THE PHARMACOGENOMICS OF CISPLATIN-INDUCED HEARING LOSS

by

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Abstract

Cisplatin is a widely used chemotherapeutic agent for the treatment of solid tumours. A serious complication of cisplatin treatment is permanent hearing loss. The study hypothesis is that genetic variants in genes involved in drug metabolism and transport can contribute to increased susceptibility to hearing loss in pediatric oncology patients treated with cisplatin. Patients were recruited from across Canada through the Canadian Pharmacogenomics Network for Drug Safety (CPNDS). Recently, our group identified several predictive genetic variants that were highly associated with cisplatin-induced hearing loss in children. We evaluated whether we could replicate these findings in a new independent cohort of 155 pediatric patients. Associations were replicated for genetic variants in *TPMT* (rs12201199, P=0.0013, Odds Ratio, OR 6.1) and *ABCC3* (rs1051640, P=0.036, OR 1.8). A predictive model combining variants in *TPMT*, *ABCC3* and *COMT* with clinical variables significantly improved the prediction of risk of developing hearing loss compared to clinical risk factors alone (P=0.00048). We next evaluated whether we could identify additional genetic variants that confer susceptibility to cisplatin-induced hearing loss. We identified novel variants in *ABCB5* (rs10950831, $P=1.06\times10^{-6}$, OR 2.0) and *DPYD* (rs6667550, P=0.0047, OR 1.9) that were significantly associated with cisplatin-induced hearing loss. We included these variants into the initial genetic model that consists of variants in *TPMT, ABCC3* and *COMT* to evaluate whether we could improve the prediction of risk. We demonstrate that the risk of prediction of hearing loss significantly improves by including genetic variants in *ABCB5* and *DPYD* (P=0.0023). We also demonstrate that by combining the clinical and genetic factors we can significantly improve the prediction of risk of hearing loss compared to clinical factors alone ($P=2.63\text{x}10^{-7}$). We were able to replicate previously described findings and also provide evidence for novel genetic variants in the

prediction of cisplatin-induced hearing loss in children. Furthermore, this study demonstrates that predictive models can classify patients based on predicted risk of cisplatin-induced hearing loss. These findings have the potential to influence treatment modifications for cisplatin therapy and may improve safety in children.

Preface

This dissertation is comprised of both published and unpublished material as follows:

This study was approved by the University of British Columbia Clinical Research Ethics Board (certificate number H04-70358).

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List of Symbols, Abbreviations

SNP	Single nucleotide polymorphism
COMT	Catechol O-methyltransferase
TPMT	Thiopurine S-methyltransferase
ABCC2	ATP-binding cassette transporter, subfamily C, member 2; multiple drug resistance protein 2 (ABCC2)
ABCC3	ATP-binding cassette transporter, subfamily C, member 3; multiple drug resistance protein 3 (ABCC3)
ABCB5	ATP-binding cassette transporter, subfamily B, member 5; multiple drug resistance protein 5 (ABCB5)
ABCG2	ATP-binding cassette transporter, subfamily B, member 2; multiple drug resistance protein 2 (ABCG2)
ADME	Absorption, Distribution, Metabolism, Excretion, and Toxicity
ADR	Adverse Drug Reaction
AUC	Area Under the Curve
CADRMP	Canadian Adverse Drug Reaction Monitoring Program
CI	Confidence Interval
CPNDS	Canadian Pharmacogenomics Network for Drug Safety
CTCAE	Common Terminology Criteria for Adverse Events
CTR1	Solute Carrier 31, Member 1
CYP	Cytochrome P450
DYPD	Dihydropyrimidine Dehydrogenase
FDA	U.S. Food and Drug Administration
GST	Glutathione S-Transferase
LD	Linkage Disequilibrium
MAF	Minor Allele Frequency

MAPK	Mitogen-Actived Protein Kinase
NOX-3	NADPH oxidase 3
NPV	Negative Predictive Value
OR	Odds Ratio
PC	Principal Component
PD	Pharmacodynamics
PK	Pharmacokinetics
PPV	Positive Predictive Value
ROC	Receiver Operating Characteristics
SJS	Stevens-Johnson Syndrome
SLC31A1	Solute Carrier 31, Member 1
TEN	Toxic Epidermal Necrolysis
UGT	Uridine Diphosphate Glucuronosyltransferase
VKORC1	Vitamin K Epoxide Reductase

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Chapter 1: Introduction

1.1 Adverse Drug Reactions

In modern drug development, clinical trials focus on the safety and efficacy of medications at standard doses. The inherent differences within the population are not taken into account or investigated in the drug development pipeline. It is now well recognized individual variability in response to medications range from a lack of therapeutic benefit to life-threatening adverse drug reactions(ADRs)[1]. An ADR is defined as a harmful, undesirable reaction to a medication which justifies methods for prevention, dose modifications or cessation of therapy[2].

ADRs can vary from mild to severe reactions, those that are permanent to ones that reverse once medication is discontinued. The variability in drug response has a major impact on health care costs and quality of life. In some cases, severe ADRs can result in hospitalizations and even put patients at risk of morbidity and mortality. Each year greater than 2 million severe ADRs claim over 100,000 lives in the United States, ranking them as the 5th leading cause of death[3-5]. In the United States alone, ADRs are estimated to cost between 30 to 130 billion dollars annually[3, 4, 6]. The economic burden associated with ADRs can be higher than for conditions such as obesity, where the annual costs ranges from 24 to 70 billion dollars[7]. A more recent prospective study conducted in the United Kingdom to address the current burden of ADRs through an analysis of 18,820 hospital admissions, attribute 1225 admissions (6.5%) to an ADR[8]. The costs associated with ADRs can pose a significant burden on the healthcare system not only by the treatment required for the ADR itself but also by the monitoring required to screen all patients for potential ADRs. Clozapine-induced agranulocytosis is an example in

which patients are frequently monitored for their white blood cell count through frequent blood tests[6, 9].

ADRs can range from minor reactions to severe-life threatening events such as heart failure. These reactions can occur within minutes after treatment or years after first exposure to the medication. For example, most carbamazepine-induced Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) cases occur within the first 8 weeks of exposure to the drug[10, 11]. Severe adverse effects may not even be observed until the drug is on the market as clinical trials are limited by study size. It may take millions of individuals exposed to the drug before unexpected adverse events emerge[12]. When regulatory authorities deem that the risk of these adverse events outweigh the benefit, medications are forced to be removed from the market.[12] However, drug withdrawals potentially leave patients that benefitted from the therapeutic effect of the medication without access to treatment. An example of this is Vioxx, a potent arthritis pain killer. Once released into the market, reports of heart attack and stroke resulted in the drug being pulled from the shelves[12].

This highlights the importance of post-market surveillance of drugs once available to the general public in order to identify and assess of adverse events of medications. Health Canada encourages health professionals to report ADRs to identify unknown adverse events and to assess the balance between risks and benefits of medications to improve safety once on the market. However, despite the obvious benefit of reporting ADRs, it is estimated that approximately 90% of all ADRs are never reported to regulatory agencies[13]. There are several studies that assess reasons of under-reporting which include doubt of association with an ADR,

the reaction is too well known to report, failure to recognize an adverse event, lack of time, and not knowing how to report an ADR to regulatory agencies[14-16]. A study evaluating TEN highlights how even life-threatening ADRs are under-reported in Canada. They identified that only 4-10% of cases of TEN were reported to the Canadian Adverse Drug Reaction Monitoring Program (CADRMP)[17].

ADRs can significantly impact patient health and result in morbidity and mortality, permanent disability and treatment cessation. It is suggested that many ADRs can be prevented; suggesting that increased effort should be placed on monitoring and reporting of adverse events[5]. Although many risk factors can contribute to the development of an ADR, in most cases it remains unclear how they influence the efficacy and toxicity of the drug.

1.1.1 The Genetic Contribution to Drug Toxicity

Drug toxicity is often associated with drug concentrations both in the plasma and target location. Drug concentrations are dependent on key processes involved in absorption, distribution, metabolism and excretion (ADME). Drug metabolism and excretion are processes that inactivate and detoxify drugs to protect the human body from potential harmful adverse effects. Metabolism can be divided into phase I and phase II reactions which convert the parent compound into metabolites that are usually more water soluble for excretion through the kidney.

Phase I metabolism involves functional group modifications through oxidation, reduction and hydrolysis reactions. A majority of Phase I metabolism enzymes belong to the cytochrome P450 (CYP) family of enzymes (**Figure 1.1**)[18]. Phase II enzymes are involved in conjugation reactions and include enzymes such as uridine diphosphate glucuronosyltransferases (*UGTs*),

thiopurine *S*-methyltransferase (*TPMT*) and glutathione *S*-tranferases (*GSTs*)[18]. These enzymes are known to exhibit genetic polymorphisms that may contribute to variability in drug response and lead to clinical consequences including ineffective drug treatment and drug-induced toxicity.

1.2 Pharmacogenomics

Pharmacogenomics is the study of the influence of individual genetic variation on drug response. The field of study dates back to the 1900's when individuals such Sir Archibald Garrod observed growing clinical evidence that individuals responded differently to standard doses of medications usually accompanied by variations in the concentrations of drug metabolites[1]. It was not until the 1950s that Arno Motulsky wrote an article on the genetic basis of adverse drug reactions[19]. In 1959, Friedrich Vogel introduced the field as "pharmacogenomics"[19]. Research is now focused on the potential to shape the practice of medicine to treat patients at the individual level.

In current practice patients are dosed based on standard protocols, adjusting for body surface area[20]. Many non-genetic factors can contribute to large population differences observed with response to therapy such as age, concomitant medications, drug interactions, organ function, patient health and environment[20]. Unlike non-genetic factors that change throughout a patient's lifetime, clinicians can utilize genetic variants that remain stable to reliably identify patients at risk of adverse events[21]. Genetic factors such as single nucleotide polymorphisms (SNPs) are known to play a role in the incidence and severity of ADRs[1, 22]. For example, clinical factors such as age, body surface area, gender, and dietary intake of vitamin K account for approximately 12% of the dose variability in patients receiving warfarin[23, 24]. However,

one study has described that 25% of the variability in warfarin dose is due to variation in vitamin K epoxide reductase (*VKORC1*)[25].

Genetic variation plays a role in drug response through pharmacokinetics (PK) and pharmacodynamics (PD). PD is the study of the effect of drugs on the body. This involves the interaction of the drug with its target site and is responsible for the relationship between drug concentration and effect[26]. In contrast, PK is the study of how the body affects the extent and rate of the ADME of drugs[27]. Differences in the genetic variants in genes encoding drug-metabolizing enzymes and transporters can substantially change the PK and PD pathways involved in drug metabolism[28]. For example, a study examined the role of *CYP2C19* polymorphisms on the PK of clopidogrel and evaluated whether genotypic differences influenced the variability of response to the drug. It is estimated that up to 4-30% of patients do not show an adequate anti-platelet response with clopidogrel[28]. This study was able to show that individuals that were poor metabolizers (i.e., *CYP2C19*2/*2* or *CYP2C19*2/*3*) responded poorly to the anti-platelet effect of clopidgrel[28]. This highlights how genetic variants can influence drug response, particularly through changes in the expression and activity of genes that influence PK and drug ADME. Genetic variation may also explain why some individuals experience ADRs whereas the drug is safe and effective in other individuals[29].

A major goal of pharmacogenomic studies is to identify genetic factors involved to predict and prevent severe ADRs from occurring after treatment. With the sequencing of the human genome and advancement in genomic technology, it is becoming increasingly more possible to screen individuals for key variants involved in drug metabolism and identifying patients at risk prior to implementing therapy[30]. Pharmacogenomics research has advanced our understanding of the mechanisms of actions of drugs including warfarin and clopidogrel[31]. Pharmacogenomics not

only has the potential to advance our knowledge of the PK and PD of medications, but also may improve the management and safety of these medications in patients[31].

1.2.1 From Scientific Discoveries to the Clinic

The variability in drug response has led to the increasing concerns by patients, clinicians, and policy makers about the safety of medications. The withdrawal of medications from the market is not an ideal solution as it leaves individuals that benefit from treatment without access to an effective therapy. By identifying patients that are at high risk of adverse events prior to treatment we can improve the safety of medications. Furthermore, these medications remain accessible to patients for whom there is a therapeutic benefit.

The translation of pharmacogenomic scientific discoveries are of importance not only to patients and medical research centers, but also within the pharmaceutical industry governed by drug regulatory agencies including Health Canada and the U.S. Food and Drug Administration (FDA). The release of the FDAs' pharmacogenomic guidelines in 2004 highlighted the importance of genetic testing for medications. The FDA has established a list of approximately thirty different pharmacogenomic markers that are mentioned in approved drug labels[32]. However, of these drugs, only four have pharmacogenomic tests available with a level of evidence where a recommendation can be made for their use in practice. Currently, FDA requires genetic testing mainly for oncology related drugs that affect efficacy and response to therapy such as *Her2/neu* for Trastuzmab (**Table 1.1**)[32]. The FDA has also recommended pharmacogenomic testing in their label for five drugs including carbamazepine, where *HLA-B*1502* is recommended prior to therapy to prevent life-threatening reactions such as SJS and TEN (**Table 1.1**)[32]. The over-arching goal of pharmacogenomics, is to implement pharmacogenomic evidence-based criteria to

help guide clinicians to better evaluate the risk-benefit profile of medications in order to provide optimal treatments for patients.

1.2.2 Pharmacogenomic Studies in Children

ADRs are of particular concern in the pediatric population because less than 25% of approved pharmaceuticals have been tested in children. As a result, there is a gross lack of guidelines for safety and efficacy in children[3]. In drug development, clinical trials that evaluate toxicity and efficacy of medications are often not carried out in children due to ethical concerns or challenges such as informed consent[33]. Children have a limited capacity to understand the risks associated with participating in research which justifies why a child under the age of 18 cannot legally give consent to participate in a clinical trial. A guardian or parent has the ability to consent the child; however there has been debate as to whether the parent is acting only in the child's best interest[33]. Consequently, dosing guidelines in children are frequently extrapolated from adult clinical data and adjusted based on weight and/or body surface area[34]. We now understand that this simplified dosing approach is not an adequate long-term solution. Children cannot be treated as small adults because age-related differences in the expression and activity of drug metabolizing enzymes and transporters contribute to the altered metabolism and excretion of drugs in children compared to adults. For example, the level of activity of some enzymes in the CYP family increase within the first three months of development[35]. These changes can explain why drug clearance of carbamazepine for example, is greater in children than adults[35]. The Phase II enzyme UGT2B7 is another example that highlights the difference in activity of enzymes during development. The increase in activity of UGT2B7 with age increases the clearance of morphine from plasma[35]. Furthermore, developmental changes in renal function can also alter clearance of drugs from the plasma resulting in differences in

elimination of compounds[35]. For this reason, inferring the PK and PD behavior of a drug extrapolated from adult data may not provide accurate dosing guidelines in children. Furthermore, these changes can significantly impact drug concentrations and can result in the exposure of infants and children to toxic drug levels.

Safe and effective drug therapy in pediatric patients requires an understanding of the developmental differences in drug metabolism and clearance in children. The contribution of genetic variants to the variability in drug response and ADRs has been investigated primarily in adult patients. Pharmacogenomic studies in children can help elucidate the mechanisms that affect the variability of drug response and toxicity in this unique population.

1.3 Cisplatin

Cisplatin (PLATINOL®) is among the most potent anti-cancer drugs. It is an effective and widely used chemotherapeutic agent for the treatment of solid tumours. In adult patients, cisplatin is used to treat several cancers including ovarian, testicular, lung and head and neck tumours[36, 37]. Cisplatin is currently used as a standard of care treatment for many types of cancer in children, including neuroblastoma, osteosarcoma, germ cell tumours, brain tumours and hepatoblastoma[38]. In fact, cisplatin has shown such an efficacy in standard risk hepatoblastoma that it can be used as monotherapy with an over 80% 3-year event-free survival[39]. Cisplatin is often incorporated in combination with other chemotherapeutic agents such as vincristine, etoposide and doxorubicin. Combination therapies have resulted in high cure rates including testicular cancer patients where the overall cure rate is over 90%[40].

1.3.1 Cisplatin Use in Children

There are approximately 16 new children treated with cisplatin annually in British Columbia alone which translates to an estimated 136 children annually treated with cisplatin in Canada. A number of different factors are used to determine how cisplatin should be administered in children including the tumour type, the stage of the tumour and the treatment protocol. Overall, children are treated with a bolus of cisplatin for most tumour types. However, the treatment of all extra-cranial germ cell tumours is unique as these patients are treated at low individual doses of 20mg/m^2 over five days[41]. These differences in how cisplatin is delivered to patients can affect duration of treatment, dose intensity and dose frequency and can influence the toxicity associated with treatment.

1.3.2 Mechanisms of Cisplatin Action

Cisplatin is a cell-cycle non-specific antineoplastic agent which is part of a class of antitumour agents that act at all stages in cell cycle[42]. Cisplatin forms covalent bonds to the N^7 positions of purine bases in DNA forming intra-strand and inter-strand crosslinks[43]. These modifications damage and distort the structure of the double-stranded DNA. This affects proteins including hMSH2, a component of the mismatch repair complex and transcription binding factors such as the TATA binding protein which are associated with cellular processes including cell-cycle arrest, transcription, and DNA repair[44, 45]. Cisplatin activates signal transduction pathways including those involved in p53, p73 and mitogen-actived protein kinase (MAPK) which then activate programmed cell death (apoptosis) in tumour cells[44, 45]. However, when this signalling pathway is altered, resistance may occur, a limitation for cisplatin chemotherapy[44].

1.4 Cisplatin-Induced ADRs

Cisplatin is limited in its' clinical application due to serious adverse effects including nephrotoxicity, neurotoxicity, and ototoxicity. Although nephrotoxicity has been reported, it can be prevented by saline hydration therapy or diuresis[46, 47]. However, there are no proven therapeutic treatment options available for neurotoxicity and ototoxicity[47].

Cisplatin is one of the most ototoxic drugs currently in the market. Cisplatin is responsible for the severe irreversible hearing loss, which affects 10-25% of adults and 26-90% of children depending on dose, treatment regimen and degree of hearing loss measured[48-53]. Furthermore, patients show no improvement in hearing and often the hearing loss continues to progress and increase in severity after the end of therapy[54]. Current clinical practice involves dosing to toxicity. Dose reductions or termination of cisplatin treatment are suggested in standard treatment protocols once signs of significant hearing loss (grade 2 or greater) are observed, thereby significantly limiting the use of cisplatin therapy in susceptible patients[55]. Although alternative chemotherapy agents exist that are less ototoxic, such as carboplatin and oxaliplatin, these treatments may not be as effective in treating certain tumours[41]. For example, cisplatin is more effective to treat adult extracranial germ cell tumours than carboplatin, and therefore is the therapy of choice[41]. Furthermore, other adverse effects including neurotoxicity and nephrotoxicity may be more prominent in these other chemotherapeutic agents. Therefore, discontinuation of cisplatin is often not a simple solution to prevent severe ototoxicity from occurring. However, there are cases where alternative medications for certain tumour types have been shown to be equally effective, less ototoxic, and are options within the current standards of care. For example, in Europe, germ cell tumours are treated using carboplatin, etoposide and

bleomycin, a treatment regimen that has similar outcomes to treatment with cisplatin, etoposide and bleomycin but with less ototoxicity[56].

There are also significant costs associated with cisplatin-induced hearing loss. Health-care costs for patients that develop severe hearing loss are mainly due to education and development, hearing aids and/or cochlear implants[57]. An economic cost analysis estimated lifetime cost of serious cisplatin-induced hearing impairment is between $445,447 and $562,198 per patient[57]. Assuming that an alternative medication to cisplatin exists that is as effective for treatment, the costs avoided due to cisplatin therapy in Canada are estimated to be $19.6 million by administering a genetic test for cisplatin-induced hearing loss[57].

1.4.2 Pathophysiology of Cisplatin-Induced Hearing Loss

Pure-tone audiometry measurements are used to determine the degree of hearing loss in patients. Toxicity can result in significant disability, highlighting the importance of careful and frequent monitoring of auditory function in patients. In patients receiving the same dose of cisplatin, the inter-individual variability of cisplatin-induced effects on hearing is profound, ranging from no hearing loss, to high frequency hearing loss and progresses to severe hearing impairment in the speech frequencies[58, 59]. It is estimated that 50% of consonants in the English language are pronounced in the high frequency range including the sounds “th”, “f”, “k”, and “s”[60]. With signs of hearing loss above 4000Hz, these sounds become indistinguishable to the affected individual[60]. In clinical practice, it is recommended that audiograms be obtained prior to therapy, prior to each round of treatment, and upon any signs of hearing loss. These are usually offered as standard care by medical service plans. However, follow-up tests may be missed by patients in rural areas, until severe hearing loss occurs, at which time there are no preventative

options to consider. There are currently no guidelines as to when audiogram hearing tests should be conducted post therapy.

Several different grading criteria for hearing loss have been developed over the years. The most commonly used methods are the Common Terminology Criteria for Adverse Events (CTCAE)[61] and the Brock scale[49] (**Table 1.2**). However, various other grading schemes exist such as the Muenster classification for early detection of hearing loss[62] and the Chang scale (**Table 1.2**)[55]. Patients are classified into different categories from individuals who do not develop hearing loss (Grade 0) to individuals who develop mild to severe hearing loss (Grade 1-4). Individuals diagnosed with hearing impairment of grade 3 or grade 4 generally require intervention including hearing aids and in extreme cases, a cochlear implant. Furthermore, patients show no improvement in hearing and often the progression and severity of hearing loss continues long after the end of therapy[54].

1.4.3 Cisplatin-Induced Hearing Loss in Children

It is well known that children are at the greatest risk of developing hearing loss from cisplatin treatment [48]. This is because cisplatin-induced hearing loss is dependent on age. Studies have shown that hearing loss with therapy is more prevalent in younger children[55, 63]. It is particularly devastating in children as they are at a critical stage of development. In children, even mild losses in hearing can significantly influence the overall quality of life including speech and language development, social-emotional development and increase the risk of learning difficulties[64, 65]. Furthermore, once detected, children show no improvement in hearing and often hearing loss progresses in severity long after treatment has ended[58, 59]. In patients

experiencing severe hearing loss, hearing aids and other assistive devices are recommended. The use of hearing aids may be delayed until hearing loss has stabilized and treatment is complete. Although these devices can improve a child's hearing, they do not completely correct hearing loss. This suggests that by detecting hearing loss early or prior to treatment will allow children at risk of cisplatin-induced hearing loss to potentially have improved quality of life.

1.4.4 Mechanism of Cisplatin-Induced Hearing Loss

Cisplatin damage occurs by apoptosis at three sites in the cochlea: the outer hair cells in the organ of Corti, the spiral ganglion and the stria vascularis[45, 66]. Cisplatin-induced hearing loss is difficult to treat, primarily as the mechanisms involved in this process are not well understood. The generation of reactive oxygen species (ROS) are involved in the toxicity associated with cisplatin therapy[67, 68]. Damage to the hair cells occurs as a consequence of antioxidant depletion, increased lipid peroxidation and an increase in ROS such as superoxide and hydrogen peroxide in the cochlea[69]. This can lead to calcium influx and activation of apoptotic pathways[69]. Rybak et al have demonstrated that NADPH oxidase 3 (NOX-3), expressed in the inner ear generates low levels of ROS species[69]. When activated, NOX-3 increases the production of ROS in the cochlea leading cell death[69]. A study has shown that nitroxide can react with ROS leading to the activation of caspase-3[70]. Studies in cisplatin treated guinea pigs have shown that activation of caspase-3 can lead to apoptosis in cells of the stria vascularis and spiral ligament in the cochlea[70]. The increase in free radicals can lead to morphological and functional changes in the organ of Corti[71]. The production of ROS changes acoustic

transduction by modulating the outer hair cell motility in the organ of Corti leading to cell death and consequently leading to hearing loss[72].

1.4.5 Clinical Risk Factors

Several studies have suggested that several clinical risk factors contribute to cisplatin-induced hearing loss. Non-genetic factors such as high cumulative cisplatin dose, younger age at time of treatment, concomitant use of aminoglycosides, pre-existing inner hearing impairment, and cranial irradiation are known to influence cisplatin-induced ototoxicity (**Table 1.3**)[59].

High cumulative dose has been described as a risk factor for cisplatin-induced hearing loss(**Table 1.3**)[48, 73, 74]. Studies have shown cumulative doses greater than 400mg/m^2 in pediatric patients[48, 75]. Many studies have confirmed that younger patients are at increased risk of developing hearing loss[48, 55, 73]. One study has demonstrated that pediatric patients less than 5 years of age were at significantly higher risk of developing hearing loss with cisplatin therapy than patients between 15 or 20 years of age (P=0.001) (**Table 1.3**)[48]. They found that 40% of children younger than 5 years would develop severe hearing loss, compared to 5% of patients between 15 to 20 years[48]. Bokemeyer et al has also observed that patients treated with concomitant vincristine were at significantly higher risk of developing hearing loss (P=0.001)[73]. Several studies have also shown aminoglycoside antibiotics, which are widely used for the treatment of gram negative bacterial infections, can cause dose-related hearing loss[76, 77]. Aminoglycosides are also suggested to affect sensory inner ear hair cells, and thus may be a confounding risk factor to cisplatin ototoxicity[77]. In children, radiotherapy is often part of the treatment protocol for brain tumours, such as medulloblastoma, because of the

positive progression free survival (86% at 3 years and 79% at 5 years)[78]. A serious adverse event associated with radiotherapy is sensorineural hearing loss that affects the inner ear and cochlea. Studies have observed that the incidence of hearing loss with concurrent cisplatin therapy and cranial irradiation increases with increasing radiation dose (**Table 1.3**)[74]. In fact hearing loss may be observed at doses as low as 45Gy at frequencies greater than 2000Hz[74, 79]. Other potential risk factors have been evaluated, such as gender[74], but have not been significantly associated with cisplatin-induced hearing loss.

1.5 Cisplatin Transport – Uptake and Efflux

Early studies have suggested that cisplatin uptake occurs by passive diffusion. However, more recent studies suggest a number of active uptake and efflux mechanisms play a role in drug resistance and transport of cisplatin[80]. The ATP-binding cassette (ABC) family of genes are well known for their ability to transport drugs across the cell membrane. Studies have shown ATP-binding cassette transporter C 2 (ABCC2) overexpression enhance the resistance to cisplatin and therefore can decrease the intracellular drug concentrations, suggesting it may play a role in promoting cisplatin efflux (**Figure 1.2**)[81, 82]. There have been some controversial results about whether ATP-binding cassette transporter G 2 (ABCG2) is a transporter for platinum compounds. Several studies have shown that ABCG2 does not transport cisplatin[83]. However, a recent study with oxaliplatin has reported that with increased expression of ABCG2, there is increased resistance to chemotherapy, suggesting it may be a transporter of platinum compounds[84]. Copper efflux transporters ATP7A and ATP7B seem to be involved in the accumulation of cisplatin in tumour cell lines[85]. Recent studies have demonstrated that copper transporter 1 (CTR1; also known as solute carrier 31 (copper transporters), member 1,

SLC31A1) mediates the influx of cisplatin and is responsible for the accumulation of the drug intracellularly[86]. With controversial results and the limited number of studies investigating cisplatin transport, more research is required to fully understand the transporters involved in cisplatin influx and efflux.

1.6 Pharmacogenomic Studies of Cisplatin-Induced Hearing Loss

Clinical criteria are not currently used to identify individuals who are at an increased risk of developing hearing loss. Genetic variation in the genes involved in ADME have been recognized to influence patient drug response and susceptibility to adverse drug events, including ototoxicity[29]. The following studies have described genetic variants that were associated with cisplatin-induced hearing loss.

1.6.1 Polymorphisms with Megalin and Cisplatin-Induced Hearing Loss

There is a three to five-fold increased accumulation of platinum-DNA adducts in the marginal cells of the stria vascularis compared to the organ of Corti[87]. This suggests that a membrane pump expressed in the stria vascularis is responsible for the high accumulation of these platinum-DNA adducts. Megalin is an endocytic receptor expressed in marginal cells of the stria vascularis of the inner ear and renal proximal tubular cells[88]. Megalin has also been shown to transport aminoglycosides which are known ototoxic drugs[89]. Several studies have been carried out to determine whether polymorphisms in megalin are associated with cisplatin-induced ototoxicity.

Riedemann et al graded patients based on the Muenster classification for early detection of cisplatin-induced hearing loss[90] (**Table 1.4**). Seventy-four pediatric patients treated with cisplatin were recruited. Patients with risk factors (prior hearing loss, <5 years of age, cranial irradiation, ototoxic medications and renal impairment) were excluded from the study. The study cohort consisted of 50 patients; 25 controls and 25 cases treated for a variety of malignancies. The controls consisted of patients with no hearing loss (Grade 0) and mild hearing loss (Grade 1). Patients in the study ranged from children to young adults. Two non-synonymous SNPs (rs2075252 and rs4668123) in the megalin gene (*LRP2*) that occur with a high frequency in the Caucasian population were evaluated. A significant association was discovered between the A-allele of *LRP2* rs2075252 (Glu4094Lys) and cisplatin-induced hearing loss. The risk 'A' allele of rs2075252 was carried by 13 (52%) cases and 6 (24%) controls.

A subsequent independent study carried out in children did not show an association with the Megalin rs2075252 variant and cisplatin-induced hearing loss[64]. Several differences in methodology and phenotyping may explain the discrepant results between studies. Unlike Riedmann et al, patients with confounding risk factors were not excluded in the latter study. Rather the authors corrected the genetic associations for these clinical cofounders in the analysis. Furthermore, patients were phenotyped using a different grading scheme (CTCAE) and those with mild hearing loss (Grade 1) were excluded in these subsequent studies to evaluate more extreme phenotypes. Cisplatin-induced hearing loss often increases in severity over time, even after completion of cisplatin treatment (i.e. patients with Grade 1 hearing loss progress to Grade 2 to 4)[91, 92]. Therefore, patients with Grade 1 hearing loss may have been misclassified in the Riedmann et al cohort.

Overall, given the lack of replication and the low number of patients (n=25 ototoxicity cases) in the Riedmann et al study, it is difficult to determine whether the association of rs2075252 with cisplatin ototoxicity is a true association or whether it has been over-estimated resulting in a false-positive finding.

1.6.2 Polymorphisms with Glutathione S-Transferases (GSTs) and Cisplatin-Induced Hearing Loss

GSTs play a role in the amount of free platinum compounds that bind to DNA by catalyzing conjugation of these compounds to glutathione. GSTs have been found to play a significant role in the detoxification of platinum drugs through conjugation of the active metabolite to glutathione and exporting it out of the cell[93, 94]. GSTs are expressed in mammalian cochlea and animal studies have suggested that GSTs can also protect against oxidative damage in the cochlea that leads to ototoxicity[95]. This suggests GSTs may play a role in the development of cisplatin ototoxicity. Several studies have therefore examined whether polymorphisms that decrease expression in GST genes such *GSTM1* and *GSTT1* are associated with cisplatin-induced hearing loss (**Tables 1.5-1.9**) [96-99]

1.6.2.1 *GSTM3*B*

Two studies have found associations between cisplatin-induced hearing loss and genetic variants in GST genes. Peters et al identified an association between cisplatin-induced hearing loss and the *GSTM3*B* variant, a three-base pair deletion that creates a recognition site for a transcription factor[96]. *GSTM3*B* was carried at a higher frequency in control patients and thus proposed to

be protective against cisplatin-induced hearing loss (OR 5.37, *P*=0.02). This study was carried out in a relatively small cohort of pediatric patients (n=39) treated for a variety of tumour types in children and did not correct the genetic findings for multiple testing and clinical cofounders (**Table 1.5**). Conversely, in a second study carried out in 100 adult patients treated for ovarian cancer, there was no significant association with *GSTM3*B* or any other GST genes in the context of cisplatin-induced ototoxicity[99]. The lack of replication of GSTM3*B with cisplatin ototoxicity may be due to differences between cohorts including age of patients and the ototoxicity grading criteria. Furthermore, Khrunin et al did not evaluate ototoxicity by audiometric tests prior to start of treatment, suggesting that individuals may have had undetected pre-existing hearing loss which could compromise results. Other studies in both children and adults have not investigated the *GSTM3*B* deletion. Therefore, it is difficult to conclude that *GSTM3*B* is important in identifying patients at risk of hearing loss due to these contradictory results. However, there is a possibility that this variant may be important in children and not in adults. Further studies need to be conducted to investigate whether this is a finding is a true association.

1.6.1.2 *GSTP1*

Oldenburg et al. identified an association of *GSTP1* with cisplatin-induced hearing loss in a cohort of 173 adult testicular cancer patient (OR 4.21, P<0.001) (Table 6) [97]. This study used significantly different ototoxicity grading criteria than most studies of cisplatin-associated ototoxicity. They ranked patients based on the threshold of hearing at 4000Hz to separate patients into specific percentile groups rather than using a grading scheme. An additional follow-up study by Oldenburg et al did not find an association with *GSTP1* in an extended cohort of

adult testicular cancer patients[98]. However, as all the patients from the previous study were included in this extended cohort, this cannot be considered an independent replication study. The effect of *GSTP1* rs1695 was in the same direction in all studies. These studies have demonstrated a similar direction of association with the *GSTP1* rs1695 variant and cisplatin-ototoxicity. However, this association only achieved statistical significance in one study of adult patients ($p < 0.001$)[97], and trended towards significance in two other studies (*P*-values= 0.055 and 0.16) [98] (**Table 1.6**).

In these studies the association of the *GSTP1* rs1695 A-allele has shown a similar trend in association with cisplatin-induced ototoxicity (odds ratios for protective GG allele in pediatric studies: no association and 0.78; and in adult studies: 0.23, 0.71, and no association). However, this association only achieved statistical significance ($p < 0.05$) in one study of adult patients[97]. It is possible that the relatively small effect size of the *GSTP1* rs1695 variant has been undetectable in the underpowered studies to date. Additional studies should further investigate the potential role of *GSTP1* variants in cisplatin-induced hearing loss. The small effect size will require large studies to confirm or refute.

1.6.1.3 *GSTM1* and *GSTT1*

Oldenburg et al. also identified an association of *GSTM1* with cisplatin-induced hearing loss and developed two multi-marker models consisting of *GSTM1*, *GSTP1*, and *GSTT1* in testicular cancer patients (**Table 1.7**)[97]. In the first cohort, the *GSTM1* positive odds ratio was 2.36 ($p = 0.022$, $n = 173$), and in the second study that combined the first cohort with a second cohort, the odds ratio was 1.81 ($p = 0.025$, $n = 238$) [98]. The second association observed with *GSTM1* may therefore have been largely due to the effect from the original 173 testicular cancer patients. The

weaker association in the new patient cohort could also be due to the fact that hearing loss of some patients in the follow-up study was self-reported, as opposed to audiometric grading. Other studies that investigated *GSTM1* and *GSTT1* have found no significant association with hearing loss after cisplatin therapy including three independent studies in children[64, 96] treated for various malignancies, as well as an adult study in ovarian cancer patients[99] (Table 4.6). Furthermore, the effect of *GSTM1* was observed in opposite directions. Therefore, further replication of the original findings in an independent patient cohort would significantly strengthen this association.

At this time, there is no consistent evidence of a strong association between genetic variants in, *GSTM1*, *GSTT1* and cisplatin-induced hearing loss. However, it cannot be excluded that the effect of some of these variants may apply only to adult testicular cancer patients, in whom they were identified. The treatment protocols for this group are often unique, with longer and slower infusions of cisplatin. Replication of this finding in another independent testicular patient cohort is required prior to use in the clinic to predict risk.

1.6.2 Variations in Genes in DNA Repair Pathways and Cisplatin-Induced Hearing Loss

The cross-links that form between cisplatin and DNA impede DNA replication. A major pathway involved in DNA repair is the nucleotide excision repair (NER) pathway which functions to remove platinum adducts. Polymorphisms in genes involved in NER (*ERCC1*, *ERCC2*, *ERCC4*, *ERCC5*, *XPA* and *XPC)* have been investigated in the context of susceptibility to cisplatin-induced ototoxicity[99, 100]. Other DNA repair genes that have been investigated include *XPD* and *XRCC1*[99].

Caronia et al reported 32 patients (adults and children) diagnosed with osteosarcoma (**Table 1.8**) [100]. Several genetic variants in the NER pathway were investigated and a weak association of a non-synonymous SNP in *XPC* rs2228001 (Lys939Gln) with cisplatin-mediated hearing loss was observed (P=0.042) [100]. The polymorphism is hypothesized to reduce the activity of *XPC*, and thus affect the capacity for DNA repair. This decrease in DNA repair capacity may increase apoptosis in the cochlea resulting in ototoxicity[100]. However, no multiple testing corrections were applied in this study and the number of patients in the study was limited (n=32). Furthermore, the study did not evaluate differences in clinical factors between cases and controls, and therefore did not take potential confounding factors into account.

Functional studies evaluating DNA repair capacity in *XPC* rs2228001 have also shown inconsistent results. For example, one study observed that individuals that carry the C-risk allele had significantly higher DNA damage compared to the A-allele[101], where as Khan et al found similar DNA repair capacity between the two alleles[102]. There are currently no replication studies to confirm this finding and due to inconsistent findings from functional studies, it is difficult to draw any conclusions about an effect of rs2228001 on cisplatin ototoxicity at this time and the use in clinical practice would require additional supporting evidence.

None of the variants in other genes in the DNA repair pathway investigated in the aforementioned studies were associated with cisplatin-induced hearing loss, including polymorphisms in *ERCC1, ERCC2, ERCC4, ERCC5* and *XPA*. Another independent study by

Khrunin et al carried out in 100 adult ovarian cancer patients (**Table 1.8**) also did not observe any association of variants in *ERCC1*, *XPD* and *XRCC1* with cisplatin ototoxicity*[99]*.

1.6.3 Genes Associated with Hereditary Deafness and Cisplatin-Induced Hearing Loss

Genes involved in hereditary deafness have also been studied in the context of cisplatin ototoxicity. Mutations in the mitochondrial genome are thought to be responsible for a number of clinical abnormalities including hearing loss. Previously, variants in *GJB2*, *SLC26A4* and mitochondrial polymorphisms in *MTRNR1* (A1555G), *MTTL1* (A3243G) and *MTTS1* (A7445G) have been shown to be associated with aminoglycoside- mediated ototoxicity and progressive nonsyndromic high frequency hearing loss (hereditary deafness). As the molecular basis of cisplatin-induced ototoxicity remains unclear, Knoll et al hypothesized that genes associated with hereditary deafness may also be associated with cisplatin-induced ototoxicity. Knoll et al carried out mutation screening in the *GJB2* and *SLC26A3* genes by sequencing and genotyping of the mitochondrial DNA (mtDNA) polymorphisms in 11 children that were selected based on self-report of clinically apparent hearing loss (**Table 1.9**). No evidence for a contribution of these variants to the susceptibility to cisplatin-induced hearing loss was detected. Given the very small cohort of patients in this study, it may have been underpowered to find any association. However, a separate independent study in 39 patients also investigated the role of mtDNA polymorphisms and also did not observe any association with cisplatin-induced hearing loss. Therefore, there is currently no evidence to suggest an association of genes involved in hereditary deafness with cisplatin-induced hearing loss. However, a larger study would be required to adequately assess whether these variants are potential predictors of cisplatin-induced ototoxicity.

1.7 Predictive Modelling in Pharmacogenomics

1.7.1 Measures of Predictive Tests

Both sensitivity and specificity are measures for assessing the results of diagnostic and screening tests (**Table 1.10**). Sensitivity represents the proportion of truly diagnosed people in a screened population who are identified as being affected and therefore, it is a measure of the probability of correctly diagnosing a disease or condition[103]. Specificity is the proportion of truly non-affected individuals identified. Thus, specificity measures the probability of correctly identifying a person without the disease or condition[103]. Generally there are trade-offs between specificity and sensitivity. Ideally, a clinical test would have high specificity and high sensitivity.

An ideal clinical test will be accurate, producing a positive result in the presence of the disease and a negative result in the absence of the disease. A test may produce false positive and false negative results (**Table 1.10**). Positive and negative predictive values represent such errors. Positive predictive value (PPV) is defined as the proportion of individuals with a positive test result who truly have the disease. Negative predictive value (NPV) is defined as the proportion of individuals with a negative diagnostic test being truly disease-free.

These measures are used in pharmacogenomics to identify whether a genetic test can accurately predict adverse events in patients. Furthermore, they allow clinicians to evaluate the risks and benefits associated with the use of a genetic test in identifying patients at risk of adverse drug reactions.

1.7.2 Genomic Profiling

Among the pharmacogenomic biomarkers currently in drug labels, FDA recommends testing *HLA-B*1502* for carbamazepine, *HLA-B*5701* for abacavir, G6PD for rasburicase and several variants in *TPMT* including *TPMT*2, *3A,*3B,* and **4* for azathioprine and 6-mercaptopurine.(**Table 1.1**)[32]. With studies identifying multiple variants associated with a disease it suggests that more than one genetic variant may contribute to a complex disease or condition. Genetic profiling, which evaluates genetic risk by interpreting the state of multiple variants and/or multiple genes, can be a useful in clinical care[104]. Genetic profiles can provide useful risk classifications by identifying patients as high, intermediate and low risk[103].

The receiver operating characteristic (ROC) curve is a method that has been established in the context of genomic profiling[105, 106]. The curve is constructed using a combination of sensitivity and specificity for each threshold of a continuous test result[104]. The magnitude of the area under the ROC curve (AUC) is a measure of the proportion of variation that contributes to the risk of a disease or condition. It remains undetermined whether combining a high number (>20 susceptibility genes) of weak predictors that generate a high discriminative value will be useful as a predictive test[104]. Determining the level of discrimination for clinical use depends on a variety of factors such as the goal of testing and the prevalence of the condition among others[104]. Screening programs usually seek a discriminative value (AUC) of greater than 0.80[104, 107].

The benefit of using genetic profiling in pharmacogenomics is to identify patients at high versus low risk of ADRs to select appropriate treatment procedures such as increased follow-up and alternative treatments. The balance between risk and benefit of a test is measured by evaluating sensitivity, specificity, positive and negative predictive values. Genomic profiling may have the

potential to convince FDA to include pharmacogenomic testing for multiple variants that have an additive effect. Furthermore, by including clinical risk factors into this risk prediction model combining genetic factors, it is possible to potentially improve the discriminative value to identify patients at greatest risk of adverse events.

1.8 Potential Confounders

1.8.1 Role of Ancestry in Drug Response and Toxicity

It is well recognized that ancestry differences influence drug response, efficacy and toxicity[108]. There are several examples of drugs, such as warfarin, which exhibit different responses in patients of different ancestries because of genetic differences[109]. The four major distinct ancestral populations are European, Indian, African and Asian. Given these differences, it is not surprising that genetic polymorphisms in genes involved in drug metabolism and drug transporters vary significantly in allele frequencies between different ethnicities. Genetics may contribute to the understanding of how ethnic differences affect drug response.

This leads to questions as to whether ancestry-specific variants are associated with severe ADRs. Several studies have suggested that there are cases in which this occurs. For example, the variant *HLA-B*1502* is associated with carbamazepine-induced SJS only in Asian populations[110]. It is evident that ethnicity can play a significant role in pharmacogenomic findings.

1.8.1.1 Ancestry as Confounder in Pharmacogenomic Studies

Most studies are carried out using a homogenous cohort of patients that consist of individuals that are of similar ancestral origins. The reason to select a homogenous cohort for association

studies is to limit the amount of population stratification in the study. Population stratification is the differences in allele frequencies between cases and controls due to ancestry[111]. Population stratification is suggested to lead to false positive associations as the effect observed is due to allele frequency differences between ethnic groups rather than with disease or drug toxicity, confounding the results. In addition, by combining multiple different subpopulations into a study the effect of a true association is masked, leading to false negative findings[112]. It has been proposed that ethnic differences must be sufficiently controlled for in genetic association studies.

1.8.1.2 Controlling for Differences in Ancestry

Many genetic association studies aim to maximize power by including as many patient samples as possible into the analysis. This may mean that patients that are included in association studies are not strictly from one ethnic group. The level of ethnic diversity and the number of inter-ethnic unions in Canada showcases this important fact. Patient self-reported ethnicity is insufficient in determining genetic ancestry, especially in populations with inter-ethnic admixture[113].

Several methods have been described to identify and correct for population stratification. Principal component analysis (PCA) is being increasingly used to detect and correct for stratification in genetic association studies[112]. The method requires genotyping patient samples for a large number of genetic markers. This data is then translated into individual principal components that capture a portion of variability in the population. The first principal component (PC1) explains the greatest amount of variability in data and distinguishes between individuals of European and East-Asian ancestry. The second principal component (PC2)

distinguishes between individuals of Africans and non-African ancestry. By reliably determining genetic ancestry of patients, we can control and correct for these ethnic differences, thereby improving power of an association study and minimizing false positive results.

1.8.2 The Importance of Clinical Characterization

Phenotype is defined as the observable characteristics or traits of an individual such as physiological or biological characteristics. Phenotyping patients are a key step in classifying individuals as cases or controls for genetic association studies. In particular, accurate and consistent phenotyping of patients is crucial to obtain reproducible findings in pharmacogenetic studies. In the context of cisplatin-induced hearing loss, patients are phenotyped based on clinical criteria (grading schemes) which are used by clinicians to identify the severity or degree of hearing loss.

There are limitations in the use of each grading scheme in terms of being able to objectively distinguish between the different degrees of hearing loss among patients. In fact, there is no consensus on which grading scheme is optimal. Challenges exist in identifying a grading scheme that is used consistently which often leads to discrepancies in patient phenotyping. Consequently, many genetic studies have used different definitions of cases and controls, making it difficult to compare findings and also results in different reported incidences of hearing loss[97, 99, 100]. Audiologic grading schemes need to be standardized so that reproducible grading classifications can be used across multiple sites in order to produce comparable results. It is particularly important that the system used to classify hearing loss be sensitive, reliable, feasible and

applicable across all ages of pediatric patients. Accurate and consistent phenotyping of patients play an important role in genetic association studies.

1.9 Importance of Replication Studies

A single association study is often inadequate to prove that a true association exists between causative genetic variants and a disease or condition. Replication of findings in multiple independent cohorts of patients is required for validation to minimize false positive results (type 1 errors) and to ensure findings are consistent and precise[114]. Replication is defined as the ability of another study to statistically confirm the same hypothesis[115]. It is now well recognized that replication of genetic associations are required before any causal inferences can be drawn[114]. An increasing number of studies attempting to replicate findings have raised awareness that consistent replication is challenging and difficult to achieve. Nevertheless, there is significant lack of replication of research findings, particularly in the field of genetic association studies. Lack of replication can be due to several factors including Type I errors, lack of statistical power, population stratification, population heterogeneity[114].

In studying/investigating the pharmacogenomics of ADRs, replication of findings in multiple independent cohorts increases the confidence that these genetic variants are truly associated with adverse events. This is important when implementing genetic findings into a predictive model to identify patients at risk of ADRs, especially if these findings will then be used in clinical diagnosis and treatment.

1.10 Hypothesis and Thesis Objectives

The study hypothesizes that genetic variants contribute to increased susceptibility to cisplatin-induced hearing loss in pediatric oncology patients treated for a variety of tumours. The aim of this study was to identify genetic factors that confer susceptibility to cisplatin-induced hearing loss to develop pharmacogenetic predictive tests to identify individuals at risk of developing hearing loss.

Specific Aims of the study were as follows:

1. To investigate the replication of the association of prior findings of genetic variants with cisplatin-induced hearing loss in a new independent cohort of well characterized pediatric patients
2. To genotype patients using an enhanced candidate gene panel to identify novel genetic variants that confer susceptibility to cisplatin-induced hearing loss

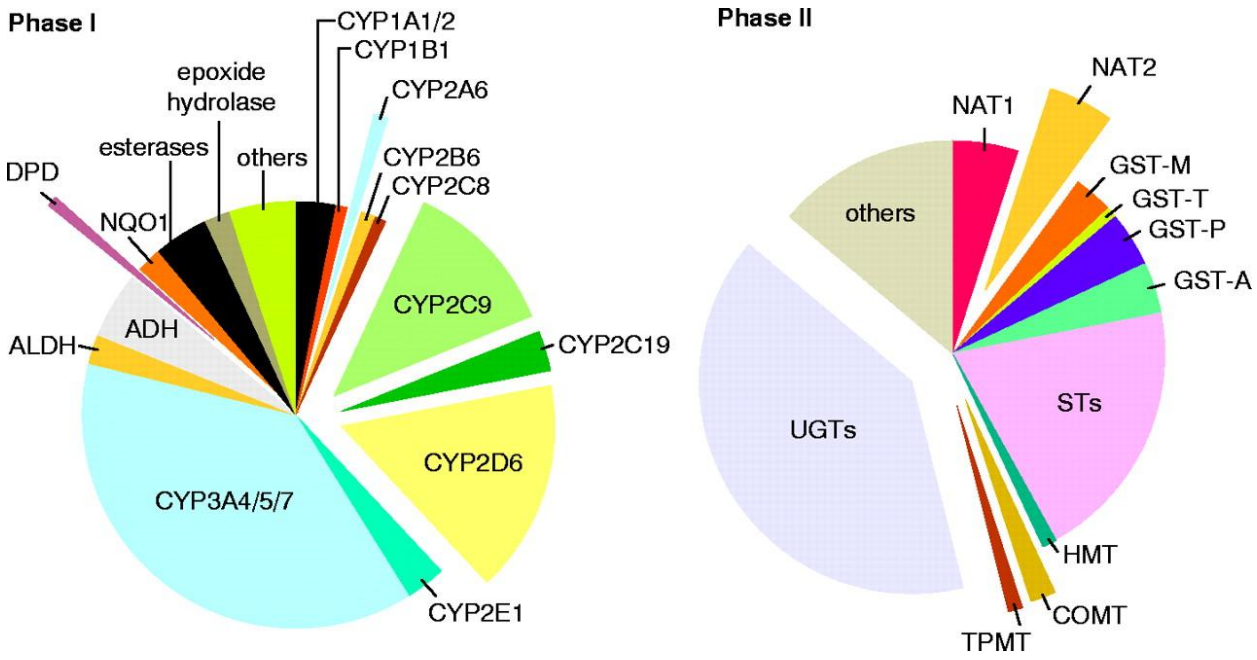

Figure 1.1: Phase I and II drug metabolizing enzymes

Drug-metabolizing enzymes are classified into phase I and phase II reactions. Phase I reactions involves functional group modifications through oxidation, reduction and hydrolysis reactions. Phase II metabolism are involved in conjugation reactions. The relative contribution of each enzyme to metabolism is observed by the size of each section of the pie chart.
ALDH, aldehyde dehydrogenase; ADH, alcohol dehydrogenase; CYP, cytochrome P45; DPD, dihydropyrimidine dehydrogenase, NQO1, NADPH: quinine oxidoreductase or DT diaphorase; COMT, catechol *O*-methyltransferase; GST, glutathione-S- transferase; HMT, histamine methyltransferase; NAT, *N*-acetyltransferase; STs, sulfotransferases; TPMT, thipurine methyltransferase; UGTs, uridine 5'-triphosphate glucuronosyltransferases.
From Evans, W.E. and M.V. Relling, *Pharmacogenomics: translating functional genomics into rational therapeutics.* Science,1999. **286**(5439): p. 487-91[18]. Reprinted with permission from AAAS.

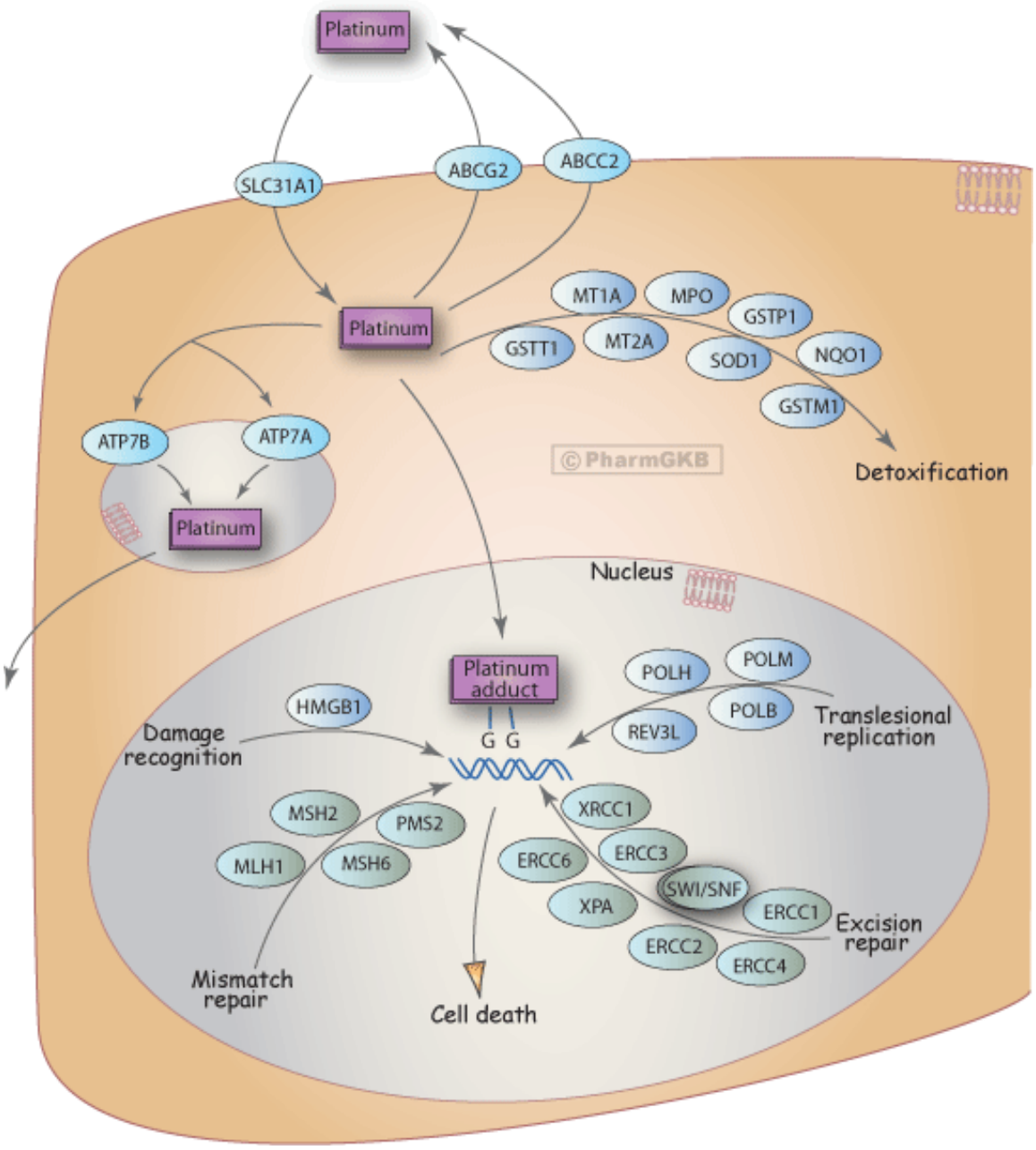

Figure 1.2: Candidate genes involved in the metabolism of platinum drugs

Several genes are involved in the transportation of cisplatin. The influx of cisplatin is suggested to be mediated by *SLC31A1*, whereas efflux is mediated by several drug transporters including *ATP7A, ATP7B, ABCC2* and *ABCG2*. This figure is reproduced with permission from PharmGKB and Stanford University[116]. Copyright PharmGKB.

Table 1.1: Pharmacogenomic biomarkers in FDA drug labels

	Drug (s)	Genetic Biomarker(s)	Use in Clinic
Required for Testing:			
	Trastuzumab	Her2/neu	Efficacy for breast cancer treatment
	Maraviroc	CCR5	Efficacy for CCR5-tropic HIV-1 infection
	Dasatinib	Philadelphia Chromosome	Efficacy for acute lymphoblastic leukemia
	Vemurafenib	BRAF V600E	Efficacy for treating metastatic melanoma
***Recommended for Testing*:**			
	Carbamazepine	HLA-B*1502	Risk of Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis
	Abacavir	HLA-B*5701	Risk of severe hypersensitivity reactions
	Azathioprine, 6-mercaptopurine	TPMT	Risk of severe, life-threatening myelosuppression
	Rasburicase	G6PD	Risk of severe hemolysis

Adapted from U.S. Food and Drug Administration. Table of Pharmacogenomic Biomarkers in Drug Labels. http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm Accessed 04/03/2012.

Table 1.2: Criteria used to determine the grade of cisplatin-induced hearing loss in patients

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[61]	<20 dB at all frequencies	Hearing loss of 20-25 dB at 4 - 8 kHz	Hearing loss of > 25 dB at 4 - 8 kHz	Hearing loss of > 25 dB at 2 - 8 kHz	Hearing loss of > 40 dB at 1 - 8 kHz
Brock Criteria[49]	< 40 dB 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[62]	<10 dB at all frequencies	Hearing loss of >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[55]	<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

Table 1.3: Select studies describing cisplatin-induced hearing loss clinical risk factors

Risk Factors	Grading scheme	Age (years)	Tumour Type	Refs.
High cumulative dose (P<0.0001) History of noise exposure (P=0.006) Cumulative vincristine dose (P=0.001)	Grading according to guidelines at Department for Otorhinolaryngology at the Hannover University Medical School	31 (21-53)	Testicular Cancer Patients	Bokemeyer (1997) [73]
Age (P=0.001) - Children <5 years are at higher risk Higher individual dose (P<0.0001) Cumulative dose (P<0.005) - Patients with >400mg/m^2 are at higher risk	Brock criteria	unknown (0-20)	Hepatoblastoma, neuroblastoma, and osteosarcoma	Li et al (2004) [48]
Age (P=0.018) Dose (P<0.0001) Cranial irradiation (P=0.028)	Based on their own specific grading criteria		Head and neck tumours	Bhandare et al (2007) [74]
Younger age and smaller body surface area (P=0.0078)	Chang criteria	11.3 (0.33-24)	Medulloblastoma, osteosarcoma, germ cell tumours	Chang et al (2010) [55]

Table 1.4: Studies associated with megalin (*LRP2*) and cisplatin-induced hearing loss

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Refs.
	Cases	Controls		Cases	Controls		
LRP2: **rs2075252** **A-allele; OR 3.45, *P*=0.016** ***LRP2:*** **rs46681234** T allele; OR2.32, *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)
LRP2**: rs2075252** A-allele; 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, hepatoblastoma, lymphoma, etc.	Ross et al (2009)

Table 1.5: Studies associated with *GSTM3*B* and cisplatin-induced hearing loss

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Refs.
	Cases	Controls		Cases	Controls		
***GSTM3*B*: OR 5.37, *P*=0.02** Carried at a higher frequency in controls (protective)	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)
No association with variants in: *GSTM3*B*	38 **Grade** 1-4	62 **Grade:** 0	National Cancer Institution Criteria No baseline audiometric tests	Overall age: 52 (23-65)		Ovarian cancer	Khrunin et al (2010)

Table 1.6: Studies associated with *GSTP1* and cisplatin-induced hearing loss

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Refs.
	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)
***GSTP1* rs1695:**AA & AG risk genotype: **OR 4.21**, ***P*<0.001**	Total number: 173 Number of cases and controls unknown		Ranked patients by 10th, 25th, 75th and 90th percentiles at 4000Hz to phenotype cases and controls	**Adults** Overall age: 42 (24-73)		Testicular cancer	Oldenburg et al (Jan 2007)
***GSTP1* rs1695:** GG protective genotype; OR 0.81, *P*=0.055 Note: 173 patients were included in previous study (not an independent study)	Total: 238 (**173 were included in previous study**)		Self-reported hearing loss	**Adults** Overall age: 29 (15-64)		Testicular cancer	Oldenburg et al (Feb 2007)
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)
No association with variants in *GSTP1*	38 **Grade** 1-4	62 **Grade:** 0	National Cancer Institution Criteria No baseline audiometric tests	Overall age: 52 (23-65)		Ovarian cancer	Khrunin et al (2010)

Table 1.7: Studies associated with *GSTM1* or *GSTT1* and cisplatin-induced hearing loss

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Refs.
	Cases	Controls		Cases	Controls		
No association with *GSTT1, 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)
***GSTM1* positivity[a] (+): OR 2.36**, ***P*=0.022** **GSTT1 positivity (+): OR 1.23, *P*=0.64** **Model 1:** (*GSTT1 +*, *GSTM1 +* and AA genotype *GSTP1*) **OR 2.76, *P*=0.005** **Model 2:**(*GSTT1 +*, *GSTM1 +* and GG genotype *GSTP1*) **OR 5.35, *P*<0.001**	Total number: 173 Number of cases and controls unknown		Ranked patients by 10th, 25th, 75th and 90th percentiles at 4000Hz to phenotype cases and controls	**Adults** Overall age: 42 (24-73)		Testicular cancer	Oldenburg et al (Jan 2007)
***GSTM1* positivity: OR 1.81, *P*=0.025** *GSTT1* positivity[a]: OR 1.20, *P*=0.60 Note: 173 patients were included in previous study (not an independent study)	Total: 238 (**173 were included in previous study**)		Self-reported hearing loss	**Adults** Overall age: 29 (15-64)		Testicular cancer	Oldenburg et al (Feb 2007)
No association with *GSTM1*, *GSTT1*,or *GSTM1GSTT1* combined model	19 **Grade** 3-4	15 **Grade** unknown	CTCAE v3	Overall age: 6.8 (1.6-18)		Medulloblastoma	Barahmani et al (June 2009)
***GSTM1* positivity[a]**: 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)
No association with variants in *GSTM1* and *GSTT1*	38 **Grade** 1-4	62 **Grade:** 0	National Cancer Institution Criteria No baseline audiometric tests	Overall age: 52 (23-65)		Ovarian cancer	Khrunin et al (2010)

[a]Positive indicates if an individual carries the *GSTM1* or *GSTT1* gene or not. A patient carries at least on copy of the gene. Effect is calculated comparing positive vs. negative.

Table 1.8: Studies associated with DNA repair pathway and cisplatin-induced hearing loss

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Refs.
	Cases	Controls		Cases	Controls		
***XPC* rs2228001**: **CC genotype: OR 17.2, *P*=0.042** No association with variants in: *ERCC1, ERCC2, ERCC4, ERCC5, XPA*	15 **Grade** unknown	17 **Grade** unknown	Unknown	Overall 15 (4-34)		Osteosarcoma	Caronia et al
No association with variants in: *ERCC1, XPD, XRCC1*	38 **Grade** 1-4	62 **Grade:** 0	National Cancer Institution Criteria No baseline audiometric tests	Overall age: 52 (23-65)		Ovarian cancer	Khrunin et al (2010)

Table 1.9: Studies associated with hereditary deafness genes

Association	Patients (n)		Grading scheme	Age (years)		Tumour Type	Refs.
	Cases	Controls		Cases	Controls		
No association with: *MTRNR, MTTS1*, 7472insC, variations in the D-loop region	20	19	Modified version of the Muenster Criteria	11.3 (3-22)	13.6 (7-19)	Osteosarcoma, neuroblastoma, medulloblastoma, germ cell tumour	Peters U et al (2003)
No association with: *GJB, SLC26A4, MTRNR1, MTTL1, MTTS1*	11	0	Self reported	8.7 (1-16)		Osteosarcoma, soft tissue sarcoma, CNS tumour	Knoll et al (2006)

Table 1.10: Calculation of sensitivity, specificity, positive and negative predictive value

	Condition Present	**Condition Absent**	
Positive outcome	True Positive (TP)	False Positive[a] (FP)	**Positive Predictive Value (PPV)** = TP/(TP+FP)
Negative outcome	False Negative[b] (FN)	True Negative (TN)	**Negative Predictive Value (NPV)** = TN/(TP+FP)
	Sensitivity = TP/(TP +FN)	**Specificity** = TN/(FP+TN)	

[a]False positive rate represents the type I error
[b]False negative rate represents the type II error

[1]Chapter 2: Replication of *TPMT* and *ABCC3* genetic variants highly associated with cisplatin-induced hearing loss in children

2.1 Introduction

Cisplatin is a widely used chemotherapeutic agent for the standard treatment of a variety of solid tumours. It is one of the most effective chemotherapeutic agents for children and has contributed to the dramatic increase in survival of many solid tumors including hepatoblastoma, brain tumors, neuroblastoma, osteosarcoma, and germ cell tumors. Cisplatin has shown efficacy in standard risk hepatoblastoma such that it can be used as monotherapy with >80% 3-year event-free survival[39]. A major complication that limits the use of cisplatin is the risk of drug-induced ototoxicity that can result in life-long disability[117]. Cisplatin is known to cause severe ototoxicity that manifests as permanent, bilateral sensorineural hearing loss, which affects 10-25% of adults and 26-90% of children[48-53]. In children in particular, even mild losses in hearing can significantly influence speech and language development, social-emotional development and increase the risk of learning difficulties[58, 64]. In adults, the rate of hearing loss may be higher than that reported due to a lack of baseline and follow-up audiometry in cisplatin protocols.

In patients receiving the same dose of cisplatin, the inter-individual variability of cisplatin-induced effects on hearing is profound, from no hearing loss, to high frequency hearing loss which progresses to severe hearing impairment in the speech frequencies[58, 59, 91].

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[1]A version of this chapter will be submitted for publication. Pussegoda K, Ross C.J, Visscher H, Yazdanpanah M, Brooks B, Rassekh S.R, Feroz Zada Y, Dubé M.P, Carleton B.C, Hayden M.R. *Replication of TPMT and ABCC3 genetic variants highly associated with cisplatin-induced hearing loss in children.*

Furthermore, patients show no improvement in hearing and often the progression and severity of hearing loss continues long after the end of therapy[54]. Higher cumulative cisplatin dose[48, 73, 74], younger age[48, 55, 73], cranial irradiation[74, 79], and concomitant use of aminoglycosides and vincristine [73, 76, 77] are known to influence cisplatin-induced ototoxicity[59]. In standard treatment protocols, the occurrence of moderate to severe cisplatin ototoxicity leads to dose reduction or the termination of cisplatin treatment, which may affect survival rates[64]. Inter-individual variability in hearing loss suggests that clinical risk factors alone are insufficient predictors of safety.

There is evidence that mechanisms such as the generation of reactive oxygen species are involved in the occurrence ototoxicity associated with cisplatin treatment[67]. Currently there are no means to identify individuals who are at an increased risk of developing hearing loss. Genetic variation in the genes involved in drug biotransformation, transport, and receptors have been recognized to influence patient drug response and susceptibility to adverse drug events, including ototoxicity[29]. The identification of genetic markers that confer increased susceptibility to cisplatin-induced ototoxicity has important implications for improving patient care during cisplatin treatment.

Recently, a candidate gene study in children receiving cisplatin identified genetic variants in thiopurine S-methyltransferase (*TPMT)* (rs12201199, rs1800460, rs1142345), catechol O-methyltransferase (*COMT)* (rs9332377, rs4646316) and several other variants including in the ATP-Binding Cassette transporter C3 (*ABCC3)* (rs1051640) conferring increased risk of developing cisplatin-induced hearing loss[64].

For clinical application, it is essential to replicate these genetic findings in multiple independent cohorts of patients to reduce the number of false positive results and to ensure that the genetic risk prediction is consistent[114]. It is now well recognized that replication of genetic associations are required before any causal inferences can be drawn[114]. Increasing number of studies attempting to replicate findings raises awareness that consistent replication is challenging and difficult to achieve. The aim of this study was to investigate replication of genetic risk factors for cisplatin-induced hearing loss in an independent cohort of patients recruited from across Canada and to assess a predictive multi-SNP model. We also report the results of combined analyses summarizing both the replication and initial findings.

2.2 Material and Methods

2.2.1 Patients

Study participants were recruited through the CPNDS, a national multicenter active surveillance consortium for studying adverse drug reactions[118]. Between June 2008 and March 2011, a new independent replication cohort of 155 pediatric oncology patients with cisplatin-induced hearing loss and drug-matched control patients were recruited at pediatric oncology units across Canada: BC Children's Hospital (Vancouver), Alberta Children's Hospital (Calgary), Stollery Children's Hospital (Edmonton), Winnipeg Health Sciences Centre (Winnipeg), Children's Hospital at the London Health Sciences Centre (London), McMaster Children's Hospital (Hamilton), Hospital for Sick Children (Toronto), Children's Hospital of Eastern Ontario (Ottawa), Hôpital Sainte-Justine (Montreal) and IWK Health Centre (Halifax). The previous

cohort (discovery and replication) included in the combined analyses has been described previously[64].

Cisplatin-induced hearing loss was diagnosed based on audiometric findings using criteria described by the CTCAEv3 (Common Terminology Criteria for Adverse Events)[61]. To better differentiate between cases and controls, subjects with serious cisplatin-induced hearing loss were defined as those with grade 2 or higher hearing impairment. Control patients were defined as children who received cisplatin with normal audiometric data who did not develop hearing loss (grade 0). Patients with grade 1 (n=8) hearing loss were excluded from the analysis. Serious hearing impairment was defined at the point at which cisplatin chemotherapy protocols recommend reducing or terminating cisplatin treatment which occurs at grades 2-4 hearing loss. Baseline and the most recent audiological assessment were used. Informed written consent or assent was obtained from each subject or their parents or legal guardians. 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 participants provided informed consent.

2.2.2 Genotyping

DNA of patients was extracted from blood, saliva or buccal swabs using the QIAmp DNA purification system (Qiagen, Toronto, ON, Canada) according to the manufacturer's protocol. We selected 11 single nucleotide polymorphisms (SNPs) to assess whether prediction of risk of hearing loss can be improved. We selected genetic variants for genotyping based on evidence of a significant association (P<0.01) in the initial combined cohort[64] as well as P<0.01 in either the initial discovery or replication cohort[64]. We first assessed whether variants in *TPMT* (rs12201199, rs1800460, rs1142345) and *COMT* (rs9332377, rs4646316) could be replicated in

the current replication cohort. We then assessed whether other variants in *ABCC3* (rs1051640), *MTHFR* (rs373964), *VKORC1* (rs17884333, rs8050894), and *SLCO1A2* (rs4115170, rs2306231) were replicated. Patient DNA was genotyped using a custom Illumina GoldenGate SNP genotyping assay (Illumina, San Diego, CA, USA). This assay included additional non-study SNPs for principal component analysis (PCA) to determine patient ancestry and for quality control purposes. All SNP genotype data were clustered manually using GenomeStudio software (Illumina, San Diego, CA, USA). Samples with a call rate below 95% were excluded. The average genotyping call rate for included samples was 98.3%. The overall genotype call rate of the 11 study SNPs was 99.9% and were all in Hardy-Weinberg equilibrium (P>0.05).

2.2.3 Statistical Analysis

Clinical characteristics of patients with and without cisplatin-induced hearing loss were compared using the Wilcoxon-Mann-Whitney U-test for continuous variables, and Fisher's exact test for categorical variables. Hardy-Weinberg equilibrium tests were conducted in controls using the permutation version of the exact test of Hardy-Weinberg of Guo and Thompson[119].

The study was calculated to have a statistical power between 65-99% (mean 86%) based on the previous effect sizes and genotype frequencies in order to replicate previously associated polymorphisms with cisplatin-induced hearing loss at P=0.05. Two genetic variants in *SLCO1A2* (rs11045913 and rs11045912) were excluded prior to genotyping due to inadequate power (<10%) to detect an association. Homozygous and heterozygous odds ratios (ORs) were calculated using the homozygous genotype of the protective allele as identified in the initial cohort as a reference[64]. Calculation of ORs in the presence of empty cells was done by adding 0.5 to the empty cells. Association between genetic polymorphisms and cisplatin-induced

hearing loss was assessed by computing Fisher's exact test for unadjusted (non-regression) p-values and adjusted p-values determined by logistic regression. Independent factors for the prediction of cisplatin-induced hearing loss in the combined cohort were identified by forward logistic regression analysis. In the forward logistic regression model the following factors were included: the first principal component, age, cisplatin dose, treatment duration, vincristine treatment and germ cell tumour. None of the variables were retained in combined cohort at a threshold of 0.10 except concomitant vincristine treatment (P=1.4×10^{-9}). However, we also included variables that are known to increase risk of hearing loss that were significantly different (P=0.05) between cases and controls. Therefore, age, concomitant vincristine treatment, germ cell tumour, and cranial irradiation were included as covariates. The treatment of all non-CNS germ cell tumours is unique as these patients are treated at low individual doses of 20mg/m^2 over five days and are known to be at less risk of hearing loss[41]. In an additional analysis, we combined the initial cohort[64] and the current cisplatin replication cohort of patients to determine the overall significance of associations. Variants were considered to be statistically significant, if they had P<0.05 in the new replication cohort.

Potential population stratification in the dataset was assessed by PCA using SVS/HelixTree software[112, 120]. Patients of African ancestry (n=8), as determined by PCA, were excluded in the analysis to match the patient ancestry of the previous cohort[64, 120]. Graphical display of principal components was constructed with GraphPad Prism 5 software (GraphPad Software Inc., California, USA). A secondary stratified analysis of individuals of only European ancestry was based on the first two principal components.

Genetic variants were further evaluated in a multivariate logistic regression model including clinical variables using an additive model. A genetic risk score was calculated by multiplying

each variable with the estimated beta (log odds ratio) from the current combined cohort. The genetic only model includes variants that were selected based on statistical evidence from a forward logistic regression model in the combined cohort. Variants that were retained in this model include *TPMT* rs12201199, *ABCC3* rs1051650 and *COMT* rs4646316.

Association between the genetic risk score and cisplatin-induced hearing loss was assessed using logistic regression. Three prediction models were investigated: (i) clinical variables only; (ii) genetic variables only; and (iii) clinical and genetic variables. The contribution of the genetic and/or clinical risk scores to the prediction of cisplatin-induced hearing loss was investigated by comparing the area under the ROC curves of the prediction models. AUC estimates were obtained using the ROC plot function on the basis of the linear predictors obtained from the logistic regression model for clinical factors and genotypic scores. The statistical difference between the curves was calculated using DeLong's method[120] implemented in the *R*-package pROC[121]. Two-sided *p*-values <0.05 were considered statistically significant.

Risk groups for cisplatin-induced hearing loss were defined based on the predictive values for each patient sample from the multi-SNP logistic regression model that included three SNPs (*TPMT* rs12201199, *ABCC3* rs1051640, *COMT* rs4646316). The threshold for genetic variables used to determine lower risk was the median predicted value of controls minus one standard deviation (predictive value <0.4) and high risk was defined as the median predicted value of cases plus one standard deviation (>0.8). Intermediate risk was defined as a predictive value between 0.4-0.8. To assess the threshold for the combination of clinical and genetic variables for lower risk, median predicted value of controls (predictive value <0.45) and high risk was defined as the median predicted value of cases (>0.8). Intermediate risk was defined as a predictive value between 0.45-0.8. To assess the predictive performance of risk groups for cisplatin-

induced hearing loss, we calculated the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for each defined threshold. Kaplan-Meier curves for cisplatin-induced hearing loss were generated for each risk group using time from start of treatment to the time of first evidence of toxicity or last audiogram. Log-rank test was used to compare the trend of survival curves. Hazard ratios for each curve were calculated using the Cox regression model considering the lower risk group as the reference.

Statistical genetic analyses were conducted using SNP and Variation Suite 7.4.5 (Golden Helix, Bozeman, USA), SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA) and R 2.13.0 (R Development Core Team).

2.2 Results

2.2.1 Patient Characteristics

Patient characteristics of the initial cohort[64], current replication cohort and the combined cohort are listed in Table 2.1. Canadian Pharmacogenomics Network for Drug Safety (CPNDS) adverse drug reaction (ADR) surveillance clinicians routinely update patient information in the CPNDS patient database as new information became available (i.e., relapase, death and additional data on concomitant medication use). In the current replication cohort 87 (56%) of the 155 pediatric oncology patients developed hearing loss. Concomitant vincristine treatment was significantly higher in cases than controls in the replication cohort (66.7% compared to 27.9%, P=2.1x10^{-6}), similar to the initial cohort (50.9% vs. 17.9%, P = 4.1 x 10^{-5}; combined cohort P=1.1x10^{-9}). Fewer osteosarcoma patients in the replication cohort developed hearing loss (11.5% in cases vs. 29.4% in controls, P=0.0073), whereas this was not significantly different in the initial cohort (22.6% in cases vs. 28.6% in controls, P=0.45). Furthermore, follow-up after

therapy was longer in cases than controls in the replication cohort (5.0 years vs. 2.0 years, P=2.1x10^{-4}) but was not significantly different in the initial cohort (3.0 years vs. 2.0 years, P=0.10).

2.2.2 Genetic Results

In the replication cohort, all genetic variants assessed in *TPMT* (rs12201199, rs1142345, rs180046) showed a significant association with cisplatin-induced hearing loss (**Table 2.2**). *TPMT* rs12201199 was the most strongly associated variant (P=0.0013, OR 6.1). The risk allele (A) was observed in 21 (12%) cases and 3 (2%) controls. The combined analysis of all 317 patients showed a stronger association with rs12201199 (P=8.7x10^{-7}, OR 8.9) than in the initial cohort alone[64]. The other *TPMT* variants, rs1142345 and rs1800460 were also stronger in the combined cohort than in the initial cohort (**Table 2.2**). All three *TPMT* variants remained significantly associated in the combined cohort after correcting for clinical factors including age, vincristine treatment, germ cell tumour and cranial irradiation.

The protective 'A' allele of *COMT* rs4646316 was carried by 33 (19%) cases and 32 (24%) controls in the replication cohort. Even though this variant showed an effect in the same direction as in the initial cohort, it was smaller (OR 1.3) and was not significantly associated with hearing loss in the replication cohort (P=0.33). A similar effect was seen for *COMT* rs9332377 (P=0.28, OR 1.4).

Next, we assessed whether other genetic variants that previously were associated with cisplatin-induced hearing loss (P<0.01) could be replicated in the new cohort of patients. Additional variants could improve a model that predicts the risk of cisplatin-induced hearing loss. In total, 3

of the 6 variants showed an effect in the same direction as the initial cohort (**Table 2.2, Table 2.3**). One synonymous variant in *ABCC3* (rs1051640) (E1503E) was significantly associated with cisplatin-induced ototoxicity in the current replication cohort (P=0.036, OR 1.8), and was more strongly associated in the combined cohort (P=7.8x10^{-4}, OR 2.0; **Table 2.2**). The protective 'A'allele of *ABCC3* rs1051640 was carried by 28 (16%) cases and 35 (26%) controls in the replication cohort. The association of *ABCC3* rs1051640 genotype with cisplatin-induced hearing loss remained significant in the combined cohort after adjusting for clinical factors (P=0.0033).

Using principal component analysis, it was observed that the majority (80%) of patients had European genetic ancestry[112] (**Figure 2.1**). To reduce potential bias caused by population stratification, we assessed whether associations in the replication cohort remained significant in a more homogenous subset of patients of only European ancestry (n=124). In the European ancestry subset, similar results were observed, though with larger effect sizes for the *TPMT* and *ABCC3* variants (**Table 2.4**).

A model that combined the effects of the associated risk genotypes and clinical risk factors was generated and Receiver Operating Characteristic (ROC) analyses were performed. The genetic-only model was constructed to include the three independent SNPs (*TPMT* rs12201199, *ABCC3* rs1051640, *COMT* rs4646316) that were selected based on forward logistic regression. Although there was a lack of significant association with variants in *COMT* in the current replication cohort; the combined cohort logistic regression retained *COMT* rs4646316 because it significantly added to the prediction model. The clinical-only model included age, vincristine

treatment, germ cell tumour and cranial irradiation. Combining both the clinical and genetic variables significantly improved the ability to predict cisplatin ototoxicity (**Figure 2.2**) resulting in an area under the curve (AUC) of 0.786 compared to the clinical-only model (AUC 0.708, P=0.00048).

A predictive multi-marker model using these genetic variants could stratify patients by risk of ototoxicity. Risk groups were defined based on predictive values from the model as lower (predictive value <0.4), intermediate (0.4-0.8) and high risk (>0.8). Based on these groups, there were 30 (9.5%) individuals classified as lower risk, 245 (77.3%) as intermediate and 42 (13.2%) as high risk (**Table 2.5**). In the high risk group 39 (92.9%) individuals developed hearing loss (positive predictive value, 92.9%) compared to 3 (7.1%) controls conferring a specificity of 97.6% (**Table 2.6**). In the lower risk group 8 (26.7%) individuals developed hearing loss (positive predictive value, 73.3%) compared to 22 (73.3%) controls conferring a sensitivity of 95.9% (**Table 2.6**).

Individuals in the high risk group also had a significantly higher risk of cisplatin-induced ototoxicity when compared to lower to intermediate risk group (P=1.3x10^{-4}, OR 11.0; **Table 2.6**). Severity of hearing loss also increased with higher risk group classifications (**Table 2.5**). A Kaplan-Meier plot illustrates that the intermediate and high risk groups have significantly increased risk of hearing loss over time (P_{trend}=5.3x10^{-9}; **Figure 2.3**). After 1 year of treatment, 71% of patients developed significant hearing loss in the high risk group compared to 27% of patients in the lower risk group (**Figure 2.3**). The risk of hearing loss in the high risk group was 5.9 times higher compared to the lower risk group (**Figure 2.3**).

A model combining the effect of *TPMT, ABCC3* and *COMT* was able to discriminate between patients that are at lower versus high risk compared to a model with *TPMT* alone (**Table 2.6**). By including both *ABCC3* and *COMT* into the model, patients at lower risk could be identified with more certainty. However, the ability to identify patients at high risk similar in the *TPMT* only model and the combined model that includes *TPMT*, *ABCC3*, and *COMT* (PPV 91.5% vs PPV 92.7%) (**Table 2.6**). A Kaplan-Meier plot combining both the clinical and genetic variables illustrate that we can identify 97 (50.3%) patients at high risk compared to 39 (20.2%) patients using genetics alone (**Figure 2.3**, **Table 2.6**). In the high risk group 97 (91.5%) of individuals developed hearing loss (positive predictive value, 91.5%) compared to 9 (8.5%) controls conferring a specificity of 92.7% and a sensitivity of 50.3% (**Table 2.6**).

2.4 Discussion

The development of hearing loss after treatment with cisplatin is known to cause serious lifelong disability, particularly in children[117]. A proven approach to identify those patients at high risk of developing serious cisplatin-induced hearing loss would significantly improve the safety and efficacy of pediatric cancer therapy.

Recently, we identified genetic variants that were associated with cisplatin-induced hearing loss in children after cancer therapy[64]. Replication of genetic associations for validation in independent populations is critically important before clinical implementation and development of guidelines. In this replication study, we recruited a large independent cohort of pediatric oncology patients from across Canada who received cisplatin therapy. We confirmed the

associations of genetic variants in *TPMT* and *ABCC3* with moderate to severe cisplatin-induced hearing loss. These results strengthen the evidence for the association between *TPMT* and *ABCC3* variants with cisplatin-induced hearing loss. Variants in *COMT* exhibited smaller effect sizes in the replication cohort (OR: 2.5 vs. 1.3 and 5.5 vs. 1.4; previous vs. new cohorts). We generated a novel predictive model combining *TPMT*, *ABCC3* and *COMT* genetic risk factors with clinical risk factors. The combined model of genetic plus clinical risk factors was more predictive than clinical risk factors alone (AUC 0.786 vs. 0.708; P= 0.00048). A predictive model that incorporates the overall most highly associated COMT variant was included as it added significantly to a predictive model with *TPMT* and *ABCC3*. The *COMT* variants do not contribute to the prediction of patients at high risk of cisplatin-induced ototoxicity, instead they stratify patients between intermediate and lower risk. The model was based on results from the combined cohort and requires further studies in children to validate these findings and assess the clinical utility.

Our study is the first to replicate the reported association between *TPMT* (rs12201199, rs1142345, rs1800460) and cisplatin-induced hearing loss in a large independent cohort of pediatric patients (n=155) treated for a variety of malignancies. Evidence indicates that patients that are heterozygous or homozygous for these *TPMT* variants have an increased risk of cisplatin-induced hearing loss, likely through reduced activity of the gene[64]. *TPMT* is likely to increase cisplatin toxicity by inactivating the binding of the compound to purines in DNA, thereby regulating cisplatin cross-linking. Loss of function mutations in *TPMT* can increase the efficiency of cisplatin cross-linking leading to increased toxicity[64]. Another potential

mechanism is that cisplatin toxicity is due to the accumulation of *S*-adenosylmethionine due to reduced activity of *TPMT*[64, 122-124].

The association between the synonymous variant in *ABCC3* rs1051640 (E1503E) and hearing loss was also significantly replicated. The minor allele frequency (MAF) of rs1051640 ranges from 20% in the European population to <1% in the Nigerian Yoruban population[125]. Patients carrying the G-allele of rs1051640 were at increased risk of developing hearing loss after cisplatin treatment. This is the first study to describe the role of genetic variation in *ABCC3* in the context of cisplatin-ototoxicity. *ABCC3* is a transporter that mediates the efflux of organic anions, xenobiotics and glutathione *S*-conjugates[126, 127]. One of the mechanisms by which cancer chemotherapies, including platinum drugs are detoxified is through conjugation of the active metabolite to glutathione making the compound more anionic[93, 94]. 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[128, 129]. Other studies carried out in lung cancer cells lines have also shown that *ABCC3* mRNA expression levels are significantly correlated to resistance to cisplatin and other platinum drugs[130, 131]. 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. Further functional studies are required to assess the exact mechanisms by which variants in *ABCC3* affect cisplatin-induced hearing loss.

Based on our a priori power calculations considering the effect sizes and genotype frequencies of the original studies, we had sufficient power for all polymorphisms to find similar effect sizes in the replication cohort. However, several SNPs, that were previously reported to be associated, could not be replicated, limiting the use of these variants in the prediction of risk of developing hearing loss. This is not surprising as many original studies report stronger genetic associations with larger effect sizes than in follow-up studies[132, 133]. There are several other potential reasons for the failure to replicate previous findings. Significant inter-study heterogeneity can arise due to differences in the cohorts such as population diversity, gender and treatment protocols [132, 133]. This could lead to differences in the effects of variants in specific subpopulations or lead to masking of the effects by factors that have not been sufficiently controlled for. We aimed to control for these factors by first evaluating power to ensure that the sample size is large enough to detect an association, then adjusting for clinical variables in the cohort and finally carrying out a subgroup analysis in patients of only European ancestry. Nevertheless, a combination of these factors may explain why we could not significantly replicate the association between *COMT* as well as other polymorphisms and cisplatin-induced hearing loss.

Clinical factors alone cannot accurately predict the risk of developing hearing loss. Combining the effect of *TPMT*, *ABCC3* and *COMT* with clinical factors significantly improved the ability to predict risk compared to clinical factors alone (AUC 0.786 vs. 0.708, P=0.00048). Including the clinical risk factors with these genetic markers we can identify a greater number of patients at high risk compared to using genetic risk factors alone (50.3% for combined vs. 20.2% for genetic alone). This is of particular importance in children, for whom even mild losses in hearing can

cause difficulties in school performance[65, 134]. Several strategies can be proposed to prevent hearing loss in individuals at high risk of adverse effects from cisplatin. Patients might be placed on alternative medications (e.g. carboplatin) which are less ototoxic[55], receive increased monitoring for hearing loss to minimize the onset of severe stages of hearing loss, or receive otoprotective agents although concerns about the possibility of compromise of antitumour activity have been raised[135, 136]. The placement of hearing aids or cochlear implantation are also options used to manage cisplatin-induced hearing loss.

In conclusion, this study confirms previous findings and provides further evidence in an independent patient cohort for the associations between *TPMT* and *ABCC3* in cisplatin-induced hearing loss and with *COMT* in the combined cohort. A combination of *TPMT, ABCC3* and *COMT* with clinical variables provides a novel tool to improve the risk prediction of patients with hearing loss from cisplatin therapy. The discovery of additional variants through genome-wide studies may also improve and refine the current predictive model. The combination of clinical and genetic risk factors can potentially improve risk classifications for hearing loss and may allow for individualized treatment.

Table 2.1:Patient demographics

	Initial Cohort[a] (n=162)			**Replication Cohort** (n=155)			**Combined Cohort** (n=317)		
	Cases (n= 106)	**Controls** (n= 56)	p-value	**Cases** (n= 87)	**Controls** (n= 68)	p-value	**Cases** (n= 193)	**Controls** (n=124)	p-value
Age, years (median (min, max))	6.0 (0, 16)	9.0 (0, 19)	0.086	6.0 (1, 25)	11.0 (0, 18)	0.050	6.0 (0, 25)	10.0 (0, 19)	**0.012**
Dose, cumulative mg/m2 (median (min, max))	400 (120, 720)	400 (100, 720)	0.61	400 (92, 800)	400 (20, 768)	0.36	400 (92, 800)	400 (20, 768)	0.71
Treatment duration, weeks (median (min, max))	2.4 (1,11)	2.3 (1, 6)	0.96	2.1 (0, 8)	2.4 (0, 9)	0.65	2.2 (0, 11)	2.3 (0, 9)	0.90
Gender (Male n, (%))	71 (67.0%)	28 (50.0%)	**0.043**	43 (49.4%)	34 (50.0%)	1.00	114 (59.1%)	62 (50.0%)	0.13
Caucasian ethnicity (n (%))	80 (75.5%)	48 (85.7%)	0.16	70 (80.5%)	54 (79.4%)	1.00	150 (77.7%)	102 (82.3%)	0.39
Concomitant medication (n, (%))									
Tobramycin	32 (30.2%)	15 (26.8%)	0.72	23 (26.4%)	15 (22.1%)	0.58	55 (28.5%)	30 (24.2%)	0.44
Vancomycin	25 (23.6%)	11 (19.6%)	0.69	26 (29.9%)	14 (20.6%)	0.20	51 (26.4%)	25 (20.2%)	0.23
Vincristine	54 (50.9%)	10 (17.9%)	**4.1×10^{-5}**	58 (66.7%)	19 (27.9%)	**2.1×10^{-6}**	112 (58.0%)	29 (23.4%)	**1.1×10^{-9}**
Gentamicin	21 (19.8%)	7 (12.5%)	0.28	21 (24.1%)	19 (27.9%)	0.71	42 (21.8%)	26 (21.0%)	0.89
Tumor type (n, (%))									
brain tumor	25 (23.6%)	8 (14.3%)	0.22	26 (29.9%)	11 (16.2%)	0.058	51 (26.4%)	19 (15.3%)	**0.026**
endodermal sinus tumor of thymus	0	1 (1.8%)	0.35	0	1 (1.5%)	0.44	0	2 (1.6%)	0.15
germ cell tumor	7 (6.6%)	15 (26.8%)	0.00063	7 (8.0%)	11 (16.2%)	0.14	14 (7.3%)	26 (21.0%)	**0.00046**
hepatoblastoma	22 (20.8%)	5 (8.9%)	0.075	17 (19.5%)	7 (10.3%)	0.12	39 (20.2%)	12 (9.7%)	**0.013**
lymphoma	0	1 (1.8%)	0.35	1 (1.1%)	2 (2.9%)	0.58	1 (0.5%)	3 (2.4%)	0.30
nasopharyngeal carcinoma	1 (0.9%)	0	1.00	0	2 (2.9%)	0.19	1 (0.5%)	2 (1.6%)	0.56
neuroblastoma	26 (24.5%)	9 (16.1%)	0.23	24 (27.6%)	12 (17.6%)	0.18	50 (25.9%)	21 (16.9%)	**0.073**
osteosarcoma	24 (22.6%)	16 (28.6%)	0.45	10 (11.5%)	20 (29.4%)	0.0073	34 (17.6%)	36 (29.0%)	**0.019**
sarcoma	1 (0.9%)	1 (1.8%)	1.00	0	1 (1.5%)	0.44	1 (0.5%)	2 (1.6%)	0.56
carcinoma	0	0		0	1 (1.5%)	0.44	0	1 (0.8%)	0.39
retinoblastoma	0	0		1 (1.1%)	0	1.00	1 (0.5%)	0	1.00
mesencymal tumour of liver	0	0		1 (1.1%)	0	1.00	1 (0.5%)	0	1.00
Follow-up, years (median (min, max))	3 (0, 18)	2 (0, 15)	0.10	5 (0, 25)	2 (0, 16)	**0.00021**	4 (0, 25)	2 (0, 16)	**9.3×10^{-5}**
Cranial irradiation (n, (%))	23 (21.7%)	7 (12.5%)	0.20	20 (23.0%)	8 (11.8%)	0.093	43 (22.3%)	15 (12.1%)	**0.025**

For age, dose, treatment duration and follow-up, the Wilcoxon-Mann-Whitney test with normal approximation was used. For gender, ethnicity, concomitant medication, tumor type, cranial irradiation the Fisher exact test was used. In bold are statistically significant values at $p<0.05$ type-I error rate.

[a]Results from the initial combined cohort [64] compared to the current replication cohort as well as all cohorts combined; [b]Caucasian ethnicity assessed by principal component analysis

Table 2.2 Genetic variants associated with cisplatin-induced hearing loss

		Initial Cohort[a] (n=162) 106 cases, 56 controls				Replication Cohort (n=155) 87 cases, 68 controls				Combined Cohort(n=317) 193 cases, 124 controls				
SNP	**Allele**	**Case**	**Ctrl**	**OR (95%CI)**	***p*-value**	**Case**	**Ctrl**	**OR (95%CI)**	***p*-value**	**Case**	**Ctrl**	**OR (95%CI)**	***p*-value**	***p*-value[b]**
TPMT														
rs12201199	A	28	1	16.9 (2.3-125.9)	0.00022	21	3	6.1 (1.8-20.9)	0.0013	49	4	8.9 (3.2-24.9)	**8.7×10^{-7}**	**4.0×10^{-5}**
	T	184	111			153	133			337	244			
rs1142345	G	19	1	10.9 (1.4-82.7)	0.0017	16	3	4.5 (1.3-15.7)	0.011	35	4	6.1 (2.1-17.3)	**0.00014**	**0.00039**
	A	193	111			158	133			351	244			
rs1800460	A	16	0	18.0 (1.1-302.7)	0.0031	13	3	3.6 (1.0-12.8)	0.038	29	3	6.6 (2.0-21.8)	**0.00043**	**0.00073**
	G	196	110			161	133			357	243			
COMT														
rs4646316	G	176	74	2.5 (1.5-4.3)	0.00055	141	104	1.3 (0.8-2.3)	0.33	317	178	1.8 (1.2-2.6)	0.0021	0.0068
	A	36	38			33	32			69	70			
rs9332377	A	36	4	5.5 (1.9-16.0)	0.00018	38	23	1.4 (0.8-2.4)	0.28	74	27	1.9 (1.2-3.1)	0.0054	0.043
	G	176	108			136	113			312	221			
ABCC3														
rs1051640	G	182	83	2.1 (1.2-3.8)	0.0092	146	101	1.8 (1.0-3.3)	0.036	328	184	2.0 (1.3-2.9)	**0.00078**	**0.0033**
	A	30	29			28	35			58	64			

[a]Results from the initial combined cohort [64] compared to the current replication cohort as well as all cohorts combined
[b] Adjusted for age, vincristine treatment, germ cell tumor and cranial irradiation
In bold indicates results more significant in current combined cohort than in previous combined cohort[64].
SNP, Single Nucleotide Polymorphism; Ctrl, Control; OR, Odds Ratio; CI, Confidence Interval.

Table 2.3 Association between additional top ranked SNPs and cisplatin-induced hearing loss

			Previous Cohort[a] (n=162) 106 ototox., 56 controls		Replication (n=155) 87 ototox., 68 controls		Combined (n=317) 193 ototox., 124 controls	
Gene	**SNP**	**Allele[b]**	**OR (95% CI)**	**p-value[b]**	**OR (95% CI)**	**p-value[b]**	**OR (95% CI)**	**p-value[b]**
MTHFR_CLCN6	rs3737964	A/G	2.64 (1.40, 4.98)	0.0022	0.82 (0.46, 1.46)	0.50	1.00 (0.73, 1.37)	1.00
VKORC1	rs17884333	A/G	2.07 (1.28, 3.35)	0.0027	1.30 (0.83, 2.05)	0.25	1.61 (1.16, 2.23)	0.0041
VKORC1	rs8050894	C/G	2.07 (1.28, 3.35)	0.0027	1.10 (0.70, 1.73)	0.68	1.47 (1.06, 2.03)	0.020
SLCO1A2	rs4115170	G/A	2.09 (1.28, 3.41)	0.0028	0.93 (0.58, 1.48)	0.75	1.40 (1.00, 1.96)	0.048
SLCO1A2	rs2306231	G/A	2.08 (1.27, 3.36)	0.0034	0.93 (0.58, 1.48)	0.75	1.39 (0.99, 1.95)	0.053

[a]Results from discovery and replication cohorts from previous study[64] compared to the current replication cohort as well as all cohorts combined
[b]SNP alleles assayed; minor allele is mentioned first
SNP, Single Nucleotide Polymorphism; Ototox, Ototoxicity; OR, Odds Ratio; CI, Confidence Interval.

.

Table 2.4 Genetic variants associated with cisplatin-induced hearing loss in patients of European ancestry

		Previous Cohort[a] (n=127) 80 ototox., 48 controls		**Replication (n=124)** 70 ototox., 54 controls		**Combined (n=252)** 150 ototox., 102 controls		
Gene	**Allele[b]**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	***p*-value[c]**
TPMT								
rs12201199	A	16.0 (2.1, 120.1)	3.95E-04	6.8 (1.5, 30.4)	0.0039	10.0 (3.1, 32.9)	**4.29E-06**	**1.18×10^{-4}**
	T	1		1		1		
rs1142345	G	9.1 (1.2, 70.4)	0.011	5.4 (1.2, 24.6)	0.015	6.6 (2.0, 22.2)	**0.00045**	**0.0010**
	A	1		1		1		
rs1800460	A	17.7 (1.0, 300.6)	0.0065	5.0 (1.1, 22.79)	0.023	8.7 (2.0, 37.2)	**5.10E-04**	**0.0016**
	G	1		1		1		
COMT								
rs4646316	G	2.5 (1.4, 4.4)	0.0022	1.3 (0.7, 2.5)	0.38	1.8 (1.2, 2.8)	0.0057	0.010
	A	1		1		1		
rs9332377	A	7.5 (2.2, 25.1)	0.00021	1.4 (0.7, 2.6)	0.35	2.2 (1.3, 3.8)	0.0027	0.017
	G	1		1		1		
ABCC3								
rs1051640	A	2.3 (1.2, 4.4)	0.0081	1.9 (1.0, 3.4)	0.043	2.1 (1.4, 3.2)	**8.31E-04**	**0.0059**
	G	1		1		1		

[a]Results from discovery and replication cohorts from previous study[64] compared to the current replication cohort as well as all cohorts combined
[b]SNP alleles assayed; minor allele is mentioned first
[c]Adjusted for age, vincristine treatment, germ cell tumours and cranial irradiation
In bold indicates results more significant in current combined cohort than in previous cohort[64].
SNP, Single Nucleotide Polymorphism; OR, Odds Ratio; CI, Confidence Interval.

Table 2.5: Risk group comparisons and grade of hearing loss

	Patients with Hearing Loss Number (%[a])	**Normal Hearing Controls** Number (%[a])	**Total** Number (%[b])	**Grade of Hearing Loss** Mean ± SEM
Lower Risk (<0.4)	8 (26.7%)	22 (73.3%)	30 (9.5%)	0.77 ± 0.24
Intermediate Risk (0.4-0.8)	146 (59.6%)	99 (40.4%)	245 (77.3%)	1.69 ± 0.092
High Risk (>0.8)	39 (92.9%)	3 (7.1%)	42 (13.2%)	2.71 ± 0.13

[a]Percentage in risk group
[b]Percentage of total

Table 2.6: Comparison of risk groups

Model	Risk Group	Ototox. (Number, %)	Controls (Number, %)	OR (95% CI)[a]	P-value[a]	Sens	Spec	PPV	NPV
TPMT only	Risk haplotype carriers[c]	43 (91.5%)	4 (8.5%)	9.3 (3.1, 27.4)	$5.5x10^{-5}$	22.3%	96.8%	91.5%	44.4%
	Non carriers	150 (55.6%)	120 (44.4%)						
TPMT, ABCC3, COMT	High (>0.8)	39 (92.9%)	3 (7.1%)	11.0 (3.2, 37.6)	$1.3x10^{-4}$	20.2%	97.6%	92.9%	44.0%
	Low plus Intermediate (<0.8)	154 (56.0%)	121 (44.0%)						
TPMT, ABCC3, COMT	High plus Intermediate(>0.4)	185 (64.5%)	102 (35.5%)	4.8 (1.9, 11.9)	$7.1x10^{-4}$	95.9%	17.7%	64.5%	73.3%
	Low (<0.4)	8 (26.7%)	22 (73.3%)						
Clinical + TPMT, ABCC3, COMT	High (>0.80)	97 (91.5%)	9 (8.5%)	8.8 (4.0, 19.2)	$5.5x10^{-8}$	50.3%	92.7%	91.5%	54.5%
	Low plus Intermediate (<0.8)	96 (45.5%)	115 (54.5%)						
Clinical + TPMT, ABCC3, COMT	High plus Intermediate(>0.45)	161 (73.2%)	59 (26.8%)	3.1 (1.6, 5.9)	$4.3x10^{-4}$	83.4%	52.4%	73.2%	67.0%
	Low (<0.45)	32 (33.0%)	65 (67.0%)						

Ototox, Ototoxicity; OR, Odds Ratio; Sens, Sensitivity; Spec, Specificity; PPV, Positive Predictive Value; NPV, Negative Predictive Value
[a]Adjusted for age, vincristine treatment, germ cell tumor and cranial irradiation
[b]Percentage of total
[c]Risk haplotype carriers are individuals that are heterozygous or homozygous for TPMTrs12201199 risk variant

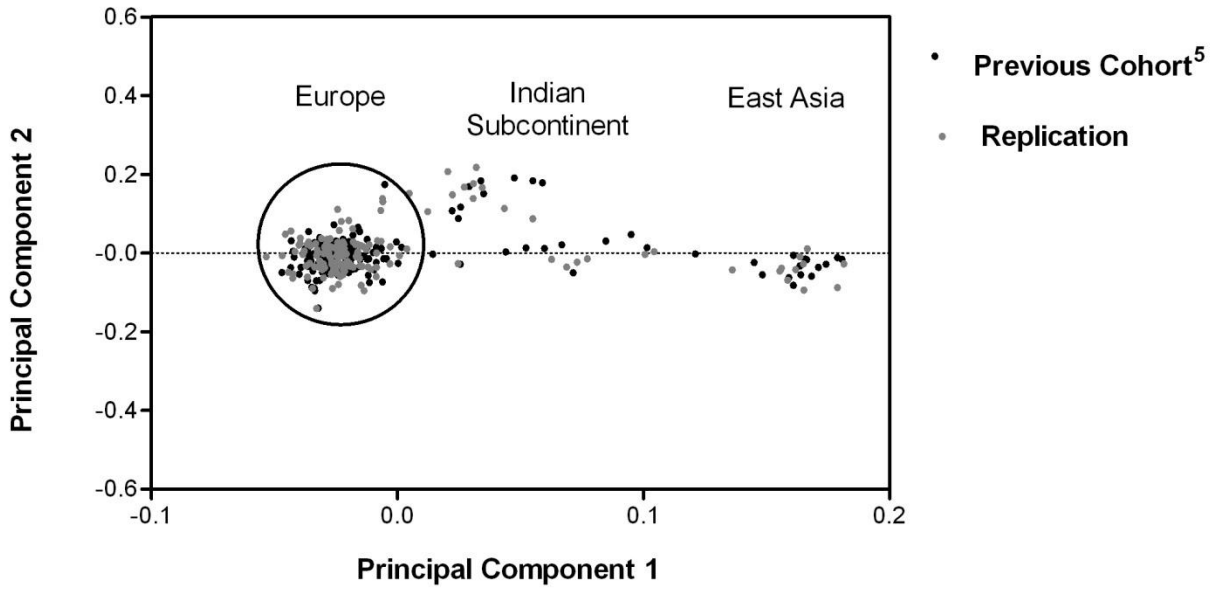
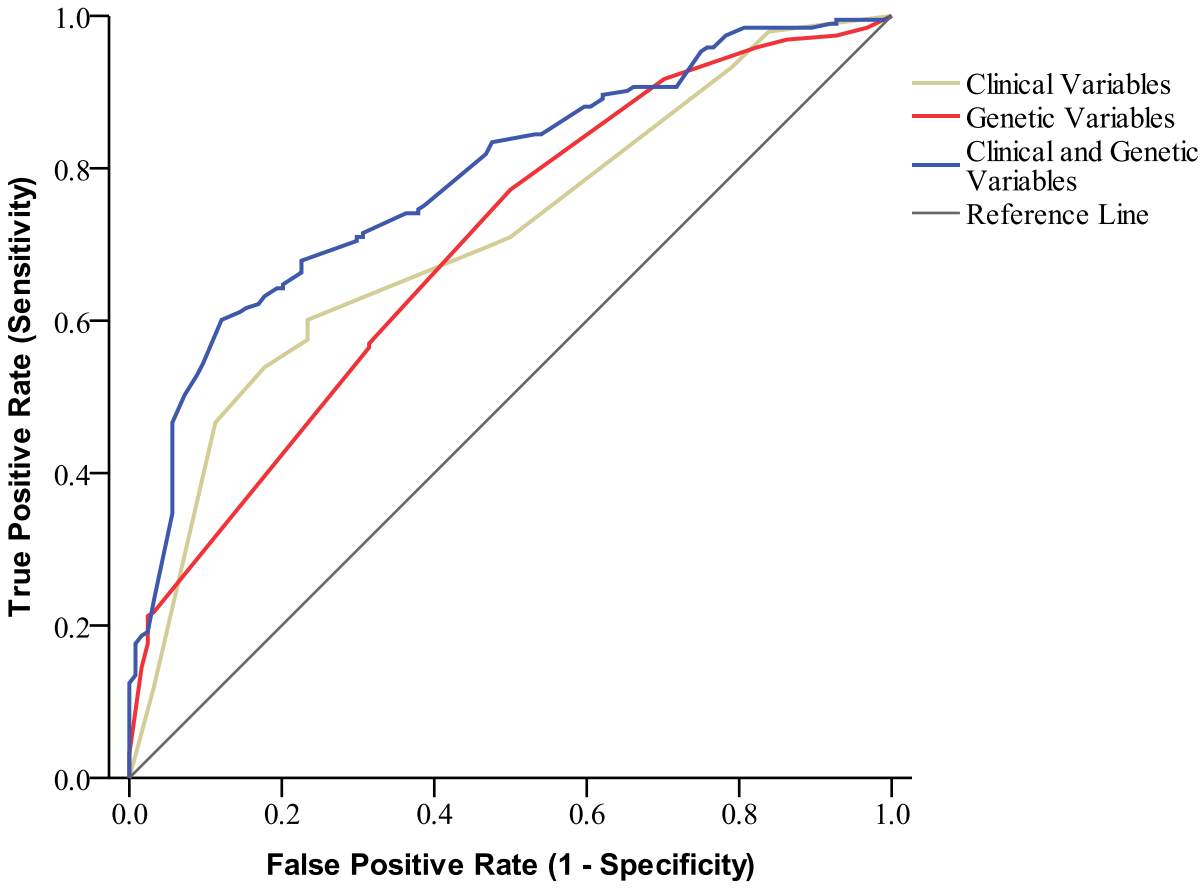


Figure 2.1: Distribution of ancestry of patients treated with cisplatin

The first two principal components were generated using SVS/Helixtree to visualize the distribution of population ancestry. The majority (80%) of the patients in replication cohort were of European ancestry after the removal of patients of African ancestry (n=8). A subgroup analysis of these patients (n=124), *TPMT* remained highly associated with cisplatin-induced hearing loss

A



B

Model	AUC (95% CI)	p-value
Clinical variables	0.708 (0.651-0.765)	-
Genetic variables (3 SNPs)	0.696 (0.638-0.754)	0.77
Clinical and genetic variables	0.786 (0.736-0.836)	0.00048

Figure 2.2: Receiver operating characteristic (ROC) curves of clinical and genetic variables for the prediction of cisplatin-induced hearing loss in the combined cohort

(a) The clinical variables include age, vincristine treatment, germ cell tumours and cranial irradiation while the genetic variables combine the effect of *TPMT* rs12201199, *COMT* rs4646316 and *ABCC3* rs1051640 **(b)** The area under the curve (AUC) for the combined cohort for each model. The p-values indicate the statistical significance between the curves for the combination of genetic and clinical variables compared to the clinical variables alone.

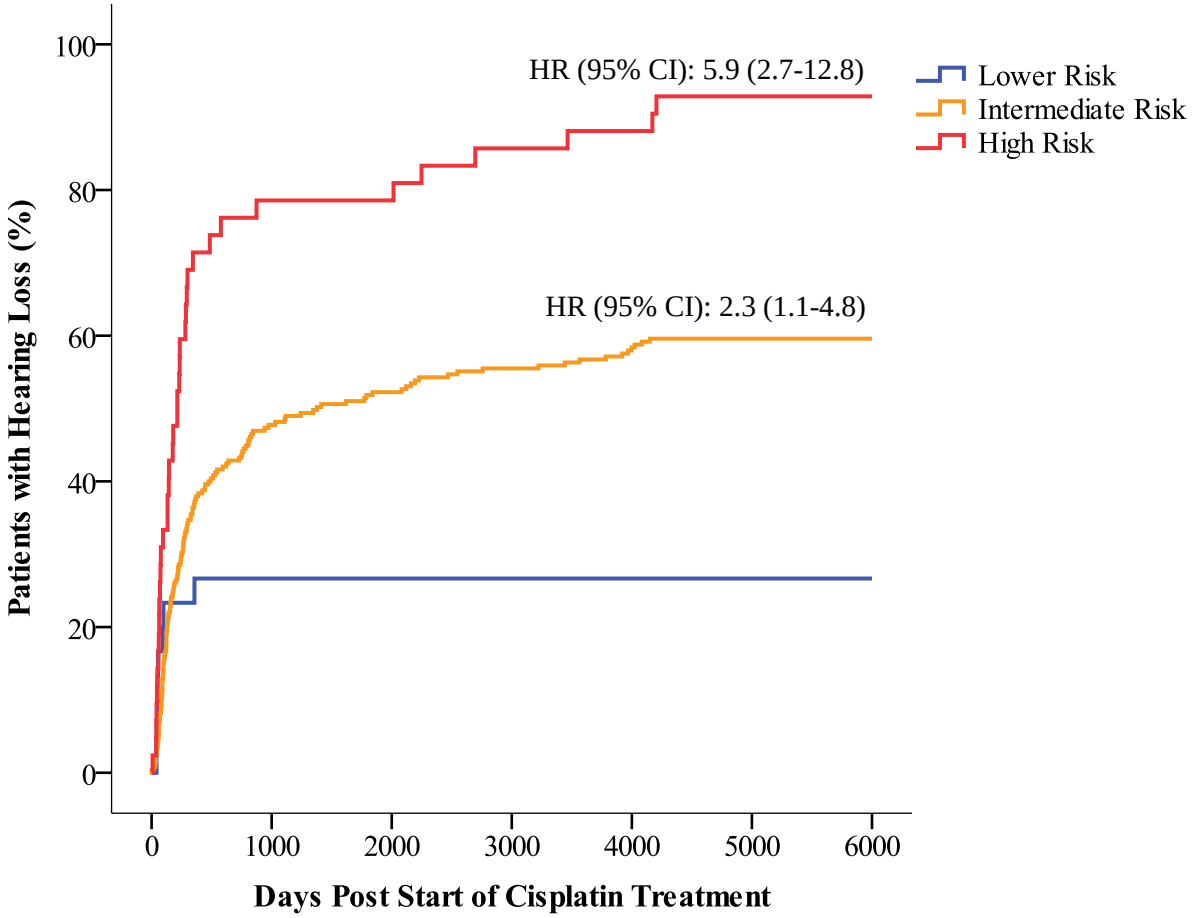


Figure 2.3: Kaplan-Meier curve of cisplatin-induced hearing loss in three different risk groups (Table 2.6) combining genetic factors.

The genetic variables combine the effect of *TPMT* rs12201199, *COMT* rs4646316 and *ABCC3* rs1051640. The Kaplan-Meier curve shows that the incidence of hearing loss increases with increasing risk group status. Hazard ratios (HR) were used to compare curves to the lower risk group and were adjusted for clinical variables (P_{trend}=5.3x10^{-9}).

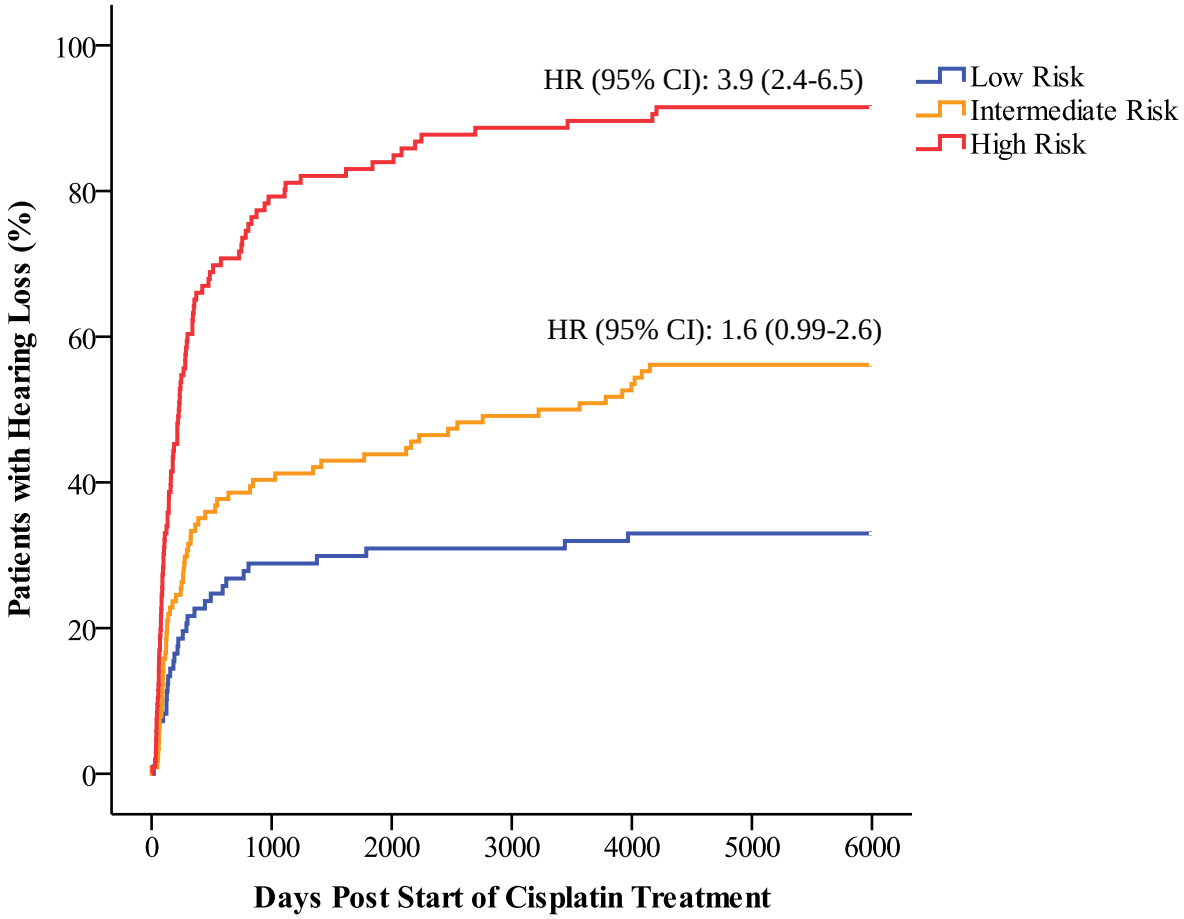


Figure 2.4: Kaplan-Meier curve of cisplatin-induced hearing loss in three different risk groups (Table 2.6) combining both clinical and genetic information.

The clinical variables include age, vincristine treatment, germ cell tumours and cranial irradiation while the genetic variables combine the effect of *TPMT* rs12201199, *COMT* rs4646316 and *ABCC3* rs1051640. The curves show that the incidence of hearing loss increases with increasing risk group status. Hazard ratios (HR) were used to compare curves to the low risk group and were adjusted for clinical variables (P_{trend}=3.4x10^{-19}).

[1]Chapter 3: Genetic Variants in *ABCB5* and *DPYD* are Associated with Cisplatin-Induced Hearing Loss in Children

3.1 Introduction

Cisplatin is a chemotherapeutic agent that is effective and widely used to treat a variety of solid tumours. A major limitation of its use is the risk of drug-induced ototoxicity that can result in life-long disability[117]. Cisplatin-induced ototoxicity presents itself as hearing loss and once detected, no improvement in hearing is observed and often hearing loss progress in severity long after treatment has ended[58, 59]. Cisplatin is responsible for the severe irreversible hearing loss which affects 10-25% of adults and 26-90% of children[48-53]. It is particularly devastating in children as they are at a critical stage of development. Even mild losses in hearing can significantly influence the overall quality of life including speech and language development, social-emotional development and increase the risk of learning difficulties[64, 65]. In patients experiencing severe hearing loss, hearing aids and other assistive devices are recommended. The use of hearing aids may be delayed until hearing loss has stabilized and treatment is complete. Although these devices can improve a child's hearing, they do not completely correct hearing loss. This suggests that by detecting hearing loss early or prior to treatment will allow children at risk of cisplatin-induced hearing loss to potentially have improved quality of life.

Clinical risk factors of cisplatin-induced hearing loss include high cumulative cisplatin dose[48, 73, 74], younger patient age at treatment[48, 55, 73], cranial irradiation[74, 79], and concomitant use of aminoglycosides[77]and vincristine[73]. It is now recognized that there is significant

[1]A version of this chapter will be submitted for publication. Pussegoda K, Ross C.J, Yazdanpanah M, Brooks B, Rassekh S.R, Carleton B.C, Hayden M.R. *Genetic variants in ABCB5 and DPYD are associated with cisplatin-induced hearing loss in children.*

inter-individual variability in hearing loss with patients receiving similar doses of cisplatin suggesting that clinical risk factors alone are insufficient predictors of safety[58, 64]. Genetic variation in the genes involved in drug biotransformation, transport, and drug targets have been recognized to influence patient drug response and susceptibility to adverse drug events, including ototoxicity[29]. Genetic markers may help to identify patients that confer increased susceptibility to cisplatin-induced ototoxicity.

A recent genetic study in children receiving cisplatin developed a multi-marker prediction model that include genetic variants (*TPMT* rs12201199, *COMT* rs4646316 and *ABCC3* rs1051640) conferring increased risk of developing cisplatin-induced hearing loss[64]. The genetic model was able to identify patients at high risk with a specificity of 97.6% and a sensitivity of 20.2% (**Chapter 2**). However, 79.8% of patients with hearing loss are not classified as high risk and wrongly predicted 2.4% of patients that developed hearing loss using this model. The aim of this study was to investigate whether additional genetic risk factors improve the prediction of risk for cisplatin-induced hearing loss in pediatric patients and to refine the current predictive multi-SNP model.

3.2 Material and Methods

3.2.1 Patients

Study participants were recruited through the Canadian Pharmacogenomics Network for Drug Safety (CPNDS)[118]. This cohort of children was recruited at pediatric oncology units across Canada: BC Children's Hospital (Vancouver), Alberta Children's Hospital (Calgary), Stollery Children's Hospital (Edmonton), Winnipeg Health Sciences Centre (Winnipeg), Children's

Hospital at the London Health Sciences Centre (London), McMaster Children's Hospital (Hamilton), Hospital for Sick Children (Toronto), Children's Hospital of Eastern Ontario (Ottawa), Hôpital Sainte-Justine (Montreal) and IWK Health Centre (Halifax). The initial cohort and replication cohort that are included in the combined analyses have been described previously[64].

Cisplatin-induced hearing loss was defined based on audiometric findings using CTCAEv3 (Common Terminology Criteria for Adverse Events) criteria as described previously[61, 64]. Briefly, to better differentiate between cases and controls, subjects with serious cisplatin hearing loss were defined as those with CTCAE grade 2 or higher hearing impairment after treatment with cisplatin. Control patients were defined as children who received cisplatin with normal audiometric data who did not develop hearing loss (grade 0). Patients with grade 1 hearing loss were excluded.

Written informed consent or assent was obtained from each subject or their parents or legal guardians. The study was approved by the ethics committees of all participating universities and hospitals.

3.2.2 Genotyping

Genomic DNA was extracted from blood, saliva or buccal swabs using the QIAmp DNA purification system (Qiagen, Toronto, ON, Canada) according to the manufacturer's protocol. DNA samples were genotyped for 4536 single nucleotide polymorphisms (SNPs) using a custom Illumina GoldenGate (SNP) genotyping assay (Illumina, San Diego, CA, USA). The panel was designed to include the genetic variants of the original custom ADME panel, increase the coverage of genetic variants and cover additional genes involved in drug biotransformation[64].

While the original panel captures the genetic variation of approximately 220 key genes, this enhanced custom panel covers 315 key genes involved in drug biotransformation including phase I and II drug metabolizing enzymes, drug transporters, drug receptors, ion channels, drug targets, transcription factors and other disease-specific genes related to the physiological pathway of cisplatin. Furthermore, the enhanced panel was optimized to avoid genomic interferences such as regions of homology to reduce the number of failed SNPs. All SNP genotype data were clustered manually using GenomeStudio software (Illumina, San Diego, CA, USA). SNPs that could not be clustered or were nonpolymorphic were excluded from further analysis. In total 4067 SNPs were available for analysis. Samples with a call rate below 95% were excluded. Two cases from the initial cohort with a call rate <95% were removed. The remaining 315 samples had an average genotyping call rate for included samples were 99.9%.

3.2.3 Statistical Analysis

Statistical genetic analyses were conducted using SNP and Variation Suite 7.4.5 (Golden Helix, Bozeman, USA), SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA) and R 2.13.0 (R Development Core Team).

Clinical characteristics of patients with and without cisplatin-induced hearing loss were compared using the Wilcoxon-Mann-Whitney U-test for continuous variables, and Fisher's exact test for categorical variables. We adjusted for multiple testing using the simple*M* correction and calculated the effective number of independent tests (M_{effG}) at 308 for a significance threshold of 0.00016. Hardy-Weinberg equilibrium tests were conducted in controls using the permutation version of the exact test of Hardy-Weinberg of Guo and Thompson[119]. Forty-six SNPs were removed due to Hardy-Weinberg disequilibrium and 71 SNPs with a completion rate less than

90%. In total, 3943 SNPs were available for analysis. To assess population stratification in the data, principal component analysis (PCA) was conducted using SVS/HelixTree software[112, 137]. Patients of African ancestry (*n*=8), as determined by PCA, were excluded in the analysis.

Homozygous and heterozygous odds ratios (ORs) using the homozygous genotype of the protective allele was used as a reference. Association between genetic polymorphisms and cisplatin-induced hearing loss was assessed by computing Fisher's exact test for unadjusted (non-regression) p-values and adjusted p-values determined by logistic regression. Independent factors for the prediction of cisplatin-induced hearing loss in the combined cohort were identified by forward logistic regression analysis. We included variables that were retained in the combined cohort and variables that are known to increase risk of hearing loss. Therefore, age, concomitant vincristine treatment, germ cell tumour, and cranial irradiation were included as covariates. In an additional analysis, we combined the initial cohort[64] and the replication cohort of patients to determine the overall significance of associations.

Genetic variants that were significant ($P<0.05$) in the initial cohort that were also significant ($P<0.05$) in the replication cohort were further evaluated in a multivariate logistic regression model including clinical variables. A genetic risk score was calculated by multiplying each variable with the estimated beta (log odds ratio) from the current combined cohort. The initial genetic model (**Chapter 2**) was constructed based on statistical evidence from a logistic regression model in the combined cohort, where *TPMT* rs12201199, *ABCC3* rs1051650 and *COMT* rs4646316 were retained. A revised model was constructed based on SNPs from the initial model (**Chapter 2**) as well as SNPs from the current study that when combined with the initial multi-marker model, independently increased the prediction of hearing loss. Genetic variants that significantly increased ($P<0.05$) the area under the curve (AUC) of the initial model

include ATP-Binding Cassette transporter B 5 (*ABCB5*) rs10950831 (*P*=0.020) and dihydropyrimidine dehydrogenase *(DPYD)* rs6667550 (*P*=0.046) and were therefore included into a new genetic prediction model. The final model combined the effects of *TPMT* rs12201199, *COMT* rs4646316, *ABCC3* rs1051640, *ABCB5* rs10950831 and *DPYD* rs6667550.

Association between the genetic risk score and cisplatin-induced hearing loss was assessed using logistic regression. Three prediction models were investigated: (i) clinical variables; (ii) genetic variables; and (iii) clinical and genetic variables. The contribution of the genetic and or clinical risk scores to the prediction of cisplatin-induced hearing loss was investigated by comparing the area under the receiver-operating-characteristic (ROC) curves of the prediction models. AUC estimates were obtained using the ROC plot function on the basis of the linear predictors obtained from the logistic regression analyses. The statistical difference between the curves was calculated using DeLong's method[120] implemented in the *R*-package pROC[121]. Two-sided *p*-values <0.05 were considered statistically significant.

Risk groups for cisplatin-induced hearing loss were defined based on the predictive values for each patient sample from the multi-SNP logistic regression model that included five SNPs (*TPMT* rs12201199, *ABCC3* rs1051640, *COMT* 4646316, *ABCB5* rs10950831 and *DPYD* rs6667550). The threshold used to determine low risk was the median predicted value of controls (predictive value <0.45) and high risk was defined as the mean predicted value of cases (>0.75). Intermediate risk was defined as a predictive value between 0.45-0.75. To assess the predictive performance of risk groups for cisplatin-induced hearing loss, we calculated the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for each defined threshold. Kaplan-Meier curves for cisplatin-induced hearing loss were generated for each risk group using time from start of treatment to the time of first evidence of toxicity or last

audiogram. Log-rank test was used to compare the trend of survival curves. Hazard ratios for each curve were calculated using the Cox regression model considering the low risk group as the reference.

3.3 Results

3.3.1 Patients

Patient characteristics of the initial cohort replication cohort have been described previously ([64], **Chapter 2**). Two cases in the initial cohort were excluded due to a call rate of <95%. Therefore, in the combined cohort 191 (61%) of the 315 pediatric oncology patients developed hearing loss (**Table 3.1**). Concomitant vincristine treatment was significantly higher in cases than controls in the combined cohort (58.6% compared to 27.9%, $P=5.5\times10^{-10}$). Fewer patients with germ cell tumour developed hearing loss (6.8% vs. 21.0%, $P=0.00035$). Furthermore, follow-up after therapy was longer in cases than controls (4.0 years vs. 2.0 years, $P=2.1\text{x}10^{-4}$). Overall median follow up time of patients was 3 years.

3.2.2 Genetic Results

We identified an intronic SNP (rs10950831) in *ABCB5* that was highly associated with cisplatin-induced hearing loss in both the initial cohort ($P=0.013$) and the replication cohort ($P=0.0012$) (**Table 3.2**). *ABCB5* rs10950831 was the most strongly associated variant that remained significant after multiple testing in the combined cohort ($P=1.06\text{x}10^{-6}$, OR 2.0). The risk allele (A) was observed in 154 (80.6%) cases and 62 (50.0%) controls in the combined cohort. The association of *ABCB5* rs10950831 genotype with cisplatin-induced hearing loss remained significant in the combined cohort after adjusting for clinical factors ($P=3.67\text{x}10^{-5}$).

In addition to *ABCB5*, we also found suggestive evidence for several additional genes associated with cisplatin-induced hearing loss (**Table 3.2**). *SLC28A3* rs17087056 was significantly associated after multiple-testing correction in the combined cohort (P=0.00011, OR 3.2). When the association with *SCL28A3* rs17087056 was adjusted for clinical factors, the significance of the effect decreased (P=0.00011 before adjustment vs. P=0.0029 after adjustment). Another genetic variant of interest that was close to the multiple testing threshold was *DPYD* rs6667550; however, this variant did not pass the threshold for multiple testing correction (P=0.00047, OR 1.9). Interestingly, the effect of *DPYD* rs6667550 became more significant in the combined cohort after adjusting for clinical factors (P=2.12x10^{-4}).

The initial predictive model which combined the effect of *TPMT*, *ABCC3* and *COMT* was able to predict 69.6% of cases (P= 7.16×10^{-5}) (**Figure 3.1**). We selected additional variants to include into a multi-marker regression model based on evidence of a significant increase (P<0.05) in prediction of hearing loss from the initial model (*TPMT*, *ABCC3*, and *COMT*). *ABCB5* rs10950831 and *DPYD* rs6667550 significantly improved (P<0.05) the prediction of hearing loss (AUC) from the initial multi-marker model and was therefore were included in the final model. In contrast, ROC analysis showed that by including *SLC28A3* rs17087056 for the prediction of cisplatin-induced hearing loss (AUC) did not significantly improve (P>0.05) the initial model. Therefore, the *ABCB5* rs10950831 and *DPYD* rs6667550 variants were selected for inclusion in the predictive model (**Figure 3.2**).

An ROC analysis that combined the effects of the associated risk genotypes and clinical risk factors was performed. The clinical-only model includes age, vincristine treatment, germ cell tumour and cranial irradiation. By including *ABCB5* and *DPYD* to the initial predictive model we can significantly increase the ability to predict cisplatin-induced hearing loss (AUC 0.775 vs AUC 0.696, P=0.0023) (**Figure 3.2**). Therefore the final genetic-only model was constructed to include five independent SNPs (*TPMT* rs12201199, *ABCC3* rs1051640, *COMT* 4646316, *ABCB5* rs10950831 and *DPYD* rs6667550). Furthermore, combining both the clinical and genetic variables significantly increased the ability to predict cisplatin ototoxicity (**Figure 3.3**) resulting in an area under the curve (AUC) of 0.841 compared to the clinical-only model (AUC 0.712, $P=2.63\text{x}10^{-7}$).

A predictive multi-marker model using these genetic variants could stratify patients by risk of ototoxicity. Risk groups were defined based on predictive values from the model as low (predictive value <0.45), intermediate (0.45-0.75) and high risk (>0.75). Based on these groups, there were 95 (30.2%) individuals classified as low risk, 122 (38.7 %) as intermediate and 98 (31.1%) as high risk. The initial model combining *TPMT*, *ABCC3* and *COMT* conferred a specificity of 97.6% and sensitivity of 20.2% (**Table 2.6**). In the new model, 87 (88.8%) of individuals in the high risk group developed hearing loss (positive predictive value, 88.8%) compared to only 11 (11.2%) controls conferring a specificity of 91.1% and a sensitivity of 45.5% (**Table 3.3**). Although the specificity of the test decreases slightly compared to the initial model (91.1% vs. 97.6%), the sensitivity of the new model is two-fold higher (45.5% vs. 20.2%).

Individuals in the high risk group also had a significantly higher risk of cisplatin-induced ototoxicity when compared to low to intermediate risk group (P=1.72x10^{-9}, OR 9.1; Table 3.3). A Kaplan-Meier plot illustrates that the intermediate and high risk groups have significantly increased risk of hearing loss over time (P_{trend}=5.7x10^{-15}; **Figure 3.4**). After 1 year of treatment, 61% of patients developed significant hearing loss in the high risk group compared to 22% of patients in the low risk group (**Figure 3.3**). A Kaplan-Meier plot combining both the clinical and genetic variables illustrates that we can identify 97 (50.8%) patients at high risk compared to 87 (45.5%) patients using genetics alone (**Figure 3.5**, **Table 3.3**). In the high risk group 97 (94.2%) of individuals developed hearing loss (positive predictive value, 94.2%) compared to 6 (5.8%) controls conferring a specificity of 95.2% and a sensitivity of 50.8% (**Table 3.3**). The initial model combining *TPMT*, *ABCC3*, *COMT* and clinical factors conferred a specificity of 92.7% and sensitivity of 50.3% (**Table 2.6**). Overall, by including *ABCB5* and *DPYD* to the initial model (*TPMT*, *ABCC3*, and *COMT*) the specificity of the test increases from 92.7% to 95.2%, with a small increase in sensitivity (50.8% vs. 50.3%).

3.3 Discussion

The ability to predict patients at risk of developing cisplatin-induced hearing loss would significantly improve the safety and efficacy of pediatric cancer therapy. The inter-individual variability in hearing loss even with treatment at similar doses suggests that there may be a genetic component. We identified genetic variants in *ABCB5* and *DPYD* that were associated with cisplatin-induced hearing loss in children treated for a variety of malignancies. Combining these genetic variants into a predictive model could improve the ability to discriminate more patients that are at high and low risk for cisplatin-induced hearing loss.

The strongest associated SNP was rs10905831, an intronic variant located in *ABCB5*. Patients carrying the A-allele of rs10905831 were at increased risk of developing hearing loss after cisplatin treatment. This is the first study to describe the role of genetic variation in *ABCB5* in the context of cisplatin-ototoxicity. ABC transporters are known to confer the efflux of a variety of drugs including cisplatin[81, 82]. Polymorphisms in *ABCB5* may regulate expression levels or affect function of the transporter which can lead to intracellular accumulation of cisplatin and toxicity.

Other SNPs that were associated occurred in genes encoding for proteins that may be involved in processes that affect cisplatin absorption, distribution, metabolism and excretion. Genetic variants in these genes can influence the pharmacokinetics of cisplatin and affect toxicity and survival[29]. Another intronic variant in *DPYD* (rs6667550) was also associated with cisplatin-induced hearing loss. One study has found that the activity *DYPD* decreased in patients treated with a combination of cisplatin-5-fluorouracil therapy[138]. This indicates that cisplatin may interact with *DPYD* and affect the activity of the enzyme. Furthermore the study suggests that individuals with low *DPYD* activity may be at increased risk of toxicity when receiving platinum compounds[138]. How intronic regions affect susceptibility to cisplatin-induced hearing loss remains unclear. Intronic SNPS can be linked to functional SNPs within the coding area of the gene or regulate function and expression. Further functional studies will be required to assess the exact mechanisms by which these variants affect cisplatin-induced hearing loss. We also found no significant associations with the previously reported variants in megalin (*LRP*) rs2075252, *GSTP1* rs1695, presence of *GSTM1*, and *XPC* rs2228001 (P=0.97, P=0.16, P=0.12, P=0.49).

The differences in the clinical characteristics between cases and controls were corrected for by including them as covariates in the logistic regression analyses. High cumulative cisplatin dose, younger patient age at treatment, concomitant use of aminoglycosides and cranial irradiation are known to influence cisplatin-induced ototoxicity[59]. Logistic regression analyses suggest that there was little to no effect of population stratification within our cohort of patients. Inter-study heterogeneity can lead to differences in the effects of variants in specific subpopulations or lead to masking of the effects by factors that were not controlled for. We aimed to control for these factors by adjusting for clinical variables in the cohort and identifying potential population stratification.

Although clinical factors have been identified, they alone cannot reliably predict the risk of developing hearing loss. We generated a refined predictive model by incorporating genetic risk in *ABCB5* and *DPYD* to the initial genetic model which combines the effect of *TPMT*, *ABCC3* and *COMT*. We were able to improve the genetic component of the risk prediction model by including *ABCB5* rs10905831 and *DPYD* rs6667550 into the initial genetic model (AUC 0.775 vs. 0.696; P=0.0023). Furthermore, by combining genetic and clinical risk factors were able better discriminate between patients at high and low risk. The combined model of genetic plus clinical risk factors was more predictive than clinical risk factors alone (AUC 0.841 vs. 0.712; $P=2.63\text{x}10^{-7}$). In the high risk group 88.8% of cases were predicted to develop hearing loss while only 11.2% of controls were predicted to be at high risk conferring a sensitivity of 45.5%. The increase in the sensitivity from the initial model is significantly improved from the initial model (20.2%) (**Chapter 2**). We are able to better identify patients at high risk with the new refined predictive model. Furthermore, including clinical and genetic risk factors we can improve the

specificity of the test (95.2% in the current model vs. 92.7% in the initial model) while maintaining a sensitivity of 50%. Whether patients at low risk require less intensive monitoring or can be treated at higher doses to improve survival without significantly increasing cisplatin-induced hearing loss needs to be further investigated. The model was based on results from the combined cohort and requires further studies in children and adults to validate these findings to assess the clinical utility.

There are an increasing number of children surviving cancer treatment. Given that cisplatin-induced hearing loss is permanent and progressive, it is increasing important to reduce or prevent hearing loss, particularly in high risk groups. In children this is of particular importance as even mild losses in hearing can cause difficulties in learning in school[65, 134]. Several preventative measures have been proposed in patients that are high risk of adverse effects from cisplatin. Carboplatin which is less ototoxic agent is suggested as alternative medication for treatment [55]. Other alternatives include increased monitoring, lowering dose and/or otoprotective agents[135, 136]. However, there are concerns about the possibility of compromise of anti-tumour activity have been raised with these alternatives strategies.

This study demonstrates that it is possible to accurately discriminate patients at higher and lower risk for cisplatin-induced hearing loss based on a patient's genetic profile in combination with clinical risk factors. This can influence treatment decisions and improve monitoring of patients at risk of adverse events. Sequencing of *ABCB5* and *DPYD* may identify functional variants that may affect expression and activity which can lead to a mechanism by which these genes influence cisplatin toxicity. Further validation of the prediction model in an independent cohort

of patients is required prior to use in clinic. Because this study includes mainly patients of European origin, it will also be important to assess these variants and the risk of cisplatin-induced hearing loss in other ethnic cohorts where allele frequencies and linkage disequilbrium may differ. In addition, functional studies can be conducted to assess the exact mechanisms by which variants in *ABCB5* and *DPYD* affect cisplatin-induced hearing loss. Identifying genetic risk factors can potentially improve risk classifications for hearing loss and may allow for individualized treatment and improve safety in children.

Table 3.1 Patient demographics of the combined cohort

	Combined Cohort (n=315)		
	Cases (n= 191)	**Controls** (n=124)	p-value
Age, years (median (min, max))	6.0 (0, 25)	10.0 (0, 19)	**0.012**
Dose, cumulative mg/m2 (median (min, max))	400 (92, 800)	400 (20, 768)	0.80
Treatment duration, weeks (median (min, max))	2.2 (0, 11.4)	2.3 (0, 8.5)	0.82
Gender (Male n, (%))	112 (58.6%)	62 (50.0%)	0.16
Caucasian ethnicity[a] (n (%))	149 (78.0%)	102 (82.3%)	0.39
Concomitant medication (n, (%))			
Tobramycin	54 (28.3%)	30 (24.2%)	0.44
Vancomycin	50 (26.2%)	25 (20.2%)	0.28
Vincristine	112 (58.6%)	29 (23.4%)	**5.5×10^{-10}**
Gentamicin	42 (22.0%)	26 (21.0%)	0.89
Tumor type (n, (%))			
brain tumor	51 (26.4%)	19 (15.3%)	**0.019**
endodermal sinus tumor of thymus	0	2 (1.6%)	0.15
germ cell tumor	13 (6.8%)	26 (21.0%)	**0.00035**
hepatoblastoma	39 (20.4%)	12 (9.7%)	**0.012**
lymphoma	1 (0.5%)	3 (2.4%)	0.30
nasopharyngeal carcinoma	1 (0.5%)	2 (1.6%)	0.39
neuroblastoma	50 (26.2%)	21 (16.9%)	**0.072**
osteosarcoma	33 (17.3%)	36 (29.0%)	**0.018**
sarcoma	1 (0.5%)	2 (1.6%)	0.56
carcinoma	0	1 (0.8%)	0.39
retinoblastoma	1 (0.5%)	0	1.00
mesencymal tumour of liver	1 (0.5%)	0	1.00
Follow-up, years (median (min, max))	4 (0, 25)	2 (0, 16)	**6.4×10^{-5}**
Cranial irradiation (n, (%))	43 (22.5%)	15 (12.1%)	**0.025**

For age, dose, treatment duration and follow-up, the Wilcoxon-Mann-Whitney test with normal approximation was used. For gender, ethnicity, concomitant medication, tumor type, cranial irradiation the Fisher exact test was used. In bold are statistically significant values at $p<0.05$ type-I error rate.

[a]Caucasian ethnicity assessed by principal component analysis

Table 3.2 Association between SNPs and cisplatin-induced hearing loss

			Previous Cohort[a] (n=160) 104 ototox., 56 controls		Replication (n=155) 87 ototox., 68 controls		Combined (n=315) 191 ototox., 124 controls		p-value[c]
Gene	**SNP**	**Allele[b]**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	
ABCB5	rs10950831	A/G	1.9 (1.2, 3.2)	0.013	2.2 (1.4, 3.6)	0.0012	2.0 (1.4, 2.9)	**1.06×10^{-6}**	**3.67×10^{-5}**
SLC28A3	rs1087056	A/T	2.6 (1.1, 5.8)	0.026	4.3 (1.7, 11.1)	0.028	3.2 (1.7, 5.9)	**0.00011**	0.0029
DPYD	rs6667550	G/A	1.7 (1.0, 2.9)	0.037	2.3 (1.3, 3.9)	0.0036	1.9 (1.4, 2.8)	0.00047	2.12×10^{-4}
XDH	rs6710015	G/A	1.9 (1.2, 3.2)	0.010	1.7 (1.0, 2.7)	0.045	1.8 (1.3, 2.5)	0.0011	0.0043
XDH	rs207425	A/G	1.8 (1.1, 3.2)	0.033	1.7 (1.0, 2.7)	0.045	1.7 (1.2, 2.4)	0.0052	0.035
RALBP1	rs3322	A/G	2.5 (1.1, 5.6)	0.023	3.5 (1.3, 9.2)	0.012	2.8 (1.5, 5.1)	0.0064	0.0058
PPARG	rs1152003	G/A	1.8 (1.0, 3.1)	0.042	2.2 (1.2, 4.0)	0.013	1.8 (1.3, 2.5)	0.0012	0.0052

[a]Results from discovery and replication cohorts from previous study[64] compared to the replication cohort as well as all cohorts combined
[b]SNP alleles assayed; minor allele is mentioned first; P-values in bold are significant after multiple testing correction in combined cohort
[c]Adjusted for age, vincristine treatment, germ cell tumor and cranial irradiation
SNP, Single Nucleotide Polymorphism; Ototox, Ototoxicity; OR, Odds Ratio; CI, Confidence Interval.

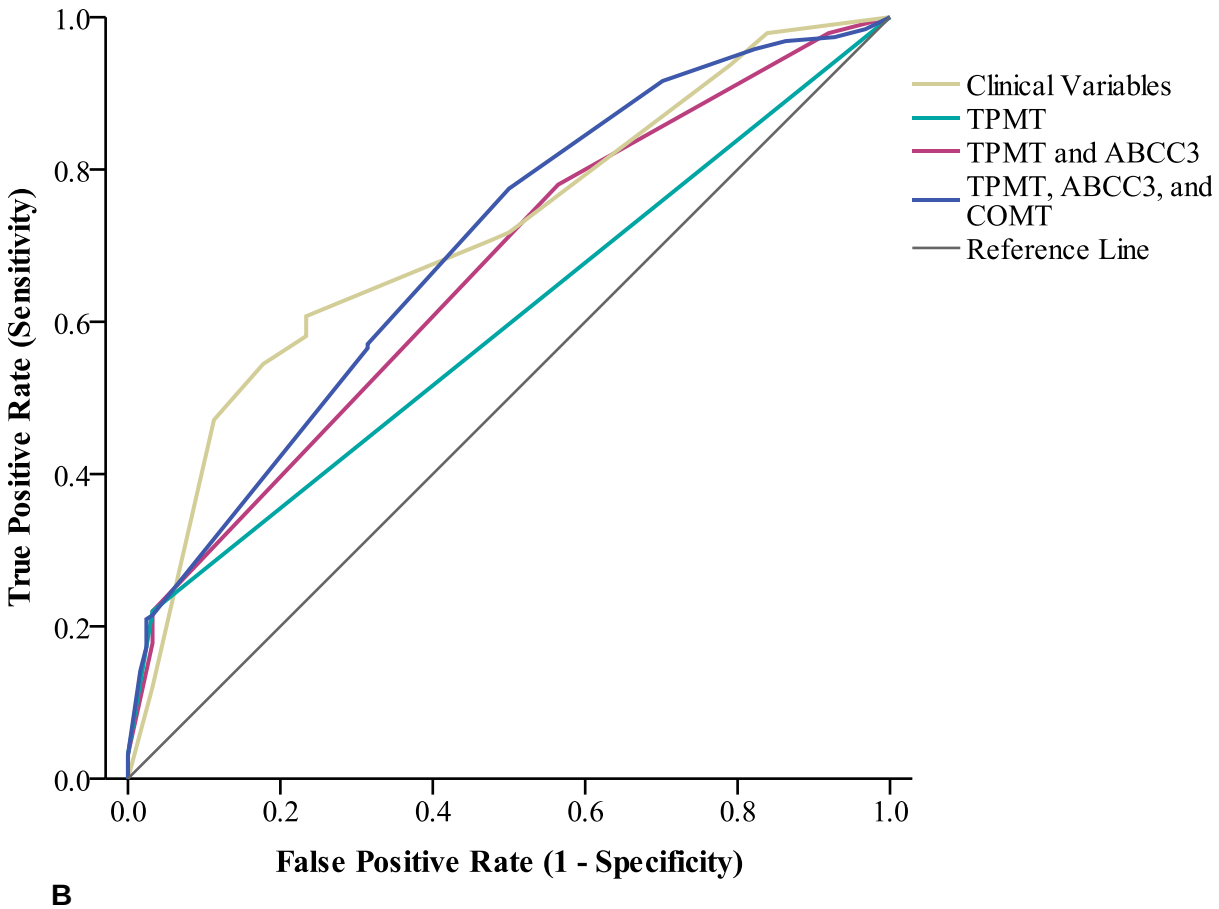
.

Table 3.3: Comparison of risk groups

Model[d]	Risk Group	Ototox. (Number, %)	Controls (Number, %)	OR (95% CI)[a]	P-value[a]	Sens	Spec	PPV	NPV
Genetic only	High (>0.75)	87 (88.8%)	11 (11.2%)	9.1 (4.4, 18.6)	$1.72x10^{-9}$	45.5%	91.1%	88.8%	52.1%
	Low plus Intermediate (<0.75)	104 (47.9%)	113 (52.1%)						
Genetic only	High plus Intermediate(>0.45)	159 (72.3%)	61 (27.7%)	6.3 (3.5, 11.3)	$5.73x10^{-10}$	83.2%	50.8%	72.3%	66.3%
	Low (<0.45)	32 (33.7%)	63 (66.3%)						
Clinical + Genetic	High (>0.82)	97 (94.2%)	6 (5.8%)	8.8 (4.0, 19.2)	$5.5x10^{-8}$	50.8%	95.2%	94.2%	55.7%
	Low plus Intermediate (<0.82)	94 (44.3%)	118 (55.7%)						
Clinical + Genetic	High plus Intermediate(>0.34)	177 (74.1%)	62 (25.9%)	3.1 (1.6, 5.9)	$4.3x10^{-4}$	92.7%	50.0%	74.1%	81.6%
	Low (<0.34)	14 (18.4%)	62 (81.6%)						

Ototox, Ototoxicity; OR, Odds Ratio; Sens, Sensitivity; Spec, Specificity; PPV, Positive Predictive Value; NPV, Negative Predictive Value
[a]Adjusted for age, vincristine treatment, germ cell tumor and cranial irradiation
[b]Percentage of total
[c]Risk haplotype carriers are individuals that are heterozygous or homozygous for TPMTrs12201199 risk variant
[d] Clinical variables include age, vincristine treatment, germ cell tumours and cranial irradiation while the genetic variables include *TPMT* rs12201199, *COMT* rs4646316, *ABCC3* rs1051640, *ABCB5* rs10950831 and *DPYD* rs6667550

A



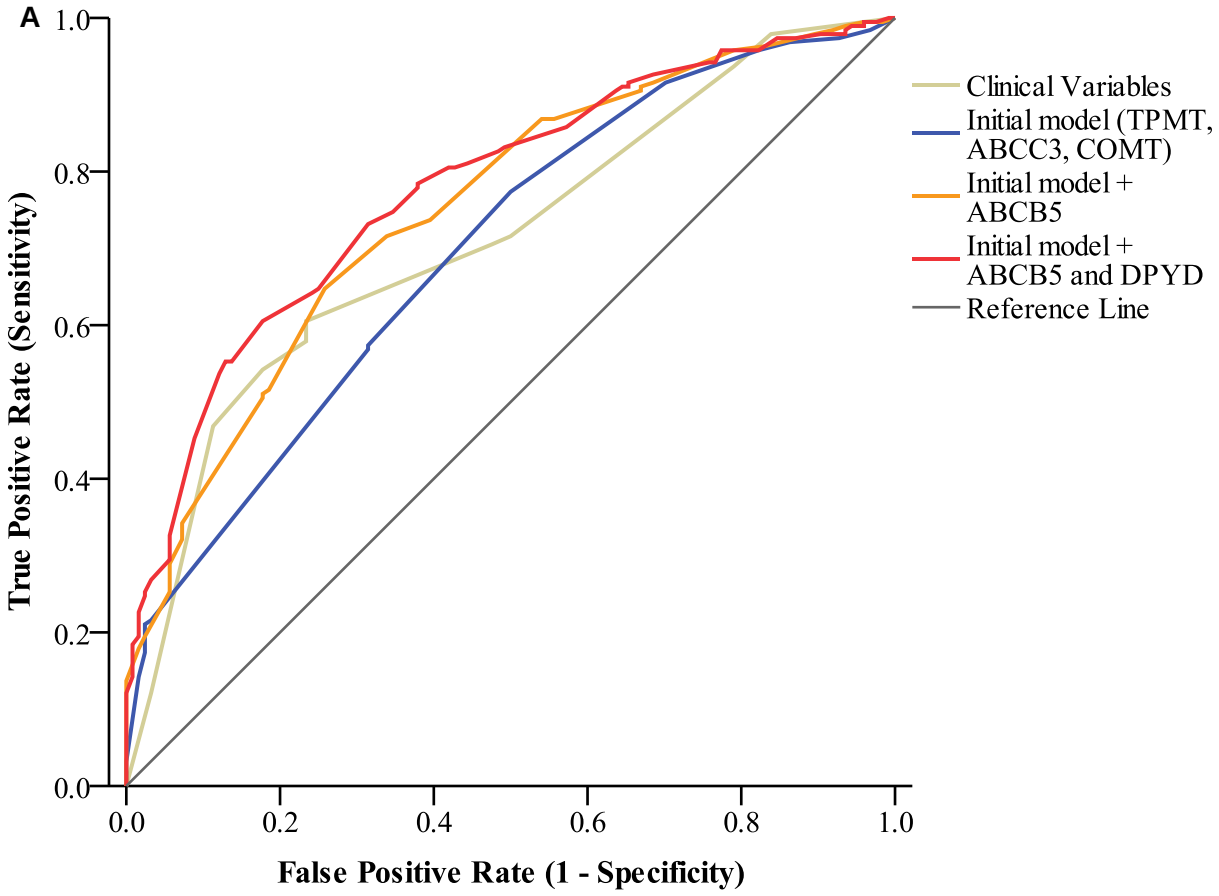
B

Model	AUC (95% CI)	p-value
Clinical Variables	0.713 (0.656, 0.770)	-
TPMT	0.594 (0.532-0.656)	-
TPMT and ABCC3	0.661 (0.601-0.722)	0.0030
Initial model (TPMT, ABCC3 and COMT)	0.696 (0.638-0.755)	7.16×10^{-5}

Figure 3.1: ROC curves of genetic variables in the initial model for the prediction of cisplatin-induced hearing loss in the combined cohort

(a) The clinical variables include age, vincristine treatment, germ cell tumours and cranial irradiation. The initial model combines the effect of *TPMT* rs12201199, *COMT* rs4646316 and *ABCC3* rs1051640 as described previously in a step-wise manner **(b)** The area under the curve (AUC) for the combined cohort for each model. The p-values indicate the statistical significance between each genetic marker in the initial model compared to TPMT rs12201199 alone.

A



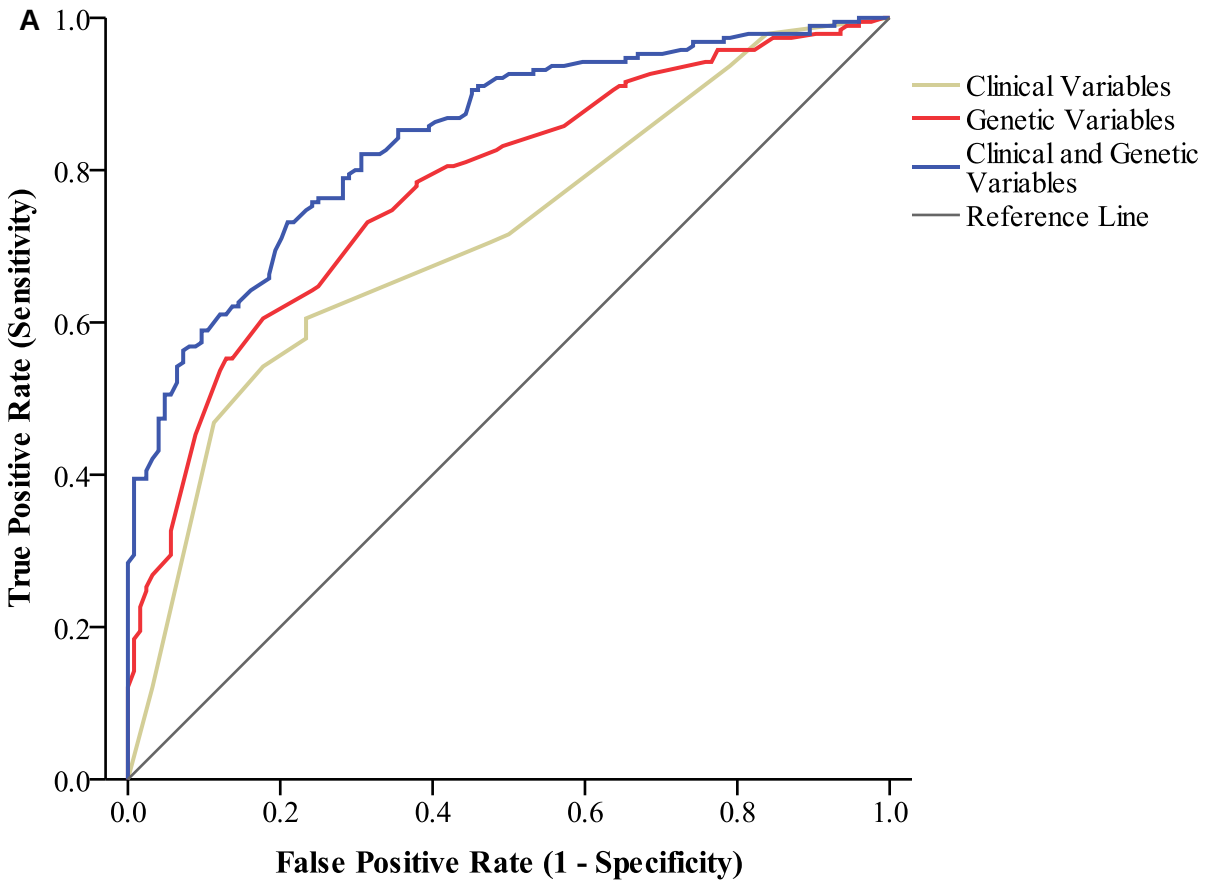
B

Model	AUC (95% CI)	p-value
Clinical Variables	0.712 (0.654, 0.769)	-
Initial Model (TPMT, ABCC3, COMT)	0.696 (0.638-0.755)	-
Initial Model + ABCB5	0.752 (0.698-0.806)	0.020
Initial Model + ABCB5 and DPYD	0.777 (0.726-0.828)	0.0023

Figure 3.2: ROC curves of new genetic variables for the prediction of cisplatin-induced hearing loss in the combined cohort

(a) The clinical variables include age, vincristine treatment, germ cell tumours and cranial irradiation. The initial model combines the effect of *TPMT* rs12201199, *COMT* rs4646316 and *ABCC3* rs1051640 as described previously. New genetic variants that statistically improve the initial model were included in a step-wise manner. The final model combines the effect of the initial variants and *ABCB5* rs10950831 and *DPYD* rs6667550 **(b)** The area under the curve (AUC) for the combined cohort for each model. The p-values indicate the statistical significance between the new genetic models compared to the initial genetic model.

A



B

Model	AUC (95% CI)	p-value
Clinical Variables	0.712 (0.654, 0.769)	-
Genetic Variables (5 SNPs)	0.777 (0.726-0.828)	0.11
Clinical and New Genetic Variables	0.841 (0.799-0.884)	2.63×10^{-7}

Figure 3.3: ROC curves of clinical and newly identified genetic variables for the prediction of cisplatin-induced hearing loss in the combined cohort

(a) The clinical variables include age, vincristine treatment, germ cell tumours and cranial irradiation while the genetic variables include *TPMT* rs12201199, *COMT* rs4646316, *ABCC3* rs1051640, *ABCB5* rs10950831 and *DPYD* rs6667550 **(b)** The area under the curve (AUC) for the combined cohort for each model. The p-values indicate the statistical significance between the curves for the combination of genetic and clinical variables compared to the clinical variables and the genetic variables alone.

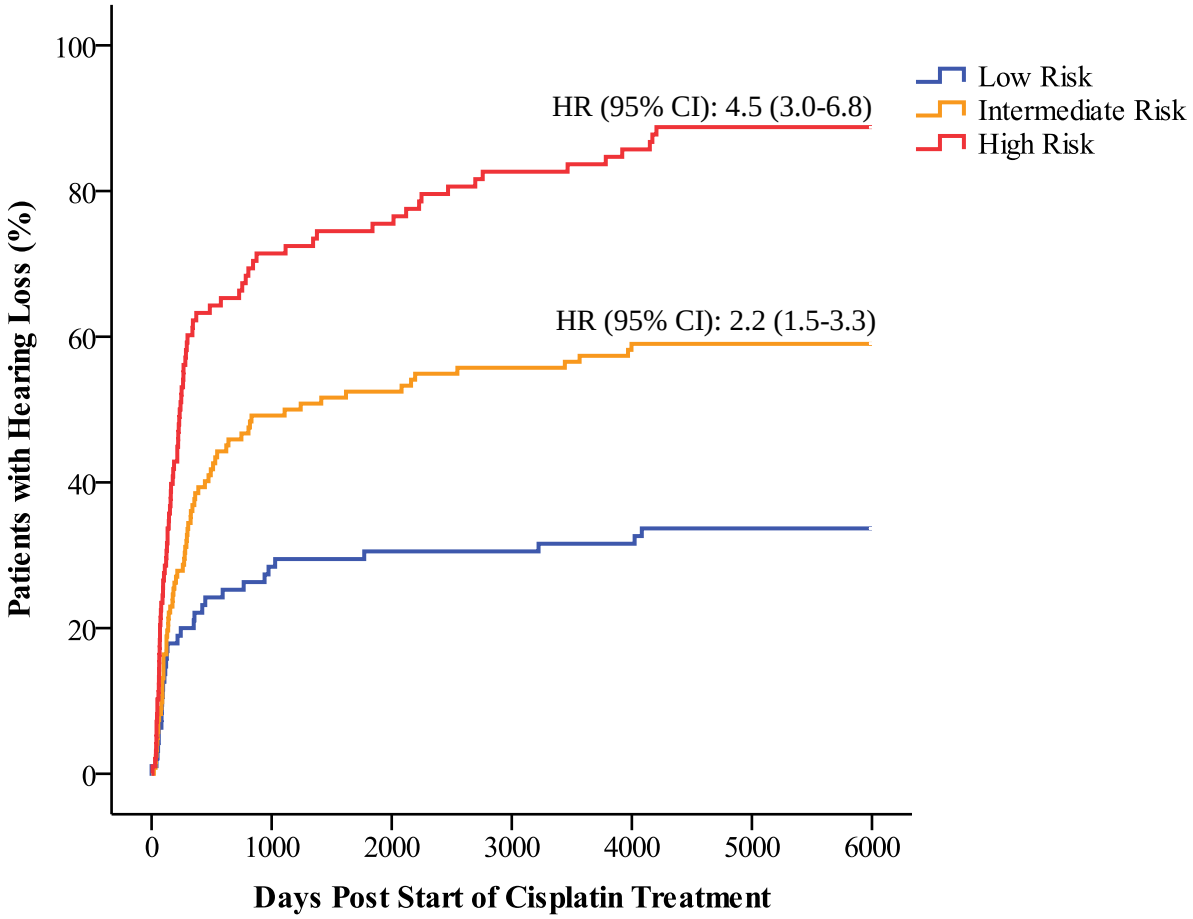


Figure 3.4: Kaplan-Meier curve of cisplatin-induced hearing loss in different risk groups (Table 3.3) combining genetic factors.

The genetic variables combine the effect of *TPMT* rs12201199, *COMT* rs4646316, *ABCC3* rs1051640, *ABCB5* rs10950831 and *DPYD* rs6667550. The Kaplan-Meier curve shows that the incidence of hearing loss increases with increasing risk group status. Hazard ratios (HR) were used to compare curves to the low risk group and were adjusted for clinical variables (P_{trend}=5.7x10^{-15}).

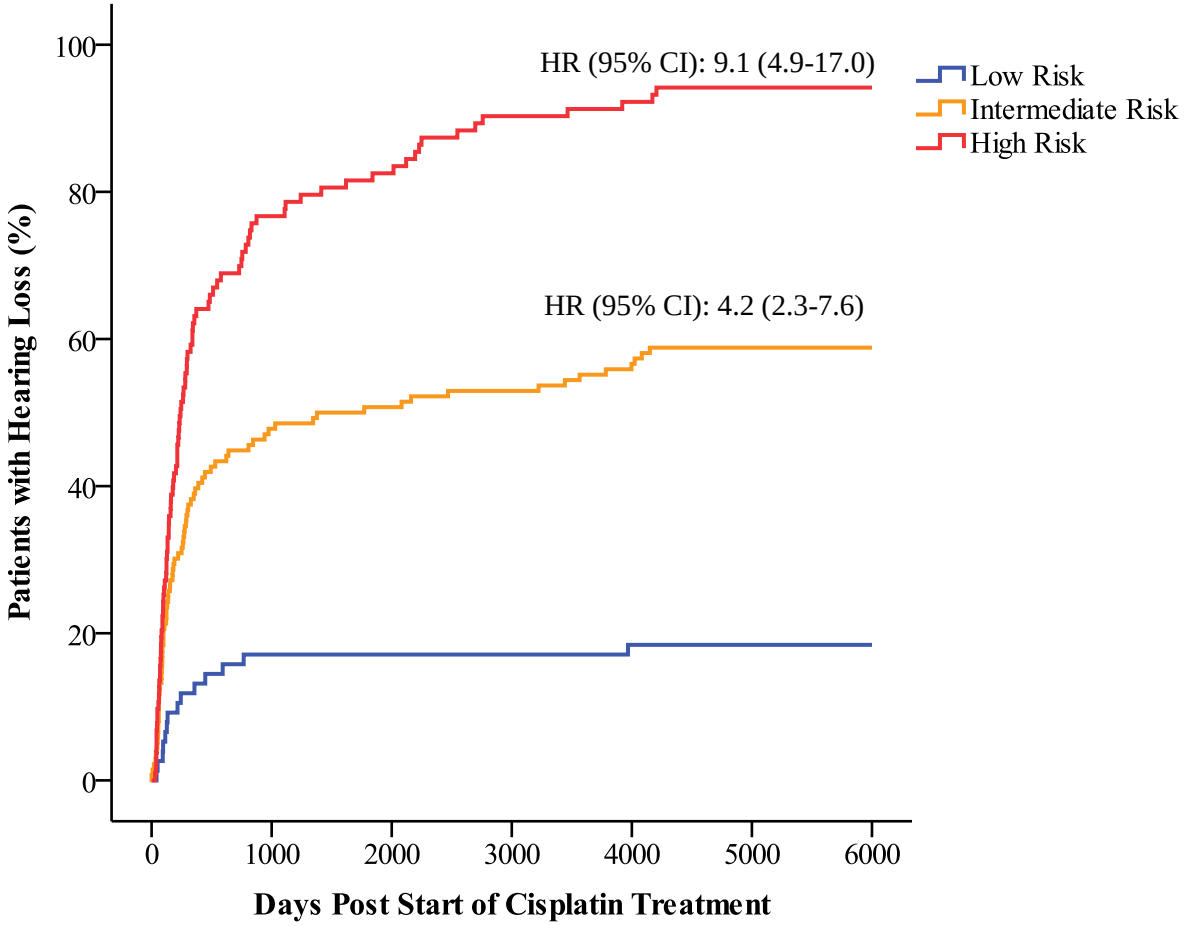


Figure 3.5: Kaplan-Meier curve of cisplatin-induced hearing loss in different risk groups (Table 3.3) combining clinical and genetic factors.

The clinical variables include age, vincristine treatment, germ cell tumours and cranial irradiation while the genetic variables include *TPMT* rs12201199, *COMT* rs4646316, *ABCC3* rs1051640, *ABCB5* rs10950831 and *DPYD* rs6667550. The Kaplan-Meier curve shows that the incidence of hearing loss increases with increasing risk group status. Hazard ratios (HR) were used to compare curves to the low risk group and were adjusted for clinical variables (P_{trend}=1.7 x10^{-23}).

Chapter 4: Discussion and Future Directions

Cisplatin is of the most effective and widely used chemotherapeutic agents for the treatment of solid tumours; yet, it's application is limited by cisplatin-induced hearing loss and other ADRs. The studies conducted as part of this thesis employed a candidate gene approach to screen genes involved in drug ADME to identify novel genetic variants associated with cisplatin-induced hearing loss. This was the first study to replicate previous associations and develop predictive models to discriminate between pediatric patients that are at high versus low risk of cisplatin-induced hearing loss using both genetic and clinical factors.

4.1 Strengths and Limitations

This study has the largest cohort of well phenotyped pediatric patients on cisplatin therapy. Patients were phenotyped using CTCAE grading scheme and those with mild hearing loss (Grade 1) were excluded to evaluate more extreme phenotypes. Cisplatin-induced hearing loss often increases in severity over time, even after completion of cisplatin treatment (i.e. patients with Grade 1 hearing loss progress to Grade 2 to 4). Furthermore, we aimed to control for confounding factors by adjusting for clinical variables in the cohort and carrying out a subgroup analyses in patients of European ancestry.

For cisplatin, the currently identified genetic variants do not independently improve the risk of prediction over clinical factors. This highlights a key limitation in the predictive models that have been generated. Ideally we would have liked to identify genetic predictors that have a greater impact on drug effects than clinical parameters alone. One possible explanation may be that additional novel genetic risk factors may be associated with cisplatin-induced hearing loss, which may increase the genetic contribution to this ADR. However, it is also possible that other

factors such as environment and clinical parameters contribute significantly to the risk of cisplatin-induced hearing loss. The clinical risk factors that have been identified in this study are currently not used to modify treatment-decisions. This study has shown that clinical factors indeed do contribute to the risk and susceptibility of hearing loss with cisplatin treatment and therefore, should be taken into account in clinical treatment decision-making. However, clinical factors alone are not adequate to predict hearing loss and by adding genetic risk factors we have shown that we can improve prediction of risk and to better classify patients into different risk groups. Another limitation of this study is that the prediction models were generated were based on the results from the combined cohort and have not yet been replicated. Therefore, it is possible that we are over-fitting the model. In addition we have selected variants that are known to affect patheways involved in ADME. Other genetic variants outside drug metabolism pathways may also affect cisplatin-induced hearing loss, but were not investigated in this study.

4.2 Replication and Functional Studies

For clinical application, it is essential to validate genetic findings in additional independent cohorts of patients to reduce the number of false positive results and to ensure that the genetic risk prediction is consistent and precise. A standardized phenotyping method would significantly facilitate accurate replication studies, and enable cross-study comparison of research findings. Many current studies have a small number of patients in which an effect was detected, but with little or no independent replication of these findings it is not possible to draw conclusions for clinical application. Furthermore, genetic risk variants may also differ with treatment for different malignancies because of differences in treatment protocols. However, due to low sample sizes many studies have found it difficult to carry out subgroup analyses to determine

whether identified variants are associated with specific treatment protocols. In addition, a majority of studies have been carried out in populations of only European ancestry. Whether these same genetic variants are also associated in different patient populations with other ancestries still needs to be investigated.

As a next step, replication of the novel variants that were identified in this study and the predictive models should be evaluated in additional independent cohorts. The replication of these findings in both pediatric and adult patients treated with cisplatin is required for validation to ensure findings are consistent and precise and to provide an unbiased selection of SNPs for the predictive model. It is important to recognize that children and adults metabolize drugs differently, which suggests that different genetic variants may be responsible for cisplatin-induced toxicity between these two groups.

Another method to validate findings is through functional validation studies to investigate the mechanisms that underlie the associations between cisplatin-induced hearing loss and the identified genes. One possible method would be to evaluate expression and activity of functional genetic variants in the genes that were identified in this study in cell culture in the presence of cisplatin. This method may confirm whether certain genotypes are associated with increased toxicity.

4.3 Investigation of Additional Gene and Gene Variants Involved in Cisplatin-Induced Hearing Loss

The identification of additional genetic risk variants for cisplatin ototoxicity may explain cases that cannot currently be predicted using known risk factors. Furthermore, these variants may have the potential to improve the positive predictive power and/or the sensitivity of genetic testing. As a next step, re-sequencing the genes that were associated with cisplatin-induced hearing loss would facilitate the identification of additional variants that may be implicated. This may include variants in linkage with the newly identified SNPs as well as unknown variants within the same gene that are independently associated. To complement this targeted candidate gene approach, a genome-wide association study (GWAS) could also be performed to investigate cisplatin-induced hearing loss at the genome-wide level. A GWAS is a hypothesis-free approach that can screen for an association with cisplatin-induced hearing loss in more than one million SNPS across the human genome. The benefit of performing a GWAS is to identify novel genes we would not suspect to contribute to cisplatin-induced hearing loss. These studies usually require large samples sizes for sufficient power to detect a true positive association due to the number of variants that are analyzed.

4.4 Implementing Genetic Testing

Pharmacogenomic testing has the potential to improve the safety and efficacy of medications. With well-established clinical applications and the variety of genotyping methods that have been established, pharmacogenomic testing is still rarely used in clinical practice. It has become widely accepted for clinicians to treat patients based on a 'trial and error' dosing approach. Therefore, there is resistance to using tests to influence medical decisions, particularly when it

comes to pharmacogenetic testing because it requires interpretation of genotypes and additional training in genetics and pharmacology to incorporate it into clinical decision-making.

The association studies for cisplatin-induced hearing loss thus far have been retrospective. To assess the real-world clinical utility of predictive pharmacogenetic testing will require prospective studies to assess safety and efficacy. This is a challenge because it is not well-known whether alternative therapies have similar efficacies in specific tumor types. Cisplatin prospective trials will allow clinicians to develop strategies to manage hearing loss such as dose modifications, use of alternate medications that are just as effective, or through the use of otoprotective agents. Whether we need to wait for prospective trials prior to implementing genetic testing for cisplatin risk variants is still under debate. However, with the information and understanding we have gained from studying and replicating genetic factors associated with cisplatin-induced hearing loss, it is difficult to not address these concerns. Patients and parents of patients undergoing cisplatin therapy should be aware of these risk factors in order to have the choice to be genotyped for these genetic risk markers. At least patients identified to be at lower risk may have one less thing to worry about with chemotherapy treatment, while those patients at high risk can monitor their hearing more closely. Knowing the risk status for cisplatin-induced hearing loss may impact the patients' quality of life, such as the social-emotional development in children. Although challenging, pharmacogenomics has the potential to translate knowledge of genetic variability into better therapeutics and improve quality of life.

4.5 Conclusions

This thesis makes important contributions to the pharmacogenomics of cisplatin. This study adds important new evidence to understanding the mechanisms behind cisplatin-induced hearing loss

given that cisplatin biotransformation in the body is not well understood. Currently in the area of cisplatin-induced hearing loss, there is a lack of replication of genetic associations, a lack of prediction models to identify patients at risk of toxicity and an absence of guidelines for pharmacogenomic testing. We were able to replicate previous associations in a large independent cohort of pediatric patients. Furthermore, this study shows that it may be possible to classify individuals at risk of cisplatin-induced hearing loss based on a patients' genetic profile in combination with clinical risk factors. It also provides new hypotheses for the mechanisms underlying the susceptibility for cisplatin-induced hearing loss in patients treated with cisplatin. These results have the potential to influence modifications to treatment decisions for cisplatin therapy to improve safety and efficacy of medications, bringing personalized medicine one step closer to reach.

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