Pharmacogenomic Evaluation of

Platinum-Induced Ototoxicity

Joseph Rothstein

Department of Otolaryngology (Head & Neck Surgery)

McGill University, Montreal

January 2015

A thesis submitted to McGill University

in partial fulfillment of the requirements of the degree of Master of Science.

Table of Contents

Abstract

Cisplatin and Carboplatin, two highly effective platinum chemotherapeutic agents, are widely used to treat a variety of malignancies. While the advent of these medications has coincided with greater survival rates among pediatric cancer patients, platinum-chemotherapy also leads to ototoxicity in more than half of these survivors. This has serious consequences on the quality of life of thousands of children.

While there are several known risk factors for ototoxicity, substantial variability remains. Genetic variants are thought to account for this variability. A literature review of randomized-controlled trials in pediatric populations was undertaken to evaluate the involvement of genetic variants in platinum-induced ototoxicity.

Based on existing literature findings, a multi-center, randomized-controlled trial was conducted at two tertiary care centers, in a combined cohort of 100 pediatric cancer patients. The association of ototoxicity (pre-treatment compared to post-treatment) and ototoxicity-progression (post-treatment compared to follow-up) with SNPs (Single Nucleotide Polymorphisms) of XPC and LRP2 was evaluated. Polymorphisms in the XPC gene, specifically rs1350344, rs2607737, rs2733533, rs3731093, and rs3731149, were found to be correlated with ototoxicity ($P \leq 0.05$). The results also showed that two XPC SNPs, rs1350344 and rs2733533, were significantly associated with progression of ototoxicity. Both SNPs of LRP2 (with p-values exceeding 0.05) were not found to be significantly associated with hearing-loss or its progression. This is the first trial to examine XPC as a primary objective and the first to find a genetic link to progressive ototoxicity.

Future studies should focus on replicating existing pharmacogenomic findings in order to establish a clinically valuable genetic profile for susceptibility to platinum-induced ototoxicity.

Résumé

Le Cisplatine et le Carboplatine sont deux agents chimiothérapiques à base de platine hautement efficaces utilisés dans le traitement d'une grande variété de tumeurs malignes. Malgré un taux de survie augmenté chez les patients atteints de cancers pédiatriques depuis l'avènement de ces traitements, la chimiothérapie à base de platine est maintenant reconnue comme étant associée à une ototoxicité chez plus de la moitié de ces patients. Il s'agit d'un effet indésirable ayant des conséquences significatives sur la qualité de vie d'un grand nombre d'enfants.

Il existe plusieurs facteurs de risques d'ototoxicité reconnus mais une variabilité substantielle persiste. Cette dernière s'expliquerait par des variations génétiques. Une revue de la littérature d'études cliniques randomisées auprès de populations pédiatriques a été entreprise afin d'évaluer l'implication de ces variations génétiques dans l'ototoxicité induite par la platine.

En se basant sur des données préétablies associant des polymorphismes génétiques à l'ototoxicité, une étude clinique randomisée pluricentrique a été réalisée auprès d'une cohorte de 100 patients provenant de deux centres tertiaires. Les associations entre l'ototoxicité (prétraitement comparé au post-traitement) et sa progression (post traitement comparé au suivi) avec les SNPs des gènes XPC et LRP2 ont été évaluées séparément. Il fût mis en évidence que des polymorphismes du gène XPC, spécifiquement rs1350344, rs2607737, rs2733533, rs3731093, and rs3731149, sont associés à une ototoxicité ($P \leq 0.05$). Les résultats obtenus ont également démontré que deux SNPs du gène XPC, (soit rs1350344 et rs3731149), sont associés de façon significative à la progression de l'ototoxicité. Quant aux deux SNP du gène LRP2, aucune association significative ne fût établie avec la perte auditive et sa progression ($P > 0.05$). Il s'agit de la première étude qui examine XPC comme objectif primaire et la première à établir une association génétique avec l'ototoxicité progressive.

Afin d'établir un profile génétique cliniquement valide en lien avec la susceptibilité de l'ototoxicité induite par la platine, les études futures devront mettre l'emphase sur la réplication des trouvailles pharmacogénomiques.

List of Abbreviations

ABCC3 - ATP-binding cassette, sub-family C
ASHA - American Speech-Language-Hearing Association Criteria
BLB - Blood Labyrinth Barrier
CAP - Compound Action Potential
Carbo - Carboplatin
CBDA - Bidentate Dicarboxylate
Cis - Cisplatin
CM - Cochlear Microphonics
COMT - Catechol-O-methyltransferase
dB - Decibles
DPOAE - Distortion Product Otoacoustic Emissions
EDTA - Ethyleediamine Tetra-Acetic Acid
EP - Endocochlear Potential
ERCC1 - excision repair cross-complementation group 1,
GSTM3 - Glutathione S-transferase M3
GSTP1 - Glutathione S-transferase P1
GSTT1 - Glutathione S-transferase T1
HZ - Hertz
IHC - Inner Hair Cell
LRP2 - low density lipoprotein-related protein 2
MSHL - Minimal Sensorineural Hearing Loss
NADPH - Nicotinamide Adenine Dinucleotide Phosphate
NER - Nucleotide Excision Repair
NO - Nitric Oxide
NSCLC – Non-small cell lung cancer
OHC - Outer Hell Cell
PNET - Primitive Neuroectodermal Tumor
PTA - Pure Tone Average
ROS - Reactive Oxygen Species
SGN - Spiral Ganglion Neurons
SIADH - Syndrome of Inappropriate Secretion of Antidiuretic Hormone
SLFs - Spiral Ligament Fibrocytes
SNPs (Single Nucleotide Polymorphisms)
SPL - Sound Pressure Level
TCSs - Testicular Cancer Survivors
TEM - Transmission Electron Microscopy
TM - Tympanic Membrane
TPMT - Thiopurine methyltransferase or thiopurine S-methyltransferase
VOR - Vestibular Ocular Reflex
XPC - Xeroderma pigmentosum complementation group C

Acknowledgements

I would like to express my deep appreciation to my supervisor, Dr. Sam Daniel, for his mentorship throughout this process. His professional and caring nature, passion for research, and ferocious work ethic have been a constant inspiration to me. I am especially thankful to Dr. Carret, my co-supervisor, whose friendly and approachable demeanor, and devotion to her patients have fueled my passion for research and medicine. I would also like to thank Dr. Bernard Segal for his continued guidance throughout my studies and in preparation of this thesis.

I am grateful to the following individuals who have made significant contributions: Dr. Nader Emami who was in charge of recruitment at the Montreal Children's Hospital, Aren Bezdjian who helped with recruitment and data collection at CHU Sainte-Justine, and Amina Barhdadi who helped with genotyping and statistical analysis (manuscript 2). I would also like to recognize Martin Morris, and Naz Torabi for helping me with the literature review (manuscript 1).

I would like to thank my committee members, Dr. Bob Sweet (committee chair), and Dr. Marie-Pierre Dubé for their insight and constructive comments.

To Mario, Aren, Isabel, Nagi, Greg and the rest of the McGill Auditory Sciences Lab team, I have truly enjoyed meeting and working with you all. Your friendship and support have been invaluable throughout this process.

I am lucky to have worked with one the most positive and supportive teams in the research and oncology ward of CHU Sainte-Justine. I am especially thankful to Marie-Saint-Jacques, Marie-Aimée, Caroline, Justine and the oncology nursing team for welcoming me into their family with open arms.

Finally, I want to thank my family and friends for their infinite love and support, which have allowed me to overcome many obstacles.

I dedicate this thesis to my cousin, Simon Goldstein, whose generosity and warm heart inspired me to help others in need.

Chapter 1 – Introduction

1.1 Introduction

In 1975, just over 50% of children diagnosed with cancer before the age of 20 survived at least 5 years.[1] Childhood cancer 5-year survival rates have increased dramatically in the past 40 years to nearly 90%.[2] This increase can be attributed to earlier detection and improved treatment of childhood cancers. Two medications that came onto the scene in between 1960-1980 were cisplatin and carboplatin. These platinum-chemotherapeutic agents are widely used to treat a variety of soft-tissue neoplasms, including testicular, ovarian, bladder, cervical, head and neck, and non-small cell lung cancers.[3] While these medications are highly effective, their use is limited by their toxicity to various organs of the body. Nephrotoxicity, neurotoxicity and ototoxicity remain a serious concern. Ototoxicity, the focus of this thesis, is seen in over 60% of children treated with platinum-chemotherapy.[4] Platinum-compounds damage structures of the inner ear, such as the cochlea, the vestibulum and the stria vascularis. This results in bilateral, sensorineural hearing-loss, which can have devastating consequences on quality of life. It has been shown, that even a mild hearing-loss, early on in life, can lead to deficits in school performance, social interaction, and cognitive development.[5] There is currently no treatment for ototoxicity, which is why dose reduction and premature stopping of platinum-treatment is often seen.

To avoid reducing or prematurely stopping treatment, ototoxicity must be prevented all together. Not everyone who receives platinum-based chemotherapy develops hearing loss; there is a large degree of individual variability in the presence and severity of ototoxicity. It is crucial to identify susceptible patients. While there are several clinical risk factors for ototoxicity including age, gender, and dosage, variability remains in patients

matched for these variables. Single Nucleotide Polymorphisms (SNPs) of specific genes expressed in the inner ear are thought to account for this variability.[6] Several pharmacogenomics evaluations of platinum-induced ototoxicity have been published in the last decade identifying these genetic variants, and examining their association with hearing-loss.[72-89] To date, however, there is no concrete genetic profile that can be used to identify susceptible patients before treatment.

Megalin, an endocytic receptor, and XPC, an important component of the nucleotide excision repair (NER) pathway, are two proteins that have been implicated in the mechanism of ototoxicity. XPC's association with ototoxicity was a secondary finding in a singular study in 2009. Megalin has been evaluated in a few pediatric, randomized-controlled trials, but the results have been inconclusive.

With growing interest in methods of otoprotection using steroidal anti-inflammatories, including intratympanic dexamethasone, it is more important than ever to establish a method of identifying susceptible patients in order to guide appropriate intervention.

1.2 Thesis rational, objectives and organization

In view of the background previously outlined, this study aims to conduct a pharmacogenomics evaluation of platinum-induced ototoxicity, specifically examining the XPC and Megalin genes. To do so, a randomized-control trial including pediatric cancer patients receiving cisplatin and/or carboplatin was designed.

The overall objective of this thesis is to study the impact of genetic variants on the susceptibility to platinum-induced ototoxicity by investigating genes identified in the literature as being involved in ototoxicity.

The working hypothesis of this thesis is that patients with SNPs of the Megalin and/or XPC are more likely to develop ototoxicity than those without.

The thesis is divided into 6 chapters. Chapter 2 begins with a literature review of the anatomy and physiology of the ear, with a focus on the inner ear. The effects of platinum-compounds on structures of the inner ear are detailed. Platinum-chemotherapy is introduced, along with ototoxicity, its characteristics, mechanisms and risk factors. Chapter 3 includes a comprehensive literature review of the genes involved in platinum-induced hearing-loss. Chapter 4 outlines the methodology and results of our randomized-control trial. Chapter 5 briefly discusses designs of a novel otoprotective trial, and Chapter 6 concludes this thesis.

Chapter 2: Background and Literature review

2.1 Introduction

Hearing is one of the major senses and is essential to communication and understanding of one's surroundings. To understand hearing and its associated pathologies it is important to first examine the anatomy and physiology of the auditory system. Perception of sound requires normal function two systems: the peripheral auditory system (outer, middle and inner ear) (Figure 1) and the central auditory system (brain stem and auditory cortex). The sensory organ for hearing, the cochlea, is deep within the temporal bone of the inner ear. This organ transduces vibrations from outside sound sources into electrical nervous impulses. These signals are then passed along to the central auditory pathways of the brain where they are processed and where sound is perceived.[8]

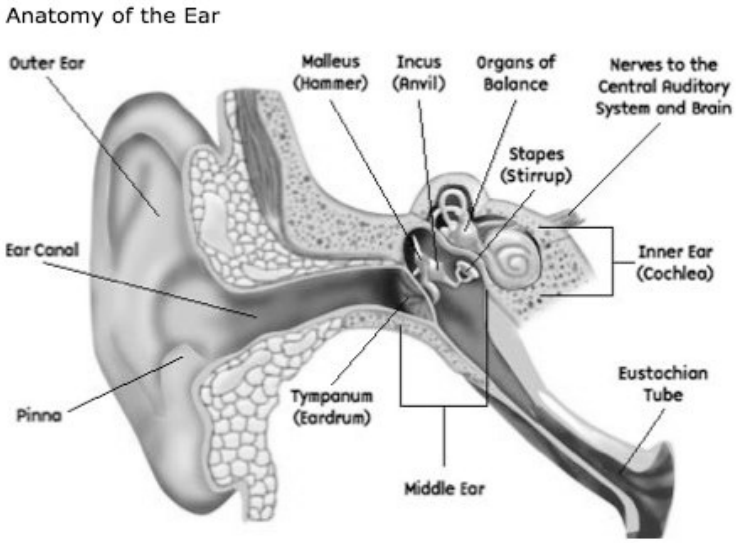


Figure 1. Diagram of the peripheral human ear, which displays the Pinna and Ear Canal, as well as the Middle and Inner ear. Adapted from Audiology Update 2010 with permission.[7]

Sound characteristics, such as frequency and intensity, determine what we perceive. Sound waves of different frequencies are processed and perceived differently by the brain. Lower frequency waves are generally in the range of audible hearing, while high frequencies waves are sometimes not perceived at all.[8]

When there is a problem with either the anatomy or physiology of the auditory pathway, a hearing-loss can occur. There are two major types of hearing loss: conductive and sensorineural. Conductive hearing loss results from a disruption in the mechanism where-by sound waves travel from the outer ear to the inner ear. The conductive pathway does not contain irreplaceable nerve ending and so it can responds to medical or surgical treatment. Sensorineural hearing-loss, on the other hand, usually results from hair cells loss in the organ of Corti, which cannot be repaired or replaced. This impairs the transduction of sound waves into electrical signals that travel to the brain, and is generally permanent.[9]

Platinum Chemotherapy has been shown to cause sensorineural hearing loss. Although, platinum compounds Cisplatin and Carboplatin have been extremely effective in treating a variety of cancers, they can lead to toxicity of different parts of the body, namely the ear. Platinum compounds damage structures of the inner ear in particular. This localized toxicity is called ototoxicity.

Ototoxicity affects over 60% of children treated with platinum-chemotherapy. Usually permanent, this side effect can have devastating consequences on children's quality of life.

Many studies have sought to elucidate the mechanism behind ototoxicity, evaluating the effect of platinum on structures of the inner ear. Reactive Oxygen Species (ROS) have been largely implicated in inner-ear damage.

Several risk factors have been reported for platinum-induced hearing-loss. Namely, genetic variants may be associated with increased susceptibility to ototoxicity, secondary to platinum treatment.[4]

2.2 Anatomy and Physiology of the Auditory System

2.2.1 The Outer Ear

The outer ear is comprised of the pinna, the inner and outer ear canal, and the tympanic membrane. The Pinnae protrude from either side of the skull and are the part of the ear that is plainly visible. They are made of cartilage and are covered by skin. The function of the pinna is to passively capture acoustic energy and to channel it into the ear canal. To this end, the pinna is angled so that it catches sound waves travelling from the front more than from behind. This is helpful in localizing the source of the waves. The ear canal, also known as the external auditory meatus, is approximately 4 centimeters long. It is made up of an outer and inner part. The outer portion is lined in skin with many hair follicles containing sweat glands, and oily sebaceous glands (which form ear wax). The hair and wax together serve as a protective barrier and a disinfectant. The inner, deeper canal is lined with a simpler, thinner layer of skin. It is a hard cavity, which absorbs little sound. Instead it functions to direct sound from the outer canal to the tympanic membrane (TM), whose skin is continuous with that of the canal. The TM separates the outer ear from the middle ear. It is the first structure of the sound transduction mechanism. The membrane is less than $1/10^{th}$ of a millimeter thick. It is a simple membrane covered by a thin layer of skin with a stiffening fibrous middle layer. The tympanic membrane spans 1 centimeter diameter round opening into the middle ear cavity. When sound waves reach the TM, it vibrates.

2.2.2 The Middle Ear

The middle ear is an open, air-filled cavity in the temporal bone. It is an extension of the respiratory air spaces of the nose and the sinuses, and is lined with a respiratory membrane. This cavity contains three ossicles connected to one another; they are the malleus, incus and stapes, but are more commonly known as the hammer, anvil and stirrup, respectively (Figure 2). These are the smallest bones in the body. These bones link the tympanic membrane (laterally) to the inner ear (medially), transmitting sound vibrations from one end to the other. The malleus is club-shaped with its handle attached to the tympanic membrane. Its head articulates with the incus. The incus is cone shaped, and has a right angle bend at its tip that is attached to the stapes. The Stapes is shaped with an arch and a foot-plate. The foot-plate of this bone articulates with and covers the oval window, which opens into the inner ear. These bones conduct vibrations from the tympanic membrane to the cochlea of the inner ear, amplifying them along the way.

10

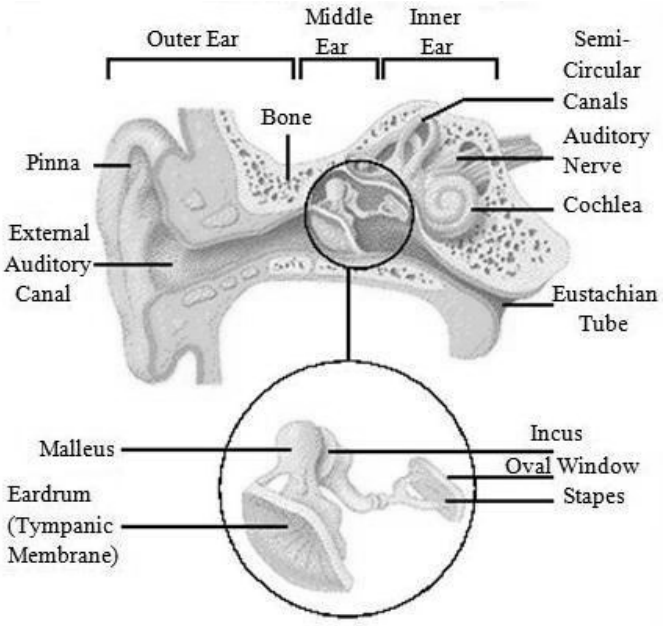


Figure 2: Diagram of the peripheral ear system, with enlarged view of middle ear. Notice the three ossicles in between the eardrum and oval window. The opening to the Eustachian tube can also be seen. Adapted from Hearing Health Clinics

The middle ear also contains the opening to the Eustachian tube. This tube runs from the pharynx to the anterior wall of the middle ear cavity, regulating the pressure within the middle ear.

The impedance of water is a few thousand times that of air. If air- waves were to travel directly to the oval window, untransformed, only 0.1% of sound energy would be transmitted to the cochlea. The middle ear serves as a transformer of sound waves so that they are effectively transferred from its air filled cavity to the fluid filled ducts of the inner ear. This mechanical transformation process is accomplished in three ways: an area ratio advantage from the eardrum to the oval window, a curved tympanic membrane (creating a buckling effect), and a lever action in the ossicular chain. The largest transformation effect comes from the area ratio advantage. In all, the middle ear provides a 46:1 pressure advantage.

2.2.3 The Inner Ear

Anatomy:

The inner ear in a complex system of fluid filled ducts in the temporal bone called he labyrinth. It consists of a bony outer osseous labyrinth and a membranous labyrinth found within this bony casing. The bony labyrinth is separated into three sections: the semicircular canals (superior lateral and posterior), the vestibule, and the cochlea. The cochlea contains the sensory organ for hearing. While the external ear and middle ear act as mechanical transformers and amplifiers of sound waves, the cochlea is the site of sound wave transduction into action potentials.[8,11]

The auditory part of the labyrinth, the cochlea, is shaped like a snail; it is encased in temporal bone and has two and a half turns. The largest turn is referred to as the basal turn. The smallest turn is referred to as the apical turn. The unwound human cochlea is approximately 34mm.[12] The winding channel running through these turns is subdivided into three compartments. The middle compartment is the *scala media*. It is filled with a fluid called endolymph. The other sections, the *scala vestibuli* (superior) and the *scala tympani (inferior),* are filled with a fluid called perilymph. The floor of the scala media is called the basical membrane, and the roof is called Reissner's membrane.

The highly specialized sensory receptor for hearing is the organ of Corti, which is found resting on the basilar membrane, and is covered by the tectorial membrane. This organ holds around 30,000 nerve receptors, which each have their own hair cell. These hair cells contain stereocilia that project from the top of the cell into the fluid-filled scale media. There are two types of hair cells: Inner hair cells (IHC) and outer hair cells (OHC). The IHCs are oriented in a single line closest to the bony core of the cochlea, and their cilia are freestanding (not contacting any other structure). These cells make up 90-95% of the connection to auditory nerve fibers that carry information to the brain. There are many more OHCs in comparison, which are organized in three rows. Unlike the IHC, the cilia of the OHCs are embedded in the gelatinous tectorial membrane.[13] (see figure 3)

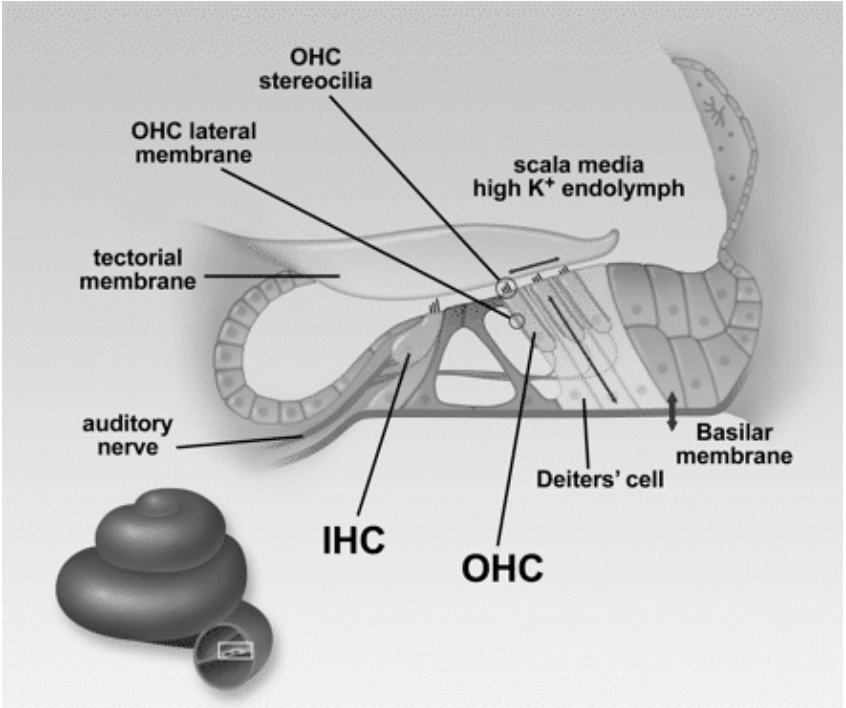


Figure 3: Organization of the mammalian organ of Corti. This schematic shows the organization of hair cells on the basilar membrane. Abbreviations: IHC – Inner Hair Cell, OHC – Outer Hair Cell. Adapted from Ashmore 2008[12]

It is important that the chemical environment of the scala media be maintained. This is accomplished by the stria vascularis, a network of capillaries attached to the spiral ligament that covers the outer wall of the scala media. This network is made up of three types of cells: marginal cells, intermediate cells and basal cells. Marginal cells play a large role in generating the endocochlear potential of the scala media.

Function:

When the stapes footplate of the middle ear vibrates it generates a wave within the cochlear fluids via the oval window. This wave displaces the entire scale media, but we will focus on the displacement of the basilar membrane. This displacement increases as it goes

from the base to the apex. The basilar membrane displaces at different locations in response to different frequencies. In this way, it is able to act as a frequency analyzer. The stiffness of the membrane is greatest at the base and decreases toward the apex. Thus, low frequency sounds cause the largest displacement at the more giving apex. High frequency waves cause the greatest displacement at the base of the basilar membrane.[9,11]

The IHCs and OHCs serve as transducers of mechanical stimuli into electrical signals. When the fluid with basilar membrane within the scala media are displaces, there is a shearing force applied to the cilia of the hair cells. This shearing force bends the stereocilia, opening a pore on the cilia, which allows ions to flow into the cell, depolarizing it from apex to the base of the hair cell. This depolarization gives rise to electrical potentials or receptor potentials (produced in response to a stimulus). At their basal pole, hair cells synapse with dendrites from spiral ganglion neurons (SGNs). They release neurotransmitters when activated. The signal is then carried to these neurons, which eventually send it to the auditory cortex, where it is processed and perceived as sound.[9] [9]

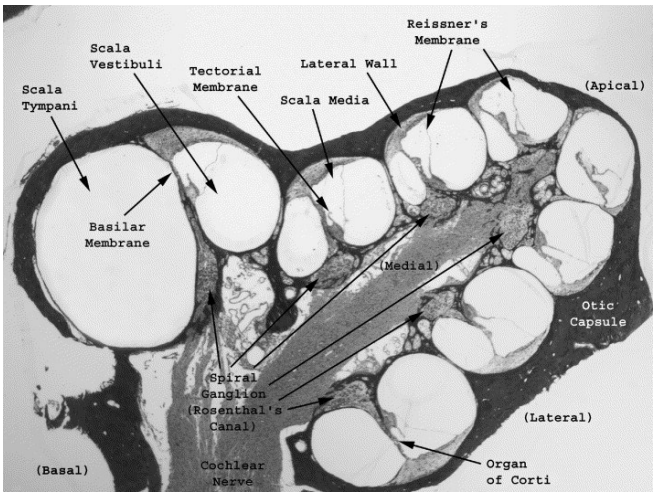


Figure 4: Cross Section of the mole rat cochlea. Adapted from Raphael et al. 2003 with permission.[14]

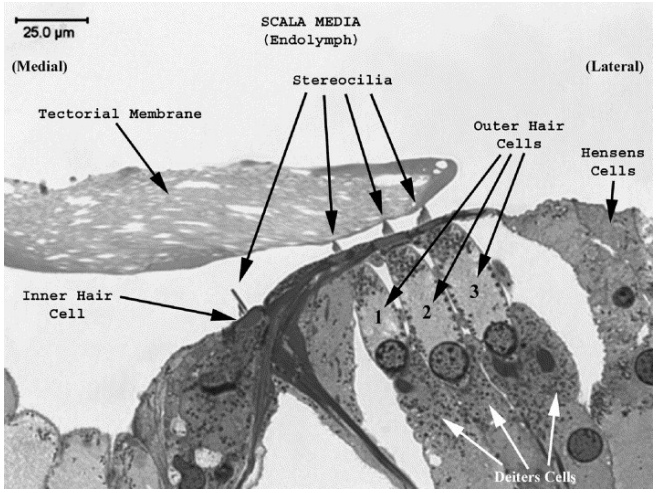


Figure 5: Cross section of the mole rat cochlea. The organ of Corti is shown. Adapted from Raphael et al. 2003 with permission.[14]

2.2.4 The Vestibular Apparatus

The sensory receptor organs of the vestibular system, the system for balance, are found in the bony labyrinth. These organs are the utricle and saccule (located within the vestibule) and the semicircular canals. These endorgans are found posterior to the cochlea. There are three semicircular canals per ear, which are able to respond to angular acceleration such as turning of the head. Each canal joins the vestibule at the ampulla, its anterior widening. The ampulla contains a receptor organ called the crista, which contains vestibular hair cells and supporting cells at its base. Hair cells of the vestibular system function quite similarly to those of the auditory system. Their steriocilia extend into the top portion of the crista called the cupula. Mechanical shear force is transduced into electrical signals at their apex and neurotransmitters are released at the basal pole, which synapses with vestibular neurons (part of the eighth cranial nerve).[15,16]

2.3 Properties of Sound

2.3.1 Introduction

Sound is produced from the vibrations of objects, which, in turn, vibrates our hearing apparatus (eardrum, ossicles, etc.) in order for us to receive and interpret the sound. Sounds such as speech, music, and environmental noises are complex; they vibrate in complicated recurring patterns. These complex patterns can be broken down and analyzed according to frequency and intensity and phase.

2.3.2 Frequency, Intensity, Phase

Frequency is determined by the number or recurring oscillations (cycles) in 1 second. A frequency of 1 cycle per second is referred to as a hertz (Hz) after the German physicist Heinrich Hertz (who's work led to the development of the radio). Every living creature has a different range of frequencies in which they can perceive sound; for young humans that range is between 20-2000 Hz. The frequencies most important for speech are found between 250-8000Hz.

Sound intensity is measured using the decibel (dB) scale. Intensity is measured against a threshold sound pressure level (SPL). Each order of magnitude increase in sound pressure is a 20dB increase in intensity. Human hearing optimally functions at sound levels between 0 and 80dB SPL. Table 1 shows decibel level of common sounds.

Table 1: Decibel (HL) Levels of a Few Common Sounds

Description	Decibels (dB HL)	Sound Source
Pain	140	Shotgun blast
Discomfort	130	Jet taking off
	120	Loud music
	100	Lawnmower
	80	Cocktail party
Conversational	60	
	30	Inside library
Whisper (5 feet)	20	
	10	
Threshold of hearing	0	
Source: Data based on Bess & McConnell (1981)		

Adapted from Hearing and Deafness – An Introduction for Health and Education Professionals[17]

The phase of a pure tone is the area or point of its progression in a cycle. If two pure tone waveforms have the same starting phase and frequency, they are considered "in phase". When a listener hears 2 puretones that have the same phase, they will be heard as being louder than its constituent waves. When waveforms are out of phase, the result is a deconstructive combination in which the sound is heard as being quieter its constituents.[17]

2.3.3 Conductive and Sensorineural hearing loss

Conductive hearing loss is caused by abnormalities or malfunctions of the outer and middle ear. These include absence or structural abnormalities of the pinna or external auditory meutus, obstruction of the meutus, and tympanic membrane or ossicle restriction (e.g. otisis media). Conductive hearing loss is, for the most part, medically treatable.

Sensorineural hearing loss (see figure 6) originates in the inner ear. It is the result of damage to the cochlea, the auditory nerve, or a combination of both. Generally, the sensory

unit (audiotry nerve fiber and hair cell) is damaged. Sensorineural hearing loss is generally permanent.[17]

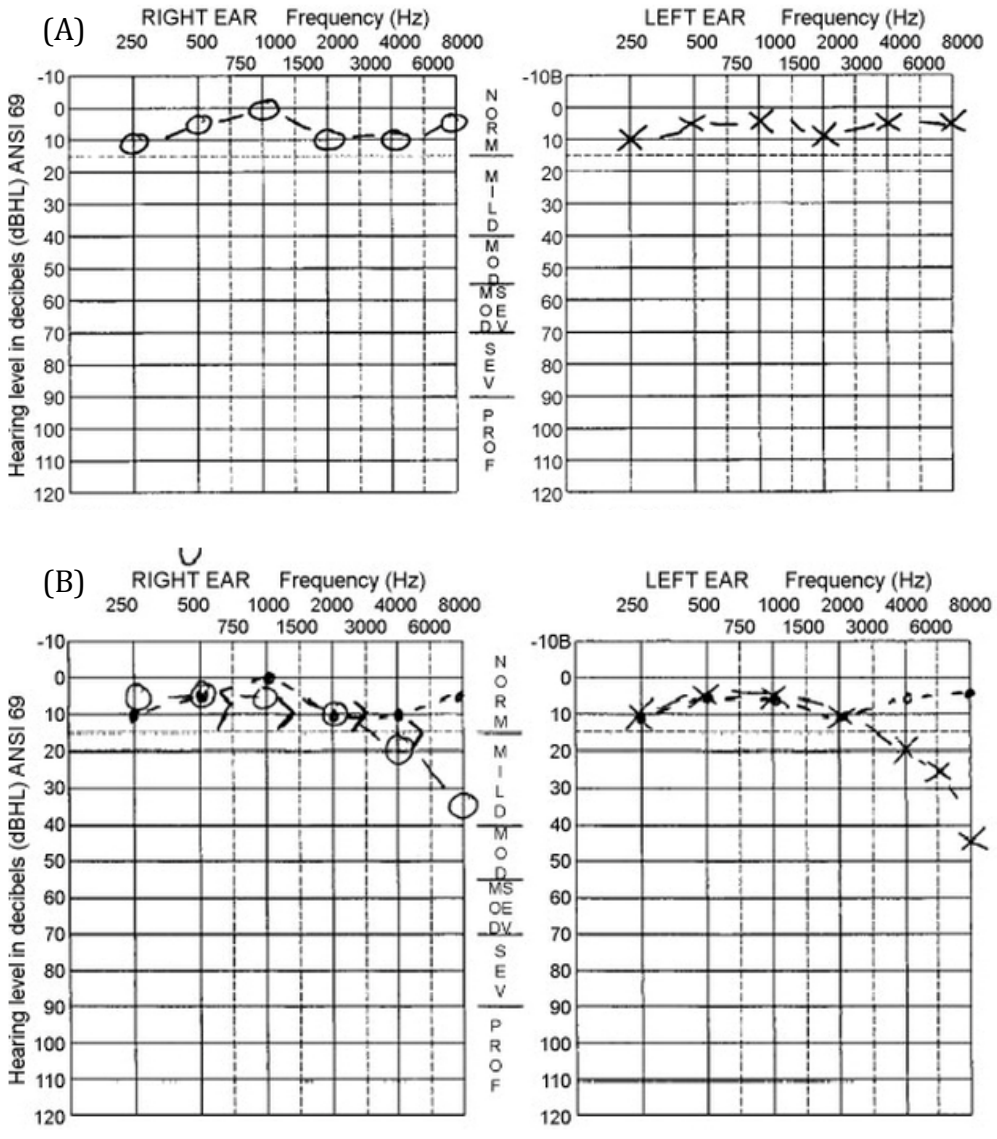


Figure 6: Case report audiogram of pediatric patient treated with cisplatin showing (A) baseline audiogram with hearing threasholds on pure tone audiometry within normal limits bilaterally, and (B) audiogram performed after initiation of cisplatin therapy showing bilateral mild high-frequency sensorineural hearing loss. Adapted from Truong 2007 with permission.[27]

2.3.4 Audiologic Descriptions of Hearing Acuity

Audiograms indicate an individual's hearing threshold across a range of frequencies between 250-8000Hz. The pure tone average (PTA) is reflective of an audiograms overall results, and is designed to char hearing sensitivity from 0-110dB. Hearing loss can be grouped into five categories: slight, mild, moderate, sever, and profound (see table 2).

Table 2: Categories of Hearing Loss

Degree of Hearing Loss in dB	**Description**
0 to 26	Normal
27-40	Slight
41-54	Mild
55-69	Moderate
70-89	Severe
>90	Profound
Source: Adapted from Paul & Whitelaw 2011[17]	

2.4 Effects of Platinum on Structures of the Inner Ear

2.4.1 Introduction

Platinum-based chemotherapy is known to cause sensorineural hearing loss. Platinum compounds can enter tumor cells through passive diffusion or active transport.[18] Healthy cells can also take up these compounds by the same mechanisms. Animal Model Research has shown that cisplatin, a platinum compound and commonly used

chemotherapeutic agent, targets three sites in the inner ear: the organ of Corti, the stria vascularis, and the spiral ganglion neurons (SGNs).

Through a variety of postulated but unconfirmed mechanisms, accumulation of platinum disrupts the morphology of these structures leading to hearing-loss. This is a "sensorineural hearing-loss", because it impairs sound transduction from the sensory receptor to the auditory nerve. The conductive pathway of the outer and middle ear remains unaffected. The phenomenon of platinum-induced hearing loss is termed "ototoxicity" (toxicity of the ear). This section will examine the effects of cisplatin and carboplatin (commonly used platinum-compounds) on individual structures of the inner ear.

2.4.2 Organ of Corti

The organ of Corti is the sensory receptor for hearing, and so any damage to it results in hearing impairment. Various animal studies, have shown that Cisplatin administration often results in loss of outer hair cells (OHC) followed by loss of inner hair cells (IHC), protrusion of supporting cells into Nuel's space and the tunnel of Corti, and damage to the organ's microarchitecture.[19,20,21,22,23] These platinum-induced changed have been shown to be dose-dependent. As the dose of administered cisplatin is increased the damage progresses from affecting the first row of OHCs in the basal cochlear turn to affecting more apical parts of the cochlear turn and IHC's.[24,25] As well, a more prolonged particular dosage correlates with the same progression. The OHC's, which are initially affected, are involved in the perception of high-frequency sound waves. As the damage progresses to the IHC's and more apical areas, low-frequency perception is affected, which is where the speech range is found. This platinum-induced progression has also been shown to correspond with

electrophysiological changes. Permanent frequency–dependent elevation of compound action potential (CAP) thresholds, as well as irreversible suppression of cochlear microphonics (CM) and distortion product otoacoustic emissions (DPOAEs), have been reported in the literature.[20,21] There have been some reports of recovery of OHC's and auditory function. It is unclear whether or not this can be attributed to generation of new hair cells or to repair of damaged ones.[26] These results were almost exclusively found, however, in animal studies. There has yet to be substantial hair-cell recovery in human studies.[27]

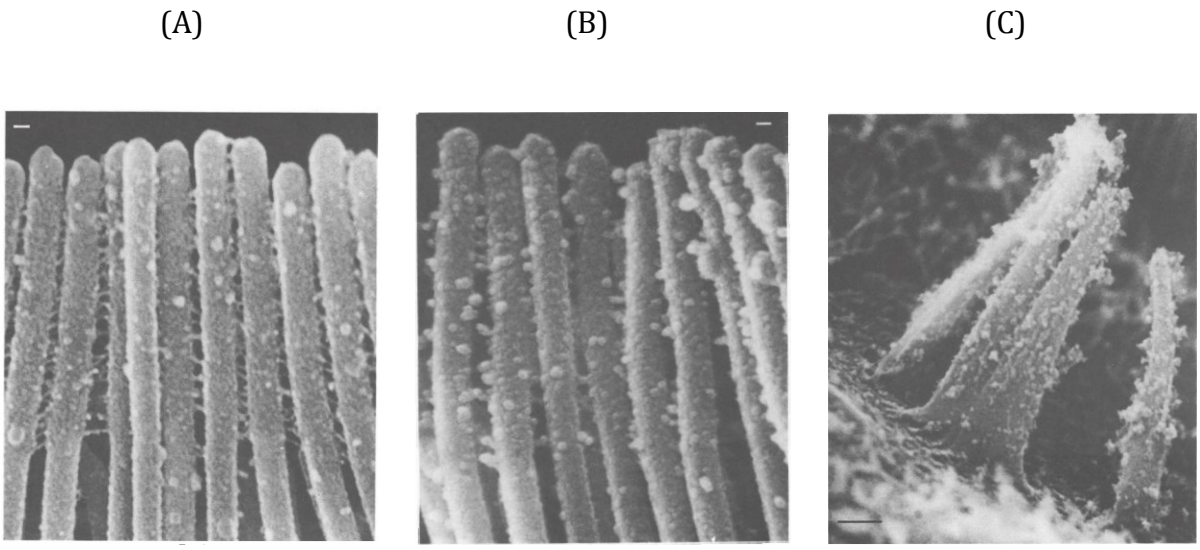


Figure 7: (A) - the tallest row of stereocilia from an OHC of a control guinea pig cochlea. Their surface membranes are rough along their upper halves and the stereocilia are connected by cross-links at the level of this roughness. (B) – steoeocilia from an OHC of a guinea pig cochlea treated with cisplatin. The membranes are rough throughout their lengths and there is marked reduction of cross-links. (C) – A hair bundle of an OHC of a cisplatin-treated cochlea. There is a reduction of the number of stereocilia. Those that remain are fused and rough all along their lengths. Adapted from Comis 1986 with permission.[25]

2.4.3 Stria Vascularis

Similar to the Organ of Corti, damage to the stria vascularis is dose and duration dependent.[20,23,24]Of the three types of strial cells, the marginal cells are most sensitive to the effects of cisplatin.[28] Effects range from normal strial morphology to cystic degeneration with protrusions into the endolymphatic space, followed by cell death. Changes in strial volume, swelling and blebbing of marginal cells and vacuolation of their cytoplasm, intermediate cell atrophy and collapse of Reisner's membrane are also commonly reported effects. In a 2009 study, Kohn et al. used light and electron microscopy studies to find that damage to strial cells was uniformly distributed. Damaged cells, appearing in translucent areas, would often appear next to normal strial cells.[29]

In a semi-quantitative analysis of the effects of cisplatin on the rat stria vascularis, Meech et al. used transmission electron microscopy (TEM), to further explore these translucent areas. It was found that their translucency was a result of damage or depleted organelles. In particular, most of the marginal cell nuclei appeared damaged. As well, it was noted that there was a disappearance of marginal cell processes, presence of autophagic vacuoles, deformed organelles, dense and shrunken intermediate cells, and vacuolization and rupture of strial cells. Meech et al. proceeded to use higher TEM to take a closer look and the highly effected marginal cells of the stria vascularis. Even the cells that seemed unaffected under lower magnification, showed changes in cellular architecture.[28] In addition to depletion of organelles in marginal cells, intracellular strial edema has been reported secondary to cisplatin administration.

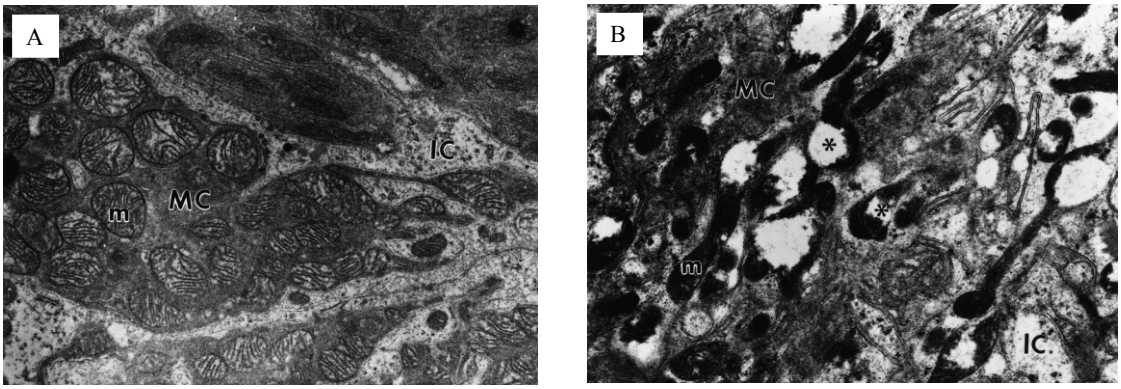


Figure 8: A - TEM micrograph of the stria vascularis of a normal control showing normal characteristics, B – TEM micrograph of a cisplatin treated stria vasularis showing several degenerating marginal cells (MC) and reduced mitochondria (m). Adapted from Meech 1998 with permission.[28]

The Stria Vascularis is responsible for the endocochlear potential (EP), the positive voltage seen in the edolymphatic space of the cochlea. Strial damage, therefore, affects the EP, decreasing it shortly after cisplatin administration.[28,21,23] Some groups have found that the decrease in EP following cisplatin administration is transient. This suggests that the damaging effects on the stria secondary to cisplatin may be reversible. These findings are inconsistent, however. Another reported effect, secondary to cisplatin administration, is endolymphatic hydrops, which is caused as a result of strial damage.[21,30]

Many groups have sought to explore whether or not damage to the cochlea, stria vascularis, and spiral ganglion neurons occur simultaneously. While there have been reports of a correlation between marginal cell damage, hair cell loss, and hearing loss, some groups have reported significant damage to the stria vascularis without OHC loss or spiral ganglion cell damage.[28,30]

2.4.4 Spiral Ganglion Neurons

The spiral ganglion is a collection of cell bodies of the auditory nerve, which carries sensory information from the cochlea to the brain. Cisplatin is known to damage peripheral nerves, and indeed also adversely affects the auditory nerve at the level of spiral ganglion neurons (SGNs).[20,21,31] It has been shown that mitochondrial swelling in these cells leads to vacuolation of the cytoplasm (see figure 9).[30] Other observed effects are cell and nuclear shrinkage, detachment of the myelin sheath, and neuronal death.[21,22] Following cisplatin administration, Alam et al. observed an increase in the bax/bcl-2 ratio in SGNs, which is a player in apoptosis regulatory pathways. This would suggest a possible mechanism for upregulation of apoptosis in SGNs secondary to cisplatin.[32]

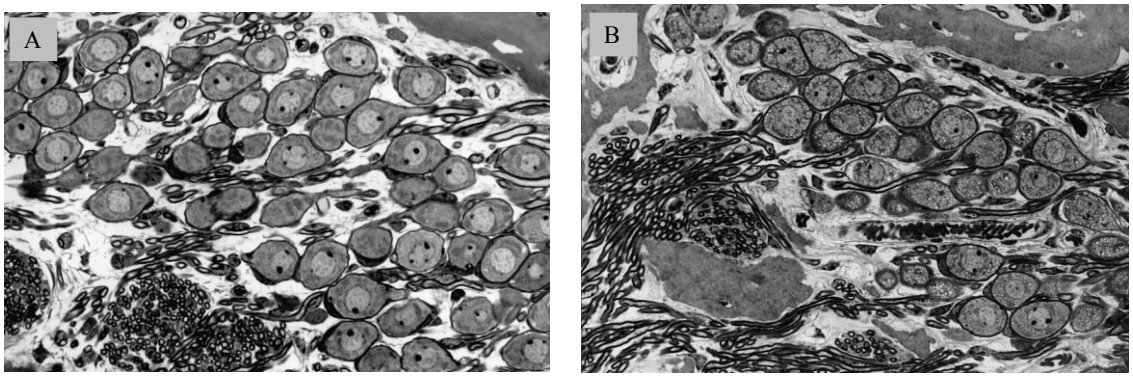


Figure 9: (A) - Light micrographs of the spiral ganglia in the middle turn of the cochlea in a normal non-treated animal, (B) - Light micrographs of the spiral ganglia in the middle turn of the cochlea of a cisplatin-treated animal. Abundant vacuolation of the cytoplasm can be seen in the neurons. Adapted from Cardinal 2000 with permission.[30]

2.4.5 Vestibular Tissues

Far fewer studies have examined vestibular toxicity as a result of cisplatin administration. Sergi et al. examined both vestibular and cochlear cisplatin toxicity in 12

albino guinea pigs, evaluating the change in vestibular ocular reflex (VOR), as well as morphological changes in sensorineural epithelium. They reported vestibular functional impairment, commencing after the third day, as well as slight hair cell loss of the cristae ampullares and maculae. They reported however, that evidence of toxic effect in the organ of Corti was more pronounced than that in the vestibular epithelium. This suggests that vestibular tissues are more resistant to platinum-toxicity than structures of the auditory system.[33]

In a 2008 study Kim HJ et al. performed a study to evaluate the role of inflammation-related events in cisplatin-mediated vestibular dysfunction. They found that increased production of pro-inflammatory cytokines following cisplatin administration caused significant vestibular utricular epithelial cell death.[34]

2.5 Platinum-Chemotherapy

2.5.1 Introduction

Cisplatin was discovered in the 1960's in Dr. Barnet Rosenberg's laboratory while studying the growth of Escherichia coli.[35] Initially known for its antibacterial effects, cisplatin is now known for its antineoplastic efficacy.[36] The US Food and Drug administration approved its clinical use in 1978, and cisplatin quickly became one of the most widely used chemotherapy drugs in North America and Europe. Carboplatin, a cisplatin derivative, was developed in the 1980s (approved in 1989), and has found its way into a variety of cancer treatment protocols. Both drugs are widely used against a number of malignancies including cancers of the bone, connective tissue and muscles, brain and nervous tissues, head and neck, lungs, bladder, kidneys, adrenal glands, lymph tissue,

ovaries, prostate, and liver. While both are extremely effective, their use is dose-limited by their toxicity.[4]

2.5.2 Cisplatin and Carboplatin

Advancements in the treatment and diagnosis of childhood cancers have coincided with an increased survival rate that has reached close to 80% in pediatric cancer patients.[37] The use of cisplatin and carboplatin has contributed to this phenomenon. Cisplatin's molecular structure consists of a central platinum atom attached to two chlorine atoms and two ammonia groups in a cis configuration (see figure 10). The platinum core has fixed bond angles, such that DNA must bend to fit the fixed structure of the drug. Other platinum compounds also have platinum at their core, but possess different leaving groups.[36] Carboplatin, for instance, has a bidentate dicarboxylate (CBDA) leaving group. This difference in molecular structure in believed to make carboplatin less toxic than cisplatin. Seeing as it is just as effective in the treatment of certain malignancies, such as ovarian and lung cancer, and other cancers that are resistant to cisplatin, this difference in toxicity should be taken into consideration.[38] Oxaliplatin, a second-generation cisplatin derivative, often used in the treatment of colorectal cancer, is taken up by the cochlea at a lower rate than previously mentioned derivatives. It is generally not ototoxic and will not be discussed further.[39]

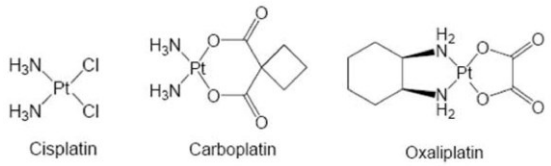


Figure 10: Two dimensional structures of cisplatin, carboplatin and oxaliplatin. Cis- and carboplatin show high degrees of cross-resistance, unlike oxaliplatin. Adapted from Eckstein 2011[40]

Platinum chemotherapy involves intravenous administration of platinum compounds dissolved in saline. These compounds will remain neutral or biologically inactive in the bloodstream due to the high concentration of chloride. Tumor cells, like all cells, have a low intracellular chloride concentration (approximately $1/13^{th}$ the concentration of extracellular fluid). Once the compounds enter tumor cells (through passive diffusion or active transport) they are activated by an aquation reaction, where two chloride leaving-groups are exchanged for water or hydroxyl ligands.[39]

The primary antineoplastic action of platinum compounds in tumor cells is believed to arise from inhibition of DNA synthesis. This is accomplished through binding of active compounds to DNA. Each active compound is able to bind to two sites in DNA. If the two sites are on the same DNA strand, a DNA adduct is formed (this occurs over 90% of the time). If the sites are on different strands a DNA cross-link is formed (this occurs less than 2% of the time). It has been reported that cisplatin can bind to all DNA bases. It does have, however, a specific affinity for the N-7 position of adenine and guanine due to these purine bases' high nucleophilicity at these sites (see Figure X). Since the platinum core of these compounds is fixed, the DNA will kink in order to fit the molecule. This kinking leads to the activation of DNA repair mechanisms. When the DNA is not properly repaired, as in the case with platinum-bound DNA, downstream apoptotic cascades are triggered leading to cell death.[38]

Platinum compounds have also been shown to inhibit RNA and protein synthesis. This inhibition requires a much larger dose, however, compared to those necessary to activate DNA synthesis inhibition. Carboplatin and cisplatin have similar mechanisms of action once active in the tumor cell. Carboplatin, however, requires esterase activity to

become active, replacing its cyclobutane moiety. It will also take longer to form DNA adducts.[36,38]

2.5.3 Toxicity of Platinum Compounds

While platinum chemotherapy is extremely effective, it can also lead to a number of dose-limiting side effects. Platinum compounds do not only target tumor cells. They can also be toxic to normal cells. This attack on normal cells often leads to a number of pathophysiological changes including nephrotoxicity, hypomagnesaemia and hypocalcaemia, gastrointestinal toxicity and myelosuppression, neurotoxicity, syndrome of inappropriate secretion of antidiuretic hormone (SIADH), and ototoxicity. Carboplatin remains less neurotoxic, emetogenic, nephrotoxic, and ototoxic than cisplatin at low doses. It still results, however, in other toxicities such as myelosuppression.[38,41] Cisplatin is reported as the most ototoxic of the platinum compounds.[42]

For the most part, these toxicities are dose and duration dependent. As well, they may reverse after the completion of treatment. Some, such as nephrotoxicity can even be prevented or treated (using hyperhydration and diuretics). Ototoxicity and Neurotoxicity remain, however, dose-limiting side effects of platinum-based chemotherapy. Ototoxicity, in particular, has been shown to progress post-chemotherapy, and in some cases to only develop post-chemotherapy. It remains an important concern in the treatment of cancer patients, and will be examined more closely in the following section.[36]

2.6 Ototoxicity

2.6.1 Characteristics

Toxicity of the inner ear can be caused by relative high doses of a variety of drugs, including: aminoglycosides (ex: gentamicin, neomycin), some diuretics, quinine, platinum-based drugs, and aspirin. Ototoxicity, caused by platinum-chemotherapy (cisplatin and carboplatin), presents as permanent and bilateral, symmetrical, sensorineural hearing-loss, which may be accompanied by vertigo and/or tinnitus.[3,4] It can manifest in both children and adults, with children being more susceptible. The incidence of ototoxicity in pediatric patients is varies greatly from 13% to 96%, largely due to differences in protocols and cumulative doses.[4] Initially, hearing at high frequencies (4-8 kHz) is affected. With increasing time and cumulative dose, ototoxicity progresses to affect the lower frequencies (1-2 kHz), which are essential for speech recognition. While ototoxicity generally presents hours or days after administration of platinum-chemotherapy, there have been reports of more delayed hearing-loss manifestations. Ototoxicity can be progressive even after treatment. This might be explained by the prolonged retention of platinum in the body even up to 20 years after administration.[43] A pilot study by our group in 2010 evaluated 21 patients long-term. The results showed that 33% of patients had progressive hearing-loss post chemotherapy. One patient, who initially did not present with ototoxicity, developed this side effect only after treatment.[44] In a recent retrospective chart review of 204 patients, our group showed that 48% (97/204) had progressive hearing loss.[45] Ototoxicity rarely recovers post-chemotherapy.[43]

Although some small improvements have been observed in patients who are followed long-term[46], platinum-induced hearing loss is effectively permanent.[47] There are no treatment or prevention methods established to date.

2.6.2 Effect on quality of life

The development of platinum-chemotherapy as a treatment for patients with malignant tumors has definitely been a positive in medical history, improving poor survival rates in cancer patients.[2] Given the large percentage of patients who suffer from hearing-loss as a result, however, it is important to consider the consequences of hearing-loss on quality of life. Unfortunately, in the case of children, sensorineural hearing-loss at a young age has exponentially greater effects than those seen in adults. As a child, the majority of learning occurs while playing and in other social interaction. Hearing-loss in these settings might go unnoticed, but will lead to serious future consequences.[5]

Speech acquisition is initially affected.[48] Ototoxicity affects higher frequencies first, which children depend upon for the formation of "fricatives" (phonetic letters s, t, and z). These phonemes constitute almost 50% of the consonant sounds in the English Language. Hearing loss at frequencies greater than 2000 Hz has been shown to slow this acquisition.[49] In a 2010 study by Judith et al., it was determined that children with unilateral hearing loss (UHL) demonstrated worse oral language scores than did their siblings with normal hearing.[50]

Even a subtle hearing deficit can go beyond speech acquisition, affecting global childhood development such as behavior, educational attainment, and overall quality of life. In a cross sectional study of 1218 children, minimal sensorineural hearing loss (MSHL) was

examined in conjunction with school behavior and performance. Those with MSHL showed greater dysfunction in behavior, energy, stress, social support, and self-esteem, and had lower scores than normal hearing children on a series of educational test measures.[51] In another study, which included 137 childhood survivors of neuroblastoma, it was reported that those with hearing loss had twice the risk of developing problems with reading, math, and attention. They also had a higher risk of general learning disability and/or special educational needs. These children also had a 10-point lower mean score on the school-functional scale of the Pediatric Quality of Life Inventory 4.0.[52]

Given the devastating effects of hearing-loss on children treated at such a young age, families and physicians are often faced with the choice of discontinuing platinum-chemotherapy to preserve residual hearing versus continuing the full course of treatment to maximize chances of survival. In order to avoid this dilemma, it is imperative to find methods of oto-protection.

2.6.3 Mechanism

If there is any hope of preventing ototoxicity, it is important to understand the mechanism behind this phenomenon. As mentioned previously, ototoxicity is caused by cochlear hair cell loss due to accumulation of cisplatin in the inner ear. Several pathways are implicated in cisplatin-induced ototoxicity including generation of toxic levels of reactive oxygen species (ROS), antioxidant depletion, lipid and protein damage, as well as DNA damage.[4,43,53, 54]

ROS are normally generated in cells as a product of cellular metabolism.[55] Ciplatin administration, however, has been shown to lead to overproduction of ROS (including

superoxide anions) in the organ of corti, lateral wall (stria vascularis, spiral ligament) and spiral ganglion cells. This can inhibit antioxidant protective molecules such as glutathiones and antioxidant enzymes. With decreased anti-oxidant action, concentrations of malondialdehyde and toxic lipid peroxidases and aldehydes (4 hydroxynonenal and peroxynitrite) are increased. This is turn, increases calcium influx into cochlear cells triggering apoptosis and cell death.

Superoxide radicals are sometimes produced by NOX-3, an isoform of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (see figure 11). This enzyme is specifically found in the cochlea. NOX-3 has been shown to be up-regulated secondary to cisplatin administration in the rat. With increased superoxide activation more hydrogen peroxide is formed, which can be catalyzed by iron to form an extremely reactive hydroxyl free radical. This radical can react with membrane lipids to form 4-hydroxynonenal, a very toxic aldehyde. Superoxide anions have also been shown to react with nitric oxide (NO) to form peroxynitrite, which damages cellular proteins, forming nitrotyrosine.[56,57] Superoxide radicals produced by NOX-3 may also cause the pro-apoptotic protein Bax to migrate to the cytosol, leading to the activation of caspases 9 and 3 and eventual apoptosis.[58]

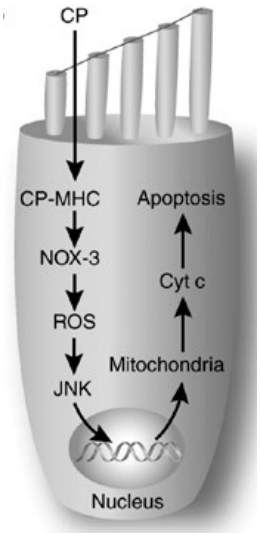


Figure 11: Potential mechanisms of outer hair cell death due to cisplatin administration. Cisplatin enters the cell, eventually activating NOX-3, which may activate JNK, which can then translocate into the nucleus of the hair cell to activate genes involves in cellular apoptosis. The subsequent release of cytochrome c from the mitochondria can trigger caspace-mediated apoptosis. Abbreviation: CP – cisplatin, CP-MHC – cisplatin monohydrate complex, NOX-3 – NADPH oxidase 3, ROS – reactive oxygen species, JNK – Jun N-terminal kinase, Cyt c – cytochrome C. Adapted from Rybak 2007 with permission.[59]

Cisplatin also causes degeneration of the stria vascularis in the cochlea. It has been shown to decrease the number of marginal and intermediate cells. As well, it can deplete spiral ganglion cells. The sensory cells for hearing, found within the inner ear, are within the blood-labyrinth barrier (BLB), meaning that cisplatin must be trafficked across this barrier in order to exact its deleterious effect. An increase in cellular permeability (decoupling of tight junctions), or a breakdown in cellular integrity in the BLB consequently decreases the endolymphatic potential crucial to normal hearing. Platinum compounds are normally blocked from crossing the BLB, but can be detected in cochlear tissues following platinum-administration. The mechanism behind their trafficking is still not fully elucidated. [60]

2.6.4 Risk Factors

Although studies have examined the possible mechanisms of ototoxicity, it is still impossible to determine who will develop hearing-loss. Without being able to identify susceptible patients, the development and implementation of otoprotection will be in vain. Platinum-induced hearing loss remains extremely variable, ranging from 0-96%. There are several known predictors for hearing-loss, reported in the literature. Cumulative dose seems to be the best predictor to date. Cumulative cisplatin doses exceeding 400mg/m^2, as well as carboplatin administered in high, myeloablative doses, have been shown to increase the risk of ototoxicity.[61,62,63] Age at exposure is another predictor. Children ≤5 years old are more likely to develop a moderate to severe hearing loss compare to older children and adolescents receiving the same cumulative dose. This might be due to the immaturity of cochlear cells or the age-related pharmacokinetics of platinum compounds.[64]

Other risk factors for platinum-induced ototoxicity include excessive noise exposure, preexisting hearing-loss, renal insufficiency, anemia, depleted nutritional state, hypoalbuminemia, irradiation of the brain or skull base, and concurrent administration of other ototoxic medications such as vincristine.[65,66,67] While these clinical risk factors are important predictors of ototoxicity,[68] large differences remain between patients matched for these variables. Genetic variants are postulated to account for these differences.[4]

Chapter 3 - Manuscript 1

Pharmacogenomic Literature Review of Platinum-Induced Ototoxicity

Joseph Rothstein, and Sam J. Daniel

Department of Otolaryngology (Head & Neck Surgery)

McGill University, Montreal

Manuscript to be submitted in

May 2015

3.0 **Abstract**

Cisplatin and Carboplatin, two highly effective platinum chemotherapeutic agents, are widely used to treat a variety of malignancies. While the advent of these medications has led to greater survival rates among pediatric cancer patients, platinum-chemotherapy also leads to ototoxicity in more than half of these survivors. This has serious consequences on the quality of life of thousands of children.

While there are several known risk factors for ototoxicity, substantial variability remains. Genetic variants are thought to account for this variability. A literature review of randomized-controlled trials in pediatric populations was undertaken to evaluate the involvement of genetic variants in platinum-induced ototoxicity. Ten articles were chosen as being relevant in this review. Based on current literature, it was concluded that there is not enough evidence to support standard genetic screening of patients to identify those who might be susceptible to platinum-induced ototoxicity. Further study is required in order to validate previous findings.

Keywords: cisplatin; carboplatin; late effects of cancer treatment; ototoxicity; pediatric oncology; pharmacogenomics; platinum.

3.1 Introduction

Platinum compounds Cisplatin and Carboplatin are two of the most widely used and successful chemotherapy drugs available. Their use is limited, however, by their secondary ototoxic effect. It is thought that certain specific genetic variants predispose patients treated with platinum-based chemotherapy to ototoxicity. This literature review focuses on recent clinical studies that have examined the correlation of specific genetic variants with

platinum-induced ototoxicity. COMT, ABCC3, TPMT, LRP2, XPC, GSTs, and most recently Otos, are the genes that have been identified to date. The potential mechanism involving each of these variants is discussed. The literature remains controversial concerning which single-nucleotide polymorphisms (SNPs) of these genes are associated with ototoxicity. It is clear that further studies are required to validate the findings in this review. With pre-treatment administration of oto-protective agents currently in clinical trial, it is vital that a concrete set of genetic biomarkers for susceptibility to be established so that these agents can be used with utmost efficacy.

3.2 Background and Significance

Ototoxicity affects approximately 60% of children treated with platinum chemotherapy.[76] While its prevalence is well documented, ototoxicity remains extremely variable, making it nearly impossible for physicians to predict which patients might be at risk of suffering a hearing deficit. Cumulative dose seems to be one of the largest risk factors for ototoxicity, yet there is still a large degree of inter-individual variability even in dose-matched patients.[68] Currently, no standard method exists for identifying patients who are at an increased risk of developing hearing impairment.

Several genetic variants have been examined in the literature, due to their potential involvement in drug biotransformation, transport and binding. If there is substantial evidence for the involvement of a specific genetic variant and ototoxicity in pediatric patients, this would support the use of routine genotyping for children treated with platinum chemotherapy. Susceptible patients could be identified before treatment based on presence of select predisposing genetic variants and measures can be taken to avoid

ototoxicity. Preliminary economic analysis of the potential financial impact of such testing showed that genotyping patients before platinum therapy could avoid $71,168 in societal costs per tested patient. This would result in a net saving of over $2.4 million annually in British Columbia alone, and around $19.6 million in Canada.[69] When impact on affected patients' quality of life is factored in, preliminary genetic testing would be extremely desirable.

To date, there is no consensus in the literature on specific genetic variants that are associated with platinum-induced ototoxicity. Based on research on TPMT variants and their predictive value (approx. 98%) for ototoxicity[87], the cisplatin product label started to include safety information regarding TPMT and risk of ototoxicity.[70] Due to inconsistent literature findings however, a consensus protocol has yet to be established, which would specify how variant genotyping should be incorporated into platinum-treatment.[71] There is a pressing need to replicate and validate previous findings in order to establish a genetic profile that can help to identify susceptible patients. This review will examine current literature of randomized control trials, seeking to do just that.

3.3 Methods

A literature review was conducted, in order to find the existing literature concerning genetic variations and platinum-induced ototoxicity. Ovid-EMBASE, and Ovid-Medline were the databases searched. The search criteria included studies evaluating the pharmacogenomics of ototoxicity in pediatric patients treated with platinum compounds carboplatin and/or cisplatin. The complete search strategy for each database can be found in Appendix 1. In total, 107 articles were found after the initial search. This number was

narrowed down to 40 after restricting papers to those considering pediatric populations (≤ 24 years). After carefully going through each article, ten were decided to be relevant for inclusion in this review.

3.4 Results

3.4.1 Introduction

Ten articles were chosen for review in this literature review of platinum-induced ototoxicity. The genes that are covered by these articles were: GSTM3, GSTP1, GSTT1, LRP2, XPC, ERCC1, TMPT, COMT, and ABCC3 (see table 3). To date, these are the genes that have been linked to platinum-induced ototoxicity in randomized control trials with pediatric cancer patients. The following will outline the possible mechanism behind platinum-induced hearing loss associated with each gene (as described in the literature). Current literature evaluating the pharmacogenomics of these genes and their genetic variants is also discussed.

Table 3: Results of Literature Review

Gene	Primary Author	Year Published
GSTM3	Peters	2000
GSTP1	Oldenburg	2007
	Rednam	2013
GSTT1	Choeypraset	2013
LRP2	Riedmann	2008
	Choeypraset	2013
XPC	Coronia	2008
ERCC1	Zehnhoff-Dinnesen	2013
TPMT, COMT, ABCC3	Ross	2009
	Pussegoda	2013
	Yang	2013

10 separate articles evaluating were found in the literature review. 9 genes were found to be involved in platinum-induced ototoxicity in pediatric patients. The earliest study was in 2000 by U. Peters et al. The latest studies were published in 2013. Abbreviations: GSTM3 - Glutathione S-transferase M3, GSTP1 - Glutathione S-transferase P1, GSTT1 - Glutathione S-transferase T1, LRP2 - low density lipoprotein-related protein 2, XPC - Xeroderma pigmentosum complementation group C, ERCC1 - excision repair cross-complementation group 1, TPMT - Thiopurine methyltransferase or thiopurine S-methyltransferase, COMT - Catechol-O-methyltransferase, ABCC3 - ATP-binding cassette, sub-family C

3.4.2 GSTs

Glutathione S-Transferase (GST) proteins have been implicated in cisplatin ototoxicity due to their role in cellular anti-oxidant processes (see figure 6). Cisplatin ototoxicity coincides with increased levels of oxygen species and free radicals. These reactive oxygen species (ROS) mediate the apoptosis of auditory neurons and hair cells.

Higher organisms have a complex variety of mechanisms developed, by which they protect against ROS activity. In humans, glutathione S-transferases (GSTs) play a role in this protection. They catalyze the conjugation of alkylating agents, platinum compounds and free radicals. They protect the cell from the deleterious effects of oxidative stress. Because the expression of many of these enzymes is genetically polymorphic, many studies have looked to link genetic defects in GSTs to susceptibility to various diseases. It is thought that single nucleotide polymorphisms (SNPs) in these genes, leading to impaired glutathione S-transferase activity, can lead to an accumulation of ROS in the presence of platinum-chemotherapy. This accumulation leads to a degeneration of hair cells and to subsequent hearing-loss.[72]

U Peters at al. conducted the first genetic association with ototoxicity test in a study in 2000. They investigated the association between the risk of hearing impairment after cisplatin therapy and SNPs in GST genes. They used a cohort of 71 children and young adults between the ages of 3 and 22 years. These patients had a variety of diagnoses including osteosarcoma, neuroblastoma, germ cell tumor, and brain tumor. All patients had a baseline audiogram done. Two groups were selected from the patient collective to be used in analysis. The first group of 20 patients were those who showed hearing loss without covariate interference from cranial radiation, severe renal insufficiency, treatment with other ototoxic drugs, pre-existing hearing loss or familial risk of hearing impairment. The second group of 20 patients were those who showed no hearing-loss during and directly after chemotherapy. GSTM1 and GSTT1 variants showed no significant association with ototoxicity. GSTM3 showed a definitive association, and they concluded that it was probably associated with sensitivity to cisplatin in cancer cell lines. A lack of GSTM3 in patients with

hearing loss supports the theory that this enzyme plays a crucial role in protection against oxidative stress. Patients with normal wild-type GSTs most likely have a better mechanism in place for anti-oxidant defense.[72]

In a 2007 study [73], Oldenburg et al. demonstrated that the GSTP1 enzyme conferred protection against cisplatin-induced ototoxicity. They identified 173 cisplatin-treated testicular cancer survivors (TCSs). The risk of developing hearing impairment was 4-fold greater in subjects with a polymorphic GSTP1 than those with a normal wild-type gene. Ross et al. sought to replicate these findings in a screen of 162 patients, but failed to do so. A self-described weakness of this study was its lack of baseline audiograms.[87]

Rednam et al. published an article in 2013 that confirmed the association between GSTP1 105 AG/GG genotype with permanent ototoxicity in 106 medullosblastoma/primitive neuroectodermal tumor (PNET) patients. They also reported that this variant strongly interacted with radiation dose, leaving patients who were positive for the SNP, and who were receiving high doses of radiation, as being 8.4 times more likely to require hearing aids.[74]

Choeyprasert et al. sought to replicate previous findings in a cohort of 68 patients with osteosarcoma, germ cell tumor, neuroblastoma, and other solid tumors. They found no association with GSTP1 but did find one with GSTT1.[78]

3.4.3 Megalin

Low-density lipoprotein-related protein 2 (LRP2), commonly known as megalin, is the largest member of the low-density lipoprotein receptor family. It has a size of approximately 600kDa. Megalin is an endocytic receptor, which binds and internalizes a

number of ligands. Megalin is highly expressed in the apical surface of the marginal cells of the stria vascularis of the inner ear, but not on the basolateral side. [75] The mechanism by which megalin polymorphisms contribute to platinum-induced hearing loss is unclear. It is postulated that a genetic variant of the megalin gene leads to a defective megalin transporter-protein. When mutated, this protein, which normally binds a transports cisplatin into the margical cells of the stria vascularis, does so at a higher rate. This leads to accumulation of platinum-DNA adducts in these cells, which, in turn, leads to increased apoptosis and cell death (see figure 6). Cell death causes degeneration of the outer hair cells of the cochlea, causing hearing loss. [76]

L. Riedemann et al. conducted a study in 2008[77] to evaluate the association between megalin genetic polymorphisms and individual sensitivity to cisplatin-induced ototoxicity. They chose to focus on two specific polymorphisms: rs2075252 and rs4668123. They used a 50-subject cohort comprised of patients receiving cisplatin therapy. 25 subjects had developed hearing loss during treatment, while the other 25 had no evidence of hearing loss post-therapy. The rs4668123 polymorphism was not significantly associated to cisplatin-induced ototoxicity. The A-allele of rs2075252, however, was recorded at a significantly higher frequency in the groups with hearing impairment than in the group with normal hearing ($p<0.016$). This author suggested that megalin might be involved in transport of cisplatin or its' adducts. Polymorphisms of the gene encoding this protein might impact individual susceptibility to cisplatin-induced ototoxicity. This study used the Muenster classification for early detection of cisplatin-induced bilateral high-frequency hearing loss.

In a 2013 study, Choeyprasert et al. genotyped 68 children diagnosed with solid tumors who received cisplatin-based chemotherapy, investigating the relationship between

megalin SNPs rs2075252 and rs2228171.[78] Unlike the previous study, they did not find a significant correlation between ototoxicity (as defined by the brock scale[79]) and rs20752525. They did, however, find that the C-allele of rs2228171 occurred with higher frequency in patients with ototoxicity.

3.4.4 XPC and ERCC1

One of the main mechanisms of resistance to platinum drugs is cellular DNA repair capacity. The NER (Nucleotide Excision Repair) pathway plays a major role in DNA repair and is involved in the removal of platinum-DNA adducts[1.] Xeroderma pigmentosum complementation group C (XPC) is a 940 amino acid protein, encoded by the XPC gene, which is a crucial component of the NER pathway (see figure 6). XPC complexes with many other proteins, but has a specific role in recognition of bulky DNA adducts. It is thought that a mutant XPC gene will lead to a defective XPC protein. This will inhibit the efficacy of the NER pathway, thus leading to a defective DNA repair system. If platinum-DNA adducts cannot be eliminated allowing for DNA repair, their accumulation in the hair cells of the organ of Corti would lead to cell death and, in turn, to hearing loss.[80] ERCC1 is the lead protein of the NER pathway. The absence of this protein has been shown to be fatal.[81] It has also been shown that increased mRNA levels of ERCC1 were directly related to platinum-based chemotherapy resistance in a variety of cancers including ovarian, cervical, colorectal, and NSCLCs.[82]

In a 2009 study, Caronia et al. investigated whether or not polymorphisms in NER genes were associated with tumor response and survival in cisplatin-treated osteosarcoma patients. XPC was among the 6 genes that were investigated (ERCC1, ERCC2, ERCC4, XPA

and XPC). The lys751Gln SNP in ERCC2 was found to be associated with tumor response. It was also found that 32 of the 91 patients had ototoxicity. As a secondary finding, a weak association was found between the CC genotype of XPC Lys939Gln (rs2228001 minor C allele) and hearing loss.[80] This was the first study to find an association between XPC and ototoxicity.

In a recent 2013 study, Zehnhoff-Dinnesen investigated SNPs of the ERCC1 gene in a pediatric cohort of 54 patients treated with cisplatin. They found an increased frequency of the rs11615 C-allele in patients who experienced hearing loss.[83]

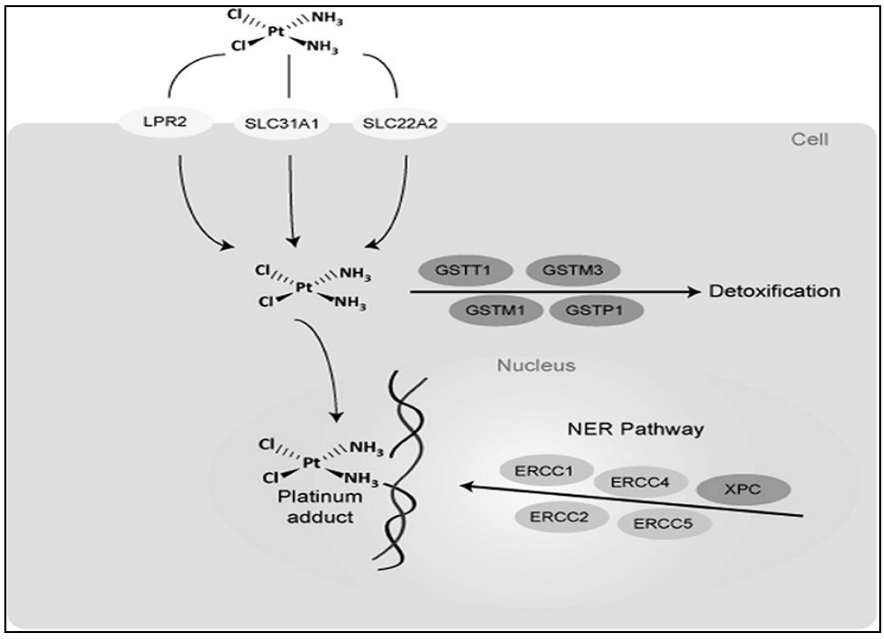


Figure 12: Established mechanisms of genes involved in platinum-induced ototoxicity. Depicted in a schematic of an inner-ear hair cell. GSTs (GSTT1, GSTM3, GSTM1, GSTP1) play an important role in detoxification of cisplatin once it enters the hair cell.[72] LRP2 is an endocytic receptor that binds and transports cisplatin into the hair cell.[76] SLCs are also cisplatin-internalizing transporters.[94] ERCCs (ERCC1, ERCC2, ERCC4, ERCC5) along with XPC are key members of the NER pathway, important for DNA repair.[80] Abbreviations: DNA, deoxyribonucleic acid; GSTs, glutathione S-Transferases; NER, nucleotide excision repair; LPR2, Low Phosphate Root2; SLC31A1 (CTR1), solute carrier family 31 (copper transporter), member 1; SLC22A2, solute carrier family 22 (organic cation transporter), member 2; ERCCs, Excision Repair Cross Complementing group of proteins; XPC, Xeroderma Pigmentosum Group C Protein. Adapted from Roco, A. et al. with permission.[84]

3.4.5 TPMT, COMT, ABCC3

Thiopurine S-methyltransferase (TPMT) and catechol O-methyltransferase (COMT) are genes that code for methyltransferase enzymes. TPMT involvement in hair cell toxicity is not clear. TPMT and COMT are dependent on this methyl donor substrate S-adenosylomethionione (SAM) in the methionine pathway. It has been shown previously that mice administered SAM and cisplatin together, exhibit increased toxicity. However, the administration of both, separately, does not lead to a significant increase in toxicity. These findings would suggest that defective TMPT and COMT enzyme activity, leading to an accumulation of SAM, could cause ototoxicity in the presence of cisplatin. The second proposed mechanism for how variants of these genes may lead to ototoxicity involves TPMT's function as an exogenous purine compound regulator. Cisplatin binds purines in DNA strands creating inter- and intra-strand crosslinks, leading to cell death. A defective TPMT gene reduces TMPT enzyme activity, the inactivation of these platinum-DNA compounds. As these compounds accumulate, platinum toxicity increases. [85]

ATP-binding cassette, sub family C, member 3 (ABCC3) is a transporter that mediates the efflux of organic anions, xenobiotics and glutathione S-conjugates. One of the ways cisplatin-toxicity is neutralized is by conjugation of the active metabolite to glutathione. This makes the compound more anionic, and more readily exported from cells through an ATP-dependent pump. Animal model studies have shown ABCC3 protein levels and mRNA expression levels to increase after cisplatin treatment. As well, lung cell lines have also shown increased mRNA expression following treatment with cisplatin. A polymorphic ABCC3 gene might affect transporter function. In this case, there would be ineffective

transport of toxic compounds out of the cell, which leads to cell apoptosis and toxicity.[86] Although this is a plausible mechanism, functional studies on ABCC3 have been inconsistent.

Ross et al. conducted an explorative genomic study in 2009[87], looking at 220 drug metabolism genes and their possible association with susceptibility to platinum induced ototoxicity. They conducted this association study in a discovery cohort of 54 pediatric cancer patients treated with cisplatin. They then did a follow-up replication study on 112 children. It was found that genetic variants in TMPT (rs12201199) and COMT (rs9332377) were associated with platinum induced hearing loss in children. As a secondary finding, in the replication cohort, they found that male gender was significantly associated with ototoxicity (67% compared 50% in females, p-value = 0.042), and that fewer children with germ-cell tumors developed ototoxicity. In the study they compared patients with no hearing-loss to those individuals with grade 2-4 ototoxicity. This is because, in standard chemotherapy protocols, there is only clinical intervention at stage 2 and above. Subjects with grade 1 hearing loss were excluded, in order to better discriminate between lack of hearing-loss and ototoxicity. All included patients had baseline hearing values of 0, meaning normal hearing. If it were possible to identify susceptible patients based the presence of these specific SNPs, than it would be possible to improve counseling and/or treatment options to avoid ototoxicity. Ross et al. suggest lowering of doses of cisplatin, and treatment with carboplatin as alternatives.

Recently, the same group from British Columbia replicated these findings combining genetic variants in TPMT, COMT and ABCC3 with clinical variables (patient age, vincristine treatment, germ-cell tumor, and cranial irradiation) to give a predictive model of platinum-induced ototoxicity.[86] The replication cohort used was comprised of 155 pediatric oncology

patients who were treated with cisplatin chemotherapy. 87 (56%) of them developed hearing loss. The three TMPT genetic variants tested (rs12201199, rs12201199, and rs1800460) all showed a significant association with cisplatin-induced hearing loss. COMT genetic variant rs12201199 showed a smaller, but still significant association in the replication cohort. Meanwhile, association between ABCC3 variant and cisplatin-induced hearing loss was also explored for the first time. It was found that the ABCC3 variant rs1051640 was significantly associated with a higher risk of hearing-loss. This study defined ototoxicity as grade 2 or higher hearing impairment. Control subjects were those who had grade 0 (normal audiometric findings) after cisplatin chemotherapy.

A separate study conducted in 2013 by Yang JJ et al.[88] also sought to replicate these findings. They used a 213 patient cohort of pediatric cancer patients and did not find an association between either TPMT/COMT variants and hearing-loss. As well, these variations did not influence hearing damage in laboratory models.

Table 4. Cisplatin study characteristics – Cases, Controls, and Age Range

Study	Ages (yrs.)	# Cases	# Controls	Pts. w/ ototoxicity (%)
Peter[72]	3-22	20	19	51
Oldenburg[73]	4-20	56	84	40
Rednam[74]	0-19	45	24	65
Choeypraset[78]	0-15	46	22	68
Riedmann[77]	5-22	25	25	50
Coronia[80]	3-34	15	17	47
Zehnhoff[83]	Children >5	15	27	38
Ross[87]	0-19	106	56	65
Pussegoda[86]	0-25	87	68	56
Yang[88]	3-22	149	64	70
Summary	0-25	564	406	58

Abbreviations: pts – patients. This table shows the age range for each study included in this gene review. The overall age range for children included is 0-25 years of age. Every study used a cohort of patients treated with cisplatin chemotherapy. The total % of subjects who displayed ototoxicity after combining these 10 studies was 58%.

3.5 Discussion

Combining the results of all of the above studies, it was found that on average 58% of children in these studies suffered from ototoxicity (see above Table 4). This is consistent with previous reports that approximately 60% of patients treated with platinum chemotherapy suffer from this side effect.[4] Considering the debilitating effect of platinum-

induced ototoxicity on quality of life of pediatric cancer patients, it is crucial to find a reliable method of identifying susceptible patients. There has been a lot of progress in identifying genetic biomarkers for this devastating side effect.

As it stands, megalin polymorphisms have not been associated conclusively to platinum-induced ototoxicity in clinical studies. Both the 2008[77] and 2013[78] studies tested a small population, leaving their results underpowered. As well, the initial results of the 2008 study were not replicated in the later study. Considering the transporter action of megalin, its localization to the stria-vascularis of the inner ear, and reports of its association to ototoxicity, future studies should continue to explore whether megalin polymorphisms are associated with platinum-induced ototoxicity in a larger cohort.

A specific variant of the XPC gene has also been implicated in platinum-induced hearing-loss. While the study does show a significant association of SNPs in XPC with ototoxicity, this was a secondary finding in a small cohort of patients. Furthermore, the study cohort was solely comprised of osteosarcoma patients. There is a need to do this study in a larger cohort, focusing on multiple XPC variants including the Lys939Gln polymorphism.

A few studies have looked at the association of GSTs with platinum-induced ototoxicity. The first described in this review looked at GSTP1 and GSTM3.[72] It was found that GSTM3 had a protective ototoxic effect. This study was done on a small cohort of patients who were not followed long term for hearing loss. The second study described sought to replicate these findings and found that while GSTM3 conferred protection, so too did GSTP1. Patients with polymorphic GSTP1 were at higher risk of platinum-induced ototoxicity. The weakness of this second study was that it was a retrospective study, and it

only included patients who had survived. This likely biased the results. As well, it only included patients who suffered from testicular cancer.

While Ross et al. found a strong association of genetic variants of TPMT and COMT with ototoxicity, more recent studies have shown inconsistent results. Pussegoda et al. confirmed the original findings but with smaller effect sizes, while also finding a significant association with an ABCC3 polymorphism. Yang et al. failed to replicate any previous findings in a cohort of 213 medulloblastoma patients. A recent 2014 randomized control study (which included adults) and meta-analysis (which included the 3 aforementioned studies and two new cohorts), also failed to confirm previous findings. This suggests that TMPT and COMT might be less influential on the development of platinum-induced ototoxicity than previously reported.[89]

Other genes have been identified in randomized control trials with adults. Otos is a gene expressed specifically by spiral ligament fibrocytes (SLFs). SLFs are known to play a key role in maintaining the chemical environment of cochlear fluids. Otos encodes a 6.4kDa protein called Otospiralin. Although the exact function of this protein is unknown, animal studies have shown that suppression of Otos gene expression leads to inner ear cell apoptosis and to permanent deafness.[90] It has also been reported that excessive noise stimulation might cause hearing-loss through down-regulation of the Otos gene.[91] It was postulated that Otos may provide a survival signal for SLFs.[92]

Timothy F. Spracklen's group out of South Africa tested 29 genetic variants of Otos in a cohort of 100 South-African cisplatin-receiving patients. The results showed an association between ototoxicity-free patients and G alleles of Otos SNPs rs77124181, and rs2291767, indicating a potential protective role for these variants.[93] SLC31A1, an influx

copper transporter, is involved in cochlear uptake of cisplatin, and has been shown to be associated with severe ototoxicity in a cohort of 204 Chinese, non-small cell lung cancer patients.[94] Mitochondrial DNA mutations may also play a role in susceptibility to platinum induced ototoxicity. Future studies should explore these genes in pediatric populations.

The accuracy of prediction models for patients who are susceptible to platinum induced hearing loss may be greatly enhanced by the use of genetic biomarkers associated with this side effect. It has been shown in the existing literature that certain genetic variants might be useful predictors. There is a need, however, to replicate these studies, addressing the weaknesses in each one, before these biomarkers may be used in medical practice.

Moreover, while most of these studies included subjects treated with cisplatin chemotherapy, very few have looked at the association of carboplatin-induced hearing loss and ototoxicity. Carboplatin is a newer form of platinum-induced chemotherapy, which is less ototoxic at lower doses. Considering the proposed mechanisms by which cisplatin causes toxicity, careful consideration should be given to this alternate platinum compound and how it is handled. Future studies should consider patients treated with carboplatin as well as cisplatin.

All of the above studies were done using a candidate gene approach. The approach chooses genes based on their potential mechanism of action. A genome-wide approach should be considered to find novel genes involved in platinum-induced ototoxicity.

3.6 Conclusion

To date, there is insufficient data in the literature to support the use of routine genotyping in all children before administration of platinum-chemotherapy.

Chapter 4

Pharmacogenomic evaluation of XPC and LRP2 as genetic biomarkers for platinum-induced ototoxicity

Rothstein, J., Emami, N., Daniel, S.J., Carret, A.S., Dube, M.-P., Bezdjian, A.

Department of Otolaryngology (Head & Neck Surgery)

McGill University, Montreal

Manuscript in preparation for submission in

May 2015

4.0 Abstract

Cisplatin and Carboplatin, two highly effective platinum chemotherapeutic agents, are widely used to treat a variety of malignancies. Their use is limited, however, by their debilitating side effects, which include ototoxicity. Sixty percent of children develop ototoxicity following administration of platinum chemotherapy. It is thought that genetic variation is an important risk factor.

Based on existing literature findings, a multi-center, randomized-controlled trial was conducted in two tertiary care centers, in a combined cohort of 100 pediatric cancer patients. The association of ototoxicity (pre-treatment compared to post-treatment) and ototoxicity-progression (post-treatment compared to follow-up) with SNPs of XPC and LRP2 was evaluated. It was found that five polymorphisms in the XPC gene, specifically rs1350344, rs2607737, rs2733533, rs3731093, and rs3731149, were correlated with ototoxicity ($P \leq 0.05$). It was also found that two XPC SNPs, rs1350344 and rs2733533, were associated with progression of ototoxicity ($P \leq 0.05$). With the growing incidence of platinum-induced ototoxicity, there is an urgent need to validate these findings.

Keywords: late effects of cancer treatment; LRP2; megalin; ototoxicity; pediatric oncology; pharmacogenomics; platinum; progressive hearing-loss; SNP; XPC.

4.1 Introduction

Platinum compounds Cisplatin and Carboplatin are two of the most widely used and successful chemotherapy drugs available. They are used to treat a variety of soft-tissue neoplasms including testicular, bladder, ovarian, head and neck, and non-small cell lung cancers.[3] Their use is limited, however, by their secondary ototoxic effect. About 60% of

children receiving platinum-chemotherapy develop ototoxicity, a secondary effect causing hearing-loss.[95] Ototoxicity most often presents as permanent, bilateral and progressive. It has been shown that even a mild hearing loss, at a young age, can be detrimental to school performance, social interaction and cognitive development.[96,97] Thus, while 85% of these cancer patients treated with platinum-chemotherapy survive[98], their quality of life can be substantially reduced. There is currently no prevention or treatment for ototoxicity, which is why dose reduction or premature termination of treatment is often seen in cancer patients. This affects overall survival rates.

Although more than half of all of patients treated with platinum-chemotherapy develop ototoxicity, it is currently not possible to predict who is at risk.[99,100] There is a large degree of individual variability in the appearance and degree of hearing-loss. The variability persists even when patients are matched with respect to age, gender and dosage. Without a validated method of identifying susceptible patients, it is impossible to prevent this devastating side effect. With the growing incidence of platinum induced ototoxicity, there is a pressing need to find a method that works.

It is hypothesized that genetic variants, previously identified in the literature, are involved in platinum-induced ototoxicity. Examples of these candidate genes are: TPMT, COMT, LRP2, XPC, GSTs and Otos. It would be extremely beneficial if it could be determined whether one of these genes might be used as biomarkers for susceptibility.

The primary objective of this study is to better understand the role of genetic variations in ototoxicity. To this end, hearing loss and the incidence of specific genetic variants is evaluated in a pediatric cohort. This pharmacogenomic evaluation identifies specific genetic variants that might be involved in ototoxicity, contributing to the

understanding of the nature and extent of platinum-induced hearing loss. As well, few studies have evaluated the long-term consequences of platinum induced hearing loss. A secondary objective of this study is to evaluate the incidence of hearing loss that progresses post-chemotherapy (after the end of platinum-treatment), and to understand the role of genetic variants concerning this phenomenon.

More specifically, this study will look to validate previous findings that single nucleotide polymorphisms (SNPs) of the LRP2 and XPC genes are related to platinum-induced hearing loss.

4.2 Methods

A detailed methods section can be found in Appendix 2

4.2.1 Patients and Variables

Pediatric cancer patients were recruited from CHU Sainte-Justine and the Montreal Children's Hospital in Montreal, Quebec. Pharmacy records at both hospitals were used to identify patients who were being prescribed Cisplatin and/or Carboplatin. Patients who were currently receiving platinum-chemotherapy, and who met eligibility criteria (see table 5) were designated as group II subjects and were recruited retrospectively. Patients who had completed platinum chemotherapy treatment , and who met eligibility were designated as group III subjects and were recruited retrospectively. Patients who had not yet received chemotherapy were designated as group I subjects, and were recruited prospectively. In all, 17 patients were excluded from the final analysis. 10 patients lacked genetic material (blood) for analysis, and 7 did not have sufficient audiology data. 83 patients were included in the final analysis. Age, Sex, Gender, type of cancer and dosages of chemotherapeutic

agents were considered in the analysis. Additional concurrent chemotherapeutic agents, and radiation therapy to the head and neck were also considered.

Table 5: Inclusion and exclusion criteria

Subject Inclusion Criteria	Subject Exclusion Criteria
Treated at the CHU Sainte-Justine, Montreal Children's Hospital, Montreal General Hospital, Jewish general Hospital	With congenital hearing loss
Treated with Cisplatin and/or Carboplatin	With a tympanic perforation With persistent otorrhea
Able to provide informed consent	≥ 25 years of age

4.2.2 Assessing ototoxicity and hearing-loss progression

Two paired t-tests were used to compare (a) pre-treatment (baseline before the start of treatment) audiograms to post-treatment (<14 days after the end of treatment) to assess ototoxicity, as well as (b) post-treatment to follow-up (≥6 months after the end of treatment) audiograms at conventional frequencies (0.25-8kHz) to assess hearing-loss progression. Bone conduction thresholds were used to compare hearing loss. Otoacoustic emission (OAE) testing was also used when needed as an alternative for detecting hearing loss. The ASHA criteria (see Table 6) were used to define the incidence of hearing loss, based on audiograms done between baseline and post-chemotherapy. ASHA defines ototoxicity in three categories: (A) 20 dB or greater hearing loss in pure tone threshold in at least one frequency, (B) 10 dB or greater decrease at two adjacent test frequencies, and (C) loss of responses at three consecutive frequencies where responses were previously obtained. [101] In order to determine the incidence of progressive ototoxicity, The Chang grading system,

which evaluates severity of ototoxicity, was used to compare post-treatment audiograms with follow-up audiograms. (see Table 7).[102]

A	B	C
20 dB or greater hearing loss in pure tone threshold in at least one frequency	10 dB or greater decrease at two adjacent test frequencies	Loss of responses at three consecutive frequencies where responses were previously obtained*

Table 6 American Speech-Language-Hearing Association Criteria. Changes are computed relative to baseline assessment. Results indicating significant change in hearing must be confirmed by repeat testing. dB = decibel.

Chang Grade	Sensorineural Hearing Loss (dB HL) bone conduction or air conduction with normal tympanogram
0	≤ 20 dB at 1, 2, and 4 kHz
1a	≥ 40 dB at any freq 6 to 12 kHz
1b	> 20 and < 40 dB at 4 kHz
2a	≥ 40 dB at 4 kHz and above
2b	> 20 and < 40 dB at 1, 2, or 3 kHz
3	≥ 40 dB at 2 or 3 kHz and above
4	≥ 40 dB at 1 kHz and above

Table 7. Chang Assessment and Grading of Ototoxicity Criteria**.** This chart shows different grades of hearing loss according to the Chang criteria. Adapted from Chang 2011[103]

4.2.3 DNA Extraction and Genotyping

Blood samples were collected from consenting patients, before, during or after treatment, at a patient's convenience. 5-10 ml venous blood samples were collected from each patient into a polypropylene tube containing ethylenediamine tetra-acetic acid (EDTA). Samples were stored at -70ºC, until DNA extraction. Staff of the Montreal Heart Institute

performed the DNA isolation and genotyping. 18 SNPs of XPC (16 SNP2) and LRP2 (2 SNPs) were analyzed.

4.2.4 Statistical Analysis

Descriptive statistics for continuous and clinical categorical variables are shown in Tables 8 and 9, For each clinical continuous variable, the number of observations, the number of missing values, the mean, the standard deviation, the median, the minimum and the maximum were calculated. For clinical categorical variables, the number of observations and the frequency for each category were calculated.

In a univariate analysis, clinical variables were tested separately for association with ototoxicity. These clinical variables were: age, diagnosis, cisplatin, carboplatin, vancomycin, cyclophosphamide, vincristine, tobramycin, gentamycin, radiation and recruitment type (see table 9).

In a covariate analysis, variables from the above list which showed significant associations (univariate P-value <0.05) were tested in the same model for association with hearing loss using a stepwise procedure (see table 10).

Association between genetic factors and hearing loss was done using a logistical regression to test for association with hearing loss with both additive and genotypic models. For the additive model genotypes were recorded as as g=0, 1 or 2 (0 for homozygotes major allele, 1 for heterozygotes and 2 for homozygotes minor allele).

All individuals were tested (a) without adjusting for cisplatin and also (b) with adjusting for cisplatin. A subgroup of patients using only cisplatin was also tested

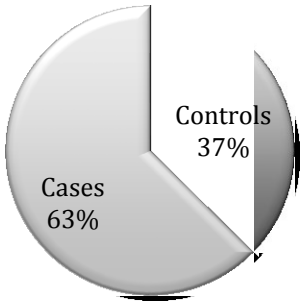
separately. Association between genetic factors and hearing loss progression was tested using the same analysis, using only patients with hearing loss.

4.3 Results

4.3.1 Study population

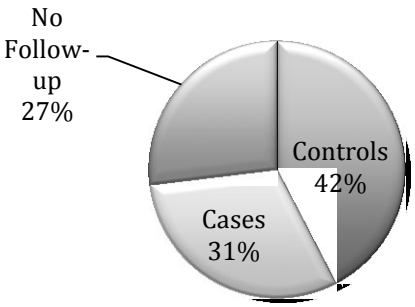
100 patients were recruited for this study, 17 of which were excluded for clinical reasons or due to insufficient data. The clinical features of the remaining 83 patients are described in Tables 8 and in Figure 15. The age range for this patient population was 0-22 years. The mean age of the case group was 6.92 years, and of controls was 7.65 years. Age was not significantly associated with ototoxicity. About 70% of patients were male, and

Figure 13: Incidence of Ototoxicity



Out of 52 Patients who presented with ototoxicity as defined by the ASHA criteria (see section 4.2.2), 16 (31%) had hearing-loss progression according to the Chang criteria (see section 4.2.2), 22 (42%)had no progression, and 14 (27%) lacked follow up data for analysis.

Figure 14. Incidence of Hearing-Loss Progression



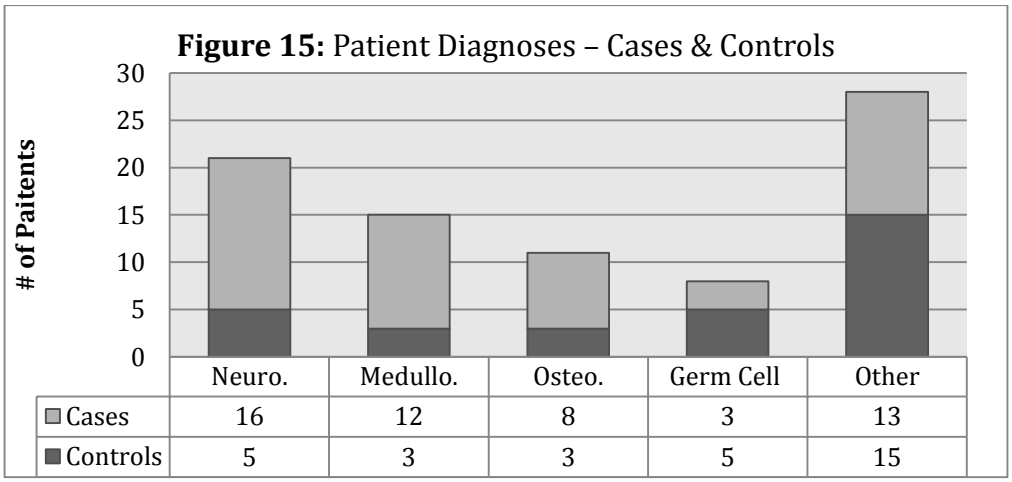
Out of 83 patients who were included in this study, 52 (63%) developed ototoxicity (cases), while 31 (37%) had normal hearing (controls). This is consistent with literature findings that approximately 60% of patients treated with platinum-chemotherapy develop ototoxicity.

about 30% were female. Gender also showed no significant correlation with ototoxicity. Overall, subjects were diagnosed with 33 different types of cancer, the most common diagnoses being neuroblastoma (25.3%), medulloblastoma (18.1%), and osteosarcoma (13.25%). 63 subjects (75.9%) were treated with cisplatin, and 20 (54.22%) with carboplatin. 52 patients (63%) were reported as cases (having ototoxicity), while 31 (37%) were controls (no ototoxicity) (see figure 13). After a stepwise selection (see table 10), only cisplatin remained associated with ototoxicity, among all other variables (including concurrent medications). 16 case subjects (30.76%) demonstrated hearing-loss progression post-chemotherapy (see figure 14).

Table 8- Descriptive statistics for continuous variable: Age

Variable	Cases				P-value	Controls				Cases + Controls			
	N	**Min**	**Max**	**Mean**		**N**	**MIN**	**Max**	**Mean**	**N**	**Min**	**Max**	**Mean**
Age (Years)	52	0.44	20.29	6.92	0.1582	31	0.02	22.06	8.86	83	0.02	22.06	7.65

Abbreviations: min – minimum, max – maximum. The P-value for the association of age with ototoxicity is 0.1582 which is >0.05. This supports that age is not significantly associated with hearing-loss.



The 83 patient cohort is comprised of 21 (25.3%) neuroblastoma patients (16 cases- 76%), 15 (18.1%) medulloblastoma patients (12 cases – 80%), 11 (13.25%) osteosarcoma patients (8 cases- 73%), 8 germ cell tumor patients (3 cases – 38%), and 28 patients with other diagnoses (13 cases – 46%). After univariate analysis, ototoxicity is not significantly associated with any one diagnosis (see appendix 3). Abbreviations: Neuro – Neuroblastoma, Medullo – Medullosblastoma, Osteo – Osteosarcoma, Germ Cell – Germ Cell Tumor.

Table 9: Descriptive statistics for categorical variables – Univariate analsis

Variable	Category	Cases	Controls	Fisher P-value	Cases + Controls
Gender	F	19(36.54%)	6(19.36%)	0.1384	25(30.12%)
	M	33(63.46%)	25(80.64%)		58(69.88%)
Carboplatin	N	27(51.92%)	11(35.48%)	0.1757	38(45.78%)
	Y	25(48.08%)	20(64.52%)		45(54.22%)
Cisplatin	N	3(5.76%)	17(54.84%)	7.51E-07	20(24.1%)
	Y	49(94.24%)	14(45.16%)		63(75.9%)
Cyclophosphomide	N	15(28.84%)	20(64.52%)	0.0026	35(42.16%)
	Y	37(71.16%)	11(35.48%)		48(57.84%)
Gentamycin	N	50(96.16%)	30(96.78%)	1	80(96.38%)
	Y	2(3.84%)	1(3.22%)		3(3.62%)
Hearing loss progression	N	22(42.3%)	0(.%)		22(42.3%)
	Y	16(30.76%)	0(.%)		16(30.76%)
	no follow up	14(26.92%)	0(.%)		14(26.92%)
Lasix	N	13(25%)	18(58.06%)	0.0045	31(37.34%)
	Y	39(75%)	13(41.94%)		52(62.66%)
Radiation	N	50(96.16%)	30(96.78%)	1.0000	80(96.38%)
	Y	2(3.84%)	1(3.22%)		3(3.62%)
Recruitment type	prospective	21(40.38%)	17(54.84%)	0.2564	38(45.78%)
	retrospective	31(59.62%)	14(45.16%)		45(54.22%)
Tobramycin	N	13(25%)	17(54.84%)	0.0092	30(36.14%)
	Y	39(75%)	14(45.16%)		53(63.86%)
Vancomycin	N	26(50%)	25(80.64%)	0.0097	51(61.44%)
	Y	26(50%)	6(19.36%)		32(38.56%)
Vincritine	N	12(23.08%)	16(51.62%)	0.0155	28(33.74%)
	Y	40(76.92%)	15(48.38%)		55(66.26%)

Abbreviations: N=NO Y=YES. Highlighted cells are those where the variable shows a significant correlation with ototoxicity (p-value < 0.05) after univariate analysis. Cisplatin, Cyclophosphomide, Lasix, Tobamycin, Vancomycin, and Vinristine are medications that are significantly asscciated with hearing loss.

Table 10 Stepwise selection analysis - association of clinical variables with ototoxicity

Step	Effect Entered	Effect Removed	Number In Model	ProbChiSq	Label
1	Cisplatin		1.0000	4.2708E-07	Cisplatin
2	Cycl		2.0000	0.045806007	Cycl
3		Cycl	1.0000	0.05124501	Cycl

Abbreviations: Cycl=Cyclophosphomide. Highlighted cells are those where the variable shows a significant correlation with ototoxicity. After a stepwise selection, only cisplatin remains significantly associated with ototoxicity.

4.3.2 Single nucleotide polymorphic (SNP) frequencies

The genotypic frequencies of 18 SNPs of LRP2 (2 SNPs) and XPC (16 SNPs) are shown in Appendix 3.

4.3.3 Association with ototoxicity

The results of the association test of ototoxicity with genotype are shown in Table 11. A significant correlation was found in the additive model with the XPC SNPs rs1350344 (P-value = 0.0350), rs2607737 (P-value = 0.0083), rs2733533 (P-value = 0.0186), rs3731093 (P-value = 0.0449), and rs3731149 (P-value = 0.0219). The association remained consistent in the genotypic model as well. In particular, the A allele of rs1350344 was associated with ototoxicity. It was found that 70% (44 of 62) of subjects, with at least one 'A' allele, had ototoxicity, compared to 40% (8 of 20) who were homozygous for the 'G' allele. Similarly, the 'T' allele of rs2607737 was also associated with ototoxicity. 71% (46 of 65) of subjects with at least one 'T' allele had ototoxicity, compared to 33% (6 of 18) who were

homozygous for the 'C' allele. The 'A' allele of rs2733533 was also associated with ototoxicity. 71% (44 of 62) of subjects with at least one 'A' allele had ototoxicity, compared to 38% (8 of 21) who were homozygous for the 'C' allele. For the rs3731093, a homozygous 'A' allele was associated with ototoxicity. 66% (50 of 76) of subjects who were homozygous (A/A) had significant hearing loss, compared to 40% (2 of 5) who were heterozygous (A/G). Finally, for rs3731149, the 'G' allele correlated with ototoxicity. 77% (27 of 35) of subjects with at least one 'G' allele had ototoxicity, compared to only 52% (25 of 48) who were homozygous for the 'T' allele. These results remained consistent after adjusting for cisplatin, and in the cisplatin subgroup.

Table 11. Summary of significant results for SNPs associated with ototoxicity. (p value additive model <=0.05). This was designated as objective 1.

SNP name	Variant	Case	Control	P value	OR	LCL	UCL	P value	Effect*	Analysis
XPC_rs1350344_AG_F	A/A	16	6	0.0350	0.49	0.25	0.95	0.7152	1 vs 0	objective 1 without adjusting for cisplatin
	A/G	28	13					0.0361	2 vs 0	
	G/G	8	12							
XPC_rs1350344_AG_F	A/A	16	6	0.0309	0.41	0.18	0.92	0.8842	1 vs 0	objective 1 adjusting for cisplatin
	A/G	28	13				0.92	0.0351	2 vs 0	
	G/G	8	12							
XPC_rs1350344_AG_F	A/A	16	6	0.0230	0.34	0.13	0.86	0.5999	1 vs 0	objective 1 cisplatin subgroup
	A/G	28	13					0.0327	2 vs 0	
	G/G	8	12							
XPC_rs2607737_CT_F	C/C	6	12	0.0083	0.40	0.20	0.79	0.4650	1 vs 0	objective 1 without adjusting for cisplatin
	C/T	27	13					0.0071	2 vs 0	
	T/T	19	6							
XPC_rs2607737_CT_F	C/C	6	12	0.0194	0.38	0.17	0.85	0.7762	1 vs 0	objective 1 adjusting for cisplatin
	C/T	27	13					0.0162	2 vs 0	
	T/T	19	6							
XPC_rs2607737_CT_F	C/C	6	12	0.0152	0.31	0.12	0.80	0.3215	1 vs 0	objective 1 cisplatin subgroup
	C/T	27	13					0.0168	2 vs 0	
	T/T	19	6							
XPC_rs2733533_AC_F	A/A	17	6	0.0186	0.45	0.23	0.88	0.6951	1 vs 0	objective 1 without adjusting for cisplatin
	A/C	27	12					0.0195	2 vs 0	
	C/C	8	13							
XPC_rs2733533_AC_F	A/A	17	6	0.0247	0.40	0.18	0.89	0.9334	1 vs 0	objective 1 adjusting for cisplatin
	A/C	27	12					0.0277	2 vs 0	
	C/C	8	13							
XPC_rs2733533_AC_F	A/A	17	6	0.0201	0.34	0.13	0.84	0.5231	1 vs 0	objective 1 cisplatin subgroup
	A/C	27	12					0.0273	2 vs 0	
	C/C	8	13							
XPC_rs3731093_CT_R	A/A	50	26	0.0449	0.15	0.02	0.96	0.0449	1 vs 0	objective 1 adjusting for cisplatin
	A/G	2	5							
XPC_rs3731149_AC_R	G/G	5	2	0.0411	2.73	1.04	7.16	0.0391	1 vs 0	objective 1 adjusting for cisplatin
	G/T	22	6					0.2383	2 vs 0	
	T/T	25	23							
XPC_rs3731149_AC_R	G/G	5	2	0.0219	6.00	1.30	27.75	0.0384	1 vs 0	objective 1 cisplatin subgroup
	G/T	22	6							
	T/T	25	23							

*: heterozygotes (g=1) are compared to homozygotes for comm allele (g=0) and homozygotes for the minor allele (g=2) are compared to homozygotes for the common allele (g=0)

We hav empty cells when the maximum likelihood does not converge because of quasi complete separation of data points.

4.3.4 SNPs associated with hearing loss progression

In addition to being correlated with ototoxicity, the XPC SNPs rs1350344 and rs2733533 were also associated with hearing loss progression post-chemotherapy. The results of this association are shown in Table 12. This association is shown for both SNPs when adjusting for cisplatin and in the cisplatin subgroup, but not without adjusting for cisplatin.

Table 12. Significant results for SNPs associated with progressive hearing loss (association with post-treatment hearing loss compared to 6-month follow-up hearing loss) (p value additive model <=0.05). This was designated as objective 2.

SNP name	Variant	Case	Control	P value	OR	LCL	UCL	P value	Effect*	Analysis
XPC_rs1350344_AG_F	A/A	3	11	0.0216	0.38	0.17	0.87	0.0170	1 vs 0	objective 2 adjusting for cisplatin
	A/G	10	9					0.0393	2 vs 0	
	G/G	3	2							
XPC_rs1350344_AG_F	A/A	3	11	0.0187	0.37	0.16	0.85	0.0147	1 vs 0	objective 2 cisplatin subgroup
	A/G	10	9					0.0349	2 vs 0	
	G/G	3	2							
XPC_rs2733533_AC_F	A/A	3	11	0.0436	0.44	0.20	0.98	0.0506	1 vs 0	objective 2 adjusting for cisplatin
	A/C	10	9					0.0699	2 vs 0	
	C/C	3	2							
XPC_rs2733533_AC_F	A/A	3	11	0.0388	0.43	0.20	0.96	0.0455	1 vs 0	objective 2 cisplatin subgroup
	A/C	10	9					0.0635	2 vs 0	
	C/C	3	2							

*: heterozygotes (g=1) are compared to homozygotes for comm allele (g=0) and homozygotes for the minor allele (g=2) are compared to homozygotes for the common allele (g=0). We have empty cells when the maximum likelihood does not converge because of quasi complete separation of data points.

4.4 Discussion

It is crucial to identify genetic variations capable of serving as biomarkers for patient susceptibility to platinum-induced ototoxicity. This study focused on XPC and LRP2, examining their potential association with hearing loss in a cohort of 83 pediatric cancer patients.

The XPC gene encodes a protein that is 940 amino acids long. The role of this protein is to recognize DNA damage.[104] The gene itself has been previously linked to cancer risk, most recently to bladder cancer.[105,106] XPC is a crucial component of the NER (nucleotide excision repair) pathway, which is a major DNA repair system involved in the removal of platinum-DNA adducts. XPC complexes with many other proteins, but has a specific role in recognition of bulky DNA adducts. It is thought that a mutant XPC gene will lead to a defective XPC protein. This will inhibit the efficacy of the NER pathway, thus leading to a defective DNA repair system. If platinum-DNA adducts cannot be eliminated and the DNA repaired, their accumulation in the outer hair cells of the organ of Corti will lead to increased apoptosis and cell death. The loss of hair cells, in turn, causes hearing loss. While this is the postulated method of action of XPC polymorphisms, all the studies to date that have sought to explore the function of specific XPC polymorphisms have been inconsistent.[107,108]

Coronia et al, in a 2009 study focusing on tumor response outcomes, found weak evidence of an association between the minor allele of XPC rs2228001 and ototoxicity in a sample of 32 patients with hearing loss following cisplatin chemotherapy.[109] This was a secondary finding, but was the first study to look at XPC and its possible involvement in platinum-induced ototoxicity. This current study, sought to validate this previous

association of XPC and ototoxicity. The previous search was expanded to include 15 polymorphisms of this gene in the analysis, including rs2228001, which was previously identified. Unfortunately, the findings of this study did not show an association with the specific variant in question. It was found, however, that the other polymorphisms rs1350344, rs2607737, rs2733533, rs3731093, and rs3731149 correlated with ototoxicity. This further suggests that XPC plays a role in platinum-induced hearing loss. Future studies are required to validate these findings.

This study also sought to replicate previous findings, which implicated the LRP2 gene in platinum-induced ototoxicity. LRP2 (commonly known as megalin) is a member of the low-density lipoprotein receptor family.[110] It is highly expressed in the marginal cells of the stria vascularis of the inner ear. The mechanism by which megalin polymorphisms contribute to platinum-induced hearing loss is unclear. It is postulated that a genetic variant of the megalin gene leads to a defective megalin transporter-protein, which binds cisplatin and transports it into the marginal cells of the stria vascularis. The mutated transporter binds this this platinum compound at a higher-rate than the wild-type receptor. This leads to accumulation of platinum-DNA adducts in these cells, which, in turn, leads to increased apoptosis and cell death. Cell death causes degeneration of the outer hair cells of the cochlea, causing hearing loss.[111,112]

L. Riedemann et al. conducted a study in 2008 to evaluate the association between Megalin genetic polymorphisms and individual sensitivity to cisplatin-induced ototoxicity. They found a strong association between the A-allele of rs20752525, and ototoxicity, indicating that SNPs of the Megalin gene might impact individual susceptibility to cisplatin-induced ototoxicity.[113] Despite these previous findings, our study, which included both LRP2

polymorphisms examined in this 2008 study, showed no evidence of a correlation with platinum-induced ototoxicity. The results remain inconsistent and must be further explored in a larger cohort of patients to determine to relationship between polymorphic LRP2 and ototoxicity.

While the incidence of ototoxicity following chemotherapy is well documented, few studies have looked at the long-term consequences of platinum-based chemotherapy on hearing. A pilot study performed by Peleva et al showed that platinum-induced ototoxicity progresses even after the completion of chemotherapy.[44] Of the 31 patients evaluated in the 2010 study, 21 were followed long-term, 7 (33%), showed progressive hearing loss, and one patient, who had normal hearing throughout treatment, developed severe ototoxicity only after the end of chemotherapy. Considering the serious effects of hearing-loss it is imperative to evaluate the long-term consequences of platinum-induced chemotherapy. In this study it was found that, of the 52 patients who had ototoxicity, 16 (31%) had a progression of hearing-loss following the end of platinum-chemotherapy. This percentage is consistent with previous findings.[45] The association of XPC and LRP2 genetic variants with progression of hearing-loss following platinum-based chemotherapy was also explored. The results showed that two of the XPC SNPs, rs1350344 and rs2733533, that were significantly associated with ototoxicity, were also associated with progression of ototoxicity. This is the first study to find a genetic association for progression of hearing loss, and there is a pressing need to validate these findings. If platinum-induced hearing loss does indeed progress, and in some cases present, post-chemotherapy, then monitoring practices must routinely include long-term follow up for hearing loss. With these biomarkers for

progression in hand, genetic screening would make it easier to know which patients are at risk and should be followed, more closely, long-term.

In conclusion, this study found that polymorphisms in the XPC gene, specifically rs1350344, rs2607737, rs2733533, rs3731093, and rs3731149, are correlated with ototoxicity. If replicated in future studies, these SNPs could be used in clinical practice as biomarkers for susceptibility to platinum-induced ototoxicity. Identifying susceptible patients before treatment, physicians would be able to modify their treatment plans to avoid ototoxicity while providing the best treatment possible. Furthermore, this is the first study to show the involvement of specific polymorphisms in hearing-loss progression following platinum chemotherapy. Further studies, however, with a larger cohort of patients, are required to validate these findings, and to explore the functional activity of XPC in ototoxicity.

Chapter 5: Conclusion and future directions

5.1 Conclusion

While platinum-chemotherapy has played a large role in increasing cancer-patient survival rates over the past 40 years, it can also cause ototoxicity. Platinum compounds, cisplatin and carboplatin, damage structures of the inner ear in a cumulative, dose-dependent manner, leading to hearing-loss in over 60% of treated patients. Pediatric patients are particularly at risk. Currently, no method of identifying susceptible patients exists.

After reviewing the literature concerning pharmacogenomics studies on pediatric, platinum-induced ototoxicity, it was found that that there is not enough evidence to support the regular use of genotyping as part of treatment protocols to identify susceptible patients. Further studies are required in order to replicate findings involving GST, TMPT, COMT, ABCC3, LRP2, XPC and ERCC1 genetic variants. .

Results reported in the current thesis do not validate previous findings, but suggest a genetic link between several novel XPC SNPs (rs1350344, rs2607737, rs2733533, rs3731093, and rs3731149) and ototoxicity. Meanwhile, no replication or novel association was found with variants of the Megalin gene.

As a secondary objective, the current study explored the pharmacogenomics of hearing-loss progression post platinum-based chemotherapy. Such an evaluation is not currently found in the literature. It was found that that two of the XPC SNPs, rs1350344 and rs2733533, which are significantly associated with ototoxicity, were also associated with progression of ototoxicity. Few studies have examined progression of hearing-loss altogether, but late-onset ototoxicity has been documented. Genetic biomarkers for

progressive hearing-loss would improve monitoring and management of this phenomena in susceptible patients who may not initially present with hearing-loss.

5.2 Future Directions

With recent advances in the administration of anti-inflammatory agents, such as intratympanic dexamethasone, it is essential that a genotypic profile be established to guide otoprotective methods. To this end, there is a pressing need to replicate existing literature findings, including associations found by our group.

Gene therapy could be the next step in preventing ototoxicity. Gene therapy is a new and innovative process that has been successful in animal studies thus far. It has shown to be effective in targeting specific genes within the cochlea. [114] With a clearer understanding of the role of genetic variants on susceptibility to ototoxicity, gene therapy could potentially be used in tandem with genetic profiling to prevent platinum-induced hearing-loss.

References

[1] Ries LAG, Smith MA, Gurney JG, et al. (eds). Cancer Incidence and Survival among Children and Adolescents: United States SEER Program 1975-1995. National Cancer Institute, SEER Program. NIH Pub. No. 99-4649. Bethesda, MD; 1999.

[2] Howlader N, Noone AM, Krapcho M, et al. (eds). SEER Cancer Statistics Review, 1975-2011, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2011/ , based on November 2013 SEER data submission, posted to the SEER web site, April 2014.

[3] Rybak, L. P. Mechanisms of cisplatin ototoxicity and progress in otoprotection. *Curr Opin Otolaryngol Head Neck Surg* **15**, 364-369, doi:10.1097/MOO.0b013e3282eee45200020840-200710000-00014 [pii] (2007).

[4] Langer. T. Understanding Platinum-Induced Ototoxicity. Trends in Pharmacological Sciences. Volume 34, Issue 8, Augist 2013, p.458-469

[5] Orgel. Hearing loss among survivors of childhood brain tumors treated with an irradiation-sparing approach. Pediatric Blood and Cancer 58(6):953 Wiley 2012 1545-5009

[6] Huang, R. S. & Dolan, M. E. Approaches to the discovery of pharmacogenomic markers in oncology: 2000-2010-2020. *Pharmacogenomics* **11**, 471-474, doi:10.2217/pgs.10.11 (2010).

[7] http://www.ssc.education.ed.ac.uk/courses/deaf/dnov10i.html

[8] Gelfand SA. Essentials of audiology. New York: Thieme 2009

[9] Cole EB, Flexer C. Children with Hearing Loss – Developing Hearing and Talking. Birth to Six. Plural Publishing. 2007

[10] Hearing Health Clinics. http://www.hhclinics.com/facts.php

[11] Bess FH, Humes L. Audiology : the fundamentals. Philadelphia: Lippincott Williams & Wilkins; 2008.

[12] Ashmore, J. Cochlear Outer Hair Cell Motility. Physiological Reviews. Published 1 Janurary 2008 Vol. 88 no. 1, 173-210

[13] Hudspeth AJ. How the ear's works work. *Nature.* 1989;341(6241):397- 404.

[14] Raphael Y, Altschuler RA. Structure and innervation of the cochlea. Brain Res Bull. Jun 15 2003;60(5-6):397-422.

[15] Hain TC, Helminski JO. Chapter 1: Anatomy and Physiology of the Normal Vestibular System. *Vestibular Rehabilitation 3rd Edition.* 3 ed: F.A. Davis Co.; 2007.

[16] Tascioglu AB. Brief review of vestibular system anatomy and its higher order projections. *Neuroanatomy.* 2005;4:24-27.

[17] Paul, Peter V., Whitelaw, Gail M. Hearing and Deafness – An Introduction for Health and Educational Professionals. Jones and Bartlett Publishers 2011.

[18] Ramirez-Camacho R, Fernandez DE, Verdaguer JM, et al. Cisplatin- induced hearing loss does not correlate with intracellular platinum concentration. Acta Otolaryngol. May 2008;128(5):505-509.

[19] Sluyter S, Klis SF, de Groot JC, Smoorenburg GF. Alterations in the stria vascularis in relation to cisplatin ototoxicity and recovery. Hearing research. Nov 2003;185(1-2):49-56.

[20] Cardinaal RM, de Groot JC, Huizing EH, Veldman JE, Smoorenburg GF. Dose-dependent effect of 8-day cisplatin administration upon the morphology of the albino guinea pig cochlea. *Hearing research.* Jun 2000;144(1-2):135-146.

[21] van Ruijven MW, de Groot JC, Smoorenburg GF. Time sequence of degeneration pattern in the guinea pig cochlea during cisplatin administration. A quantitative histological study. *Hearing research.* Nov 2004;197(1-2):44-54.

[22] de Franceschi CM, Tochetto T, da Silveira AF, Fantinel MR, Algarve TD. Cisplatin effects on guinea pigs: cochlear histology and genotoxicity. *Braz J Otorhinolaryngol.* Nov-Dec 2011;77(6):728-735.

[23] Laurell G, Bagger-Sjoback D. Degeneration of the organ of Corti following intravenous administration of cisplatin. *Acta Otolaryngol.* 1991;111(5):891-898.

[24] Saito T, Aran JM. Comparative ototoxicity of cisplatin during acute and chronic treatment. *ORL J Otorhinolaryngol Relat Spec.* Nov-Dec 1994;56(6):315-320.

[25] Comis, SD, Rhys-Evans, PH, Osborne, MP, Pickles, JO, Jeffries, DJR, Pearse, HAC. Early morphological and chemical changes induced by cisplatin in the guinea pig organ of Corti. J Laryngol Otol 1986;100:1375–83

[26] Stengs CH, Klis SF, Huizing EH, Smoorenburg GF. Cisplatin-induced ototoxicity; electrophysiological evidence of spontaneous recovery in the albino guinea pig. *Hearing research.* Sep 1997;111(1-2):103-113.

[27] Truong MT, Winzelberg J, Chang KW. Recovery from cisplatin-induced ototoxicity: a case report and review. *International journal of pediatric otorhinolaryngology.* Oct 2007;71(10):1631-1638.

[28] Meech RP, Campbell KC, Hughes LP, Rybak LP. A semiquantitative analysis of the effects of cisplatin on the rat stria vascularis. *Hearing research.* Oct 1998;124(1-2):44-59.

[29] Kohn S, Fradis M, Podoshin L, et al. Toxic effects of cisplatin alone and in combination with gentamicin in stria vascularis of guinea pigs. *Laryngoscope.* Jul 1991;101(7 Pt 1):709-716.

[30] Cardinaal RM, de Groot JC, Huizing EH, Veldman JE, Smoorenburg GF. Dose-dependent effect of 8-day cisplatin administration upon the morphology of the albino guinea pig cochlea. *Hearing research.* Jun 2000;144(1-2):135-146.

[31] van Ruijven MW, de Groot JC, Smoorenburg GF. Time sequence of degeneration pattern in the guinea pig cochlea during cisplatin administration. A quantitative histological study. *Hearing research.* Nov 2004;197(1-2):44-54.

[32] Alam SA, Ikeda K, Oshima T, et al. Cisplatin-induced apoptotic cell death in Mongolian gerbil cochlea. *Hearing research.* Mar 2000;141(1-2):28-38.

[33] Sergi, B. Cisplatin ototoxicity in the guinea pig: vestibular and cochlear damage. Hear Res. 2003 Aug;182(1-2):56-64.

[34] Kim, H.J., et al. Role of proinflammatory cytokines in cisplatin-induced vestibular hair cell damage. Head Neck. 2008 Nov; 30 (11): 1445-56

[35] *Platinum-Based Drugs in Cancer Therapy.* New Jersey: Humana Press; 2000.

[36] Barabas K, Milner R, Lurie D, Adin C. Cisplatin: a review of toxicities and therapeutic applications. *Vet Comp Oncol.* Mar 2008;6(1):1-18.

[37] Siegel, R., Naishadhamc, D., & Jemal, A. (2013). Cancer statistics, 2013. CA: A Cancer Journal for Clinicians, 63(1), 11–30.

[38] Chabner B, Longo DL. Cancer chemotherapy and biotherapy principles and practice. 2006

[39] Hellberg V, Wallin I, Eriksson S, et al. Cisplatin and oxaliplatin toxicity: importance of cochlear kinetics as a determinant for ototoxicity. *J Natl Cancer Inst.* Jan 7 2009;101(1):37-4

[40] Eckstein, N. et al. Platinum resistance in breast and ovarian cancer cell lines. Journal of Experimental & Clinical Cancer Research. 2011

[41] Colby C, Koziol S, McAfee SL, Yeap B, Spitzer TR. High-dose carboplatin and regimen-related toxicity following autologous bone marrow transplant. *Bone marrow transplantation.* Mar 2002;29(6):467- 472.

[42] A.H. Rossof, R.E. Slayton, C.P. Perlia. Preliminary clinical experience with cis-diamminedichloroplatinum (II) (NSC 119875 CACP) Cancer, 30. (1972) p. 1451.

[43] Campbell KCM. *Pharmacology and Ototoxicity for Audiologists.* Clifton Park, NY: Delmar, 2007.

[44] Al-Khatib T, Cohen N, Carret AS, Daniel S. Cisplatinum ototoxicity in children, long-term follow up. *International journal of pediatric otorhinolaryngology.* Aug 2010;74(8):913-919.

[45] Peleva E1, Emami N, Alzahrani M, Bezdjian A, Gurberg J, Carret AS, Daniel SJ. Incidence of platinum-induced ototoxicity in pediatric patients in Quebec. Pediatr Blood Cancer. 2014 Nov;61(11):2012-7. doi: 10.1002/pbc.25123. Epub 2014 Jun 29.

[46] Truong, M.T. Recovery from cisplatin-induced ototoxicity: A case report and review. International Journal of Pediatric Otorhinolaryngology. Volume 71, Issue 10, October 2007, P. 1631-1638.

[47] Zuur CL, Simis YJ, Lansdaal PE, et al. Audiometric patterns in ototoxicity of intra-arterial Cisplatin chemoradiation in patients with locally advanced head and neck cancer. *Audiology & neuro-otology.* 2006;11(5):318-330.

[48] P.G. Stelmachowicz, A.L. Pittman, B.M. Hoover, D.E. Lewis, M.P. Moeller. The importance of high-frequency audibility in the speech and language development of children with hearing loss. Arch. Otolaryngol. Head Neck Surg., 130 (2004), pp. 556–562

[49] Stelmachowicz PG, Pittman AL, Hoover BM, et al. The importance of high-frequency audibility in the speech and language development of children with hearing loss. Arch Otolaryngol Head Neck Surg 2004; 130: 556–562.

[50] Lieu JEC, Tye-Murray N, Karzon RK, et al. Unilateral hearing loss is associated with worse speech-language scores in children.Pediatrics 2010; 125: e1348–e1355.

[51] Bess FH, Dodd-Murphy J, Parker RA. Children with minimal sensorineural hearing loss: Prevalence, educational performance, and functional status. Ear Hear 1998; 19: 339–354.

[52] Gurney JG, Tersak JM, Ness KK, et al. Hearing loss, quality of life, and academic problems in long-term neuroblastoma survivors: A report from the Children's Oncology Group. Pediatrics 2007; 120: e1229–e1236.

[53] M S Gonçalves, A F Silveira, A R Teixeira and M A Hyppolito (2013). Mechanisms of cisplatin ototoxicity: theoretical review. The Journal of Laryngology & Otology, 127, pp 536-541. doi:10.1017/S0022215113000947.

[54] So H, Kim H, Lee JH, et al. Cisplatin cytotoxicity of auditory cells requires secretions of proinflammatory cytokines via activation of ERK and NF-kappaB. J Assoc Res Otolaryngol. Sep 2007;8(3):338-355.

[55] Barreiros, ALBS, David, JM, David, JP. Oxidative stress: relations between the formation of reactive species and the organism's defense [in Brazilian Portuguese]. Quim Nova 2006;29:113–23

[56] Mukherjea, D, Jajoo, S, Whitworth, CA, Bunch, JR, Turner, JG, Rybak, LP et al. Short interfering RNA against transient receptor potential vanilloid-1 attenuates cisplatin-induced hearing loss in the rat. J Neurosci 2008;28:13056–65

[57] Bánfi, B, Malgrange, B, Knisz, J, Steger, K, Dubois-Dauphin, M, Krause, K-H. NOX3, a superoxide-generating NADPH oxidase of the inner ear. J Biol Chem 2004;279:46065–72

[58] Rybak, LP, Mukherjea, D, Jajoo, S, Ramkumar, V. Cisplatin ototoxicity and protection: clinical and experimental studies. Tohoku J Exp Med 2009;219:177–86

[59] Rybak LP. Ototoxicity. Official journal of the International Society of Nephrology. Kidney International (2007) 72, 931–935; doi:10.1038/sj.ki.5002434; published online 25 July 2007

[60] Rybak LP. Mechanisms of cisplatin ototoxicity and progress in otoprotection. Curr Opin Otolaryngol Head Neck Surg. Oct 2007;15(5):364-369.

[61] Biro K, Noszek L, Prekopp P, et al. Characteristics and risk factors of cisplatin-induced ototoxicity in testicular cancer patients detected by distortion product otoacoustic emission. *Oncology.* 2006;70(3):177-184.

[62] Dean JB, Hayashi SS, Albert CM, King AA, Karzon R, Hayashi RJ. Hearing loss in pediatric oncology patients receiving carboplatin- containing regimens. *J Pediatr Hematol Oncol.* Feb 2008;30(2):130-134.

[63] Gunes D, Kirkim G, Demiral P, Uysal KM, Serbetcioglu B, Olgun N. Platinum-Induced

Ototoxicity in Children and Adolescents with Cancer. *Int. Adv. Otol.* 2009;5(3):345-355.

[64] Li Y, Womer RB, Silber JH. Predicting cisplatin ototoxicity in children: the influence of age and the cumulative dose. *Eur J Cancer.* Nov 2004;40(16):2445-2451.

[65] Laurell GF. Combined effects of noise and cisplatin: short- and long-term follow-up. *Ann Otol Rhinol Laryngol.* Dec 1992;101(12):969-976.

[66] Bokemeyer C, Berger CC, Hartmann JT, et al. Analysis of risk factors for cisplatin-induced ototoxicity in patients with testicular cancer. *Br J Cancer.* Apr 1998;77(8):1355-1362.

[67] Dell'Aringa AH, Isaac ML, Arruda GV, et al. Audiological findings in patients treated with radio- and concomitant chemotherapy for head and neck tumors. *Radiat Oncol.* 2009;4:53.

[68] Schacht J. Cisplatin and Aminoglycoside antibiotics: hearing loss and its prevention. Anat Rec. 2012;295:1837-1850

[69] Dionne, F. Economic impact of a genetic test for cisplatin-induced ototoxicity. The Pharmacogenomics Journal (2012) 12, 205–213; doi:10.1038/tpj.2011.15; published online 19 April 2011

[70] Platinol [package insert] Sermoneta-Latina, Italy: Corden Pharma Latina SpA; 2011.

[71] Wyatt, L.E. Cisplatin-Induced Ototoxicity and the Role of Pharmacogenomic Testing. J Pediatr Pharmacol Ther. 2012 Oct-Dec; 17(4): 395-399.

[72] Peters U., et al. Glutathione S-transferase genetic polymorphisms and individual sensitivity to theototoxic effect of cisplatin. Anti-Cancer Drugs. 11 (8) (pp 639-643), 2000. Date of Publication: 2000.

[73] Oldenburg, J., Kraggerud, S. M., Cvancarova, M., Lothe, R. A. & Fossa, S. D. Cisplatin-induced long-term hearing impairment is associated with specific glutathione s-transferase genotypes in testicular cancer survivors. J Clin Oncol 25, 708-714, (2007).

[74] Rednam S. et al. Glutathione S-transferase P1 single nucleotide polymorphism predicts permanent ototoxicity in children with medulloblastoma. Pediatric Blood and Cancer. 60 (4) (pp 593-598), 2013. Date of Publication: April 2013.

[75] 18. Tauris J, Christensen EI, Nykjaer A, Jacobsen C, Petersen CM, Ovesen T. Cubilin and megalin co-localize in the neonatal inner ear. Audiol. Neurootol. 2009; 14(4):267–278.

[76] Mukherjea D., et al. Pharmacogenomics of cisplatin-induced ototoxicity. Pharmacogenomics. Jul 2011; 12(7): 1039–1050.

[77] Riedemann, L. et al. Megalin genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin. Pharmacogenomics J 8, 23-28, doi:6500455 [pii]10.1038/sj.tpj.6500455 (2008).

[78] Choeyprasert W. et al. Cisplatin-induced ototoxicity in pediatric solid tumors: The role of glutathione S-trasnferases and megalin genetic polymorphisms. Journal of Pediatric Hematology/ Oncology. 35 (4) (pp e138-e143), 2013.

[79] Penelope R Brock, et al. Platinum-Induced Ototoxicity in Children: A Consensus Review on Mechanisms, Predisposition, and Protection, Including a New International Society of Pediatric Oncology Boston Ototoxicity Scale. Journal of Clinical Oncology 07/2012; 30(19):2408-2417

[80] Caronia, D. et al. Common variations in ERCC2 are associated with response to cisplatin chemotherapy and clinical outcome in osteosarcoma patients. Pharmacogenomics J 9, 347-353, doi:tpj200919 [pii]10.1038/tpj.2009.19 (2009).

[81] Wilson MD, Ruttan CC, Koop BF, Glickman BW. ERCC1: a comparative genomic perspective. Environ Mol Mutagen, 38: 209-15, 2001.

[82] Zhou W, Gurubhagavatula S, Liu G, Park S, Neuberg DS, Wain JC et al. Excision repair cross-complementation group 1 polymorphism predicts overall survival in advanced non-small cell lung cancer patients treated with platinum-based chemotherapy. Clin Cancer Res 2004; 10: 4939–4943.

[83] Am Zehnhoff-Dinnesen. Pharmacogenomics link rs11615 of ERCC1 to cisplatin-induced ototoxicity. Pediatric Blood and Cancer. Conference: 45th Congress of the International Society of Paediatric Oncology, SIOP 2013 Hong Kong China. Conference Publication: (var.pagings). 60 (pp 164), 2013. Date of Publication: September 2013.

[84] Roco, A. et al. Can pharmacogenomics explain efficacy and safety of cisplatin pharmacotherapy? Front. Genet., 14 November 2014. Doi 10.3389/fgene.2014.00391

[85] Debashree Mukherjea, Leonard P Rybak. Pharmacogenomics of cisplatin-induced ototoxicity. Pharmacogenomics. Jul 2011; 12(7): 1039–1050.

[86] Pussegoda. Replication of TPMT and ABCC3 genetic variants highly associated with cisplatin-induced hearing loss in children. Clinical Pharmacology and Therapeutics 94(2):243, 2013, 0009-9236

[87] C.J. Ross, et al. Genetic variants in TPMT and COMT are associated with hearing loss in children receiving cisplatin chemotherapy. Nat. Genet., 41 (2009), pp. 1345–1349

[88] Yang JJ. et al. The role of inherited TPMT and COMT genetic variation in cisplatin-induced ototoxicity in children with cancer. Clin Pharmacol Ther. 2013 Aug;94(2):252-9. doi: 10.1038/clpt.2013.121. Epub 2013 Jun 11

[89] Hagleitner MM et al. Influence of Genetic Variants in TMPT and COMT Associated with Cisplatin-Induced Hearing-Loss in Patients with Cancer: Two New Cohorts and a Meta-Analysis Reveal Significant Heterogeneity between Cohorts. 2014 Dec 31;9(12):e115869. doi: 10.1371/journal.pone.0115869. eCollection 2014.

[90] B. Delprat et al. Downregulation of otospiralin, a novel inner ear protein, causes hair cell degeneration and deafness. J. Neurosci., 22 (2002), pp. 1718–1725

[91] A. Caravelli.Down-regulation of otospiralin mRNA in response to acoustic stress in guinea pig Hear. Res., 198 (2004), pp. 36–40

[92] Xian-Lu Zhuo. Adenoviral-mediated up-regulation of Otos, a novel specific cochlear gene, decreases cisplatin-induced apoptosis of cultured spiral ligament fibrocytes via MAPK/mitochondrieal pathway. Toxicology Volume 248, Issue 1, 3 June 2008, p.33-38

[93] Spracklen TF. Genetic Variation is Otos is associated with cisplatin-induced ototoxicity. Pharmacogenomics. 2014 Sep;15(13):1667-76. doi: 10.2217/pgs.14.112.

[94] Xu X., et al: Prediction of copper transport protein 1 (. Lung Cancer 2012; 77: pp. 438-442

[95] Rabik CA, Dolan ME. Molecular mechanisms of resistance and toxicity associated with platinating agents. Cancer Treatment Reviews. 2007; 33(1):9-23

[96] Rajendran V, Roy FG. Comparison of health related quality of life of primary school deaf children with and without motor impairment. Italian journal of pediatrics. 2010;36:75.

[97] Umansky AM, Jeffe DB, Lieu JEC. The HEAR-QL: Quality of Life Questionnaire for Children with Hearing Loss. Journal of the American Academy of Audiology. 2011;22(10):644-653.

[98] McKeage MJ. Comparative adverse effect profiles of platinum drugs. Drug Saf. 1995;13:228–244.

[99] Li Y, Womer RB, Silber JH. Predicting cisplatin ototoxicity in children: the influence of age and the cumulative dose. Eur. J. Cancer. 2004;40:2445–2451.

[100] Coradini PP, Cigana L, Selistre SG, Rosito LS, Brunetto AL. Ototoxicity from cisplatin therapy in childhood cancer. J. Pediatr. Hematol. Oncol. 2007;29:355–360. [PubMed]

[101] Durrant SAFMTJWKRBSMFKSHVAMJMTJ. Guidelines for the audiologic management of individuals receiving cochleotoxic drug therapy. ASHA, 36 (1994), p. 11

[102] Chang KW, Chinosornvatana N. Practical grading system for evaluating cisplatin ototoxicity in children. J Clin Oncol 2010;28:1788–1795.

[103] Chang, W. Clinically accurate assessment and grading of ototoxicity. The laryngoscope, Volume 121 Issue 12, p.2649-2657, December 2011

[104] Hu Z, Wang Y, Wang X, Liang G, Miao X, Xu Y et al. DNA repair gene XPC genotypes/haplotypes and risk of lung cancer in a Chinese population. Int J Cancer 2005; 115: 478–483.

[105] Vogel U, Overvad K, Wallin H, Tjønneland A, Nexø BA, Raaschou- Nielsen O et al. Combinations of polymorphisms in XPD XPC and XPA in relation to risk of lung cancer. Cancer Lett 2005; 222: 67–74.

[106]Zhang Y, Wang X, Zhang W, Gong S. An association between XPC Lys939Gln polymorphism and the risk of bladder cancer: a meta-analysis. Tumour Biol. 2013 Apr;34(2):973-82. doi: 10.1007/s13277-012-0633-7. Epub 2012 Dec 27.

[107] Khan SG, Metter EJ, Tarone RE, Bohr VA, Grossman L, Hedayati M et al. A new xeroderma

pigmentosum group C poly (AT) insertion/deletion polymorphism. Carcinogenesis 2000; 21: 1821–1825.

[108] Zhu Y, Yang H, Chen Q, Lin J, Grossman HB, Dinney CP et al. Modulation of DNA damage/DNA repair capacity by XPC polymor- phisms. DNA Repair (Amst) 2008; 7(2): 141–148.

[109] Caronia D, Patino-Garcia A, Milne RL, et al. Common variations in ERCC2 are associated with response to cisplatin chemotherapy and clinical outcome in osteosarcoma patients. Pharmacogenomics J. Oct 2009;9(5):347-353.

[110] May P, Woldt E, Matz RL, Boucher P. The LDL receptor related protein (LRP) family: an old family of proteins with new physiological functions. Ann. Med. 2007;39:219–228. [PubMed]

[111] *Tauris J, Christensen EI, Nykjaer A, Jacobsen C, Petersen CM, Ovesen T. Cubilin and megalin co-localize in the neonatal inner ear. Audiol. Neurootol. 2009;14(4):267–278. [PubMed]*

[112] Koenig O, Ruettiger L, Mueller M, et al. Estrogen and the inner ear: megalin knockout mice suffer progressive hearing loss. FASEB J. 2008;22:410–417. [PubMed]

[113] Riedemann, L. *et al.* Megalin genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin. *Pharmacogenomics J* **8**, 23-28, doi:6500455 [pii]10.1038/sj.tpj.6500455 (2008).

[114] Waissbluth, S. Gene Therapy for Cisplatin-Induced Ototoxicity: A Sytematic Review of In Vitro and Experimental Animal Studies. Ototlogy & Neurotology. Issue: Volume 33(3), April 2012, p. 302-310.

Appendix 1: Gene Review Search Strategy

Embase 1996 to 2015 Week 02

#	Searches	Results	Search Type
18	limit 17 to updaterange="emef(20150109074836-20150109074836]"	0	Advanced
17	limit 16 to (infant <to one year> or child <unspecified age> or preschool child <1 to 6 years> or school child <7 to 12 years> or adolescent <13 to 17 years>)	23	Advanced
16	4 and 9 and 15	115	Advanced
15	10 or 11 or 12 or 13 or 14	238800	Advanced
14	single nucleotide polymorphism.mp.	102257	Advanced
13	SNP.ti,ab.	46774	Advanced
12	dna polymorphism/ or genetic polymorphism/ or single nucleotide polymorphism/	210035	Advanced
11	(Pharmacogenetic* or toxicogenetic* or pharmacogenomic*).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]	25474	Advanced
10	pharmacogenetics/ or pharmacogenomics/ or toxicogenetics/	19993	Advanced
9	5 or 6 or 7 or 8	148337	Advanced
8	carboplatin.mp. or carboplatin/	43385	Advanced
7	cisplatin/ or cisplatin.mp.	104049	Advanced
6	Platinum.mp.	39178	Advanced
5	platinum derivative/	5946	Advanced
4	1 or 2 or 3	29351	Advanced
3	ototoxic*.mp.	7274	Advanced
2	ototoxicity/	6114	Advanced
1	hearing impairment/ or bilateral hearing loss/ or functional hearing loss/ or high frequency hearing loss/	22798	Advanced

Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily, Ovid MEDLINE(R) and Ovid OLDMEDLINE(R) 1946 to Present

#	Searches	Results	Search Type
20	limit 19 to updaterange="pmoz(20150109130404-20150111001210]"	0	Advanced
19	limit 18 to ("infant (1 to 23 months)" or "preschool child (2 to 5 years)" or "child (6 to 12 years)" or "adolescent (13 to 18 years)" or "young adult (19 to 24 years)")	19	Advanced
18	4 and 9 and 17	170	Advanced
17	10 or 11 or 12 or 13 or 14 or 15 or 16	182107	Advanced
16	genetic variation.ti,ab.	23293	Advanced
15	SNP.ti,ab.	34444	Advanced
14	Polymorphism, Single Nucleotide/	67707	Advanced
13	genetic phenomena/ or genetic variation/	87975	Advanced
12	pharmacogenomic*.mp.	4215	Advanced
11	(Pharmacogenetic* or toxicogenetic*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]	13130	Advanced
10	pharmacogenetics/ or toxicogenetics/	9353	Advanced
9	5 or 6 or 7 or 8	84357	Advanced
8	Platinum/ or Platinum.mp.	31197	Advanced
7	cisplatin.mp.	55735	Advanced
6	carboplatin.mp. or Carboplatin/	13049	Advanced
5	platinum compounds/ or cisplatin/	42135	Advanced
4	1 or 2 or 3	434241	Advanced
3	(toxic or toxicity).ab,ti.	387371	Advanced
2	ototoxic*.mp.	4867	Advanced
1	hearing loss/ or deafness/ or hearing loss, bilateral/ or hearing loss, functional/ or hearing loss, high-frequency/ or hearing loss, sensorineural/	44726	Advanced

Appendix 2: Pharmacogenomic Evaluation of XPC and LRP2 - Methods

2.1 Study design

In this project it is hypothesized that genetic variants of the ERCC2 and LRP2 genes are associated with increased susceptibility to platinum-induced hearing loss following chemotherapy in pediatric cancer patients. To determine whether or not these genetic variants are associated with ototoxicity a mixed prospective/retrospective cohort experimental design was used. The design was chosen because of the large number of participants that could be recruited for the study.

2.2 Study Population

The selection criteria, the recruitment process and the demographic characteristics of the study participants are included in this section.

2.2.1 Selection Criteria

A number of factors were considered, in selecting which participants to recruit, Table 1 shows inclusion criteria for participants. Table 2 shows exclusion criteria for participants.

Table 1. Inclusion Criteria

1	Patients treated with cisplatin and/or carboplatin
2	Treated at CHU Sainte Justine or the Montreal Children's Hospital
3	Able to Provide informed consent

Table 2. Exclusion Criteria

1	Patients with congenital hearing loss
2	With tympanic perforation
3	With persistent otorrhea
4	≥25 years

2.2.2 Recruitment and Sample Size

Patients who met the selection criteria were identified using CHU Sainte-Justine and The Montreal Children's pharmacy lists, which contained information on patients who have undergone, will undergo, or are currently undergoing treatment with cisplatin and/or carboplatin. Hospital databases provided further information on the patient, including when they would be in the oncology clinic. The treating physician, or primary nurse of the patient, introduced the person in charge of recruitment for the study to the patient and their family/legal guardian during a visit to the clinic (initial or follow-up). The patients were

then introduced to the study and explained its purpose and nature. If they agreed to participate, a baseline questionnaire (see appendix 1) was filled out in order get some background information on the patient, and to further ensure that they met the selection criteria. Verbal consent was obtained from the patient if they were able to understand the nature of the study, or by a parent or legal guardian in the event that they were not. A written consent form (see appendix 2) was also read and obtained from the patient if they were over the age of 18, or a parent/legal guardian if the patient was under age, as is deemed necessary by Quebec civil Code (article 21). Patients who needed more time to decide whether to participate or not were given a written consent form and approached again at a subsequent visit to the clinic. Patients who refused participation were not approached a second time.

2.2.3 Demographic characteristics of study participants

Recruited patients' demographic characteristics were collected from patient charts, the database, and from pharmacy records.

2.3 Study Protocol and procedures

In this study there are three groups of patients. Group 1 subjects are those who will be receiving platinum-based chemotherapy, Group 2 subjects are those who are currently undergoing treatment with platinum-based chemotherapy, and Group 3 subjects are those have received platinum based chemotherapy in the past. This section will describe the protocol for each group of patients.

2.3.1 Group I: Subjects who will receive platinum-based chemotherapy:

Assessed for eligibility:

The respective pharmacies at CHU Sainte-Justine and at the Montreal Children's Hospital will identify those patients who are being prescribed carboplatin and/or cisplatin. Patients who meet the eligibility criteria and who consent to participate will be recruited into the study.

Baseline Hearing Evaluation:

Within 7 days prior to the commencement of platinum-based chemotherapy patients will be asked to:

1) Respond to questions about other possible causes of hearing loss as well as other background information (birthdate, age, sex, eye color, ethnicity, etc.)
2) Provide a blood sample for genetic analysis
3) Undergo an air- and bone-conduction audiometry test, performed by a trained technician.

Post-Therapy Evaluation:

Within 14 days after the completion of platinum-based chemotherapy subjects will be asked to:

1) Undergo an air- and bone-conduction audiometry test, performed by a trained technician.

Follow-Up Evaluation:
Six months after the completion of all platinum-based chemotherapy cycles subjects may be asked to:

1) Undergo an air- and bone-conduction audiometry test, performed by a trained technician.

We will also gather information about non-genetic determinants of ototoxicity, including age at diagnosis, cumulative dose of cisplatin and carboplatin, tumour site and stage, and other platinum-induced toxicities (nephrotoxicity, hypomagnesemia and hypocalcemia, gastrointestinal toxicity, myelosuppression, neurotoxicity), other ototoxic medication that they may be taking, and concurrent radiotherapy.

If a blood sample cannot be obtained prior to the start of treatment it can be obtained during treatment or after treatment.

2.3.2 Group 2: Subjects who are currently receiving platinum-based chemotherapy:

Assessed for eligibility:
The respective pharmacies at CHU Sainte-Justine and at the Montreal Children's Hospital will identify those patients who are being prescribed carboplatin and/or cisplatin. Patients who meet the eleigibility criteria and who consent to participate will be recruited into the study.

Hearing Evaluation:
At the time of recruitment the patients will be asked to:

4) Respond to questions about other possible causes of hearing loss, as well as other background information (birthdate, age, sex, eye color, ethnicity, etc.)
5) Provide a blood sample for genetic analysis

Post-Therapy Evaluation:
Within 14 days after the completion of platinum-based chemotherapy subjects will be asked to:

2) Undergo an air- and bone-conduction audiometry test, performed by a trained technician.

Follow-Up Evaluation:
Six months after the completion of all platinum-based chemotherapy cycles subjects were asked to:

2) Undergo an air- and bone-conduction audiometry test, performed by a trained technician.

Chart-Review (retrospective component):
A chart review was conducted to collect data concerning the patients' chemotherapy and audiology tests that had been performed prior to recruitment. It is normal practice for patients receiving platinum-based chemotherapy to undergo air- and bone-conduction

audiometry (this includes baseline and post-therapy hearing evaluations). It was expected that the data in the charts would be accurate and complete.

We will also gather information about non-genetic determinants of ototoxicity, including age at diagnosis, cumulative dose of cisplatin and carboplatin, tumour site and stage, and other platinum-induced toxicities (nephrotoxicity, hypomagnesemia and hypocalcemia, gastrointestinal toxicity, myelosuppression, neurotoxicity), other ototoxic medication that they may be taking, and concurrent radiotherapy.

If a blood sample cannot be obtained prior to the start of treatment it can be obtained during treatment or after treatment.

2.3.3 Group 3: Subjects who have received platinum-based chemotherapy in the past:

Assessed for eligibility:

The respective pharmacies at CHU Sainte-Justine and at the Montreal Children's Hospital will identify those patients who have received platinum based chemotherapy in the past.

Chart-Review (retrospective component):

A chart review was conducted to collect data concerning the patients' chemotherapy and audiology tests that had been performed prior to recruitment. It is normal practice for patients receiving platinum-based chemotherapy to undergo air- and bone-conduction audiometry (this includes baseline and post-therapy hearing evaluations). It was expected that the data in the charts would be accurate and complete.

We will also gather information about non-genetic determinants of ototoxicity, including age at diagnosis, cumulative dose of cisplatin and carboplatin, tumour site and stage, and other platinum-induced toxicities (nephrotoxicity, hypomagnesemia and hypocalcemia, gastrointestinal toxicity, myelosuppression, neurotoxicity), other ototoxic medication that they may be taking, and concurrent radiotherapy.

If a blood sample cannot be obtained prior to the start of treatment it can be obtained during treatment or after treatment.

2.4 Data Collection

2.4.1 Effects of Platinum-Based Chemotherapy on Hearing

In order to evaluate and quantify the effects of platinum-based chemotherapy on hearing, we will compare the results of hearing tests performed at several time-points:

Table 1: Time-points

Baseline evaluation	-performed prior to beginning platinum-based chemotherapy
Post-Therapy evaluation	-performed soon after completion of chemotherapy
Follow-up evaluation	-long-term follow-up evaluation performed at least 6 months after completion of chemotherapy

The hearing evaluations that will be performed include:
Table 2: Hearing evaluations

Hearing test	Purpose
Air- and bone- conduction audiometry	-to determine the hearing thresholds at different frequencies
Distortion Product Otoacoustic Emissions (DPOAE) test	-to evaluate outer hair cell function
Speech, Spatial, and Qualities of Hearing Scale (SSQ)	-a self-assessment tool used to quantify the patient's perceived hearing handicap

2.4.2 Air- and bone-conduction audiometry

Air- and bone-conduction audiometry tests are normally performed in all children who are receiving platinum-based chemotherapy, to monitor changes in hearing.

Audiometry tests determine the hearing thresholds at different frequencies. A threshold is defined as the lowest level at which responses occur at least 50% of the time. The audiometry test consists of two parts: 1) Air-conduction audiometry and 2) Bone-conduction audiometry. Air-conduction audiometry detects both conductive and sensorineural hearing loss, but cannot distinguish between the two. Bone-conduction audiometry is not affected by conductive hearing loss. Hence, we will use bone-conduction audiometry results in determining the incidence of hearing loss. The test will be performed by a trained technician at the hospital.

Hearing loss is determined by comparing the audiograms performed at two time-points, for example Baseline and Post-Therapy, and using the American Speech-Language-Hearing Association (ASHA) criteria for hearing loss. The ASHA criteria for hearing loss is defined as:

(A) 20 dB or greater hearing loss in pure tone threshold in at least one frequency, OR
(B) 10 dB or greater decrease at two adjacent test frequencies, OR
(C) loss of responses at three consecutive frequencies where responses were previously obtained.[11]

2.4.2 Blood Sampling for Genetic Analysis

Sample collection

Blood samples will be collected from patients who consent to the study. Only one sample is required from each patient. A sample can be collected whenever possible. We will collect our sample at the same time as blood is being collected for the patient's cancer care.

5-10ml of venous blood will be collected into a polypropylene tube containing ethylenediamine tetra-acetic acid (EDTA). The tube will be gently inversed several times in order to mix the blood and EDTA.

Sample storage and DNA isolation

Blood Samples will be stored at -70ºC in respective freezers at CHU Sainte-Justine research center and the Montreal Children's Hospital Auditory Sciences Laboratory, until such time that they are delivered, frozen on dry ice, by the study coordinator to the Montreal Heart institute.

At the Montreal Heart Institute laboratory, DNA from the blood samples will be isolated and stored under Good Laboratory Practice conditions.

Bio-Bank Option
As part of the written consent form, subjects were explained and given an "Open Consent" option. If this was signed, DNA that is isolated from their blood sample is kept at the Montreal Heart Institute for up to 50 years in a Bio-Bank. It is to be used for further analysis in experiments concerning ototoxicity. After 50 years all remaining samples are to be de-identified. The link between the research code and the patients will be destroyed. If a closed consent is signed, patients' DNA is used only for this study, and excess cells are disposed of once they are no longer needed for the study's purposes, at which time samples will be de-identified.

Dr. Marie Pierre-Dube (director of the Montreal Heart Institute lab) is responsible for the samples, and will supervise a group of highly-trained technical staff in the handling of them.

2.5 Genetic Analysis

2.5.1 Candidate Gene Approach

We have chosen a candidate gene (CG) approach in order to test our primary hypothesis. In this approach a set of Single Nucleotide Polymorphisms (SNPs) are selected, based on current literature. With the knowledge of specific genes and their biological mechanisms of action, we can deduce which genes might be implicated in the phenotype under study.

There have already been a number of genetic variants, implicated in susceptibility to platinum-induced ototoxicity. The approach used in this study to further verify their implication is one consisting of a limited number of genetic tests. These tests are evaluated at a significance threshold of $p = 0.05$, adjusted for the number of genetic tests tested under the primary hypothesis. The study experts and investigators have determined which SNPs of the target genes will be identified and tested, prior to initiation of statistical analysis. This choice was based on the best available evidence at that time, based on the current literature.

Due to the extensive progress that has been made in the identification and understanding of genetic polymorphisms association with ototoxicity, a candidate gene approach was used to test our primary hypothesis that is limited to a restricted selection of SNPs.

The CG approach was chosen based on our current knowledge of genes and their biological action, which alludes to their possible implication in hearing loss. A hardy Weinberger equilibrium was calculated for each SNP, with adjustment for multiple testing. The null hypothesis is absence of genotypic effect.

Advantages

The approach offers several advantages over several genome wide approaches. Firstly, it significantly increases the coverage of potentially important genes. This decreases the likelihood of false negatives, while also affording the opportunity to test rare variants. As well, the dense coverage allows for the testing of more specific haplotypes, increasing the chances of finding nearby casual variants in linkage disequilibrium (LD). The CG approach offers a means of validating the accumulating reports of genetic predictors of susceptibility to platinum-induced ototoxicity.

Disadvantages

The weakness of this approach lies in the fact that it only allows us to test the genetic variants that we already know.

Appendix 3: Single nucleotide polymorphic (SNP) frequencies of LRP2 and XPC genes

SNP name	Variant	Case	Control
LRP2_rs2075252_AG_R	C/C	26	17
LRP2_rs2075252_AG_R	C/T	21	13
LRP2_rs2075252_AG_R	T/T	5	1
LRP2_rs2075252_AG_R	C	73	47
LRP2_rs2075252_AG_R	T	31	15
LRP2_rs2228171_AG_R	C/C	25	19
LRP2_rs2228171_AG_R	C/T	23	12
LRP2_rs2228171_AG_R	T/T	4	
LRP2_rs2228171_AG_R	C	73	50
LRP2_rs2228171_AG_R	T	31	12
XPC_rs1124303_GT_F	G/T	9	3
XPC_rs1124303_GT_F	T/T	43	28
XPC_rs1124303_GT_F	G	9	3
XPC_rs1124303_GT_F	T	95	59
XPC_rs1126547_CG_F	C/C	1	
XPC_rs1126547_CG_F	C/G	9	9
XPC_rs1126547_CG_F	G/G	42	22
XPC_rs1126547_CG_F	C	11	9
XPC_rs1126547_CG_F	G	93	53
XPC_rs1350344_AG_F	A/A	16	6
XPC_rs1350344_AG_F	A/G	28	13
XPC_rs1350344_AG_F	G/G	8	12
XPC_rs1350344_AG_F	A	60	25
XPC_rs1350344_AG_F	G	44	37
XPC_rs2227999_AG_R	C/C	43	28
XPC_rs2227999_AG_R	C/T	9	2
XPC_rs2227999_AG_R	T/T		1
XPC_rs2227999_AG_R	C	95	58
XPC_rs2227999_AG_R	T	9	4
XPC_rs2228000_CT_R	A/A	4	1
XPC_rs2228000_CT_R	A/G	21	12
XPC_rs2228000_CT_R	G/G	27	18
XPC_rs2228000_CT_R	A	29	14
XPC_rs2228000_CT_R	G	75	48
XPC_rs2228001_AC_R	G/G	6	6
XPC_rs2228001_AC_R	G/T	23	17
XPC_rs2228001_AC_R	T/T	23	8
XPC_rs2228001_AC_R	G	35	29

XPC_rs2228001_AC_R	T	69	33
XPC_rs2279017_GT_F	G/G	23	8
XPC_rs2279017_GT_F	G/T	23	17
XPC_rs2279017_GT_F	T/T	6	6
XPC_rs2279017_GT_F	G	69	33
XPC_rs2279017_GT_F	T	35	29
XPC_rs2470352_AT_R	A/A	28	21
XPC_rs2470352_AT_R	A/T	19	8
XPC_rs2470352_AT_R	T/T	3	1
XPC_rs2470352_AT_R	A	75	50
XPC_rs2470352_AT_R	T	25	10
XPC_rs2607737_CT_F	C/C	6	12
XPC_rs2607737_CT_F	C/T	27	13
XPC_rs2607737_CT_F	T/T	19	6
XPC_rs2607737_CT_F	C	39	37
XPC_rs2607737_CT_F	T	65	25
XPC_rs2733533_AC_F	A/A	17	6
XPC_rs2733533_AC_F	A/C	27	12
XPC_rs2733533_AC_F	C/C	8	13
XPC_rs2733533_AC_F	A	61	24
XPC_rs2733533_AC_F	C	43	38
XPC_rs2733537_CT_R	A/A	26	14
XPC_rs2733537_CT_R	A/G	21	15
XPC_rs2733537_CT_R	G/G	5	2
XPC_rs2733537_CT_R	A	73	43
XPC_rs2733537_CT_R	G	31	19
XPC_rs3731093_CT_R	A/A	50	26
XPC_rs3731093_CT_R	A/G	2	5
XPC_rs3731093_CT_R	A	102	57
XPC_rs3731093_CT_R	G	2	5
XPC_rs3731127_CT_R	A/G	2	2
XPC_rs3731127_CT_R	G/G	50	29
XPC_rs3731127_CT_R	A	2	2
XPC_rs3731127_CT_R	G	102	60
XPC_rs3731143_AG_F	A/A	43	28
XPC_rs3731143_AG_F	A/G	9	2
XPC_rs3731143_AG_F	G/G		1
XPC_rs3731143_AG_F	A	95	58
XPC_rs3731143_AG_F	G	9	4
XPC_rs3731146_AC_F	A/A	23	20

XPC_rs3731146_AC_F	A/C	24	9
XPC_rs3731146_AC_F	C/C	5	2
XPC_rs3731146_AC_F	A	70	49
XPC_rs3731146_AC_F	C	34	13
XPC_rs3731149_AC_R	G/G	5	2
XPC_rs3731149_AC_R	G/T	22	6
XPC_rs3731149_AC_R	T/T	25	23
XPC_rs3731149_AC_R	G	32	10
XPC_rs3731149_AC_R	T	72	52