped define the pharmacogenomics of the anti-folate drug methotrexate (12, 195-199). Whilst this may indicate genes of potential importance in osteosarcoma, it must be emphasised that methotrexate administered within MAP is at a significantly higher dose than that used for ALL and particularly RA and the extent to which this influences target and metabolizing enzymes is unclear.

Methotrexate is actively transported into the cell by the RFC protein. A common functional SNP in this gene c.80G>A p.Arg^{27}His, influences plasma folate and homocysteine levels in healthy individuals (137). Patino-Garcia et al did not observe any association between c.80G>A p.Arg^{27}His and disease response or toxicity in osteosarcoma, although in children with ALL, variant homozygotes had higher plasma methotrexate levels and inferior event-free survival (126, 138). *In vitro* studies have shown no genotypic effect on methotrexate transport in erythroleukaemia cell lines (136). Seeking to identify other RFC mutations in childhood leukaemia, Kaufman et al screened the entire coding region for sequence alterations (200). Somewhat surprisingly, only 1.2% patient samples harbored mutations although RFC inactivation is thought to be a frequent mechanism of methotrexate resistance in human leukaemia cell lines. They concluded the evidence did not suggest a significant role of RFC mutations in intrinsic or acquired resistance in childhood leukaemia, contrary to resistance mechanisms seen in osteosarcoma.

Pharmacogenomic effects of DHFR, TYMS and MTHFR, the three main target enzymes of methotrexate and its polyglutamates, have been investigated in various malignancies. Decreased DHFR expression was observed in diagnostic compared to metastatic biopsy specimens from osteosarcoma patients (126). A number of functional polymorphisms have been reported in the DHFR gene, some associated with clinical outcome. Promoter SNPs are associated with higher DHFR mRNA levels and worse ALL outcome in children (201, 202). A 19bp insertion/deletion polymorphism in intron 1 has also been described whereby the deletion allele removes a potential Sp1 transcription factor binding site and is associated with lower plasma homocysteine levels (203, 204). This allele is associated with increased hepatotoxicity in adult ALL and increased risk of breast cancer, albeit it only in multivitamin users (132, 138).

A variable number tandem repeat (VNTR) polymorphism within the 5'-untranslated region (UTR) of the TYMS gene influences mRNA levels, with the three-repeat (3R) showing greater translational efficiency than the two-repeat sequence (139). The

TYMS 3R genotype showed no association with histological response, survival or chemotherapy toxicity in osteosarcoma although 3R homozygosity was associated with inferior outcomes in 205 children with ALL (126, 141). Methotrexate exposure was the major difference between these studies, with nearly four-fold higher doses administered to the osteosarcoma patients. Of note in both studies, germline rather than tumoural DNA was genotyped. Similar association of 3R with adverse outcomes has been reported in gastric cancer. The presence of a G/C snp within the triple repeat may further influence transcriptional activation, reducing the deleterious effect of this genotype (205). A small study reported superior survival in gastric cancer patients with favourable TYMS genotypes (2R/2R, 2R/3C, 3C/3C) using DNA extracted from paraffin-embedded tumour DNA (140). Two other studies in advanced gastric cancer, one enrolling 175 patients, also found poor survival was significantly associated with TYMS 3G genotypes (2R/3G, 3G/3C, 3G/3G) although in contrast to the former, genotyping was performed on DNA extracted from peripheral blood samples (22, 206). Patients received similar chemotherapy with 5-fluourouracil, another drug acting on the folate pathway. Patino Garcia et al did not genotype the G>C SNP in osteosarcoma patients although did analyse an additional 6-bp deletion/insertion polymorphism in the TYMS 3'-UTR that was not significant (126). This deletion polymorphism is associated with decreased TYMS mRNA stability *in vitro* and lower intratumoral levels in colorectal cancer (207). In platinum-treated gastroesophageal cancer, the TYMS 3R/+6 haplotype was identified as an independent predictor of superior overall survival and was also associated with the lowest incidence of treatment-related leucopenia (208).

Several functional polymorphisms have been found in the MTHFR gene, with c.677C>T p.Ala^{222}Val the most heavily investigated in cancer. Reduced enzyme activity is associated with the variant allele of MTHFR c.677C>T p.Ala^{222}Val, hetero- and homozygotes having 60% and 30% of wild-type activity respectively (128). This leads to impaired remethylation of homocysteine to methionine, adversely affecting DNA replication and repair but also likely to enhance methotrexate toxicity. Increased risk of relapse and leukaemia-related death associated with the variant allele was described in 201 Canadian children with ALL, findings validated by 2 subsequent studies including the largest single-trial paediatric oncology pharmacogenomics study published to date (129, 130, 134). Aplenc et al genotyped 520 paediatric patients enrolled onto a Children's Cancer Study Group ALL trial (CCG-1891), finding the variant allele of c.677C>T p.Ala^{222}Val more predictive of relapse than day 7 bone marrow status, initial white cell count and treatment regimen. In concordance, in adult ALL it was associated with decreased survival (132). In all cited ALL studies , genotyping was performed on DNA extracted from peripheral blood or bone marrow at diagnosis, possibly leading to

confusion whether findings represent germline or tumoural associations. Over-representation of the variant allele has also been found in early-onset breast cancer (209).

Enhanced methotrexate toxicity has been repeatedly associated with c.677C>T p.Ala^{222}Val variants in solid and haematological malignancies. In osteosarcoma, grade 3-4 haematological toxicity was significantly higher in patients with variant homozygote genotypes (126). In ovarian cancer, variant homozygotes were 40 times more likely to experience grade 3-4 toxicity, particularly haematological and mucositis, than other genotypes (131). This study consisted of 43 heavily pre-treated patients with refractory disease, receiving low-dose M ordinarily unlikely to cause severe toxicity. Similarly, increased haematological and gastrointestinal toxicity was observed in variant homozygotes receiving methotrexate maintenance therapy for ALL, high-dose for NHL and prophylaxis for graft-versus-host-disease (133, 210, 211). Although these studies are all small with different methotrexate dosing and administration schedules, concordant results increase confidence of a real association. Furthermore, a meta-analysis investigating the effect of folate pathway polymorphisms in RA found c.677C>T p.Ala^{222}Val exerted a small but significant increase in toxicity (212). However, the association was not replicated by the large Children's Cancer Study Group ALL trial (129). A second reduced-function SNP in the MTHFR gene, c.1298A>C p.Glu^{429}Ala was not associated with disease outcome or toxicity in osteosarcoma or childhood ALL (126, 129). Reduced *in vitro* sensitivity of lymphoblasts to methotrexate was observed in heterozygotes but in adult NHL, variant homozygotes were at significantly increased risk of mucositis (133, 213).

3.2.2 ATP-Binding Cassette Transporters (MTX, DOX, cisplatin)

The ATP-Binding Cassette (ABC) superfamily of membrane efflux-transporters regulate the bioavailability of endogenous and exogenous molecules with ATP hydrolysis enabling active transport of substrates against steep concentration gradients. Three human ABC transporters are primarily associated with the multidrug resistance phenomenon, namely ABCB1 (P-glycoprotein, MDR1), ABCC1 (MRP1) and ABCG2 (breast cancer resistance protein, BCRP) (214).

ABCB1 is the best characterized human drug transporter, with substrates including steroids, lipids, peptides and cytotoxics (16). Several SNPs have been reported in this gene, a functional effect first demonstrated in exon 26 c.3435C>T p.Ile^{145}Ile. Although not causing an amino acid change, the T allele is associated with decreased intestinal protein expression and drug transport activity in healthy volunteers and decreased

protein expression in breast cancer patients (144, 215). Supporting the functional data, infectious complications in childhood ALL were significantly increased in TT homozygotes (216). Other results are conflicting, superior responses to cisplatin-based chemotherapy of lung cancer were reported in CC homozygotes by two studies, each genotyping 54 patients (13, 146). However, patients with this genotype experienced inferior survival in acute myeloid leukaemia (AML), likely due to increased relapse (226). Sample size and ethnicity illustrate fundamental differences between these studies; the AML cohort was 10-fold larger and included European patients whereas the lung cancer studies were conducted on Korean and Chinese patients. In particular, ethnic variations in allele frequency distributions may preclude direct comparisons.

ABCB1 c.3435C>T p.Ile^{145}Ile is in strong linkage disequilibrium with both c.1236C>T p.Gly^{412}Gly and c.2677G>A/T. In Asian breast cancer patients, those harboring the wild-type homozygote haplotype of all 3 SNPs had significantly decreased DOX exposure levels and increased clearance although patient outcome was not included as an end-point (142). In cisplatin-treated NSCLC, patients carrying at least one variant T allele of c.2677G>A/T had significantly increased drug resistance and gastrointestinal toxicity (217). In contrast, patients from the Australian Ovarian Cancer Study who carried the minor T/A alleles were significantly less likely to relapse, although subsequently this effect was not confirmed in an independent validation set (218). One of the largest published studies in pharmacogenomics, the Scottish Randomised Trial in Ovarian Cancer (SCOTROC) also failed to validate this observation (143). Genotyping of 27 polymorphisms from 16 key genes in taxane and platinum cellular pathways was performed in over 900 women, with no significant association between genotype and chemotherapy toxicity or disease outcome found for any gene including ABCB1.

ABCG2 was first detected in cross-resistant breast cancer cell lines but is now known to be widely expressed in normal tissues including the placenta, blood-brain barrier, testis and intestine (214). Methotrexate is a high-capacity, low-affinity substrate of ABCG2 which unlike other drug efflux pumps, is able to transport M-di and triglutamates (219). Overexpression correlates with resistance to M and a p.Arg^{482}Gly mutation confers high-level resistance to antifolates (15, 220, 221). More than one study has shown the variant allele of ABCG2 c.421C>A p.Gln^{141}Lys reduces protein expression and enhances chemosensitivity (147, 148). This SNP was included in the SCOTROC study although no association was found with chemotherapy toxicity or disease outcome (143). Doxorubicin is also an established ABCG2 substrate; protein overexpression correlates with resistance and a tendency towards decreased DOX cytotoxic activity in childhood and adult leukaemia (222, 223). Cisplatin is not a

recognised substrate, although in a subgroup analysis of patients treated for lung cancer, worse survival was seen for variant carriers of c.421C>A p.Gln^{141}Lys treated with platinum-based drugs (150, 214).

The substrate specificity of ABCC2 (multidrug resistance-related protein, MRP2) includes glutathione, glucuronide and sulfide conjugates as well as conferring resistance to a number of cytotoxic drugs (224). Cisplatin-resistant melanoma cells overexpress ABCC2 mRNA and protein in association with decreased DNA platination and accelerated re-entry into the cell cycle (225). In ovarian carcinoma, the cellular location of ABCC2 appears to be of importance in mediating resistance. Nuclear, as opposed to cytoplasmic membrane expression, correlates with decreased chemotherapy response and survival (226). Methotrexate is also an important substrate, with transfection of ABCC2 into ovarian cancer cell lines causing marked resistance to short-term M exposure (227).

Polymorphisms in the ABCC2 gene appear to influence the activity of all three MAP chemotherapy drugs. When all 32 exons were analysed in 59 healthy white subjects, 8 common SNPs were identified, 5 of which were in complete linkage disequilibrium (152). The same group analysed 44 paediatric ALL patients treated with HD-M (5g/m^2), reporting a 2-fold increase in M area under the curve (AUC) and 9-fold increased risk of additional folinic acid rescue with the variant allele of c.24C>T (152). However, this was observed in female patients alone, of which there were only 15 in the study. Increased response to platinum-based chemotherapy with c.24C>T was recently reported in 113 Chinese patients with NSCLC but this was not gender-specific (154). In nearly 1700 patients with NHL, the ABCC2 c.3563T>A p.Val^{1188}Glu-c.4544G>A p. Cis^{1515}Tyr haplotype was associated with acute anthracycline cardiotoxicity in addition to a non-synonymous ABCC1 SNP, ABCC1 c.2012G>T p.Gly671Val (160). ABCC2 c.3563T>A p.Val^{1188}Glu has previously been implicated in poor survival in diffuse large B-cell lymphoma (DLBL) (156). Many ABCC2 polymorphisms exhibit strong linkage disequilibrium thus attributing an effect to a single SNP may be misguided. The identification of reliable associations will require large numbers of patients .

3.2.3 Glutathione S-transferases (cisplatin, DOX)

GST enzymes are implicated in cisplatin and DOX resistance both through glutathione conjugation and a regulatory role in apoptosis. Seven classes of cytosolic GSTs have been identified, namely alpha, mu, pi, theta, sigma, omega, theta and zeta, based on sequence similarities, substrate specificities and immunoreactivity. Highly polymorphic, GSTs have a number of enzymatic (glutathione conjugation) and non-enzymatic

functions (regulation of cellular responses to genotoxic, metabolic and oxidative stress) and a wide range of substrates (114). For this reason, attention has focussed on a potential role of GST polymorphisms, mainly those of pi (GSTP), mu (GSTM), theta (GSTT) and alpha, in response to and toxicity of cancer chemotherapy.

GSTP1 is widely expressed in human epithelium, with overexpression contributing to both cisplatin and DOX resistance in osteosarcoma (228). GSTP1 has two common polymorphisms, c.313A>G p.Ile^{105}Val and c. 341C>T p.Ala^{114}Val, the four alleles showing significant differences in their abilities to protect against anti-cancer agents (229). The variant allele of c.313A>G p.Ile^{105}Val is associated with significantly decreased enzyme activity although the functional effect of c.341C>T p.Ala^{114}Val is less clear (177, 181). There are no published data on the influence of GSTP1 c.313A>G p.Ile^{105}Val in osteosarcoma disease response although extensive research exists in other malignancies, much of it conflicting. The variant allele has been associated with superior outcomes in ALL, NSCLC, gastric and breast cancer (22, 140, 154, 178, 230, 231). However, others report the opposite or no association (17, 143, 208, 232).

The association of c.313A>G p.Ile105Val with chemotherapy toxicity is similarly conflicting. The SCOTROC study reported no association but in gastroesophageal cancer and AML, wild-type homozygotes were at risk of severe haematological toxicity (143, 208, 233). Similarly, testicular cancer survivors with this genotype were at increased risk of cisplatin-induced hearing impairment and patients with gastrointestinal cancer were at increased risk of oxaliplatin-induced neuropathy (234, 235). In contrast, in oxaliplatin-treated colorectal cancer, wild-type homozygotes were at decreased risk of neurotoxicity (17).

GSTT1 and GSTM1 are also polymorphic, both demonstrating common null allleles (GSTT1 null, GSTM1 null) with absent enzyme activity (183). Emerging evidence implicates GSTT1 and GSTM1 null alleles as predictors of cancer risk as a consequence of decreased detoxification of carcinogens. Individuals with GSTT1-null genotype are at significantly increased risk of bladder cancer , meningioma, AML and squamous cell carcinoma (114, 236). The GSTM1-null allele is particularly associated with lung and bladder cancer risk in smokers (237). Salinas-Souza et al recently investigated the influence of GSTT1 and GSTM1 on clinical outcome in a case-control study of 80 osteosarcoma patients and 160 normal controls (127). The presence of at least one allele of GSTM1 was associated with good histological response and superior survival whereas patients null for GSTM1 were at increased risk of pulmonary relapse. In contrast, GSTT1 null correlated with improved overall survival. In lung cancer,

GSTM1 deletions have also been associated with poor survival suggesting a particular role in lung protection (238).

The role of GSTT1 and GSTM1 deletions in response to cancer chemotherapy has also been extensively investigated in AML. A number of studies have shown an association between GSTT1-null genotype and inferior survival due to increased relapse rate, resistance to induction chemotherapy or increased treatment-related mortality (21,239-241). GSTM1-null enhanced resistance to induction chemotherapy and decreased survival in some (241, 242) but not all studies (21, 233, 239). In ovarian cancer, mean survival time was significantly improved in GSTM1-null and GSTT1-null patients (185, 186). The combination of both GSTT1-null and GSTM1-null alleles appears additive, associated with decreased risk of death in breast cancer and decreased risk of relapse in ALL but unresponsiveness to primary chemotherapy in ovarian cancer, (178, 184, 243).

The anti-oxidant functions of GST enzymes suggest they are of potential importance in protecting against anthracycline cardiotoxicity. Only one published study has investigated the role of GST polymorphisms in cardiac toxicity. Archived bone marrow DNA was genotyped for GSTT1 and GSTM1 from 76 long-term survivors of childhood ALL, with cardiac toxicity assessed using echocardiography and serial electrocardiogram measurements (244). No genotypic association was observed with cardiac damage.

3.2.4 Nucleotide excision repair (cisplatin, DOX)

A cytotoxic mechanism common to both cisplatin and DOX is DNA damage due to intercalation, strand-breaks or adduct formation. Multiple cellular pathways process DNA damage and restore genomic integrity; DNA repair pathways enzymatically restore DNA, cell cycle checkpoint control mechanisms allow adequate time for repair, and apoptosis pathways mediate programmed cell death when damage is excessive (104). Enzymatic restoration of bulky cisplatin or DOX-induced damage is primarily by NER genes such as ERCC1, and to a lesser extent BER genes such as XRCC1. Polymorphic functional variants of these enzymes may theoretically lead to suboptimal DNA repair in normal cells thereby enhancing toxicity or in malignant cells, enhanced repair leading to drug resistance.

ERCC1 has two well-characterized functional polymorphisms, c.354T>C p.Asn^{118}Asn and c.8092C>A (now known as CD3EAP c.1510C>A p.Gln^{504}Lys). The C allele of c.354T>C p.Asn^{118}Asn is associated with both decreased mRNA levels and increased

cisplatin sensitivity in ovarian cancer cell lines (164). This SNP was not associated with clinical outcome or chemotherapy toxicity in osteosarcoma (19). Similarly, no association with outcome was seen in NSCLC, oesophageal or gastric cancer (22, 159, 160, 245). These studies differed in size, enrolling between 65-175 patients but platinum-based chemotherapy was administered in all. However, the Spanish Lung Cancer Group reported significantly increased median survival in NSCLC patients homozygous for the C allele (18). Improved response and longer survival with the CC genotype was also seen in two separate studies of metastatic colorectal cancer treated with FOLFOX-4 (fluorouracil, oxaliplatin) chemotherapy, enrolling 166 European and 168 Asian patients respectively (17, 165). Although a similar genotypic influence was seen, the prevalence of CC in the Asian cohort was twice that of the Euorpean cohort, highlighting the difficulties of comparing different ethnicities. In complete contradiction, the TT genotype conferred a more favourable response to platinum-based chemotherapy in ovarian cancer, although in this study DNA was extracted from paraffin-embedded tumour (246).

The variant allele of c.8092C>A is associated with increased DNA adduct levels, suggesting reduced DNA repair capacity (158). Improved survival with the variant allele was seen in patients with oesophageal cancer but in NSCLC, the same allele both improved and decreased survival (159-161). Conflicting results were also seen in ovarian cancer; no association was observed in SCOTROC but a much smaller Korean study found the variant allele was prognostic for poor progression-free survival (143, 185). The influence of c.8092C>A on chemotherapy toxicity is also unclear. The SCOTROC study found no association but grade 3-4 gastrointestinal toxicity was significantly increased in carriers of the variant allele treated with platinum chemotherapy for NSCLC (143, 163).

ERCC2 (XPD) is one of a group of proteins required for DNA unwinding prior to lesion removal (166). The ERCC2 gene contains several SNPs, with c.2251A>C p.Lys^{751}Gln extensively studied. Functional studies are conflicting with decreased mRNA associated with the C allele in one study but suboptimal DNA repair associated with the A allele in another (166, 167). This SNP was one of 8 NER polymorphisms investigated for associations with clinical outcomes in 70 patients with osteosarcoma. The odds ratio of poor histological response was increased nearly 5-fold and median survival significantly shortened in the presence of the variant allele (19). Inferior outcomes with the variant allele were observed in colorectal and oesophageal cancer but not lung or ovarian cancer (143, 159, 175, 247). The variant allele also negatively influenced survival in elderly but not childhood AML patients (174, 248).

XRCC3 is a key player in the Homologous Recombination Repair pathway, mending DNA double strand breaks in replicating cells (104). A functional SNP in exon 5, c.722C>T p.Thr^{241}Met is associated with decreased DNA repair capacity (173). In oesophagogastric cancer, variant homozygotes had improved survival although no association was seen in gastric or colorectal cancer (17, 22, 170).

3.2.5 Carbonyl reductases, NAD(P)H oxidase (DOX)

As anthracyclines are unique in their ability to cause significant cardiotoxicity, investigating the role of genetic polymorphisms in cardiac damage may provide insight into DOX toxicity *per se*. This is important as treatment strategies for most malignancies now involve multi-drug chemotherapy regimens, making the identification of pharmacogenomic influences for individual drugs extremely challenging. Carbonyl reductase (CBR) enzymes catalyse 2-electron conversion of DOX to the cardiotoxic metabolite, DOXol and are implicated in cardiotoxicity (249). Recent evidence suggests non-synonymous SNPs in CBR1 and CBR3 significantly alter *in vitro* metabolism of DOX (250, 251). In Asian patients with breast cancer, variant homozygotes of CBR3 c.11G>A p.Cys^{4}Tyr showed reduced conversion of DOX to DOXol, decreased CBR3 mRNA and experienced significantly greater tumour reduction and myelosuppression (99). The same study also showed the variant allele of another CBR3 SNP, c.730G>A p.Val^{244}Met, was associated with increased levels of DOXol suggesting higher catalytic activity, a finding replicated in a larger ethnically-diverse study (187). In contrast, two more recent studies demonstrated reduced catalytic activity with the variant allele, one also noting a trend towards increased congestive heart failure (188, 250). In Asian breast cancer patients, one diplotype of CBR1 SNPs (at least one variant allele of c.672C>T and +967G>A) was associated with significantly higher DOX exposure (252).

Polymorphisms in genes involved in oxidative stress-protection have also been associated with anthracycline cardiotoxicity. A large study of patients treated for NHL implicated polymorphisms in two distinct processes: production of reactive oxygen species and anthracycline transmembrane transport (155). Chronic ACT was associated with the c.212A>G variant of NAD(P)H oxidase subunit NCF4, responsible for downregulation of the enzyme. Acute ACT was associated with 2 SNPs in additional subunits of the same enzyme: p22phox and RAC2 (ras-related C3 botulinum toxin substrate 2). CYBA encodes p22phox, the c.242C>T p.His^{72}Tyr SNP affecting a haem-binding site thought to be important for stability of NAD(P)H oxidase. The functional consequences of this SNP are unclear with superoxide production both increased and decreased by the variant allele (253, 254). In diffuse large B-cell lymphoma treated with cyclophosphamide and doxorubicin, variant homozygotes of c.242C>T p.His^{72}Tyr

displayed poorer event-free survival (156). The same study showed a protective effect of the NCF4 c.212A>G variant allele on haematological and infectious complications and more importantly, cardiotoxicity. The variant allele of a further CYBA SNP, c.640A>G, is associated with decreased NAD(P)H oxidase activity, lower mRNA and protein expression and inferior outcome in non-Hodgkin lymphoma (273). Superoxide dismutase and catalase both play an important role in the metabolism of reactive oxygen species. Polymorphisms in these genes were investigated for a role in ACT in survivors of childhood ALL, with the finding of an association between a CAT intronic SNP and late cardiac toxicity, possibly mediated by modification of SOD transcription (244).

4.0 Copy number variation

The aim of cancer genetics is to elucidate all variant alleles that may carry a predisposition to malignancy. Single nucleotide polymorphisms have been widely used as the basis for genome-wide association studies, uncovering common SNPs associated with malignant and non-malignant disease (256, 257). However, the effect of implicated genes is small and insufficient to explain disease heritability (258). The presence of large-scale CNV is becoming increasingly recognized as a potential modifier of disease susceptibility (26). Amplification of large genomic regions has been demonstrated in a number of cancers and is thought to promote oncogenesis through gene overexpression (259) whereas deletion may remove tumour suppressor genes. A CNV is defined as a duplication or deletion of a DNA segment larger than 1000 bases (1kb) up to several Mb in size, present in variable copy number in relation to a reference genome (260). The term CNV relates specifically to germline DNA whereas *somatic copy number alteration* (SCNA) is a sequence found at different copy numbers in an individual's germline DNA and in the DNA of a clonal subpopulation of cells. A copy number polymorphism (CNP) is a locus that exhibits CNV above a specified frequency (typically 1-5%) among individuals within a population (261).

The study of CNV disease-causing mechanisms in humans has been problematic due mainly to population heterogeneity and lack of tissue samples. By using the mouse genome as a model, altered gene expression in genes mapping to CNV was shown to highly correlate with copy number (262). Effects were also observed in genes flanking CNV, suggesting that CNVs can also influence gene expression through disruption of regulatory sequences. Altered gene dosage and disruption of coding or regulatory regions may then impart potentially catastrophic phenotypic consequences.

A recent comprehensive map of the human genome found CNV regions (merged CNV, CNVR) accounted for 12% (360Mb) and were ubiquitously distributed, with approximately one-quarter located near previously known segmental duplications (263). Although current data suggests CNV tend to be located outside genes, a significant number of genes have been found within CNVR. More specifically, of 1447 HapMap CNVRs, Redon et al found 2908 protein-coding genes and 285 OMIM genes (genes associated with human disorders, listed in the Online Mendelian Inheritance in Man database, www.nci.nlm.nih.gov/omim) (263). Furthermore, genes particularly enriched within CNVs included cell adhesion, smell perception and neurophysiological processes whereas genes relating to cell signalling, proliferation and phosphorylation/kinase activity were under-represented. Negative selection against

CNV in the latter genes most likely provides some protection against dosage-sensitive genes such as tumour-suppressors or copy-number modification in those critical for normal development.

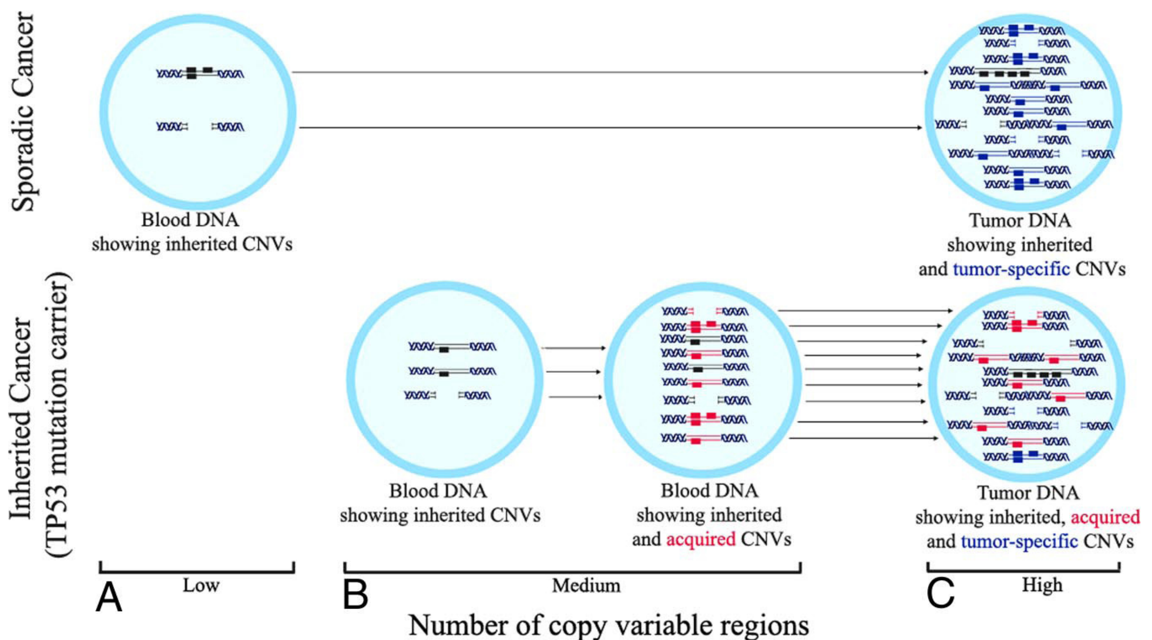
In recognition of the potential importance of CNV in oncogenesis, a comprehensive analysis of high-resolution copy number was recently undertaken in 3131 cancer samples from more than two-dozen cancer types, identifying a total of 357 significant regions of SCNA (261). Further genomic and functional studies are now underway to identify target genes within these regions. In lung adenocarcinoma, a novel proto-oncogene ($NKX_{2\text{-}1}$) was recently identified after analysis of 300 samples found 31 recurrent focal events, including 7 homozygous deletions and 24 amplifications (264). Copy number profiling of paediatric acute lymphoblastic leukaemia patients showed an average of 6 copy-number alterations per leukaemia genome with significant differences between subtypes (265). It is proposed that the number of CNVs in genomes of healthy individuals is maintained at a figure greatly below the number present in genomes of those genetically predisposed to malignancy. More CNVR are acquired as a tumour grows. This model is shown in Figure 7.

4.1 CNV in osteosarcoma

Cytogenetic studies reveal complex chromosomal abnormalities in the majority of osteosarcoma tumour samples (266). Certain chromosomal regions appear to be affected more frequently than others with loss of chromosomes 9, 10, 13 and 17 and gains of 1q, 3q, 6p, 8q, 12q, 14q, 17p, Xp and Xq commonly reported (267-269). Furthermore, evidence suggests resistance to doxorubicin and methotrexate by human osteosarcoma cell lines may be mediated through gene copy number and expression changes (270).

Using comparative genomic hybridization (CGH) on tumour samples from 47 patients registered on the Cooperative Osteosarcoma Study, Ozaki et al detected genomic imbalances in 40 of 41 primary tumours and 6 of 6 relapsed tumours (271). Gains predominated over losses with the median number of aberrations significantly higher in primary high-grade osteosarcoma than lower grade subtypes. Loss of 13q was observed in 41% of tumours and was strongly associated with poor prognosis. This is not unexpected as this locus harbors the RB1 gene, previously established as a prognostic predictor in osteosarcoma (272). Frequent high-level gains were observed in 1p, 8q and 17p, supporting observations from previous studies (273, 274).

Figure 7: Proposed model for CNVs in tumourigenesis



A model of copy-number-variable DNA regions in patients with sporadic (top) or inherited (bottom) cancer. It is proposed that healthy people maintain a similar low number of CNVs in their genomes (left; black blocks indicate inherited CNVs), whereas those at risk of developing early onset cancer have an excess of CNVs and a greater overall genomic burden of copy-number-variable DNA (middle; red blocks indicate somatically acquired CNVs). As a tumor grows, it acquires more copy-number-variable regions, including tumor-specific regions (blue). Reproduced with permission from Schlien et al. Excessive genomic DNA copy number variation in the Li-Fraumeni cancer predisposition syndrome. PNAS 2008: 105 (32); 11264-11269 Copyright (2008) National Academy of Sciences, USA.

More recent studies have replicated these findings. CGH array of 48 osteosarcoma samples found that high-level amplification and homozygous deletions constituted 28.6% and 3.8% of the tumour genome respectively with recurrent amplifications mapping to 1p36.32, 6p21.1, 8q24, 16p13 and 17p11.2 (275). Remarkably, amplification of 6p, 8q and 17p was also found in a study of only 9 osteosarcoma tumours (276). A further study of CGH array of 22 tumour samples reported similar findings, with large amplified regions recurrently detected at 12q11-q15, 8q24, 6p12-13 and 17p11-p13 (269). A much smaller CGH array study of only 10 tumours again reported consistent amplification of 6p22, 8q24 and 17p12 (259). Protein expression and immunohistochemistry studies have now identified CDC5L, a cell cycle regulator important for G2-M transition, as the likely candidate oncogene for the 6p12-21 amplicon in osteosarcoma (277).

Genetic alterations in the 8q24 chromosome region have been associated with cancers other than osteosarcoma. Genome-wide association studies have found associations between risk of breast, bladder, prostate and colorectal cancer and SNPs at 8q24, a

region approximately 300kb away from the MYC oncogene (278-280). Mirabello et al investigated this region in osteosarcoma by genotyping 214 tag SNPs in germline DNA from 99 cases and 1430 controls (281). Analyses of nine SNPs previously associated with cancer were non-significant and although a further 7 SNPs showed a significant association, the susceptibility observed was modest. Array studies clearly pinpoint that this region is of interest but further definition is required.

Amplification of the chromosome region 17p is consistently reported, the putative oncogene or genes remain uncharacterised, reflecting the low resolution of CGH arrays. To address this, van Dartel et al undertook semiquantitative PCR of candidate genes in this region known to encode signal transduction pathways or involved in growth regulation. Findings suggested that multiple amplification targets contributed to osteosarcoma tumourigenesis including TOP3A, MAPK7 and PMP22 (282). Subsequent expression profiling of a further 11 samples confirmed overexpression of PMP22 but also implicated COPS3 (283).

Yan et al performed quantitative real-time PCR for COPS3, PMP22, NCOR1 and TOM1L2 in 155 osteosarcoma samples, finding COPS3 amplification in 48 tumours (267). Amplification was strongly associated with large tumour size but the study was underpowered to detect an effect on survival. Furthermore, COPS3 amplification and p53 mutation frequently co-existed suggesting they are not mutually exclusive events. PMP22 amplification was seen in 12.2% of cases but never in isolation suggesting COPS3 was the likely target gene for the amplicon. As PMP22 is an integral membrane protein constituting a major component of myelin in the peripheral nervous system, explanation of a role in osteosarcoma tumourigenesis is difficult. A role for COPS3 is more logical, as it encodes a subunit of the COP9 signalosome implicated in the ubiquitination and ultimately degradation of the p53 tumor suppressor, possibly resulting in a phenotype similar to p53 mutation (284). In search of novel copy number alterations in osteosarcoma, Pasic et al analysed DNA from primary biopsies of 27 patients (285). The most common deletion was within chromosome 3q13.31, observed in 14 patients. Further copy number analysis on paired blood and osteosarcoma tumour DNA confirmed these were *de novo* events in tumour DNA. Cell lines from other malignancies also contained regions of focal copy number loss in this region, supporting the hypothesis that it may be involved in tumourigenesis. The deletions mapped to two non-coding RNAs (ncRNAs) and the *limbic-system associated membrane protein (LSAMP)* tumour suppressor and were frequently accompanied by loss of heterozygosity in flanking DNA. Expression profiling showed that dysregulation of 3q13.31 was ubiquitous, with a change in expression of at least one gene evident in 100% samples. Furthermore, proliferation of osteosarcoma cell lines was promoted by

depletion of *LSAMP* and one of the ncRNAs, the latter also upregulating transcription of CCND1, VEGF and VEGR1 genes. Two other groups have also reported deletions at 3q13.31 in osteosarcoma, suggesting *LSAMP* is a novel candidate tumour suppressor gene (286, 287).

Using p53+/- mice as a model for human osteosarcoma, Ma et al found seven frequent regions of copy number gain and loss but focused on a recurrent amplification event on mouse chromosome 9A1 (288). Increased expression was noted in three genes on this amplicon: Birc2 and Birc3, anti-apoptotic genes and MMP13, matrix metalloproteinase. High MMP13 RNA expression was also observed in human osteosarcoma relative to normal osteoblasts. Transplantation of immunodeficient recipient mice with osteosarcoma cell lines transduced with lentiviral shRNA vectors to downregulate Birc2, Birc3 and MMp1, resulted in enhanced apoptosis and reduced tumour growth.

5.0 Materials and Methods (i): Candidate polymorphism study

5.1 Study population

5.1.1 Patient identification

Enrollment criteria for this retrospective study comprised 1) age >16 years at study entry and; 2) completion of **M**ethotrexate, **A**driamycin, Cis**P**latin (MAP) chemotherapy for histologically proven osteosarcoma.

A database of eligible patients was created from hospital coding, outpatient, inpatient and pathology records, multidisciplinary team meeting transcripts, Chemocare (computerised chemotherapy prescribing software) and clinical trial recruitment data. The potential series consisted of approximately 100 patients who had received MAP chemotherapy (treated on previous randomised European Osteosarcoma Intergroup (EOI) trials or according to MAP guidelines). Eligible patients were invited to participate while attending routine outpatient follow-up appointments or whilst inpatients for end-of-treatment investigations. Patients ordinarily attend for outpatient review 2-6 monthly dependent on time from completion of treatment.

SNP-array genotyping was performed on the first 50 recruited patients.

5.1.2 Ethics

The study protocol (Appendix 1) was approved by the local research ethics committee (reference no: 07/H0715/105). Patient and carer information sheets were provided (Appendix 2) and written informed consent obtained (Appendix 3).

5.2 Data collection

5.2.1 Demographic data

At study entry, the following clinical data were collected from sources referred to above, coded and recorded onto a secure database:

- age and date at diagnosis, gender, ethnic group
- tumour site and histology, presence of metastases
- treatment protocol, date of commencement

- number of administered chemotherapy cycles, delays and dose modifications
- total cumulative doxorubicin and cisplatin exposure (mg/m^2)
- surgical intervention: limb salvage, amputation, tumour resection
- histological response[2]
- date of completion of chemotherapy
- adjuvant radiotherapy dose and rationale
- date of completion of treatment
- length of follow up
- time to relapse
- site of relapse
- treatment at relapse
- disease status at final analysis

To ensure completeness, missing data was sought from imaging, pathology, computerised chemotherapy records and hospital record archives.

5.2.2 Chemotherapy toxicity

Guidelines for safe administration of MAP chemotherapy dictate assessment of toxicity prior to each M, AP and A cycle to determine whether treatment delay or modification is required. Measurement of renal, cardiac and auditory function is mandatory at diagnosis, pre-operatively and at end of treatment (EoT) using EDTA creatinine clearance to estimate isotopic glomerular filtration rate (GFR $ml/min/1.73m^2$), cardiac ejection fraction/fraction shortening (EF/FS %) and audiogram respectively.

Chemotherapy-induced toxicities are graded according to Common Terminology Criteria for Adverse Events v3.0 (CTCAE) (290). The CTCAE grading system is a descriptive terminology widely used for reporting medical adverse events. An adverse event is defined as any unfavourable or unintended sign, symptom or disease temporally associated with the use of a medical treatment or procedure that may be related or unrelated to its use. It is system-based with grade assigned according to severity. CTCAE grading of cardiac, nephro and ototoxicity is presented in Table 2.

For the current study, the following toxicity data were obtained from patient records:

- GFR, EF and audiogram results at diagnosis, pre-operatively and at EoT

[2] Histological response, determined by the degree of tumour necrosis found histologically in the surgical resection specimen was defined as good (GR≥90% necrosis) or poor (PR≤90%) 307. Bacci G BF, Longhi A, Ferrari S, Forni C, Biagini R, Baccini P, Donati D, Manfrini M, Bernini G, Laris S. Neoadjuvant chemotherapy for high grade central osteosarcoma of the extremity. Histologic response to pre operative chemotherapy correlates with histologic subtype of the tumour. Cancer. 2003 Jun 15;97(12):3068-75.

- CTCAE grades from all pre-chemotherapy assessments

Cardio-, nephro- and ototoxicity were defined as:

1) **early**: decrease in EF/FS, GFR, audiogram by ≥1 CTCAE grade from diagnosis to cycle 2
2) **EoT**: decrease in EF/FS, GFR, audiogram by ≥1 CTCAE grade between diagnosis and EoT

Table 2: CTCAE grades for cardiac, renal and ototoxicity

CTCAE Grade	Cardiac	Renal	Auditory
1	Aysmptomatic EF<60-50%; FS<30-24%	GFR 60-89	Threshold shift or loss of 15 - 25dB relative to baseline, averaged at 2 or more contiguous test frequencies in at least one ear; or subjective change in the absence of a Grade 1 threshold shift
2	Asymptomatic EF<50-40%; FS<24-15%	GFR 40-59	Threshold shift or loss of >25 - 90 dB, averaged at 2 contiguous test frequencies in at least one ear
3	Symptomatic, congestive heart failure responsive to intervention; EF<40-20%; FS<15%	GFR 20-39	Adult: Threshold shift or loss of >25–90 dB, averaged at 3 contiguous test frequencies in at least one ear Paediatric: Hearing loss sufficient to indicate therapeutic intervention
4	Severe refractory CHF; EF<20%; Operative intervention required	GFR≤19	Adult only: Profound bilateral hearing loss (>90 dB) Paediatric: audiological indication for cochlear implant

EF - ejection fraction; FS - fractional shortening; GFR – glomerular filtration rate (ml/min/1.73m^2); CHF – congestive heart failure; dB - decibels

5.3 Selection of candidate polymorphisms

Firstly, examination of currently known intracellular pathways of methotrexate, doxorubicin and cisplatin identified 22 important target genes implicated in drug transport, detoxification, cell cycle modulation and DNA replication and repair (291) (See Figures 4-6: Drug pathways for MAP). For each target gene, polymorphisms were selected on the basis of functional consequences identified from the National Center for Biotechnology Information (NCBI) (292) or previously reported clinical associations. This yielded a total of 36 candidate polymorphisms from 22 genes most advantageous for analysis (see Table 1: Functional and clinical effects of candidate polymorphisms).

5.4 Preparation of DNA

5.4.1 Extraction of genomic DNA from whole blood

At study entry, a 20ml venous blood sample was obtained and placed in two EDTA tubes. This was transferred to the designated laboratory and stored at 4^0C prior to DNA extraction. Lymphocytic genomic DNA was extracted from 5ml whole blood by digestion with Protease K, precipitation with isopropanol and resuspension in 70% ethanol using the Qiagen FlexiGene kit (Qiagen, UK). Detailed methods are presented in Appendix 4.

5.4.2 Quantification

Extracted DNA was stored at room temperature prior to quality assurance. Presence of DNA was confirmed by electrophoresis on 0.8% agarose gel followed by nanodrop spectrophotometry quantification. This provides a measure of DNA concentration (ng/µl) and DNA purity by examining optical density for protein contamination. Once DNA quality was assured, the remaining sample was stored at -70°C.

5.5 SNP microarray analysis

A total of 52 patient DNA samples including two duplicates, were analysed with the Illumina 610-Quad SNP microarray. All laboratory work was performed by staff at the Institute of Child Health Microarray Centre (UCL Genomics) in accordance with Illumina guidelines (Illumina Inc, USA). The Illumina 610 Quad microarray was chosen in order to provide additional data for genome-wide copy number analysis.

5.5.1 Illumina 610-Quad SNP array

The Illumina 610 Quad SNP array (Figure 8), an Infinium whole-genome genotyping BeadArray, assays 610,000 SNP loci simultaneously. Data from the International HapMap project (293) were used to derive tag SNPs from all four HapMap populations (Caucasian, Han Chinese/Japanese, Yoruban). Detection of known and novel CNV regions is also possible due to 60,000 additional markers developed in collaboration with deCODE Genetics, specifically targeting regions known or likely to contain CNVs, including segmental duplications and regions in the unSNPable genome.

Figure 8: Illumina 610-Quad microarray

The Illumina 610-Quad microarray is a four sample chip built on the 550,000-SNP Illumina HumanHap550 with an additional 60,000 copy number markers. Reproduced with permission from Illumina Inc.

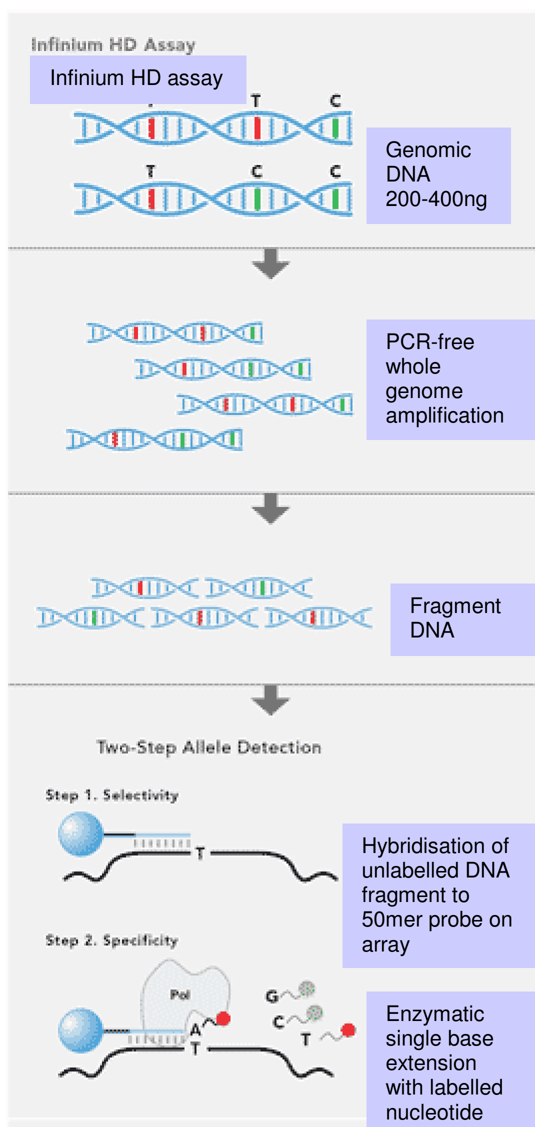
5.5.2 Infinium HD whole-genome genotyping single base extension assay (WGG-SBE)

WGG-SBE is unique among genotyping methods in that the entire complexity of the genome is present in the sample during array hybridisation. It consists of four modular components: 1) a single-tube whole genome amplification step, 2) an array-based hybridisation capture step, 3) single-base extension and 4) immunohistochemistry-based signal amplification (294).

Following initial DNA quality assurance by gel electrophoresis, whole-genome amplification, fragmentation and low-concentration resuspension was performed (295). Samples were denatured and loaded onto the arrays using a Tecan liquid handling robot (Tecan Group Ltd, Switzerland). Fragmented patient DNA was allowed to

hybridise to 50 mer probes on the array for 16-24 hours at 48^{0}C. Probes were extended by a single base complementary to the hybridised DNA (SBE), bases labelled (A and T with dinitrophenol (DNP), C and G with Biotin) and the DNA sample removed from the chip with formamide. The staining procedure allowed signal amplification by a polyclonal antibody and streptavidin conjugated to Cy3 (green) for Biotin labelled nucleotides and Cy5 (red) for DNP labelled nucleotides. Finally, stained chips were coated to protect the dyes, scanned with the iScan scanner and autoloader and genotypes assigned by BeadStudio v3 software (Illumina Inc, USA). An outline of the WGG-SBE assay is shown in Figure 9.

Figure 9: Infinium HD WGG-SBE assay



Reproduced with permission from Illumina Inc.

5.5.3 Microarray quality control

Illumina standard quality control procedures detailed below ensured reliability of genotype calls for the candidate polymorphism study. Further more stringent quality control procedures were applied for genome-wide copy number analysis (see section 6.2).

- each array contains both sample dependent and sample independent control probes. Sample independent probes assess quality of processing, sample dependent probes assess quality of DNA
- sample call rate should be >98% and >99% average across the sample set. Call rates <98% dictate checking of control probes to identify possible processing errors
- contaminated samples are identified by checking B-allele frequency (BAF) plots. This plot would show more than three modes if the sample had been contaminated at source. A noisy BAF plot suggests degradation of the DNA sample.

5.6 Polymerase chain reaction (PCR)

Five candidate polymorphisms comprised genetic alterations more complex than single nucleotide changes and were manually genotyped by PCR techniques (TYMS VNTR, 3R G/C SNP, DHFR 19bp indel, GSTT1 null, GSTM1 null). First described in 1984, PCR is used to amplify specific regions of DNA to produce millions of copies within a short time period (296). This is achieved by: 1) initial denaturation at 94-96^{0}C to separate DNA into single strands; 2) annealing of primers to their complementary sequence at 50-65^{0}C; 3) synthesis of new DNA strands by Taq polymerase at 72^{0}C; 4) repetition of the cycle to exponentially increase DNA.

5.6.1 Standard PCR

Standard PCR was used to manually genotype all patients for TS VNTR and DHFR in/del, with restriction fragment length polymorphism (RFLP) analysis to genotype the TS 3R G/C SNP. Conditions were as previously described with minor modifications (83, 201, 297). RFLPs are phenotypically neutral alterations in DNA sequence occurring throughout the genome in all individuals (298). Restriction enzymes recognize and splice regions of homologous DNA differing in restriction sites, resulting in fragments separatable by gel electrophoresis. Although superseded by newer techniques, RFLP remains of use in VNTR. Multiplex PCR was used to detect homozygous deletions of

GSTT1 and GSTM1 with primers for the housekeeping gene BCL2 as an internal control (241). Multiplex PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. Targeting multiple genes simultaneously provides additional information in a shorter and less labour intensive manner. Primer sequences and fragment size are presented in Table 3.

5.6.1.1 TS VNTR final amplification

A standard PCR 25µl reaction mixture containing 1µl of patient DNA (100-500ng), 10 picomols of each primer, 2.5µl buffer (10x), 200µM of each dNTP (dATP, dCTP, dGTP, dTTP), 1 unit AmpliTaq Gold (Applied Biosystems), 25mM$MgCl_2$ and 10% DMSO was used. Amplification conditions consisted of initial denaturation at 94^0C for 10 minutes followed by 30 cycles at 94^0C for 30 seconds, 55^0C for 30 seconds, 72^0C for 30 seconds and a final extension step of 72^0C for 7 minutes. See Table 4 for optimisation steps.

5.6.1.2 DHFR in/del

A standard PCR reaction mixture was used as above. Amplification conditions differed only in annealing temperature (60^0C) and number of cycles (35).

Table 3: PCR Primer sequences and fragment size

Name	Sequence 5'-3'	Fragment size (bp)
TS (forward F)	[6FAM]AGGCGCGCGGAAGGGGTCCT	141/113
TS (reverse R)	TCCGAGCCGGCCACAGGCAT	
DHFR F	[6FAM]ATGGGACCCAAACGGGCGCA	118/137
DHFR R	AAAAGGGGAATCCAGTCGG	
GSTT1 F	TTCCTTACTGGTCCTCACATCTC	480
GSTT1 R	TCACGCGATCATGGCCAGCA	
GSTM1 F	GAACTCCCTGAAAAGCTAAAGC	219
GSTM1 R	GTTGGGCTCAAATATACGGTGG	
BCL2 F	GCAATTCCGCATTTAATTCATGG	154
BCL2 R	GAAACAGGCCACGTAAAGCAAC	

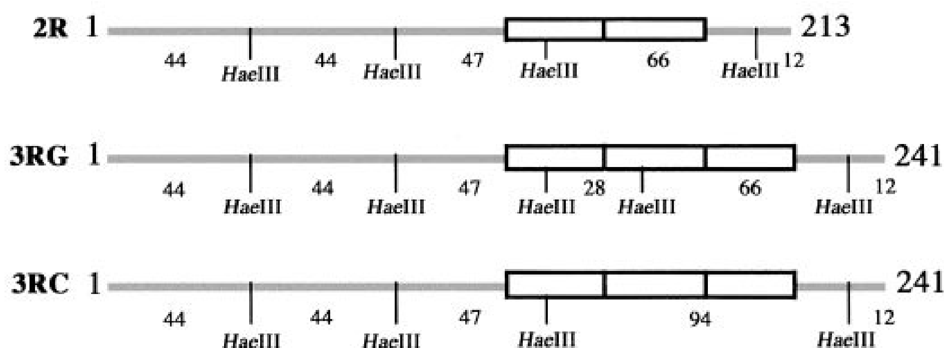
Table 4: TS VNTR optimisation steps

Trial	Conditions	Optimisation	Result
1	25µl reaction mixture, GoTaq 1unit, 10pmol each primer, 2.5µl buffer, 200µM each dNTP	Annealing temperature 55^{0}C	Non-specific amplification
2	As (1)	Annealing temp 58^{0}C	Non-specific amplification
3	As (1)	Annealing temp 60^{0}C 10% DMSO	No amplification
4	25µl reaction mixture, GoTaq 1unit, 2.5µl buffer, 200µM each dNTP	Annealing temp 59^{0}C 20pmol each primer	No amplification
5	As (1)	$MgCl_2$ titration	Optimal with 1µl $MgCl_2$ but non-spec amplification
6	25µl reaction mixture, 10pmol each primer, 2.5µl buffer, 200µM each dNTP	Annealing temp 60^{0}C AmpliTaq Gold 1 unit 1µl $MgCl_2$, +/- 10% DMSO	No amplification without DMSO. Bands clear with DMSO and Amplitaq Gold
7	25µl reaction mixture, 10pmol each primer, 2.5µl buffer, 200µM each dNTP	Annealing temp 55^{0}C AmpliTaq Gold 1 unit 10% DMSO, 1µl $MgCl_2$	Final mixture

5.6.1.3 RFLP analysis: TS VNTR G/C SNP

16µl of PCR mixture was digested with the *HaeIII* restriction enzyme (Promega) in a 20µl reaction volume at 37 ^{0}C for 2 hours. One unit of enzyme was added to the initial mixture with a further 1 unit added after 1 hour. All were sized using 3% agarose gel electrophoresis. The G→C base change in 3RC removes a *HaeIII* restriction endonuclease site and alters the electrophoretic gel band pattern. Hence fragment sizes were 94, 37 and 10 bp for 3RC and 66, 37, 28 and 10 bp in length for 3RG (see Figure 10: *Hae III* restriction sites).

Figure 10: *Hae III* restriction sites



The presence of the G/C SNP in the TYMS 3R removes a restriction site thereby producing DNA fragments of different size visible by gel electrophoresis. Reproduced from Mandola et al. Cancer Res 2003; 63; 2898-2904. Permission requested.

5.7 Multiplex PCR: GSTT1 and GSTM1 null alleles

A 25µl reaction mixture was used for multiplex PCR, containing 1µl of patient DNA (100-500ng), 10 picomols of each primer, 2.5µl buffer (10x), 200µM dNTPs, 1 unit AmpliTaq Gold (Applied Biosystems), 25mM MgCl2 and 10% DMSO with amplification conditions identical to TS VNTR. No optimisation steps were required.

5.7.1 Gel electrophoresis

All fragments were analysed by gel electrophoresis on 3% (GSTT1/GSTM1) and 4% (TYMS, DHFR) agarose gels stained with ethidium bromide (see Images 1-4). Final assignment of genotypes was confirmed by a clinician scientist blinded to patient outcomes. To ensure sizing accuracy, TYMS VNTR fragments were analysed on the ABI 3730xl automated DNA sequencer and sized using LIZ 500 size standard. Results were analysed using Genemapper software (ABI) and compared with those obtained from agarose gel electrophoresis.

Image 1: TS VNTR gel electrophoresis

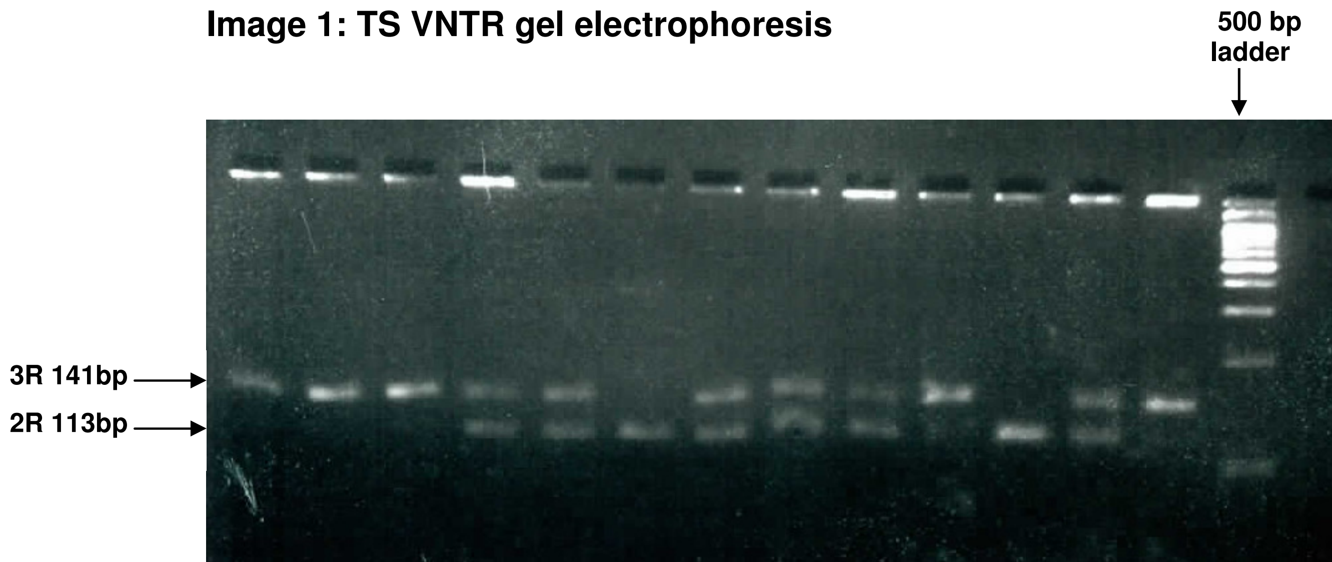


Image 2: TS Triple repeat G/C SNP

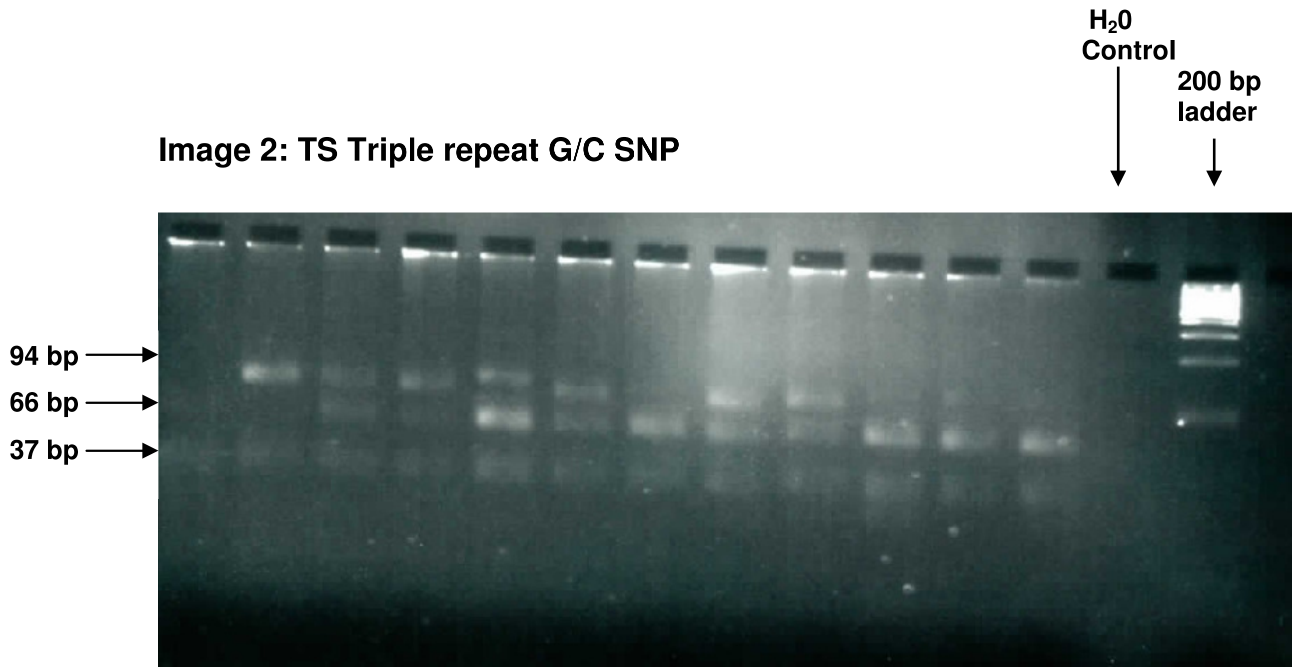


Image 3: DHFR electrophoresis

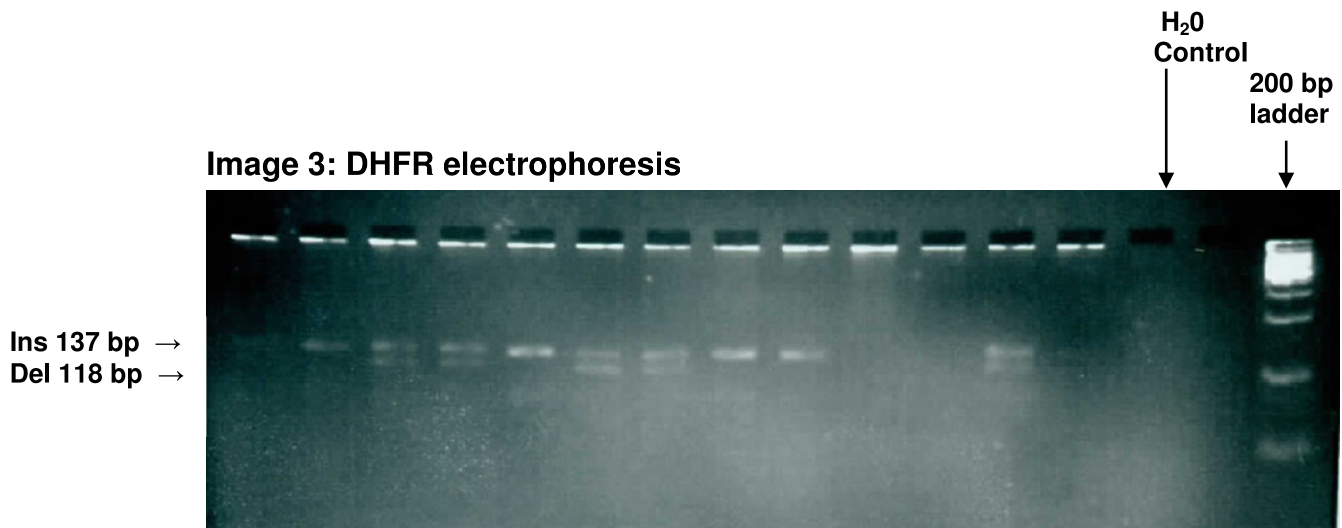
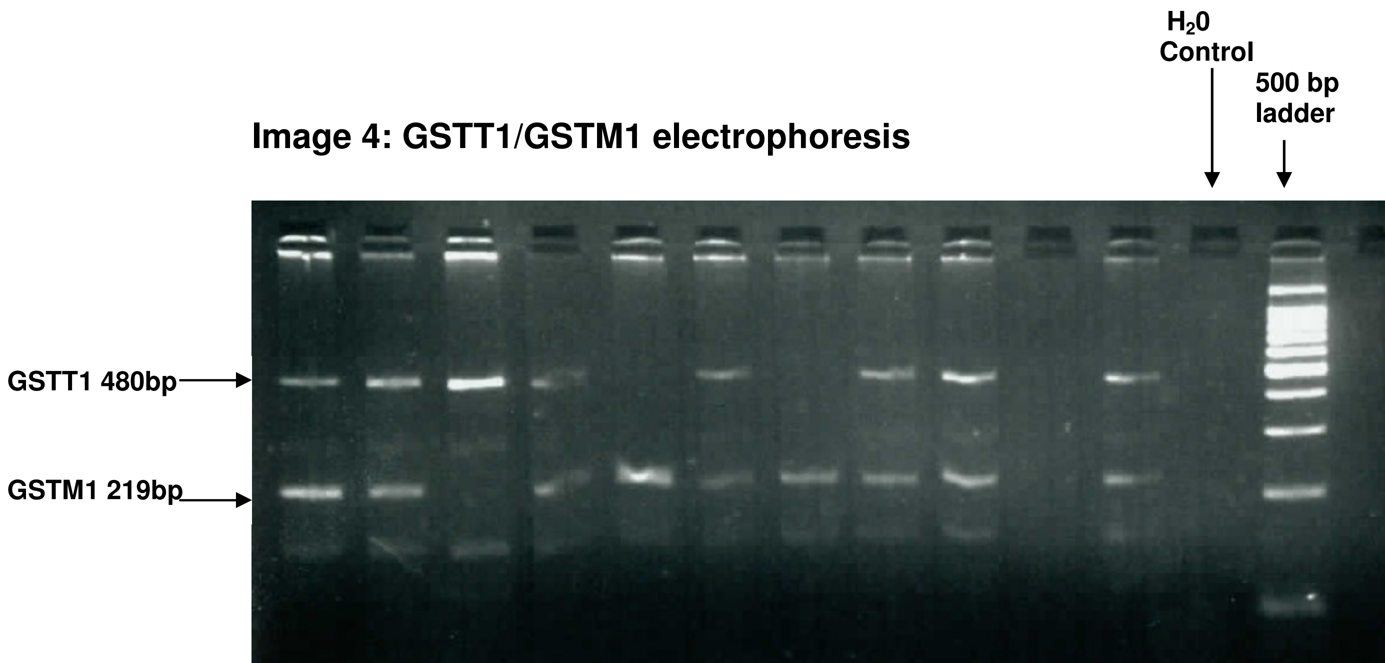


Image 4: GSTT1/GSTM1 electrophoresis



5.8 Statistical analysis

Candidate SNPs with minor allele frequency (MAF)<0.05 or deviating from Hardy Weinberg Equilibrium [3](HWE) $p<0.001$ were excluded from further analysis (300). Pearson's chi-square or Fisher's Exact tests were used to analyse demographic, clinical and genotypic data. In addition to wild-type (WT), heterozygote and variant homozygote forms, genotypes were also grouped as dichotomous variables on the basis of their phenotypic consequences.

The highest recorded CTCAE grade was grouped as a binary variable (grade 0-2 vs grade 3-4) for each drug cycle and associations investigated with Pearson's chi-square or Fisher's Exact test.

Logistic regression analysis was used to further investigate significant associations by calculation of the odds ratio (OR) and its 95% confidence interval (CI). For some analyses, small sample size resulted in the statistical phenomenon of separation necessitating the use of penalized log-likelihood regression for calculation of OR and CI (301). Multivariate logistic regression analysis of cardiotoxicity and nephrotoxicity included doxorubicin and cisplatin exposure (mg/m^2) as covariates respectively. Student's T-test was used to investigate differences between mean diagnostic and EoT EF and GFR with respect to genotype.

Progression-free survival (PFS) was calculated from the start of chemotherapy to first disease recurrence. Patients without disease recurrence at final analysis were censored at last follow up. The Kaplan-Meier method was used to calculate survival probabilities and log rank test used to compare differences in PFS. Overall survival was not informative due to small patient numbers. Cox proportional hazards model was used for calculation of hazard ratios (HR) in multivariate analysis with predictors of survival identified in univariate analysis as covariates (tumour primary site, metastasis at diagnosis). Analysis of the influence of cardio/nephrotoxicity on survival was performed using time-limited covariates.

All statistical analyses were performed using SPSS 14.0 and Stata 10. Reported p values were two-sided and a value of 0.05 was considered statistically significant.

[3] Hardy-Weinberg equilibrium states that both allele and genotype frequencies within a population remain constant. In a single locus with two alleles, the dominant allele is denoted A and recessive a, with frequencies denoted by p and q such that, $p+q=1$. If the allele frequency is in HWE, the frequency will be $p^2+q^2+2pq=1$ (ie. freq AA homozygotes+freq aa homozygotes+freq heterozygotes). 319. Hardy G. Mendelian proportions in a mixed population. Science. 1908;28(706):49-50.

Bonferroni correction was not used as this implies all variables are independent and may lead to over-correction (302, 303). Interpretation of data with confidence intervals was felt to be more informative. Results are discussed for genotype with the narrowest confidence intervals.

6.0 Materials and methods (ii): Genome wide CNV analysis

6.1 Study population

CNV data was analysed from 50 patients genotyped with the Illumina 610-Quad microarray for the candidate polymorphism study detailed above. All patients were aged >16 years at study entry and had completed MAP chemotherapy for histologically proven osteosarcoma. The procedures for DNA extraction and genotyping are as previously documented (see Section 5.4-5.5).

6.2 CNV analysis

6.2.1 Quality control procedures

Samples with call rates <99% were excluded. Concordance rate for duplicate genotyping was >99.99%.

6.2.2 CNV detection

All 50 subjects passed QC procedures and were entered into the CNV analysis. CNV calls incorporating 10 SNPs were generated using PennCNV (2009Aug27 version), a hidden Markov model (HMM) based algorithm for detection of CNVs using whole-genome SNP genotyping data (see Table 5: Hidden states, copy numbers and descriptions). This software incorporates multiple sources of information including total signal intensity (log R ratio LRR) and alleleic intensity ratio (BAF), distance between neighbouring SNPs and the allele frequency for all SNPs and CNV probes included on the array (304) (Figure 11: Signal intensity profiles of different CNV types). All analyses were restricted to autosomes due to the complications of hemizygosity in males and X-chromosome inactivation in females. Visual inspection of chromosomal copy number changes was also performed using dChip software to ensure regions of CNV had not been missed by Penn CNV and to confirm PennCNV novel CNV regions (305) (version 2010.01, available at https://sites.google.com/site/dchipsoft/htm). Similar to PennCNV, dChip infers CNV using a HMM algorithm once all arrays have been normalised to a common baseline.

Table 5: Hidden states, copy numbers and descriptions

Copy number state	Total copy no	Description (autosome)	CNV genotypes
1	0	Deletion of two copies	Null
2	1	Deletion of one copy	A, B, AB
3	2	Normal state	AA,AB, BB
4	2	Copy-neutral with LOH	AA, BB
5	3	Single copy duplication	AAA, AAB, ABB, BBB
6	4	Double copy duplication	AAAA, AAAB, AABB, ABBB, BBBB

6.2.3 Filtering CNV calls

In order to minimise the number of false positive CNV calls without compromising detection of true CNVs, standard PennCNV quality control checks were used to exclude unreliable samples, including LRR standard deviation>0.28, BAF median>0.55 or <0.45, BAF drift>0.002 or a waviness factor of greater than 0.04 or less than -0.04. In addition, samples that were outliers in terms of number of CNV calls were excluded. The threshold for this was calculated as (upper quartile + 1.5 x interquartile range) (306). Individual CNVs were excluded if the PennCNV generated confidence score was <50, if the CNV spanned a centromere and finally, if the CNVs overlapped at least 50% with regions previously described as being prone to false positives due to somatic mutations (307). Therefore, a CNV was excluded if it overlapped the following regions by 50% or more of its length: chr2 87.0-92.0, chr14: 18-23.6 Mb, chr14: 104.5-106.5 Mb, chr15: 17.0-21.0, chr16: 31.8-36.0 Mb, chr22: 20.5-21.8 Mb (immunoglobulin regions); chr1: 12.1-12.8Mb; chr2: 91-95.7Mb; chr3: 89.4-93.2Mb; chr4: 48.7-52.4Mb; chr5: 45.8-50.5Mb; chr6: 58.4-63.4Mb; chr7: 57.4-61.1Mb; chr8: 43.2-48.1Mb; chr9: 46.7-60.3Mb; chr10: 38.8-42.1Mb; chr11: 51.4-56.4 Mb; chr12: 33.2-36.5Mb; chr13: 13.5-18.4Mb; chr14: 13.6-19.1Mb; chr15: 14.1-18.4Mb; chr16: 34.4-40.7Mb; chr17: 22.1-23.2Mb; chr18 15.4-17.3 Mb; chr19: 26.7-30.2Mb; chr20: 25.7-28.4Mb; chr21: 10.0-13.2Mb; chr22: 9.6-16.3Mb (centromeric regions NCBI36/hg18).

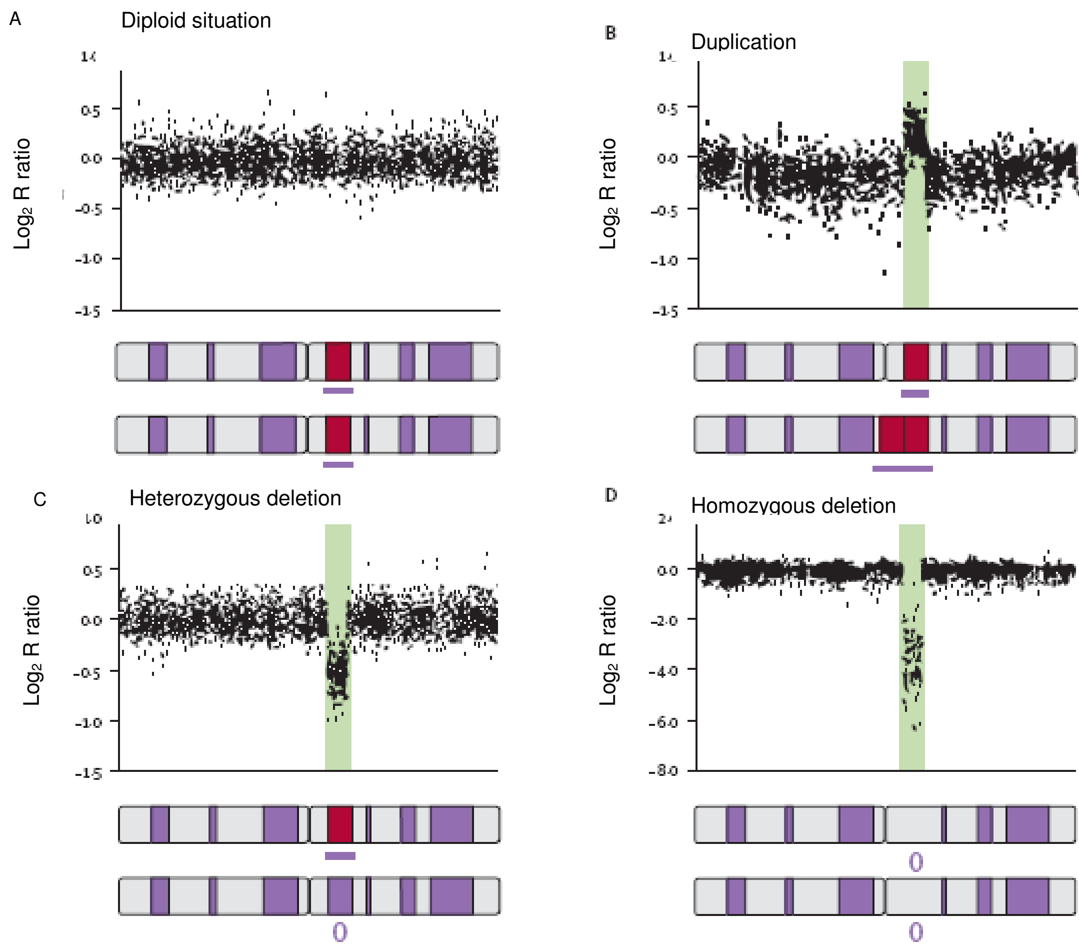


Figure 11: SNP signal intensity profiles of different CNV types.

The effect of CNVs on SNP intensity probes can be used to detect CNVs. The signal intensity of each SNP (black dot) is expressed as the $\log_2$ R ratio. The x-axis corresponds to chromosomal positions (not to scale). In the normal (diploid) situation (A), the $\log_2$ R ratio has a value of about 0. A duplication of a chromosomal segment (B) increases intensity because there are three rather than two copies. A heterozygous deletion (C) is marked by a negative deflection in signal intensity and a homozygous deletion (D) by an even sharper decrease in intensity, because at most there is only one copy. Note the different scales on the y-axis. Reproduced with permission from Blauw et al. Copy-number variation in sporadic amyotrophic lateral sclerosis: a genome-wide screen. Lancet Neurol 2008; 7: 319-26

6.2.4 Defining CNV regions

To avoid inclusion of false positives, only recurrent CNV calls (present in at least two patients) were included for further analysis. PennCNV calls were clustered into CNV loci using a reciprocal overlap threshold of >70% and sorted by length with the largest assigned the initial CNV locus identifier (308). Subsequent calls were compared to the locus and allocated to it if it showed a reciprocal overlap of >70% with calls allocated to that locus. If overlap criteria were not met for a previously assigned locus, a new locus was allocated. To identify novel CNV regions in osteosarcoma patients, data was then compared with the Database of Genomic Variants (available at http://projects.tcag.ca/variation), a comprehensive summary of structural variation in the human genome, containing over 11000 CNVs from 2500 healthy controls (26). Novel CNVs were confirmed in dChip together with visual inspection of the BAF and log R ratio plots in BeadStudio.

6.2.5 Statistical analysis of CNV

The recurrent CNV identified in osteosarcoma patients were analysed using the chi-square test. Control data for CNV was provided from a genome-wide association study of patients with Parkinson's disease (with permission) (309, 310). All statistical analyses were performed using SPSS 14.0 and Stata 10.

7.0 Results (i): Candidate polymorphism study

7.1 Patient characteristics

Sixty patients entered the study. Two patients who received chemotherapy not adhering to MAP guidelines were excluded. The median age at diagnosis was 18 years (range 10-51) with 46 patients aged <25 yrs (79%). Thirty-four patients were male (59%) and 41 (70%) were Caucasian. Primary tumour site was extremity in 50 patients (86%), axial in 4 (7%) and maxillofacial in 4 (7%) patients. Osteoblastic osteosarcoma was the most common histological subtype (n=37, 64%) followed by chondroblastic (n=7, 12%) and telangiectatic (n=6, 10%). Surgical resection and reconstruction was performed in 53 (91%) patients, the remainder undergoing amputation. Demographic data for the microarray samples did not differ significantly from the entire cohort. Clinical and pathological characteristics of study patients are shown in Table 6.

Table 6: Clinical and pathological characteristics of study patients

Patient characteristics	n (%)	Patient characteristics	n (%)
Total no.patients	58	**Metastasis at diagnosis**	
		Absent	52 (90)
Age at diagnosis (yrs)		Present	6 (10)
Median (range)	18 (10-51)		
		Histological subtype	
Follow-up (mths)		Osteoblastic	37 (64)
Median (range)	41.5 (12-93)	Chondroblastic	7 (12)
		Telangiectatic	6 (10)
Gender		Other	5 (9)
Male	34 (59)	Unknown	3 (5)
Female	24 (41)		
		Histological response	
Ethnic group		Good	38 (65)
Caucasian	41 (71)	Poor	12 (21)
Afro-Caribbean	8 (14)	Not evaluable	8 (14)
Indian/Asian	9 (15)		
		Relapse	
Primary site		No	45 (78)
Femur	26 (45)	Yes	13 (22)
Tibia/Fibula	21 (36)		
Humerus/Radius	3 (5)	**Death**	
Axial	4 (7)	No	53 (91)
Maxillofacial	4 (7)	Yes	5 (9)

7.2 Disease outcomes

Thirty-eight patients (65%) showed a good histological response, 12 patients (21%) poor response and 8 patients (14%) could not be evaluated due to early surgical resection for diagnostic purposes or disease control. No significant associations were observed between histological response and gender, age group, ethnic group, primary site, histological subtype, metastasis at diagnosis and relapse.

Median follow-up was 41.5 months (range 12-93 months). At time of final analysis, 13 patients (22%) had relapsed and 5 (9%) had died. Decreased PFS was significantly associated with tumour site, presence of metastasis at diagnosis and type of surgery (see Table 7: Disease characteristics and progression free survival). There was no evidence of a difference in survival according to age group, gender, histological subtype and histological response.

Table 7: Disease characteristics and progression-free survival

Disease characteristic	Hazard ratio (95% CI)	p
Tumour site		
Extremity	Ref	
Axial	10.0 (2.4–42.6)	0.002
Metastases at diagnosis		
No	Ref	
Yes	6.3 (1.8-21.7)	0.004
Type of surgery		
Limb salvage/Reconstruction	Ref	
Amputation	5.5 (1.2-25.3)	0.028

7.3 Toxicity outcomes

Chemotherapy toxicity was recorded for a total of 211 AP courses, 575 M courses and 94 A courses. Three patients were excluded due to incomplete toxicity data. Two patients receiving additional ifosfamide/etoposide were excluded from renal toxicity analysis. At least one episode of infective, myelosuppressive or gastrointestinal grade 3-4 toxicity was experienced by 46 (79%) patients following M chemotherapy, 54 (93%) following AP and 40 (69%) following A chemotherapy. Table 8 summarises the incidence of grade 3-4 toxicities for each drug.

Table 8: Grade 3-4 chemotherapy toxicity by cycle

Chemotherapy toxicity (grade 3-4)	M n (%)	AP n (%)	A n (%)
Any toxicity	46 (79)	54 (93)	40 (69)
Recurrent (>2 episodes)	30 (52)	48 (83)	25 (43)
Infection	26 (45)	49 (85)	20 (35)
Mucositis	18 (31)	22 (38)	3 (5)
Anaemia	16 (28)	26 (45)	13 (22)
Neutropenia	34 (59)	54 (93)	35 (60)
Thrombocytopenia	16 (28)	37 (64)	16 (28)
Treatment delay/dose reduction	47 (81)	31 (53)	33 (57)

7.3.1 Cardiac toxicity

Cardiotoxicity data was available for 55 patients. Median diagnostic EF was 63% (Interquartile range IQR 57-66) and doxorubicin exposure 444mg/m^2 (IQR 386-453). Neither showed any association with cardiotoxicity. Early and EoT cardiotoxicity was recorded in 16 (29%) and 25 (45%) patients respectively. A deterioration in cardiac function by ≥ 2 CTCAE grades from diagnosis to EoT was seen in 10 (18%) patients. An improvement in cardiac function (denoted by a decrease in CTCAE grade) from diagnosis to EoT was seen in 2 patients (4%). Cardiac function by change in CTCAE grade is presented in Table 9.

7.3.2 Nephrotoxicity

Nephrotoxicity data was available for 55 patients. Median diagnostic GFR was 110ml/min/1.73m^2 (IQR 97.5-127) and cisplatin exposure 474mg/m^2 (IQR 443-480 mg/m^2). Early and EoT nephrotoxicity was recorded in 18 (33%) and 33 (60%) patients respectively. A deterioration in renal function by ≥2 CTCAE grades from diagnosis to EoT was seen in 5 (9%) patients. Neither showed any association with nephrotoxicity. Early and EoT nephrotoxicity was recorded in 18 (33%) and 33 (60%) patients respectively. A deterioration in renal function by ≥2 CTCAE grades from diagnosis to EoT was seen in 5 (9%) patients. An improvement in renal function (denoted by a decrease in CTCAE grade) between diagnosis and EoT was seen in 1 patient (2%). Renal function by change in CTCAE grade is presented in Table 10.

Table 9: Cardiac toxicity by change in CTCAE grade

	Change in CTCAE grade				
Time of measurement	**-1**	**0**	**1**	**2**	**Missing**
Diagnosis	N/A	38 (69)	16 (29)	1 (2)	0
Post 2 cycles	5 (9)	31 (56)	13 (24)	3 (6)	3 (5)
End of treatment	2 (4)	24 (44)	15 (27)	10 (18)	4 (7)

Table 10: Nephrotoxicity by change in CTCAE grade

	Change in CTCAE grade				
Time of measurement	**-1**	**0**	**1**	**2**	**Missing**
Diagnosis	N/A	49 (89)	5 (9)	0	1 (2)
Post 2 cycles	1 (2)	32 (58)	15 (27)	3 (6)	4 (7)
End of treatment	1 (2)	19 (34)	28 (51)	5 (9)	3 (6)

7.4 Genotype information

Genotype frequencies were consistent with previously reported studies. Two SNPs with MAF<0.05 (NQO1 c.415C>T p.Arg^{139}Trp and GSTP1 c.341C>T p.Ala^{114}Val) were excluded from further analysis. Genotype showed no association with age, primary site or histology. TS group 2 (2R/3G, 3G/3C, 3G/3G) was over-represented in males (p=0.016) and wild-type MTHFR c.677C>T p.Ala^{222}Val over-represented in non-caucasians (p=0.015). Metastasis at diagnosis was associated with CCND1 c.870A>G p. Pro^{241}Pro wild-type homozygotes (Odds ratio (OR) 9, 95% CI 1.4-57.9, p=0.021) and ERCC1 c.354T>C p.Asn^{118}Asn wild-type (CC) homozygotes (OR 12, 95% CI 1.3-112, p=0.03).

7.5 Histological response

Poor histological response was significantly increased in variants of ABCC2 c.24C>T (OR 6.3 95% CI 1.4-28.5, p=0.017) and GSTP1 c.313A>G p.Ile^{105}Val heterozygotes (OR 7.8, 95% CI 1.6-37.5, p=0.01). Carriers of MTHFD1 c.1958G>A p.Arg^{653}Gln were more likely to be good responders (OR 0.2, 95% CI 0.05-0.8, p=0.03). The complete data is shown in Table 11: Genotypic associations with histological response.

Table 11: Genotypic associations with histological response

Polymorphism	Genotype	Patients n (%)	HR p	Poor histol response OR (95% CI) p
Folate pathway				
MTHFR				
677C>T Ala^{222}Val	CC	27 (54)		
	CT	16 (32)		
	TT	7 (14)		
	CT/TT		0.5	
1298A>C Glu^{429}Ala	AA	23 (46)		
	AC	22 (44)		
	CC	5 (10)		
	AC/CC		0.63	
1305C>T Phe^{435}Phe	CC	1 (2)		
	CT	1 (2)		
	TT	48 (96)		
	CT/TT	49 (98)	1	
1781G>A Arg^{594}Gln	GG	46 (92)		
	AG	4 (8)		
	AA	0		
	AG/AA	4 (8)	0.56	
MTHFD1				
401A>G Lys^{134}Arg	AA	4 (8)		
	AG	14 (28)		
	GG	32 (64)		
	AG/GG		0.31	
1958G>A Arg^{653}Gln	GG	19 (38)		Reference
	AG	20 (40)		0.22 (0.05-1.07) 0.03
	AA	11 (22)		0.16 (0.02-1.61) 0.12
	AG/AA		**0.03**	**0.2 (0.05-0.9) 0.03**
RFC				
80G>A Arg^{27}His	GG	15 (30)		
	AG	22 (44)		
	AA	13 (26)		
	AG/AA		0.44	
DHFR				
19bp indel	Ins/Ins	23 (40)		
	Del/Del	28 (48)		
	Ins/Del	6 (10)		
	Failed	1 (2)		
	I/I, I/D		0.14	
TS VNTR and G/C SNP				
2R/2R, 2R/3C, 3C/3C		28 (48)		
2R/3G, 3C/3G, 3G/3G		26 (45)		
Other		4 (7)	0.56	
ABC efflux				
ABCB1				
1236C>T Gly^{412}Gly	CC	18 (36)	0.63	
	CT	17 (34)		
	TT	15 (30)		
	CT/TT		1	
3435C>T Ile^{145}Ile	CC	17 (34)	0.9	
	CT	17 (34)		
	TT	16 (32)		
	CT/TT		1	

Table 11: Genotypic associations with histological response continued

Polymorphism	Genotype	Patients n (%)	HR p	Poor histol response OR (95% CI) p
ABCG2 421C>A Gln^{141}Lys	CC	45 (90)	0.56	
	AC	5 (10)		
	AA	0		
	AC/AA		0.57	
ABCC1				
rs 246240 A>G	AA	38 (76)	0.7	
	AG	12 (24)		
	AG/GG		0.7	
rs 3784862 A>G	AA	26 (52)	0.63	
	AG	22 (44)		
	GG	2 (4)	0.7	
	AG/GG			
ABCC2				
24C>T	CC	28 (56)	0.013	Reference
	CT	18 (36)		4.7 (0.9-22.9) 0.058
	TT	4 (8)		21 (1.6-273) 0.02
	CT/TT		**0.011**	**6.3 (1.4-28.5) 0.017**
1249G>A Val^{417}Ile	GG	36 (72)	0.14	
	AG	14 (28)		
	AA	0		
	AG/AA		0.14	
3563T>A Val^{1188}Glu	TT	1 (2)	0.57	
	AT	9 (18)		
	AA	40 (80)		
	AT/AA		1	
4544G>A Cis^{1515}Tyr	GG	39 (78)	0.12	
	AG	9 (18)		
	AA	2 (4)		
	AG/AA		0.24	
DNA repair				
ERCC1				
1510C>A Gln^{504}Lys	CC	24 (48)	0.79	
	AC	20 (40		
	AA	6 (12)		
	AC/AA		1	
354T>C Asn^{118}Asn	TT	11 (22)	0.91	
	CT	21 (42)		
	CC	18 (36)		
	CT/CC		0.69	
ERCC2 2251A>C Lys^{751}Gln	AA	20 (40)	0.56	
	AC	23 (46)		
	CC	7 (14)		
	AC/CC		0.25	
ERCC4 1244G>A Arg^{415}Glu	GG	45 (90)	1	
	AG	4 (8)		
	AA	1 (2)		
	AG/AA		1	

Table 11: Genotypic associations with histological response continued

Polymorphism	Genotype	Patients n (%)	HR p	Poor histol response OR (95% CI) p
XRCC3				
722C>T Thr[241]Met	CC	22 (44)	0.65	
	CT	22 (44)		
	TT	6 (12)		
	CT/TT		0.5	
XPC				
2886A>C Lys[939]Gln	AA	24 (48)	0.58	
	AC	20 (40)		
	CC	6 (12)		
	AC/CC		0.44	
GST enzymes				
GSTP1				
313A>G Ile[105]Val	AA	25 (50)	0.01	Reference
	AG	23 (46)		7.8 (1.6-37.5) 0.01
	GG	2 (4)		1.6 (0.06-42.7) 0.8
	AG/GG		**0.009**	**7.9 (1.5-42.5) 0.016**
GSTT1	Non-null	37 (64)	0.73	
	Null	20 (34)		
	Failed	1 (2)		
GSTM1	Non-null	43 (74)	0.26	
	Null	14 (24)		
	Failed	1 (2)		
Others				
CBR3				
11G>A Cys[4]Tyr	GG	21 (42)	0.09	
	AG	16 (32)		
	AA	13 (26)		
	AG/AA		0.37	
730G>A Val[244]Met	GG	14 (28)		
	AG	15 (30)		
	AA	21 (42)		
	AG/AA		0.11	
CCND1				
870A>G Pro[241]Pro	AA	12 (24)	0.55	
	AG	23 (46)		
	GG	15 (30)		
	AG/GG		0.24	
NQO1				
609C>T Pro[187]Ser	CC	31 (62)	0.37	
	CT	19 (38)		
	TT	0		
	CT/TT		0.37	
NADPH NCF4				
212G>A	GG	23 (46)	0.21	
	AG	18 (36)		
	AA	9 (18)		
	AG/AA		0.08	
CYBA p22phox				
242C>T His[72]Tyr	CC	24 (48)	0.7	
	CT	19 (38)		
	TT	7 (14)		
	CT/TT		0.7	

7.6 Progression free survival (PFS)

In univariate analysis, decreased PFS was observed in wild-type homozygotes of CCND1 c.870A>G p.Pro^{241}Pro (p=0.018), variant carriers of GSTP1 c.313A>G p.Ile^{105}Val (p=0.025) and RFC c.80G>A p.Arg^{27}His (log rank p=0.02) and patients with the GSTT1 null allele (p=0.0006) (See Figure 12: Kaplan Meier curves for PFS i-iv). Significance was not retained for any polymorphism in multivariate analysis although the GSTT1 null allele suggested a trend (HR 3.2, 95% 0.9-11.6, p=0.075). It was not possible to calculate the HR for RFC c.80G>A p.Arg^{27}His using the Cox model, although logistic regression for relapse suggested an association (OR 14.5, 95% CI 0.8-265, p=0.071), unchanged by multivariate analysis (OR 17.7, 95% CI 0.8-417, p=0.075). Univariate and multivariate analysis is presented in Table 12: Genotypic associations with PFS.

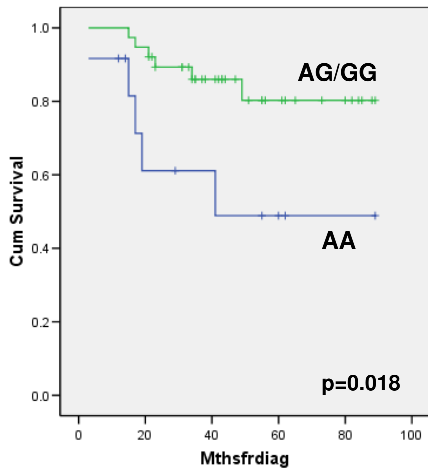
Table 12: Genotypic associations with PFS

Polymorphism	Genotype	PFS Unadj HR (95% CI)	p	PFS Adj HR (95% CI)	p
CCND1					
870A>G Pro^{241}Pro	AA	Reference		Reference	
	AG	0.07 (0.008-0.6)	0.35	0.09 (008-1.1)	0.06
	GG	0.55 (0.16-1.9)	0.06	0.7 (0.2-2.7)	0.6
	AG/GG	0.26 (0.08-0.9)	0.028	0.4 (0.1-1.9)	0.28
RFC					
80G>A Arg^{27}His	GG	See text		See text	
	AG				
	AA				
	AG/AA				
GSTP1					
313A>G Ile^{105}Val	AA	Reference		Reference	
	AG	4.6 (1-21.9)	0.05	3.1 (0.6-16.4)	0.18
	GG	7 (0.6-77.6)	0.11	10 (0.9-116)	0.066
	AG/GG	4.8 (1-22.4)	0.04	3.6 (0.7-18)	0.22
				*4.9 (0.9-27.3)	0.071
GSTT1					
	Non-null	Reference		Reference	
	Null	4.1 (1.4-12.4)	0.012	3.2 (0.9-11.6)	0.075
				*3.6 (0.9-13.9)	0.066
				*cardiotoxicity as covariate	

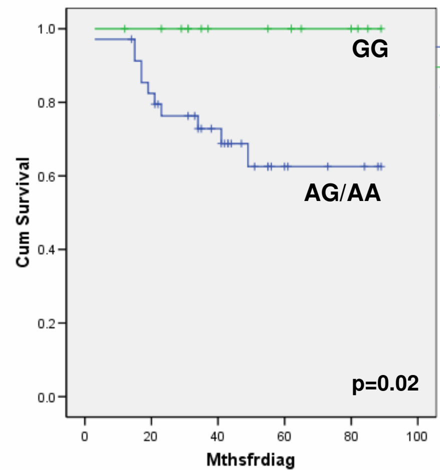
Figure 12: Kaplan Meier curves for PFS

The graphs depict Kaplan-Meier curves for the four polymorphisms associated with PFS in univariate analysis: i) CCND1 is a cell-cycle regulator protein, its oncogenicity enhanced by the wild-type allele of 870A>G; ii) RFC 80G>A encodes a reduced-function variant of the reduced folate carrier protein, critical for intracellular transport of methotrexate; iii) GSTP1 313A>G encodes GSTP1 with reduced catalytic activity; iv) GSTT1 null allele abolishes enzyme activity

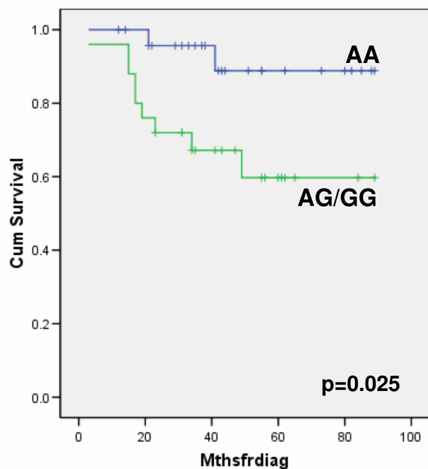
i) CCND1 870A>G



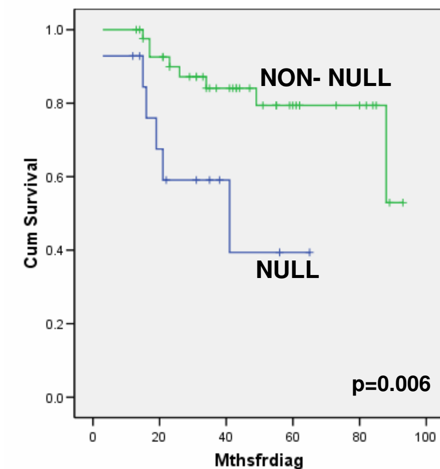
ii) RFC 80G>A



iii) GSTP1 313A>G



iv) GSTT1 null



7.7 Chemotherapy toxicity: Genotypic associations

For complete data see Table 13: Genotypic associations with chemotherapy toxicity.

7.7.1 Methotrexate (M) cycles

Two folate pathway SNPs were significantly associated with grade 3-4 haematological toxicity; MTHFD1 c.1958G>A p.Arg^{653}Gln (OR anaemia 5.4, 95% CI 1-27.5, p=0.044) and MTHFR c.1298A>C p.Glu^{429}Ala (OR anaemia 4.6, 95 CI 1.1-19.2, p=0.038; OR thrombocytopenia 3.3 CI 0.9-12.2, p=0.08; OR recurrent toxicity 4.8, 95% CI 1.3-17.1, p=0.015).

Several ABC efflux transporter SNPs influenced methotrexate toxicity; ABCB1 c.3435T>C Ile^{145}Ile (OR severe mucositis 6.2, 95% CI 1.2-31.8, p=0.027; OR leucopenia 4.9, 95% CI 1-25, p=0.057); ABCC2 c.3563T>A p.Val^{1188}Glu (variant allele T) (OR leucopenia 5.2, 95% CI 1.2-22.3, p= 0.028); ABCG2 c.421C>A p.Gln^{141}Lys heterozygotes (OR recurrent gr 3-4 toxicity 0.1, 95% CI 0.01-1, p=0.05).

7.7.2 Doxorubicin/cisplatin (AP) cycles

A decreased risk of grade 3-4 mucositis was observed in variant homozygotes (TT) of ERCC1 c.354T>C p.Asn^{118}Asn (OR 0.1, 95% CI 0.01-0.9, p=0.045), a NER enzyme. Variant carriers of CYBA c.242C>T p.His^{72}Tyr, a doxorubicin pathway enzyme, were also protected against severe mucositis and haematological toxicity (OR grade 3-4 mucositis 0.3, 95% CI 0.09-0.9, p=0.038; OR anaemia 0.3, 95% CI 0.08-0.9, p=0.028).

7.7.3 Doxorubicin (A) cycles

Variants of the NER enzyme, ERCC1 c.1510C>A p.Gln^{504}Lys showed an increased risk of grade 3-4 infection (OR 3.7, 95% CI 1-13.7, p=0.047) and leucopenia (OR 5.4, 95% CI 1.1-26, p=0.036). Significantly fewer episodes of grade 3-4 infection were seen in variants of the DNA repair enzyme, XPC c.2886A>C p.Lys^{939}Gln (OR 0.2, 95% CI 0.06-08. p=0.024).

GST enzymes were also associated with increased toxicity, with GSTP1 c.313A>G p.Ile^{105}Val variants experiencing significantly more grade 3-4 leucopenia (OR 7.8, 95% CI 1.3-47, p=0.024) and GSTM1 null, treatment delays due to doxorubicin toxicity (OR 9.4, 95% CI 1.9-46.9, p=0.006).

Table 13: Genotypic associations with chemotherapy toxicity

Cycle	Pathway	Toxicity (gr 3-4)	Polymorphism	Genotype	OR (95%) CI	p
Methotrexate	**Folate pathway**	**Anaemia**	MTHFD1 c.1958G>A p.Arg^{653}Gln	GG	Ref	
				AG	3.6 (0.6-20.9)	0.15
				AA	10.2 (1.5-67.2)	0.016
				AG/AA	5.4 (1-27.5)	0.044
			MTHFR c.1298A>C p.Glu^{429}Ala	AA	Ref	
				AC	3.8 (0.8-16.9)	0.08
				CC	10 (1.1-86.9)	0.037
				AC/AA	4.6 (1.1-19.2)	0.038
		Thrombocytopenia	MTHFR c.1298A>C p.Glu^{429}Ala	AA	Ref	
				AC	4.7 (1.2-18.6)	0.025
				CC	1.3 (0.5-3.2)	0.5
				AC/AA	3.3 (0.9-12.2)	0.08
		Recurrent (any)	MTHFR c.1298A>C p.Glu^{429}Ala	AA	Ref	
				AC	4.9 (1.3-19.1)	0.022
				CC	4.4 (0.4-45.3)	0.2
				AC/AA	4.8 (1.3-17.1)	0.015
	ABC efflux	**Leucopenia**	ABCC2 3563T>A Val^{1188}Glu (wild-type AA)	AA	Ref	
				AT	4 (0.96-17.1)	0.057
				TT	9.9 (0.4-265)	0.17
				AT/TT	5.2 (1.2-22.4)	0.028
			ABCB1 c.3435T>C p.Ile^{145}Ile	TT	Ref	
				CT	0.4 (0.1-1.7)	0.2
				CC	7.5 (1.3-44)	0.026
				CT/CC	4.9 (1-25)	0.057
		Mucositis	ABCB1 c.3435T>C p.Ile^{145}Ile	TT	Ref	
				CT	5.2 (0.9-30.6)	0.06
				CC	7.5 (1.3-44)	0.026
				CT/CC	6.2 (1.2-31.8)	0.027
		Recurrent (any)	ABCG2 c.421C>A p.Gln^{141}Lys	CC	Ref	
				AC	0.1 (0.01-1)	0.05
				AA	Nil	

Table 13: Genotypic associations with chemotherapy toxicity continued

Cycle	Pathway	Toxicity (gr 3-4)	Polymorphism	Genotype	OR (95%) CI	p
Doxorubicin/Cisplatin	**A metabolism**	**Anaemia**	CYBA c.242C>T p.His^{72}Tyr	CC	Ref	
				CT	0.2 (0.06-0.9)	0.029
				TT	0.5 (0.9-2.7)	0.4
				CT/TT	0.3 (0.09-0.9)	0.038
		Mucositis	CYBA c.242C>T p.His^{72}Tyr	CC	Ref	
				CT	0.2 (0.5-0.75)	0.018
				TT	0.5 (0.1-2.9)	0.47
				CT/TT	0.3 (0.08-0.9)	0.028
	DNA repair	**Mucositis**	ERCC1 c.354T>C p.Asn^{118}Asn	CC	Ref	
			(wild-type CC)	CT	1.1 (0.3-3.9)	0.9
				TT	0.1 (0.01-0.9)	0.045
				CT/TT	0.6 (0.2-1.9)	0.4
Doxorubicin	**DNA repair**	**Infection**	XPC 2886A>C Lys^{939}Gln	AA	Ref	
				AC	0.2 (0.04-0.8)	0.03
				CC	0.3 (0.04-2)	0.2
				AC/CC	0.2 (0.06-0.8)	0.024
			ERCC1 c.1510C>A p.Gln^{504}Lys	CC	Ref	
				AC	3.7 (0.9-15.2)	0.06
				AA	3.7 (0.5-28.4)	0.2
				AC/AA	3.7 (1-13.7)	0.047
		Leucopenia	ERCC1 c.1510C>A p.Gln^{504}Lys	CC	Ref	
				AC	5.4 (1-29.6)	0.05
				AA	5.4 (0.4-66.7)	0.19
				AC/AA	5.4 (1.1-26)	0.036
	GST enzymes	**Treatment delay**	GSTM1	Non-null	Ref	
				Null	9.4 (1.9-46.9)	0.006
		Leucopenia	GSTP1 c.313A>G p.Ile^{105}Val	AA	Ref	
				AG	6.8 (1.5-31.2)	0.013
				GG	4 (0.2-91)	0.39
				AG/GG	7.8 (1.3-47)	0.024

7.7.4 Cardiotoxicity

Diagnostic EF showed no significant association with genotype. The GST enzyme SNP GSTP1 c.313A>G p.Ile^{105}Val was associated with cardiotoxicity, variant carriers at increased risk of early cardiac damage (OR 9.4, 95% CI 1.8-49.3, p=0.008) and decreased EoT EF (OR 4.8, 95% CI 1.4-16.4, p=0.011). Accordingly, the mean fall in EF from beginning to EoT was significantly greater in variant carriers (10% vs. wild type 5%, p=0.033). Results of the multivariate analysis of cardiotoxicity are summarised in Table 14.

7.7.5 Nephrotoxicity

Diagnostic GFR showed no significant association with genotype. Variant carriers of ERCC2 c.2251 A>C p.Lys^{751}Gln showed an increased risk of early nephrotoxicity (OR 4.4, 95% CI 1-1.8, p=0.044) and in accordance, a greater mean fall in GFR from diagnosis to end of cycle 2 (23ml/min/1.73m^2 vs. wild-type homozygotes 4 ml/min/1.73m^2 p=0.021).

Variant carriers of the folate pathway enzyme, MTHFR c.677C>T p.Ala^{222}Val showed a trend towards early nephrotoxicity (OR 3.1, 95% CI 0.9-11.6, p=0.085). Although a significant association with EoT nephrotoxicity was not observed, variant carriers demonstrated a greater mean fall in GFR from diagnosis to EoT (variants 33 ml/min/1.73m^2, wild-type 20.5 ml/min/1.73m^2, p=0.043). Results of the multivariate analysis of nephrotoxicity are summarised in Table 14.

7.7.6 Ototoxicity

Meaningful analysis of ototoxicity was not possible due to incomplete audiogram data in 27 patients (47%).

Table 14: Multivariate analysis of genotypic associations with cardio- and nephrotoxicity

Toxicity	Measure	Polymorphism	Genotype	OR (95% CI)	p
Cardiotoxicity	**Early**	GSTP1 c.313A>G p.Ile^{105}Val	AA	Ref	
			AG	9.2 (1.7-49)	0.01
			GG	11.5 (0.5-264)	0.12
			AG/GG	9.4 (1.8-49)	0.008
	EoT	GSTP1 c.313A>G p.Ile^{105}Val	AA	Ref	
			AG	5.1 (1.5-18)	0.01
			GG	2.6 (0.1-47.4)	0.5
			AG/GG	4.8 (1.4-16.4)	0.011
Nephrotoxicity	**Early**	ERCC2 c.2251A>C p. Lys^{751}Gln	AA	Ref	
			AC	4.1 (0.9-18.4)	0.064
			CC	5.8 (0.8-45)	0.091
			AC/CC	4.4 (1-18.8)	0.044
		MTHFR c.677C>T p. Ala^{222}Val (gender additional covariate)	CC	Ref	
			CT	3.2 (0.8-13)	0.10
			TT	3 (0.4-20.5)	0.26
			CT/TT	3.1 (0.9-11.6)	0.085

7.8 Chemotherapy toxicity and histological response

No significant associations were observed between chemotherapy toxicity or dose modifications and histological response.

7.9 Chemotherapy toxicity and PFS

Considering first the analysis of all three drugs together, severe toxicity did not show any influence on PFS (Table 15: Chemotherapy toxicity and PFS). Further analysis of each drug separately demonstrated an association between methotrexate toxicity and PFS. In univariate analysis, improved PFS was associated with recurrent severe toxicity in any system (grade 3-4 in >2 cycles) (p=0.005) and presence of mucositis (p=0.004) (Figure 13 i-ii). Recurrent severe toxicity remained highly significant as a predictor of improved PFS in multivariate analysis (HR 0.1, 95% CI 0.02-0.5, p=0.004). No measure of cardiac or nephrotoxicity was associated with PFS.

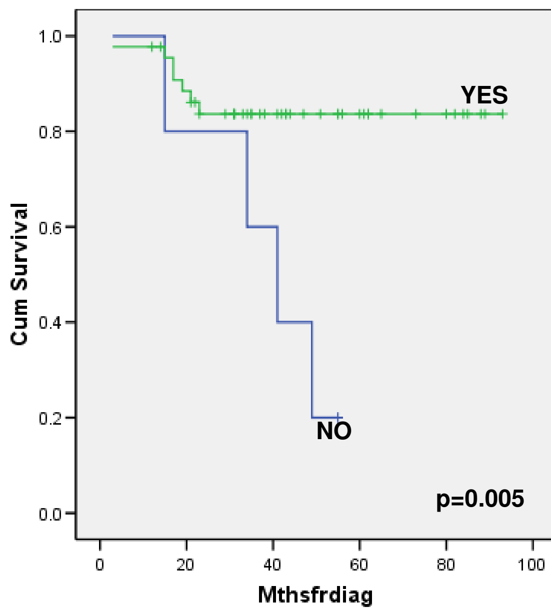
Table 15: Chemotherapy toxicity and PFS

Chemotherapy toxicity	Hazard ratio (95% CI)	p (log rank)
Infection	0.4 (0.04 - 4)	0.44
Mucositis	0.4 (0.12 –1.4)	0.15
Anaemia	0.4 (0.09 – 1.5)	0.16
Neutropenia	Constant	
Thrombocytopenia	0.5 (0.1 – 2.8)	0.44

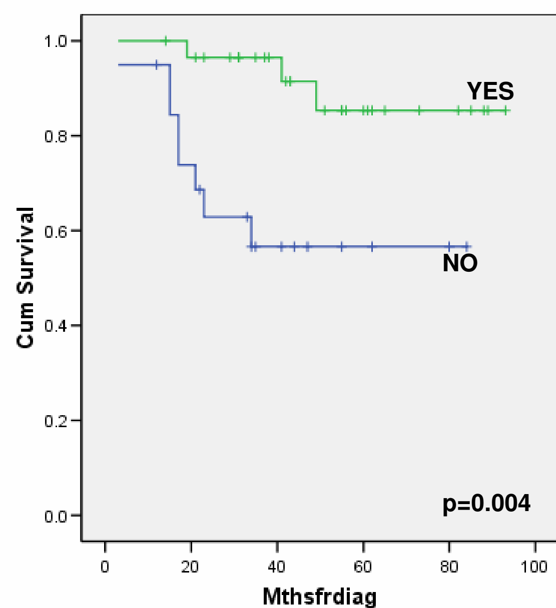
Figure 13: Kaplan-Meier curves for methotrexate toxicity and PFS

The graphs depict Kaplan-Meier curves for the univariate analysis of methotrexate mucositis and PFS; 1) Absence of methotrexate-induced mucositis was predictive of poor PFS ii) Recurrent episodes of grade 3-4 methotrexate-induced mucositis were associated with improved PFS in univariate analysis.

i) Any methotrexate mucositis



ii) Recurrent severe methotrexate mucositis



8.0 Results (ii): Genome-wide CNV analysis

8.1 Study population

Analysis of genome-wide CNV was performed using data from germline DNA of 50 osteosarcoma patients genotyped by the Illumina 610-Quad microarray. No samples were excluded following application of quality control criteria detailed in Section 6.0.

8.2 GWAS of CNV

8.2.1 Unfiltered CNV

In total, PennCNV called 1049 CNVs. After exclusion of calls with confidence <50, there were 142 CNV remaining for further analysis (median 3, IQR 2-5). One patient was an outlier in number of CNV calls and was excluded from further analysis (upper quartile + (1.5 x IQR) (308). Median CNV size was 115kb (IQR 64-186kb) with no significant difference seen in CNV number or size according to ethnic group. Figure 14: PennCNV 10-SNP CNV calls.

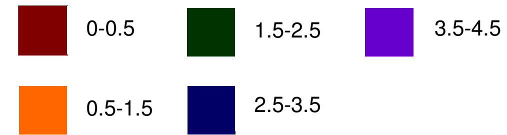
8.2.2 Analysis of single CNV

Ninety-six single CNV were identified in 35 patients. Median size was 149kb (IQR 72-100kb). A complete list of CNV and associated genes is presented in Table 16.

8.2.3 Analysis of recurrent CNV regions

Eight recurrent CNV were identified by PennCNV. The most frequent CNV was a 140kb deletion in chromosome 8, present in 8 patients. The largest was a 238kb duplication in chromosome 7 observed in 2 patients. The presence of the listed recurrent CNV was not significantly different in osteosarcoma patients compared to control data. No recurrent CNV were identified. The complete list of recurrent CNV is presented in Table 17.

Figure 14: Unfiltered PennCNV 10-SNP CNV calls



This figure illustrates the unfiltered CNV calls from 50 osteosarcoma patients, calculated by PennCNV using SNP data from the Illumina 610-Quad array.

chr1 0M
chr2 245M
chr3 488M
chr4 688M
chr5 879M
chr6 1060M
chr7 1231M
chr8 1389M
chr9 1536M
chr10 1674M
chr11 1810M
chr12 1944M
chr13 2076M
chr14 2191M
chr15 2297M
chr16 2397M
chr17 2486M
chr18 2565M
chr19 2641M
chr20 2705M
chr21 2767M
chr22 2814M
chrX 2864M
chrY 3019M

Table 16: Single CNV and associated genes

chromosome	start	end	length	gene
1	103969301	104099390	130089	AMY2A,AMY1A,RP5-1108M17.5,AMY1B,AMYP1,AMY1C
1	164802822	164848916	46094	FMO8P,FMO9P
1	186213171	186875734	662563	RP11-134C1.1,7SK,RP11-405P11.1
1	187802905	187875295	72390	none
1	195030562	195199868	169306	CFHR3,CFHR1,CFHR4,CFHR2
1	229771352	229879757	108405	DISC1,U5
1	245895420	246344261	448841	RP11-634B7.1,OR13G1,OR6F1,OR14A2,OR14K1,OR1C1,OR9H1P,OR14A16,HSD17B7P1,
				RP11-438H8.3,OR11L1,TRIM58,OR2W3,OR2AJ1,RP11-438H8.8,OR2L13,RP11-438H8.9,
				OR2L8,OR2AK2,RP11-430I15.2,OR2L1P,Y_RNA,RP11-430I15.4,OR2L5,Y_RNA,OR2L2,
				Y_RNA,OR2L3,AL513488.11
2	32487194	33181898	694704	BIRC6,hsa-mir-558,TTC27,Y_RNA,LTBP1,AL133244.1
2	76793083	76802609	9526	none
2	129892216	130149783	257567	AC019050.4
2	242568597	242656041	87444	AC093642.5
3	2004929	2145519	140590	7SK,AC087427.3-1
3	8294151	8524665	230514	AC023481.6,LMCD1
3	8677963	8788494	110531	C3orf32,AC034187.6,CAV3,OXTR
3	26021365	26132149	110784	none
3	101840361	101916282	75921	GPR128,TFG
3	146536518	146597501	60983	none
3	164190672	164376517	185845	none
3	165049762	165509032	459270	AC084017.14,hsa-mir-1263
4	11851124	12005487	154363	U6
4	34434019	34746213	312194	AC020589.4
4	69064675	69163188	98513	UGT2B17
4	69085989	69163188	77199	UGT2B17
4	69097539	69163188	65649	UGT2B17

Table 16: Single CNV and associated genes continued

chromosome	start	end	length	gene
4	114718478	115392116	673638	CAMK2D,ARSJ
4	115407950	115554158	146208	none
4	136185613	136399366	213753	AC104619.5
4	151418530	152106730	688200	LRBA,MAB21L2,AC110813.4,AC092544.4
4	161271979	161290509	18530	none
4	188698152	189096902	398750	AC097521.2-1,AC097521.2-2,AC115540.3
4	189381793	189499240	117447	none
4	189607314	189654606	47292	none
5	341014	424958	83944	PDCD6
5	457713	559051	101338	PDCD6,AC010442.7-2,EXOC3,AC010442.7-3,SLC9A3
5	8756085	8800106	44021	none
5	59758639	59807480	48841	AC109486.2-1
5	97074222	97125076	50854	none
5	101031711	101313163	281452	none
5	104538869	104859087	320218	none
5	115066422	115158825	92403	U2
5	115624019	115737055	113036	COMMD10
6	29972182	30004400	32218	HLA-16,BAT1P1,MCCD1P1,HCG4P6,HLA-K
6	29976032	30003207	27175	BAT1P1,MCCD1P1,HCG4P6,HLA-K
6	30025570	30045812	20242	HLA-80
6	31473378	31562344	88966	MICA,Y_RNA,HCP5
6	63434153	63826855	392702	AL589736.6
6	66460447	66507460	47013	EYS
6	67075448	67105019	29571	none
6	77248982	77397823	148841	none
6	79029649	79090197	60548	none
6	152424075	153088816	664741	ESR1,SYNE1,C6orf98,AL138832.10,5S_rRNA,MYCT1,RP1-9B16.2
6	162154323	162225392	71069	PARK2,5S_rRNA,AP000886.3-1
6	162644237	162829925	185688	PARK2
6	168169995	168339100	169105	KIF25,FRMD1

Table 16: Single CNV and associated genes continued

chromosome	start	end	length	gene
7	1934771	2199983	265212	MAD1L1
7	5999101	6504180	505079	PMS2,Y_RNA,JTV1,SNORA42,EIF2AK1,ANKRD61,AC004895.2-1,SRP_euk_arch,
				AC004895.2-2,USP42,CYTH3,Y_RNA,AC009412.5,RAC1,DAGLB,KDELR2,GRID2IP
7	76147604	76385939	238335	AC004980.5-3
7	76223675	76387981	164306	none
7	108310080	108467430	157350	C7orf66
7	119057622	119473254	415632	U1
7	141419097	141435188	16091	MGAM
8	13663608	13694172	30564	none
8	15994142	16065839	71697	MSR1
8	39356825	39497557	140732	ADAM5P,AC106011.3
8	113714803	114832205	1117402	CSMD3,AC107890.3
8	137757137	137926509	169372	none
9	581094	598622	17528	KANK1
9	30410638	30548222	137584	none
9	118051212	118306516	255304	PAPPA,AL137024.27-2,AL137024.27-1,ASTN2
10	44530696	44679489	148793	RP11-18M11.1
10	44568949	44679489	110540	RP11-18M11.1
10	47007374	47122505	115131	AL450388.13,RP11-292F22.2
10	47025300	47218918	193618	AL450388.13,RP11-292F22.2,ANTXRL,AL603965.10-1,FAM25HP,ANXA8L2,AL603965.10-2
10	55048674	55124158	75484	none
10	67748487	67772339	23852	CTNNA3
10	67754983	67829658	74675	CTNNA3
10	135106317	135204241	97924	AL161645.14-1,AL161645.14-2,CYP2E1
10	135199216	135204241	5025	CYP2E1
11	21145601	21173428	27827	NELL1
11	55124465	55209499	85034	OR4C11,OR4P4,OR4S2,OR4C6,OR4P1P
11	55127597	55209499	81902	OR4C11,OR4P4,OR4S2,OR4C6,OR4P1P
11	84127155	84152565	25410	DLG2
11	84782761	84909123	126362	DLG2,AP003035.2-1,U6

Table 16: Single CNV and associated genes continued

chromosome	start	end	length	gene
11	133889790	134227062	337272	AP001999.4
12	7891603	7990569	98966	SLC2A14,Y_RNA,AC006517.46-1,NANOGP1,SLC2A3
12	31157554	31298174	140620	AC008013.8-3,AC008013.8-2,AC024940.39-1
12	84767389	86411270	1643881	NTS,MGAT4C
12	130296270	130343129	46859	none
13	22257525	22381716	124191	SNORD36,RP11-363G2.1,RP11-363G2.2,RP11-363G2.3,AL162853.17-1,RP11-124N19.1
13	103069986	103348841	278855	none
14	42897456	43318929	421473	none
14	44251087	44294325	43238	none
14	86082980	86293375	210395	none
15	19095051	19545168	450117	AC068446.22-1,U6,AC068446.22-2,POTEC,U6,AC126335.16-3
15	28253440	28410705	157265	AC135731.6-1,U6
15	30255140	30544756	289616	U6,AC139426.2-3,SRP_euk_arch,AC139426.2-1,AC139426.2-2,FAM7A1,U8,
				AC135983.4-3,AC135983.4-7,SRP_euk_arch,AC135983.4-2,AC135983.4-4
15	79795368	79875169	79801	AC104041.5
15	83616952	83935336	318384	AKAP13
16	32281925	32505195	223270	AC138915.2
16	32396948	32505195	108247	none
16	32413490	32505195	91705	none
16	32415046	32505195	90149	none
16	86228369	86592857	364488	JPH3,AC010536.8,KLHDC4,SLC7A5,CA5A,BANP
17	21480206	22138035	657829	AC144838.2,AC138761.4-1,AC138761.4-2,FAM27L,AC131055.10-1,AC131055.10-2,
				AC131055.10-3,SSU_rRNA_5,AC131055.10-4,AC131055.10-5,AC131055.10-6,AC131055.10-7,
				AC131055.10-8,AC131055.10-9,AC131055.10-10,AC131055.10-11,AC131055.10-12,
				AC131055.10-13,AC131055.10-14
17	41785962	42137359	351397	ARL17,LRRC37A2,ARL17P1,SRP_euk_arch,NSF
18	64897188	64906488	9300	none
19	47997996	48157656	159660	PSG10,PSG1,PSG6,CEACAMP7
19	48354926	48531928	177002	PSG5,PSG4,PSG9,CEACAMP11,CEACAMP4,AC005392.8
19	48498399	48531928	33529	CEACAMP4,AC005392.8

Table 16: Single CNV and associated genes continued

chromosome	start	end	length	gene
20	14679595	14733705	54110	C20orf133,RPS10P2
22	17092563	17398986	306423	AC008132.35-2,GGT3P,AC008132.12,AC008132.13,AC008132.35-1,AC008103.29,DGCR6,
				PRODH
22	17334629	17529580	194951	DGCR2,Y_RNA,AC004461.4,TSSK1A,DGCR14,AC004471.2,TSSK2,GSC2
22	17337129	17388108	50979	none
22	17660403	18078129	417726	HIRA,AC000092.1-1,SRP_euk_arch,MRPL40,C22orf39,UFD1L,CDC45L,CLDN5,AC000077.2
22	18240875	18395615	154740	TXNRD2,AC000078.5,COMT,ARVCF,C22orf25
22	19066315	19366850	300535	USP41,ZNF74,U6,SCARF2,KLHL22,AC007731.16-1,Y_RNA,Y_RNA,AC007731.16-2,
				XXbac-B562F10.10,MED15,AC007050.18,AC007050.11,FAM108A5
22	19418146	19795780	377634	PI4KA,SERPIND1,SNAP29,CRKL,AC002470.17-1,AC002470.17-2,AIFM3,LZTR1,THAP7,
				AC002472.8-1,P2RX6,SLC7A4,hsa-mir-649,P2RX6P,AC002472.8-2,AC002472.14,
				AP000550.1-2
22	20645312	20903637	258325	TOP3B,PRAMEL,IGLVI-70,IGLV4-69,IGLVI-68,LL22NC03-23C6.12,IGLV10-67,
				LL22NC03-23C6.15,IGLVIV-66-1,IGLVV-66,IGLVIV-65,IGLVIV-64,IGLVI-63,IGLV1-62,IGLV8-61,
				LL22NC03-88E1.17,FAM108A6,LL22NC03-30E12.10,LL22NC03-30E12.11,IGLV4-60,
				LL22NC03-30E12.13,SOCS2P2,IGLVIV
22	23994408	24165514	171106	IGLL3,CTA-246H3.8,LRP5L
22	24087268	24244593	157325	LRP5L,CRYBB2P1
22	24198241	24240667	42426	none

Table 17: Recurrent CNVs in osteosarcoma patients

Chr	No of patients	Copy number phenotype	Start Mb	End Mb	Size kb	Gene
1	2	Hemizygous deletion	103.96	104.09	130	AMY 1A, AMY 2A
4	5	Homozygous deletion	69.06	69.16	98	UGT2B17
5	2	Hemizygous deletion	8.75	8.80	44	
6	3	Hemizygous deletion	29.97	30.0	32	HCG4P6
7	2	Duplication	76.14	76.38	238	
8	8	Homozygous deletion	39.35	39.49	140	AK128178 BC026083
10	2	Duplication	44.53	44.67	148	
10	2	Duplication	135.10	135.20	97	BC038300 AK126713 CYP2E1
19	2	Hemizygous deletion	48.35	48.53	177	
22	2	Duplication	17.33	17.52	194	DGCR2, 13, 14 TSSK2
22	2	Dupl/Hemi del	24.08	24.24	157	

9.0 Discussion (i): Candidate polymorphism study

Understanding and overcoming inter-individual variation in drug response and toxicity remains one of the major challenges in cancer chemotherapy. This study represents the most comprehensive investigation to date examining the role of genetic polymorphisms on chemotherapy toxicity and disease outcome in osteosarcoma. As a "proof of principle" exploratory study, the aim was to gain some understanding of potential pharmacogenomic influences on the complex treatment protocols used in this rare disease. Thirty-six MAP pathway polymorphisms were selected on the basis of putative functional effects or previously reported associations in haematological and solid malignancies.

A number of novel polymorphic associations were observed as well as confirming several previously reported findings. While limited by small sample size secondary to retrospective recruitment and the rarity of this tumour, several positive associations warrant further prospective study. Ultimately, it is hoped that pharmacogenomic profiling may be used to predict aggressive disease, poor clinical outcome or severe toxic effects. Findings for metastatic disease, histological response and survival will be discussed by gene whereas toxicity will be discussed using a pathway-based approach.

9.1 Patient characteristics

Patient and disease characteristics did not differ significantly from previously reported data in osteosarcoma with the majority of patients aged below 25 years and a prevalence of males and caucasians (32). A lower age limit of 16 years was selected for two reasons. Firstly, to ensure younger children would not be subjected to further blood sampling having completed intensive chemotherapy and secondly, within the first incidence peak, evidence suggests the biology and natural history of osteosarcoma does not differ with age (310, 311).

In concordance with others, the most frequent primary site in this study was in extremity long bones (3). Maxillofacial osteosarcoma is uncommon and the inclusion of 4 patients reflects the expertise at the recruiting centre. Osteoblastic histology was the most common subtype as previously reported although there were more telangiectatic tumours than may be expected from other studies (2, 312). However, the outcome of patients with telangiectatic histology has been shown to be similar to that of other high-grade osteosarcoma subtypes, which was true in the current study (313). Consistent with larger series, most patients underwent reconstructive surgery reflecting the high

level of surgical expertise available within specialist sarcoma services. Inferior outcome was strongly associated with amputation in this study, a finding previously reported in osteosarcoma (314).

Histological response is a well-established predictor of poor prognosis, the observed lack of association in this study most likely due to small numbers of poor responders. The observed association of axial tumours and metastatic disease with poor survival is as expected from other studies (36, 314). Complete excision of disease is of fundamental importance in seeking a cure for osteosarcoma, explaining why metastatic disease is frequently incurable as its multifocal nature compromises the likelihood of complete resection. Likewise, axial tumours may only be resectable with mutilating or disabling surgery.

9.2 Genotypic associations

Genotype frequencies were in keeping with other reports with no deviations from HWE. A significant association of TYMS group 2 “risk” genotypes (2R/3G, 3G/3C, 3G/3G) with male gender warrants discussion. The TYMS 3R and “risk” genotypes are associated with inferior survival in childhood ALL and gastric cancer respectively but not histological response or survival in osteosarcoma although the VNTR alone was genotyped in the latter study (22, 126, 141). Methotrexate exposure was a major difference between these studies; the drug being an integral component of treatment protocols for both ALL and osteosarcoma but not gastric cancer. Furthermore, nearly four-fold higher doses were administered to osteosarcoma compared to ALL patients. Of note however, germline rather than tumoural DNA was genotyped in all. None reported a gender influence in TYMS genotype, suggesting this may be a spurious observation secondary to small patient numbers in the present study. Nonetheless, as male gender is known to be a strong predictor of unfavourable outcome in osteosarcoma a disproportionate increase in unfavourable TYMS genotypes is intriguing.

9.3 Markers of metastasis

9.3.1 Cyclin D1 (CCND1)

Although multi-drug chemotherapy has significantly improved survival in non-metastatic osteosarcoma, curing those with metastatic disease is a continuing challenge. The identification of metastatic markers may allow early treatment intensification, potentially salvaging some patients.

The observation that the wild-type homozygote genotype of CCND1 c.870A>G p. Pro^{241}Pro was over-represented in patients with metastatic disease at diagnosis has not previously been reported in osteosarcoma (OR 9, 95% CI 1.4-57.9, p=0.021).

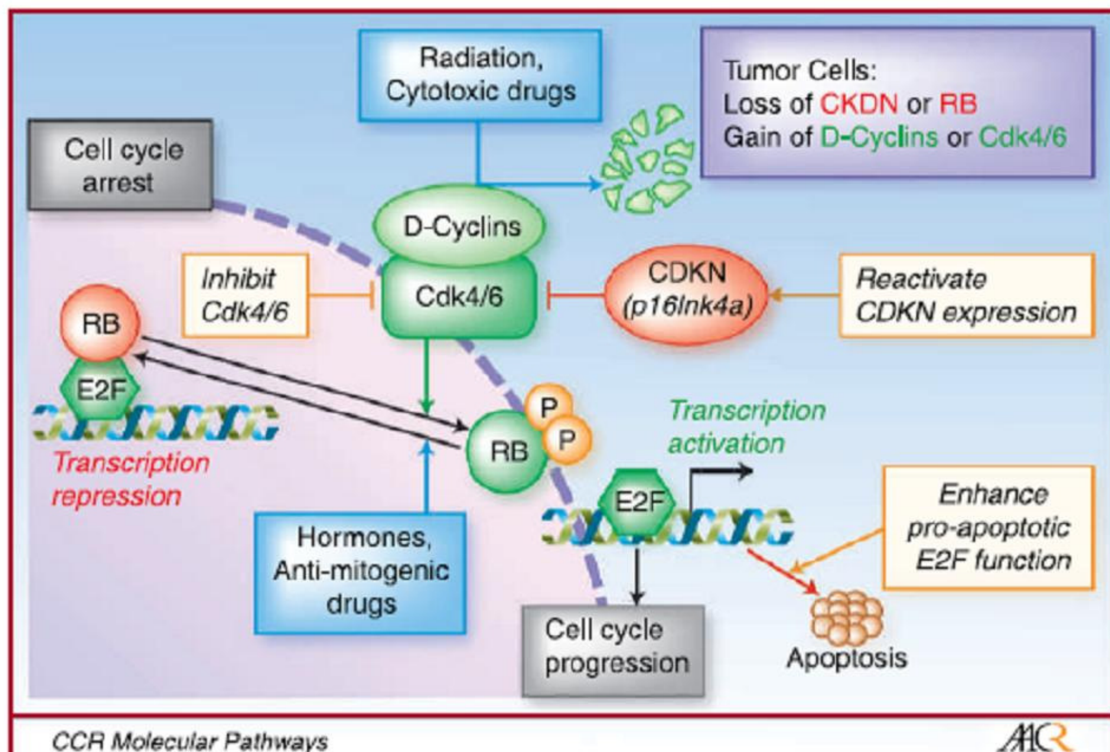
Cyclin D1 assumes a key role in cell cycle progression from G1 to S phase by acting as a mitogenic sensor communicating extracellular signals to core cell cycle machinery. Expression is frequently altered in human cancers, driven by multiple mechanisms comprising genomic alterations, posttranscriptional regulation, and post-translational protein stabilization. The c.870A>G p. Pro^{241}Pro wild-type allele enhances the oncogenicity of CCND1 by encoding a truncated protein (termed CCND1b), rendering it refractory to nuclear export, facilitating a prolonged influence on cell cycle control (315). A large number of epidemiological studies have investigated the influence of this polymorphism in cancer susceptibility and disease outcome (316). The majority of studies have implicated the A allele in increased cancer risk and poor outcome in diverse tumour types including leukaemia, colorectal, breast, bladder, ovarian and lung cancer. However, as over 100 SNPs have been identified in the CCND1 gene, it is not yet clear whether this represents a causal variant or is simply in linkage disequilibrium wth the true culprit.

9.3.1.2 CCND1, the cell cycle and osteosarcoma pathogenesis

If this observed association is genuine, by what mechanism may CCND1 c.870A>G p. Pro^{241}Pro promote metastatic disease in osteosarcoma?

CCND1 regulation of the cell cycle is mediated through its interaction with cyclin-dependent kinases 4 and 6 (CDK 4/6) (316); the predominant function of the D1/CDK4 enzyme being the phosphorylation-dependent deactivation of the retinoblastoma protein (pRB) (315). The active form of pRB negatively regulates cycle progression from G0/G1 to S phase by suppression of E2F transcription factors. Phosphorylation of pRB interrupts E2F binding allowing transcription activation and unhindered progression of the cell cycle (35) (see Figure 15: The RB pathway in cancer therapy).

Figure 15: The RB-pathway in cancer therapy



The RB-pathway in cancer therapy. The components of the RB-pathway ie. RB, E2F, D-type cyclins, Cdk 4/6, p16Ink4a (CDKN2a), and their functional interactions are depicted in the diagram. Genetic and epigenetic alterations in the RB-pathway are consistently detected in the majority of sporadic human cancers, and these defects are summarized in the upper right-hand corner of the diagram. The status of the RB-pathway affects tumour cell responses to radiation and genotoxic drugs, which cause cell-cycle arrest through the degradation of cyclin D1 and the consequent RB dephosphorylation. The status of the RB-pathway also affects tumour responses to hormone and other therapeutic strategies that block mitogenic signalling. Defects in the RB-pathway cause deregulated E2F activity, which stimulates gene expression to promote G1/S transition and apoptosis. Potential therapeutic strategies that directly target the RB-pathway defects are depicted in the orange boxes, and these include the reactivation of p16Ink4a expression in cases in which the gene is silenced but not mutated, the inhibition of Cdk4/6 kinase activity, and the enhancement of E2F-dependent apoptosis. Reprinted with permission from the American Association for Cancer Research. Knudsen E, Wang J. Targeting the RB-pathway in Cancer Therapy. Clin Cancer Res 2010; 16(4): 1094-9.

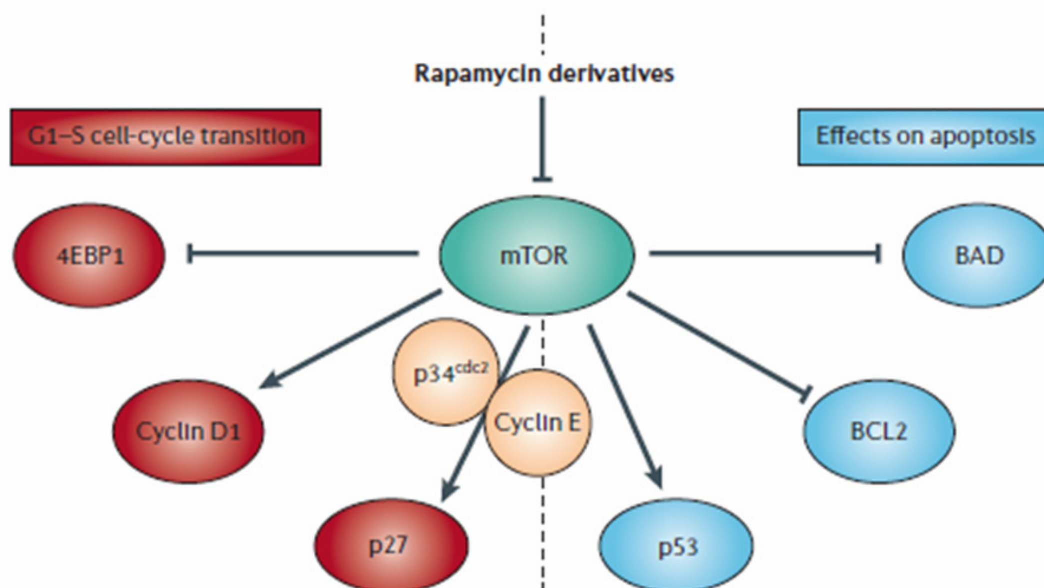
The retinoblastoma protein is the product of the retinoblastoma tumour suppressor (RB1), a gene already implicated in the pathogenesis of osteosarcoma. Inherited mutations of RB1 not only cause retinoblastoma, a developmental malignancy of the retina but are associated with approximately 200-fold higher incidence of osteosarcoma (317). Moreover, up to 70% of sporadic osteosarcomas have genetic alterations in the RB1 gene (318). Even tumour samples negative for RB1 mutations implicate the CCND1/CDK/pRB pathway in osteosarcoma, showing decreased expression of the CDK inhibitor $p16^{INK4a}$ suggesting that it acts as a tumour suppressor (319). Considering the above, a potential mechanism by which CCND1 c.870A>G p. $Pro^{241}Pro$ may promote metastatic osteosarcoma is through increased pRB

deactivation leading to uncontrolled cell-cycle progression, further augmented by the presence of underlying RB1 mutations. Certainly in osteosarcoma cell lines, proliferation is inhibited by RNA interference of CCND1 with concomitant decreased mRNA and protein expression (320). Interestingly, CCND1 may also play a role in the second most common malignant bone tumour, Ewing's sarcoma. In this disease, the diagnostic EWS-FLI1 translocation directly stimulates CCND1 transcription particularly favouring the D1b isoform, the same isoform encoded by the c.870A>G p. $Pro^{241}Pro$ SNP (321).

Several other cellular signalling pathways include CCND1 as a key component and may therefore also be influenced by the presence of the functional polymorphism c.870A>G p. $Pro^{241}Pro$. Rapamycin-inhibition of mTOR (mammalian target of rapamycin, a protein kinase central to the PI3K-AKT signalling pathway) disrupts downstream messengers, consequently preventing cyclin-dependent kinase (CDK) activation, inhibiting retinoblastoma protein phosphorylation and accelerating CCND1 turnover. This leads to a deficiency of active CDK4/cyclin D1 complexes, all of which may help cause G1 phase arrest (322) (see Figure 16: Effects of mTOR inhibitors on cancer cells). In osteosarcoma tumour samples, mTOR expression correlates with surgical stage, presence of metastasis, histological response and survival (323). In addition, rapamycin strongly inhibits osteosarcoma cell line growth concomitant with down-regulation of CCND1 (324). Cell cycle regulators including CCND1 are also fundamental elements of the Notch signalling pathway. A Notch target gene, HES1 is expressed in osteosarcoma cells and is associated with invasive and metastatic potential (325). Furthermore, suppression of osteosarcoma growth by Notch inhibition has recently been demonstrated, mediated by reduced expression of CCND1 and other regulatory proteins (326).

CCND1 also appears to influence cellular sensitivity to several chemotherapeutic agents, particularly antifolates. Phosphorylation of pRB ultimately increases transcription of E2F and the methotrexate target genes, DHFR and TYMS. Osteosarcoma cells lacking pRB exhibit methotrexate resistance together with increased activity and mRNA of DHFR and TYMS (329). Similarly, overexpression of CCND1 in human fibrosarcoma cell lines reduces sensitivity to methotrexate (190). Furthermore, gastric carcinoma cells transfected with CCND1 antisense oligonucleotides show increased chemosensitivity to methotrexate (328). Recently, ALL cell lines treated with mTOR inhibitors (MTI) showed a marked reduction in DHFR and CCND1 expression together with increased sensitivity to methotrexate (329). The observation of *in vitro* and *in vivo* synergy between MTI and methotrexate offers an exciting potential treatment option for tumours resistant to methotrexate.

Figure 16: Effect of mTOR inhibitors on cancer cells



Cellular effects of mTOR activation include facilitation of G1-S cell cycle transition and inhibition of apoptosis through interaction with key molecules of cell-cycle control (4EBP1, cyclin D1 and p27) and apoptosis (BAD,BCL2 and p53). These processes are reversed by mTOR inhibitors. 4EBP1, eukaryotic initiation factor 4E-binding protein; BAD, BCL2-anatgonist of cell death; BCL2, B-cell lymphoma 2; mTOR, mammalian target of rapamycin. Reprinted by permission from Macmillan Publishers Ltd: Faivre S, Kroemer G, Raymond E. Current development of mTOR inhibitors as anticancer agents. Nature Rev Drug Disc 2006; 5: 671-688, copyright 2006.

The effect of CCND1 on cisplatin and doxorubicin sensitivity appears more variable. The osteosarcoma and fibrosarcoma cell lines referred to above showed no resistance to doxorubicin or cisplatin although cisplatin sensitivity was enhanced in gastric carcinoma cells (190, 327, 328). Cisplatin resistance in association with CCND1 overexpression is found in pancreatic, testicular germ cell, ovarian and prostatic tumours whilst RNA interference of CCND1 sensitises human breast cancer cells to cisplatin (330-332).

There is persuasive evidence that CCND1 may be important in both the pathogenesis and chemosensitivity of osteosarcoma but it must be emphasised that the majority of studies relate to tumour samples or cell lines rather than germline DNA as in the current study. Direct comparison of tumoural and germline DNA is required before drawing further conclusions. Nonetheless, it appears that c.870A>G p. Pro^{241}Pro may impose far-reaching cellular consequences, suggesting the association with metastatic disease deserves more extensive investigation.

9.3.2 ERCC1

The reduced-function genotype of ERCC1 c.354T>C p.Asn^{118}Asn was associated with metastatic disease at diagnosis. This SNP has previously been investigated for an association with clinical outcome of osteosarcoma but not the presence of metastatic disease (19). A recent meta-analysis found no association between this SNP and susceptibility to solid tumours including breast, bladder, endometrial and colorectal cancer although ERCC2 c.2251A>C p.Lys^{751}Gln, a SNP in strong linkage disequilibrium with ERCC1 c.354T>C p.Asn^{118}Asn, was significantly associated with lung cancer (333). Another linked SNP, ERCC1 c.1510A>C was associated with adult onset glioma in a large study (334).

ERCC1, a NER enzyme located on chromosome 19q13, maintains genomic integrity by recognizing and restoring bulky helix-distorting DNA damage (104). Unrepaired DNA damage may cause cell cycle arrest and apoptosis but the accumulation of misrepairs may lead to chromosome instability and carcinogenesis (335). It is plausible that a reduced-function polymorphism in a DNA repair enzyme may be associated with aggressive disease and chemoresistance by permitting persistent misrepair.

Genetic changes are complex in osteosarcoma, reflecting chromosomal instability secondary to both numerical and structural aberrations (336). Intriguingly, one chromosomal region frequently affected is 19q13, the location of ERCC1 and ERCC2. Aberrations are seen in 17% of sporadic osteosarcomas, although mutations also appear in giant cell tumour of bone (337). Further inference that defective DNA repair may have a role in osteosarcoma pathogenesis is demonstrated by sufferers of the inherited DNA repair disorders Rothmund Thomson, Werner's and Bloom syndrome, who carry a striking predisposition to osteosarcoma. These disorders are characterised by germline mutations in RecQ helicases, proteins that maintain genomic integrity at the interface between DNA replication and recombination by repairing damaged replication forks (35). Cells devoid of functional RecQ proteins show abnormal DNA replication, shortened telomeres, hyper-recombination, hypersensitivity to DNA-damaging agents and meiotic defects suggesting a tumour-suppressor role (338).

RecQ mutations are found only infrequently in sporadic osteosarcoma (339). However, increased gene expression in tumour samples is associated with a greater frequency of structural chromosome change; elevated levels may maintain DNA in a prolonged single stranded vulnerable state continually promoting structural instability (336). Overexpression of RecQL4 has been found in primary extremity soft tissue sarcoma, promotes metastasis in breast carcinoma and is associated with late stage disease in

laryngeal carcinoma (340-342). RB1 pathway disruption and Ras activation synergistically induce RecQ genes, driving cells to increase DNA replication that is then maintained in a state vulnerable to structural damage (343). In a similar manner to dysfunctional RecQ, it is possible that ERCC1 c.354T>C p.Asn^{118}Asn increases cellular vulnerability to RB1 pathway disruption by compromising its ability to maintain DNA integrity.

9.4 Genotypic associations with histological response

The relationship between poor histological response and inferior survival is well established in osteosarcoma with the concept of later treatment intensification to "rescue" these patients currently being explored in an international randomised clinical trial (5, 6). Lacking at present is a means of identifying patients at risk of poor response at diagnosis. This study observed three novel polymorphic associations with histological response but did not replicate a reported association between ERCC2 c.2251A>C p.Lys^{751}Gln (19).

9.4.1 MTHFD1

Good responders were more likely to carry the reduced-function variant allele of MTHFD1 c.1958G>A p.Arg^{653}Gln. In contrast, evidence from other studies suggests a trend towards non-response to methotrexate with the variant allele. A Canadian study enrolled over 200 children with ALL and found a lower probability of event-free survival in variant carriers in univariate but not multivariate analysis. A trend towards increased relapse and decreased survival was seen when c.1958G>A p.Arg^{653}Gln was combined with the TYMS 3R variant, suggesting a pathway-based approach to analysis may be more informative (134). In rheumatoid arthritis, a trend towards non-response to methotrexate was seen in variant homozygotes although its use as a disease-modifying agent at low dose with folic acid supplementation is not comparable to use in cancer chemotherapy (344). *In vitro* evidence is not persuasive of a significant influence on drug resistance, with no effect on sensitivity to methotrexate in leukaemic blasts nor to a range of cytotoxics in human tumour cell lines (213, 345).

MTHFD1 catalyses the formation of substrates for purine and pyrimidine synthesis and is therefore not a component of cisplatin or doxorubicin metabolising pathways. In the folate pathway it is possible that reduced enzyme function conferred by the c.1958G>A p.Arg^{653}Gln variant allele synergises with methotrexate inhibition of DHFR by further decreasing the availability of reduced intracellular folates. Subsequent impaired purine and thymidine synthesis and DNA replication leads to cell death and tumour necrosis. Small patient numbers in this study increase the likelihood that this is a chance

association. Alternatively, if methotrexate were to predominate over doxorubicin and cisplatin in determining early tumour cell necrosis, a synergistic polymorphism could then increase the likelihood of a good response. Enhanced methotrexate efficacy in variants of MTHFHD1 c.1958G>A p.Arg^{653}Gln may also be expected to increase toxicity, a concept supported by data discussed below (Section 9.6.1: Methotrexate cycles).

9.4.2 ABCC2

Poor response was significantly associated with ABCC2 c.24C>T in a dose dependent manner. Located in the epithelial apical luminal membrane of excretory organs including the liver and kidney, ABCC2 is an important element of detoxification pathways and effluxes all three MAP drugs (16). The c.24C>T SNP is located in the 5'-UTR, an essential part of the promoter but does not induce an amino acid change (346). The functional influence of ABCC2 SNPs is ill defined, particularly as many exhibit strong linkage disequilibrium. In 44 paediatric ALL patients treated with HD-M (5g/m^2), female carriers of the c.24C>T variant allele showed a 2-fold increase in M area-under-the-curve, suggesting reduced efflux (152). Other functional studies are conflicting, with lower mRNA demonstrated in normal kidney and no significant effect found in duodenal cells (153, 347).

Han et al investigated the role of ABCC2 polymorphisms in pharmacokinetics and treatment outcome of NSCLC, genotyping 107 Korean patients receiving irinotecan/cisplatin therapy. Two SNPs of which c.24C>T was one, were associated with higher response rates and improved PFS, in contrast to findings reported here (348). However, Han et al appeared to disregard the co-administration of cisplatin, concluding that common polymorphisms in ABCC2 (and ABCB1) may be important in irinotecan pharmacokinetics and the resulting tumour response. As ABCC2 is known to be an effluxer of cisplatin, this inference may be questioned (16). In concordance with Han et al, another similar-sized study of Asian NSCLC patients also found significantly increased treatment responses with the c.24C>T variant allele (154). Although all patients received platinum-based therapy, additional drug combinations included taxanes, vinorelbine and gemcitabine. These studies again illustrate two fundamental difficulties encountered when comparing pharmacogenomic analyses. Firstly, ethnic populations will always be heterogeneous and secondly, polymorphisms are likely to exert differential effects within multi-drug combination regimens. Thus potential significant polymorphisms can only validated if drug regimens are identical.

In this small study, the observed association of c.24C>T and poor response in osteosarcoma may be questioned. Nonetheless, a number of potential biological explanations may be proposed to account for such an association.

Supposing the functional consequence of c.24C>T was reduced drug efflux, the resultant high plasma levels of methotrexate, doxorubicin and cisplatin may increase toxic side effects. Certainly, further findings in this study show an association between ABCC2 SNPs and increased methotrexate toxicity as discussed below. Enhanced toxicity may compromise timely delivery of treatment, reducing chemotherapy dose intensity (DI) thereby increasing the likelihood of poor response. Although appealing, this hypothesis is complicated by ill-defined relationships between drug levels, dose intensity, toxicity and response in osteosarcoma.

Considering first the relationship between drug levels and toxicity, there is no doubt that high plasma methotrexate levels predispose to significant side effects, particularly renal damage (80). Similarly, cisplatin plasma levels correlate with degree of kidney damage with magnitude of decline in GFR directly associated with peak plasma concentration and infusion rates in children (349). However, although high cumulative anthracycline dose is a well-recognised risk factor for cardiac damage, the relationship between cardiotoxicity and peak serum levels is unclear (47).

The influence of drug levels and toxicity on histological response must also be considered. Within MAP chemotherapy, methotrexate levels are routinely monitored as an aid to folinic acid rescue (61). Cisplatin and doxorubicin levels are not monitored, therefore the studies referred to below all relate to methotrexate. The relationship between peak serum methotrexate, AUC and histological response has been extensively investigated with conflicting results. An early study evaluating the role of pharmacokinetic measurement of methotrexate levels as an aid to dose calculation found improved histological responses in patients who were dose-escalated to a level of ≥1000μM at the end of a 6 hour infusion (89). Similarly, methotrexate peak level was a significant predictive factor for histological response in a retrospective study of 272 patients treated with MAP (90). Conversely, a US study reported no association between mean peak levels and histological response after recording peak methotrexate levels after a 4-hour infusion in 52 patients treated with MAP plus ifosfamide and/or liposomal muramyl tripeptide phosphatidyl ethonolamide (350). The Institute Rizzoli concurred with the US study, reporting that increased methotrexate dose correlated with serum peak level but not histological response in 336 patients treated with MAP (351).

There appears to be a stronger consensus on the positive influence of peak levels and AUC on survival although again, not all studies concur. Graf et al analysed nearly 7000 serum methotrexate levels from patients treated in Cooperative Osteosarcoma Study Group clinical trials COSS-80, COSS-82 and COSS-86 and found disease-free survival correlated significantly with a threshold concentration of 1000µM after a 4 hour infusion (352). The Scandinavian Sarcoma Group also reported that the 24hr methotrexate level was an independent prognostic factor in patients treated on the SSG VIII clinical trial (312). Two other small studies found significantly improved DFS in patients with higher methotrexate peak levels and AUC respectively although in the latter, the highest DFS (and dose-density) was actually seen in the least exposed patients (353). In a study of 140 patients from St Judes, 96% achieved a threshold concentration of ≥1000µM but those with mean peak levels of ≥1500µM had significantly worse outcomes, possibly due to decreased dose-intensity of other agents or increased folinic acid requirement compromising methotrexate efficacy (356). In a small patient cohort, Zelcer et al reported no association between peak methotrexate levels and event-free survival (350).

The effect of DI on response and survival in osteosarcoma remains similarly unclear. In EOI clinical trials from 1983-1993, no clear survival benefit was seen with higher received DI of doxorubicin/cisplatin (355). A later trial compared the same doses of the two-drug regimen given as 3-weekly or 2-weekly cycles and found the more dose-intense regimen improved histological responses but not survival (3). However, in patients treated with triple-drug MAP, DFS was significantly higher in those who had received >90% of the scheduled dose-intensity (356). Is it possible that DI of methotrexate is particularly critical in determining response and survival? A comprehensive analysis of 30 clinical trials administering combination chemotherapy (doxorubicin, cisplatin, ifosfamide, methotrexate) for high-grade osteosarcoma certainly supports this concept as methotrexate was the only drug whose DI correlated with disease-free survival (357). Moreover, a regimen devoid of methotrexate has been documented as one of the major poor prognostic factors in osteosarcoma (358). Contrary to this, a recent international collaboration examined prognostic factors in osteosarcoma and found a 32% increase in mortality in patients treated with methotrexate and a recent study presented comparable survival without its use, albeit with major limitations (314, 359). Debate therefore continues on the role of high-dose methotrexate in current treatment schedules, a question that cannot be definitively answered at present (360-362).

An alternative explanation for the observed association of c.24C>T with poor response could be proposed if the functional consequence was enhanced drug efflux with

subsequent decreased intracellular drug concentration. Evidence suggests ABCC2 may mediate resistance to all three components of the MAP regimen. Overexpression of ABCC2 in human ovarian carcinoma cells reduces intracellular methotrexate and long-chain polyglutamate accumulation by 50% and 2-4 fold respectively after 24 hours (227). Of note, methotrexate resistance in soft tissue sarcoma cell lines is associated with greatly reduced levels of polyglutamated derivatives as is poor response to induction therapy in ALL (363, 364). Cisplatin-treated hepatocytes demonstrate increased ABCC2 protein and mRNA levels and in human embryonic kidney transfected with the ABCC2 gene, resistance to cisplatin and doxorubicin increased 10- and 8-fold respectively (365, 366). Cisplatin-resistant melanoma cells also show increased ABCC2 mRNA together with decreased formation of platinum-DNA adducts, decreased G2 arrest and resistance to apoptosis (225). Furthermore, when anti-ABCC2 antisense constructs are introduced to cisplatin-resistant HepG2 cells, resistance is reversed (367). However, not all studies support a role of ABCC2 in drug resistance. Resistance in the presence of decreased ABCC2 expression was demonstrated in cisplatin-resistant liver carcinoma cells together with reduced carboplatin accumulation (surrogate marker for cisplatin), suggesting defective uptake as opposed to enhanced efflux (368). Defective uptake appeared to be due to deranged endocytic recycling with a comprehensive failure of carrier proteins to localize to the plasma membrane, possibly leading to cross-resistance by a mechanism independent of ABC efflux (369).

Both the genomics of ABCC2 and interaction with other cellular pathways are complex and yet to be fully elucidated. The conflicting findings discussed suggest the consequence of both decreased and increased functional variants and SNPs in strong linkage disequilibrium may be both tumour and drug-dependent. The relative influence of ABCC2 efflux SNPs on each component of MAP metabolism may alter the efficacy of the regimen as a whole. Further extensive analysis is required both in germline and tumour DNA, focussing particularly on haplotypes and individual drug pathways. Although it has previously been concluded that genetic variants in ABCC2 appear to be of minor importance in drug bioavailability, findings from this study may suggest otherwise.

9.4.3 GSTP1

Poor response was associated with GSTP1 c.313A>G p.Ile^{105}Val. This SNP leads to the substitution of isoleucine by valine at codon 105 in the GSTP1 gene causing a substantial reduction in catalytic activity, potentially compromising efficiency of detoxification (177). The influence of GSTP1 genotypes on clinical outcome in

osteosarcoma is unknown and data from other malignancies is conflicting. Similar to data presented here, the variant allele was associated with poor prognosis in 233 Asian patients with oesophageal cancer although the two studies are not directly comparable due to different ethnic populations and administered chemotherapy (232). In contrast to data presented here, the majority of studies report favourable outcomes with the variant allele. In ALL, decreased risk of relapse in c.313A>G p.Ile^{105}Val variant homozygotes was first described in 64 cases of relapsed childhood patients matched with 64 successfully treated controls, using DNA from remission samples of peripheral blood or bone marrow (178). Similarly, correlation of germline c.313A>G p.Ile^{105}Val genotype with clinical outcome in 106 colorectal cancer patients showed a 3-fold increased risk of death in wild-type homozygotes (230). Results from a study of 175 patients with gastric cancer were in concordance, with chemoresistance and poor survival with AA genotypes (22). A further small study in gastric cancer demonstrated inferior response and survival in wild-type homozygotes but using paraffin-embedded tumour DNA (140). Improved response to platinum-based chemotherapy in variant carriers with NSCLC has also been reported and in over 1000 patients with invasive breast cancer, variant homozygotes showed a 60% reduction in mortality risk (154, 231). More recently however, pharmacogenetic analysis of peripheral blood DNA in over 150 subjects each with metastatic gastric cancer or advanced colorectal cancer did not replicate these findings (17, 208). Likewise, no association was observed in ovarian and breast cancer (185, 370).

The validity of direct comparisons between different pharmacogenomic studies is questionable. Results may be influenced not only by the primary malignancy but also by sample size, ethnic origin, chemotherapy regimen and source of DNA, either tumour or germline. Further explanation of conflicting results may lie in the relative roles of GST enzymes in the deactivation of cytotoxics and their distribution within tumour tissue. The eight classes of GST enzymes catalyse the conjugation of endogenous and exogenous electrophiles with glutathione (229). Cisplatin and doxorubicin are not directly detoxified by glutathione-conjugation unlike the true cytotoxic substrates, melphalan and the alkylating agents. Rather, GSTP1 and GSTM1 negatively regulate the MAPK apoptotic pathway through which cisplatin and doxorubicin induce apoptosis (119). GSTP1 is the most highly expressed isoform in human cancer (114), the lowest levels occurring in lymphoma and breast cancer and the highest in lung cancer and head and neck tumours (371). Consistent with its involvement in metabolism of cytotoxics, in many solid tumours and leukemias GSTP1 amplification has repeatedly been associated with drug resistance, failure of therapy and poor clinical outcomes (113, 114, 372, 373).

Several studies have investigated GSTP1 expression in osteosarcoma. Poor histological response, high stage and poor prognosis were all associated with overexpression in surgical but not initial biopsy specimens (118). The authors suggested either failure of optimal peri-tumoural pre-operative chemotherapy concentration led to overexpression or, overexpressing tumour cells enjoyed a survival advantage. A further study demonstrated that increased GSTP1 expression in biopsy specimens was associated with higher relapse rate and inferior clinical outcome (117). A role in chemoresistance has also been demonstrated, with an inverse correlation between GSTP1 transcript levels and doxorubicin growth inhibition in osteosarcoma tumour xenografts (374). Similarly, in osteosarcoma cell lines, cisplatin resistance was associated with both increased intracellular levels and activity of GSTP1 (117).

Considering the above, how may the observed association of poor response with reduced-function variants of GSTP1 c.313A>G p.Ile^{105}Val be interpreted? As already discussed, it is probable that the functional effect of a polymorphism is tumour, drug and regimen dependent. MAP chemotherapy is a complicated regimen with co-administration of cisplatin and doxorubicin; the effect of a SNP within one pathway may modify its impact in another. Certainly, evidence now suggests differential metabolism of cytotoxics by the allelic proteins of GSTP1 (114, 229). This is also likely to be the case for other enzymes, rendering comparison of non-identical drug regimens extremely difficult. Furthermore, it is possible the influence of a SNP alters depending on whether the drug is directly detoxified or indirectly deactivated through the MAPK pathway. Finally, it must also be considered whether c.313A>G p.Ile^{105}Val enhances toxicity, impacting on chemotherapy DI in a similar manner to ABCC2. In support of this, increased myelosuppression was seen in variants of c.313A>G p.Ile^{105}Val, discussed in more detail below.

Replication of preliminary pharmacogenomic findings is straightforward in common malignancies as large numbers of patients receive similar chemotherapy. However, in rare tumours treated with complex chemotherapy such as osteosarcoma, validation of preliminary findings is more difficult and requires international collaboration.

9.5 Genotypic associations with progression-free survival (PFS)

Progression free survival was associated with polymorphisms in two GST enzymes (GSTP1 and GSTT1) and a folate pathway membrane transport protein (RFC). A recently reported association between ERCC2 c.2251A>C p.Lys^{751}Gln and poor survival in osteosarcoma was not replicated (19).

9.5.1 Glutathione-S-transferase polymorphisms

Homozygous deletion of GSTT1 was strongly associated with poor PFS with a trend retained in multivariate analysis. The GSTT1 gene is polymorphically deleted with carriers of the null allele showing absent enzyme activity (183). This is the first report of an association with inferior clinical outcome in osteosarcoma although gene deletion has been repeatedly associated with increased toxicity, relapse and shorter survival in AML (21, 239-241). The largest of these studies included 306 children enrolled on Children's Cancer Group clinical trials, the remainder all recruited over 100 patients each but with a higher median age. All patients received a comparable anthracycline/cytarabine regimen with or without additional etoposide. The GSTT1 null allele was also associated with shorter survival in a recently published Phase III trial of platinum/fluorouracil in metastatic gastric cancer but not in two studies of oxaliplatin-treated colorectal cancer, both with over 100 patients (17, 208, 375). In contrast, Salinas-Souza et al reported improved overall survival with the GSTT1 null allele in patients with osteosarcoma, albeit in a selected subgroup of those with metastatic disease (127). The null genotype was also associated with improved survival in breast cancer patients treated with primary radiotherapy or cyclophosphamide/doxorubicin/fluorouracil chemotherapy (184). In Korean patients with ovarian cancer treated with taxanes and platinums, GSTT1 non-null genotype was a prognostic factor for poor progression-free survival (185). Similarly, a small case-control study of childhood ALL demonstrated a 3-fold reduction in risk of relapse with the null genotype (178).

In AML, the negative effect on survival was mainly attributed to increased toxicity, with increased death rate in induction noted in more than one study (240, 376). There is no evidence from the present study that a negative influence on survival is due to enhanced toxicity. As with GSTP1, it is likely that the effect of the null allele of GSTT1 is disease and drug dependent, but currently little is known of the detoxification role of GSTT1 on individual chemotherapeutic drugs. Corroboration in a larger population of osteosarcoma patients is required, along with pharmacological and tumoural gene expression studies.

Variant carriers of GSTP1 c.313A>G p.Ile^{105}Val demonstrated poor PFS in univariate, although not multivariate analysis. This genotype also demonstrated a significant association with poor response that as previously discussed, is a recognised predictor of poor survival in osteosarcoma. Although no correlation between poor response and survival was seen in the current study, a possible association of GSTP1 c.313A>G p.Ile^{105}Val with both is of interest. The possibility of chance observations within small

patient numbers must certainly be considered and prospective validation is required. Evidence from other malignancies is contradictory, suggesting the effect of GSTP1 c.313A>G p.Ile^{105}Val varies with disease and therapy. As discussed above, wild-type homozygosity is associated with poor survival in gastrointestinal cancers (22, 230). In 700 Australian ovarian cancer patients, carriers of the variant allele showed a significant survival advantage. There was also evidence of an additive effect with a stronger survival advantage in patients carrying three low-function GST genotypes (GSTT1 null, GSTM1 null, GSTP1 GA/GG) (400). Furthermore, the variant allele was associated with decreased relapse in ALL and a 60% reduction in mortality risk for variant homozygotes in over 1000 patients with invasive breast cancer (178, 231). However, further large studies in ovarian and colorectal cancer reported no positive associations (22, 143). As has been emphasised previously, studies vary not only in sample size and tumour type but also source of DNA and patient ethnicity, making direct comparison impossible. In particular, the differential drug metabolising activities of GSTP1 through direct glutathione-conjugation or negative regulation of the JNK-MAPK pathway may provide additional explanation for inconsistent observations.

In the current study GSTP1 c.313A>G p.Ile^{105}Val heterozygotes were also found to have an increased risk of cardiotoxicity. One may speculate that cardiotoxicity adversely influences subsequent chemotherapy dose intensity providing a potential mechanism whereby GSTP1 c.313A>G p.Ile^{105}Val may adversely affect survival. Certainly, inclusion of cardiotoxcity as a covariate in the multivariate survival analysis model enhanced the association of GSTP1 c.313A>G p.Ile^{105}Val with poor survival. Any interpretation of this other than an intriguing observation is not possible, as detailed dose-intensity data was not recorded within this study.

9.5.2 Reduced folate carrier (RFC)

A trend towards increased relapse was observed with the variant allele of RFC c.80G>A p.Arg^{27}His in multivariate analysis. This SNP has been studied in ALL with conflicting findings. In concordance with the trend observed here, in a cohort of 204 French-Canadian children treated for ALL on Dana-Farber Cancer Institute protocols, variant allele carriers showed significantly reduced event-free survival (135). Methotrexate was administered at high-dose during induction and low-dose as maintenance therapy; genotyping was performed on DNA obtained from buccal smears, peripheral blood or remission bone marrow. In contrast, no association with outcome was seen when normal blood DNA was genotyped from 246 children with ALL receiving similar chemotherapy on the St Jude’s Hospital Total XIIIB study (378). More recently, genomic DNA from diagnostic samples of 170 children with ALL treated within

the Australia and New Zealand Children's Cancer Study Group VI trial was genotyped for RFC c.80G>A p.Arg^{27}His (379). Patients who carried the variant allele were 50% less likely to relapse or die during follow-up. In all three studies, allele frequencies were similar although ethnic origin appeared more diverse in the St Jude's cohort. In rheumatoid arthritis, patients homozygous for the variant allele showed lower disease activity (380). It must be considered whether inconsistent results may reflect the diverse pharmacolological impact of different methotrexate dosing schedules.

The variant allele of RFC c.80G>A p.Arg^{27}His causes an amino acid change in the first transmembrane domain of the protein with the expectation of altered transport properties (136). Functional studies are conflicting with reduced function in variant homozygotes suggested by higher plasma methotrexate levels in ALL (135). Additional *in vitro* transport assays showed no difference in drug uptake rates and no effect was seen on *in vivo* folate status and homocysteine levels (136, 137, 381).

The RFC protein is widely expressed in mamalian tissues and is the primary intracellular transporter of folates and antifolates. Shortly after the clinical introduction of methotrexate, impaired transport was recognized as an important method of resistance although it is now clear this is one of multiple mechanisms (382). As plasma methotrexate levels decline, the provision of sufficient drug for continued folate pathway enzyme inhibition and polyglutamate formation is reliant on efficient RFC function (383).

Decreased expression and function of RFC appear to be particularly important modes of resistance in ALL and osteosarcoma, diseases where methotrexate is pivotal in treatment (383). Thus a reduced-function variant of RFC, c.80G>A p.Arg^{27}His, exerting a negative effect on survival in osteosarcoma is entirely plausible. In ALL, a significant association was seen between treatment failure and low RFC transcripts in lymphoblasts at diagnosis (384). Decreased RFC expression is frequently found in osteosarcoma tumour samples, possibly related to promoter methylation (90, 385). Moreover, it is associated with poor histological response and *in vitro* methotrexate resistance (91, 126, 386). Suboptimal RFC expression in osteosarcoma supports the argument for use of high-dose methotrexate as at high concentrations the drug is able to diffuse across the cell membrane, encouraging eradication of tumour cells resistant to methotrexate by virtue of RFC dysfunction (387). Recent evidence suggests that the RFC may also be an important mediator of resistance to other chemotherapeutic drugs. In human tumour cell lines, RFC expression significantly correlates with sensitivity to a range of cytotoxics including taxanes, thiopurines, nitrosoureas and platinums, the latter particularly relevant to osteosarcoma (345).

In the context of defective RFC function, other folate transport mechanisms may assume relatively greater importance. The folate receptor (FOLR) is a glycosylphosphatidylinositol-anchored protein with four isoforms that transport folates and antifolates into the cytosol (388). The alpha isoform participates in physiological folate uptake when insufficient concentration gradients exist for RFC transport and *in vitro,* compensates for defective RFC function (388, 389). Upregulation in the face of defective RFC may be advantageous for malignant cell growth by ensuring a continued folate source even at nanomolar concentrations (390). Overexpression of FOLR-alpha is found in a number of malignancies including osteosarcoma (389, 391). Although this may facilitate intracellular methotrexate transport by endocytosis, the concurrent increased intracellular folate pool counteracts a beneficial effect by competitive inhibition of folylpolyglutamyl synthetase and interruption of polyglutamylation. Resistance to methotrexate mediated by FOLR-alpha overexpression has recently been demonstrated in melanoma cells (390). FOLR alpha-induced endocytic transport of methotrexate, together with melanosomal sequestration and cellular exportation prevents accumulation of the drug in the cytosol. This process impedes receptor recycling to the cell membrane, making the melanoma cell impermeable to further drug and other folates. Intracellular folate depletion causes the cells to enter a latent state, avoiding apoptosis and rendering them even more resistant to antifolates. Of potential critical importance in MAP chemotherapy, altered endocytic recycling of FOLR is one means by which cisplatin-resistant cell lines exhibit cross-resistance to methotrexate (392, 393).

There is increasing evidence that reduced expression of the RFC mediates chemoresistance in osteosarcoma, not just to antifolates. Clarifying the role of c.80G>A p.Arg^{27}His in drug resistance will be aided by investigation of sequence alterations in paired tumour and germline DNA together with definitive functional studies. Importantly, as RFC c.80G>A p.Arg^{27}His occurs in osteosarcoma tumour samples at a frequency comparable to germline DNA, genotyping can be readily and reliably performed on whole blood DNA (92). The possible association of this polymorphism with inferior outcome suggested by this study deserves further investigation. If validated, it may offer a means by which patients at risk of poor outcome may be identified at diagnosis and perhaps considered for alternative therapies.

9.6 Genotypic associations with chemotherapy toxicity

Chemotherapy for osteosarcoma carries a significant burden of both early and long-term side effects. The early identification of patients at risk of severe toxicity may facilitate improved monitoring and early intervention to minimise morbidity. The

identification of those at risk of chemoresistance is of equal importance, as preliminary evidence suggests absence of chemotherapy toxicity may predict poor outcome in osteosarcoma. A retrospective analysis of 533 patients treated within 3 consecutive European Osteosarcoma Intergroup clinical trials demonstrated improved overall survival in patients with grade 3-4 oral mucositis, grade 1-2 nausea and vomiting or grade 1-2 thrombocytopenia (394).

Within the present study, comprehensive toxicity data collection has enabled the investigation of multiple MAP pathway polymorphisms for their influence on clinically important chemotherapy toxicities. Previous studies investigating the pharmacogenomic influences of chemotherapy for osteosarcoma have not undertaken such detailed analysis. In concordance with published literature, methotrexate toxicity was influenced by polymorphisms in folate pathway enzymes but additionally in ABC transporter proteins. Furthermore, as previously reported, cisplatin and doxorubicin toxicity was influenced by polymorphisms in DNA repair pathway enzymes.

9.6.1 Methotrexate cycles

Variant carriers of the folate pathway enzyme MTHFD1 c.1958G>A p.Arg^{653}Gln experienced greater haematological toxicity. This reduced-function variant may act synergistically with methotrexate through additional depletion of intracellular folates. This mechanism may also explain the beneficial effect on histological response observed for this SNP. However, no positive association with methotrexate toxicity has previously been reported in either a disease modifying or chemotherapeutic role (191, 395, 396).

Variant carriers of MTHFR c.1298A>C p.Glu^{429}Ala were also at a greater risk of myelosuppression, notably severe anaemia and thrombocytopenia. The substitution of glutamatic acid by alanine by the A>C base change results in reduced MTHFR enzyme function (82). As DNA replication and thus cell turnover is dependent upon MTHFR activity, a reduced function variant may be expected to enhance the toxicity of methotrexate. This is the first study to report an association with increased methotrexate toxicity in osteosarcoma. Patino-Garcia et al investigated the role of both MTHFR c.1298A>C p.Glu^{429}Ala and c.677C>T p.Ala^{222}Val in toxicity of high-dose methotrexate in osteosarcoma, finding increased myelosuppression only with the latter SNP (126). Although patients were treated with MAP chemotherapy, it is not clear if toxicity was analysed according to drug or following each complete multi-drug cycle. However, in 68 adult patients treated for NHL with combination chemotherapy containing high-dose methotrexate, the risk of severe mucositis increased 9-fold in those with c.1298CC genotypes (133). This genotype was also associated with

significantly more adverse events in 205 RA patients treated with low-dose methotrexate (198). Conversely, in ALL Huang et al reported decreased haematological toxicity in association with variant genotypes of c.1298A>C p.Glu^{429}Ala but genotyped DNA from leukaemic blasts (395). Several further large studies in ALL have reported no association (12, 129, 191). Importantly, it must be emphasised that many studies have investigated methotrexate toxicity at doses rarely exceeding 5g/m^2 whereas the usual treatment dose in osteosarcoma is 12g/m^2. It is unclear to what extent this alters intracellular pharmacology as high doses increase passive and active cellular uptake, favouring formation of polyglutamates. This reduces drug efflux and facilitates prolonged inhibition of target enzymes, potentially modifying the functional effects of some of SNPs.

Methotrexate toxicity was also influenced by variants of three ABC efflux transporters; increased by ABCB1 c.3435T>C Ile^{145}Ile and ABCC2 c.3563T>A p.Val^{1188}Glu but decreased by ABCG2 c.421C>A p.Gln^{141}Lys. Few studies have investigated the role of ABC transporter SNPs in methotrexate toxicity and functional studies are inconsistent. Severe leucopenia was observed more frequently in carriers of ABCC2 c.3563T>A p.Val^{1188}Glu. Evidence of a direct association of this SNP with methotrexate toxicity is not apparent from the literature although other ABCC2 polymorphisms have been linked to raised plasma methotrexate levels in ALL (152). A single case report of a patient treated with high-dose methotrexate for large B-cell lymphoma describes how impaired elimination and nephrotoxicity was due to a loss-of-function ABCC2 mutation (397). Of potential significance is the finding that ABCC2 polymorphisms show a high degree of linkage; c.3563T>A p.Val^{1188}Glu is in 100% linkage with 4544G>A Cis^{1515}Tyr, in turn closely linked to other functional polymorphisms (152, 155).

A protective effect against recurrent toxicity was seen in variants of ABCG2 c.421C>A p.Gln^{141}Lys. However, a complete absence of variant homozygotes in this cohort makes any meaningful interpretation impossible. Data on clinical effects of this polymorphism are scant, although a large study in ovarian cancer reported no influence on toxicity (143). The functional effects of the SNP include reduced plasma membrane protein expression, drug efflux and increased sensitivity to a number of anticancer agents (16, 147, 148, 214). As ABCG2 is the only folate transporter capable of effluxing polyglutamates, altered function has particular significance for tumours treated with high-dose methotrexate especially as it is already established that overexpression confers resistance (219, 221).

Understanding the complex interactions of ABC transporters may be key to defining their role in methotrexate activity. ABCG2 and ABCC2 are both located in the apical

membrane of hepatocytes and epithelial cells of the small intestine and kidney. They have overlapping substrate specificities and together are major determinants of the pharmacokinetics of methotrexate and its toxic hydroxy-metabolite (7OH-MTX) (398). In ABCG2 and ABCC2-negative knockout mice, the plasma AUC for methotrexate was 1.6 fold and 2-fold higher respectively, but in combination was 3.3 times higher than wild-type mice. Furthermore, a dramatic reduction in biliary excretion of methotrexate was seen, leading the authors to conclude that patients with mutations or polymorphisms of ABCG2 and/or ABCC2 altering transport capacity could be at greater risk of toxicity. A role in methotrexate efflux has also been demonstrated for other members of the ABC family, namely ABCC3 and ABCC4 (399, 400).

ABCB1 transports a large number of hydrophobic substrates although not classically methotrexate. Although a synonymous SNP, the T allele of ABCB1 c.3435T>C Ile^{145}Ile is associated with decreased mRNA and protein expression (16, 144). The current study recorded significantly more leucopenia and mucositis in variant carriers. Not previously implicated in toxicity of methotrexate-containing regimens, increased mucositis has been reported following paclitaxel-based chemotherapy (401). Another ABCB1 polymorphism associated with severe haematological and gastrointestinal toxicities in ovarian cancer (c.2766G>T/A), cosegregates with c.3435T>C, thus attributing a particular toxicity to a single SNP is not possible (185, 402).

Data from this and other studies implicates ABC transport proteins particularly ABCC2, in methotrexate efficacy and toxicity. More complete understanding is required of the interactions between uptake and efflux transporters at varying doses of methotrexate.

9.6.2 Doxorubicin/Cisplatin cycles

As may be expected from their mode of action, toxicity of cisplatin and doxorubicin was influenced by several SNPs in DNA repair enzymes. Exposure of cells to cisplatin or doxorubicin results in the formation of intrastrand DNA adducts, ultimately responsible for cell death. NER enzymes remove bulky, DNA-distorting lesions with increased expression of several NER genes correlating with cisplatin resistance (111).

Carriers of the TT genotype of ERCC1 c.354T>C p. Asn^{118}Asn experienced significantly less mucositis. Enhanced repair of chemotherapy-induced damage in the presence of the wild-type allele may be expected, secondary to increased protein expression (165). A recent study of NER polymorphisms in osteosarcoma found no association with the TT genotype and cisplatin-induced hearing loss but did not investigate other chemotherapy-induced toxicities (19). Although increased protein

expression by the T allele renders a protective effect biologically plausible, this association must be viewed with some caution. Two large studies collectively enrolled over 1000 patients with colorectal or ovarian cancer and reported no association with gastrointestinal or haematological toxicity (17, 143). Similarly, no association with toxicity was reported in NSCLC, gastric and ovarian cancer (22, 163, 185, 245). In all studies chemotherapy was platinum-based but additional drugs included taxanes, fluorouracil and gemcitabine further demonstrating the problem of directly comparing complex drug regimens.

A second functional ERCC1 polymorphism, c.1510C>A p.Gly^{504}Lys, predisposed to severe leucopenia and infection following doxorubicin. This polymorphism affects mRNA stability and is associated with gastrointestinal toxicity in cisplatin-treated lung cancer and nephrotoxicity in ovarian cancer (163, 403). This SNP is closely linked to ERCC1 c.354T>C p. Asn^{118}Asn, the highest level of DNA adducts seen in the AC haplotype (158). A further polymorphism, IVS5+34C>A is associated with lung and metabolic toxicities in acute myeloid leukaemia treated with a cytarabine/anthracycline regimen (20). Greater investigation of linkage disequilibrium within the ERCC1 gene is required before attributing any effect to a particular SNP.

The XPC SNP, c.2886A>C p.Lys^{939}Gln was protective against severe infection. A predisposition to ototoxicity in variant homozygotes was recently demonstrated in osteosarcoma, a finding the current study could not confirm due to missing audiogram data (19). Functional studies are again conflicting, variant carriers showing significantly higher DNA damage induced by gamma radiation and alkaline comet assay whereas both alleles showed similar repair of ultra violet damage (175, 404). Discordant findings may reflect alternative methodologies, emphasising the need for common functional assays. This SNP has not been extensively investigated in chemotherapy toxicity, but is associated with increased lung cancer risk (405).

CYBA c.242C>T p.His^{72}Tyr was also protective against severe mucositis and haematological toxicity. CYBA is a member of the NAD(P)H oxidase multienzyme complex and is a major source of reactive oxygen species. The functional consequences of this SNP are unclear although the variant allele is implicated in both acute and chronic cardiotoxicity (155).

9.6.3 Doxorubicin cycles

Variant genotypes of two GST enzymes, GSTP1 c.313A>G p.Ile^{105}Val and GSTM1 null were associated with myelosuppression and treatment delay

The GSTM1 null allele abrogates enzyme activity, with homozygosity for the deletion decreasing capacity to detoxify possible carcinogens (406). Thus it may be suggested that decreased detoxification results in increased chemotherapy toxicity. However, previous studies investigating GSTM1 null and toxicity in AML, gastrointestinal and ovarian cancer have failed to show an association.(22, 17, 185, 208, 233). The association between GSTM1 null and delay in treatment was only observed when single-agent doxorubicin was administered in cycles 5 and 6. It is likely to be a chance finding in a small population. Of note however, when comparing to the studies cited above, the only patients group to receive anthracyclines were those with AML.

An association with severe leucopenia was seen in variant carriers of GSTP1 c.313A>G p.Ile^{105}Val, the same genotype implicated in poor histological response and poor PFS. In contrast, the c.313AA genotype was a risk factor for severe haematological toxicity in Korean women with ovarian cancer and in gastric cancer, both treated with platinum based chemotherapy (185, 208). In cisplatin-treated testicular cancer survivors, the risk of an inferior audiometric result was increased 4-fold in carriers of the c.313A allele (234). Associations with oxaliplatin neurotoxicity have been reported but with opposing genotypes; in 156 patients with gastric cancer, c.313AA was significantly associated with neurotoxicity but in colorectal cancer, c.313G carriers were more prone to neurotoxicity (17, 208). Chemotherapy regimens in these studies are all platinum-based without additional anthracyclines. This is of interest as within the current study, the association of GSTP1 c.313A>G p.Ile^{105}Val with leucopenia was only observed following doxorubicin administration alone. With small numbers there must be some doubt that this is a genuine association particularly as GST enzymes do not directly detoxify doxorubicin. However, a further association of this SNP with cardiotoxicity, the most important dose-limiting side-effect of doxorubicin, suggests these may be real observations.

9.6.4 Cardiotoxicity

The risk of early and EoT cardiotoxicity was increased 9-fold and 5-fold respectively in variant carriers of GSTP1 c.313A>G p.Ile^{105}Val. This is a novel association and requires prospective study in a larger patient cohort. Nonetheless, if validated it has potentially significant implications for patient management.

The GST enzymes play a fundamental role in protecting cardiac cells against oxidative stress, not only by direct glutathione conjugation and subsequent cellular efflux but also through glutathione peroxidase activity (407). When cardiac cells are exposed to doxorubicin, a dose-dependent decrease in glutathione peroxidase activity is seen (51

408). Treatment with probucol, a lipid-lowering agent with antioxidant properties, completely protects against doxorubicin-induced cardiomyopathy by ameliorating this effect (409).

In a cardiac cell line treated with doxorubicin, GST overexpression reduced both ROS and cell death (100). Furthermore, treatment of cardiac myocytes with *Schisandra fructus* (SFE), a traditional Chinese herbal medicine with potent antioxidant properties, reduced doxorubicin-induced cytotoxicity, intracellular ROS and lipid peroxidation together with a concurrent increase in cellular GST activity (410). Moreover, microarray data showed that SFE treatment modulates the gene expression of phase II enzymes such as GST suggesting resistance of cardiomyocytes to doxorubicin injury is mediated thorough induction of cellular detoxifying mechanisms. Similarly, pre-treatment of cardiomyocytes with Schisandrin B, a dual inhibitor of p-glycoprotein and multi-drug resistance protein significantly attenuated doxorubicin-induced cardiotoxicities by enhancing key enzymes in cardiac glutathione redox cycling, including GST (411).

GSTP is the predominant isoform expressed in cardiac cells (412). The inferior function of GSTP1 c.313A>G p.Ile^{105}Val may therefore further compromise the already reduced oxidative stress capacity of doxorubicin-exposed cardiac myocytes, facilitating cardiac damage. As high-dose doxorubicin is a fundamental component of osteosarcoma therapy and a proven cardioprotectant is now available, pre-treatment identification of individual cardiotoxicity risk is attractive and offers the potential to reduce the significant burden of late cardiac damage.

Cardiac toxicity did not directly influence PFS in survival analysis but as a covariate, increased the association of GSTP1 c.313A>G p.Ile^{105}Val with poor survival. One possible mechanism is through cardiac toxicity adversely affecting chemotherapy dose intensity. This requires more detailed evaluation of dose intensity than recorded in this study, although using treatment delay as a crude proxy shows no association with cardiac toxicity.

The present study did not confirm previously reported SNP associations with cardiotoxicity including CBR3 c.730G>A p.Val^{244}Met, NADPH NCF4 c. 212G>A, CYBA 22phox c. 242C>T p.His^{72}Tyr and ABCC2 c.3563T>A p.Val^{1188}Glu - c.4544G>A p.Cis^{1515}Tyr haplotype (155, 188). This may reflect low power but direct comparison of studies is also problematic due to differing doxorubicin exposure, administration schedules, assessment of cardiac toxicity and statistical analysis. Median doxorubicin exposure was 444 mg/m^2 in the current study and doxorubicin exposure was included as a covariate in multivariate analysis. The Childhood Cancer Survivor nested case-

control study included haematological and solid malignancies with anthracycline exposure ranging from <100 to >500 mg/m^2 (188). Cardiac toxicity was self-reported congestive heart failure rather than objectively measured and no adjustment for anthracycline exposure was made in multivariate analysis. A large study demonstrated an association with the following SNPs and cardiac impairment; NADPH NCF4 c. 212G>A, CYBA 22phox c. 242C>T p.His^{72}Tyr and ABCC2 c.3563T>A p.Val^{1188}Glu - c.4544G>A p.Cis^{1515}Tyr haplotype. Impairment was measured by ejection fraction and both anthracycline dose and scheduling were adjusted for in the analysis (155). A further study found an association between wild-type CBR3 c.11G>A p.Cys^{4}Tyr and increased levels of doxorubicinol, the less cytotoxic but more cardiotoxic metabolite of doxorubicin (99). Less leucocyte suppression and decreased tumour response was in keeping with enhanced conversion of doxorubicin but this study did not measure the clinical effect of increased doxorubicinol levels on cardiac function.

9.6.5 Nephrotoxicity

A trend towards early nephrotoxicity was observed in variant carriers of MTHFR c.677C>T p.Ala^{222}Val and although not significant, a greater mean fall in GFR through treatment was also observed. Reduced enzyme activity is associated with the variant allele, hetero- and homozygotes having 60% and 30% of wild-type activity respectively (128). Efficient DNA synthesis and repair depend on adequate MTHFR function, thus reduced-function variants may enhance the intracellular effects of methotrexate. In mice treated with methotrexate, MTHFR-deficient animals were more likely to suffer from nephrotoxicity than their MTHFR-overexpressing counterparts (413). Acute renal failure following high-dose methotrexate has previously been reported in heterozygotes and variant homozygotes of MTHFR c.677C>T p.Ala^{222}Val (414, 415).

Methotrexate is primarily excreted by the kidney with acute nephrotoxicity mediated by pH-dependent drug precipitation in the renal tubules together with direct toxic effects (61). Acute renal damage leads to delayed excretion, a rapid rise in plasma levels and intensification of other methotrexate toxicities such as mucositis and myelosuppression. Aside from these potentially life-threatening side effects, ensuing renal dysfunction has further implications for continued chemotherapy administration, particularly important when other nephrotoxic drugs are co-administered. The pathogenesis of methotrexate nephrotoxicity may echo its mechanism of small intestinal injury, wherein drug administration enhances oxidative stress, demonstrated by increased lipid peroxidation and reduced glutathione levels together with neutrophil infiltration in rat models (416). Recent evidence suggests renal damage is indeed

mediated through a similar mechanism, with significantly elevated neutrophil activity and markers of oxidative damage in the kidneys of methotrexate-treated rats (417).

Patients with diabetes may also provide insight into the mechanism of renal toxicity associated with MTHFR c.677C>T p.Ala^{222}Val. The variant allele is associated with diabetic nephropathy and faster progression to end stage renal failure but also correlates with hyperhomocysteinaemia, a well-defined risk factor for endothelial dysfunction (418, 419). Homocysteine is metabolised by MTHFR by remethylation to methionine, thus decreased enzyme function may increase plasma homocysteine levels. This may be directly toxic to glomerular cells, mediated by oxidative and endoplasmic reticulum stress, homocysteinylation and hypomethylation resulting in glomerular injury and ultimately sclerosis (420). Patients with variant genotypes of MTHFR c.677C>T p.Ala^{222}Val who are treated with methotrexate may not only be susceptible to enhanced drug toxicity but also additional renal damage due to hyperhomocysteinaemia.

A 4-fold increased risk of early nephrotoxicity was seen in variants of ERCC2 c.2251A>C p.Lys^{751}Gln. A fall in GFR of 22ml/min/1.73m^2 is of arguable clinical significance although the impact of this on early chemotherapy dose intensity is potentially important. Functional studies conflict and no previous reports associate this SNP with chemotherapy toxicity (17, 166, 167). Cisplatin nephrotoxicity is mediated through several different mechanisms including ischaemia due to renal vasculature damage, a robust TNF-α inflammatory response and activation of apoptotic cellular signalling pathways (55). NER enzymes are important in the removal of non-bulky oxidative DNA damage caused by cell death pathway-generated ROS, thus renal tubules may be exposed to greater oxidative stress in variants of ERCC2 c.2251A>C p.Lys^{751}Gln (111, 421). However, this suggested mechanism must be regarded with caution as ERCC2 c.2251A>C p.Lys^{751}Gln is strongly linked to other SNPs previously associated with toxicity in platinum-treated NSCLC and daunorubicin/cytarabine-treated AML (20, 422).

9.7 Chemotherapy toxicity and progression-free survival

Data from the present study found patients with recurrent severe methotrexate toxicity showed significantly improved PFS in multivariate analysis. Pooled data from 3 previous EOI clinical trials found a significantly reduced risk of death in patients with mild thrombocytopenia and severe mucositis (394). Evidence of a relationship between chemotherapy toxicity and disease outcome is also emerging in other malignancies. In metastatic colorectal cancer treated with cetuximab, response rates were higher in

patients with skin reactions and in early breast cancer treated with adjuvant chemotherapy, a survival advantage of 10% was seen in those with moderate neutropenia (423, 424). Similarly, a pooled analysis of three trials in NSCLC demonstrated a reduced risk of death in patients with neutropenia during chemotherapy (425).

In NSCLC, absence of neutropenia was due to chemotherapy underdosing therein exposing the complexity of using toxicity as a predictor of survival (425). Individual pharmacogenomic profiling may allow cytotoxic dosing to be optimised in order to balance manageable toxicity with maximal dose intensity.

9.8 Limitations of the candidate polymorphism study

This study represents the most comprehensive to date investigating the pharmacogenomics of disease outcome and chemotherapy toxicity in osteosarcoma. Three previous studies investigated genetic polymorphisms in osteosarcoma but have focussed only on single pathways only (19, 126, 127).

Findings must be interpreted in light of the study limitations, primarily small sample size and retrospective recruitment. Osteosarcoma is a very rare malignancy treated in only a handful of centres in the UK making recruitment of large patient numbers challenging without multi-centre collaboration. Indeed the London Sarcoma Service is one of the main UK centres for the management of bone tumours and has a large patient population. This study was always proposed as “proof of principle” with the intention that positive findings would require replication in a larger cohort. A larger number of patients would certainly have facilitated reliable haplotype analysis that may be particularly informative in the context of multiple cellular pathways such as in MAP chemotherapy.

Retrospective recruitment of mainly outpatients is a potential source of bias. The natural history of osteosarcoma is such that poor responders are at increased risk of relapse and death, resulting in over-representation of good responders in outpatient follow-up clinics. However, two-thirds of this patient cohort were good responders, a figure comparable to other published studies in osteosarcoma (38). Furthermore, most patients who develop recurrent disease will do so 2-3 years after completion of treatment (426); almost 50% of this patient cohort had completed treatment within the previous 2 years, the inference being even relapsed poor responders would not yet have succumbed to their disease and still be attending follow-up clinics. The only means to overcome these problems is through prospective recruitment of large numbers of patients. EURAMOS-1, the first international phase III clinical trial in

osteosarcoma has demonstrated large scale collaboration is achievable to address important clinical questions (5). It is hoped that results from this pilot study may demonstrate the need for prospective pharmacogenomic profiling in subsequent clinical trials.

Notwithstanding the limitations discussed, this study has a number of strengths. Careful selection of candidate polymorphisms based on pharmacological pathways, functional data and literature citations implicating a role in other malignancies optimised the value of the dataset obtained. The recording and analysis of chemotherapy toxicity according to drug is a major advantage; many studies combine toxicities of multi-drug regimens despite actions on several different pharmacological pathways. In addition, as MAP is a prolonged and intense chemotherapy regimen with strict protocol-driven toxicity monitoring, extensive data were recorded for each patient giving a comprehensive overview of individual toxicity. Information included common side-effects following each chemotherapy cycle but also cardiac and renal function at three discrete time points. Finally, predictors of survival identified by univariate analysis were used as covariates in multivariate analysis of survival.

Germline rather than tumoural DNA was selected for genotyping, in common with many other studies. An obvious significant advantage is the ready availability of high quality germline DNA opposed to tumour DNA potentially degraded by fixative measures. However, the applicability of positive germline SNP associations to tumour should be questioned. Reassuringly, several studies have demonstrated the germline DNA genotype is highly conserved in DNA from both fresh/frozen and paraffin embedded tumour tissue. Fresh frozen colorectal tumour specimens and paired normal mucosa were genotyped for 28 polymorphisms with 93% samples showing one or fewer discordant genotypes and 77% in complete concordance (427). Similarly, comparison of 21 SNPs and 2 deletions in paired pre-treatment bone marrow and buccal DNA in acute myeloid leukaemia demonstrated complete concordance in 90% samples, increasing to 97% discordant for only 1 SNP (428). Both studies selected polymorphisms highly relevant to cancer pharmacogenetics including drug metabolism and transport, oxidative stress mechanisms and DNA repair. Investigation of paraffin-embedded (PE) tissue specimens has produced comparable results. Genotyping for 6 polymorphisms in DNA extracted from 8 PE and paired viable tumour cell lines showed 100% concordance as did 10 PE breast cancer tissue and paired whole blood samples (429). Another study in breast cancer found 100% concordance for 3 polymorphisms genotyped in DNA extracted from PE primary tumour, involved and uninvolved lymph nodes, again implying germline imprints the tumour genotype (430). The clinical relevance of germline DNA polymorphisms was shown in patients with advanced

colorectal cancer where the TYMS VNTR genotype in PE normal colonic tissue predicted the sensitivity of the tumour to 5-fluorouracil (431). A recent comprehensive review of available literature concluded there was almost complete concordance between germline and somatic DNA in variants of pharmacogenetic genes even in cancer (432).

10.0 Discussion (ii): Genome-wide analysis of CNV

10.1 CNV analysis

From the available literature, this appears to be the first study describing genome-wide CNV in germline DNA from patients with osteosarcoma, although several studies describe CNV in tumour cells (see Section 4.1). As a preliminary analysis of a small patient cohort with no validation set, the findings are purely descriptive and anything other than speculative comment is precluded. Robust analysis requires a much larger patient cohort and paired germline/tumour samples.

Ninety-six single CNV were observed in 35 patients but none encompassed genes recorded in the Cancer Gene Census (www.**sanger**.ac.uk/genetics/CGP/Census/ Accessed June 6th 2011). Further comment on single CNV's is not robust and these will not be discussed further.

Several recurrent CNV were observed but all had been reported previously and listed in the DGV (available at http://projects.tcag.ca/variation). Furthermore, when compared to a control population the frequency was not significantly different. However, brief discussion is warranted.

In 5 patients, a 98kb deletion in chromosome 4 was observed in a region containing Uridine diphosphate glucuronyltransferase (UGT) 2B17. UGT enzymes participate in the metabolism of steroid hormones, cancer chemotherapy agents, carcinogens and nicotine metabolites. UGT2B17 encodes an enzyme critical for local inactivation of androgens, such that increased expression inhibits the androgen-signalling pathway (433). Gallagher et al noted 4 fold higher UGT2B17 expression in men compared to women, potentially resulting in different levels of carcinogen detoxification (434). A potential role in androgen-dependent cancers has been sought and although deletion of UGT2B17 has been associated with increased prostate cancer risk, this is not a uniform finding (435-438). Endometrial cancer (EC) is also a steroid-hormone dependent cancer and in EC cells, mRNA expression of UGT2B17 is significantly increased compared with matched normal endometrium. Furthermore, depletion of UGT2B17 induced inhibition of cell growth and apoptosis, mediated by downregulation of the Myeloid cell leukaemia-1 (Mcl-1) gene (439).

Androgen is a major source for oestrogen and has a direct effect in stimulating bone formation. Genome-wide copy number analysis of Chinese individuals with osteosporosis identified UGT2B17 as a susceptibility gene for osteoporotic hip fracture although a recent study in Caucasian women found no association (440, 441). CNV in

UGT2B17 is common in Caucasians, thus a homozygous deletion in 10% of patients in this study may be expected. However, one could speculate that CNV of UGT2B17, a gene critical in androgen metabolism thereby indirectly influencing bone growth, could contribute to the pathogenesis of osteosarcoma, a disease likely to have a hormonal influence in its development implied by its incidence peak at puberty.

Analysis of this dataset was challenging in view of limited bioinformatic support. For this reason and lack of statistical robustness due to small patient numbers, only a basic preliminary analysis was undertaken as described. However, it is anticipated that more detailed analysis will be performed in collaboration with other research groups investigating genome-wide changes in human and canine osteosarcoma.

11.0 Conclusions

Disease response and how this translates to survival is of overwhelming importance to any cancer patient. In osteosarcoma, good histological response is key to long-term cure, with this patient group achieving survival rates of 70%. Of only slightly less importance to a cancer patient is the severity of potential chemotherapy side-effects, both short and long-term. Whilst identification of novel cellular targets is of critical importance for future drug development, individual pharmacogenomic profiling allows not only optimisation of currently used chemotherapeutic agents but also the prediction and management of severe toxicity, with the aim of improving disease outcomes and minimising late-effects. The present study represents the most comprehensive investigation to date examining the role of genetic polymorphisms on chemotherapy toxicity and disease outcome in osteosarcoma. Judicious selection of candidate polymorphisms yielded several novel associations with potential implications for patient management.

Considering measures of disease outcome, repeated associations of folate pathway SNPs with histological response and progression-free survival suggests methotrexate efficacy may be of considerable importance in successful treatment. The use of high-dose methotrexate in osteosarcoma continues to be controversial but direct associations between a methotrexate-specific ABC-transporter, a folate pathway enzyme and histological response may suggest a dominant role in early tumour necrosis. The weak association of a folate pathway membrane transporter SNP with progression-free survival may be strengthened with greater patient numbers but importantly, mirrors the effect seen in ALL, another disease in which methotrexate is pivotal in treatment. The association of GST polymorphisms with disease response and survival is well described although findings in this study were contrary to others, either a chance finding or an illustration that pharmacogenomic effects are disease and drug-regimen dependent.

Several SNPs showed a significant association with chemotherapy toxicity. In particular ABC transporter SNPs influenced methotrexate and NER SNPs, cisplatin and doxorubicin toxicity. The observation that methotrexate toxicity, not cisplatin or doxorubicin positively correlated with survival was of particular interest. The concept of toxicity as a predictor of survival is only just being explored in osteosarcoma but this may indicate methotrexate has a greater role in disease outcome than previously appreciated.

Finally, the SNPs associated with cardiac and nephrotoxicity are perhaps the most significant for patient care. Cardiac and renal damage not only lead to interruption of primary treatment but also contribute to significant long-term morbidity. Pre-treatment identification of those at high risk for cardiac damage may encourage more rigorous monitoring and consideration of early administration of cardioprotectants although recent evidence suggests this may be controversial (www.ema.europa.eu). The role of pharmacogenomics in predicting and managing nephrotoxicity is less clear. Although early identification of those at risk is helpful, with no proven renoprotectants currently available, prevention of damage is not possible unless treatment is modified, perhaps compromising outcomes.

Despite small patient numbers in this study, careful selection of polymorphisms and analysis by drug pathway has demonstrated several interesting polymorphic associations with both disease outcome and chemotherapy toxicity in osteosarcoma. This warrants validation in a larger study and the organising committee of EURAMOS 2 have already agreed that prospective pharmacogenomic profiling will be undertaken in patients entered into future international clinical trials.

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Finally, thanks to my family for their unending support.

Appendix 1: Research protocol

Title: A pilot pharmacogenomic study of the influence of cytotoxic metabolising gene polymorphisms on toxicity and outcome in resectable osteosarcoma.

Short title: Can inherited differences in chemotherapy metabolism change response to treatment in osteosarcoma?

Investigators: Dr Rachael Windsor
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Study sponsor: Research and Development Department
University College Hospital

PROTOCOL DRAFT 1 30.11.07

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Appendix 1 Patient Information Sheet
Appendix 2 Patient Consent form
Appendix 3 Data collection form

List of Abbreviations

TS	Thymidylate synthase
MTHFR	Methotrexate tetrahydrofolate reductase
GFR	Glomerular Filtration Rate
SF	Shortening fraction
MTX	Methotrexate
ALL	Acute lymphoblastic leukaemia
GST	Glutathione S-transferase complex
GSTT, GSTP, GSTM	Glutathione S-transferase enzymes
DNA	Deoxyribonucleic acid
SNP	Single nucleotide polymorphism
CTCAE	Common Terminology Criteria for Adverse Events
MUGA	Multiple Gated Acquisition scan
UCLH	University College Hospital
CDR	Pathology results system

Summary

Osteosarcoma is the commonest bone tumour in children and young people. However, despite known effective chemotherapy with methotrexate, cisplatin and doxorubicin, approximately 40% patients have a poor response to treatment with a consequent 5-year survival of 45-55% compared to 75-80% for good responders. A number of patients will also experience significant long-term treatment related toxicities.

Recent research has indicated the importance of inherited enzyme polymorphisms in drug response. The action of methotrexate is critically dependent on the levels of the enzymes Thymidylate synthase and Methotrexate tetrahydrofolate reductase. Similarly, the efficacy of cisplatin depends on Glutathione S-transferase levels and doxorubicin, the efficiency of the membrane transporter ABCG2. A number of functional genetic variants, including single nucleotide polymorphisms and a triplet repeat, have been identified in the above pathways. Recent studies have demonstrated an association between their presence and inferior survival in paediatric acute lymphoblastic leukaemia and some adult solid tumours.

There is no data relating to these in osteosarcoma and this study aims to investigate a possible association between enzyme polymorphisms, response to treatment and toxicity. Blood samples from 50 osteosarcoma patients who have completed treatment will be screened for polymorphisms using a genome-wide approach. The primary objective, treatment response, is measured by percentage of viable tumour tissue in the surgical resection specimen. The secondary objective of treatment related toxicity will be recorded from protocol-driven assessment of renal, cardiac and auditory function along with degree of mucositis.

The longer term goal of this study is to establish the importance of pharmacogenomics in the treatment of osteosarcoma, provide data to support a larger prospective study and ultimately, improve survival.

Background

Osteosarcoma is the commonest bone tumour in children and adolescents. Prior to the introduction of systemic therapy, 2 year survival was 15-20%. The majority of combination chemotherapy regimens now report 3 year disease-free survival rates between 60-70%. However, despite intense pre-operative chemotherapy, 40% patients have >10% viable tumour at resection (poor responders). This is reflected in a 5 year survival of 45-55% compared to 75-80% for those with <10% viable tumour (good responders) (1).

Enzyme polymorphisms

The most active agents against osteosarcoma are doxorubicin, cisplatin and very high dose methotrexate. Methotrexate blocks the action of methylene tetrahydrofolate reductase, a crucial enzyme in folate metabolism. MTHFR has two common functional polymorphisms, C677T and A1298G (2). MTHFR C677T encodes a protein with about 30% of the wild type activity whereas heterozygotes (40% of the population) have approximately 60% activity (3). MTHFR A1298G is also a lower activity variant. Childhood acute lymphoblastic leukaemia is one of the most well developed areas of cancer pharmacogenomics and may provide a model for other human cancers. The MTHFR C677T is significantly associated with relapse in paediatric ALL (2) and the A1298G variant is associated with decreased in vitro MTX sensitivity (4). Interestingly, there is also an association between C677T and decreased treatment related toxicity in ALL (5). The active polyglutamate forms of MTX inhibit other folate pathway enzymes particularly thymidylate synthase (3). A polymorphic tandem-repeat sequence has been identified in the promoter region of the TS gene, the three 28 bp repeat being associated with higher expression of TS than the two 28bp repeat (6). An association between homozygosity for the TS triple repeat (and therefore higher TS activity) and inferior event free survival in childhood acute lymphoblastic leukaemia has been demonstrated in a number of studies (7,8).

Cisplatin, a platinum, is metabolized by the glutathione detoxification system, the main components of which are glutathione, glutathione-related enzymes and the glutathione S-conjugate complex export protein (9). GSTP1 is directly involved in the detoxification of platinum compounds and has been associated with acquired drug resistance (10). A single nucleotide substitution (A to G) at position 313 of the GSTP1 gene (isoleucine replaced by valine) results in much reduced enzyme activity (11). GSTT1 and GSTM1 have the same deletion polymorphism whereby the homozygous form renders them inactive. The prognostic significance of GST polymorphisms has been extensively studied in cancers other than osteosarcoma. In metastatic colorectal cancer, the GSTP1 Ile105Val polymorphism is associated in dose-dependent fashion with increased survival of patients with advanced colorectal cancer receiving 5-Fluorouracil/oxaliplatin chemotherapy. GSTM1 and GSTT1 genotypes were not associated with survival or clinical response (11). In gastric cancer patients, the GSTP1 Val-Val polymorphism was associated with increased response rate and significantly longer median survival (12) and in ovarian cancer, is associated with risk of disease progression (13).

Doxorubicin is an anthracycline antibiotic active in all phases of the cell cycle. It has several modes of action including intercalation into the DNA double helix and enzyme and oxygen free radical-mediated DNA and cell membrane damage. ABCG2 is a member of the ATP-binding cassette membrane transporter known to be involved in the efflux of various anticancer compounds including doxorubicin. There are several known SNP's of ABCG2: cells expressing cDNA of two major variants (V12M and Q141K) have demonstrated drug resistance to doxorubicin in vitro (14). No research has focussed on a potential role in drug metabolism in osteosarcoma.

Toxicity

It is also possible that the severity of chemotherapy-related side effects may be dependent on enzyme polymorphisms. Administration of methotrexate results in a dose-dependent decrease in renal glomerular function measured by Glomerular Filtration Rate. Cisplatin administration causes an acute, mainly proximal tubular impairment which precedes alterations in renal haemodynamics. High-dose cisplatin therapy causes a severe, progressive decrease in GFR during treatment which may persist after treatment has ceased (15). It also has the additional toxicity of destroying sensory hair cells of the inner ear which can lead to permanent sensorineural hearing loss, balance disturbance or both (16); high tone hearing loss is seen in 75-100% of patients on audiometry (17). Doxorubicin, causes myocyte damage by a number of mechanisms, free radical generation probably being the most significant. Acute toxicity is manifest by arrythmias, dilating cardiomyopathy or congestive cardiac failure. Late cardiac dysfunction (4-20 yrs) ranging from subclinical dysfunction to irreversible failure has been reported in the paediatric population (18). Routine cardiac imaging studies (echocardiogram/ MUGA) can be used to identify cardiac dysfunction by measuring SF.

Assessment of treatment-related toxicity with blood counts, isotopic GFR, SF and high-tone hearing loss on audiogram is frequent during chemotherapy and is protocol-driven. It is graded according to Common Terminology Criteria for Adverse Events (19). For the purposes of this study CTCAE data will be collected retrospectively at three time points: diagnosis, pre-surgery and completion of chemotherapy.

Justification

There are approximately 120 number of new cases of osteosarcoma diagnosed each year in the United Kingdom in children and young adults. Intensive chemotherapy leads to a good response in about 50% of patients: of the remainder, two thirds will ultimately die from their disease. Pharmacogenomics is the use of a genome-wide approach to elucidate the inherited basis of inter-individual differences in drug response. Childhood acute lymphoblastic leukaemia provides an excellent model for this and has been studied extensively as have a number of solid tumours. There are however, no data regarding the impact of inherited differences in metabolising enzymes in osteosarcoma and therefore this study is timely.

The longer term goal of this study is to establish the importance of pharmacogenomics in osteosarcoma by providing data to support large prospective evaluations. Ultimately we would wish to use this knowledge to further understand and influence management of tumour resistance, improve survival and decrease toxic long term side effects.

University College Hospital encompasses the London Bone and Soft Tissue Sarcoma Unit and provides the ideal location to undertake this study. This service treats approximately 40 new osteosarcoma cases per year and has a further 150 patients on follow-up therefore providing a large patient population.

Study Objectives

Primary:

To investigate a possible association between known polymorphisms in critical target enzymes of methotrexate, the drug-detoxification enzyme complex Glutathione S-transferase and drug transporter ABCG2 and response to treatment in osteosarcoma.

The primary endpoint for response to treatment is percentage of viable tumour in the surgical resection specimen.

Secondary:

To examine any potential association between polymorphisms and treatment related mucositis, renal, cardiac and oto-toxicity assessed by Common Terminology Criteria for Adverse Events (19).

Study Design

This will be a cross-sectional pilot study.

Study Group:

Patients aged over 16 who have completed treatment for osteosarcoma and are attending for outpatient review will be eligible for this study. This group consists of approximately 150 patients treated on previous randomised European Osteosarcoma Intergroup (EOI) trials and guidelines or the current osteosarcoma treatment protocol EURAMOS-1. Patients are reviewed every 1-3 months depending on length of time off treatment. Most patients have developed an interest in research while undergoing treatment and are usually willing to participate.

Patients will be invited to participate while attending routine outpatient clinic follow up. Patient Information Sheets will be provided and written informed consent obtained by chief investigators (Dr Windsor or Dr Whelan) in clinic. A 20 ml blood sample will be taken and transferred to two EDTA tubes. If the patient does not have a central line, a peripheral venepuncture will be required. Samples will be stored at the Institute of Neurology where DNA extraction and a genome-wide search for polymorphisms will be performed.

Data Collection

The following data will be collected retrospectively from patient notes (Appendix 3):

- date of diagnosis, sex, age
- site of primary tumour, presence of metastases
- treatment protocol
- number of cycles of chemotherapy, dose modifications and reason
- response to chemotherapy (% of viable tissue in surgical resection specimen)
- toxicity assessed by GFR, SF, blood counts, degree of mucositis and audiogram at three time points: diagnosis, post 2 cycles of chemotherapy and completion of 3 drug therapy. CTCAE grade will be recorded for each variable at each time point
- site of relapse
- length of time to relapse
- treatment at relapse

Missing data will be sought from the UCLH CDR system and data managers
Data will be entered onto a secure password protected database at UCLH. Patients will be informed that their trial based data will be stored, analysed and used for scientific publications.

Statistical considerations

As a pilot study, the primary objective is to evaluate the effect of enzyme type on the response rate. A sample of 15 patients with the enzyme of interest would allow us to estimate the proportion of patients with a poor outcome with a 95% confidence interval of width +/- 25%, assuming 40% of patients actually have a poor outcome. Since the reported prevalence of the main polymorphisms is approximately 30% (20-22) we will need to collect data on 50 patients overall.

Compliance

The subject may withdraw from the study at any time by revoking their consent.

Ethical considerations

This research proposal has been submitted to UCLH REC Alpha for ethical approval.

Finance

Costs arising from this study will be met by a charitable grant from the Bone Cancer Research Trust.

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Appendix 2: Patient information sheet for adults

Chemotherapy metabolism and outcome in osteosarcoma
Patient information sheet for adults
Version 2 January 2008

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.

Thank you for taking the time to read this information sheet.

1. What is the purpose of the study?
Osteosarcoma is the commonest bone cancer in children and young people. The most important drugs used to treat it are methotrexate, cisplatin and doxorubicin. Each patient reacts differently to chemotherapy and we measure this by seeing how many cancer cells have been killed when the tumour is removed at surgery. Also, some patients have troublesome side effects from the chemotherapy, whereas others may not.

Chemotherapy works by affecting the cell machinery or special proteins called enzymes. Different types of enzymes (polymorphisms) are found in different people. These can affect the way a person responds to a particular drug. This research will look to see if there is a link between enzyme type and response to the 3 main chemotherapy drugs. We would also like to see if different enzyme types can influence side effects..

2. Why have I been chosen?
You have completed your treatment for osteosarcoma and are now regularly attending outpatient clinics at University College Hospital. Approximately 50 other patients will be taking part in this study.

3. 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 the standard of care you receive.

4. What will happen to me if I take part?
You will not be asked to make extra visits to the hospital. A 20 ml blood test (about 4 teaspoons full) will be taken today while you are in clinic. The blood will be stored at the Institute of Neurology within University College Hospital.

We would also like to collect some clinical information from your notes about the exact chemotherapy given and tests you had through treatment. These include kidney and heart function, blood counts and hearing tests.

5. What are known risks of the study or the side effects of any treatment received?
This study does not involve treatment of any sort.

6. What are the possible benefits of taking part?

As you have completed your treatment, this research offers no direct benefit to you. However, information from this study may help us to treat future patients with osteosarcoma more effectively.

7. The information held about the research subject

If you participate in this study, information about you will be collected by the study organiser and held on a secure database at University College Hospital. This information will not be shared with any other organisation. The form will contain no identifying information about you as a code number is used. All information which is collected about you during the course of the research will be kept strictly confidential. UCLH is registered under the Data Protection Act (or equivalent national laws) to hold such information on a confidential basis.

8. Studies on tissue

The blood sample you give will be a gift and may be retained for future research in specific areas. Any new research will be reviewed by a research ethics committee but consent for future studies may only be required if the committee considers that the study is likely to substantially effect the subjects interests.

9. Gene Studies

Your blood sample will be identified by a number, not your name. DNA will be extracted and used to test for a range of enzyme types shown already to be important in drug metabolism. These have no implications for you future health and you will not be informed of the results.

10. What happens when the research study stops?

You will continue your outpatient follow up as usual.

11. What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of this study, the normal National Health Service complaints mechanisms should be available to you.

12. What will happen to the results of the research study?

When the study is complete the results may be published in a medical journal. No individual patients will be identified. You can obtain a copy of the published results by contacting the study organiser (details at the end of this sheet). It is anticipated this study will take about one year to complete.

13. Who is organising and funding the research?

This research is being organised by a Clinical Research Fellow in Oncology at UCLH. It is being funded by a charitable grant provided by the Bone Cancer Research Trust.

14. Withdrawal from the project

Your participation in the research is entirely voluntary. You are free to decline to enter or to withdraw from the study any time without having to give a reason. If you choose not to enter the trial, or to withdraw once entered, this will in no way affect your future medical care. All information regarding your medical records will be treated as strictly confidential and will only be used for medical purposes. Your medical records may be inspected by competent authorities and properly authorised persons, but if any information is released this will be done in a coded form so that confidentiality is strictly maintained. Participation in this study will in no way affect your legal rights.

15. Who has reviewed the study?

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by a NHS Research Ethics Committee before it goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However approval means that the Committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits, and that you have been given sufficient information on which to make an informed decision to take part or not.

16. Contact for further information

If you require further information, please contact:
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Clinical Research Fellow
University College Hospital
Department of Oncology
First Floor Central
250 Euston Rd
London NW1 2PG

Tel no: 0207 380 9950

Thank you for taking part in this study.

This information sheet was printed onto UCL Hospitals headed paper.

Appendix 3: Consent form

Chemotherapy metabolism and outcome in Osteosarcoma

Consent Form

Principal investigator : Dr R. Windsor
Patient Identification Number for this study:
Please initial box

1. I confirm that I have read and understood the information sheet dated …….. (version ……..) 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 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 any 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 (company name) 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. ☐
6. I understand that my blood sample will be a gift. ☐

CONSENT FORM

Can inherited differences in chemotherapy metabolism change response to treatment in Osteosarcoma?

Principal investigator :

____________________	______________	____________________
Name of patient	Date	Signature
____________________	______________	____________________
Name of Person taking consent (if different from researcher)	Date	Signature
_______________________	______________	____________________
Researcher (to be contacted if there are any problems)	Date	Signature

Comments or concerns during the study

If you have any comments or concerns you may discuss these with the investigator. If you wish to go further and complain about any aspect of the way you have been approached or treated during the course of the study, you should write or get in touch with the Complaints Manager, UCL hospitals. Please quote the UCLH project number at the top this consent form.

1 form for Patient;
1 to be kept as part of the study documentation,
1 to be kept with hospital notes

This consent form was printed onto UCLH Clinical Trials Department headed paper.

Appendix 4: DNA extraction SOP

DNA extraction from blood, using the Qiagen Flexigene DNA extraction Kit.

Safety: Refer to Neurogenetics Local Rules and Risk Assessments (5, 2, 10, 8, 17, & 4):
DNA EXTRACTION - There are hazards associated with the extraction of DNA; i.e. the handling of pathological specimens - use class II cabinet, sealed tubes and personal protective measures; for –70°C FREEZERS use thermal protective gloves; ELECTROPHORESIS - do not set up, handle or dismantle with power on; CENTRIFUGATION - only use after training, use sealed tubes/plates only and stay with machine until required speed is reached.

The method below is used to extract DNA from blood samples. The method is scalable depending on the size of the sample you are starting with.

For 5mls of blood in EDTA either fresh or frozen.
If you are using frozen blood defrost it quickly at 37°C with gentle agitation and as soon as it is defrosted put it on ice. Do not defrost samples for a long time before you set up the extraction.

The kit uses Guanidine HCl in Buffer FG2; this must not be mixed with bleach or presept. The initial supernatant (blood plus FG1) can be treated with presept but the supernatant generated after precipitating the DNA (FG2 plus isopropanol) must not be added to this.

1. Have one 50ml Falcon tube for each blood sample to be extracted. On each 50ml Falcon, and on the lid, write the number of each samples position in that particular run. Also write the DNA number and surname on the tube, if more than one person has the same surname in that run, include the first name. All labeling must be done in permanent marker. The maximum number of samples you can extract in one run is 32.

2. Prepare a solution of Protease K and buffer FG2 mix consisting of 2.5mls (0.5ml per 1ml of blood) buffer FG2 and 25µl (5µl per 1ml of blood) of Protease K from the kit. Only make up as much as you need and do not prepare it more than an hour in advance.

3. Working in the Class II cabinet put 12.5mls (2.5ml per 1ml of blood) of Buffer FG1 into each Falcon.

4. Add blood, cap tubes and invert the tubes 5 times. The blood tube labeling and blood transfer must be checked and the extraction sheet signed.

5. Place the tubes in centrifuge canisters and spin in the Beckman bench top centrifuge at 2900rpm for 8 minutes.

6. Remove from the centrifuge and pour off the supernatant into 500mls presept solution (presept solution = 1L water + 1 presept tablet), making sure that you don't lose the cell pellet at the bottom of the tube. Invert the tubes onto tissue to get rid of as much liquid as possible also watching that pellets do not begin to slide out.

7. Add 2.5mls (0.5ml per 1ml of blood) of buffer FG2 and Protease K solution to the cell pellets, cap tube and vortex immediately until the pellet is dissolved and the solution is clear. Make sure there are no streaks visible in the solution. During this step only add the solution to one tube at a time and don't delay before vortexing.

8. Once all the tubes have been vortexed invert the tubes 3 times and incubate at 65°C for 15 to 20mins, or until the solution changes colour from red to olive green.

9. Remove from the incubator and add 2.5ml (0.5ml per 1ml of blood) of isopropanol to each tube, replace cap and invert thoroughly to mix, DNA should be visible as condensing white threads.

10. Centrifuge at 2900 rpm in the Beckman centrifuge for 8 mins to pellet the DNA.

11. Remove from the centrifuge and pour off the supernatant into a beaker taking care not to disturb the DNA pellet. Invert tubes onto tissue to remove as much liquid as possible.

12. Add 2.5mls (0.5ml per 1ml of blood) of 70% ethanol and re-suspend the pellet by tapping the tube until it is floating freely.

13. Centrifuge at 2900 rpm for 8 mins to pellet the DNA.

14. Remove from the centrifuge and carefully pour off the ethanol trying to aspirate as much as possible. Invert onto tissue for ten minutes, then allow to air dry for ten minutes and re-suspend in 500µl (100ul per ml of blood) of buffer FG3 and leave to dissolve overnight.

15. DNA tubes are labeled as follows:

- On the laboratory PC, open shortcut to 'Neuro3' (N:\Shared\Label Printer\Neuro\Neuro3) and click on the Print icon. Now click on the browse symbol to bring up a list of all samples in the PIMS database.
- Using your extraction sheet, highlight samples in groups depending on whether they are first, second or single extractions. Once all are selected, click on OK.
- Type in the extraction type (1st, 2nd, All) then select Print. All those will now be printed. Repeat until all labels are printed.
- Stick each label to a DNA tube and also write the DNA number onto the tube with a permanent marker pen. Wrap this writing and the label in Sellotape.

16. When the DNA has dissolved, spin the tubes briefly to collect the DNA at the bottom of the tube and remove condensation. Using a 1000µl pipette, transfer the samples into the DNA tubes (**this must be witnessed**).

17. On completion, shake at 55C for 2hrs then leave at room temperature until analysis by gel electrophoresis and spectrophotometry. Store at -70°C in order of DNA number and extraction type. Single extractions are stored with the first extractions.

Making an extraction sheet

1. Open the PIMS database.
3. Click on '**Open PIMS**' and the Neurogenetics main switchboard comes up.
4. Click on **'Samples for Extraction'** and in 'SAMPLE INFORMATION- Extraction' box, click on '**New Extraction'.**
5. Click on '**Add New Worksheet'** at the bottom of the screen.
6. In the 'SAMPLE INFORMATION- New Extraction worksheet' box, an **Extraction Sheet ID** is assigned as soon as the **Extraction Sheet Name** (usually the day of the week) is entered. Enter **Extraction Date** only if the date of extraction differs from the date the extraction sheet is made.
7. Enter appropriate initials in **Personnel Initials.**
8**.** Select **Qiagen Flexigene Kit** from the drop down menu in **Instrument Description.** Then click on **'Proceed'.**
9**.** In Sample Information / Select Extraction Worksheet**.** Click in the **'Select to Proceed'** box next to the extraction sheet desired.
10. Back in Sample Information / Select Extraction Worksheet, the Worksheet name and Extraction date can be changed but the only useful option for Instrument description is Autogen NA3000. Click in the **'Select to Proceed'** box next to the extraction sheet desired and proceed to step 10.
11. In 'Sample Information Select Sample Type for Extraction' click on '**Blood'**
12. From the list of Samples For Extraction – Blood, click in the **'Select'** box next to samples to be extracted. A tally of the 'Total samples selected' appears on the bottom right hand corner.
13. After all the samples to be extracted have been selected, click **'Proceed'** at the bottom left hand corner.
14. Click '**OK'** in the Sample Information Extraction Sheet pop up box and the DNA Extraction sheet can be viewed. If this list needs to be amended, click on **'Close'** at the top of the screen return to step 12; otherwise, click Print **(make sure the extraction sheet prints),** then click on **'Continue'** and exit the database.