A GENOME-WIDE ASSOCIATION STUDY OF CISPLATIN-INDUCED HEARING LOSS IN CHILDREN

by

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Abstract

Cisplatin is an effective chemotherapeutic agent used for a variety of solid organ malignancies in children and adults. However, its clinical use is limited by the high incidence of cisplatin-induced ototoxicity (CIO), which can affect up to 40-60% of children treated. To date, the genetic basis for CIO has been studied with only focused candidate-gene approaches.

Here we report the findings of the first genome-wide association study (GWAS) of cisplatin-induced ototoxicity in children. We examined 738,432 genetics markers in a discovery cohort of 282 Canadian paediatric patients treated with cisplatin, followed by a replication study in an independent Canadian cohort of 82 children. In addition, clinical, therapeutic, and demographic characteristics of cases and controls were analysed to identify clinical factors that may also contribute to the susceptibility to CIO.

The genome-wide analyses identified a significant association within the toll-like receptor 4 (*TLR4*) gene on chromosome 9. The most highly associated single nucleotide polymorphism (SNP) rs960312 conferred a highly protective effect against cisplatin-induced hearing loss ($P = 1.19 \times 10^{-8}$, odds ratio = 0.22). This variant was subsequently replicated in an independent paediatric cohort ($P = 0.018$, odds ratio = 0.25). This variant is a tag SNP for a *TLR4* promoter haplotype reported to have significantly altered transcriptional efficiency of *TLR4*. In both cohorts, CIO is significantly associated with younger age ($P = 3.41 \times 10^{-6}$), concomitant vincristine use ($P = 2.03 \times 10^{-12}$), and germ-cell tumour type ($P = 4.50 \times 10^{-6}$). After correcting for these clinical factors, *TLR4* rs960312 remains highly associated (Uncorrected $P = 1.16 \times 10^{-9}$; Corrected $P = 1.01 \times 10^{-9}$).

Several lines of evidence from *in vitro* and *in vivo* studies have implicated TLR4 in cisplatin-induced cochlear toxicity and hearing loss. Here we provide the first evidence linking TLR4 and CIO in human patients treated for cancer, leading to new insights into the mechanism underlying this pervasive and clinically limiting adverse drug reaction. The identification of

additional markers that contribute to the susceptibility of CIO can be used to develop individualized patient treatments, which can potentially improve safety and treatment outcome of cisplatin.

Preface

Parts of chapter 1 have been published in a review article, (Lee JW., Aminkeng F., Bhavsar AP., Shaw K., Carleton BC., Hayden MR., Ross CJ. (March 2014) The emerging era of pharmacogenomics: current successes, future potential, and challenges, *Clinical Genetics*). JL, FA, KS, AP conducted the literature search and JL wrote the draft manuscript. CR, MH and BC supervised the manuscript. All authors edited the manuscript.

The study was approved by the University of British Columbia / Children & Women's Health Centre of British Columbia Research Ethics Board **(H04-70358)** and all subjects provided informed consent. Informed written consent was obtained from each participant and/or their parents or legal guardians.

Table of Contents

List of Tables

List of Figures

List of Symbols, Abbreviations

ABCC3	ATP-binding cassette transporter, subfamily C, member
ADME	Absorption, Distribution, Metabolism, Excretion and Toxicity
ADRs	Adverse Drug Reactions
AUC	Area Under the Curve
CIO	Cisplatin-induced Ototoxicity
COMT	Catechol O-methyltransferase
CPNDS	Canadian Pharmacogenomic Network for Drug Safety
CTCAE	Common Terminology Criteria for Adverse Events
CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4
CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7
FDR	False Discovery Rate
GSTM	Glutathione S-transferase mu
GSTP	Glutathione S-transferase pi
GSTs	Glutathione S-Transferase
GSTT	Glutathione S-transferase theta
hiPSCs	Human Induced Pluripotent Stem Cell
HMGB1	High mobility group box 1
hMSH2	Human DNA mismatch repair protein Msh2
HWE	Hardy Weinberg Equilibrium
LD	Linkage Disequilibrium
LRP2	Megalin
MAF	Minor Allele Frequency
MAPK	Mitogen-activated protein kinase
NHGRI	National Human Genome Research Institute

NPV	Negative Predictive Value
OR	Odds Ratio
PCA	Principal Component Analysis
PCs	Principal Components
PD	Pharmacodynamic
PK	Pharmacokinetic
PPV	Positive Predictive Value
PTA	Pure Tone Audiometry
QC	Quality Control
ROC	Receiver Operating Curve
ROS	Reactive Oxygen Species
SAM	*S*-Adenosyl methionine
SIOP	International Society of Pediatric Oncology
SJS	Steven-Johnsons Syndrome
SLC01B1	Solute Carrier 1, Member B1
SNP	Single Nucleotide Polymorphism
SNPs	Single Nucleotide Polymorphisms
STS	Sodium Thiosulfate
TBP	TATA-binding Protein
TEN	Toxic Epidermal Necrosis
TPMT	Thiopurite S-methyltransferase
VKORC1	Vitamin K Epoxide Reductase
λ_{GC}	Genomic Control Inflation Factor

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Dedication

To my mother, thank you for your sacrifice and love.

Chapter 1: Introduction

1.1 Variability of Drug Response

A significant limitation of evidence-based drug therapy is that clinical trials only provide information on the average response at standard doses in relatively small, specific populations. Individuals, however, vary significantly in their response to a specific medication; at the same dose, a group of patients can experience no therapeutic effect while others develop serious adverse drug reactions (ADRs). Accordingly, it is estimated that most major medications are effective in only 25-60% of patients[1]. Unfortunately, this flawed "one size fits all" paradigm can have detrimental, costly and fatal consequences.

1.2 Adverse Drug Reactions (ADRs)

ADRs are defined as any undesirable experience or outcome concerned with a particular drug beyond its anticipated therapeutic effects[2]. These events may occur during drug development, leading to the withdrawal of otherwise valuable drugs, or may emerge after the drug has been licensed due to the rare nature of some ADRs. Despite this, drugs with serious known ADRs continue to be prescribed because of the absence of effective alternatives[3]. ADRs can vary from mild to severe and can lead to significant events such as hospitalization, disability or permanent damage, congenital abnormalities and in some instances, death.

1.2.1 ADRs in Clinical Practice

In the clinical setting, ADRs are a common and significant public health issue. Each year, ADRs account for 4.2-30% of all hospital admissions in the USA and Canada, 2.5-10.6% of admissions in Europe and 5.7-19.8% of admissions in Australia[4]. In the United States alone, up to 2 million people suffer serious drug-related toxicities claiming over 100,000 lives[5, 6]. In an in-patient hospital setting, it is estimated that one in seven individuals experience an ADR, resulting in a significant increase in morbidity[7]. Furthermore, a majority of drug-induced side effects are unreported and ADR-related deaths are often

misidentified. In a ten-year longitudinal study conducted in the UK, the annual number of diagnosed ADRs and the in-hospital mortality rate increased by 76.8% and 10% respectively[8]. In vulnerable populations such as children and the elderly, drug-induced toxicities can be even more significant and dangerous; up to 39% of ADRs suffered by paediatric patients are found to be potentially life threatening or fatal[9].

ADRs can lead to increased hospitalization, longer hospital stay and more clinical investigation for serious toxicities. The economic burden of these consequences can be substantial with ADRs estimated to cost up to 30–130 billion dollars annually in the United States [3, 10]. In an inpatient setting alone, ADRs are found to cost up to $2,262 (US dollars) per patient suffering a reaction[11]. In addition, ADRs often lead to the prescription of new medication for the treatment of conditions that are a result of an initial drug. Prescribing cascades not only increase the cost of pharmacotherapy but also compound the risk for additional ADRs[12]. The cost on patients and their families are also substantial and long lasting considering the permanent and significant effects ADRs can have. For example, the estimated lifetime cost of serious cisplatin-induced hearing impairment in children is approximately $500,000 per patient[13].

1.2.2 Types of ADRs

ADRs are classified into two categories, type A (pharmacological) and type B (idiosyncratic). The majority of drug-induced toxicities are type A, in which the underlying mechanism of toxicity is dose dependent[14]. These reactions are often reversible on reducing the dose or withdrawing the therapy and are often due to the augmentation of a medication's therapeutic action. Bleeding episodes with the anti-coagulant warfarin or hypotension with anti-hypertensive therapy are examples of type A pharmacological reactions[15].

Idiosyncratic ADRs, on the other hand, are drug-induced events that are dose independent and not predictable from the known pharmacology of a drug[16]. These reactions are usually rare and severe, and are often only discovered after the drug development process or

licensing. As a result, millions of patients may be exposed to the medication before unexpected ADRs emerge and gain significant attention[17]. For example, carbamazepine-induced Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are potentially fatal skin reactions that manifest in only a very small group of patients and are caused by an unknown immunological reaction to the anti-epileptic drug[14].

It is thought that the majority of drug toxicities are preventable[3, 5, 18]. Furthermore, drug withdrawals often leave patients that benefited from the therapy without suitable alternative. As a result, understanding the mechanisms causing ADRs and identifying the patients who are at the most risk is of great importance for patient safety and the healthcare system. Serious and life threatening ADRs can arise from both type A and B mechanisms. Regardless of type, genetic factors have been found to play a significant role in contributing to the susceptibility to these reactions.

1.2.3 Genetic Contribution to Drug Variability

A familial component to drug action variability was first described as "inborn errors of metabolism" by the 20th century British physician Sir Archibald Garrod[19]. Through his pioneering work on the monogenic disorder Alkaptonuria, Garrod proposed that defects in metabolic pathways of drugs could alter drug concentrations and therefore effect. In the late 1950's, it was recognized that patients with extreme levels of drug concentrations in their blood or urine inherited the biochemical traits that led to such variation[20]. On a population level, this concept was further supported by drug response differences being larger among members in a population than within the same person at different times[21]. Since then, mounting evidence has demonstrated that variation in a patient's genome can significantly affect inter-patient disparities in drug response. In fact, an individual's genome has been estimated to account for 20-95% of the variability in drug disposition and effects[21].

Drug efficacy and ADRs are often associated with concentration levels of a drug's active metabolites in the plasma and target location. An individual's therapeutic window is defined by the drug dosages in which the patient will experience a therapeutic effect without an

adverse reaction. This range of drug concentrations is required for effective and safe therapy, but may vary significantly between individuals. Drug concentrations in plasma can vary more than 600-fold between two individuals of the same body weight on the same dosage of a drug[22]. As a result, a drug dose in the average therapeutic window for the majority of a patient population may be too low or too high for a small minority of outlier individuals, resulting in non-optimal drug therapy.

While physiological and environmental factors such as age, disease status and concomitant medications can lead to atypical dose response curves, genetic polymorphisms in drug targeting, metabolism and transport are suggested to be the most importance sources of inter-patient differences in drug efficacy[23]. For example, warfarin is one of the most widely prescribed oral anticoagulants in North America and Europe, but has a narrow therapeutic window that often leads to inappropriate dosing[24]. As a result, its use is often complicated by serious adverse events including hemorrhaging or undesired thrombosis as a result of overdosing and underdosing. Clinical factors such as age, gender, body surface area and vitamin K dietary intake are shown to explain approximately 12% of dose variability. In comparison, genetic variants in enzymes involved in the metabolism warfarin, cytochrome P450 gene (*CYP2C9)* and vitamin K epoxide reductase (*VKORC1),* are found to account for more than 30-40% of total variation in the final warfarin dose[25].

1.3 Pharmacogenomics

Pharmacogenomics is the study of genetic variation underlying inter-individual differences of drug response. It aims to identify useful, predictive and biologically informative genetic variants to optimize drug therapy on a patient-by-patient level. Specifically, pharmacogenomics can allow clinicians to target drug therapies to genetically defined subsets of ideal responders, leading to improved response and a clinically important reductions in ADRs[22]. In the case of warfarin, incorporating *CYP2C9* and *VKORC1* genotyping into dosing algorithms has been demonstrated to reduce hospitalizations for hemorrhaging by 28% in a prospective study of 900 patients[26].

1.3.1 Types of Genetic Variation

Several types of genetic variation can influence drug response ranging from single-nucleotide polymorphisms (SNPs), deletions, insertions or duplication of DNA sequences. The most common genetic variants are SNPs, which are single nucleotide changes that occur with a frequency greater than 1% in the population[27]. Conversely, single base-pair changes that are rare (ie < 1%) are referred to as mutations. Most SNPs are located outside of the coding regions of genes and appear to have no effect on gene function; of the 3.6 million SNPs each individual on average carries, only 24,000 are located in the exons of a gene[28]. Nonetheless, SNPs both inside and outside of coding regions can have functional consequences by causing changes to amino acid sequences, mRNA stability and transcription factor binding affinity[29].

1.3.2 Pharmacokinetics and Pharmacodynamics

To date, pharmacogenomic studies have focused on genes that encode proteins involved in either the pharmacokinetics (PK) or pharmacodynamics (PD) of a drug[23]. PD is the study of the biochemical and physiological effect drugs have on the human body[30]. It depicts the relationship between drug concentration and effect through the interaction of the drug with its target site. On the other hand, PK refers to the extent and rate of which the body metabolizes and transports drugs to and out of its target location. The level of drug at the target site is dependent on key processes involved in the bodily absorption, distribution, metabolism and excretion (ADME) of a drug[31]. Metabolism can be classified into phase I and phase II reactions, which reflect the functional changes that occur to a parent drug. While some drugs may undergo only one of the two types of metabolism, both phase I and phase II reactions are effective defense mechanisms for the body to inactivate, detoxify and excrete drugs[32]. In phase I metabolism, the parent drug is converted to more polar active metabolites by oxidation, reduction and hydrolysis reactions of the functional groups. Phase II metabolism involves conjugation reactions such as glucuronidation, acetylation and sulfation.

Genetic variation in genes of the PK and PD pathways can significantly influence an

individual's susceptibility to drug-induced toxicities[23]. For example, one drug-metabolizing enzyme, cytochrome P450 2D6 (CYP2D6), is responsible for the metabolism of approximately 20-25% of all marketed drugs[33]. However, the *CYP2D6* gene is highly polymorphic with over 100 genetic variants identified[34]. Specific combinations of these alleles result in a wide range of enzyme activities, which in turn, can have significant effects on the overall metabolism of many drugs. For example, CYP2D6 converts the widely used opioid drug codeine, into its pharmacologically active metabolite, morphine, which has an approximately 600-fold greater affinity to the opioid receptor than the pro-drug[35]. Patients with 3 or more functional copies of *CYP2D6* are classified as ultra-rapid metabolizers and therefore can rapidly convert codeine to toxic levels of morphine. As a result, severe life-threatening adverse events such as respiratory depression, and in rare cases death, have been reported in individuals carrying these high activity alleles[36, 37]. Furthermore, neonates have developed serious or fatal adverse reactions after receiving breast milk from mothers taking standard prescribed doses of codeine for post-partum pain[38, 39]. Conversely, poor metabolic activity of CYP2D6 is frequently caused by genetic variant*s* that disrupt enzyme function or cause *CYP2D6* deletions. Poor metabolizers are unable to effectively convert codeine into morphine, resulting in minimal analgesic effect and pain relief[40]. Consequently, the amount of morphine produced from the parent drug codeine can be highly variable between individuals, ranging from 0% to nearly 75% of the total codeine dose[37].

1.3.3 Translation of Pharmacogenomics Discoveries to Bedside

By understanding a clear relationship between an individual's genotype and drug response, patients that are at high risk of ADRs can be identified prior to treatment and given genotype-appropriate doses or alternative therapies. The translation of pharmacogenomic scientific discoveries to clinical practice is a priority for patients, clinicians, policy makers and regulatory agencies. As of 2014, approximately 160 pharmacogenomic markers are mentioned in approved drug labels for nearly a 140 drugs[41]. However, only a few of these have pharmacogenomic tests readily available and with a level of evidence where recommendations can be made for their use in practice. For one of these drugs, carbamazepine, testing for *HLA-B*15:02* is recommended in populations at high risk for

carbamazepine -induced SJS and TEN[42, 43]. In fact, pharmacogenomic testing for this variant is now standard practice in at least 50 hospitals in Taiwan and it is estimated that selectively prescribing carbamazepine for non–*HLA-B*1502* carriers versus prescribing higher-cost alternative drugs for all patients would save the Taiwanese government $1 billion (US dollars) per year[44].

1.3.4 Pharmacogenomics of ADRs in Paediatric Oncology

Paediatric cancer is a rare disease that accounts for only 1% of the annual incidence rate of cancer in the United States[45]. This results in approximately 13,500 new cases per year but this rate has been increasing by an average of 0.5% per year since 1975[46]. Fortunately, the survival rate in children with cancer has greatly improved over the last 30 years, with cancer specific mortality rates decreasing by more than 50% and 5-year survival rates approaching nearly 80%[46]. In 2013, the number of children surviving cancer is estimated to be just over 420,000 and this is predicted to approach 500,000 individuals by 2020[46]. While the cures for primary malignancies have become highly effective and successful, the incidence of adverse health related effects and long-term chronic health conditions as a result of chemotherapeutic treatments has significantly increased in paediatric cancer survivors as well[47, 48]. In fact, nearly three quarters of childhood cancer survivors suffer an ADR related to their cancer therapy[49]. Furthermore, 40% of cancer survivors experience a severe life threatening or permanently disabling ADR[49].

Despite this, pharmacogenomic studies in children have been relatively late in terms of the amount of research investigating ADRs of chemotherapeutic agents. The conventional approach to dosing in children is to extrapolate from adult clinical data and adjust by weight and/or body surface area[50]. Children however cannot be represented as small adults as the ontogeny of drug receptors, transporters and metabolizing enzymes are key determinants in the differences of drug response in children compared to adults[51]. Developmentally induced changes of pathways involved in drug metabolism, activation, disposition and clearance are recognized to have a major impact on drug safety in children[52]. For example, the enzyme CYP3A7 primarily drives oxidative drug metabolism during fetal development.

The expression of CYP3A7, however, rapidly declines after birth and is replaced by the increase in activity of a different metabolizing enzyme CYP3A4 in young neonates[53].

The appreciation of age dependent differences in drug response and safety strongly suggests that paediatric-specific pharmacogenomic studies are of great relevance and need. There are several barriers however, ranging from ethical considerations to technical difficulties in obtaining sufficient biological samples from young patients[51]. While these challenges do exist, the potential of pharmacogenomics in improving drug therapy is promising and substantial, especially for highly vulnerable patients such as children treated for cancer.

1.4 Cisplatin

Cisplatin (CDDP, PLATINOL®) is a potent chemotherapeutic agent for the standard treatment of a variety of solid organ tumours. Discovered in the early 1970s, the platinum compound is still one of the most widely used anti-tumour drugs today. With a cure rate of up to 85%, it is also one of the most effective chemotherapeutic agents for children and has contributed to the dramatic increase in survival from many cancers including neuroblastoma, hepatoblastoma, brain tumors, osteosarcoma, and germ cell tumours[54]. For the treatment for standard risk hepatoblastoma, cisplatin as a monotherapy alone has been reported to show an efficacy of over 80% for 3-year event-free survival[55]. Cisplatin is also used to treat a variety of adult cancers including ovarian, gastrointestinal, testicular, lung, and head and neck tumours[56, 57].

1.4.1 Cisplatin Mechanism of Cytotoxic Action

Cisplatin is a non-specific antineoplastic agent that acts at all stages in the cell cycle and induces cytotoxicity through drug-mediated cellular effects, including the inhibition of DNA synthesis, suppression of RNA transcription and the induction of apoptosis and cell death [58]. The primary mechanism of action occurs through the formation of covalent bonds between cisplatin and the nucleophilic N7-sites of purine bases in DNA, resulting in DNA-protein and DNA-DNA inter-strand and intra-strand crosslinks[59]. These DNA lesions

distort the structure and integrity of double stranded DNA**.** The intra-strand adducts are shown to have the biggest role in the cytotoxic action of cisplatin, accounting for 85-90% of total lesions[60, 61].

The formation of these DNA crosslinks initiates the binding of several proteins such as hMSH2 of the mismatch repair system, HMGB1 and HGMB2, and the transcription factor TATA binding protein (TBP)[62]. These damage recognition proteins transduce DNA damage signals to several downstream effectors, activating several signaling pathways that ultimately end with the induction of apoptosis **(Figure 1.1)**[63]. Specifically, cisplatin induces the activation of p53, p21 and the mitogen activated protein kinase (MAPK) signaling pathway, which then activate both the cell survival and apoptotic mechanisms in the cell. DNA damage that exceeds the cellular repair capacity results in a net biological effect which favors the activation of apoptosis over cell survival[63]. Unfortunately, neither cisplatin-mediated cytotoxicity nor apoptosis are exclusively induced in cancer cells. As a result, cisplatin can also target normal healthy cells leading to diverse and clinically limiting ADRs.

1.4.2 Cisplatin-induced ADRs

ADRs of cisplatin therapy include general cell-damaging effects, such as nausea, vomiting, decreased appetite, alopecia, myelosuppression and immunosuppression[58]. A major complication that limits the clinical use of cisplatin however is the risk of more specific and serious ADRs, which include nephrotoxicity, peripheral neurotoxicity and ototoxicity. Although nephrotoxicity is treatable with saline hydration or mannitol diuresis, there are no proven cures or preventative treatments available for cisplatin-induced neurotoxicity and hearing loss[64, 65].

1.5 Cisplatin-induced Ototoxicity

Cisplatin-induced ototoxicity (CIO) is a clinically pervasive ADR that has been reported to affect approximately 10-25% of adults and up to 40-60% of children, depending on treatment protocol, dose and the grading of hearing loss[66-71]. It manifests as permanent,

sensorineural hearing impairment that can either occur shortly after or months and years after the initiation of cisplatin[72, 73]. One study of CIO in children found that only 5% of patients developed ototoxicity before the end of treatment, compared to 44% after 2 years of follow up[72]. Furthermore, cisplatin-induced hearing impairment usually shows no improvement and can even progress in severity years after the end of treatment[72, 74]. This is due to the fact that cisplatin can remain in the body for long periods of time. In fact, patients given cisplatin for testicular cancer were found to have traces of platinum compounds within their bodies for up to 20 years after treatment[75].

The clinical implications of CIO are huge as current clinical practice involves dosing to toxicity. Standard treatment protocols suggest dose reductions or early termination of cisplatin treatment once signs of significant ototoxicity manifests[76]. The switch to an alternative chemotherapeutic agent however, can result in the decrease of effectiveness in treating for certain tumours and can also increase the risk of ADRs specific for other drugs[77]. For example, although carboplatin is a less ototoxic platinum agent, cisplatin is found to be much more effective for the treatment of extra-cranial germ cell tumours[77].

1.5.1 Cisplatin-induced Ototoxicity in Children

Children are more susceptible to hearing loss following cisplatin treatment than adults[78, 79]. As the ototoxic effects occur during the critical developmental period of a child, the consequences of hearing loss can be much more significant and detrimental. First, hearing impairment in early childhood can interfere with speech discrimination, language acquisition, social-emotional development and increase the risk of learning difficulties[80, 81]. Ototoxicity usually begins in the higher frequencies between 4000-8000 Hz but can also progress to involve speech frequencies (500-2000 Hz) with increasing cumulative dose[67]. Nonetheless, high frequency hearing is thought to be critical for understanding speech as nearly 50% of English consonants contain energy in the 8000 Hz range[82]. Relevant sounds in the English language such as “th”, “f”, “k” and “s” also lie in this range. As a result, even hearing loss restricted to the high frequencies is reported to significantly influence verbal ability and reasoning skills in young children[83]. Older children who develop ototoxicity

even after the language acquisition years can also experience academic difficulties as hearing loss can impair a child's ability to cognitively gather and process information at school[84]. In fact, children with hearing impairments secondary to cancer treatment are found to be more than twice as likely to have difficulties with math or reading skills than those who do not suffer hearing loss from cisplatin[85].

1.5.2 Measuring Hearing Loss in Children

Hearing thresholds are primarily measured by pure-tone audiometry (PTA). The degree of hearing loss is determined by an individual's ability to respond to pure tone stimuli at the lowest intensity at each frequency threshold. As PTAs are subjective behavioural measurements of hearing however, baseline measurements are often difficult to obtain in young children, particularly very ill patients[86]. As a result, the Children's Oncology Group also recommends using evoked otoacoustic emissions or auditory brainstem response test for younger patients[84].

Several grading criteria have been developed to measure and quantify the severity of hearing loss in children **(Table 1.1)**[67, 76, 87-89]. These may differ depending on the purpose the scale tries to achieve. For example, certain grading schemes can be used to guide treatment decisions, identify ototoxicity at the soonest possible opportunity during treatment or report the severity of ototoxicity at the completion of treatment for the comparison of clinical trials[87]. In general, the classification of ototoxicity needs to be objective, sensitive, accurate, functionally relevant and applicable to results obtained at any age.

Currently, the most commonly used grading criteria are the Brock Scale[67], Chang[76] and the Common Terminology Criteria for Adverse Events (CTCAE)[90]. Ototoxicity is graded on a scale from 0 to 4, with grade 0 indicating no hearing loss, grade 1 and 2 indicating mild to moderate hearing loss and grade 3 and 4 referring to severe hearing loss that generally requires therapeutic interventions such as hearing aids and cochlear implants. One limitation for the Brock and CTCAE classification is the requirement of a baseline audiometric measurement, which can be especially difficult to obtain for young and sick children. Based

on absolute hearing levels rather than changes from baseline, the Society of Paediatric Oncology (SIOP) Boston Ototoxicity Grade Scale was recently developed in order to further refine and simply the characterization of hearing loss[87].

1.5.3 Pathophysiology of Cisplatin-induced Ototoxicity

The anatomy of the human ear can be divided into three segments, the outer, middle and inner ear. The external auditory canal and pinna form the outer ear, which transmits sound waves to the tympanic membrane of the middle ear. The vibration of the tympanic membrane causes the ossicles (malleus, incus and stapes) of the middle ear to vibrate, which in turn oscillates the fluid (perilymph) housed in the cochlea of the inner ear. The fluid moves along the Organ of Corti, which is lined with several thousand auditory nerve receptors called hair cells. When these hair cells are stimulated by this fluid movement, nerve impulses are sent to the auditory processing centers of the brain, in which audition is perceived[84]. Depending on which segment of the ear is compromised, hearing loss can be classified into two types, conductive and sensorineural[84]. While conductive hearing loss occurs through an impairment of the auditory pathway in the outer or middle ear sections, sensorineural ototoxicity occurs when there is damage to the components of the inner ear. As cisplatin is found to primarily damage the cochlear tissues of the inner ear, patients with CIO suffer from sensorineural hearing loss[91].

1.5.4 Mechanism of Cisplatin-induced Ototoxicity

The mechanisms underlying cisplatin-induced cytotoxicity of inner ear cells have not been fully elucidated. Cisplatin damage causes apoptosis at three main sites in the cochlea: the outer hair cells in the organ of Corti, the spiral ganglion and the lateral wall (stria vascularis and spiral ligament)[92]. Several lines of evidence suggest that the generation of reactive oxygen species (ROS) are involved in the toxicity associated with cisplatin[93, 94]. This ROS-mediated damage occurs as a consequence of the depletion of the antioxidant system (glutathione and antioxidant enzymes) and increased lipid peroxidation in the cochlea[95]. The accumulation of toxic lipid peroxides and ROS, such as superoxide and hydrogen peroxide, can lead to calcium influx within cochlear cells, resulting in the activation of

apoptotic pathways[96]. The accumulation of ROS has also been reported to lead to morphological and functional changes in the organ of Corti[97]. The production of ROS changes acoustic transduction by modulating the outer hair cell motility in the organ of Corti resulting in cell death, which has been demonstrated as a mechanism for cisplatin-induced ototoxicity[98].

1.5.5 Management of Hearing Loss

CIO is irreversible, leading to permanent hearing loss that can progressively worsen months or years after the end of treatment. In addition, an improvement in hearing thresholds after hearing loss occurs is rarely observed. Depending on the severity of ototoxicity, therapeutic options to manage hearing loss in children include hearing aids, cochlear implants, assistive listening devices, and education accommodations[84]. Although these therapeutic options help in partially improving hearing, they fail to restore the same quality of life that unaffected patients experience.

1.5.6 Otoprotective Agents

Several otoprotective agents are in preclinical and clinical stages of testing and may become viable treatment options to prevent hearing loss. These include amifostine, sodium thiosulfate (STS), D-methione N-acetyl cysteine, ebselin and dexamethasone with allopurinol. However, the efficacy and safety of these interventions are still not well known in both children and adults. An ideal otoprotective agent must be effective in protecting against hearing loss, with minimal adverse events and without reducing anti-tumour efficacy (response and survival)[87, 99]. Currently, the potential impact of otoprotective agents on anti-tumour efficacy has not been well studied.

1.5.6.1 Amifostine

In preclinical and clinical studies there is conflicting evidence about the protective effect of amifostine for hearing loss during cisplatin therapy. Animal studies have investigated the protective effect of amifostine in combination with cisplatin therapy. Although high doses of

amifostine resulted in protection against ototoxicity, it was also associated with neurotoxicity in hamsters[100]. In one small prospective trial in children with germ cell tumours treated with amifostine during cisplatin therapy, patients developed significant ototoxicity[101]. A prospective cohort study in children with medulloblastoma (n=97) showed a reduction in the incidence of grade 3 and 4 hearing loss when amifostine was administered with cisplatin, without altering patients' survival outcome[102]. Currently only two randomized control trials[103, 104] and one clinical controlled trial[105] have investigated the protective effect of amifostine in children with osteosarcoma and hepatoblastoma. These three trials however showed no significant difference in ototoxicity between patients treated with and without amifostine[99].

1.5.6.2 Sodium Thiosulfate

Several animal studies have shown that sodium thiosulfate (STS) significantly protects against cisplatin-induced hearing loss[106-108]. Delayed administration of STS has also shown reduced platinum ototoxicity without reduction of anti-tumour activity[109]. In one prospective randomized phase III trial consisting of 137 adult patients with head and neck cancer who were treated with high dose cisplatin and STS resulted in no significant difference in overall survival at 2 years of follow-up, but also no overall significant difference in hearing was observed between patients treated with STS and those without STS[110]. A subgroup analysis by different frequency ranges (low, medium, high) showed a significant protective effect of STS at frequencies vital for speech perception. However, the route of cisplatin administration varied between treatment groups, which limited the conclusions that could be drawn from this study. To date, there are no published clinical phase III trials assessing the efficacy and safety of STS in children.

1.5.6.3 Other Otoprotectants

Several other ototoprotective agents including D-methione N-acetyl cysteine, ebselin and dexamethasone with allopurinol are currently undergoing preclinical testing. Large-scale clinical trials are needed to determine efficacy of these agents in terms of protection against

hearing loss in children as well as anti-tumour efficacy, prior to implementing these into standard care.

There currently is no strong evidence to suggest that otoprotective agents such as amifostine and STS significantly protect against hearing loss. There could be several reasons as to why significant differences between treatment groups in the trials to date have not been observed, including power to detect effect (i.e., low sample size), length of follow-up and underlying genetic susceptibilities to CIO that have not yet been accounted for in these studies[99].

1.6 Clinical Risk Factors of Cisplatin-induced Ototoxicity

Several clinical risk factors have been consistently reported to play a role in cisplatin-induced hearing loss. These include age, cumulative cisplatin dose, concomitant aminoglycoside treatment, pre-existing hearing impairment and cranial radiotherapy[111, 112].

1.6.1 Age

Mounting evidence has shown that younger children are at higher risk of developing hearing loss[72, 78, 113]. Within paediatric patients, cisplatin therapy is also reported to have an age-dependent influence on both the incidence and severity of ototoxicity. Patients younger than the age of 5 were found to be at a significantly higher risk (up to 21 times more likely in one study) to develop moderate to severe high frequency hearing loss after cisplatin exposure compared to older children given the same cumulative dose[67, 78]. Furthermore, age is reported to have an inverse relationship with hearing loss severity[114].

1.6.2 Cisplatin Cumulative Dose

Patients receiving higher cumulative doses have been found to be at a significantly greater risk of developing ototoxicity[66, 113, 115, 116]. In a study by Bokemeyer *et al.*, cisplatin cumulative dose was identified to be the greatest predictor of CIO (p-value < 0.0001)[115]. In this cohort of testicular cancer patients, the mean cumulative cisplatin dose of patients with ototoxicity was more than twice that of patients without hearing loss. Simon *et al*. found

that cisplatin-induced hearing impairment was associated with the stage at diagnosis of the cancer, as patients with more advanced disease received higher total cumulative dosages of cisplatin. Only 1% of patients treated for stage 1 neuroblastoma developed hearing loss compared to 26.9% of patients who received twice the cumulative dose for metastatic stage 4 disease[116]. Cumulative dose is also correlated with the severity of ototoxicity, with patients at risk of developing hearing loss in the speech frequencies when doses exceed 400 mg/m^2[113].

1.6.3 Other Clinical Risk Factors

Aminoglycoside antibiotics have also been reported to cause sensorineural hearing loss in patients[117-119]. In fact, the toxicity profiles of cisplatin and aminoglycosides are similar, with both causing nephrotoxicity, neuropathy and ototoxicity[117]. Unlike cisplatin, aminoglycosides are found to be both cochleotoxic (loss of hearing) and vestibulotoxic (loss of balance)[117]. The severity of hearing loss varies among different aminoglycosides: amikacin is considered the least toxic, gentamicin, kanamycin and tobramycin somewhat more and neomycin is regarded as highly toxic.

The combination of cisplatin and radiotherapy is shown to be highly effective for the treatment of paediatric brain tumours[120]. The risk of hearing loss, however, is found to significantly increase when cranial irradiation is concomitantly administered with platinum-based chemotherapy[82, 121]. Cranial radiotherapy has also been reported to cause irreversible high frequency hearing loss independently[122]. The incidence and severity of ototoxicity is shown to increase with higher radiation doses and hearing loss is found to be both conductive and sensorineural[86].

In standard treatment protocols, the occurrence of moderate to severe cisplatin ototoxicity will cause the clinician to reduce the cisplatin dose or terminate cisplatin treatment, which may adversely affect survival rates[80]. It is now recognized that there is significant variability in hearing loss between individual patients receiving similar cumulative doses and application schedules of cisplatin, which suggest that clinical risk factors alone are

insufficient predictors of CIO[80, 123].

1.7 Pharmacogenomic Studies of Cisplatin-induced Ototoxicity

CIO demonstrates significant inter-patient variation, which may be related to factors such as dose, age, exposure to cranial irradiation and other ototoxic insults [65]. However, it is widely recognized that clinical risk factors alone are insufficient predictors for CIO [80]. Genetic variation in the genes involved in drug biotransformation, transport, and drug targets has been shown to influence drug response and the susceptibility to adverse drug events, including ototoxicity [124]. Using cell lines derived from HapMap European pedigrees, the heritability for susceptibility to cisplatin-induced cytotoxicity has been estimated to be approximately 38-47%[125]. The identification of genetic variants that alter gene expression, disrupt protein function, and subsequently influence the susceptibility to drug-induced toxicity could be used to identify patients at higher or lower risk of CIO.

Although only a few studies have examined the hereditary basis for CI, several genes and polymorphisms have been identified through knowledge driven candidate gene studies. Specifically genetic variants in *TPMT, COMT, ABCC3, GSTs* and *LRP2* and have been investigated in several studies for their influence on the susceptibility to cisplatin-induced hearing loss (**Table 1.2-1.7)**.

1.7.1 Methyltransferases (*TPMT* and *COMT*)

A candidate gene study in children receiving cisplatin identified genetic variants in two methyltransferase genes, thiopurine S-methyltransferase (*TPMT)* (rs12201199, rs1800460, rs1142345) and catechol O-methyltransferase (*COMT)* (rs9332377, rs4646316), conferring increased risk of developing cisplatin-induced hearing loss in two independent cohorts of various paediatric tumour patients **(Table 1.2)**[80]. An independent replication study in 155 paediatric patients showed that all genetic variants assessed in *TPMT* (rs12201199, rs1800460, rs1142345) were significantly associated with cisplatin-induced hearing loss in the replication cohort[126]. Recently, a study by Yang *et al*. reported a lack of significant association with the *TPMT* and *COMT* variants and hearing loss in a cohort of

medulloblastoma only patients **(Table 1.2)**[127]. However, there were key differences in the patient demographics and treatment protocols between these studies[128]. In fact, Yang *et al*. did observe a strong trend of association with the *TPMT* functional variants (rs1800460, rs1142345) in a second cohort of patients treated similarly to the cohorts of the previous studies, but due to the small number of patients, their analysis was underpowered to reach statistical significance[128].

Although the mechanisms of *TPMT* and *COMT* in CIO are currently unknown, these have been postulated to increase cisplatin toxicity by influencing the binding of cisplatin to purines in DNA, thereby modulating cisplatin cross-linking. Alternatively, cisplatin toxicity may be due to the increased accumulation of the key methyl donor substrate of TPMT, *S*-adenosylmethionine (SAM), due to reduced activity levels of TPMT and COMT. In line with this hypothesis, SAM is demonstrated to significantly increase the toxicity of cisplatin in rats, as measured by cisplatin-induced nephrotoxicity[80, 129-131]. Furthermore, the administration of cisplatin to mouse embryonic stem cells has been reported to significantly up-regulate *TPMT* gene expression, in addition to the increased expression of metabolic genes, and the corresponding metabolites, in pathways clustered around SAM[132].

1.7.2 Cisplatin Efflux Transporter (*ABCC3*)

ABCC3 is a transporter that mediates the efflux of organic anions, xenobiotics and glutathione *S*-conjugates, including glutathione S-conjugated cisplatin[133, 134]. One of the mechanisms by which platinum drugs are detoxified is through conjugation of the active metabolite to glutathione making the compound more anionic[135, 136]. This enables the compounds to be more readily exported from cells through an ATP-dependent pump. Studies in rat hepatocyte cell lines have shown that both ABCC2 and ABCC3 protein levels and mRNA expression increased after treatment with cisplatin[137, 138]. Similarly, studies carried out in lung cancer cells lines have shown that *ABCC3* mRNA expression levels are significantly correlated to resistance to cisplatin and other platinum drugs[139, 140]. Reduced activity of ABCC3 can affect the detoxification pathway resulting in ineffective transport of toxic compounds out of the cell leading to toxicity. This suggests that ABCC3

levels might affect cisplatin transport. In turn, polymorphisms may regulate ABCC3 levels or affect function of the transporter.

An association between the synonymous variant in *ABCC3* rs1051640 (E1503E) and hearing loss is reported to contribute to susceptibility of hearing loss in paediatric patients. The association of this variant with cisplatin ototoxicity was initially discovered by Ross *et al.* (*p*-value=0.0092, OR 2.1)[80]. An additional independent replication study replicated this association (*p*-value=0.036, OR 1.8) **(Table 1.3)**[141]. In each study, the association of *ABCC3* with cisplatin-induced hearing was not significant after correction for multiple testing. However, when these cohorts were combined the association was significant after multiple testing corrections (*p*-value=0.00078, OR 2.0)[141]. Further replication and functional validation studies are required to assess the exact mechanisms by which variants in *ABCC3* affect cisplatin-induced hearing loss and to determine if a similar association is also observed in adult patients.

1.7.3 Glutathione S-Transferases (*GSTs*)

One of the mechanisms by which cisplatin is detoxified is through the conjugation of the active platinum metabolite to glutathione by glutathione s-transferases (GSTs), resulting in its export out of the cell[135, 136]. This protects the cell from the negative effects associated with increased oxidative stress. In the cochlea, the level and activity of glutathione decreases when hearing loss develops after treatment with cisplatin, suggesting a role of glutathione in the development of cisplatin ototoxicity[142]. Several studies have therefore examined whether polymorphisms in GST genes (*GSTM, GSTP, GSTT)* are associated with cisplatin-induced hearing loss **(Tables 1.4-1.6)**[143-147].

1.7.3.1 *GSTMs*

To date, three studies have reported significant associations between CIO and genetic variants in *GSTM* **(Table 1.4)**. The first study by Peters *et al.* found that the *GSTM3*B* allele was present at a higher frequency in paediatric patients with normal hearing(18%) than patients with significant hearing ototoxicity(2.5%)[143]. Proposed as a protective variant

against CIO, *GSTM*3* is a 3 base-pair deletion that forms a transcription factor recognition site. This allele is demonstrated to increase the transcription potential of the gene thus enhancing the detoxification activity of the enzyme[148]. In a second study investigating GST polymorphisms in a 100 adult patients treated for ovarian cancer, Khrunin *et al*. was not able to replicate the association of *GSTM3*B* in the context of CIO[146]. Differences in age of patients and the grading criteria for hearing loss are important considerations to make when assessing the lack of replication. No other studies have investigated the influence of *GSTM3*B* on cisplatin-induced ototoxicity.

Conversely, Oldenburg *et al.* published two separate studies reporting an association of *GSTM1* with CIO in adult testicular cancer survivors **(Table 1.4)**[144, 145]. In both studies, the presence of *GSTM1* was associated with an increased risk for hearing impairment. However, patients of the first cohort (n=173) were included into the overall analysis of the second study (n=238) and thus the observed association may have largely been influenced by the effect of the original 173 patients. Aside from these two studies, one adult study in ovarian cancer patients[146] and four independent studies in children treated for various cancers[80, 143, 149, 150] have failed to observe a significant association for *GSTM1* and CIO. In fact, the effect of *GSTM1* was observed in the opposite direction with a protective effect.

1.7.3.2 *GSTPs*

In the first of two studies published by Oldenburg *et al.*, a non-synonymous variant in *GSTP1* rs1965 was also identified to be significantly associated with cisplatin-induced hearing loss (*GG* genotype; OR 0.24, *p*-value < 0.001) (**Table 1.5**)[144]. The overall association of rs1965 did not achieve statistical significance when examining the extended cohort (n=238) of the second study (OR 0.81, *p*-value=0.055)[145]. Recently, Rednam *et al*. reported a significant association of *GSTP1* rs1965 with CIO in a paediatric cohort of 69 medulloblastoma patients[150]. Contrary to the protective effect observed in the previous studies, patients carrying the *G* allele of rs1965 were found to be four times more likely to require hearing aids than non-carriers (AG/GG genotype: OR 4.0, *p*-value=0.03)[150]. All

69 children were treated with craniospinal irradiation and when correcting for this factor, *GSTP1* rs1695 demonstrated a statistical significance of 0.05. Four studies (3 paediatric, 1 adult) reported no significant association of *GSTP1* with hearing loss in patients treated with cisplatin[80, 126, 143, 146].

1.7.3.3 *GSTTs*

A study by Choeyprasert *et al.* was the first to provide evidence for *GSTT1* having a significant influence on the susceptibility to CIO[147]. In a cohort of 68 paediatric patients, individuals with wild-type *GSTT1* were found to be at a higher risk for hearing loss than patients with null genotypes (OR 10.1, *p*-value=0.023) (**Table 1.6**). However, six previous studies in both children (4) and adults (2) were unable to find a significant association with *GST* and cisplatin-induced ototoxicity[143-146, 149, 150].

1.7.4 Megalin (*LRP2*)

Megalin (LRP2) is a multiligand endocytic receptor thought to play a crucial role in the development of the inner ear and is widely expressed within the marginal cells of the cochlear tissues and the proximal tubular cells of the kidney[151]. Megalin has been demonstrated to mediate the uptake of aminoglycosides in renal tubular cells, which are drugs known to have a similar ADR profile as cisplatin[147]. Due to the possibility that megalin can also bind to cisplatin, studies have examined genetic polymorphisms in the megalin gene (*LRP2*) in the context of cisplatin-induced ototoxicity (**Table 1.7**)[80, 126, 147, 152].

Associations between two common non-synonymous SNPs (rs2075252 and rs2228171) in *LRP2* were examined in four different studies of children treated with cisplatin for various malignancies. First, Riedemann *et al*. reported a significant association between rs2075252 (Glu4094Lys) and CIO (OR 3.45, *p*-value=0.016)[152]. In a cohort of 50 patients, the risk 'A' allele of rs2075252 was found in 52%(13) of cases compared to 24%(6) of controls. A strong trend to association was also reported for rs2228171 but did not pass the significance threshold (OR 2.32, *p*-value=0.087). On the other hand, Choeyprasert *et al*. found that

rs2228171 was significantly associated genetic risk variant for CIO but rs2075252 failed to show any trends of association[147]. In two large pharmacogenomic studies conducted by Ross *et al.* and Pussegoda *et al*., both variants did not exhibit statistically significant effects on cisplatin-induced hearing loss in children[80, 126]. Several differences in methodology and phenotyping may be possible reasons for the discrepant results between the several studies.

1.8 Genome-wide Association Studies (GWAS)

Initial pharmacogenomic studies have focused on candidate genes that encode proteins hypothesized to be involved in the absorption, distribution, metabolism and excretion of most prescription and nonprescription drugs. This hypothesis driven candidate-gene approach has identified several genetic variants associated with cisplatin-induced hearing loss [80, 143, 146, 153, 154]. However, the inability to replicate and validate these findings along with the complexity of cisplatin cytotoxicity in the auditory system suggests that multiple genetic factors play a role in the inter-individual differences observed in the susceptibility for CIO.

Completion of the Human Genome Project, combined with advances in high-throughput genetic technologies, has allowed for the investigation of a broader spectrum of genetic contributions. A genome-wide association approach is a powerful and unbiased method to examine common genetic variation across the entire genome for complex disease associations[155]. In contrast to the hypothesis driven candidate-gene approach, genome wide association studies (GWAS) make no assumptions about the genomic locations of causative variants and thus can identify novel and unexpected genes and pathways and allow for a potential breakthrough in our biological understanding of phenotypes[156]. Since 2007 to 2014, the National Human Genome Research Institute (NHGRI) GWAS catalog has recorded 1,751 published studies and 11,912 SNPs with complex trait associations[157].

1.8.1 Genetic and Statistical Principles of GWAS

For the most part, genetic association studies are performed using a case-control study design[158]. Other types include cohort and randomized clinical trial designs. In a pharmacogenomics case-control study, the frequency of an allele (A, a) and genotypes (AA, Aa, aa) is compared between individuals with the outcome of interest (cases) versus those without (controls). In the past, candidate-gene association studies would test tens to thousands of genetic variants that are pre-selected based on prior biological knowledge. GWAS, on the other hand, utilize genotyping arrays that contain anywhere from hundreds of thousands to millions of markers. SNPs are the ideal variants to use as markers of genomic regions in GWAS as they are the most common form of genetic variation, with a density of one every 1000 base pairs on average in the genome[159].

1.8.1.1 Linkage Disequilibrium

SNPs can have important functional consequences by altering amino acid sequences, mRNA stability and transcription factor binding affinity[29]. They can also exert an indirect functional effect, in which the associated SNP is in linkage disequilibrium (LD) with a second, not directly genotyped, polymorphism that is the functional or causal variant[160]. LD refers to the correlation between SNP alleles at one loci with specific alleles carried at loci nearby, reflecting specific combination of alleles (haplotypes) descended from single, ancestral chromosomes[161]. This phenomenon is due to the fact that SNPs in closer proximity on the same chromosome are less likely to be separated during a crossover event (recombination) in meiosis, and thus inherited together[162]. Several factors influence the extent and distribution of LD, including mutation, genetic drift, admixture, inbreeding, bottlenecks and natural selection[163]. An important consideration to make is that LD observed in a population is dependent upon ancestry. More ancestral populations such as that of the African descent have undergone greater extensions of recombination and thus show smaller regions of LD compared to Asian and European populations[160].

LD between ancestral genomic variants is extremely advantageous for GWAS study designs. Once the patterns of LD are known for a given genomic region, one can genotype only a

minor proportion of SNPs in that region (tag SNPs) and still unambiguously infer the alleles at the other polymorphic sites. Tag SNPs, which are specifically selected for as they are in strong LD with other variants surrounding them, can capture most of the genetic variation within a specific region. In populations of European descent, a set of 500,000 to a million SNPs spread across the genome can capture more than 80% of commonly occurring SNPs[164]. The majority of SNPs on GWAS genotyping arrays are in intergenic and intron sequences of genes. As a result, outcome-associated SNPs typically found in GWAS are flags that indicate regions of the genome where the functional and causal variant likely resides.

1.8.1.2 Multiple Testing Correction

A significant consequence of testing for such an extensive number of markers is the need to correct for multiple testing[158]. A GWAS that performs 1 million statistical tests at the standard significant threshold of 0.05 would result in approximately 50,000 genetic variants passing the significance threshold by chance. In order to avoid false positive results, imposing stringent statistical thresholds by correcting for multiple testing correction is essential for GWAS.

There are several approaches to correct for multiple testing. The most common procedure used is the Bonferroni correction, in which the 0.05 *p*-value threshold is divided by the total number of statistical tests performed to determine the new significance threshold[160]. For example, a GWAS with 500,000 markers would correspond to a statistical significance threshold of $1x10^{-8}$. The Bonferroni correction is considered to be too conservative of an approach however, as it assumes that all statistical tests performed are independent[165]. The presence of LD between SNPs infers that testing all markers for association will not completely yield independent statistics[158].

Other methods of multiple testing correction include the adjustment of the false discovery rate (FDR) and permutation testing. The FDR approach estimates the proportion of statistically significant associations at *p*-value = 0.05 that are false positive results[166]. On

the other hand, permutation testing generates an empirical distribution of test statistics for a given dataset when the null hypothesis is true[167]. This is done by rearranging the phenotype labels for all the individuals *N* times, while keeping the genotype architecture of the dataset intact. Each rearrangement represents a dataset or permutation that is tested for each marker, allowing for the comparison of the original test result from an individual marker with the most significant permuted results from all tested markers. Permutation testing is currently computationally challenging for large data sets such as GWAS.

1.8.1.3 Correcting for Covariates

Statistical tests of associations for genetic variants should also be adjusted for factors known to influence the outcome of interest[168]. For pharmacogenomic studies, this includes known clinical covariates such as age, gender, cumulative dose, concomitant medication and any other relevant drug-specific factors that may be significantly different between cases and controls. Including covariates in logistic regression models can serve to reduce false positive and false negative associations due to sampling artifacts in study designs[160]. This correction can come at a price of using additional degrees of freedoms, which may impact statistical power. However, for diseases or outcomes that is common (prevalence of >20%), correcting for confounding factors that are independent of the tested genotypes in the population not only protects against spurious associations but is also shown to increase the statistical power of a GWAS[169]. Non-confounding covariates can account for some of the phenotypic variation that would otherwise appear as noise and thus help in detecting the effects of the genetic associations.

1.8.1.4 Correcting for Population Stratification

Population substructure is one of the most important considerations to address in GWAS, especially when studies are carried out using an ethnically heterogeneous cohort of patients[168]. Allele frequencies can vary significantly between different human sub-populations. This can lead to population stratification in a study, in which spurious associations arise due to population-specific differences in SNP allele frequencies, rather

than drug toxicity associated differences[170]. As a result, it is imperative to detect and account for population stratification in genetic association studies.

The presence of population substructure differences between cases and controls can be first evaluated by computing the genomic control inflation factor (λ_{GC})[171]. This is calculated by comparing the chi-squared distribution of statistics from association tests with the theoretical null distribution. With this, confounding associations by stratification will be more widely distributed than expected[172]. Values of $\lambda_{GC} < 1.05$ generally indicate a benign influence from population stratification[172]. Although the genomic control can be used to correct the test statistics of all genetic association tests, it is considered widely inadequate to evaluate ancestral differences that are specific to each SNP[173].

Principal component analysis (PCA) has been demonstrated to be a powerful method to correct for population stratification, especially in large data sets like GWAS[172, 173]. PCA applies genotype data to infer continuous axes of genetic variation and generates a set of linearly uncorrelated variables called principal components (PCs). PCs ascertained by genotypes represent systemic variation dependent on ethnicity. Plotting the first two PC allows for a general visualization of clusters representing population sub-structures, allowing for the removal of outlier individuals from the overall sample if desired[158]. To correct for population stratification, PCs can be used to either calculate ancestry-adjusted genotypes to compute association statistics or can be used in logistic regression analysis as covariates[173, 174].

1.8.1.5 Replication

Replication of genetic findings discovered in GWAS is essential to minimize false positive results and prove the existence and validity of genetic associations[158]. Replication is defined as the ability to confirm a significant association in the same direction of the original signal in independent and comparable patient populations. Replication of genetic associations is required for the publication of significant GWAS findings, emphasizing the need to differentiate true positive results before any causal inferences can be drawn[175, 176].

Failure to replicate genetic associations, however, has been a reoccurring problem for GWAS[177]. As a result, it is critically important that a replication study repeat the ascertainment and study design of the original GWAS as closely as possible[168]. Specifically, minimizing inter-study heterogeneity and ensuring sufficient statistical power to detect the true effect size of the associated variants are important considerations to ensure the success of replication[175].

1.8.2 Post-GWAS Considerations

Nearly 90% of the genetic variants genotyped in GWAS lie in intergenic or intronic regions [178]. As a result, the majority of SNPs identified in GWAS does not exert a direct functional effect and are instead, thought to flag the genomic regions in which the causal variant exists. However, precise identification of functional variants has been achieved for only a small fraction of these associations[179]. This can be attributed to the fact that examining variation within coding sequences is the conventional approach for identifying causal SNPs. However, the non-coding nature of GWAS hits has led to the understanding and appreciation that genetic variants residing in regulatory regions can exert functional effects to drive the observed associations[180]. For example, non-coding SNPs can influence transcriptional output by altering transcription factor binding sites, enhancer regions, microRNA elements and other regulatory sequences. Currently, there still remains a lack of understanding and consistency on how to define causality for GWAS SNPs, especially for those in non-protein coding regions. In order to further delineate the real genetic variant driving the associations seen in GWAS, different applications and strategies can be utilized such as imputation, targeted sequencing and functional validation.

1.8.2.1 Imputation and Targeted Sequencing

Fine mapping the genetic region implicated by GWAS allows a narrowing of the region of the association and potentially the identification of the true causal variant. Fine mapping involves additional genotyping or sequencing of the region, or the use of known LD patterns and haplotype frequencies, for example, from the 1000 Genomes Project, in order to impute

genotypes for SNPs not directly genotyped in the initial study[168]. This can potentially uncover genetic variants with more obvious functional consequences and importantly, provide the needed evidence that ensures the right SNPs or regions are being pursued in subsequent sequencing and functional studies. Imputation can serve other purposes as well, including the identification of the most highly associated SNP with the phenotype and the facilitation of meta-analysis studies between multiple GWAS utilizing different genotyping platforms[181]. With high-density reference datasets made available by the 1000 Genomes Project, imputation techniques for GWAS can now infer the genotypes of all common variants with minor allele frequencies greater than 5% at an impressive accuracy of 90-95%[28].

Indirect LD-based methods, however, are not equipped to assess potentially deleterious variation such as rare (MAF<1%) and structural variants, and therefore may require targeted sequencing of the phenotype-associated genetic regions[182]. The specific regions to be sequenced can be guided by either the LD structure within the associated loci or an arbitrary physical boundary across the risk variant[180]. It is important to note that the GWAS approach is well powered to detect common variants with modest effects, but less effective in testing rare variation with much more significant deleterious consequences[155]. This is an inherent limitation of genome-wide genotyping arrays, which are designed to primarily capture common variation. However, with the constant improvement of next generation sequencing technologies resulting in more cost-effective and faster techniques, sequencing of whole regions flagged by initial GWAS discoveries is becoming an invaluable tool to uncover rare variants with direct functional consequences.

1.8.2.2 Functional Validation in Model Systems

Once sufficient evidence implicating the gene with the phenotype has been established, functional validation through *in vitro* and *in vivo* genotype-phenotype studies should be performed to support a mechanistic understanding of a genetic association. Species-specific phenotypic differences, however, can be a considerable caveat to studies employing non-human models. In addition, technical challenges of large DNA transfer (>15kb) into cultured cells and the ensuing loss of native genomic context complicate the study of genetic variants

identified in non-coding regions. Technical advances in the generation of human induced pluripotent stem cells (hiPSCs) and precision genome engineering are opening new avenues to investigate variants that were previously intractable to conventional study[183, 184]. hiPSCs derived from somatic cells of patients with the phenotype of interest, or from matched controls, can be differentiated into relevant cell-types of interest to facilitate functional validation. One key advantage of patient-derived hiPSCs is that they can be generated from patients who suffered the ADR, and likely contain the causal variant and thus, mechanistic studies can proceed without knowing the specific causal variant *a priori*. Similarly, advances in genomic editing using site-specific mutagenesis, e.g. CRISPR/Cas9 and TALEN technologies, will facilitate targeted approaches that not only preserve genomic context, but also validate the causality of a variant.

1.9 GWAS in Pharmacogenomics

Even with a thorough understanding of the PK and PD pathways of a drug, the pathophysiological mechanism of several ADRs remains unidentified. Genetic variation can affect drug response by modulating the function of less obvious proteins that influence the biological context of a drug reaction[23]. As a result, a genome-wide approach can be a suitable and effective approach to uncover novel pathways involved in the pathophysiology of serious ADRs. Such biological discoveries could not only make it possible to guide drug therapy on a personalized level, but also instigate the development of safer and more effective drug therapies[185]. Furthermore, GWAS in pharmacogenomics can also be used to rule out contributions of unidentified genes to drug response phenotype prior to initiating prospective clinical trials based on known pharmacogenomic variants[155]. For example, a GWAS on warfarin dosing provided necessary evidence for the National Heart, Lung and Blood Institute in the US to proceed with a large clinical trial examining the influence of *VKORC1* and *CYP2C9* on warfarin dosing[186].

1.9.1 Extreme Phenotypes Approach

The extreme phenotypes approach is a specific strategy proposed for pharmacogenomic GWAS on ADRs, as it can enrich the likelihood of observing a genomic signal by reducing noise arising from inaccurate phenotyping[44, 187]. Binary case-control dichotomization is convenient for genetic association studies, but ignores differences that may exist in the severity of drug toxicities. Phenotyping can thus be imprecise and inaccurate leading to misclassifications of cases and controls. In the extreme phenotypes approach, only patients with the most severe drug toxicities are compared to patients with no adverse effects. In fact, the severity in itself can be influenced by genetic variation. As the genetic variants that influence these traits may have larger genetic effect sizes, the extreme phenotypes approach can also compensate for the limited sample sizes available for pharmacogenomic studies by increasing the statistical power of a study[188].

Studies on adverse drug reactions have demonstrated the strength of this approach when performing GWAS with smaller cohorts. For example, Daly *et al.* performed a GWAS on flucloxacillin-induced liver injury in 51 cases and 282 controls, reporting an odds risk ratio of 45.0 (p-value = 8.7×10^{-33}) for carriers of the *HLA-B*5701* variant[189]. Another study investigating statin-induced myopathy identified a significantly associated SNP in *SLC01B1* (rs4363657, p-value = 4.1×10^{-9}) in a cohort of only 85 cases and 90 controls[190]. As pharmacogenomic studies shift from hypothesis-driven to more unbiased high-throughput approaches such as whole-genome genotyping and sequencing, the utilization of an extreme phenotypes approach can be especially useful for pharmacogenomic studies.

1.9.2 Considerations For GWAS on ADRs

As of 2012, at least 6 GWAS on ADRs have been published with significant genome-wide associations[191]. For the most part, these studies have only examined drug-induced liver injuries and skin hypersensitivity reactions. Indeed, a significant challenge of utilizing a genome-wide approach for studying ADRs is the limited sample sizes available for pharmacogenomic studies[155]. Sample size requirements for conventional GWAS are usually in the thousands in order to have sufficient statistical power to detect and replicate

genetic variants[192]. While similarly sized cohorts have been recruited for GWAS relating to drug response such as studies on warfarin and clopidogrel, obtaining adequate number of cases for rare idiosyncratic ADRs is significantly more challenging[191]. Depending on the precise drug involved, serious drug toxicity could occur in only 1 out of every 10,000 to 100,000 patients treated[192].

The application of an extreme phenotypes approach can help alleviate such sample size restrictions inherent to pharmacogenomic studies on ADRs. Another potential solution proposed by pharmacogenomics researchers is the formation of consortiums that would pool patient populations and expedite both discovery and replication efforts[193]. However since the effect sizes of pharmacogenomic variants have been historically reported to be much greater than that of complex disease variants, GWAS studies can still be a powerful approach to study ADRs even if carried out on smaller sample sizes.

1.10 Hypothesis and Thesis Objectives

This study hypothesizes that genetic variants in unidentified genes contribute to the susceptibility to cisplatin-induced hearing loss in children treated for a variety of malignancies. The overall aim of this study was to uncover novel gene(s) involved in the pathophysiological mechanism of cisplatin-induced ototoxicity.

Specific objectives of the study were:

1. To perform a genome-wide association study on cisplatin-induced hearing loss in a paediatric cohort
2. To replicate these genetic findings in a new independent cohort of well characterized paediatric patients
3. To develop a multi-marker prediction model incorporating clinical and genetic risk factors for the identification of individuals at risk of developing cisplatin-induced hearing loss

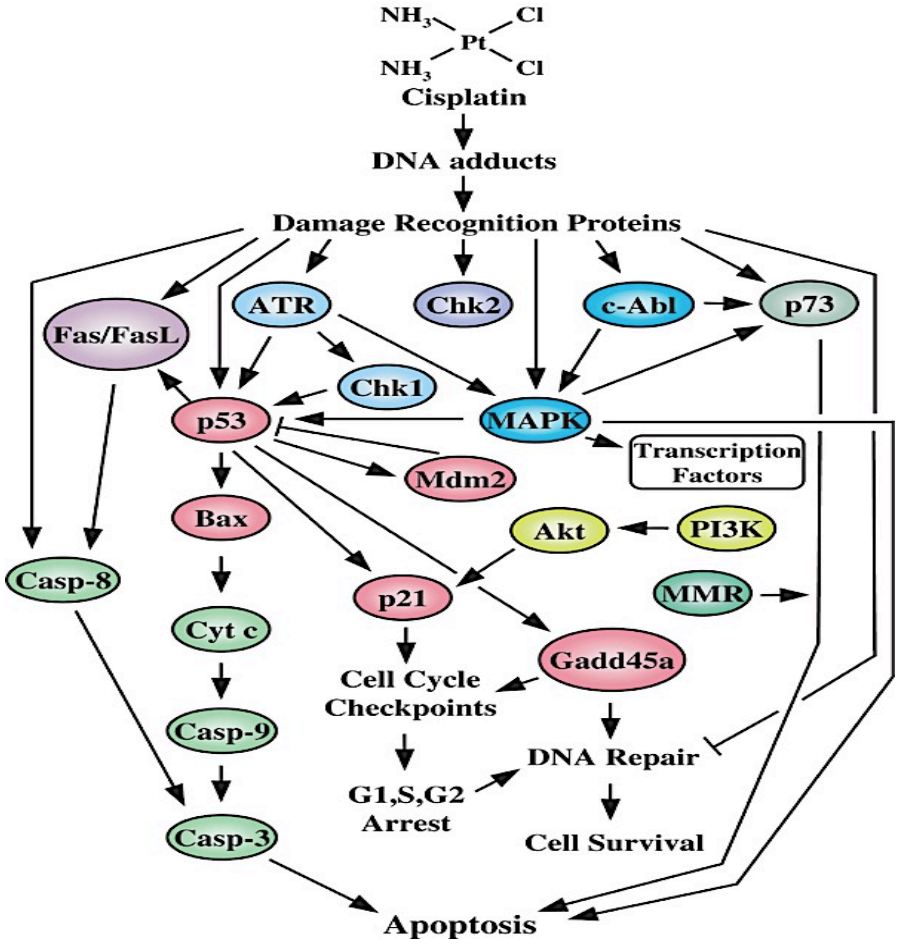


Figure 1.1 Cytotoxic Mechanism of Cisplatin

Formation of DNA crosslinks initiates the binding of several proteins such as hMSH2 of the mismatch repair system, HMGB1 and HGMB2, and the transcription factor TATA binding protein (TBP) [62]. These damage recognition proteins transduce DNA damage signals to several downstream effectors, activating several signalling pathways that ultimately end with the induction of apoptosis.
From Siddik, Z.H., *Cisplatin: mode of cytotoxic action and molecular basis of resistance.* Oncogene, 2003. **22**(47): p. 7265-79. Reprinted with permission from NPG.

Table 1.1 Ototoxicity Grading Criteria Used in Children

Grading Scheme	**Grade 0** Normal Hearing	**Grade 1** Mild Hearing Loss	**Grade 2** Moderate Hearing Loss	**Grade 3** Severe Hearing Loss	**Grade 4** Severe Hearing Loss
CTCAE Criteria 4.03[89]	<20 dB hearing loss at all frequencies	Hearing loss of ≥ 20 dB at 8 kHz	Hearing loss of ≥20 dB at 4 - 8 kHz	Hearing loss of ≥20 dB at 2 - 8 kHz	Hearing loss of ≥ 40 dB at 1 - 8 kHz Audiologic indication for cochlear implant and additional speech language related services indicated.
Brock Criteria[67]	< 40 dB hearing loss at all frequencies	Hearing loss of ≥ 40 dB at 8 kHz	Hearing loss of ≥40 dB at 4 kHz and above	Hearing loss of ≥40 dB at 2 kHz and above	Hearing loss of ≥40 dB at 1 kHz and above
Muenster Criteria[88]	<10 dB at all frequencies	>10 to <20 dB at all frequencies	Hearing loss ≥4 kHz, >20 dB **2a:** >20 to ⩽40 dB **2b:** >40 to ⩽60 dB **2c:** >60 dB	Hearing loss <4 kHz; >20 dB **3a:** >20 to ⩽40 dB **3b**: >40 to ⩽60 dB **3c:** >60 dB	Mean hearing loss <4 kHz; ≥80 Db
Chang Criteria[76]	< 20 dB at 1, 2, and 4kHz	Hearing loss of **1a:** ≥ 40 dB at 6-12 kHz **1b:** >20 to< 40 dB at 4kHz	Hearing loss of **2a:** ≥ 40 dB at 4 kHz and above **2b:** >20 to< 40 dB below 4kHz	Hearing loss of ≥ 40 dB at 2 or 3 kHz	Hearing loss of ≥ 40 dB at 1 kHz
SIOP Boston Criteria[87]	<20 dB hearing loss at all frequencies	Hearing loss of >20 dB above 4 kHz	Hearing loss of >20 dB at 4 - 8 kHz	Hearing loss of >20 dB at 2 - 8 kHz	Hearing loss of > 40 dB at 2 - 8 kHz

Table 1.2 Pharmacogenomic Associations of Methyltransferases (*TPMT* and *COMT*) and CIO

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Study
	Cases	Controls		Cases	Controls		
TPMT: **rs12201199:** A-allele: **OR 14.3, *P*=0.0097** **rs1142345:** G-allele: **OR 11.0, *P*=0.022** **rs1800460:** A-allele: **OR 11.0, *P*=0.022** ***COMT:*** **rs4646316:** G-allele: **OR 2.5, *P*=0.044** **rs9332377:** A-allele: **OR 5.1, *P*=0.024**	33 **Grade** 2-4	20 **Grade** 0	CTCAE v3	5 (0-16)	9 (0-16)	Osteosarcoma, neuroblastoma, brain tumour, germ cell tumour, etc.	Discovery Cohort Ross *et al.* (Dec 2009)[80]
TPMT: **rs12201199:** A-allele: **OR 10.0, *P*=0.0071** **rs1142345:** G-allele: **OR 5.8, *P*=0.044** **rs1800460:** A-allele: **OR 8.1, *P*=0.046** ***COMT:*** **rs4646316:** G-allele: **OR 2.5, *P*=0.0059** **rs9332377:** A-allele: **OR 6.2, *P*=0.0087**	73 **Grade** 2-4	36 **Grade** 0	CTCAE v3	5 (0-16)	9 (0-16)	Osteosarcoma, neuroblastoma, brain tumour, germ cell tumour, etc.	Replication Cohort Ross *et al.* (Dec 2009)[80]
TPMT: **rs12201199:** A-allele: **OR 6.1, *P*=0.0013** **rs1142345:** G-allele: **OR 4.5, *P*=0.011** **rs1800460:** A-allele: **OR 3.6, *P*=0.038** *COMT:* rs4646316: G-allele: OR 1.3, *P*=0.33 rs9332377: A-allele: OR 1.4, *P*=0.28	87 **Grade** 2-4	68 **Grade** 0	CTCAE v4	6 (1-25)	11 (0-18)	Osteosarcoma, neuroblastoma, brain tumour, germ cell tumour, etc.	Pussegoda *et al.* (2013)[154]
TPMT: rs12201199: *P*=0.50 rs1142345: *P*=0.27 rs1800460: *P*=0.40 *COMT:* rs4646316: *P*=0.062 rs9332377: *P*=0.61	149 **Grade** >0	64 **Grade** 0	CTCAE v4 & Chang Criteria	8 (3-21)	10 (3-20)	Medulloblastoma	Yang *et al.* (2013)[127]

Table 1.3 Pharmacogenomic Associations of Cisplatin Efflux Transporter (*ABCC3)* and CIO

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Study
	Cases	Controls		Cases	Controls		
ABCC3 rs1051640: OR 1.2, *P*=0.74	33 **Grade** 2-4	20 **Grade** 0	CTCAE v3	5 (0-16)	9 (0-16)	Osteosarcoma, neuroblastoma, brain tumour, germ cell tumour, etc.	Discovery Cohort Ross *et al.* (Dec 2009)[80]
***ABCC3* rs1051640: OR 2.8**, ***P*=0.0037**	73 **Grade** 2-4	36 **Grade** 0	CTCAE v3	5 (0-16)	9 (0-16)	Osteosarcoma, neuroblastoma, brain tumour, germ cell tumour, etc.	Replication Cohort Ross *et al.* (Dec 2009)[80]
***ABCC3* rs1051640: OR 1.8**, ***P*=0.036**	87 **Grade** 2-4	68 **Grade** 0	CTCAE v3	6 (1-25)	11 (0-18)	Osteosarcoma, neuroblastoma, brain tumour, germ cell tumour, etc.	Pussegoda *et al* (2013)[154]

Table 1.4 Pharmacogenomic Associations of *GSTM* and CIO

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Study
	Cases	Controls		Cases	Controls		
***GSTM3*B*:** **OR 5.37, *P*=0.023** (protective) No association with *GSTM1*	20 **Grade** 2-3	19 **Grade:** 0	Modified version of the Muenster Criteria	11.3 (3-22)	13.6 (7-19)	Osteosarcoma, neuroblastoma, medulloblastoma, germ cell tumour	Peters *et al.* (2000)[143]
***GSTM1* (+)[a]:** **OR 2.36**, ***P*=0.022**	Total number: 173 Number of cases and controls unknown		Ranked patients by 10th, 25th, 75th and 90th percentiles at 4000Hz	**Adults** Overall age: 42 (24-73)		Testicular cancer	Oldenburg *et al.* (Jan 2007)[144]
***GSTM1* (+):** **OR 1.81**, ***P*=0.025**	Total number: 238 (173 Patients of previous study included)		Self-reported Audiometry	**Adults** Overall age: 29 (15-64)		Testicular cancer	Oldenburg *et al.* (Dec 2007)[145]
No association with *GSTM1*	19 **Grade** 3-4	15 **Grade** unknown	CTCAE v3	Overall age: 6.8 (1.6-18)		Medulloblastoma	Barahmani *et al.* (June 2009)[149]
GSTM1 (+): OR 0.78, *P*=0.51	106 **Grade** 2-4	56 **Grade** 0	CTCAE v3	6 (0-16)	9 (0-19)	Osteosarcoma, neuroblastoma, brain tumour, germ cell tumour, etc.	Ross *et al.* (Dec 2009)[80]
No association with *GSTM3*B, GSTM1*	38 **Grade** 1-4	62 **Grade:** 0	National Cancer Institution Criteria	Overall age: 52 (23-65)		Ovarian cancer	Khrunin *et al.* (2010)[146]
No association with *GSTM1*	24	45	Ototoxicity if requiring hearing aids	Overall age: 6 (0.5-18)		Medulloblastoma	Rednam *et al.* (2012)[150]

[a] *GSTM1* positivity indicates if an individual carries at least one copy of the gene

Table 1.5 Pharmacogenomic Association of *GSTP* and CIO

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Study
	Cases	Controls		Cases	Controls		
No association with *GSTP1*	20 **Grade** 2-3	19 **Grade:** 0	Modified version of the Muenster Criteria	11.3 (3-22)	13.6 (7-19)	Osteosarcoma, neuroblastoma, medulloblastoma, germ cell tumour	Peters *et al.* (2000)[143]
***GSTP1* rs1695: GG genotype, OR 0.24, *P*<0.001**	Total number: 173 Number of cases and controls unknown		Ranked patients by 10th, 25th, 75th and 90th percentiles at 4000Hz	**Adults** Overall age: 42 (24-73)		Testicular cancer	Oldenburg *et al.* (Jan 2007)[144]
GSTP1 rs1695: GG genotype, OR 0.81, *P*=0.055	Total number: 238 (173 Patients of previous study included)		Self-reported Audiometry	**Adults** Overall age: 29 (15-64)		Testicular cancer	Oldenburg *et al.* (Dec 2007)[145]
GSTP1 rs1695: GG genotype, OR 0.71, *P*=0.61	106 **Grade** 2-4	56 **Grade** 0	CTCAE v3	6 (0-16)	9 (0-19)	Osteosarcoma, neuroblastoma, brain tumour, germ cell tumour, etc.	Ross *et al.* (Dec 2009)[80]
No association with *GSTP1*	38 **Grade** 1-4	62 **Grade:** 0	National Cancer Institution Criteria	Overall age: 52 (23-65)		Ovarian cancer	Khrunin *et al.* (2010)[146]
***GSTP1* rs1695: AG/GG risk genotype: OR 4.0, *P*=0.03**	24	45	Ototoxicity if requiring hearing aids	Overall age: 6 (0.5-18)		Medulloblastoma	Rednam *et al.* (2012)[150]
GSTP1 rs1695: *P*=0.16	87 **Grade** 2-4	68 **Grade** 0	CTCAE v3	6 (1-25)	11 (0-18)	Osteosarcoma, neuroblastoma, brain tumour, germ cell tumour, etc.	Pussegoda *et al* (2013)[154]

Table 1.6 Pharmacogenomic Associations of *GSTT* and CIO

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Study
	Cases	Controls		Cases	Controls		
No association with *GSTT1*	20 **Grade** 2-3	19 **Grade:** 0	Modified version of the Muenster Criteria	11.3 (3-22)	13.6 (7-19)	Osteosarcoma, neuroblastoma, medulloblastoma, germ cell tumour	Peters *et al.* (2000)[143]
GSTT1 positivity (+): OR 1.23, *P*=0.64	Total number: 173 Number of cases and controls unknown		Ranked patients by 10th, 25th, 75th and 90th percentiles at 4000Hz	**Adults** Overall age: 42 (24-73)		Testicular cancer	Oldenburg *et al.* (Jan 2007)[144]
GSTT1 positivity (+): OR 1.20, *P*=0.60 Note: 173 patients were included in previous study	Total number: 238 (173 Patients of previous study included)		Self-reported Audiometry	**Adults** Overall age: 29 (15-64)		Testicular cancer	Oldenburg *et al.* (Dec 2007)[145]
No association with *GSTT1*	19 **Grade** 3-4	15 **Grade** Unknown	CTCAE v3	Overall age: 6.8 (1.6-18)		Medulloblastoma	Barahmani *et al.* (2009)[149]
No association with *GSTT1*	38 **Grade** 1-4	62 **Grade:** 0	National Cancer Institution Criteria	Overall age: 52 (23-65)		Ovarian cancer	Khrunin *et al.* (2010)[146]
No association with *GSTT1*	24	45	Ototoxicity if requiring hearing aids	Overall age: 6 (0.5-18)		Medulloblastoma	Rednam *et al.* (2012)[150]
***GSTT1* positivity (+): OR 10.1, *P*=0.023**	54 **Grade** 1-4	14 **Grade:** 0	Brock	Overall age: 8.3 (0.3-15.3)		Osteosarcoma, Germ cell tumour, Neuroblastoma, Medulloblastoma, other	Choeyprasert *et al.* (2013)[147]

Table 1.7 Pharmacogenomic Associations of Megalin (*LRP2*) and CIO

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Study
	Cases	Controls		Cases	Controls		
***LRP2:* rs2075252** **A-allele; OR 3.45, *P*=0.016** *LRP2:* rs2228171 T allele; OR 2.32, *P*=0.087	25 **Grade** 2-4	25 **Grade** 0-1	Muenster	12.6 (6-22)	13.6 (5-19)	Osteosarcoma, neuroblastoma, medulloblastoma, germ cell tumour, teratoma, testicular cancer	Riedemann *et al.* (2008)[152]
LRP2: rs2075252 OR 1.2, *P*=0.55	106 **Grade** 2-4	56 **Grade** 0	CTCAE v3	6 (0-16)	9 (0-19)	Osteosarcoma, neuroblastoma, brain tumour, germ cell tumour, etc.	Ross *et al.* (Dec 2009)[80]
LRP2: rs2075252 OR 1.0, *P*=0.97	87 **Grade** 2-4	68 **Grade** 0	CTCAE v3	6 (1-25)	11 (0-18)	Osteosarcoma, neuroblastoma, brain tumour, germ cell tumour, etc.	Pussegoda *et al* (2013)[154]
No association with rs2075252 ***LRP2:* rs2228171** **C allele; OR 4.33 *P*=0.034**	54 **Grade** 1-4	14 **Grade:** 0	Brock	Overall age: 8.3 (0.3-15.3)		Osteosarcoma, Germ cell tumour, Neuroblastoma, Medulloblastoma, other	Choeyprasert *et al.* (2013)[147]

Chapter 2. Materials and Methodology

2.1 Study Populations

Study participants were recruited by the Canadian Pharmacogenomics Network for Drug Safety (CPNDS), a multicenter active surveillance consortium studying adverse drug reactions in children and adults in Canada[194]. 416 paediatric patients enrolled in the network were selected for the study. Study cohorts consist of patients who developed ototoxicity during or after the completion of cisplatin treatment (cases) and patients who received cisplatin and did not develop ototoxicity (controls). All patients were children (≤ 18 years of age) at the start of cisplatin therapy.

The discovery cohort used in this study (n = 282 patients; 188 cases, 94 controls) was recruited from 13 paediatric oncology units across Canada between July 2005 and November 2010. Paediatric patients (n = 82 patients; 55 cases, 27 controls) recruited after the first cohort formed a second, independent replication cohort. Patients with grade 1 ototoxicity (n=32; see **Table 2.1** for grading criteria) and signs of hearing impairment prior to cisplatin therapy (n=1) were excluded to better differentiate between ototoxic cases and normal hearing controls. To prevent the misclassification of late-onset cisplatin-induced ototoxicity cases as controls, patients that had not developed ototoxicity but had less than 8 months of follow up audiogram data (n=19) were excluded.

All 282 patients of the discovery cohort and 6 patients of the replication cohort were part of an original combined cohort (n=317) of a candidate gene study to identify and replicate SNPs significantly associated with cisplatin-induced hearing loss[80, 154]. From this original cohort, patients that had developed grade 1 ototoxicity (n=17) or had less than 8 month follow up audiogram data (n=12) were excluded.

2.2 Patient Clinical Characterization

All clinical, therapeutic and demographic information available as of February 28th 2014

were extracted from patients' medical records. Clinical characterization included: demographics (birth date, gender, self-reported ancestries of patient and family members), disease characteristics (diagnosis, date of diagnosis and date of relapse/death if applicable), medications (cumulative dose of cisplatin, all concomitant medications, start and end of treatments, radiation therapy) and audiological evaluations (audiograms, date of ototoxicity, level of intervention).

Patient records of all study participants were routinely updated through the ongoing active surveillance of the CPNDS Network in order to evaluate the late-effects of drug therapy, and may contain additional updated details than previously described in publications and other works. Data extraction and quality control was performed by highly trained ADR surveillors through the CPNDS network.

2.2.1 Evaluation of Cisplatin-induced Ototoxicity

Age and developmentally appropriate audiological evaluations (e.g., pure tone audiometry, otoacoustic emissions or brain stem auditory response) were performed prior to, during and following the completion of cisplatin therapy. Cisplatin-induced ototoxicity was classified using the Common Terminology Criteria for Adverse Events Version 4.0 (CTCAEv4) grading system (**Table 2.1**)[89]. Patients that developed moderate to severe hearing loss (grade 2, 3, or 4) were defined as cases.

Patients who exhibited normal hearing function with at least 8 months of follow-up audiometric testing from the start of cisplatin treatment were defined as controls. This threshold of follow-up time to define controls was the time at which 90% of cases had developed hearing loss (**Figure 2.1**).

2.3 Molecular Methods, Genotyping and Quality Control

2.3.1 DNA Extraction

Blood or saliva samples were collected from patients using Oragene DNA collection kits

(DNA Genotek, Ottawa, Canada). Genomic DNA was extracted using the QIAmp DNA purification system (Qiagen, Toronto, Ontario, Canada) according to the manufacturer's protocol. DNA samples were quantified using the Quanti-iT PicoGreen assay (Invitrogen, Eugene, OR, USA).

2.3.2 Discovery Stage Genotyping

DNA samples from 288 patients of the discovery cohort were genotyped using the Illumina Infinium HumanOmniExpress assay containing 738,432 SNPs (Illumina, San Diego, CA, USA). Samples were processed using the standard protocol on the Illumina Tecan Freedom EVO 150 liquid handler (Illumina, San Diego, CA, USA) and arrays were scanned using the Illumina HiScan system (Illumina, San Diego, CA, USA). Prior to genotyping, DNA samples were normalized to 50ng/µl according to the Illumina standard protocol. Every set of 96 samples included a negative control (1x Tris-EDTA buffer) and positive control samples of replicates.

In order to validate the genotype calls of the Illumina Infinium HumanOmniExpress assay, the most highly associated SNP in the GWA analysis was re-genotyped in 92 randomly selected patients from the discovery cohort using the Taqman SNP genotyping assay (Life Technologies, Streetsville, ON, Canada). The concordance rate between the two genotyping assays was 100%.

2.3.3 Genetic Markers (SNPs) Quality Control Procedure

All SNPs were initially clustered using the Illumina 740K cluster file and marker statistics were calculated. Thresholds of various quality control (QC) metrics available on GenomeStudio (Illumina, San Diego, CA, USA) were sequentially assessed to allow visual inspection of cluster plots. Manual re-clustering was subsequently performed for markers at the boundaries of each metric. In order, these filtering steps examined mean normalized intensity, mean normalized theta, cluster separation, heterozygote excess, replication errors and finally the SNP call rate. Markers unable to be improved by manual re-clustering and beyond the threshold of each of these measures were automatically zeroed. Additional details of the specific procedure used are outlined **(Table 2.2).**

After removing SNPs that failed QC filtering steps, marker statistics were re-calculated. Additional filtering of SNPs was then performed for the genetic association analysis, which included the removal of non-autosomal and intensity only SNPs, SNPs with call rate <95%, minor allele frequency (MAF) < 1% in both cases and controls, and SNPs that significantly deviate from Hardy-Weinberg equilibrium (HWE) within controls (p-value $<10^{-8}$). A total of 643,314 SNPs remained for the GWA discovery analysis.

2.3.4 Sample Quality Control Results

Patient samples (n=6) with <95% call rates were excluded from the discovery cohort for the GWA analysis and subsequently included in the replication cohort. Samples were then checked for gender mismatch and cryptic relatedness using identity by descent estimation. No samples had a reported gender opposite of what is determined using the heterozygosity rate and no duplicates or cryptic relationships were identified.

2.4 Patient Demographic Analysis

Clinical variables in cases and controls were compared using Fisher's exact test for categorical variables (age, gender, ethnicity, concomitant medication, tumour type, cranial irradiation) and Wilcoxon-Mann-Whitney rank-sum test for continuous variables (Cumulative dose, treatment duration, audiogram follow-up).

2.4.1 Principal Component Analysis to Assess Population Stratification

To assess for population stratification in the discovery dataset, principal component analysis was applied using the EIGENSTRAT method implemented in SVS/HelixTree 8.1.1[173].

2.5 Genetic Association Analysis

Power calculations were performed using QUANTO v1.2.4[195]. The statistical significance of association for each genetic marker was tested using the Cochram-Armitage trend test for

the primary analysis of the GWA stage. Logistic regression with an additive model was performed to estimate the effect size (odds ratio). The following were further examined using the Chi-squared test: allelic, genotypic, and dominant model. Fisher's exact test was used if allele or genotype values were less than 5. For each test, Bonferroni correction was applied for multiple testing resulting in an overall genome-wide significance level of *p*-value = 7.77×10^{-8}. The top selected variants were investigated in the replication cohort using a significance level of *p*-value =0.05.

To correct for significant clinical factors and population stratification, genetic variants were further evaluated in a multivariate logistic regression model using PCA-adjusted genotype data and age, vincristine and germ cell tumour as regression covariates. These clinical variables were selected through forward logistic regression of all significant clinical factors in the combined cohort.

2.5.1 Imputation Analysis

Imputation analysis in regions of interest that showed significant associations in the genome-wide analysis was performed using the software package BEAGLE 3.3.2 and 1072 patients from the "Genomes.phase1_release_v3" of the 1000 Genomes Project as the reference population[196]. BEAGLE produces posterior genotype probabilities for imputed genotypes, and those imputed SNPs with maximum posterior probability lower than 0.9 were removed. We further removed imputed SNPs with MAF < 0.01. A total of 17,756 SNPs were imputed from 720 directly genotyped SNPs in the discovery cohort.

2.5.2 Multivariate Prediction Models

Genetic variants of interest were further evaluated in a multivariate logistic regression model, including previously reported clinical risk factors (age, concomitant vincristine, germ cell tumour type and cranial irradiation) and established genetic risk markers (*TPMT* rs12201199, *COMT* rs46430 and *ABCC3* rs1051640). Predicted probabilities of each patient were calculated for the following prediction models: (i) old genetic variables (ii) new genetic

variables (iii) clinical variables only (iv) clinical and old genetic variables (v) clinical and new genetic variables. Receiver operating characteristic (ROC) curves for each model were generated and area under the curve (AUC) estimates with 95% confidence intervals were calculated using the ROC plot function based on the predicted probabilities of the logistic regression model for the respective clinical and genetic variables. ROC curves of the new and old prediction models were statistically compared using DeLong's method[197] implemented in the *R*-package pROC[198].

2.5.3 Comparison of Prediction Models

To directly compare prediction models of previous work[154] with our results, risk groups for cisplatin-induced hearing loss were designated for each patient sample based on the predicted probabilities from the logistic regression analysis of each respective model (i-v). The thresholds used to determine low or high risk was the average of the median predicted value of controls and the median predicted value of cases. The average value for each model was approximately 0.65 and therefore patients with predicted probabilities (<0.65) were defined as low risk and (>0.65) as high risk. To examine the predictive performances of risk groups from the previous and new models, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for each defined threshold **(Figure 2.2)**.

2.5.4 Clinical Multi-Risk Group Prediction Model

To improve the granularity of predictive test results for clinical application, we developed a preliminary multi-risk-group risk predictive model that incorporates both clinical and new genetic variables. Individuals were stratified into five risk groups based on thresholds determined from the inter-quintile ranges of the predicted probabilities for controls and cases: **Risk I** (< 20^{th} percentile of controls [< 0.15]), **Risk II** (between 20^{th} and 60^{th} percentile of controls [0.15-0.50]), **Risk III** (> 60^{th} percentile of controls and < 40^{th} percentile of cases [0.50-0.75]), **Risk IV** (between 40^{th} and 80^{th} percentile of cases [0.75-0.90]) and **Risk V** (> 80^{th} percentile of cases [>0.90]). Risk I corresponds to the group of patients with the lowest risk; Risk V corresponds to the group with the highest risk. The retrospective risk of

ototoxicity in each patient group was calculated by dividing the number of cases by the total number of patients **(Figure 2.3)**.

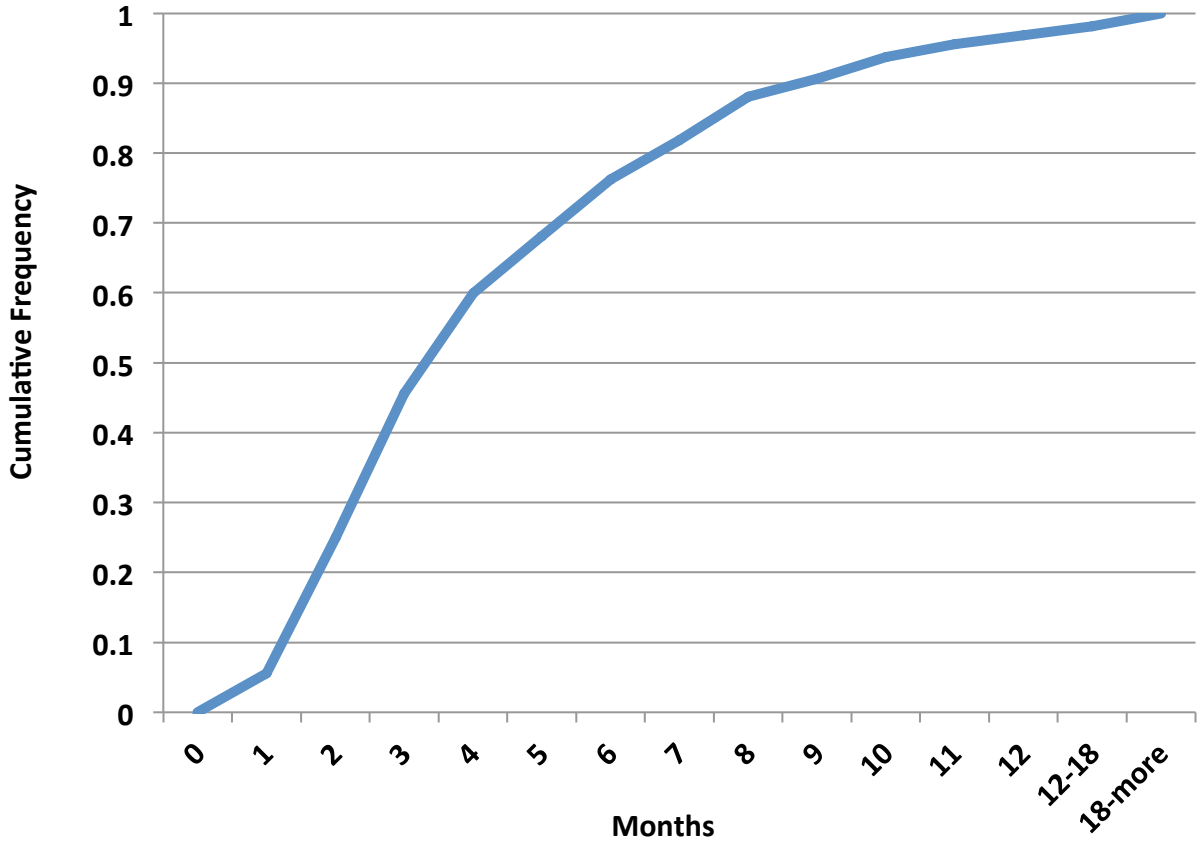


Figure 2.1 Time to First Audiometric Symptoms of Grade 2 or Higher Ototoxicity in the Cases of the Discovery Cohort

Audiogram dates indicating the first sign of hearing loss were used to identify time to ototoxicity. Patients that had no audiogram data within the first 6 months of treatment were excluded from this analysis as the specific date ototoxicity first occurs could not be accurately captured (n=28).

	Phenotype Present	Phenotype Absent	
Positive Test	True Positives (TP)	False Positivevs (FP)	**Positive Predictive Value (PPV)** = TP/(TP+FP)
Negative Test	False Negatives (FN)	True Negatives (TN)	**Negative Predictive Value (NPV)** = TN/(TP+FP)
	Sensitivity = TP/(TP+ FN)	**Specificity** = FP/(FP+TN)	

Figure 2.2 Calculation of Sensitivity, Specificity, Positive and Negative Predictive Value

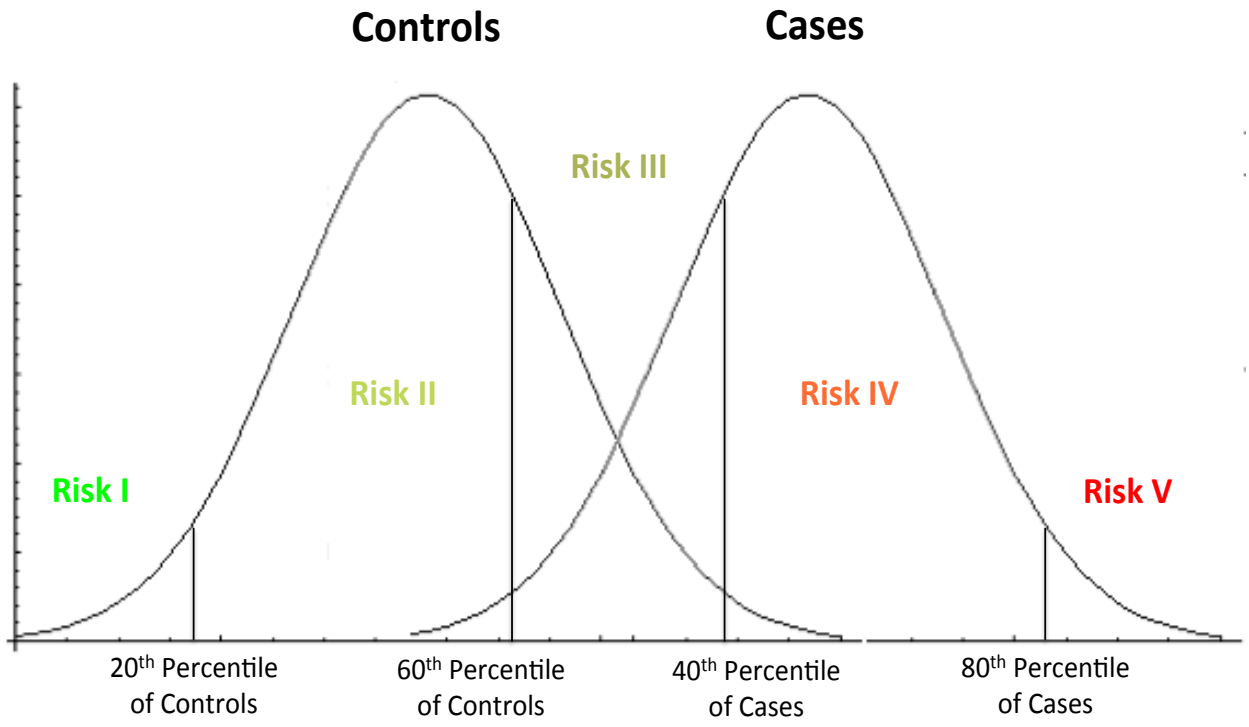


Figure 2.3 Probability Thresholds for Clinical Multi-Risk Group Modelling

To improve the granularity of predictive test results for clinical application, we developed a preliminary multi-risk-group risk predictive model that incorporates both clinical and new genetic variables. Individuals were stratified into five risk groups based on thresholds determined from the inter-quintile ranges of the predicted probabilities for controls and cases: Risk I (< 20th percentile of controls [< 0.15]), Risk II (between 20th and 60th percentile of controls [0.15-0.50]), Risk III (> 60th percentile of controls and < 40th percentile of cases [0.50-0.75]), Risk IV (between 40th and 80th percentile of cases [0.75-0.90]) and Risk V (> 80th percentile of cases [>0.90]).

Table 2.1 CTCAEv4 Criteria for the Classification of Cisplatin-induced Ototoxicity

Grade of Hearing	Audiological Criteria
Grade 0 – Normal Hearing	<20 dB hearing loss at all frequencies
Grade 1 – Mild Hearing Loss	Hearing loss of ≥ 20 dB at 8 kHz
Grade 2 – Moderate Hearing Loss	Hearing loss of ≥20 dB at 4 - 8 kHz
Grade 3 – Severe Hearing Loss	Hearing loss of ≥20 dB at 2 - 8 kHz
Grade 4 – Severe Hearing Loss Requiring Medical Intervention	Hearing loss of ≥ 40 dB at 1 - 8 kHz Audiologic indication for cochlear implant and additional speech language related services indicated

Table 2.2 GenomeStudio SNP QC Procedure for Discovery Stage Genotyping

Criteria	Quality Control Procedure
Mean Normalized Intensity	Exclusion of low-intensity markers • Removed markers with AB R mean <= 0.25
Mean Normalized Theta	Heterozygote clusters shifted too close to homozygote clusters • Removed markers with AB T mean < 0.2 or ≥ 0.8 • Manually inspect plots for markers on the boundaries (0.2 ≤ AB T mean ≤ 0.25; 0.75 ≤ AB T mean < 0.8)
Cluster Separation	Poor separation on theta axis • Removed markers with cluster separation < 0.3 • Manually inspect plots for markers markers with cluster separation between 0.3-0.32
Heterozygous Excess	More heterozygotes called than expected under HWE • Removed markers with het excess ≥ 0.2
Heterozygous Excess (False Homozygotes)	Fewer heterozygotes called than expected under HWE - Visually inspected subset of intensity plots for markers with het excess ≤ -0.3 i. Multiple minor-allele homozygotes • Removed markers with R dev ≥ 0.05 ii. Wide homozygote clusters • Manually inspect markers with AA or BB T Dev ≥ 0.05 and removed markers with multiple clusters on T axis called together as homozygotes iii. Wide heterozygous clusters • Manually inspect markers with AB T Dev ≥ 0.05 and removed markers with all three clusters being called as a single heterozygous cluster
Replication Error	Removed any remaining markers with >3 replication errors
Call Rate	Removed all remaining markers with call rate < 95%

Chapter 3: Results

3.1 Patient Clinical Characteristics

Patient characteristics are provided in **Table 3.1** for the discovery, replication and the combined cohorts. The proportion of patients who developed cisplatin-induced hearing loss was 66.6% (188 cases) and 67.1% (55 cases) in the discovery and replication cohorts respectively. In the discovery cohort, 106 patients (56.4%) were diagnosed with hearing impairment of grade 3 or higher compared to 25 patients (45.5%) of the replication cohort. For all individuals who developed hearing loss, the median time to ototoxicity was 3.7 months (**Figure 2.1**).

In both the discovery and replication cohort, the median age at initiation of cisplatin therapy was significantly lower in cases compared to controls (Combined, 4.0 vs 10.1 years; *p*-value = 3.41×10^{-6}). Furthermore, the proportion of patients treated with concomitant vincristine was significantly higher in cases than controls in both the discovery (59.6% vs 20.2%, *p*-value = 2.70×10^{-10}) and replication cohort (58.2% vs 22.2%, *p*-value = 0.0024; Combined, *p*-value = 2.03×10^{-12}). Patients diagnosed and treated for germ-cell tumours developed less ototoxicity in both the discovery (*p*-value = 1.90×10^{-4}) and replication cohort (*p*-value = 0.0078; Combined, *p*-value = 4.50×10^{-6}). In the combined analysis, there was a significant difference in the rate of hearing loss between patients treated for brain tumours (*p*-value = 0.028), hepatoblastomas (*p*-value = 0.015), neuroblastomas (*p*-value = 0.033) and osteosarcomas (*p*-value = 0.036). However, these were not significantly different in the individual discovery and replication cohorts. Follow up audiological testing after cisplatin therapy was longer in cases than controls in the discovery cohort (6.1 vs 4.7 years, $p = 0.010$) but not in the replication cohort (3.9 vs 3.7 years, *p*-value = 0.58; Combined, 5.7 vs 4.3 years, *p*-value = 0.038). This is an expected finding that occurs after hearing loss is detected. Once clinically relevant hearing loss is detected, patients receive follow-up rehabilitation services (e.g., hearing aid placement, cochlear implants) and long term audiometric monitoring.

3.2 Power Calculations

Assuming an unmatched case-control design (2:1) for gene only effects using a log-additive genetic model, the GWAS discovery analysis of this study was calculated *a priori* to have a statistical power between 33-93% (mean 75%) based on allele frequencies ranging 10-25%, an effect size of 5.0 and a two-sided type 1 error rate of *p*-value = 7.77×10^{-8} (0.05/632,314 SNPs) using QUANTO v1.2.4 (**Figure 3.1**).

3.3 Genome-wide Association Results

A genome-wide association analysis in the discovery cohort of 282 patients yielded a significant association in the toll-like receptor 4 (*TLR4*) locus of chromosome 9 (**Figure 3.2**, **Table 3.2)**. The most highly associated SNP, rs960312, conferred a strong protective effect against cisplatin-induced hearing loss (*p*-value = 1.19×10^{-8}, OR = 0.22) (**Table 3.3**). The next most significant association with rs5030717 (*p*-value = 2.85×10^{-7}, OR = 0.19) was located in the second intron of *TLR4*, which was in high linkage disequilibrium (LD) with rs960312 (r^2 = 0.84 using the CEU component of HapMap) (**Figure 3.3**). As a validation step, rs960312 was re-genotyped in 92 randomly selected patients using a TaqMan SNP assay. The concordance rate was found to be 100%.

3.4 Detection of Population Stratification

To assess for population stratification in the discovery dataset, the top 10 eigenvalues or principal components (PCs) were calculated using PCA. Graphical representation of the top 2 PCs demonstrated that the PCA-generated clusters correlated accurately with self-reported ethnicities, as previously described (**Figure 3.4)**[199]. Association statistics accounting for population structure were calculated with genotype data adjusted for the top PCs (*K*=5*)*. The number of significant PCs was determined by plotting the top eigenvalues against each other until no discernible clusters of groups were identifiable by visual inspection (**Figure 3.5**). The proportion of variation explained by the top 5 PCs was 75.1%. The genomic control factor (λ_{GC} = 1.012) was estimated from the median chi-squared statistics of the trend test. A genomic

control factor close to 1.0 indicates a minimal or benign influence from stratification and other confounding factors such as family structure or cryptic relatedness[172].

3.5 Correction for Ancestry and Clinical Covariates

To account for population stratification, an association analysis was performed on PCA-adjusted genotype data using the top 5 PCs (**Table 3.3**). As there were significant clinical variables that differed between cases and controls, we further examined the *TLR4* association in the discovery cohort using logistic regression analysis with the top 5 PCs, age, vincristine and germ cell tumour as regression covariates. The association of *TLR4* rs9603132 remained significant with a similar effect size after accounting for population stratification (*p*-value = 2.90×10^{-8}, OR = 0.23) and the association remained strongly associated with an even larger effect size (lower odds ratio) when furthering correcting for these significant clinical variables (*p*-value = 3.20×10^{-7}, OR = 0.20). Similarly, the association and effect size of rs5030717 (*p*-value = 2.85×10^{-7}, OR = 0.19) became stronger when correcting for PCA-ancestry (*p*-value = 4.71×10^{-8}, OR = 0.17) and when accounting for clinical factors as well (*p*-value = 6.12×10^{-7}, OR = 0.15). *TLR4* rs960312 and rs5030717 remained the most highly associated GWAS findings when correcting all SNPs for ancestry and clinical factors.

3.6 Replication of TLR4 rs960312 and rs5030717

Replication of genetic findings discovered in GWAS is an essential step to minimize false positive results and prove the existence and validity of genetic associations[158]. *TLR4* rs960312 and rs5030717 were selected for genotyping in the replication stage based on evidence of a significant or nearly significant association on a genome wide level (*p*-value < 7.77×10^{-8}) in the initial discovery cohort. Independent evidence in literature supporting the involvement of *TLR4* in cisplatin-induced ototoxicity in cell-based and mouse models was also considered in the selection of these two SNPs[200].

We confirmed the discovery associations of *TLR4* rs960312 and rs5030717 in an independent paediatric cohort of 82 children (*p*-value = 0.018, OR = 0.37; *p*-value = 0.033, OR = 0.36,

respectively) (**Table 3.4**). After correcting for age, concomitant vincristine and germ cell tumour, the associations for rs960312 and rs5030717 in the replication cohort became more significant with even stronger odds ratios (*p*-value = $7.30x10^{-4}$, OR = 0.14; *p*-value = 0.0066, OR = 0.18, respectively). Combining the discovery and replication cohorts, *TLR4* rs960312 and rs5030717 were significantly associated with a decreased risk of cisplatin-induced hearing loss after adjusting for all clinical factors (*p*-value = $1.01x10^{-9}$, OR = 0.20 and *p*-value = $5.32x10^{-8}$, OR = 0.18, respectively) (**Table 3.4**). The protective 'G' allele of rs960312 was carried by 40 (16.5%) cases and 55 (45.5%) controls in the combined cohort **(Table 3.5**).

3.7 Subgroup Analysis of Severe Ototoxicity

The extreme phenotypes approach is a specific strategy proposed for pharmacogenomic GWAS on ADRs, as it considers the differences that may exist in the severity of drug toxicities. In fact, the severity of ototoxicity in itself can be influenced by genetic variation. To examine the potential role of TLR4 in patients with more severe cisplatin-induced hearing loss, a subgroup analysis of only patients with severe ototoxicity (grade 3 and higher) was conducted (**Table 3.6**).

In this extreme phenotype subgroup (131 cases, 121 controls), the *TLR4* associations were found to be strongly associated with severe CIO, with a nearly 50% increase in their effect (decrease in odds ratios) as protective variants (combined, rs960312 *p*-value = $3.84x10^{-10}$, OR = 0.11; rs5030717 *p*-value = $6.41x10^{-8}$, OR = 0.10) **(Table 3.6)**. These results provide further evidence to validate the *TLR4* findings as it examines only patients with the most definitive phenotype. Furthermore, the lower frequency of rs960312 and rs5030717 in the most extreme cases of hearing loss suggests the possibility that the protective effects of the *TLR4* variants can mitigate the severity of CIO.

3.8 Imputation of TLR4 Locus

To fine-map the association signal around the *TLR4* locus, we performed a genotype imputation analysis of all known SNPs in a 2.3Mb Chromosome 9 sub-region (Chr9: 119198.3 – 121525.4Kb) centred on rs960312 (**Figure 3.6**). A total of 17,756 SNPs were

imputed using 720 markers genotyped in the discovery cohort. An intergenic SNP (rs1329060) 5.3kb upstream of rs960312 showed a stronger association ($P = 4.63x10^{-9}$, OR = 0.22) than rs96012, but the two SNPs were in complete LD ($r^2 = 1.0$, D' = 1.0).

3.9 TLR4 SNPs Genotyped on GWAS Panel

As rs960312 and rs5030717 are located in the intergenic and intronic regions of the *TLR4* gene, we sought to identify additional, potentially causal variants in *TLR4*. In total, 142 SNPs in the *TLR4* locus was genotyped and analysed in the discovery cohort (**Table 3.7).** All of the *TLR4* variants that passed a significance level of ($P < 0.05$) were in intronic or outside of the *TLR4* coding region.

3.10 Linkage Disequilibrium Analysis of TLR4 Region

Two coding polymorphisms in *TLR4*, rs4986790 and rs4986791, have been reported in literature to have functional consequences[201]. Performing a linkage analysis using the CEU component of HapMap, both SNPs were in low LD with the rs5030717 intronic variant (r^2=0.0004) (**Figure 3.3)**. These two coding variants were genotyped in the discovery cohort in the Illumina OmniExpress GWAS panel. Both rs4986790 and rs4986791 were not significantly associated with cisplatin-induced ototoxicity (*p*-value = 0.096, OR = 2.13; *p*-value = 0.52, OR = 1.29, respectively) (**Table 3.7)**. Interestingly, rs496790 did show a trend to association but was [10^7]-fold less significant that rs960312. The genotyping of the coding SNPs was validated using a TaqMan assay.

The distal regulatory region between nucleotides -3228 and -743 in human *TLR4* is considerably variable with eight common SNPs identified in this region[202]. The SNP rs10759932 in the promoter region of *TLR4* (-1604) was the only non-intergenic and non-intronic polymorphism in high LD with rs960312 (($r^2 = 1.0$, D' = 1.0). This variant was subsequently genotyped in the discovery cohort and demonstrated the same significance level and effect sizes as rs960312.

3.11 Descriptive Characteristics of Prediction Model

To assess whether *TLR4* rs960312 could better predict CIO susceptibility beyond the currently established clinical (age at treatment, concomitant vincristine treatment, germ-cell tumours and cranial irradiation) and genetic (*TPMT* rs12201199, *COMT* rs4646316 and *ABCC3* rs1051640) risk factors, we compared multi-marker prediction models incorporating these factors with and without *TLR4.* Including *TLR4* rs960312 in the old genetic model (*TPMT, COMT, ABCC3)* significantly increased the AUC of the prediction model from 0.669 to 0.745 (*p*-value = 0.0054) **(Figure 3.7)**. The AUC of a combined model including both clinical and genetic variables was also significantly improved when incorporating the *TLR4* variant (AUC, increased from 0.802 to 0.830, *p*-value = 0.038) (**Figure 3.8**). A prediction model including both genetic and clinical variables was shown to be significantly more predictive than clinical factors alone (AUC, clinical 0.753 vs. clinical + genetic 0.830, *p*-value = 0.00078).

Using predicted probabilities generated from a logistic regression analysis, risk groups (high and low) were developed to further evaluate the previous and new prediction models (**Table 3.9**). Compared to the previous genetic model, a new model including *TLR4* rs960312 was able to better capture patients who had developed ototoxicity (sensitivity increased from 20.7% to 60.6%) at the cost of missing patients who were controls for CIO (specificity decreased from 95.7% to 86.2%). The accuracy of patients predicted in the high risk group was similar in the old and new genetic model (PPV, 90.7% vs 89.8%) but the differentiation of low risk patients was greatly improved (NPV 37.7% to 52.3%). When combining clinical and genetic variables, including the *TLR4* variant improved the sensitivity (20.2% to 30.3%) of the prediction model with a slight decrease in specificity (98.9% to 96.8%). Furthermore, the accuracy of predicting patients slight decreased in the high risk group (PPV, 97.4% to 95.0%) but increased in the low risk group (NPV, 38.3% to 41.0%).

3.12 Clinical Multi-Risk Prediction Model

In order to present more clinically actionable information about an individual patient's risk of CIO, we developed a risk prediction model that classifies patients into five risk groups (I, II, III, IV, V) to better translate the granular risk information derived from the predictive test to patients (**Figure 3.9**). The retrospective incidence of hearing loss in each of the risk groups was 95.7% (n=90), 82.8% (n=58), 63.0% (n=81), 36.2% (n=58) and 6.7% (n=15) respectively.

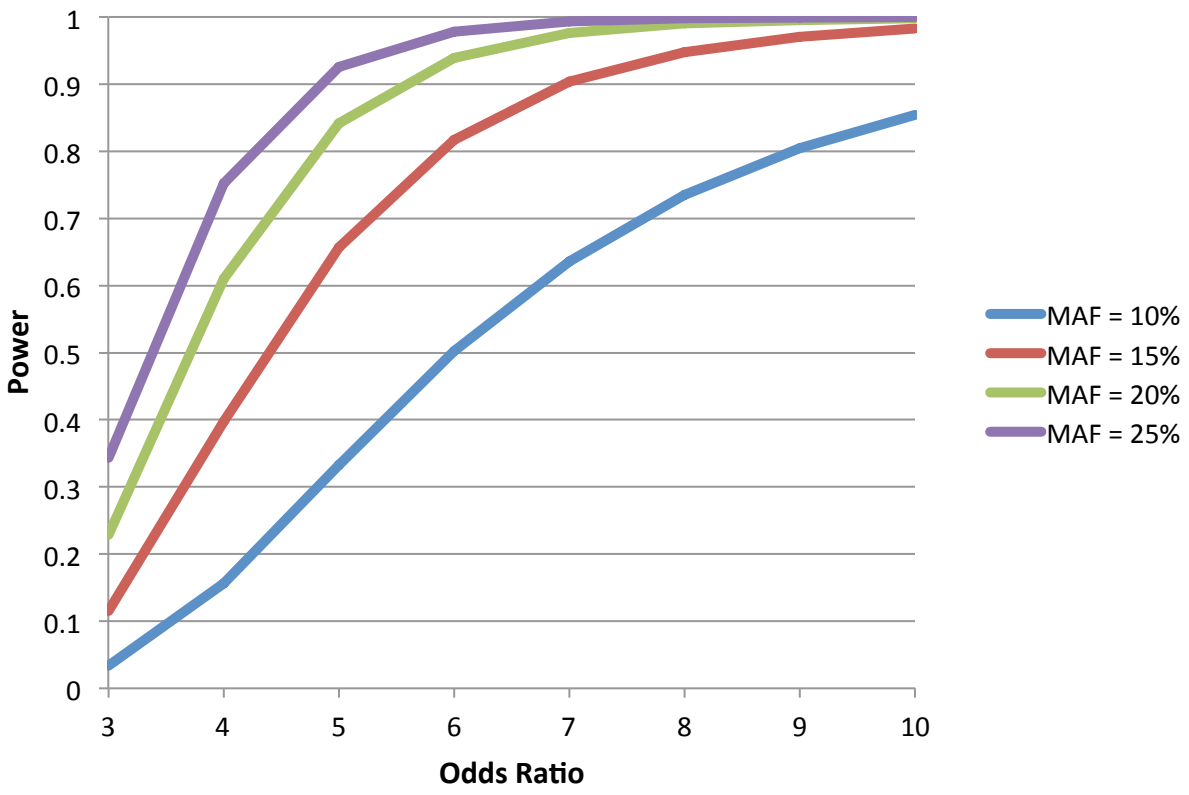


Figure 3.1 GWAS Statistical Power of Detection in Discovery Cohort (n=282)

Statistical power for the GWA study was calculated based on allele frequencies ranging 10-25%, effect sizes ranging from 3 to 10, a two-sided type 1 error rate of 7.77×10^{-8} (0.05/632,314 SNPs) and assuming a case-control design (2:1) for gene only effects using a log-additive genetic model.

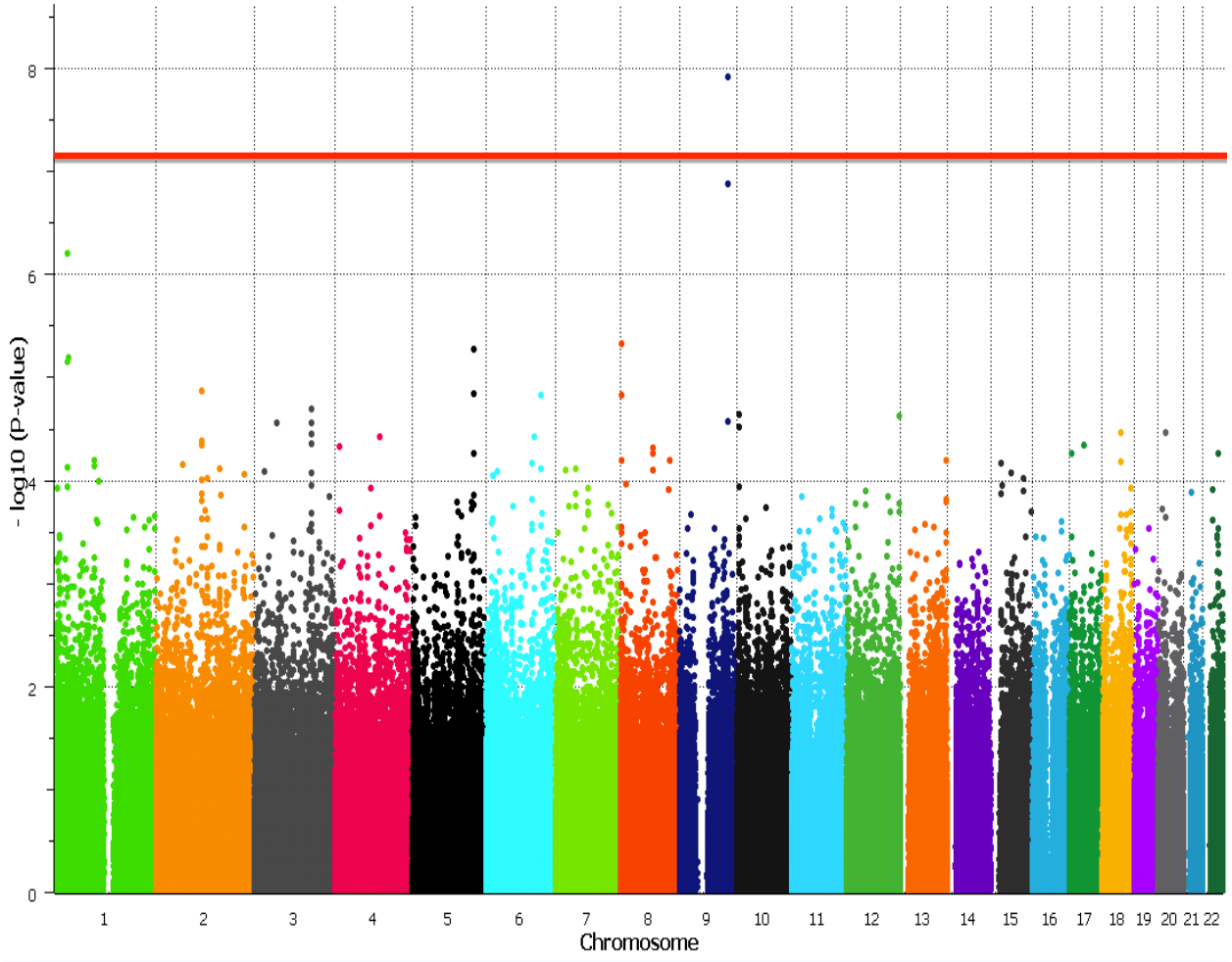


Figure 3.2 Manhattan Plot of all SNPs of Genome-wide Association Analysis

This figure shows the genome-wide association results of CIO in the discovery cohort of 282 patients. Chromosomal position of each SNP is plotted on the x-axis and *p*-values ($-\log_{10}$ Cochrane-Armitage trend test p-value) are plotted on the y-axis. The horizontal red line indicates the Bonferroni threshold of statistical significance (*p*-value < 7.77×10^{-8}).

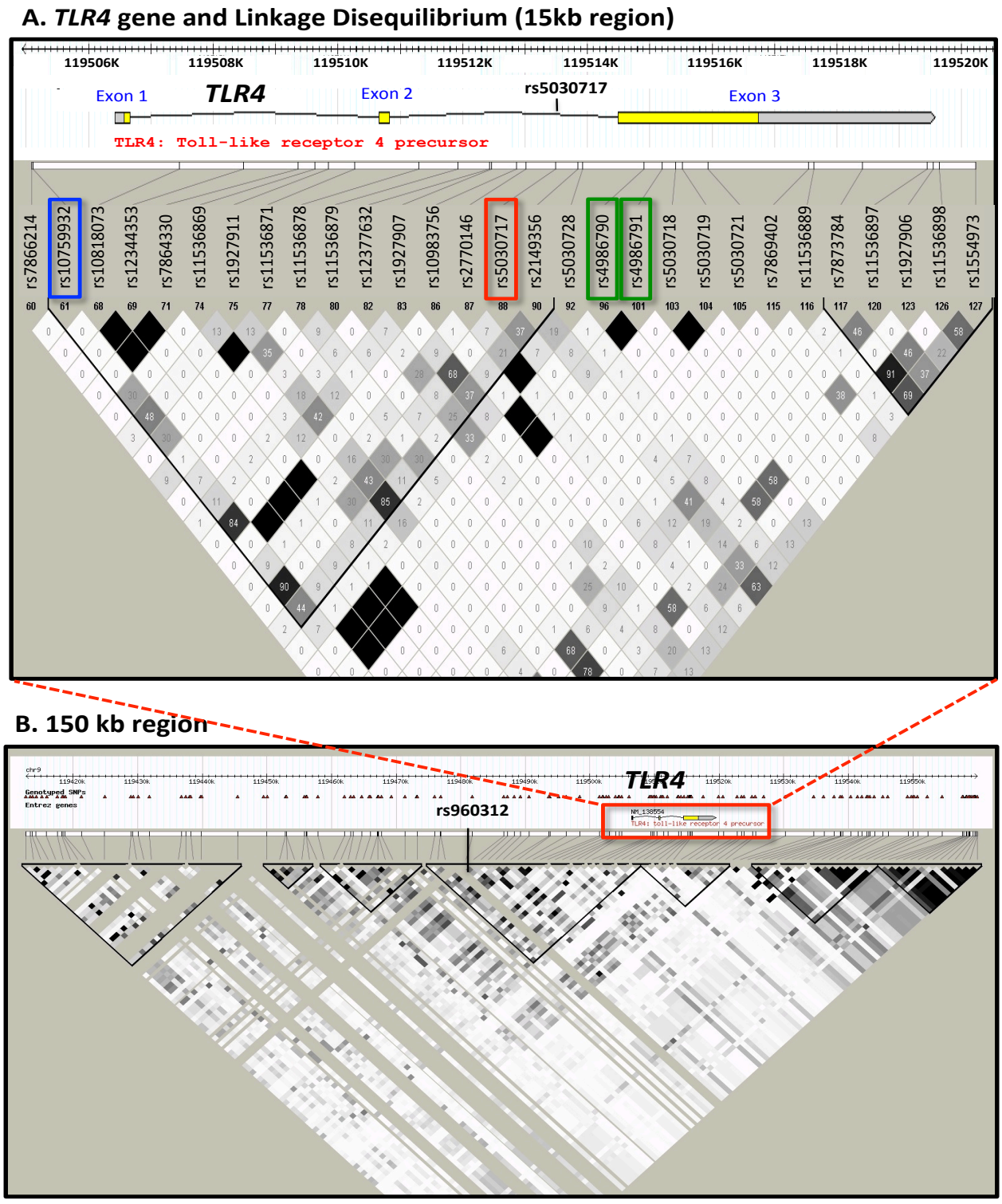


Figure 3.3 Linkage Disequilibrium (LD) Plots of 15kb and 150kb regions containing TLR4

(**A)** A LD plot of a 15kb region encompassing the *TLR4* gene is shown. Rs5030717 (boxed in red) is located in the 2nd intron between exon 2 and 3. Two coding polymorphisms rs4986790 and rs4986791 (boxed in green) are not in LD with rs5030717 (r^2=0.0004). Located in the *TLR4* promoter, rs10759932 (boxed in blue) is in high LD with rs5030717 and is reported in literature to have functional consequences *in-vitro*. (**B)** A LD plot of a larger 150kb region containing *TLR4*.

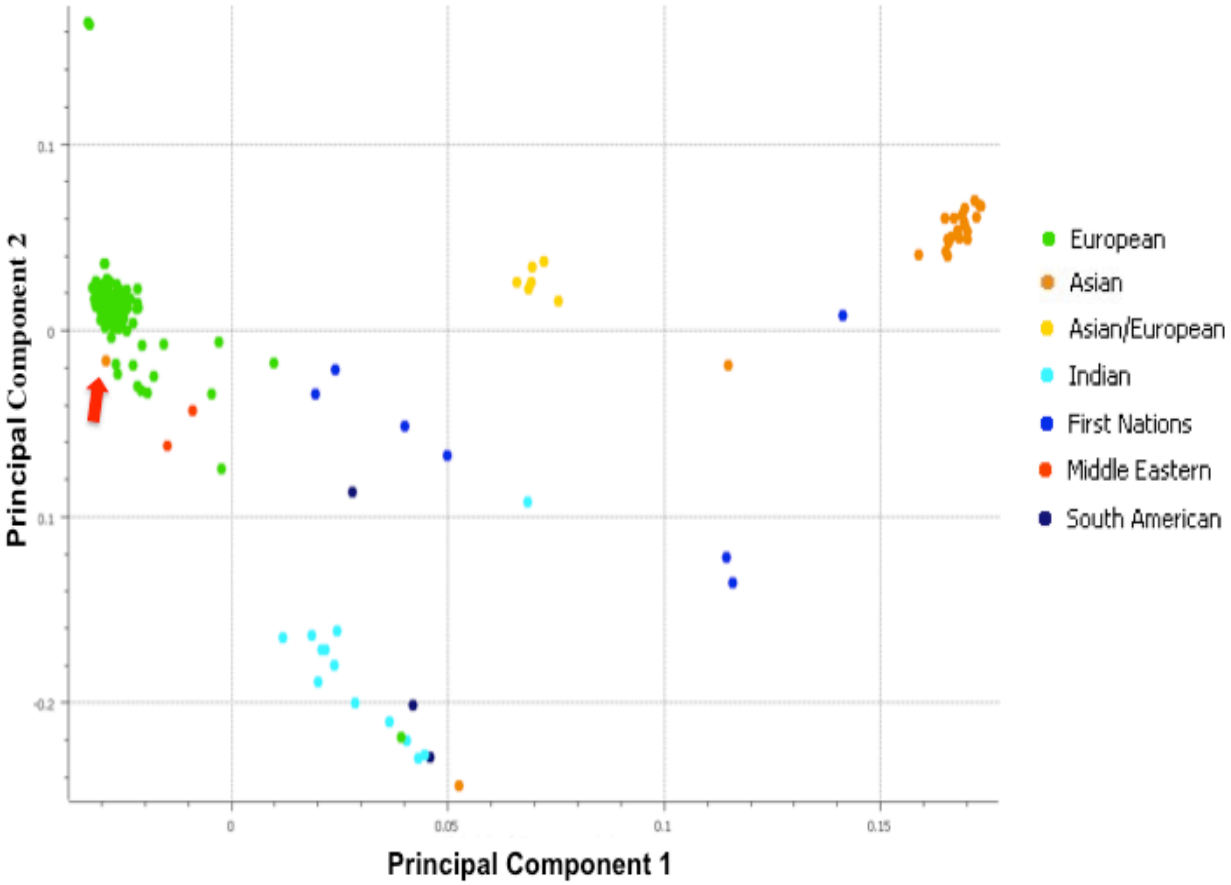


Figure 3.4 Patient Ancestry Determined by Principal Component Analysis (PCA) vs Self-reported Ethnicity

The first two principal components are plotted to visualize the distribution of population ancestry in the discovery cohort. Patients are colour differentiated by self-reported ethnicities as provided in the legend. The clustering of patients based on self-reported ancestries demonstrates the accuracy of PCA to infer population structure using only genotype data. The proportion of variation explained by the 1st PC is 45.4%. One patient with self-reported Asian ethnicity was unexpectedly ascertained into the European cluster (Red Arrow). By examining more PCs, this patient was found to have significant variation in the 3rd, 4th and 5th PC suggesting that using the top 2 PCs alone is inadequate to sufficiently correct for population stratification.

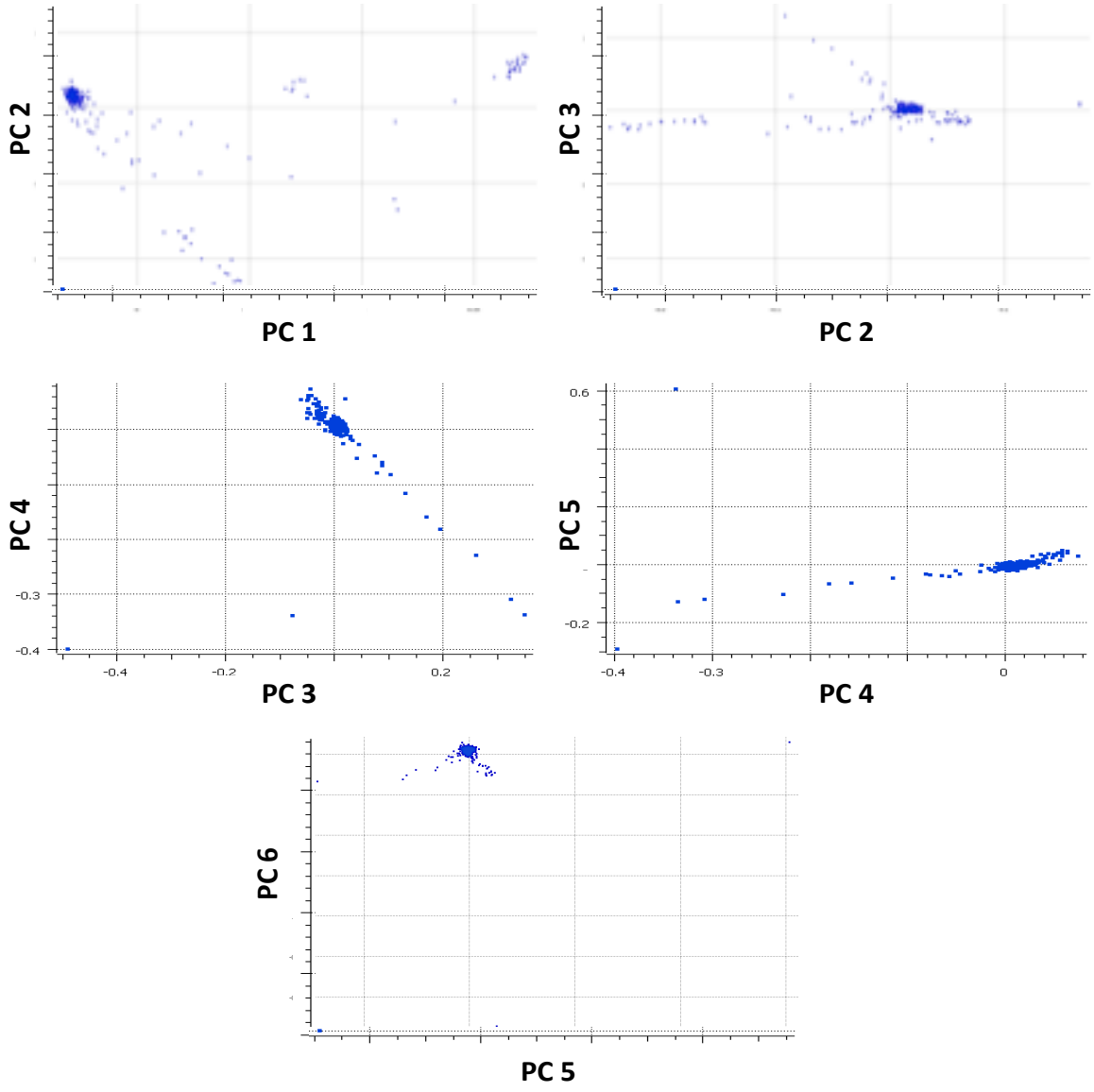


Figure 3.5 Graphical Plots of the Top 6 Principal Components (PCs)

The number of significant principal components was determined by plotting the top eigenvalues against each other until no discernible clusters of groups were identifiable by visual inspection. The proportion of variation explained by the top 5 PCs is 75.1%.

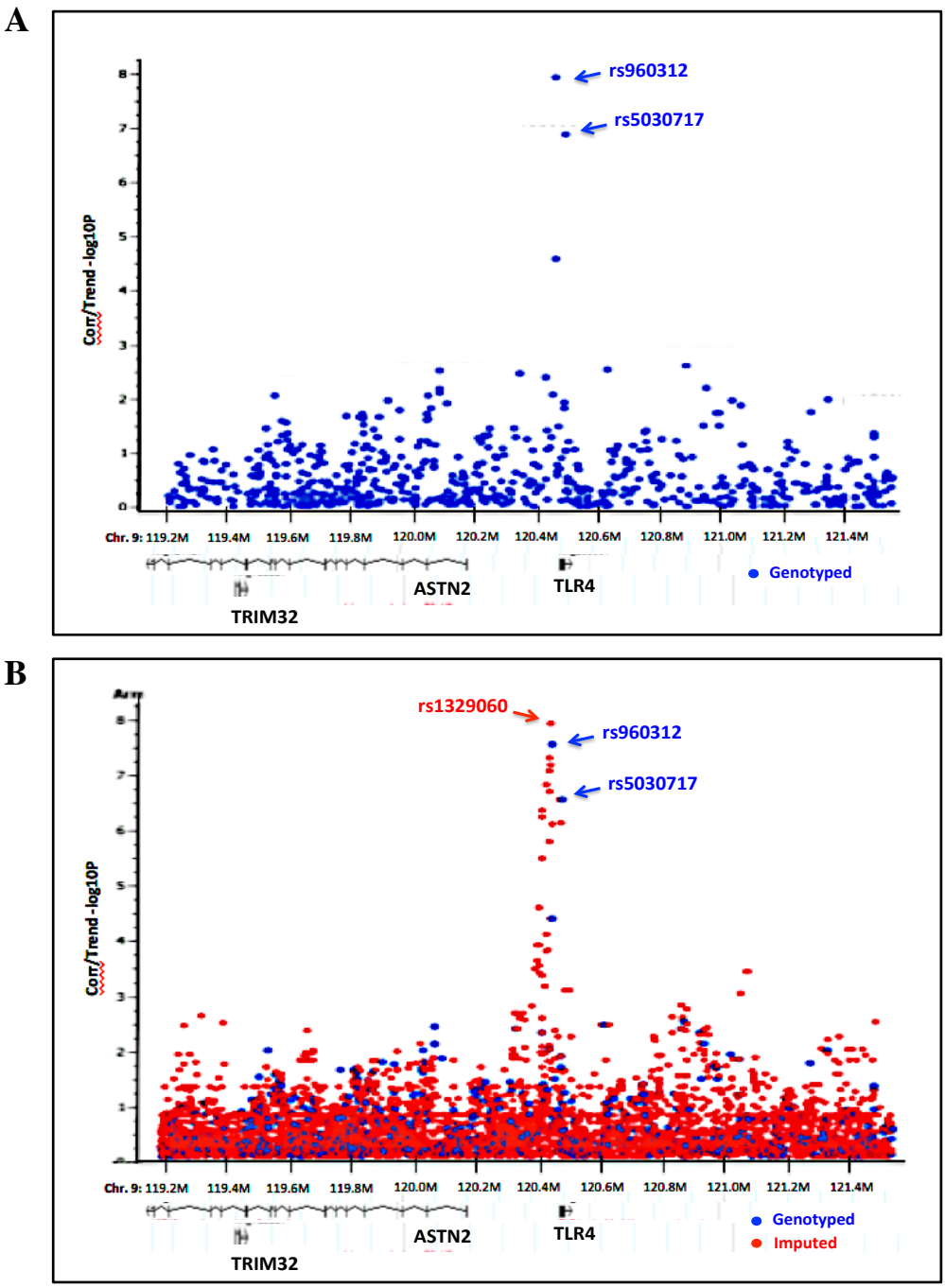
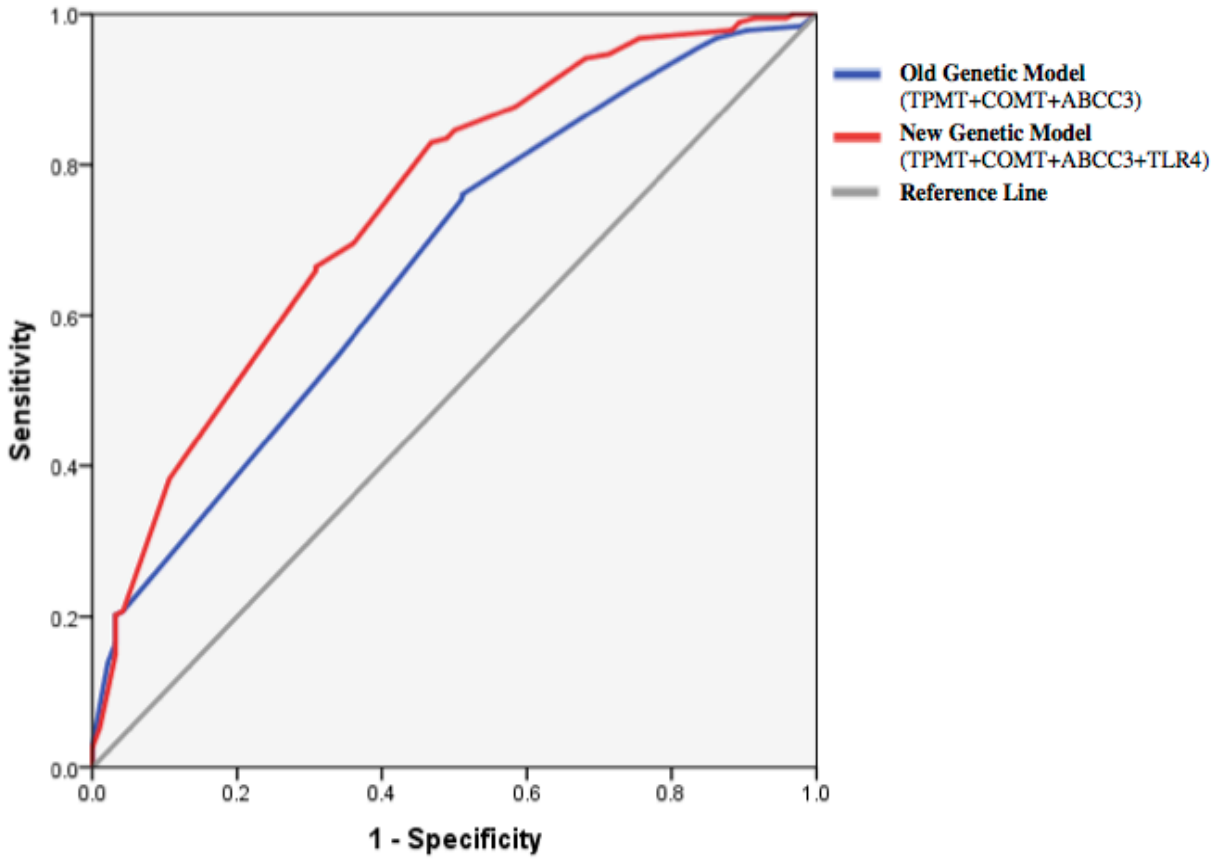


Figure 3.6 Genotype Imputation Analysis of 2.3Mb Region around TLR4 (Chr9: 119198.3 – 121525.4Kb)

Manhattan plots for Discovery cohort association results showing the observe distributions of – log10 (*P*-values) against SNP chromosomal position: **(A)** Genotyped SNPs only **(B)** Genotyped (blue) and Imputed (red) SNPs.

A



B

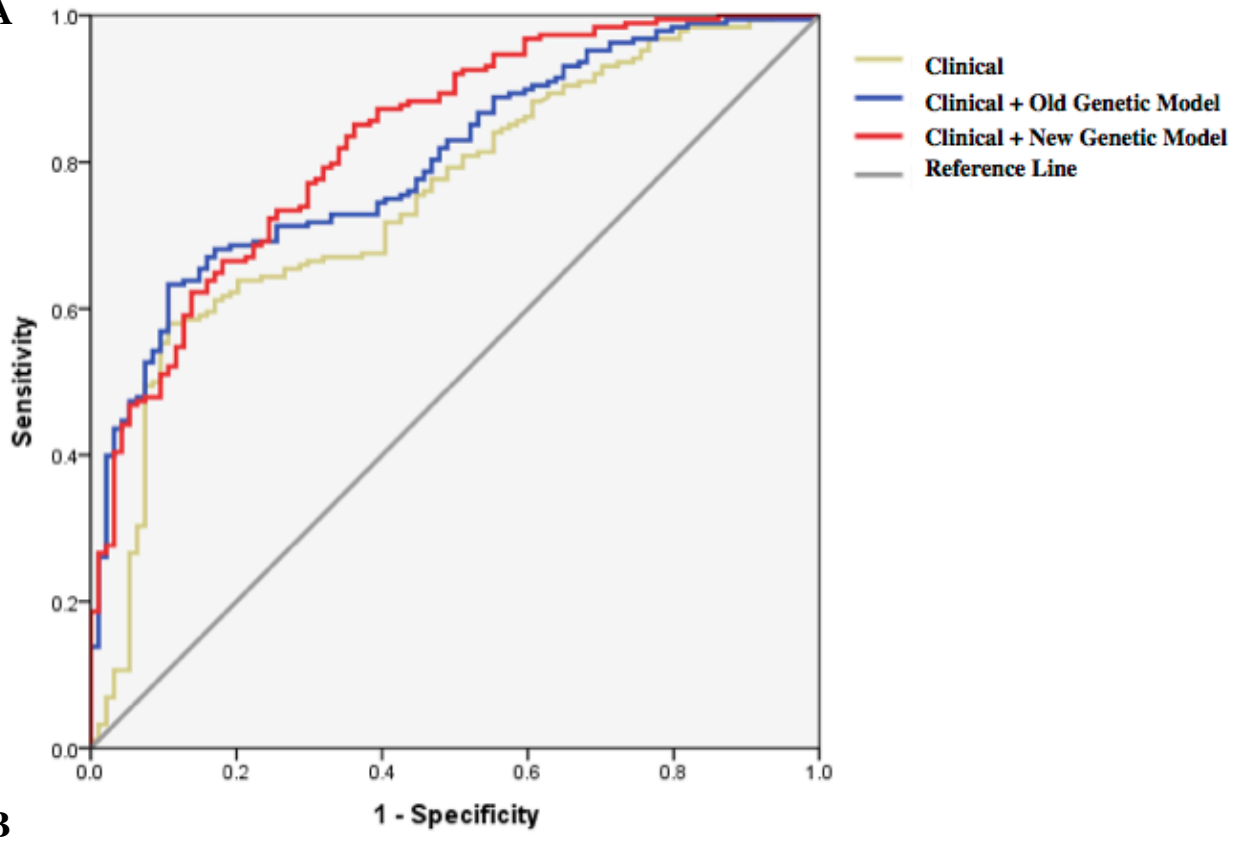
Model	AUC (95% CI)	p-value
Old Genetic Model (TPMT+COMT+ABCC3)	0.669 (0.603-0.735)	-
New Genetic Model (TPMT+COMT+ABCC3+TLR4)	0.745 (0.684-0.806)	0.0054

AUC, Area Under the Curve; CI, Confidence Interval

Figure 3.7 Receiver operating characteristic (ROC) curves of genetic variables in models with and without TLR4 for the prediction of CIO in the discovery cohort

(A) The genetic variables of the old genetic model include *TPMT* rs12201199, *COMT* rs464316 and *ABCC3* rs1051640. The effect of *TLR4* rs9060312 is added in the new genetic model.
(B) The area under the curve (AUC) is calculated for each model and the *p*-value indicates the statistical difference between the old and new genetic models calculated using DeLong's method.

A



B

Model	AUC (95% CI)	p-value vs Clinical + New Genetic Model
Clinical Variables	0.753 (0.694-0.813)	0.00078
Clinical + Old Genetic Model (TPMT+COMT+ABCC3)	0.802 (0.751-0.853)	0.038
Clinical + New Genetic Model (TPMT+COMT+ABCC3+TLR4)	0.830 (0.783-0.879)	-

AUC, Area Under the Curve; CI, Confidence Interval

Figure 3.8 ROC curves of clinical and genetic variables in models with and without TLR4 for the prediction CIO in the discovery cohort

A) The clinical variables include age, concomitant vincristine treatment, germ cell tumour type and cranial irradiation. The genetic variables of the old genetic model include *TPMT* rs12201199, *COMT* rs464316 and *ABCC3* rs1051640. The effect of *TLR4* rs9060312 is added in the new clinical and genetic combined model. **(B)** The area under the curve (AUC) is calculated for each model. P-values indicate the statistical difference between the curves of the clinical variables alone and the combination of old genetic and clinical variables with the new clinical and genetic model.

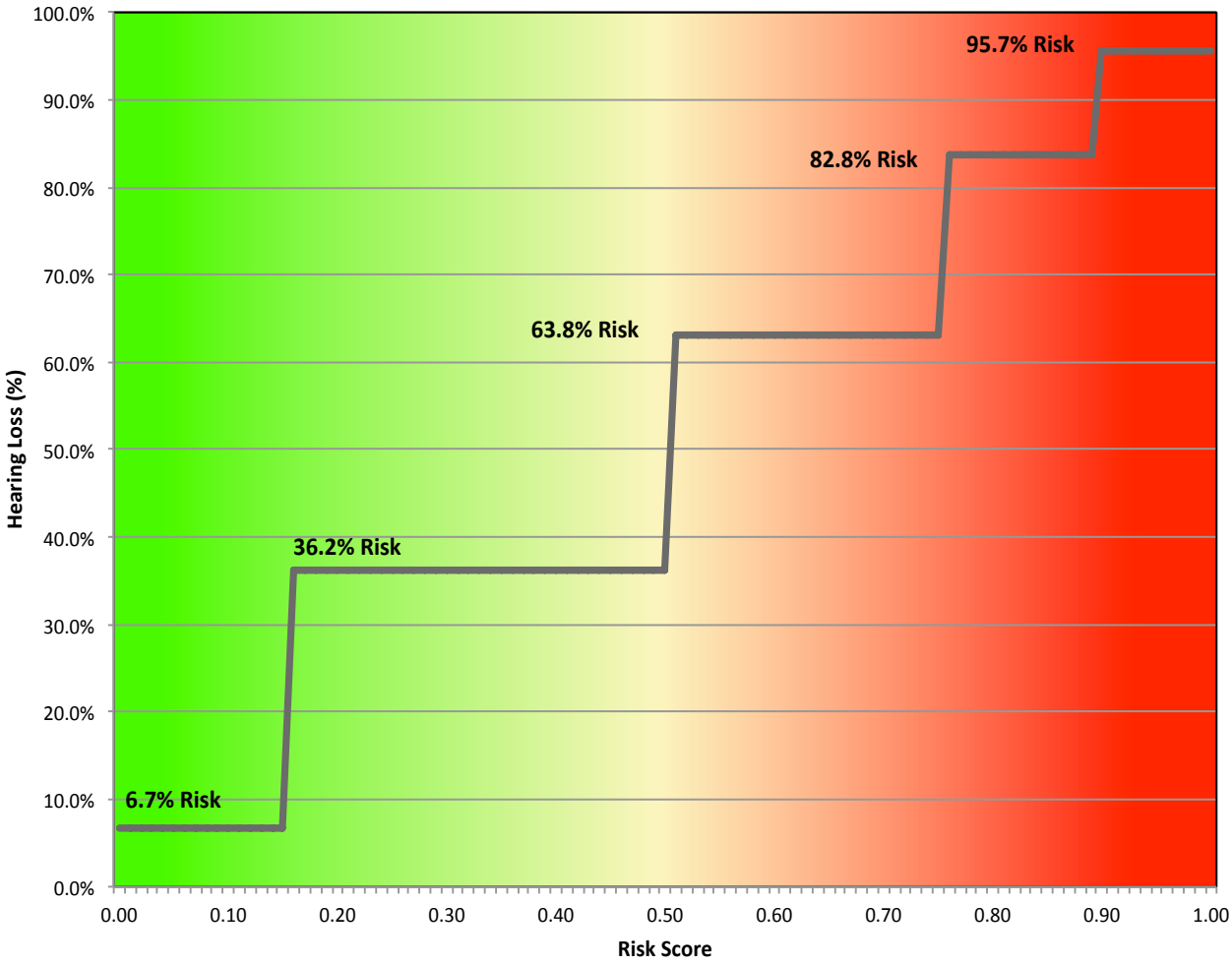


Figure 3.9 Clinically Actionable Risk Prediction Model

Probabilities generated from logistic regression analysis using clinical and genetic variables were used to distinguish patients into 5 different risk groups: Risk I (6.7% risk of ototoxicity), Risk II (36.2%), Risk III (63.8%), Risk IV (82.8%), and Risk V (95.7%). The thresholds of each group were determined by calculating the inter-quintile ranges for cases and controls independently (see Methods in Chapter 2).

Table 3.1 Patient Demographics of Discovery and Replication Cohort

	Discovery Cohort (n=282)			Replication Cohort (n=82)			Combined Cohort (n=364)		
	Cases (n=188)	**Controls** (n= 94)	p-value	**Cases** (n=55)	**Controls** (n=27)	p-value	**Cases** (n=243)	**Controls** (n=121)	p-value
Age, years (median (min, max))	4.2 (0.04-16.2)	9.9 (0.08-18.8)	**6.31E-05**	3.1 (0.1, 17.5)	11.2 (0.7, 15.8)	**0.013**	4.0 (0.05, 17.5)	10.1 (0.08, 18.8)	**3.41E-06**
Gender (Male n, (%))	109 (57.9%)	47 (50.0%)	0.21	27 (49.1%)	10 (37%)	0.35	136 (56.0%)	57 (47.1%)	0.12
Caucasian ethnicity[a] (n, (%))	138 (73.4%)	75 (79.8%)	0.30	45 (81.8%)	23 (85.2%)	1.0	183 (75.3%)	98 (81.0%)	0.24
Dose, cumulative mg/m2 (median (min, max))	400 (120, 800)	400 (55, 760)	0.83	400 (100, 625)	400 (100, 630)	0.96	400 (100, 800)	400 (55, 760)	0.81
Treatment duration, months (median (min, ma	3.6 (0, 22.5)	4.4 (0.03,14.8)	0.17	3.7 (0, 14.3)	3.8 (0, 10.2)	0.92	3.7 (0, 22.5)	4.3 (0, 14.8)	0.28
Concomitant medication[b] (n, (%))									
Tobramycin	63 (33.5%)	31 (32.9%)	1.0	11 (20.0%)	9 (33.3%)	0.27	74 (30.5%)	40 (33.1%)	0.63
Gentamicin	46 (24.5%)	24 (25.5%)	0.88	18 (32.7%)	3 (11.1%)	0.058	64 (26.3%)	27 (22.3%)	0.44
Vancomycin	60 (31.9%)	22 (23.4%)	0.17	14 (25.5%)	7 (25.9%)	1.0	74 (30.5%)	29 (24.0%)	0.22
Vincristine	112 (59.6%)	19 (20.2%)	**2.70E-10**	32 (58.2%)	6 (22.2%)	**0.0024**	144 (59.3%)	25 (20.7%)	**2.03E-12**
Tumour type (n, (%))									
brain tumour	53 (28.2%)	17 (18.1%)	0.079	16 (29.1%)	4 (14.8%)	0.183	69 (28.4%)	21 (17.4%)	**0.028**
germ cell tumour	11 (5.9%)	20 (21.3%)	**1.90E-04**	6 (10.9%)	10 (37%)	**0.0078**	17 (7.0%)	30 (24.8%)	**4.50E-06**
hepatoblastoma	37 (19.7%)	10 (10.6%)	0.063	9 (16.4%)	1 (3.7%)	0.15	46 (18.9%)	11 (9.1%)	**0.015**
neuroblastoma	49 (26.1%)	15 (16.0%)	0.070	14 (24.5%)	4 (14.8%)	0.40	63 (25.9%)	19 (15.7%)	**0.033**
osteosarcoma	33 (17.6%)	26 (27.7%)	0.062	7 (12.7%)	6 (22.2%)	0.34	40 (16.5%)	32 (26.4%)	**0.036**
other sarcoma	1 (0.5%)	2 (2.1%)	0.26	0	1 (3.7%)	0.33	1 (0.4%)	3 (2.5%)	0.11
other carcinoma	2 (1.1%)	2 (2.1%)	0.6	1 (1.8%)	0	1.0	3 (1.2%)	2 (1.7%)	1
lymphoma	1 (0.5%)	1 (1.1%)	1.0	2 (3.6%)	1 (3.7%)	1.0	3 (1.2%)	3 (1.7%)	1.0
retinoblastoma	1 (0.5%)	0	1.0	0	0	-	1 (0.41%)	0	1.0
endodermal sinus tumour of thymus	0	1 (1.1%)	0.330	0	0	-	0	1 (0.8%)	0.33
Ototoxicity Grade ≥ 3	106 (56.4%)	-	-	25 (45.5%)	-	-	131 (53.9%)		
Follow up, years (median (min, max))	6.1 (0.13-25.4)	4.7 (0.75-17.5)	**0.010**	3.9 (0.15-21.0)	3.7 (1.5-14.6)	0.58	5.7 (0.13-25.4)	4.3 (0.75-17.5)	**0.038**
Cranial irradiation (n, (%))	45 (23.9%)	14 (14.9%)	0.089	9 (16.4%)	4 (14.8%)	0.86	54 (22.2%)	18 (14.9%)	0.12

[a] Caucasian ethnicity assessed by principal component analysis in the discovery cohort and by self-reported ethnicity in the replication cohort

[b] Concomitant medications indicated for drug-induced ototoxicity and taken during cisplatin therapy

Age, dose, treatment duration and follow-up time was tested for statistical signifcance using the Wilcoxon-Mann-Whitney test with normal approximation. Gender, ethnicity, concomitant modication, tumor type, cranial irradiation was tested for statistical significance using the Fisher Exact test. Statistically signficant differences ($p<0.05$ type-I error)) between cases and controls are highlighted in bold.

Table 3.2 Genome-wide Association Results in Discovery Cohort

Discovery Cohort (n=282) 188 ototoxicity, 94 controls								
SNP rs-ID	Chr	Position	Gene	Type	Minor Allele	MAF		Odds Ratio (95% CI)[a]	Trend *p*-value[b]
						Cases	Controls		
rs960312	9	120443779	ASTN2 | TLR4	INTERGENIC	G	7.7%	24.5%	0.22 (0.13-0.38)	**1.19E-08**
rs5030717	9	120473834	TLR4	INTRON	G	4.5%	17.6%	0.19 (0.1-0.37)	1.32E-07
rs4381243	1	30675881	LOC100288450 | MATN1	INTERGENIC	G	27.1%	48.4%	0.38 (0.26-0.57)	6.28E-07
rs4875509	8	5187640	CSMD1 | LOC100287015	INTERGENIC	A	27.3%	10.1%	3.33 (1.94-5.7)	4.71E-06
rs969835	5	150664002	SLC36A3	INTRON	A	19.0%	36.2%	0.38 (0.25-0.59)	5.32E-06
rs6669405	1	33920466	PHC2 | ZSCAN20	INTERGENIC	G	27.7%	46.8%	0.42 (0.29-0.62)	6.40E-06
rs10799085	1	30669469	LOC100288450 | MATN1	INTERGENIC	G	23.7%	42.0%	0.41 (0.28-0.62)	7.02E-06
rs4849007	2	112856165	TMEM87B	INTRON	C	54.8%	34.6%	2.2 (1.53-3.18)	1.36E-05
rs17659864	5	150656548	SLC36A3	UTR	G	19.8%	36.2%	0.4 (0.26-0.61)	1.43E-05
rs7016311	8	5170509	CSMD1 | LOC100287015	INTERGENIC	A	31.6%	14.4%	2.75 (1.71-4.41)	1.46E-05
rs4896134	6	135566943	MYB | AHI1	INTERGENIC	A	29.8%	47.9%	0.43 (0.29-0.64)	1.49E-05
rs1026716	3	140983891	ACPL2	INTRON	C	52.9%	34.6%	2.32 (1.56-3.45)	2.00E-05
rs4237340	10	6714979	LOC439949 | SFMBT2	INTERGENIC	C	51.6%	32.4%	2.22 (1.52-3.24)	2.27E-05
rs1874520	12	130771845	FZD10 | PIWIL1	INTERGENIC	A	31.9%	50.5%	0.46 (0.32-0.66)	2.36E-05
rs1329057	9	120441946	ASTN2 | TLR4	INTERGENIC	G	13.6%	28.2%	0.39 (0.25-0.62)	2.64E-05

[a] Odds ratios are calculated using logistic regression with an additive model

[b] p-values are generated from the Cochran-Armitage trend test, in bold indicates SNPs that are signficant after multiple testing correction

Chr, Chromosome; MAF, Minor Allele Frequency; CI, Confidence Interval

Table 3.3 TLR4 Associations Correcting For Ethnicity and Clinical Factors in Discovery Cohort

Discovery Cohort (n=282) 188 ototoxicity , 94 controls										
SNP rs-ID	Chr	Gene	OR (95% CI)[a]	Allelic *p*-value	Genotypic *p*-value	Trend *p*-value	PCA-adjusted OR ((95% CI))[b]	PCA-adjusted *p* -value[b]	PCA + Clinical adjusted OR (95% CI)	PCA + Clinical adjusted *p*-value[c]
rs960312	9	TLR4	0.22 (0.13-0.38)	3.30E-08	8.40E-08	1.19E-08	0.23 (0.13-0.40)	2.90E-08	0.20 (0.11-0.38)	3.20E-07
rs5030717	9	TLR4	0.19 (0.1-0.37)	2.85E-07	9.03E-07	1.32E-07	0.17 (0.08-0.35)	4.71E-08	0.15 (0.06-0.33)	6.12E-07

[a] Odds ratios are calculated using logistic regression with an additive model

[b] p-values and odds ratio are adjusted for population stratification using PCA-corrected genotype data with the top 5 principal components

[c] p-values and odds ratio are adjusted for population stratification and signficant clinical factors (age, concomittant vincristine, germ cell tumour)

[b,c] p-values are generated from the Cochran-Armitage trend test

Chr, Chromosome; OR, Odds Ratio; CI, Confidence Interval

Table 3.4 TLR4 Associations in Discovery, Replication and Combined Cohorts

	Discovery (n=282) 188 ototoxicity , 94 controls				Replication (n=82) 55 ototoxicity, 27 controls				Combined (n=364) 243 ototoxicity, 121 controls			
Gene / SNP -rs ID	OR (95% CI)[a]	*p*-value[b]	Clinical Adjusted OR (95% CI)[c]	Clinical Adjusted p-value[c]	OR (95% CI)	*p*-value	Clinical Adjusted OR (95% CI)	Clinical Adjusted p-valuec	OR (95% CI)	*p*-value	Clinical Adjusted OR (95% CI)	Clinical Adjusted p-valuec
TLR4												
rs960312	0.22 (0.13-0.38)	1.19E-08	0.21 (0.12-0.39)	3.27E-07	0.37 (0.15-0.87)	0.018	0.14 (0.04-0.52)	7.30E-04	0.25 (0.16-0.41)	1.16E-09	0.20 (0.12-0.35)	1.01E-09
rs5030717	0.19 (0.1-0.37)	1.32E-07	0.18 (0.1-0.38)	2.64E-06	0.36 (0.14-0.97)	0.033	0.18 (0.05-0.68)	0.0066	0.24 (0.14-0.41)	3.20E-08	0.18 (0.09-0.35)	5.32E-08

[a] Odds ratios are calculated using logistic regression with an additive model

[b] p-values are generated from the cochrane-armitage trend test

[c] p-values and odds ratio are adjusted for signficant clinical factors (age, concomittant vincristine, germ cell tumour)

Chr, Chromosome; OR, Odds Ratio; CI, Confidence Interval

Table 3.5 Genotype and Allele Counts of TLR4 SNPs in the Discovery, Replication and Combined Cohorts

		Discovery (n=282)			Replication (n=82)			Combined (n=364)		
SNP	Allele/ Genotype	Cases (n=188)	Controls (n=94)	*p*-value[a]	Cases (n=55)	Controls (n=27)	*p*-value	Cases (n=243)	Controls (n=121)	*p*-value
rs960312	G	29 (7.7%)	46 (24.5%)	3.30E-08	12 (10.9%)	14 (25.9%)	0.013	41 (8.4%)	60 (24.8%)	1.80E-09
	A	347 (92.3%)	142 (75.5%)		98 (89.1%)	49 (74.1%)		445 (91.6%)	182 (75.2%)	
	Total (2n)	**376**	**188**		**110**	**54**		**486**	**242**	
	G/G	0 (0%)	3 (3.2%)		1 (1.8%)	2 (7.4%)		1 (0.4%)	5 (4.1%)	
	A/G	29 (15.4%)	40 (42.6%)	8.40E-08	10 (18.2%)	10 (37%)	0.058	39 (16.0%)	50 (41.3%)	8.90E-09
	A/A	159	51		44	15		203	66	
	Total (n)	**188**	**94**		**55**	**27**		**243**	**121**	
rs5030717	G	17 (4.5%)	33 (17.6%)	2.85E-07	8 (7.3%)	10 (18.5%)	0.030	25 (5.1%)	43 (17.8%)	
	A	359 (95.5%)	155 (82.4%)		102 (92.7%)	44 (81.5%)		461 (94.9%)	199 (82.2%)	3.50E-08
	Total (2n)	**376**	**188**		**110**	**54**		**486**	**242**	
	G/G	0 (0%)	1 (1.1%)		0 (0%)	2 (7.4%)		0 (0%)	3 (2.5%)	
	A/G	17 (9.0%)	31 (32.9%)	9.03E-07	8 (14.5%)	6 (22.2%)	0.074	25 (10.3%)	37 (30.6%)	2.12E-07
	A/A	171 (91.0%)	62 (66.0%)		47 (85.5%)	19 (70.4%)		218 (89.7%)	81 (66.9%)	
	Total (n)	**188**	**94**		**55**	**27**		**243**	**121**	

[a]Allelic and genotypic *p*-values were calculated using the chi-squared test. If value of alleles or genotypes were less than 5, *p*-values were calculated using the fishers exact test

Table 3.6 Sub-group Analysis of TLR4 Variants in Patients with Extreme Phenotypes (Grade ≥3 ototoxicity)

	Discovery (n=200) 106 ototoxicity, 94 controls				Replication (n=52) 25 ototoxicity, 27 controls				Combined (n=252)[a] 131 ototoxicity, 121 controls			
Gene / SNP -rs ID	OR (95% CI)[b]	*p*-value[c]	Clinical Adjusted OR (95% CI)[d]	Clinical Adjusted p-value[d]	OR (95% CI)	*p*-value	Clinical Adjusted OR (95% CI)	Clinical Adjusted p-valuec	OR (95% CI)	*p*-value	Clinical Adjusted OR (95% CI)	Clinical Adjusted p-valuec
TLR4												
rs960312	0.13 (0.06-0.27)	6.89E-09	0.13 (0.05-0.31)	1.90E-07	0.31 (0.10-0.99)	0.038	0.029 (0.002-0.41)	2.70E-04	0.16 (0.09-0.31)	1.32E-09	**0.11 (0.05-0.25)**	**3.84E-10**
rs5030717	0.010 (0.04-0.26)	1.17E-07	0.09 (0.03-0.29)	2.37E-06	0.33 (0.08-1.2)	0.079	0.12 (0.019-0.79)	0.010	0.14 (0.06-0.31)	5.85E-08	**0.10 (0.04-0.27)**	**6.41E-08**

[a] Cases with grade 2 hearing loss (n=112) were excluded from the extreme phenotype sub-group analysis

[b] Odds ratios are calculated using logistic regression with an additive model

[c] p-values are generated from the cochrane-armitage trend test

[d] p-values and odds ratio are adjusted for signficant clinical factors (age, concomittant vincristine, germ cell tumour)

Chr, Chromosome; OR, Odds Ratio; CI, Confidence Interval

Table 3.7 TLR4 SNPs Genotyped on Illumina Panel in Discovery Cohort

Discovery Cohort (n=282) 188 ototoxicity, 94 controls						
SNP rs-ID	Gene	Type	*Minor Allele Frequency*		Odds Ratio[a]	Trend *p* -value
			Cases	Controls		
rs960312	TLR4	INTERGENIC	7.7%	24.5%	0.22	1.19E-08
rs5030717	TLR4	INTRON	4.5%	17.6%	0.19	1.32E-07
rs1329057	TLR4	INTERGENIC	13.6%	28.2%	0.39	2.64E-05
rs12339926	TLR4	INTERGENIC	0.3%	3.2%	0.08	0.0029
rs476999	TLR4	INTERGENIC	25.3%	37.2%	0.57	0.0035
rs7034845	TLR4	INTERGENIC	37.8%	50.5%	0.59	0.0041
rs10818066	TLR4	INTERGENIC	32.2%	20.7%	1.68	0.0084
rs11536869	TLR4	INTRON	2.9%	7.4%	0.36	0.012
rs1927911	TLR4	INTRON	23.1%	32.8%	0.62	0.015
rs4837496	TLR4	INTERGENIC	24.3%	32.8%	0.65	0.033
rs10983664	TLR4	INTERGENIC	28.7%	37.8%	0.68	0.036
rs540178	TLR4	INTERGENIC	21.8%	30.3%	0.67	0.036
rs10983737	TLR4	INTERGENIC	9.8%	4.8%	2.17	0.039
rs1577808	TLR4	INTERGENIC	13.3%	7.4%	1.83	0.047
rs10818038	TLR4	INTERGENIC	29.9%	38.3%	0.69	0.048
rs4837254	TLR4	INTERGENIC	38.3%	46.8%	0.69	0.048
rs4986790	TLR4	CODING	6.6%	3.2%	2.13	0.096
rs4986791	TLR4	CODING	6.6%	5.3%	1.29	0.523

[a] Odds ratios are calculated using logistic regression with an additive model

In total, 142 SNPs in the *TLR4* locus were genotyped and analysed in the discovery cohort, including two coding polymorphisms rs4986790 and rs4986791. SNPs that passed a significance level of $p < 0.05$ in addition to the coding SNPs are listed.

Table 3.8 Comparisons of Prediction Models

MODEL	Risk Group[a]	Cases (n, %)	Controls (n, %)	Sensitivity	Specificity	PPV	NPV	AUC	*p*-value[b]
Old Genetic Model (TPMT, ABCC3, COMT)	High Risk	39 (90.7%)	3 (9.3%)	20.7%	95.7%	90.7%	37.7%	0.669	-
	Low Risk	149 (62.3%)	90 (37.3%)						
New Genetic Model (TPMT, ABCC3, COMT, TLR4)	High Risk	114 (89.8%)	13 (10.2%)	60.6%	86.2%	89.8%	52.3%	0.745	0.0054
	Low Risk	74 (47.7%)	81 (52.3%)						
Clinical[c] + Old Genetic Model	High Risk	38 (97.4%)	1 (2.6%)	20.2%	98.9%	97.4%	38.3%	0.802	-
	Low Risk	150 (61.7%)	93 (38.3%)						
Clinical + New Genetic Model	High Risk	57 (95.0%)	3 (5.0%)	30.3%	96.8%	95.0%	41.0%	0.830	0.038
	Low Risk	131 (59.0%)	91 (41.0%)						

[a] Thresholds for high and low risk groups were calculated using inter-quartile ranges of predicted probabilities in cases and controls (see Methods in Chapter 2)
[b] The statistical difference between the ROC curves of the two models, p-values calculated using DeLong's Method
[c] Clinical variables included into model: age, concomittant vinctristine, germ cell tumour, cranial irradiation

OR, Odds Ratio; PPV, Positive Predicted Value; NPV, Negative Predicted Value; AUC, Area Under the Curve

Chapter 4. Discussion

4.1 Overall Review of Key Issue and Findings

We describe here the results of the first genome-wide pharmacogenomic association study to investigate the genetic basis of cisplatin-induced hearing loss in children. Cisplatin chemotherapy exhibits significant individual variation, both in terms of efficacy in tumour response and the incidence and severity of adverse reactions[203]. Cisplatin ototoxicity can have significant repercussions, often restricting the clinical use of the anti-cancer drug with potential reductions in survival, as well as detrimental and expensive long-term consequences for patients and their families[204, 205]. This inter-individual variation may be due in part to underlying genetic susceptibility to CIO. To date, pharmacogenomic studies in both children and adults have examined cisplatin-induced hearing loss using narrow candidate gene studies, limited primarily by the cost of genome-wide analyses and the lack of sufficiently large and well-characterized patient cohorts. As a result, the full genetic architecture of CIO and its pathophysiological mechanism have remained incomplete.

We describe here the results of a genome-wide analysis of 738,432 variants genotyped in a discovery cohort of 282 paediatric patients that identified a significant association with a variant in *TLR4* (rs960312, OR = 0.22, *p*-value = 1.19×10^{-8}), and replicated these findings in a second independent cohort of 82 children (OR = 0.37, p-value = 0.018). Patients carrying the protective '*G*' minor allele were conferred a nearly five-fold decreased risk of cisplatin mediated ototoxicity. Taken together, a new genetic and clinical predictive model that incorporates *TLR4* has an approximately 33% improvement in sensitivity (20.2 to 30.3%) with a high specificity of 96.8%.

Several lines of evidence from *in vitro* and *in vivo* studies have also implicated TLR4 in cisplatin-induced cochlear toxicity and hearing loss[200]. Here we provide the first level of evidence linking TLR4 and CIO in human patients treated for cancer, leading to new insights into the mechanism underlying this pervasive and clinically limiting adverse drug reaction.

4.2 Clinical Factors Associated With Cisplatin-induced Ototoxicity

Significant clinical differences between cases and controls were observed in both the discovery and replication cohort of children treated with cisplatin. Specifically, younger age, concomitant vincristine use and patients treated for germ cell tumours were significantly associated with cisplatin-induced hearing loss. Theses differences exhibited no significant effects on the *TLR4* genetic associations when corrected for in a logistic regression analysis.

Our research group has previously published the significant associations of these clinical risk factors with CIO for patients in our discovery cohort[80, 154]. We showed here the replication of three key clinical covariates in our new, independent, paediatric cohort, including: age, concomitant vincristine, and germ cell tumour type. These findings significantly strengthen the evidence implicating these clinical risk factors with cisplatin-induced hearing loss.

4.2.1 Age

Mounting evidence suggests that age has a substantial influence on susceptibility to CIO, with younger children being at higher risk (see section 1.6.1)[78]. For children, age-related hearing loss due to cisplatin therapy is also reported to have a role in both the incidence and severity of ototoxicity. We show that patient age at the start of treatment is significantly lower in children developing hearing loss compared to control patients. In our combined cohort, children who developed more severe ototoxicity were also younger (median 3.1 years +/- 4.1) than those that experienced milder forms of toxicity (median 5.7 years +/- 4.5) (*p*-value = 0.002). Compensatory methods for hearing and the difficulty of audiometric testing in especially young patients are important considerations in regards to age of treatment and severity of ototoxicity. Still, our findings in two independent cohorts support the notion that age can be a considerable influence on the risk of CIO, even within children.

4.2.2 Vincristine

In our study, we found that concomitant vincristine treatment was a highly significant risk factor for CIO. Vincristine is a chemotherapeutic agent that is frequently administered concomitantly with cisplatin in many paediatric protocols. Whether vincristine itself, or in combination with cisplatin, can cause hearing loss is unclear. Systematic clinical trials have demonstrated that vincristine alone is not ototoxic[206, 207]. On the other hand, case studies have reported incidences of vincristine-induced hearing impairment, suggesting that the drug may be ototoxic or transiently ototoxic at high doses[208, 209]. A large cohort study of adult males treated with cisplatin for testicular cancer found that patients receiving high doses of concomitant vincristine experienced greater but reversible symptoms of hearing loss[115].

Our results provide further evidence that vincristine treatment in combination with cisplatin may influence the susceptibility to cisplatin-induced hearing loss in children. Previous studies examining the ototoxic profile of vincristine have been performed primarily in adults and limited with small sample sizes. These findings suggest that vincristine should be considered as a relevant clinical characteristic to be assessed for in future studies on cisplatin-induced hearing loss in children and adults.

4.2.3 Germ-cell Tumour Type

In contrast to the majority of tumour types treated with cisplatin, a significantly lower proportion of patients treated for germ-cell tumours experienced hearing loss in both the discovery and replications cohorts. This tumour specific phenomenon may be due to the dose and method of cisplatin administration for patients with germ cell tumours. For most standard protocols, children are treated with a high dose bolus of cisplatin over short periods of time (i.e., over 1 day). For the treatment of extra-cranial germ cell tumours however, patients are administered cisplatin at low individual doses of $20mg/m^2$ over five days[77].

Several studies have reported that cisplatin given over a longer period of time has a safer ototoxicity profile than that administered by bolus dosing[210, 211]. The cumulative doses of cisplatin in these germ cell tumour protocols are generally the same or even higher than what

is given for other tumour types. A study by Gupta *et al*. found that paediatric germ cell tumour patients treated with a continuous infusion of cisplatin had a significantly lower incidence of ototoxicity at even high cumulative doses[212]. Additional work identifying all patients treated with continuous cisplatin administration and the impact this has on treatment efficacy and safety will be of importance to understanding this association.

4.3 Population Stratification

Population stratification can lead to spurious associations as a result of ethnic-specific differences in SNP allele frequencies rather than drug toxicity associated differences[170]. We first assessed the presence of population structure differences between cases and controls by computing the genomic control factor (λ_{GC}). Values of $\lambda_{GC} < 1.05$ generally indicate a benign influence from population stratification[172]. In our discovery cohort, the genomic control was calculated to be $\lambda_{GC} = 1.012$, suggesting that confounding due to population stratification is minimal.

To further ensure that our discovery findings were not confounded by inter-patient heterogeneity as a result of population stratification, we utilized the principal component analysis to correct for population structure in our GWAS. Using the top 5 PCs as covariates in a logistic regression analysis, the genetic associations of *TLR4* remained highly associated with even stronger associations and effect sizes for rs5030717. This result strongly suggests that the TLR4 findings are not spurious associations as a result of population stratification.

4.4 Pharmacogenomic Prediction of Cisplatin-induced Ototoxicity

Building upon a previously published multi-marker prediction model including clinical factors (age, vincristine, germ cell tumour, cranial irradiation) and genetic variants (*TPMT* rs12201199, *ABCC3* rs1051640 and *COMT* rs4646316) [80, 154], we examined whether incorporating the *TLR4* rs960312 variant would significantly improve our ability to predict patients at risk. For a genetic only model, a significant increase in the predictive performance (AUC, 0.669 to 0.745; *p*-value = 0.0054) was observed when including the protective

rs960312 variant. Specifically, we found that the addition of the *TLR4* SNP greatly enhanced the ability to capture patients who developed hearing loss. This is a result of a large proportion of cases, non-carriers for the rs960312 SNP, being previously predicted at low risk when utilizing only the *TPMT, ABCC3* and *COMT* variants. By testing for *TLR4*, patients without the protective variant can be better stratified into the high risk group, as indicated by the nearly three-fold increase in sensitivity and 15% increase in our ability to predict patients at low risk (NPV).

Furthermore, clinical factors associated with CIO alone cannot fully account for the risk of developing hearing loss in patients (AUC, 0.753). Compared to clinical factors alone, combining clinical and genetic variables was significantly better at predicting patients at risk of CIO (AUC, 0.753 to 0.830; *p*-value = 0.00078). As these models are based on the results from the combined cohort, further studies to validate these findings and prospectively assess the clinical utility of pharmacogenetic testing are needed. Nonetheless, our findings provide further evidence for the potential benefit of pharmacogenomic testing for cisplatin-induced hearing loss.

A major barrier to the implementation of genetic testing is the interpretation of genetic information for appropriate clinical decision-making. We developed a preliminary multi-risk group predictive model in order to provide clinicians with more clinically actionable information **(Figure 3.9).** Although based on retrospective data, this model is able to provide varying levels of risk based on genetic and clinical risk factors. As pharmacogenomic testing is but one facet of clinical care decision-making, such information can be much more informative than a relatively insensitive dichotomization of risk (high vs low). As decisions are evaluated based on risk and benefit, this presentation of risk for CIO can be a more appropriate method of incorporating pharmacogenomic information within the current standards of care. Of importance, clinicians will need to carefully balance the information generated from genetic testing with other critical factors such as the patients' stage of cancer, prognosis, expectations and desires for the outcome of their treatment.

4.5 Linking TLR4 to Cisplatin-induced Ototoxicity

Toll-like receptor 4 (TLR4) is a pivotal component of the innate immune system, responsible for detecting pathogen associated molecular patterns (PAMPs) such as endotoxins or lipopolysaccharides (LPS) expressed on gram-negative bacteria[213]. Upon TLR4 activation, downstream signalling via the MyD88- and TRIF-dependent pathway mediates the expression of pro inflammatory cytokines (including TNF-α, IL-1β, and IL-6) and the ensuing innate immune response[214]. TLR4 is primarily known for its role in detecting foreign pathogens and initiating the defence response against infectious agents. Recently, several lines of evidence from *in vitro* and *in vivo* studies have implicated TLR4 in cisplatin-induced cochlear toxicity and hearing loss[200, 215].

4.5.1 Pro-inflammatory Cytokines and Cisplatin-induced Cochlear Toxicity

The inner ear is considered an immunologically privileged organ, capable of rapidly generating an active immune response that can ultimately lead to cochlear degeneration and permanent hearing loss[216]. Pro-inflammatory cytokines are potent signalling molecules that are key regulators of this immune response and can induce inflammation, trauma and cell death[217]. A part of the TLR4 signalling cascade is the activation of the transcription factor NF-kB, which is involved in regulating the expression of these cytokines and other inflammatory mediators that participate in acute inflammatory responses[217].

Interestingly, murine cochlear cells (HEI-OC1) treated with cisplatin exhibit up-regulated mRNA expression and increased secretion of pro-inflammatory cytokines TNF-α, IL-1B, and IL-6[215]. Upstream of this, cisplatin is shown to induce the nuclear translocation of NF-kB, resulting in the up-regulation of genes controlled by this transcription factor[215]. In line with these findings, inhibition of these cytokines with neutralizing antibodies markedly attenuates cisplatin-induced cochlear cell death. Furthermore, activation of TLR4 in cisplatin-treated cochlear cells result in significantly higher levels of pro-inflammatory cytokines and subsequent cell toxicity compared to cells treated with cisplatin alone[200].

4.5.2 Pro-inflammatory Cytokines and ROS Generation

Mechanistically, cisplatin ototoxicity manifests through the acute and chronic accumulation of ROS in cochlear tissues, resulting in the activation of pro-apoptotic pathways and subsequent inner ear cell death[65]. This oxidative stress, in part, is caused by the markedly increased expression of NADPH oxidase (NOXs) in the organ of Corti of the cochlear and vestibular system upon cisplatin exposure[218-220]. Interestingly, cisplatin-induced pro-inflammatory cytokine production is shown to directly activate these NOXs, inducing intracellular ROS generation in murine cochlear cells[220]. Together, these findings suggest the interaction of inflammation and ROS generation play a key role in the mechanism underlying cisplatin ototoxicity.

4.5.3 TLR4 Signalling and Cisplatin-induced Hearing Impairment

The transcriptional and translational expression of TLR4 has been demonstrated to increase in mouse cochleae *in vivo* and organ of Corti explants *ex vivo* after cisplatin exposure[200]. Therefore, it was postulated that cisplatin could potentiate the TLR4 signalling cascade in the inner ear cells, leading to increased expression of pro-inflammatory cytokines and subsequent ROS generation upon activation. To examine the role of TLR4 in CIO *in vivo*, TLR4 mutant (C3H/HeJ) and TLR deficient ($TLR^{-/-}$) mice were compared to wild-type (C57BL/6) mice after administration of cisplatin (5mg/kg) and LPS[200]. Strikingly, hearing as measured by auditory brain response threshold of mice with functional TLR4 showed significant hearing loss, whereas TLR4 deficient and mutant mice were effectively protected from cisplatin-induced hearing impairment.

4.6 Future Work

Published results provide strong evidence for the role of TLR4 signalling in cisplatin-mediated ototoxicity in cell-based and mouse model work. The link between TLR4 and cisplatin-induced hearing loss in humans, however, has yet to be reported. Here we provide the first level of evidence associating TLR4 genetic variation and CIO in paediatric patients. In our study, we discovered and replicated *TLR4* variants that were significantly associated

with a decreased risk for CIO in patients. Taken together with the published data, we hypothesize that decreased activity variants of *TLR4* can attenuate the downstream effects of *TLR4* signalling, resulting in less oxidative stress and cisplatin-induced cytotoxicity of inner ear cells.

4.6.1 Identifying the Causal Variant

As *TRL4* rs960312 and rs5030717 are intergenic and intronic SNPs respectively, we examined whether these were tag SNPs for functional variants in the coding regions of the gene. Sequencing of the human *TLR4* gene has revealed that the frequency of most nonsynonymous polymorphisms is rare in humans (<1%)[221]. Two co-segregating SNPs in exon 3 of *TLR4* (rs4986790, Asp299Gly and rs4986791, Thr399Ile) are described to have population frequencies greater than 5%; however, these SNPs were neither in LD with rs960312, nor strongly associated with CIO. Interestingly, the rs4986790 (Asp299Gly) variant showed a trend to association (p-value = 0.096) in our cohort. Although this variant is unlikely the causal variant driving our GWAS association, it can potentially be an independent genetic risk factor and thus will be of interest to genotype in additional patients.

Evidence linking a functional effect to these two coding SNPs has been largely based on genetic association studies and has been highly inconsistent and controversial. A comprehensive review of all literature on TLR4 polymorphisms found that 62% of studies (n=157 studies) demonstrate no association between the coding SNPs and susceptibility to infection[201]. Studies examining the phenotypic consequences of Asp299Gly and Thr399Ile are limited. Furthermore, the reported effects of these two haplotypes are also inconsistent. Two studies have reported that transfected cells with any of the *TLR4* haplotypes have decreased NF-kB activity compared with normal TLR4[222, 223]. On the other hand, the one study investigating cytokine production by individuals with the Asp299Gly haplotype reported that TNF-α cytokine response was stronger rather than blunted[224].

We hypothesize that the causal SNP driving the association of TLR4 and CIO may exist in genomic sequences modulating gene expression. Although the proximal promoter (-75bp

upstream) exhibits a high degree of conservation between human and mouse genomes, a distal regulatory region between nucleotides -3228 and -743 in human *TLR4* is considerably variable, with eight common SNPs identified in this region[202]. One of these SNPs, rs10759932 (-1607T>C), is in complete LD with the rs960312 variant identified in our study. Upon genotyping of this variant in our cohort, we found that this variant was equally associated with CIO. This variant (-1607 T<C) is predicted *in silico* to abrogate the binding of the Nkx-2 transcription factor[225]. In line with our hypothesis, promoter haplotypes of patients with this rs10759932 variant have been shown to have significantly lower baseline TLR4 expression levels[226].

Additional studies to assess the influence of this promoter SNP on transcriptional activity in the presence of cisplatin will be of great interest and are on-going. The identification of a functional *TLR4* variant or haplotype could potentially improve the accuracy and reliability of a pharmacogenomic predictive test for CIO. In addition to the role of these variants in CIO, it will be interesting to see if these variants better account for the wide inter-individual variability in TLR4 activity and cytokine production observed in people.

4.7 Strengths and Limitations

Understanding the pharmacogenomics of CIO in children presents multiple challenges. Ototoxicity is an adverse drug reaction that is difficult to characterize in young children, which can lead to in inaccurate phenotypes. Furthermore the number of paediatric patients treated with cisplatin in total is small. It is estimated that less than 100 children are treated with cisplatin annually in British Columbia.

Even with nation-wide patient recruitment, the number of patients required for a GWAS would be too large to identify variants of small effect sizes (odds ratios of 1.5 - 2)[191]. Therefore, this study sought to identify only genetic variants (should they exist) of clinically significant odds ratios (>3). To address these limitations, we implemented a nation-wide recruitment process that also incorporated detailed patient clinical characterization methods. This strategy built upon the advantage of a nation-wide universal health care system with

motivated collaborators across Canada working to address this important healthcare problem. To further address the limitations of statistical power, we sought to increase power by comparing patients of phenotypic extremes, a strategy to enrich the presence of a genomic signal. A demonstration of this effect can be observed in the sub-group extremes analysis for *TLR4* rs960312 and rs5030717, which resulted in higher effect sizes and more significant results. Finally, to address the limitations of sample size and statistical power, this study incorporated an independent replication. This is a critical tool for the validation of GWAS findings[187]. In this thesis, the variants discovered in the initial were shown to be significantly associated in an independent, similarity treated patient cohort.

An inherent limitation of GWAS and our study is the assumption that the genetic architecture of cisplatin-induced ototoxicity is solely caused by common variation. This may limit our ability to capture the full genetic architecture of the ADR phenotype, as we are unable to identify rare variants (MAF<1%) contributing to the susceptibility CIO. Current genome-wide arrays have improved the coverage to genotype up to 5 million markers. Until high throughput sequencing platforms become more cost-effective and computationally feasible, GWAS may not be able to appropriately handle rare variation.

4.8 Conclusion

In conclusion, this thesis describes the first study to perform a GWAS on cisplatin-induced hearing loss in humans. In a total of 364 children, we identified and replicated a highly protective variant in the *TLR4* gene. Combined with significant evidence from model systems reported in the literature, these findings implicate a role of the innate immune response in the pathogenic mechanism of cisplatin-induced hearing loss in humans. These findings provide evidence for exciting new hypotheses, such as the development of novel drug therapies that can provide protection from CIO by targeting this novel pathological pathway. Furthermore, by incorporating this variant in a prediction model that incorporates both clinical and genetic risk factors, we are able to significantly improve our ability to stratify patients at risk of CIO. These results demonstrate the potential of using pharmacogenomic information in order to improve safety and efficacy of cisplatin in children. .

References

1. Wilkinson, G.R., *Drug metabolism and variability among patients in drug response.* The New England journal of medicine, 2005. **352**(21): p. 2211-2221.
2. Pirmohamed, M., et al., *Adverse drug reactions.* Bmj, 1998. **316**(7140): p. 1295-8.
3. Sultana, J., P. Cutroneo, and G. Trifirò, *Clinical and economic burden of adverse drug reactions.* Journal of pharmacology & pharmacotherapeutics, 2013. **4**(Suppl1).
4. Howard, R.L., et al., *Which drugs cause preventable admissions to hospital? A systematic review.* British journal of clinical pharmacology, 2007. **63**(2): p. 136-147.
5. Lazarou, J., B.H. Pomeranz, and P.N. Corey, *Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies.* JAMA : the journal of the American Medical Association, 1998. **279**(15): p. 1200-1205.
6. Bonn, D., *Adverse drug reactions remain a major cause of death.* Lancet, 1998. **351**(9110): p. 1183.
7. Davies, E.C., et al., *Adverse drug reactions in hospital in-patients: a prospective analysis of 3695 patient-episodes.* PLoS One, 2009. **4**(2): p. e4439.
8. Wu, T.-Y.Y., et al., *Ten-year trends in hospital admissions for adverse drug reactions in England 1999-2009.* Journal of the Royal Society of Medicine, 2010. **103**(6): p. 239-250.
9. Impicciatore, P., et al., *Incidence of adverse drug reactions in paediatric in/out-patients: a systematic review and meta-analysis of prospective studies.* British journal of clinical pharmacology, 2001. **52**(1): p. 77-83.
10. Johnson, J.A. and J.L. Bootman, *Drug-related morbidity and mortality. A cost-of-illness model.* Arch Intern Med, 1995. **155**(18): p. 1949-56.
11. Classen, D.C., et al., *Adverse drug events in hospitalized patients. Excess length of stay, extra costs, and attributable mortality.* Jama, 1997. **277**(4): p. 301-6.
12. Kalisch, L.M., et al., *The prescribing cascade* Aust Prescr, 2011(34): p. 162-166.
13. Dionne, F., et al., *Economic impact of a genetic test for cisplatin-induced ototoxicity.* Pharmacogenomics J, 2012. **12**(3): p. 205-13.
14. Pirmohamed, M., et al., *Adverse drug reactions.* BMJ (Clinical research ed.), 1998. **316**(7140): p. 1295-1298.
15. Wilke, R.A., et al., *Identifying genetic risk factors for serious adverse drug reactions: current progress and challenges.* Nature reviews. Drug discovery, 2007. **6**(11): p. 904-916.
16. Aronson, J.K. and R.E. Ferner, *Clarification of terminology in drug safety.* Drug Saf, 2005. **28**(10): p. 851-70.
17. Greener, M., *Drug safety on trial. Last year's withdrawal of the anti-arthritis drug Vioxx triggered a debate about how to better monitor drug safety even after approval.* EMBO Rep, 2005. **6**(3): p. 202-4.
18. White, T.J., A. Arakelian, and J.P. Rho, *Counting the costs of drug-related adverse events.* Pharmacoeconomics, 1999. **15**(5): p. 445-58.
19. Motulsky, A.G., et al., *Pharmacogenetics* 1st edn ed. 1997, New York Oxford Univ. Press
20. Kalow, W. and N. Staron, *On distribution and inheritance of atypical forms of human serum cholinesterase, as indicated by dibucaine numbers.* Can J Biochem Physiol, 1957. **35**(12): p. 1305-20.

21. Kalow, W., B.K. Tang, and L. Endrenyi, *Hypothesis: comparisons of inter- and intra-individual variations can substitute for twin studies in drug research.* Pharmacogenetics, 1998. **8**(4): p. 283-9.
22. Eichelbaum, M., M. Ingelman-Sundberg, and W.E. Evans, *Pharmacogenomics and individualized drug therapy.* Annu Rev Med, 2006. **57**: p. 119-37.
23. Ma, Q. and A.Y. Lu, *Pharmacogenetics, pharmacogenomics, and individualized medicine.* Pharmacological reviews, 2011. **63**(2): p. 437-459.
24. Daly, A.K. and B.P. King, *Pharmacogenetics of oral anticoagulants.* Pharmacogenetics, 2003. **13**(5): p. 247-52.
25. Manolopoulos, V.G., G. Ragia, and A. Tavridou, *Pharmacogenetics of coumarinic oral anticoagulants.* Pharmacogenomics, 2010. **11**(4): p. 493-6.
26. Epstein, R.S., et al., *Warfarin genotyping reduces hospitalization rates results from the MM-WES (Medco-Mayo Warfarin Effectiveness study).* J Am Coll Cardiol, 2010. **55**(25): p. 2804-12.
27. Crawford, D.C. and D.A. Nickerson, *Definition and clinical importance of haplotypes.* Annu Rev Med, 2005. **56**: p. 303-20.
28. Genomes Project, C., et al., *An integrated map of genetic variation from 1,092 human genomes.* Nature, 2012. **491**(7422): p. 56-65.
29. Griffith, O.L., et al., *ORegAnno: an open-access community-driven resource for regulatory annotation.* Nucleic Acids Res, 2008. **36**(Database issue): p. D107-13.
30. Moroney, A.C., *Overview of Pharmacodynamics* The Merck Manual, 2013
31. Le, J., *Overview of Pharmacokinetics.* The Merck Manual, 2014
32. Evans, W.E. and M.V. Relling, *Pharmacogenomics: translating functional genomics into rational therapeutics.* Science, 1999. **286**(5439): p. 487-91.
33. Williams, J.A., et al., *Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios.* Drug Metab Dispos, 2004. **32**(11): p. 1201-8.
34. Madadi, P., et al., *Clinical practice guideline: CYP2D6 genotyping for safe and efficacious codeine therapy.* Journal of population therapeutics and clinical pharmacology = Journal de la thérapeutique des populations et de la pharamcologie clinique, 2013. **20**(3): p. 96.
35. Volpe, D., et al., *Uniform assessment and ranking of opioid μ receptor binding constants for selected opioid drugs.* Regulatory toxicology and pharmacology : RTP, 2011. **59**(3): p. 385-390.
36. Ciszkowski, C., et al., *Codeine, ultrarapid-metabolism genotype, and postoperative death.* The New England journal of medicine, 2009. **361**(8): p. 827-828.
37. Gasche, Y., et al., *Codeine intoxication associated with ultrarapid CYP2D6 metabolism.* N Engl J Med, 2004. **351**(27): p. 2827-31.
38. Madadi, P., et al., *Pharmacogenetics of neonatal opioid toxicity following maternal use of codeine during breastfeeding: a case-control study.* Clin Pharmacol Ther, 2009. **85**(1): p. 31-5.
39. Koren, G., et al., *Pharmacogenetics of morphine poisoning in a breastfed neonate of a codeine-prescribed mother.* Lancet, 2006. **368**(9536): p. 704.
40. Somogyi, A., D. Barratt, and J. Coller, *Pharmacogenetics of opioids.* Clinical pharmacology and therapeutics, 2007. **81**(3): p. 429-444.

41. Administration, U.S.F.a.D., *Table of Pharmacogenomic Biomarkers in Drug Labeling* 2014.
42. Amstutz, U., et al., *Recommendations for HLA-B*15:02 and HLA-A*31:01 genetic testing to reduce the risk of carbamazepine-induced hypersensitivity reactions.* Epilepsia, 2014.
43. Leckband, S., et al., *Clinical Pharmacogenetics Implementation Consortium guidelines for HLA-B genotype and carbamazepine dosing.* Clinical pharmacology and therapeutics, 2013. **94**(3): p. 324-328.
44. Urban, T. and D. Goldstein, *Pharmacogenetics at 50: genomic personalization comes of age.* Science translational medicine, 2014. **6**(220).
45. Howlader N, N.A., Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds), *SEER Cancer Statistics Review, 1975-2010.* 2013.
46. Robison, L.L. and M.M. Hudson, *Survivors of childhood and adolescent cancer: life-long risks and responsibilities.* Nature reviews. Cancer, 2013. **14**(1): p. 61-70.
47. Hudson, M.M., et al., *Clinical ascertainment of health outcomes among adults treated for childhood cancer.* Jama, 2013. **309**(22): p. 2371-81.
48. Tai, E., et al., *Health status of adolescent and young adult cancer survivors.* Cancer, 2012. **118**(19): p. 4884-91.
49. Geenen, M.M., et al., *Medical assessment of adverse health outcomes in long-term survivors of childhood cancer.* Jama, 2007. **297**(24): p. 2705-15.
50. Leeder, J.S., et al., *Understanding the relative roles of pharmacogenetics and ontogeny in pediatric drug development and regulatory science.* J Clin Pharmacol, 2010. **50**(12): p. 1377-87.
51. Rieder, M.J. and B. Carleton, *Pharmacogenomics and adverse drug reactions in children.* Frontiers in genetics, 2013. **5**: p. 78.
52. Choonara, I. and M.J. Rieder, *Drug toxicity and adverse drug reactions in children – A brief historical review.* Paediatric and Perinatal Drug Therapy 2002(5): p. 12-18.
53. Dotta, A. and N. Chukhlantseva, *Ontogeny and drug metabolism in newborns.* J Matern Fetal Neonatal Med, 2012. **25 Suppl 4**: p. 83-4.
54. McKeage, M.J., *Comparative adverse effect profiles of platinum drugs.* Drug Saf, 1995. **13**(4): p. 228-44.
55. Perilongo, G., et al., *Cisplatin versus cisplatin plus doxorubicin for standard-risk hepatoblastoma.* N Engl J Med, 2009. **361**(17): p. 1662-70.
56. Helm, C.W. and J.C. States, *Enhancing the efficacy of cisplatin in ovarian cancer treatment - could arsenic have a role.* J Ovarian Res, 2009. **2**: p. 2.
57. Hohnloser, J.H., et al., *Cisplatin based chemotherapy in testicular cancer patients: long term platinum excretion and clinical effects.* Eur J Med Res, 1996. **1**(11): p. 509-14.
58. Florea, A.-M.M. and D. Büsselberg, *Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects.* Cancers, 2010. **3**(1): p. 1351-1371.
59. Eastman, A., *The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes.* Pharmacol Ther, 1987. **34**(2): p. 155-66.

60. Pinto, A.L. and S.J. Lippard, *Binding of the antitumor drug cis-diamminedichloroplatinum(II) (cisplatin) to DNA.* Biochim Biophys Acta, 1985. **780**(3): p. 167-80.
61. Kelland, L.R., *New platinum antitumor complexes.* Crit Rev Oncol Hematol, 1993. **15**(3): p. 191-219.
62. Bellon, S.F., J.H. Coleman, and S.J. Lippard, *DNA unwinding produced by site-specific intrastrand cross-links of the antitumor drug cis-diamminedichloroplatinum(II).* Biochemistry, 1991. **30**(32): p. 8026-35.
63. Siddik, Z.H., *Cisplatin: mode of cytotoxic action and molecular basis of resistance.* Oncogene, 2003. **22**(47): p. 7265-79.
64. Santoso, J.T., et al., *Saline, mannitol, and furosemide hydration in acute cisplatin nephrotoxicity: a randomized trial.* Cancer Chemother Pharmacol, 2003. **52**(1): p. 13-8.
65. Mukherjea, D. and L.P. Rybak, *Pharmacogenomics of cisplatin-induced ototoxicity.* Pharmacogenomics, 2011. **12**(7): p. 1039-50.
66. Li, Y., R.B. Womer, and J.H. Silber, *Predicting cisplatin ototoxicity in children: the influence of age and the cumulative dose.* Eur J Cancer, 2004. **40**(16): p. 2445-51.
67. Brock, P.R., et al., *Cisplatin ototoxicity in children: a practical grading system.* Med Pediatr Oncol, 1991. **19**(4): p. 295-300.
68. Blakley, B.W. and S.F. Myers, *Patterns of hearing loss resulting from cis-platinum therapy.* Otolaryngol Head Neck Surg, 1993. **109**(3 Pt 1): p. 385-91.
69. Ilveskoski, I., et al., *Ototoxicity in children with malignant brain tumors treated with the "8 in 1" chemotherapy protocol.* Med Pediatr Oncol, 1996. **27**(1): p. 26-31.
70. Skinner, R., et al., *Ototoxicity of cisplatinum in children and adolescents.* Br J Cancer, 1990. **61**(6): p. 927-31.
71. Montaguti, M., et al., *[Cisplatin and carboplatin-induced ototoxicity in children: clinical aspects and perspectives for prevention].* Acta Otorhinolaryngol Ital, 2002. **22**(1): p. 14-8.
72. Bertolini, P., et al., *Platinum compound-related ototoxicity in children: long-term follow-up reveals continuous worsening of hearing loss.* J Pediatr Hematol Oncol, 2004. **26**(10): p. 649-55.
73. Knight, K., D. Kraemer, and E. Neuwelt, *Ototoxicity in children receiving platinum chemotherapy: underestimating a commonly occurring toxicity that may influence academic and social development.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2005. **23**(34): p. 8588-8596.
74. Weissenstein, A., et al., *Progressive hearing loss after completion of cisplatin chemotherapy is common and more pronounced in children without spontaneous otoacoustic emissions before chemotherapy.* Int J Pediatr Otorhinolaryngol, 2012. **76**(1): p. 131-6.
75. Gietema, J.A., et al., *Circulating plasma platinum more than 10 years after cisplatin treatment for testicular cancer.* Lancet, 2000. **355**(9209): p. 1075-6.
76. Chang, K.W. and N. Chinosornvatana, *Practical grading system for evaluating cisplatin ototoxicity in children.* J Clin Oncol, 2010. **28**(10): p. 1788-95.
77. Afzal, S., et al., *Challenges in management of patients with intracranial germ cell tumor and diabetes insipidus treated with cisplatin and/or ifosfamide based chemotherapy.* J Neurooncol, 2010. **97**(3): p. 393-9.

78. Li, Y., R.B. Womer, and J.H. Silber, *Predicting cisplatin ototoxicity in children: the influence of age and the cumulative dose.* European journal of cancer (Oxford, England : 1990), 2004. **40**(16): p. 2445-2451.
79. McHaney, V.A., et al., *Hearing loss in children receiving cisplatin chemotherapy.* J Pediatr, 1983. **102**(2): p. 314-7.
80. Ross, C.J., et al., *Genetic variants in TPMT and COMT are associated with hearing loss in children receiving cisplatin chemotherapy.* Nat Genet, 2009. **41**(12): p. 1345-9.
81. Blair J.C., P.M.E., Viehweg S.H., *The effects of mild sensorineural hearing loss on academic performance of young school age children.* Volta Rev, 2001. **87**: p. 87-93.
82. Grewal, S., et al., *Auditory late effects of childhood cancer therapy: a report from the Children's Oncology Group.* Pediatrics, 2010. **125**(4): p. e938-50.
83. Davis, J.M., et al., *Effects of mild and moderate hearing impairments on language, educational, and psychosocial behavior of children.* J Speech Hear Disord, 1986. **51**(1): p. 53-62.
84. Helt-Cameron, J. and P.J. Allen, *Cisplatin ototoxicity in children: implications for primary care providers.* Pediatr Nurs, 2009. **35**(2): p. 121-7.
85. Gurney, J.G., et al., *Hearing loss, quality of life, and academic problems in long-term neuroblastoma survivors: a report from the Children's Oncology Group.* Pediatrics, 2007. **120**(5).
86. Gurney, J.G. and J.K. Bass, *New International Society of Pediatric Oncology Boston Ototoxicity Grading Scale for pediatric oncology: still room for improvement.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2012. **30**(19): p. 2303-2306.
87. Brock, P.R., et al., *Platinum-induced ototoxicity in children: a consensus review on mechanisms, predisposition, and protection, including a new International Society of Pediatric Oncology Boston ototoxicity scale.* J Clin Oncol, 2012. **30**(19): p. 2408-17.
88. Schmidt, C.M., et al., *[The "Muenster classification" of high frequency hearing loss following cisplatin chemotherapy].* Hno, 2007. **55**(4): p. 299-306.
89. *Cancer Therapy Evalution Program-Common Terminology Criteria for Adverse Events-Version 4.03.* 2010.
90. *National cancer Institute: Common Terminology Criteria for Adverse Events, Version 4.03. .*
91. Rybak, L.P., et al., *Mechanisms of cisplatin-induced ototoxicity and prevention.* Hear Res, 2007. **226**(1-2): p. 157-67.
92. Kim, H.J., et al., *Roles of NADPH oxidases in cisplatin-induced reactive oxygen species generation and ototoxicity.* J Neurosci, 2010. **30**(11): p. 3933-46.
93. Vlajkovic, S.M., G.D. Housley, and P.R. Thorne, *Adenosine and the auditory system.* Curr Neuropharmacol, 2009. **7**(3): p. 246-56.
94. Rybak, L.P., et al., *Cisplatin Ototoxicity and Protection: Clinical and Experimental Studies.* Tohoku J Exp Med, 2009. **219**(3): p. 177-86.
95. Rybak, L.P., et al., *Effect of protective agents against cisplatin ototoxicity.* Am J Otol, 2000. **21**(4): p. 513-20.
96. Clerici, W.J., D.L. DiMartino, and M.R. Prasad, *Direct effects of reactive oxygen species on cochlear outer hair cell shape in vitro.* Hear Res, 1995. **84**(1-2): p. 30-40.
97. Ravi, R., S.M. Somani, and L.P. Rybak, *Mechanism of cisplatin ototoxicity: antioxidant system.* Pharmacol Toxicol, 1995. **76**(6): p. 386-94.

98. van Ruijven, M.W., J.C. de Groot, and G.F. Smoorenburg, *Time sequence of degeneration pattern in the guinea pig cochlea during cisplatin administration. A quantitative histological study.* Hear Res, 2004. **197**(1-2): p. 44-54.
99. van As, J.W., H. van den Berg, and E.C. van Dalen, *Medical interventions for the prevention of platinum-induced hearing loss in children with cancer.* Cochrane Database Syst Rev, 2012. **5**: p. CD009219.
100. Church, M.W., et al., *WR-2721 (Amifostine) ameliorates cisplatin-induced hearing loss but causes neurotoxicity in hamsters: dose-dependent effects.* J Assoc Res Otolaryngol, 2004. **5**(3): p. 227-37.
101. Marina, N., et al., *Amifostine does not protect against the ototoxicity of high-dose cisplatin combined with etoposide and bleomycin in pediatric germ-cell tumors - A children's Oncology Group study.* Cancer, 2005. **104**(4): p. 841-847.
102. Fouladi, M., et al., *Amifostine protects against cisplatin-induced ototoxicity in children with average-risk medulloblastoma.* J Clin Oncol, 2008. **26**(22): p. 3749-55.
103. Gallegos-Castorena, S., et al., *Toxicity prevention with amifostine in pediatric osteosarcoma patients treated with cisplatin and doxorubicin.* Pediatr Hematol Oncol, 2007. **24**(6): p. 403-8.
104. Katzenstein, H.M., et al., *Amifostine does not prevent platinum-induced hearing loss associated with the treatment of children with hepatoblastoma: a report of the Intergroup Hepatoblastoma Study P9645 as a part of the Children's Oncology Group.* Cancer, 2009. **115**(24): p. 5828-35.
105. Petrilli, A.S., et al., *Results of the Brazilian Osteosarcoma Treatment Group Studies III and IV: prognostic factors and impact on survival.* J Clin Oncol, 2006. **24**(7): p. 1161-8.
106. Stocks, R.M., et al., *Ototoxic protection of sodium thiosulfate: daily vs constant infusion.* Otolaryngol Head Neck Surg, 2004. **131**(1): p. 115-9.
107. Wang, J., et al., *Local application of sodium thiosulfate prevents cisplatin-induced hearing loss in the guinea pig.* Neuropharmacology, 2003. **45**(3): p. 380-93.
108. Saito, T., et al., *The effect of sodium thiosulfate on ototoxicity and pharmacokinetics after cisplatin treatment in guinea pigs.* Eur Arch Otorhinolaryngol, 1997. **254**(6): p. 281-6.
109. Muldoon, L.L., et al., *Delayed administration of sodium thiosulfate in animal models reduces platinum ototoxicity without reduction of antitumor activity.* Clin Cancer Res, 2000. **6**(1): p. 309-15.
110. Zuur, C.L., et al., *Ototoxicity in a randomized phase III trial of intra-arterial compared with intravenous cisplatin chemoradiation in patients with locally advanced head and neck cancer.* J Clin Oncol, 2007. **25**(24): p. 3759-65.
111. Lewis, M.J., et al., *Ototoxicity in children treated for osteosarcoma.* Pediatr Blood Cancer, 2009. **52**(3): p. 387-91.
112. Zuur, C.L., et al., *Risk factors of ototoxicity after cisplatin-based chemo-irradiation in patients with locally advanced head-and-neck cancer: a multivariate analysis.* Int J Radiat Oncol Biol Phys, 2007. **68**(5): p. 1320-5.
113. Kushner, B.H., et al., *Ototoxicity from high-dose use of platinum compounds in patients with neuroblastoma.* Cancer, 2006. **107**(2): p. 417-22.
114. Yancey, A., et al., *Risk factors for cisplatin-associated ototoxicity in pediatric oncology patients.* Pediatr Blood Cancer, 2012. **59**(1): p. 144-8.

115. Bokemeyer, C., et al., *Analysis of risk factors for cisplatin-induced ototoxicity in patients with testicular cancer.* Br J Cancer, 1998. **77**(8): p. 1355-62.
116. Simon, T., et al., *The incidence of hearing impairment after successful treatment of neuroblastoma.* Klin Padiatr, 2002. **214**(4): p. 149-52.
117. Schacht, J., A.E. Talaska, and L.P. Rybak, *Cisplatin and aminoglycoside antibiotics: hearing loss and its prevention.* Anatomical record (Hoboken, N.J. : 2007), 2012. **295**(11): p. 1837-1850.
118. Fee, W.E., Jr., *Aminoglycoside ototoxicity in the human.* Laryngoscope, 1980. **90**(10 Pt 2 Suppl 24): p. 1-19.
119. Moore, R.D., C.R. Smith, and P.S. Lietman, *Risk factors for the development of auditory toxicity in patients receiving aminoglycosides.* J Infect Dis, 1984. **149**(1): p. 23-30.
120. Wahba, H.A., et al., *Adjuvant chemotherapy after reduced craniospinal irradiation dose in children with average-risk medulloblastoma: a 5-year follow-up study.* J buon, 2013. **18**(2): p. 425-9.
121. Warrier, R., et al., *Cisplatin and cranial irradiation-related hearing loss in children.* Ochsner J, 2012. **12**(3): p. 191-6.
122. Grau, C., et al., *Sensori-neural hearing loss in patients treated with irradiation for nasopharyngeal carcinoma.* Int J Radiat Oncol Biol Phys, 1991. **21**(3): p. 723-8.
123. Ekborn, A., et al., *Cisplatin-induced hearing loss: influence of the mode of drug administration in the guinea pig.* Hear Res, 2000. **140**(1-2): p. 38-44.
124. Wang, L., H.L. McLeod, and R.M. Weinshilboum, *Genomics and drug response.* N Engl J Med, 2011. **364**(12): p. 1144-53.
125. Dolan, M.E., et al., *Heritability and linkage analysis of sensitivity to cisplatin-induced cytotoxicity.* Cancer research, 2004. **64**(12): p. 4353-4356.
126. Pussegoda, K., et al., *Replication of TPMT and ABCC3 genetic variants highly associated with cisplatin-induced hearing loss in children.* Clinical pharmacology and therapeutics, 2013. **94**(2): p. 243-51.
127. Yang, J., et al., *The role of inherited TPMT and COMT genetic variation in cisplatin-induced ototoxicity in children with cancer.* Clinical pharmacology and therapeutics, 2013. **94**(2): p. 252-259.
128. Carleton, B.C., et al., *Role of TPMT and COMT genetic variation in cisplatin-induced ototoxicity.* Clin Pharmacol Ther, 2013.
129. Weinshilboum, R.M. and S.L. Sladek, *Mercaptopurine pharmacogenetics: monogenic inheritance of erythrocyte thiopurine methyltransferase activity.* Am J Hum Genet, 1980. **32**(5): p. 651-62.
130. Weinshilboum, R.M., *Pharmacogenomics: catechol O-methyltransferase to thiopurine S-methyltransferase.* Cell Mol Neurobiol, 2006. **26**(4-6): p. 539-61.
131. Ochoa, B., et al., *S-Adenosyl-L-methionine increases serum BUN and creatinine in cisplatin-treated mice.* Arch Med Res, 2009. **40**(1): p. 54-8.
132. von Stechow, L., et al., *Identification of cisplatin-regulated metabolic pathways in pluripotent stem cells.* PLoS One, 2013. **8**(10): p. e76476.
133. Ballatori, N., et al., *Molecular mechanisms of reduced glutathione transport: role of the MRP/CFTR/ABCC and OATP/SLC21A families of membrane proteins.* Toxicol Appl Pharmacol, 2005. **204**(3): p. 238-55.

134. Lee, Y.M., et al., *Identification and functional characterization of the natural variant MRP3-Arg1297His of human multidrug resistance protein 3 (MRP3/ABCC3).* Pharmacogenetics, 2004. **14**(4): p. 213-23.
135. Yang, P., et al., *Role of the glutathione metabolic pathway in lung cancer treatment and prognosis: a review.* J Clin Oncol, 2006. **24**(11): p. 1761-9.
136. Leitner, H.M., R. Kachadourian, and B.J. Day, *Harnessing drug resistance: using ABC transporter proteins to target cancer cells.* Biochem Pharmacol, 2007. **74**(12): p. 1677-85.
137. Schrenk, D., et al., *Up-regulation of transporters of the MRP family by drugs and toxins.* Toxicol Lett, 2001. **120**(1-3): p. 51-7.
138. Young, L.C., et al., *Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: correlation of protein levels with drug response and messenger RNA levels.* Clin Cancer Res, 2001. **7**(6): p. 1798-804.
139. Young, L.C., et al., *Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response.* Clin Cancer Res, 1999. **5**(3): p. 673-80.
140. Oguri, T., et al., *Association between expression of the MRP3 gene and exposure to platinum drugs in lung cancer.* Int J Cancer, 2001. **93**(4): p. 584-9.
141. Pussegoda, K., et al., *Replication of TPMT genetic variants highly associated with cisplatin-induced hearing loss in children*, in *Pharmacogenomics and Personalised Medicine 2011*. 2011: Hinxton, Cambridge, UK.
142. Lautermann, J., et al., *Glutathione-dependent antioxidant systems in the mammalian inner ear: effects of aging, ototoxic drugs and noise.* Hear Res, 1997. **114**(1-2): p. 75-82.
143. Peters, U., et al., *Glutathione S-transferase genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin.* Anticancer Drugs, 2000. **11**(8): p. 639-43.
144. Oldenburg, J., et al., *Cisplatin-induced long-term hearing impairment is associated with specific glutathione s-transferase genotypes in testicular cancer survivors.* J Clin Oncol, 2007. **25**(6): p. 708-14.
145. Oldenburg, J., et al., *Association between long-term neuro-toxicities in testicular cancer survivors and polymorphisms in glutathione-s-transferase-P1 and -M1, a retrospective cross sectional study.* J Transl Med, 2007. **5**: p. 70.
146. Khrunin, A.V., et al., *Genetic polymorphisms and the efficacy and toxicity of cisplatin-based chemotherapy in ovarian cancer patients.* Pharmacogenomics J, 2010. **10**(1): p. 54-61.
147. Choeyprasert, W., et al., *Cisplatin-induced ototoxicity in pediatric solid tumors: the role of glutathione S-transferases and megalin genetic polymorphisms.* J Pediatr Hematol Oncol, 2013. **35**(4): p. e138-43.
148. Xu, Y., J. Wang, and W. Dong, *GSTM3 A/B polymorphism and risk for head and neck cancer: a meta-analysis.* PLoS One, 2014. **9**(1): p. e83851.
149. Barahmani, N., et al., *Glutathione S-transferase M1 and T1 polymorphisms may predict adverse effects after therapy in children with medulloblastoma.* Neuro Oncol, 2009. **11**(3): p. 292-300.
150. Rednam, S., et al., *Glutathione S-transferase P1 single nucleotide polymorphism predicts permanent ototoxicity in children with medulloblastoma.* Pediatric blood & cancer, 2013. **60**(4): p. 593-598.

151. Mizuta, K., et al., *Ultrastructural localization of megalin in the rat cochlear duct.* Hear Res, 1999. **129**(1-2): p. 83-91.
152. Riedemann, L., et al., *Megalin genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin.* Pharmacogenomics J, 2008. **8**(1): p. 23-8.
153. Caronia, D., et al., *Common variations in ERCC2 are associated with response to cisplatin chemotherapy and clinical outcome in osteosarcoma patients.* Pharmacogenomics J, 2009. **9**(5): p. 347-53.
154. Pussegoda, K., et al., *Replication of TPMT and ABCC3 genetic variants highly associated with cisplatin-induced hearing loss in children.* Clin Pharmacol Ther, 2013. **94**(2): p. 243-51.
155. Motsinger-Reif, A.A., et al., *Genome-wide association studies in pharmacogenomics: successes and lessons.* Pharmacogenet Genomics, 2013. **23**(8): p. 383-94.
156. Hirschhorn, J.N. and M.J. Daly, *Genome-wide association studies for common diseases and complex traits.* Nat Rev Genet, 2005. **6**(2): p. 95-108.
157. Welter, D., et al., *The NHGRI GWAS Catalog, a curated resource of SNP-trait associations.* Nucleic acids research, 2013. **42**(Database issue): p. 6.
158. Grady, B.J. and M.D. Ritchie, *Statistical Optimization of Pharmacogenomics Association Studies: Key Considerations from Study Design to Analysis.* Curr Pharmacogenomics Person Med, 2011. **9**(1): p. 41-66.
159. Abecasis, G.R., et al., *A map of human genome variation from population-scale sequencing.* Nature, 2010. **467**(7319): p. 1061-73.
160. De, R., W.S. Bush, and J.H. Moore, *Bioinformatics Challenges in Genome-Wide Association Studies (GWAS).* Methods in molecular biology (Clifton, N.J.), 2013. **1168**: p. 63-81.
161. Reich, D.E., et al., *Linkage disequilibrium in the human genome.* Nature, 2001. **411**(6834): p. 199-204.
162. Slatkin, M., *Linkage disequilibrium--understanding the evolutionary past and mapping the medical future.* Nat Rev Genet, 2008. **9**(6): p. 477-85.
163. Ardlie, K.G., L. Kruglyak, and M. Seielstad, *Patterns of linkage disequilibrium in the human genome.* Nat Rev Genet, 2002. **3**(4): p. 299-309.
164. Li, M., C. Li, and W. Guan, *Evaluation of coverage variation of SNP chips for genome-wide association studies.* Eur J Hum Genet, 2008. **16**(5): p. 635-43.
165. Rice, T.K., N.J. Schork, and D.C. Rao, *Methods for handling multiple testing.* Adv Genet, 2008. **60**: p. 293-308.
166. Hochberg, Y. and Y. Benjamini, *More powerful procedures for multiple significance testing.* Stat Med, 1990. **9**(7): p. 811-8.
167. Hahn, L.W., M.D. Ritchie, and J.H. Moore, *Multifactor dimensionality reduction software for detecting gene-gene and gene-environment interactions.* Bioinformatics, 2003. **19**(3): p. 376-82.
168. Bush, W.S. and J.H. Moore, *Chapter 11: Genome-wide association studies.* PLoS computational biology, 2011. **8**(12).
169. Pirinen, M., P. Donnelly, and C.C. Spencer, *Including known covariates can reduce power to detect genetic effects in case-control studies.* Nat Genet, 2012. **44**(8): p. 848-51.

170. Pritchard, J.K. and N.A. Rosenberg, *Use of unlinked genetic markers to detect population stratification in association studies.* Am J Hum Genet, 1999. **65**(1): p. 220-8.
171. Zheng, G., B. Freidlin, and J.L. Gastwirth, *Robust genomic control for association studies.* Am J Hum Genet, 2006. **78**(2): p. 350-6.
172. Price, A.L., et al., *New approaches to population stratification in genome-wide association studies.* Nature reviews. Genetics, 2010. **11**(7): p. 459-463.
173. Price, A.L., et al., *Principal components analysis corrects for stratification in genome-wide association studies.* Nature genetics, 2006. **38**(8): p. 904-909.
174. Tian, C., P.K. Gregersen, and M.F. Seldin, *Accounting for ancestry: population substructure and genome-wide association studies.* Hum Mol Genet, 2008. **17**(R2): p. R143-50.
175. Hirschhorn, J.N. and D. Altshuler, *Once and again-issues surrounding replication in genetic association studies.* J Clin Endocrinol Metab, 2002. **87**(10): p. 4438-41.
176. Chanock, S.J., et al., *Replicating genotype-phenotype associations.* Nature, 2007. **447**(7145): p. 655-60.
177. Ioannidis, J.P., *Non-replication and inconsistency in the genome-wide association setting.* Hum Hered, 2007. **64**(4): p. 203-13.
178. Edwards, S.L., et al., *Beyond GWASs: illuminating the dark road from association to function.* American journal of human genetics, 2013. **93**(5): p. 779-797.
179. Cooper, G.M. and J. Shendure, *Needles in stacks of needles: finding disease-causal variants in a wealth of genomic data.* Nat Rev Genet, 2011. **12**(9): p. 628-40.
180. Freedman, M.L., et al., *Principles for the post-GWAS functional characterization of cancer risk loci.* Nat Genet, 2011. **43**(6): p. 513-8.
181. Marchini, J. and B. Howie, *Genotype imputation for genome-wide association studies.* Nat Rev Genet, 2010. **11**(7): p. 499-511.
182. Via, M., C. Gignoux, and E.G. Burchard, *The 1000 Genomes Project: new opportunities for research and social challenges.* Genome Med, 2010. **2**(1): p. 3.
183. Carroll, D., *Precision genome engineering.* Curr Biol, 2014. **24**(3): p. R102-3.
184. Thorrez, L. and M. Sampaolesi, *The future of induced pluripotent stem cells for cardiac therapy and drug development.* Curr Pharm Des, 2011. **17**(30): p. 3258-70.
185. Yap, T.A., et al., *Envisioning the future of early anticancer drug development.* Nat Rev Cancer, 2010. **10**(7): p. 514-23.
186. Kangelaris, K.N., et al., *Genetic testing before anticoagulation? A systematic review of pharmacogenetic dosing of warfarin.* J Gen Intern Med, 2009. **24**(5): p. 656-64.
187. Gurwitz, D. and H.L. McLeod, *Genome-wide studies in pharmacogenomics: harnessing the power of extreme phenotypes.* Pharmacogenomics, 2013. **14**(4): p. 337-339.
188. Li, D., et al., *Using extreme phenotype sampling to identify the rare causal variants of quantitative traits in association studies.* Genet Epidemiol, 2011. **35**(8): p. 790-9.
189. Daly, A.K., et al., *HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin.* Nat Genet, 2009. **41**(7): p. 816-9.
190. Link, E., et al., *SLCO1B1 variants and statin-induced myopathy--a genomewide study.* N Engl J Med, 2008. **359**(8): p. 789-99.
191. Daly, A.K., *Using genome-wide association studies to identify genes important in serious adverse drug reactions.* Annu Rev Pharmacol Toxicol, 2012. **52**: p. 21-35.

192. Daly, A.K., *Genome-wide association studies in pharmacogenomics.* Nat Rev Genet, 2010. **11**(4): p. 241-6.
193. Psaty, B.M., et al., *Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium: Design of prospective meta-analyses of genome-wide association studies from 5 cohorts.* Circ Cardiovasc Genet, 2009. **2**(1): p. 73-80.
194. Carleton, B., et al., *Adverse drug reaction active surveillance: developing a national network in Canada's children's hospitals.* Pharmacoepidemiol Drug Saf, 2009. **18**(8): p. 713-21.
195. Gauderman W, M.J., *QUANTO 1.1: A computer program for power and sample size calculations for genetic-epidemiology studies*. 2006.
196. Browning, B.L. and S.R. Browning, *A unified approach to genotype imputation and haplotype-phase inference for large data sets of trios and unrelated individuals.* Am J Hum Genet, 2009. **84**(2): p. 210-23.
197. DeLong, E.R., D.M. DeLong, and D.L. Clarke-Pearson, *Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach.* Biometrics, 1988. **44**(3): p. 837-45.
198. Robin, X., et al., *pROC: an open-source package for R and S+ to analyze and compare ROC curves.* BMC Bioinformatics, 2011. **12**: p. 77.
199. Visscher, H., et al., *Application of principal component analysis to pharmacogenomic studies in Canada.* Pharmacogenomics J, 2009. **9**(6): p. 362-72.
200. Oh, G.S., et al., *Activation of lipopolysaccharide-TLR4 signaling accelerates the ototoxic potential of cisplatin in mice.* J Immunol, 2011. **186**(2): p. 1140-50.
201. Ferwerda, B., et al., *Functional consequences of toll-like receptor 4 polymorphisms.* Molecular medicine (Cambridge, Mass.), 2007. **14**(5-6): p. 346-352.
202. Ragnarsdóttir, B., et al., *Genetics of innate immunity and UTI susceptibility.* Nature reviews. Urology, 2011. **8**(8): p. 449-468.
203. *Pharmacogenomics of cisplatin-induced ototoxicity.* Pharmacogenomics, 2011. **12**(7): p. 1039-1050.
204. Kling, J., *US FDA contemplates collection of pharmacogenomic data.* Nat Biotechnol, 2003. **21**(6): p. 590.
205. Knight, K.R., D.F. Kraemer, and E.A. Neuwelt, *Ototoxicity in children receiving platinum chemotherapy: underestimating a commonly occurring toxicity that may influence academic and social development.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2005. **23**(34): p. 8588-8596.
206. Riga, M., et al., *The effect of treatment with vincristine on transient evoked and distortion product otoacoustic emissions.* Int J Pediatr Otorhinolaryngol, 2006. **70**(6): p. 1003-8.
207. Lugassy, G. and A. Shapira, *A prospective cohort study of the effect of vincristine on audition.* Anticancer Drugs, 1996. **7**(5): p. 525-6.
208. Lugassy, G. and A. Shapira, *Sensorineural hearing loss associated with vincristine treatment.* Blut, 1990. **61**(5): p. 320-1.
209. Aydogdu, I., et al., *Bilateral transient hearing loss associated with vincristine therapy: case report.* J Chemother, 2000. **12**(6): p. 530-2.
210. Waters, G.S., et al., *Ototoxicity due to cis-diamminedichloroplatinum in the treatment of ovarian cancer: influence of dosage and schedule of administration.* Ear Hear, 1991. **12**(2): p. 91-102.

211. Posner, M.R., et al., *A phase I trial of continuous infusion cisplatin.* Cancer, 1987. **59**(1): p. 15-8.
212. Gupta, A.A., et al., *Low incidence of ototoxicity with continuous infusion of cisplatin in the treatment of pediatric germ cell tumors.* J Pediatr Hematol Oncol, 2006. **28**(2): p. 91-4.
213. Hoshino, K., et al., *Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product.* J Immunol, 1999. **162**(7): p. 3749-52.
214. O'Neill, L.A. and A.G. Bowie, *The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling.* Nature reviews. Immunology, 2007. **7**(5): p. 353-364.
215. So, H., et al., *Cisplatin cytotoxicity of auditory cells requires secretions of proinflammatory cytokines via activation of ERK and NF-kappaB.* Journal of the Association for Research in Otolaryngology : JARO, 2007. **8**(3): p. 338-355.
216. Satoh, H., et al., *Proinflammatory cytokine expression in the endolymphatic sac during inner ear inflammation.* Journal of the Association for Research in Otolaryngology : JARO, 2003. **4**(2): p. 139-147.
217. Dinarello, C.A., *Proinflammatory cytokines.* Chest, 2000. **118**(2): p. 503-8.
218. Bánfi, B., et al., *NOX3, a superoxide-generating NADPH oxidase of the inner ear.* Journal of Biological …, 2004.
219. Mukherjea, D., et al., *Expression of the kidney injury molecule 1 in the rat cochlea and induction by cisplatin.* Neuroscience, 2006. **139**(2): p. 733-740.
220. Kim, H.-J., et al., *Roles of NADPH oxidases in cisplatin-induced reactive oxygen species generation and ototoxicity.* The Journal of neuroscience, 2010. **30**(11): p. 3933-3946.
221. Smirnova, I., et al., *Excess of rare amino acid polymorphisms in the Toll-like receptor 4 in humans.* Genetics, 2001. **158**(4): p. 1657-1664.
222. Arbour, N.C., et al., *TLR4 mutations are associated with endotoxin hyporesponsiveness in humans.* Nat Genet, 2000. **25**(2): p. 187-91.
223. Marsik, C., et al., *The Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms influence the late inflammatory response in human endotoxemia.* Clin Chem, 2005. **51**(11): p. 2178-80.
224. Ferwerda, B., et al., *TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans.* Proc Natl Acad Sci U S A, 2007. **104**(42): p. 16645-50.
225. Heinemeyer, T., et al., *Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL.* Nucleic Acids Res, 1998. **26**(1): p. 362-7.
226. Ragnarsdottir, B., et al., *Toll-like receptor 4 promoter polymorphisms: common TLR4 variants may protect against severe urinary tract infection.* PLoS One, 2010. **5**(5): p. e10734.