
Investigating the genetic basis of cisplatin-induced ototoxicity in adult South African patients

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by

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Declaration

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Abbreviations

5-FU: 5-fluorouracil
A: adenine
ABC: ATP-binding cassette
ACYP2: acylphosphatase 2, muscle type
ADA: adenosine deaminase
ADME: drug absorption, distribution, metabolism and elimination
ADR: adverse drug reaction
AFR: 1000 Genomes Project African population
AMPK: AMP-activated protein kinase
AMR: 1000 Genomes Project admixed American population
AP: alkaline phosphatase
AQP: aquaporin
ARE: antioxidant response element
Arntl: aryl hydrocarbon receptor nuclear translocator-like
ASHA: American Speech-Language-Hearing Association
ASN: 1000 Genomes Project East Asian population
ATP: adenosine triphosphate
BA: black African
BCL2: B-cell CLL/lymphoma 2
bp: base pairs
C: cytosine
C2: complement component 2
C3: complement component 3
C5: complement component 5
CAF: Central Analytical Facility
cAMP: cyclic adenosine monophosphate
CAT: catalase
Cau: Caucasian
CCND1: cyclin D1
CDH13: cadherin 13
CFB: complement factor B
CFTR: cystic fibrosis transmembrane conductance regulator
CI: confidence interval

COCH: cochlin
COMT: catechol-O-methyltransferase
Cont.: continued
Corp.: corporation
CPDB: ConsensusPathDB
CREB: cAMP response element-binding protein
CREBBP: CREB binding protein
CTCAE: Common Terminology Criteria for Adverse Events
CTR1: copper transporter 1
CUBN: cubilin
dB: decibel
dB HL: decibels hearing loss
DFNA: deafness, autosomal dominant
DFNB: deafness, autosomal recessive
DFNX: deafness, X-linked
DNA: deoxyribonucleic acid
dNTP: 2`-deoxynucleotide-5`-triphosphate
DP: read depth
DPYD: dihydropyrimidine dehydrogenase
DYX1C1: dyslexia susceptibility 1 candidate 1
EDTA: ethylenediamine tetra-acetic acid
EIF3A: eukaryotic translation initiation factor 3, subunit A
EP300: E1A binding protein p300
ERBB: Erb-B2 receptor tyrosine kinase
ERCC: excision repair cross-complementation group
ERK: extracellular signal-regulated kinase
EUR: 1000 Genomes Project European population
ExoI: exonuclease I
FDA: Food and Drug Administration of America
FLII: flightless I homologue (Drosophila)
FRAS1: Fraser extracellular matrix complex subunit 1
FREM2: FRAS1 related extracellular matrix protein 2
Freq: frequency
G: guanine
GATK: Genome Analysis Toolkit
GJB2: gap junction protein, beta 2
GO: gene ontology

GRB7: growth factor receptor-bound protein 7
GSH: Groote Schuur Hospital
GST: glutathione S-transferase
GWAS: genome-wide association study
Gy: Gray
h: hour/hours
HER2: human epidermal growth factor receptor 2
HO-1: haem oxygenase 1
HREC: Human Research Ethics Committee
HWE: Hardy-Weinberg Equilibrium
Hz: hertz
IBM: Integrated Business Machines
ID: identifier
IDT: Integrated DNA Technologies
IFN: interferon
Inc.: incorporated
Ind: Indian
ING1: inhibitor of growth family, member 1
JNK: c-Jun N-terminal kinase
KEAP1: kelch-like ECH-associated protein 1
kHz: kilohertz
LARP1B: La ribonucleoprotein domain family, member 1B
LD: linkage disequilibrium
LRP2: low-density lipoprotein receptor-related 2
LRR: leucine rich repeat
LRRC3B: leucine rich repeat containing 3B
LRRFIP1: leucine rich repeat interacting protein 1
MA: mixed ancestry
MAF: minor allele frequency
MAPK: mitogen-activated protein kinase
Max: maximum
Min: minimum
Mins: minutes
MMR: mismatch repair
mRNA: messenger RNA
MRP: multidrug resistance associated protein
mtDNA: mitochondrial DNA

MT-RNR1: mitochondrially encoded 12S RNA
n: number of individuals
NADPH: dihydronicotinamide-adenine dinucleotide phosphate
NCD: non-communicable disease
NCI: National Cancer Institute
ND: not determinable
NER: nucleotide excision repair
NFE2L2: nuclear factor, erythroid 2-like 2
NF-κB: nuclear factor-kappaB
NGS: next-generation sequencing
No.: number
NOS3: nitric oxide synthase 3
NOX: NADPH oxidase
NRG1: neuregulin 1
OCT: organic cation transporter
OMIM: Online Mendelian Inheritance in Man
OR: odds ratio
OTOS: otospiralin
p: p-value
PCA: principal component analysis
PCR: polymerase chain reaction
PELP1: proline, glutamate and leucine rich protein 1
PER1: period 1
PGM2: phosphoglucomutase 2
PITX2: paired-like homeodomain 2
PNP: purine nucleoside phosphorylase
PolyPhen 2: Polymorphism Phenotyping version 2
RBC: red blood cell
Rcf/g: relative centrifugal force/g-force
Ref: reference allele
RELA: V-rel avian reticuloendotheliosis viral oncogene homologue A
RNA: ribonucleic acid
ROS: reactive oxygen species
Rpm: revolutions per minute
RT: radiation therapy
s: seconds
SA: South Africa/South African

SAM: S-adenosylmethionine
SAN: 1000 Genomes South Asian population
SDS: sodium dodecyl sulphate
SIFT: Sorting Intolerant From Tolerant
SIOP: International Society of Paediatric Oncology Boston
siRNA: silencing RNA
SLC22A1: solute carrier family 22, member 2
SLC22A2: solute carrier family 22, member 2
SLC26A4: solute carrier family 26 (anion exchanger), member 4
SLX4: SLX4 structure-specific endonuclease subunit
SNP: single nucleotide polymorphism
SNV: single nucleotide variant
SOD2: superoxide dismutase 2
T: thymidine
T_a: annealing temperature
TBE: Tris/borate/EDTA
TE: Tris/EDTA
TIMP: tissue inhibitor of metalloproteinase
TIMP3: TIMP metallopeptidase inhibitor 3
TP53: tumour protein P53
TPMT: thiopurine S-methyltransferase
TRP: transient receptor potential
UCSC: University of California, Santa Cruz
UCT: University of Cape Town
UK: United Kingdom
USA: United States of America
Var: variant allele
VCF: variant call format
WES: whole-exome sequencing
WFS1: Wolfram syndrome 1
WHO: World Health Organisation
w/v: weight per volume
XPC: xeroderma pigmentosum, complementation group C
ZFN: zinc finger nuclease

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Abstract

Cisplatin, a potent chemotherapeutic agent, is widely used in the treatment of numerous soft-tissue cancers. Although high cure rates can be achieved when cisplatin is incorporated in chemotherapy regimens, the therapeutic utility of the drug may be limited by the development of dose-limiting adverse reactions in patients. A prevalent reaction to cisplatin is ototoxicity, or drug-induced hearing loss, which occurs when the drug accumulates in and damages cells of the inner ear, leading to permanent and progressive hearing impairment. In this investigation, two approaches were employed to explore the role of genetics in cisplatin response amongst South African cancer patients (n = 214). Using a candidate gene approach, which investigated variants in six genes which are involved in drug transport and processing, potential modifiers in the genes *nuclear factor, erythroid 2-like 2* (*NFE2L2*) and *solute carrier family 22, member 2* (*SLC22A2*) were identified. *SLC22A2* encodes a known transporter of cisplatin, and the variant rs316019 conferred potentially protective effects against Chang- and TUNE-graded ototoxicity through a reduced transport of the drug ($p = 0.039$ and $p = 0.031$, respectively). Similarly, the variant *NFE2L2* rs6721961 was possibly protective, as it occurred more frequently in patients who did not develop hearing impairment according to four different ototoxicity grading scales during high-dose (≥ 200 mg/m^2) cisplatin treatment (ASHA, $p = 0.001$; Chang, $p = 0.022$; CTCAE, $p = 0.001$; TUNE, $p = 0.028$). When supplementing the prospective cohort with retrospective patient data, an increased susceptibility of indigenous African patients to Chang grade > 0 ototoxicity was observed ($p = 0.001$). For this reason, whole-exome sequencing was conducted on a subset of the patient cohort (n = 11), focussing on individuals of African origin who represented the phenotype extremes. Potential genetic modifiers were identified in genes involved in various biological processes, including transmembrane transport, development, hearing, the response to DNA damage, immune reactions and signalling pathways, implicating many previously unreported genes in the cellular response to cisplatin as well as its ototoxicity. The results reported in this study indicate that genetic information can improve predictive models of cisplatin response, although there are many novel genes which should be explored in the South African population. Identifying these genetic modifiers, such as those in *SLC22A2* and *NFE2L2*, has the potential to further our understanding of this adverse drug reaction, and may assist in the future personalisation of treatment plans in the management of cancer.

1. Introduction

1.1 Cancer

Non-communicable diseases (NCDs) can affect individuals from any age group and any geographical region. It is noteworthy then that up to 80% of all NCD-related deaths, and 90% of those before the age of 60 years, occur in developing countries (World Health Organisation [WHO], 2012). One of the most prevalent NCDs is cancer, a disease characterised by uncontrolled cell division and growth, leading to the development of tumours (Marx, 1994).

While the causes of cancer are diverse, chromosomal instability (Boyer et al, 1995) and genetic mutations (Salk et al, 2010) are usually involved, and lead to the formation of immortal neoplastic cells. These cells are able to evade cell death pathways and the human immune system, and may metastasise to other organs in the body (Hanahan and Weinberg, 2011). Although tumorous tissue can be surgically removed in some instances, a multimodal approach to therapy is usually required in the treatment of locally advanced cancers. These antineoplastic regimens may include the utilisation of chemotherapy and radiation therapy (RT) to target and destroy the cancerous cells.

Despite advances in our knowledge of the disease and the development of new methods to treat various tumour types, neoplasia remains a leading cause of mortality worldwide. Approximately 8.2 million deaths in 2012 were cancer-related, 65% of which occurred in developing countries (Globocan, 2012). It is estimated that 12.7 million new cases were diagnosed in 2008 (WHO, 2012) and 14.1 million in 2012 (WHO, 2014). This is projected to reach 22 million new cancer diagnoses by 2030 (WHO, 2012). Cancer is a significant disease burden in South Africa (SA), with more than 100,000 diagnoses and an estimated 40,000 cancer-related deaths every year (Mayosi et al, 2009). Aspects involved in addressing the global cancer burden may include prevention and early detection, but one focus of research is improving the management of newly diagnosed patients. Because the incidence of neoplasia is still increasing, part of this research must explore optimising the use of already existing therapies, for example by targeting treatment to those patients who are more likely to derive benefit (Hertz and McLeod, 2013).

1.2 Adverse drug reactions

An important consideration in the optimisation of therapy is the development of adverse drug reactions (ADRs), which illustrate an aspect of heterogeneity in drug response amongst patients. ADRs are detrimental and unintended reactions to therapy which occur within the approved dosage recommendations (Su et al 2014), and may or may not be accompanied by success of treatment. Adverse reactions to medications have been reported to affect between 6.5% and 14.7% of all patients in international hospitals (Lazarou et al, 1998; Pirmohamed et al, 2004; Davies et al, 2009). Although there is less published data from developing countries such as SA, the prevalence of ADRs can be expected to be comparable (Mehta et al, 2007). ADRs range from relatively mild, reversible reactions to severe, irreversible and, in some instances, fatal drug reactions (Pirmohamed et al, 2004). In addition to an increase in morbidity and mortality amongst patients, severe ADRs can lead to permanent disability and have other consequences on patient quality-of-life (Su et al, 2014). The impact of ADRs in healthcare systems can therefore be considerable, as treatment regimens may require additional methods of monitoring, preventing, or treating ADRs, and they can alter the treatment or lead to its discontinuation.

Most ADRs are influenced by both clinical and demographic factors such as patient age and drug dosages (Alomar, 2014); however, there is still variability in the response to numerous therapeutics which cannot be explained by these factors. Increasing evidence suggests that genetics plays a role in modifying the risk of ADR development (Su et al, 2014), but much of this genetic influence remains uninvestigated. In the prioritisation of ADRs for future genetic research, factors which should be considered include the severity and burden of the ADR, its irreversibility, expected incidence rate, tendency to cause life-long disability, and economic impact (Shaw et al, 2013). Ototoxicity is an adverse reaction to cisplatin therapy which meets many of these prioritisation criteria (Shaw et al, 2013), and is the subject of investigation in this submission.

1.3 Cisplatin

Cisplatin (*cis*-diamminedichloroplatinum(II), trade name Platinol) is a potent antineoplastic agent. Platinum-based compounds were originally discovered to have chemotherapeutic properties in the 1970s, when they were able to successfully regress large sarcoma tumours in mouse models (Rosenberg and VanCamp, 1970). Since its introduction into clinical practice, cisplatin has been effectively administered in the first-line treatment and management of a diversity of soft-tissue cancer types (McKeage, 1995).

In treatment regimens, cisplatin is often combined with RT and other chemotherapeutics such as etoposide, bleomycin, vincristine, doxorubicin and 5-fluorouracil (5-FU) (Homesley et al, 1999; Ortega et al, 2000). The incorporation of cisplatin into combination therapies improved the cure rate of testicular cancer from 5% to over 90% (Einhorn, 2002), and significantly lengthened progression-free survival in advanced cervical cancer patients (Rose et al, 1999). Cisplatin-containing regimens have been successfully used in the treatment of many other neoplasms, such as ovarian, lung, head and neck, bladder, and oesophageal cancers, where high cure rates are often achieved (McKeage, 1995).

1.3.1 Cisplatin's mechanism of action

Cisplatin is administered intravenously to patients and is circulated throughout the body by the cardiovascular system. In the blood, cisplatin remains a neutral molecule, but is converted into an aquated and cytotoxic form upon entry into cancer cells. The uptake of cisplatin occurs largely via passive diffusion (Gale et al, 1973), although transporters such as copper transporter 1 (CTR1) have been demonstrated to actively facilitate this process (Holzer et al, 2004). The aquated form of cisplatin is cationic and highly reactive, and in this form induces extensive cellular damage, binding to proteins (Akaboshi et al, 1992; Neault et al, 2001), membrane phospholipids (Speelmans et al, 1997), cytoskeletal microfilaments (Christen et al, 1993), RNA (Hostetter et al, 2012), and DNA (Akaboshi et al, 1992), as well as causing mitochondrial dysfunction and calcium efflux (Aggarwal, 1993).

Although the exact mechanism of cisplatin's cytotoxicity has not yet been established, it is general consensus that genomic DNA is the primary target of the drug. Platinum-based compounds have been shown to interact strongly with DNA, causing the formation of inter- and intra-strand crosslinks by covalently binding to purine bases (Plooy et al, 1984; Fichtinger-Schepman et al, 1985). These platinum-DNA adducts distort and induce structural damage to the DNA helix, including bending (Rice et al, 1988), unwinding (Scovell and Collart, 1985), and the formation of double-stranded breaks (Sorenson and Eastman, 1988). The inhibition of DNA replication and transcription has also been reported after cisplatin treatment (Johnson et al, 1980; Sorenson and Eastman, 1988), and may be due to the inability of polymerase enzymes to circumvent cisplatin-DNA adducts, or the interaction between cisplatin and proteins involved in these processes. Although the response to platinum-induced DNA damage is so far unclear, numerous proteins recognise and bind to the DNA adducts. These include those involved in DNA repair processes, namely nucleotide excision repair (NER) and mismatch repair (MMR) proteins, as well as those which play a

role in the regulation of transcription and replication, signal transduction and apoptosis (Jung and Lippard, 2007).

These effects in the nucleus lead to arrest of the cell cycle in the G_2 phase (Cullinane et al, 1999), and the activation of cell death pathways (Persons et al, 1999). Cisplatin retains its cytotoxicity in enucleated cells, implying that nucleus-independent pathways may also be involved in the drug's mechanism of action (Yu et al, 2008). Although cisplatin has been reported to induce necrosis at high concentrations *in vitro* (Lieberthal et al, 1996), the drug exerts its chemotherapeutic properties primarily through the activation of apoptosis (Persons et al, 1999). The mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways are stimulated following cisplatin treatment, and may mediate the decision between two different cell fates: DNA repair and cell survival, or the induction of apoptosis (Wang et al, 2000; Mansouri et al, 2003; Bragado et al, 2007).

1.3.2 Adverse reactions to cisplatin therapy

The effective use of cisplatin in chemotherapy can be limited by the development of ADRs in the patient, which include nephrotoxicity, neurotoxicity, and ototoxicity (McKeage, 1995), as well as anaemia, nausea, and acute, delayed or anticipatory emesis (Levi et al, 1981; McKeage, 1995). These ADRs occur when cells other than cancer cells accumulate the drug and are damaged. Nephrotoxicity, once the major dose-limiting ADR of cisplatin, can be largely prevented by hydration 24 hours before treatment (Santoso et al, 2003), while nausea and emesis can be ameliorated with antiemetic therapy (Hu et al, 2014a). For cisplatin-induced ototoxicity, however, no effective methods of prevention or treatment currently exist.

1.3.2.1 Cisplatin-induced ototoxicity

Ototoxicity is the progressive cellular degeneration of inner ear structures due to the cytotoxic effects of therapeutic agents in the region. This results in the development of sensorineural hearing loss (cochleotoxicity) and/or vestibular dysfunction (vestibulotoxicity) in patients, and is a known ADR of many drugs such as aminoglycoside antibiotics (Xie et al, 2011), loop diuretics (Gallagher and Jones, 1979), antimalarials (Jourde-Chiche et al, 2012) and platinum-containing chemotherapeutics (Ding et al, 2012).

Cisplatin is considered the most ototoxic of the platinum-based chemotherapeutics (McKeage, 1995; Ding et al, 2012). Reported incidences of hearing loss range between 25% and 100% of patients affected (Kopelman et al, 1988; Bokemeyer et al, 1998; De Jongh et al, 2003; Knight et al, 2005; Kushner et al, 2006; Coradini et al, 2007; Arora et al, 2009; Yancey et al, 2012; Choeyprasert et al, 2013; Yang et al, 2013; Peleva et al, 2014), depending on the audiometric criteria used, the sensitivity of the tests performed and, possibly, on the age of the patients being treated. Using conventional audiometry (0.25-8 kHz), significant hearing loss typically affects less than 50% of adult cancer patients (Bokemeyer et al, 1998; De Jongh et al, 2003). However, up to 100% of patients may develop some degree of hearing loss when more sensitive hearing tests (8-16 kHz) are employed (Kopelman et al, 1988; Arora et al, 2009). A retrospective analysis of SA cisplatin-receiving cancer patients at Groote Schuur Hospital (GSH) by our research group indicated an ototoxicity incidence rate of 55% using standard audiometry (Whitehorn et al, 2014a).

Hearing impairment due to cisplatin chemotherapy is typically progressive, bilateral, and irreversible in nature, and may present with other symptoms such as ear pain and tinnitus (Arora et al, 2009). In cisplatin-induced ototoxicity, the higher frequencies of hearing (6-8 kHz) are initially affected, but over repeated doses this hearing loss can progress into lower frequencies (Kopelman et al, 1988), including the speech frequencies of hearing (0.5-4 kHz) (de Andrade et al, 2013). Notably, as many as 50% of consonants spoken in the English language are heard in the 4-8 kHz range, and these are required for the understanding of spoken language (Grewal et al, 2010). As a result, the development of ototoxicity can have long-term impacts on patient quality-of-life. Hearing loss can impair verbal-auditory communication and have educational, social and economic consequences (Knight et al, 2005; Einarsson et al, 2011). These effects are particularly pronounced in paediatric patients, in whom hearing impairment can cause difficulty in language acquisition and the ability to thrive, both academically and socially (Bess et al, 1998; Al-Khatib et al, 2010).

Because of these quality-of-life considerations, ototoxicity can have an impact on the success of treatment. When hearing loss occurs, the dose of cisplatin may be reduced, or cisplatin treatment may be discontinued in favour of a less ototoxic, but often less effective drug, such as carboplatin. Carboplatin has demonstrated inferiority in terms of both overall and progression-free survival, response rate and the incidence of relapse when compared to cisplatin in the treatment of bladder, head and neck, lung, and germ cell cancers (Lokich and Anderson, 1998; Hotta et al, 2004; Sanborn, 2008). For other cancers, such as those of the ovaries, the efficacy of carboplatin may be equivalent, although less established (Lokich and Anderson, 1998).

1.3.2.2 Mechanism of cisplatin-induced ototoxicity

The inner ear is composed of the cochlea and the vestibular apparatus (Figure 1.1). The vestibular apparatus is involved in balance, while the cochlea functions in auditory perception. Both of these structures are sensitive to cisplatin, and ototoxicity may occur when they are damaged during treatment.

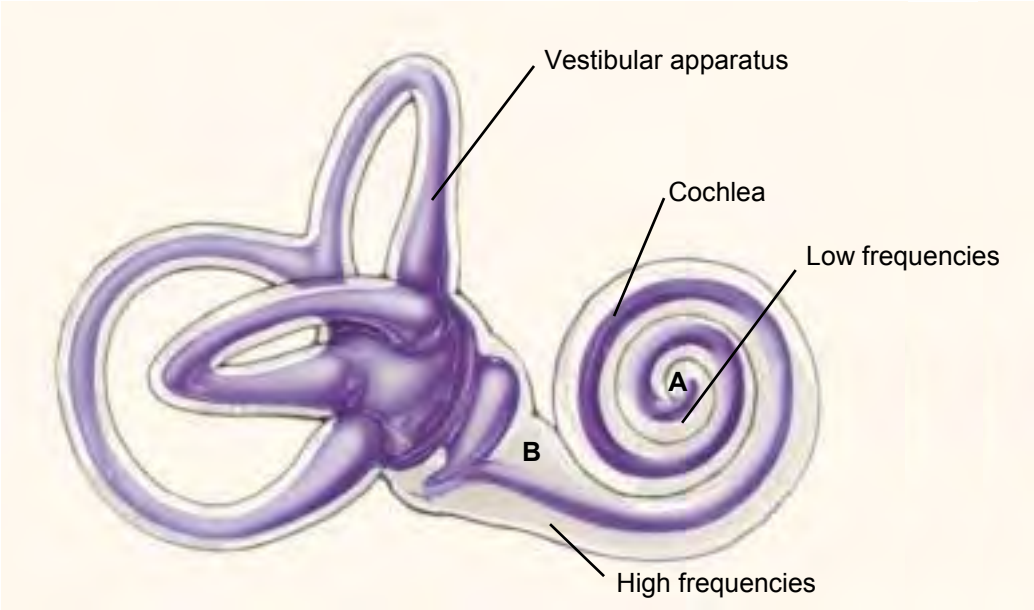


Figure 1.1: Diagram of inner ear structures. The inner ear is composed of the cochlea and the vestibular apparatus. The cochlea, involved in hearing, has a basal (B) and an apical (A) turn. High frequencies are detected at the basal turn of the cochlea, and progressively lower frequencies are perceived at the more apical regions (adapted from Willems, 2000).

Experiments in guinea pigs demonstrated an early accumulation and rapid saturation of the drug in the cochlea, as well as its delayed elimination from inner ear structures (Hellberg et al, 2013). Cochlear cells which display the greatest sensitivity to cisplatin are those in the organ of Corti (particularly the outer hair cells and supporting cells), the spiral ganglion, the stria vascularis, and the spiral ligament (Alam et al, 2000; Van Ruijven et al, 2005) (Figure 1.2). The vestibular apparatus contains a population of localised stem cells, and although these provided promise for curative interventions (for example, the stimulation of these stem cells to differentiate and replace damaged cells), it has been demonstrated that these cells are also vulnerable to cisplatin treatment (Slattery et al, 2014).

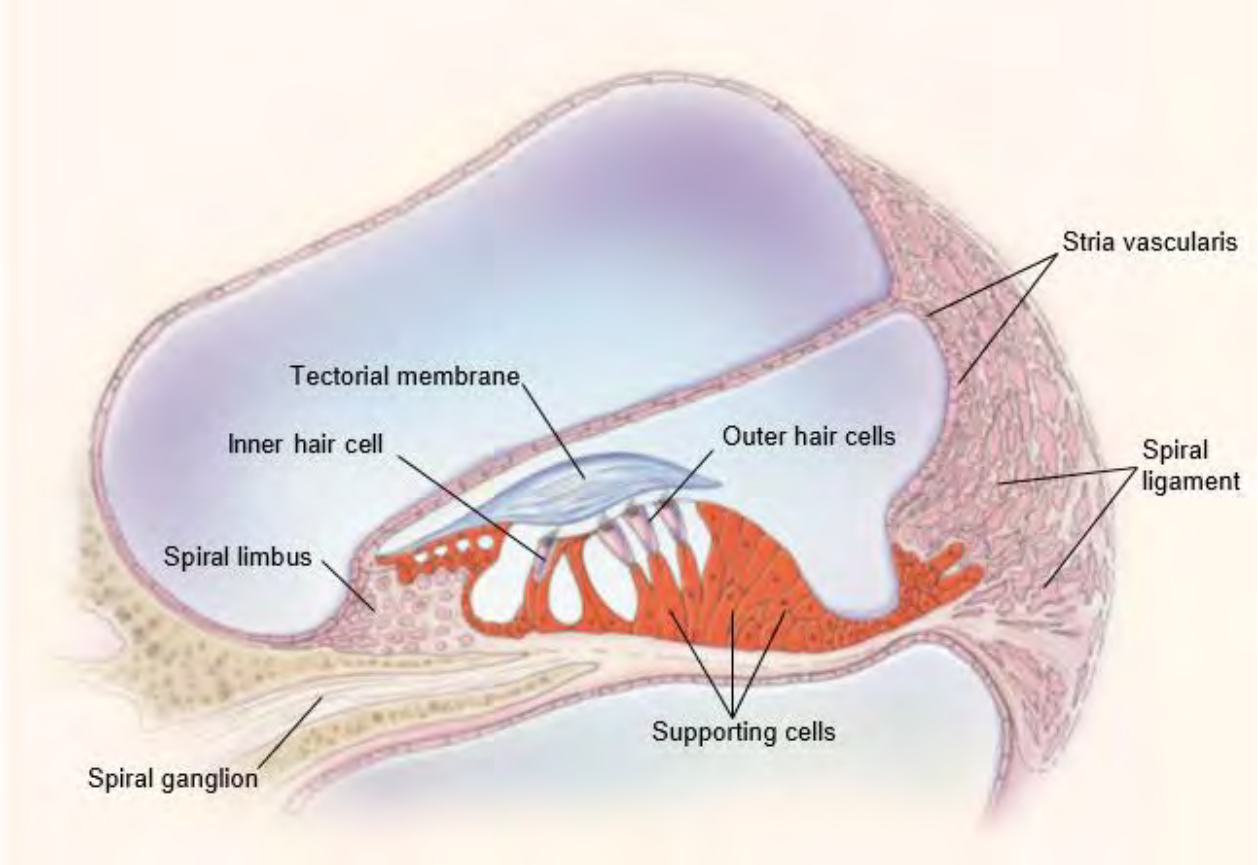


Figure 1.2: Cross-section through the human cochlea. Cells which are sensitive to cisplatin are indicated. These include the outer hair cells as well as those of the spiral ganglion, spiral ligament and stria vascularis (adapted from Willems, 2000).

Cisplatin exerts its cytotoxic effects in these cells through the mechanisms already discussed (Section 1.3.1). However, cisplatin-induced oxidative stress is an important contributor to apoptosis in the inner ear. Oxidative stress occurs when the redox balance in cells is disturbed, for example by the accumulation of reactive oxygen species (ROS) and free radicals such as superoxide anions, hydroxyl radicals, and hydrogen peroxide and its derivatives. These compounds can cause extensive damage to DNA, proteins, lipids, and organelles in the cell (Yakes and Van Houten, 1997). Consequently, prolonged oxidative stress results in DNA strand breaks, lipid and protein peroxidation, mitochondrial dysfunction and other cellular insults, which ultimately lead to the initiation of apoptotic cell death pathways (Roberg and Öllinger, 1998). These include mitochondria-dependent and -independent pathways, such as the release of pro-apoptotic factors from mitochondria and the activation of JNK and p38 pathways (Yamashita et al, 2004; Kamata et al, 2005; Matsuzawa and Ichijo, 2008).

Cellular sources of ROS include the mitochondria and NADPH oxidase (NOX) enzymes. NOX3 is a protein which is expressed in the inner ear and has been associated with the generation of cisplatin-induced oxidative stress, when silencing cochlear expression of *NOX3* attenuated apoptosis and the development of ototoxicity in rat models (Mukherjea et al, 2010). The hair cells are hypothesised to be particularly vulnerable to ROS accumulation due to a reduced concentration of glutathione and other antioxidants (Sha et al, 2001). Demonstrations that oxidative stress causes significant changes to hair cell morphology (Clerici et al, 1995), and that application of ROS to guinea pig inner ears resulted in significant hearing loss (Clerici and Yang, 1996), indicate the sensitivity of the cochlea to oxidative stress. The generation of ROS and free radicals has been demonstrated in the cochlea following exposure to cisplatin (Clerici et al, 1996); hair cells and cells of the spiral ganglion seem to be most susceptible to ROS excess, while supporting cells in the organ of Corti tend to be more resistant to oxidative stress (Sha et al, 2001; Bánfi et al, 2004).

Hair cells are sensory, detecting sound as vibrations in the endolymph (Hibino et al, 2010), and the spiral ganglion consists of neurons responsible for the transduction of auditory signals from the cochlear hair cells to the brain. Consequently, the loss of these cell types can contribute directly to hearing impairment. Upon treatment with cisplatin, cells in the spiral ganglion become detached from their myelin sheaths (Van Ruijven et al, 2005), and undergo apoptosis (Alam et al, 2000). The organisation of hair cells in the cochlea is such that those in different regions of the cochlea confer sensitivity to a specific range of sound frequencies (Figure 1.1). Cisplatin initially accumulates in the basal turn of the cochlea (Hellberg et al, 2013), inducing apoptosis in these cells (Alam et al, 2000), which is why the high frequencies are initially affected (Grewal et al, 2010). Lower frequencies of hearing are affected more gradually, as the drug concentration increases in more apical regions of the cochlea (Helberg et al, 2013).

Together, the stria vascularis and spiral ligament form the lateral wall of the cochlea (Figure 1.2). The spiral ligament consists of fibrocytes and connective tissue and is largely unaffected by cisplatin treatment, although evidence of drug-induced apoptosis has been noted in the region (Alam et al, 2000). Conversely, extensive damage to cells in the stria vascularis has been observed following exposure to cisplatin (Meech et al, 1998). This is an epithelial tissue which functions in the production of endolymph in the scala media (Hibino et al, 2010). Changes to the endolymph, such as a reduction in potassium ion concentration (Rozengurt et al, 2003) or alteration in pH (Stawicki et al, 2014), can adversely affect the environment in which the hair cells function.

1.4 Audiometry

Because there are no preventative or curative strategies for cisplatin-induced ototoxicity, current clinical practice involves monitoring patient hearing throughout treatment for signs of significant hearing loss, and altering the treatment regimen accordingly. Audiometric monitoring programmes are recommended to include a baseline audiogram performed before the first cycle of chemotherapy, and follow-up audiograms conducted before each subsequent treatment cycle, so that deterioration in hearing can be detected (American Speech-Language-Hearing Association [ASHA], 1994).

Unfortunately, audiometric monitoring strategies have several limitations in their ability to preserve patient quality-of-life. The first is a lack of awareness of and compliance with the need for audiometric tests, particularly in the SA context (de Andrade et al, 2009). Secondly, although cognisance of ototoxicity is reportedly improving, with a 300% increase in the number of audiometric tests performed on cisplatin-receiving cancer patients attending GSH between 2006 and 2010 (Whitehorn et al, 2014a), these tests are not always sufficiently available in hospitals. Thirdly, due to the progressive nature of cisplatin's ADRs, sometimes significant hearing loss is only detected after the cessation of treatment. For example, an analysis of 120 paediatric patients demonstrated that, although 5% of patients displayed signs of severe ototoxicity immediately following treatment, this increased to 44% of patients when audiometric tests were conducted two years after treatment (Bertolini et al, 2004). The progressivity of ototoxicity also implies that altering treatment due to the onset of ototoxicity will not necessarily mitigate any further deterioration in hearing. Finally, permanent hearing loss has been observed in some patients after a single cycle of treatment (Durrant et al, 1990), illustrating the inability of audiometric monitoring to prevent ototoxicity.

Due to these limitations, research into preventative measures is desirable. These include the development of otoprotective agents which could be administered concomitantly with cisplatin, and the characterisation of intrinsic risk factors which may affect the susceptibility of certain patients to ototoxicity. The identification of otoprotective compounds is an area of much research, and agents which have been investigated include antioxidants, channel blockers, and inhibitors of apoptosis (Rybak et al, 2009). However, many of these compounds have only been demonstrated as protective *in vitro*, or in animal models when using invasive methods of administration (Wimmer et al, 2004; Shafik et al, 2013). The translation of these results to clinical utility has been challenging because the inner ear is a relatively isolated organ, making localised delivery in patients problematic. While systemic delivery of these agents may be able to protect the inner ear from damage (Mohan et al,

2014), this method of administration carries the potential to interfere with cisplatin's anticancer effects, and therefore the success of treatment (Wimmer et al, 2004). Knowledge of intrinsic risk factors, however, may enable the identification of at-risk patients prior to the initiation of chemotherapy.

1.5 Risk factors of ototoxicity

Investigations into clinical risk factors of cisplatin-induced ototoxicity consistently identify the dose of cisplatin as a predictor of hearing impairment. It is the cumulative dose, rather than individual cisplatin dose, which seems to influence the risk of ototoxicity, as evidenced in numerous studies. In paediatric patients receiving total cisplatin doses exceeding 400 mg/m^2, a 3.35-fold increased risk of ototoxicity has been calculated (Li et al, 2004), a dose at which up to 44% of patients were reported to develop significant hearing loss (Bertolini et al, 2004). The dose-dependency of ototoxicity has also been demonstrated in adult patients (Bokemeyer et al, 1998), and has been replicated in the SA population (Whitehorn et al, 2014a). In this population, the cancer diagnosis was also identified as a risk factor: patients presenting with cancers of the reproductive organs were at a reduced risk of hearing impairment, while differential risks were suggested for the head and neck, lymphoma and digestive system tumours, although these have not been replicated (Spracklen et al, 2014; Whitehorn et al, 2014a). This indicates that other factors such as the method of cisplatin administration and infusion rates, which are treatment-specific, may also have an effect.

Despite the identification of cumulative cisplatin dose as a risk factor, it is evident that there are substantial inter-individual differences in patient susceptibility to ototoxicity. Certain treatment regimens and cisplatin dosages may prove highly ototoxic in one patient, while another may retain normal hearing throughout a similar treatment regimen. Additional risk factors which have been reported less consistently (and, in some instances, in isolation) include young patient age at the initiation of treatment (Li et al, 2004; Yancey et al, 2012), patient gender (Yancey et al, 2012; Kirkim et al, 2014), the use of cranial irradiation (Chen et al, 2006; Yang et al, 2013) or other ototoxic drugs during treatment (Bokemeyer et al, 1998; Harris et al, 2012), previous exposure to noise (Bokemeyer et al, 1998), pre-existing hearing loss (Bokemeyer et al, 1998), impaired renal function (Bokemeyer et al, 1998), anaemia (De Jongh et al, 2003) and diet (Lautermann et al, 1995). The roles of both patient age and gender in cisplatin-induced hearing loss have proved contentious. Although young age (< 5 years) has been reported as a risk factor in independent investigations (Li et al, 2004; Yancey et al, 2012), other studies in similar populations have shown no association between age extremes and hearing loss (Bokemeyer et al, 1998; De Jongh et al, 2003; Choeyprasert

et al, 2013). Male paediatric patients have been reported to be at a greater risk of ototoxicity (Yancey et al, 2012), but this observation has not replicated in other studies, including adult populations (De Jongh et al, 2003; Li et al, 2004; Choeyprasert et al, 2013).

1.6 Pharmacogenetics

Pharmacogenetics is the study of the effect of genetic variation on individual drug responses. These may include efficacy and toxicity of treatment, as well as the development of resistance to therapy. Genes which are of interest in pharmacogenetics include those involved in the action of drugs, as well as their metabolism, distribution and elimination from the body (Weng et al, 2013). Pharmacogenetic studies may therefore contribute to the understanding of drug action and pharmacokinetics, although their ultimate objective is to improve medical practice. The current approach to medicine is limited to the prescription of drugs based on standard procedures, in which patients with the same diagnosis tend to be prescribed the same drug at the same dose (Abul-Husn et al, 2014), and issues such as ADRs are only reacted to after their occurrence. However, research into pharmacogenetics may enable the future personalisation of treatment regimens (including drug choice and dosage schedule) based on a patient's genotype, and has the potential to optimise therapy in a way that is impossible at present.

Cellular sensitivity to cisplatin has been demonstrated to have a heritable component, estimated to be between 32% and 47% from two experiments using lymphoblastoid cell lines which were derived from 10 and 27 families (Dolan et al, 2004; Shukla et al, 2008). This indicates that a pharmacogenetic approach to cisplatin-induced ototoxicity is valid, and the role of genetics in predisposing certain individuals to cisplatin's adverse effects is a developing area of research (Rybak et al, 2007).

1.6.1 The pharmacogenetics of cisplatin-induced ototoxicity

The role of genetics in the development of cisplatin-induced ototoxicity has been under investigation since 2000 (Peters et al, 2000). A diverse group of genes has been explored to date, including those which are involved in the metabolism, detoxification and transport of drugs, as well as DNA repair genes, deafness susceptibility loci and mitochondrial genes (Table 1.1). However, despite over a decade of research on the subject, there is little consensus on the role of these genes in cisplatin-induced hearing loss.

Table 1.1: Potential genetic modifiers of cisplatin-induced ototoxicity

Gene	Variant	Effect on risk	Sample size	p-value	Reference
Drug metabolism and detoxification genes					
GSTM3	*GSTM3*B*	Reduced	39	0.020	Peters et al (2000)
GSTP1	rs1695	Reduced	173	< 0.001	Oldenburg et al (2007)
		None	162	0.610	Ross et al (2009)
		None	115	0.069	Whitehorn et al (2014b)
GSTM1, *GSTT1*	*GSTM1*0*, *GSTT1*0*	Reduced	173	0.022	Oldenburg et al (2007)
TPMT	rs12201199	Increased	162	< 0.001	Ross et al (2009)
		Increased	317	< 0.001	Pussegoda et al (2013)
		None	213	0.500	Yang et al (2013)
		None	100	0.175	Whitehorn et al (2014b)
		None	63	0.483	Lanvers-Kaminsky et al (2014)
		None	110	0.650	Hagleitner et al (2014)
COMT	rs9332377	Increased	162	0.001	Ross et al (2009)
		Increased	317	0.043	Pussegoda et al (2013)
		None	213	0.780	Yang et al (2013)
		None	115	0.564	Whitehorn et al (2014b)
		None	63	0.094	Lanvers-Kaminsky et al (2014)
		None	110	0.830	Hagleitner et al (2014)
	rs4646316, rs9332377, rs4818	Haplotype increased risk	20	0.020	Whitehorn et al (2014b)
DPYD	rs6667550	Increased	155	0.0047	Pussegoda (2012)
Membrane receptors and transporters					
LRP2	rs2075252	Increased	50	0.016	Riedemann et al (2008)
		None	68	0.763	Choeyprasert et al (2013)
		None	115	0.872	Whitehorn et al (2014b)
	rs2228171	Increased	68	0.087	Choeyprasert et al (2013)
		None	50	0.034	Riedemann et al (2008)
		None	115	0.140	Whitehorn et al (2014b)
ABCC3	rs1051640	Increased	317	0.0033	Pussegoda et al (2013)
ABCB5	rs10950831	Increased	155	1.06×10^{-6}	Pussegoda (2012)
CTR1	rs10981694	Increased	204	0.01	Xu et al (2012a)
SLC22A2	rs316019	Reduced	64	0.018	Lanvers-Kaminsky et al (2015)
Deafness-associated genes					
MT-RNR1	Screened	None	39	NA	Peters et al (2003)
			11	NA	Knoll et al (2006)
D-loop region	Screened	None	39	0.08	Peters et al (2003)
GJB2	35delG	None	11	0.35	Knoll et al (2006)
SLC26A4	Screened	None	11	NA	Knoll et al (2006)
Other genes					
XPC	rs2228001	Increased	32	0.042	Caronia et al (2009)
		None	115		Whitehorn et al (2014b)
SOD2	rs4880	Increased	71	0.04	Brown et al (2015)
EIF3A	rs77382849	Increased	282	0.01	Xu et al (2012b)
OTOS	rs77124181, rs2291767	Reduced	100	0.022	Spracklen et al (2014)
CDH13	rs17758876	Increased	27 families	< 0.0001	Shukla et al (2008)
ACYP2	rs1872328	Increased	238	3.9×10^{-8}	Xu et al (2015)

ABCB5, ATP-binding cassette, subfamily B, member 5; ABCC3, ATP-binding cassette, subfamily C, member 3; ACYP2, acylphosphatase 2, muscle type; CDH13, cadherin 13; COMT, catechol-O-methyltransferase; CTR1, copper transporter 1; DPYD, dihydropyrimidine dehydrogenase; EIF3A, eukaryotic translation initiation factor 3, subunit A; GJB2, gap junction protein, beta 2; GST, glutathione S-transferase; LRP2, low-density lipoprotein receptor-related 2; MT-RNR1, mitochondrially encoded 12S RNA; NA, not applicable; SLC22A2, solute carrier family 22, member 2; SLC26A4, solute carrier family 26 (anion exchanger), member 4; SOD2, superoxide dismutase 2; TPMT, thiopurine S-methyltransferase; XPC, xeroderma pigmentosum, complementation group C

1.6.1.1 Genes involved in drug metabolism and detoxification

1.6.1.1.1 The glutathione S-transferases (GSTs)

The GSTs are an important superfamily of detoxifying enzymes which act by catalysing the conjugation of reduced glutathione to various substrates, including drugs and their metabolites. Three of the most ubiquitous GST isoforms are GSTM1, GSTT1 and GSTP1, which are expressed in many cell types, including the cochlear hair cells (el Barbary et al, 1993). Chemotherapeutics such as cisplatin are detoxified by GSTP1 (Ban et al, 1996; Goto et al, 2002). The GST genes were of initial interest in the study of cisplatin-induced ototoxicity because, in addition to their role in drug detoxification, they are involved in antioxidant processes in cells (Baez et al, 1997). Therefore there are two mechanisms whereby GST polymorphisms may influence susceptibility to ototoxicity: by altering the detoxification of cisplatin (and therefore vulnerability to its toxic effects), and by modifying the ability of cells to respond to cisplatin-induced oxidative stress.

The GST genes are highly polymorphic in humans (Garte et al, 2001), and polymorphisms within *GSTM1*, *GSTT1* and *GSTP1* are the most frequently investigated regarding hearing loss. The variants *GSTM1*0* and *GSTT1*0* are homozygous deletions yielding null alleles. The *GSTP1* c.313A>G (rs1695) single nucleotide polymorphism (SNP), which encodes a reduced-function variant (Watson et al, 1998), has a minor allele frequency of approximately 30% in Caucasian populations. Functional effects of the G-allele include an altered substrate specificity and capacity for detoxification (Allan et al, 2001; Hohaus et al, 2005). This allele encodes a better detoxifier of cisplatin, which improved protection against the drug in *E. coli* cells (Ishimoto and Ali-Osman, 2002).

The first GST polymorphism which associated with cisplatin-induced ototoxicity, however, was the *GSTM3*B* allele, which was suggested to be protective against hearing impairment in 39 paediatric cancer patients (Peters et al, 2000). This allele encodes an intronic deletion which could potentially alter expression of the gene (Inskip et al, 1995), although evidence for this hypothesis is lacking. The authors failed to associate variation within other GST genes, including *GSTP1*, *GSTT1* and *GSTM1*, with cisplatin-induced ototoxicity. However, a subsequent analysis of 173 testicular cancer survivors demonstrated the protective effect of variants within these genes. Particularly, presence of the *GSTP1* c.313 G-allele, as well as the *GSTM1*0* and *GSTT1*0* alleles, potentially reduced the risk of hearing loss (Oldenburg et al, 2007).

1.6.1.1.2 Methyltransferases

Methyltransferase enzymes catalyse the conjugation of methyl groups in the cellular metabolism of xenobiotic compounds such as pharmaceutical drugs. After assaying 220 genes involved in drug absorption, distribution, metabolism and elimination (ADME), two methyltransferase variants were identified in association with significant hearing loss in Canadian paediatric patients (Ross et al, 2009). The genes, *thiopurine S-methyltransferase* (*TPMT*) and *catechol-O-methyltransferase* (*COMT*), had not been associated with ototoxicity before. However, the SNPs *TPMT* c.419+94T>A (rs12201199) and *COMT* c.466-367C>T (rs9332377) were both reported to increase the risk of ototoxicity in paediatric cancer patients (Ross et al, 2009).

Because there is as yet no evidence that these genes play a role in the metabolism of cisplatin, the functional basis for these associations is unclear. Two hypotheses have been proposed by the authors. Reduction or abolition of methyltransferase activity may alter the formation of DNA cross-links, and thus affect the ability of cisplatin to damage DNA and induce apoptosis. Alternatively, these variants may cause the accumulation of S-adenosylmethionine (SAM) in cells, since SAM is a substrate for methyltransferase enzymes. While SAM itself is not toxic to cells, it has been demonstrated to increase the incidence of cisplatin-induced ADRs in mouse models (Ochoa et al, 2009).

On the basis of this genetic association study alone, the Food and Drug Administration of America (FDA) altered the product label of cisplatin to incorporate the association of *TPMT* polymorphisms and ototoxicity risk in paediatric patients (Boddy, 2013). The clinical utility of this amendment is somewhat debateable, considering the lack of replication of the result at the time, and the lack of any clear functional link to cisplatin or its ototoxicity (Boddy, 2013). Although the association between the *TPMT* variant (and, indeed, the *COMT* variant) was subsequently replicated by the same research group (Pussegoda et al, 2013), neither gene could explain the development of ototoxicity in American or European cohorts of patients (Yang et al, 2013; Hagleitner et al, 2014; Lanvers-Kaminsky et al, 2014). The study by Yang et al (2013) further questioned the validity of the association by demonstrating that the variants did not alter cellular sensitivity to cisplatin *in vitro*. Both genes have also been investigated in the SA population, and while *TPMT* polymorphisms did not associate with ototoxicity, three variants in *COMT* were proposed to form a risk haplotype in Caucasian patients (Whitehorn et al, 2014b). Hagleitner et al (2014) performed a meta-analysis of all published data, together with their work in Dutch and Spanish patient cohorts, and concluded that the role of *TPMT* and *COMT* variants in ototoxicity has been largely

overstated. In the final analysis, which considered five gene variants in a total of 664 patients, only one of the SNPs, *COMT* c.*217C>T (rs4646316), showed a weak association with ototoxicity (Hagleitner et al, 2014). Although this SNP was not identified as statistically significant in the studies by Ross et al (2009), Pussegoda et al (2013), and Yang et al (2013), it was included in the risk haplotype proposed by Whitehorn et al (2014b).

1.6.1.1.3 Dehydrogenases

Using the assay which identified the *TPMT* and *COMT* modifiers, but with an additional 95 genes included in the analysis, the intronic polymorphism c.2623-43032A>G (rs6667550) within *dihydropyrimidine dehydrogenase* (*DPYD*) associated with cisplatin-induced ototoxicity in a similar cohort of patients (Pussegoda, 2012). The association has not yet been replicated and, again, there is no evidence that DPYD or other dehydrogenase enzymes are involved in the metabolism of cisplatin. Rather, DPYD metabolises 5-FU, which is often co-administered with cisplatin in chemotherapy regimens. Increased expression of *DPYD* was correlated with a reduced cellular sensitivity to both 5-FU and cisplatin *in vitro* (Minegaki et al, 2013). Conflictingly, the same study reported that inhibition of DPYD activity decreased the sensitivity of human carcinoma cells to cisplatin (Minegaki et al, 2013). Whether the enzyme is involved in the metabolism of cisplatin or is required for its toxicity remains to be investigated further.

1.6.1.2 Membrane receptors and transporters

Following the discovery of cell-surface receptors and transporters which contribute to the active uptake of cisplatin by cells (Holzer et al, 2004; Filipski et al, 2008), the role of these proteins in the accumulation of cisplatin in off-target regions in the body, such as the inner ear, has been under consideration. Megalin is a cell-surface receptor which is expressed in the stria vascularis of the inner ear, and has been implicated in the transport of aminoglycosides (Schmitz et al, 2002). Two non-synonymous SNPs within the gene encoding megalin, *low-density lipoprotein receptor-related 2* (*LRP2*), have been investigated regarding their role in cisplatin-induced ototoxicity. Riedemann et al (2008) demonstrated a significant association between *LRP2* c.12280A>G (rs2075252) and ototoxicity, and no association between *LRP2* c.8614G>A (rs2228171) and hearing impairment. Contradictory findings were reported by Choeyprasert et al (2013), in which the *LRP2* c.8614 C-allele potentially increased the risk of ototoxicity, while *LRP2* c.12280A>G showed no significant association. These differences may be explained by the different hearing loss and grading criteria used in the studies, as well as differences in methodology and statistical analyses.

Regardless, the sample sizes in both studies were small (n = 50 and n = 68, respectively), and the role of megalin in ototoxicity cannot be ascertained with any confidence. Both variants failed to associate with hearing loss in a larger analysis of 115 patients (Whitehorn et al, 2014b).

More recently, two potential genetic modifiers in the ATP-binding cassette (ABC) superfamily of transporters have been identified. These are a group of broad-specificity transporters which facilitate the ATP-dependent transport of various molecules across cell membranes. The variants *ABCC3* c.*3180A>G (rs1051640) and *ABCB5* c.1290+1532G>T (rs10950831) associated with ototoxicity in 155 paediatric cancer patients (Pussegoda, 2012). Limitations of the associations between the *ABC*s, as well as *LRP2*, and ototoxicity include the lack of a mechanistic link to cisplatin and its ototoxic effects. It is unknown whether any of these proteins actively transport cisplatin into or out of cells, and, apart from experiments correlating *ABCC3* expression and resistance to cisplatin in lung cancer cells (Young et al, 2001), these genes have not been functionally analysed regarding their role in the cellular response to cisplatin.

Known transporters of cisplatin include copper transporters such as CTR1, members of the transient receptor potential (TRP) superfamily, and the organic cation transporters (OCTs) (Waissbluth and Daniel, 2013). CTR1 was reported to transport cisplatin into cancer cells (Holzer et al, 2004), and is highly expressed in the hair cells, spiral ganglion and stria vascularis of the cochlea (More et al, 2010). It is also involved in the cellular response to oxidative stress (Kuo et al, 2001). Twenty SNPs within *CTR1* were genotyped in 204 Chinese cancer patients, and the intronic variant c.-36+2451T>G (rs10981694) was associated with an increased risk of ototoxicity (Xu et al, 2012a). OCT2, encoded by the gene *solute carrier family 22, member 2* (*SLC22A2*), is also expressed in the inner ear (Ciarimboli et al, 2010). The variant c.808T>G (rs316019) in *SLC22A2* has been associated with protection against ototoxicity in a German cohort of cisplatin-receiving patients (Lanvers-Kaminsky et al, 2015).

1.6.1.3 Deafness-associated genes

Although numerous genetic loci have been identified as causative of hearing loss in the absence of cisplatin, research into the role of these genes in the development of cisplatin-induced ototoxicity is limited. Because mitochondrial mutations such as those in the gene encoding 12S rRNA (*MT-RNR1*) have been associated with non-syndromic deafness as well as aminoglycoside-induced ototoxicity (Prezant et al, 1993; Estivill et al, 1998), Peters et al

(2003) screened patient mitochondrial DNA (mtDNA) for potential modifiers of cisplatin's ototoxicity. The authors failed to identify any known mutations in their patient cohort (Peters et al, 2003). A second investigation considered the role of five hearing genes: three in the mtDNA and two genomic genes, *gap junction protein, beta 2* (*GJB2*) and *solute carrier family 26 (anion exchanger), member 4* (*SLC26A4*). Apart from a single mutation in *GJB2*, no polymorphisms were detected, and the authors could not explain the development of hearing loss in these patients (Knoll et al, 2006). Due to the small number of patients included in these studies (n = 39 and n = 11, respectively), the involvement of deafness-associated genes in cisplatin-induced ototoxicity cannot be excluded at present.

1.6.1.4 Other genes

Alternative candidate genes have been explored based on the current knowledge of cisplatin's mechanism of action. The NER pathway is involved in the repair of cisplatin-induced DNA damage, by removing platinum-containing adducts. The *xeroderma pigmentosum, complementation group C* (*XPC*) gene encodes a component of this pathway. A weak association between the nonsynonymous variant *XPC* c.2815C>A (rs2228001) and increased hearing loss was reported in 32 osteosarcoma patients (Caronia et al, 2009). Due to a small sample size, this study was likely underpowered, and the variant did not associate with ototoxicity in a study of 115 SA patients (Whitehorn et al, 2014b). A gene which functions upstream of the NER pathway, *eukaryotic translation initiation factor 3, subunit A* (*EIF3A*), has also been implicated as a potential modifier of ototoxicity. Although *EIF3A* functions primarily in the initiation of protein synthesis, it is also involved in the regulation of the NER pathway (Yin et al, 2011). The nonsynonymous SNP *EIF3A* c.2408G>A (rs77382849) may modify the risk of cisplatin's ADRs, including ototoxicity, in Chinese lung cancer patients (Xu et al, 2013).

Apart from the GSTs, few genes involved in the response to oxidative stress have been explored to date. A novel gene, *OTOS*, was suggested to play a role in antioxidant defences and could protect cells from the cytotoxic effects of cisplatin (Zhuo et al, 2008). Two *OTOS* polymorphisms, c.-192-182C>G (rs77124181) and c.-192-22A>G (rs2291767), were shown to be protective against ototoxicity in 100 cancer patients (Spracklen et al, 2014). Variation in *superoxide dismutase 2* (*SOD2*) has also been associated with noise-induced and, possibly, age-related hearing losses (Fortunato et al, 2004; Chang et al, 2009; Liu et al, 2010; Nolan et al, 2013). The variant *SOD2* c.47T>C (rs4880) was associated with the incidence of hearing aid use following cisplatin exposure in a cohort of American paediatric cancer patients, suggesting a potential role for the gene in the development of ototoxicity (Brown et

al, 2015). In a linkage analysis of cell lines derived from 27 Caucasian families, Shukla et al (2008) identified five genes which may explain variability in cisplatin-induced cytotoxicity. The genes were *La ribonucleoprotein domain family, member 1B* (*LARP1B*), *leucine rich repeat containing 3B* (*LRRC3B*), *paired-like homeodomain 2* (*PITX2*), *zinc finger protein 385D* (*ZFN385D*), and *cadherin 13* (*CDH13*). Of these, *CDH13* is most likely to directly affect cisplatin cytotoxicity. Unlike other cadherins, it is not implicated in cell-cell adhesion, but is rather involved in numerous cellular functions, including cell survival and the regulation of apoptosis due to oxidative stress (Joshi et al, 2005). *CDH13* is expressed in many cochlear cells and has been proposed to have a role in hearing (Girotto et al, 2014). To date, only one genome-wide association study (GWAS) has been conducted into cisplatin-induced ototoxicity. Using this approach, the intronic variant c.185+29374G>A (rs1872328) in the gene *acylphosphatase 2, muscle type* (*ACYP2*) was identified in association with the phenotype in 238 paediatric patients (Xu et al, 2015). Although *ACYP2* expression has been demonstrated in the transcriptomes of murine inner and outer hair cells (Liu et al, 2014), its contribution to hearing or cisplatin response is currently unknown.

1.7 Future considerations or directions

Given the inconsistent results of the abovementioned genetic association studies, the importance of replication of these results cannot be overstated (Hirschhorn and Altshuler, 2002). Previous work in the SA patient population could not replicate the association between ototoxicity and variants within *GSTP1*, *TPMT*, *LRP2* and *XPC*, although a risk haplotype in *COMT* was reported in Caucasian patients (Whitehorn et al, 2014b). The protective *OTOS* variants were limited to Caucasian and mixed ancestry patients (Spracklen et al, 2014). Notably, the role of genetics in ototoxicity amongst indigenous Africans is unknown.

From these studies, it is also evident that the reported genetic factors cannot explain the total variation in the incidence of cisplatin-induced ototoxicity. Therefore, in addition to replication, new genes need to be explored for their involvement in ototoxicity. Such candidates may be chosen based on current knowledge of the pathway whereby cisplatin causes ototoxicity. For instance, variants in genes involved in the transport of cisplatin into or out of cells may be involved, as might variants which affect the cellular response to cisplatin treatment. However, it should be noted that current understanding of these pathways is limited and, consequently, so too is our ability to choose and analyse specific candidate genes. The study of ototoxicity may be benefitted by the utilisation of next-generation sequencing (NGS) techniques to analyse genome-scale regions of DNA for potential genetic

modifiers. This has the potential not only to be hypothesis-generating (by identifying potentially causative variants in novel genes) but also to contribute to knowledge of the mechanism of cisplatin-induced ototoxicity.

1.8 Research aim and objectives

The aim of this study is to investigate the role of genetic variation in the development of cisplatin-induced ototoxicity, by identifying genetic variants in a population of adult cisplatin-receiving SA cancer patients that may modify the risk of ototoxicity. The project aim will be addressed through the following objectives:

- The identification of candidate genes from the literature, with specific variants which may be associated with the development of cisplatin-induced ototoxicity
- The establishment of genotypic frequencies for each candidate gene variant in a cohort of SA cancer patients, comparison of these frequencies to other published populations, and analysis of the associations between given genotypes and specific drug response phenotypes
- The analysis of clinical and demographic risk factors present in the local patient population and how these may be used to accurately assess the development of ototoxicity
- The identification of clinically relevant patients and the utilisation of NGS approaches to investigate the total protein-coding region of patient DNA for potential genetic modifiers
- The investigation of potential functional effects of the genetic variants *in silico*, including how they may contribute to the development of cisplatin-induced ototoxicity in patients

In this submission, Chapter 2 contains a brief investigation of hospital data for the incidence of ototoxicity amongst the local cisplatin-receiving cancer patient population, as well as the presence of clinical and demographic risk factors for the ADR. The role of genetics in cisplatin-induced ototoxicity is subsequently explored in Chapters 3 and 4. This includes the recruitment of a prospective patient cohort, which is described in Chapter 3, together with the results of a candidate gene investigation where seven genetic variants were analysed for their association with cisplatin response. Chapter 4 discusses a pilot NGS investigation which was conducted on a subset of the patient cohort, and was used to prioritise novel genes for future molecular investigations.

2. Analysis of clinical and demographic risk factors of cisplatin-induced ototoxicity

2.1 Introduction

As one of the most effective and widely used antineoplastic agents currently in clinical use, cisplatin remains the drug of choice in the treatment of numerous cancers (Desari and Tchounwou, 2014). Cisplatin is frequently employed in first-line chemotherapy regimens, contributing to the successful treatment of a variety of lymphomas, sarcomas and carcinomas, including cancers of the lungs, oesophagus, head and neck regions, testes, ovaries, and cervix (Desari and Tchounwou, 2014). Its widespread use can be attributed to the improved success rates which are often achieved when incorporating cisplatin in treatment plans; cure rates as high as 85% have been reported in some instances (McKeage, 1995; Einhorn, 2002).

The therapeutic utility of cisplatin, however, may be restricted by the development of dose-limiting ADRs such as ototoxicity (McKeage, 1995). Ototoxicity is an adverse reaction to cisplatin which presents as irreversible, bilateral, and high-frequency hearing loss. It has the potential to be a disabling drug reaction, particularly when hearing impairment progresses into lower ranges of hearing (0.5-4 kHz) which are required for the recognition of spoken language (de Andrade et al, 2013). Therefore, the development of ototoxicity presents an important challenge in healthcare, as continuing optimal chemotherapy may then compromise patient quality-of-life. Although the protective effects of antioxidant compounds, channel blockers and apoptosis inhibitors have been extensively investigated in animal models of cisplatin response (Rybak et al, 2007), there are currently no methods of treating, attenuating or preventing ototoxicity in a clinical setting. Other platinum-based chemotherapeutics such as carboplatin and oxaliplatin may be less ototoxic than cisplatin, but these agents have their own toxicity profiles and are typically not as effective in the treatment of cancer (Lokich and Anderson, 1998; Hotta et al, 2004; Sanborn, 2008; Fakhrian et al, 2015). The lack of a safer alternative to cisplatin, with an efficacy which is comparable and well established, has contributed to the continued use of cisplatin in first-line chemotherapy regimens despite the risk of ototoxicity and other ADRs.

Dosage reductions or the discontinuation of cisplatin treatment may occur in patients who display signs of hearing impairment, which may then reduce the efficacy of chemotherapy. This is significant when considering that the reported prevalence of ototoxicity amongst cisplatin-receiving cancer patients ranges from 25% to 100% (Kopelman et al, 1988; Bokemeyer et al, 1998; De Jongh et al, 2003; Knight et al, 2005; Kushner et al, 2006;

Coradini et al, 2007; Arora et al, 2009; Yancey et al, 2012; Choeyprasert et al, 2013; Yang et al, 2013; Peleva et al, 2014). One factor which may influence this incidence is the sensitivity of the audiometric tests employed during treatment. Using standard audiometry (0.25-8 kHz), up to 50% of adult patients may develop hearing impairment over the course of chemotherapy (Bokemeyer et al, 1998; De Jongh et al, 2003). In a retrospective analysis of SA cancer patients, an ototoxicity prevalence of 55% was reported, indicating that hearing loss is a frequent adverse reaction to cisplatin in the local patient population (Whitehorn et al, 2014a). While numerous studies have demonstrated that the risk of ototoxicity is influenced by the cumulative dose of cisplatin to which the patient is exposed (Bokemeyer et al, 1998; Li et al, 2004; Oldenburg et al, 2007; Choeyprasert et al, 2013), there is still much inter-individual variation in sensitivity to cisplatin's ototoxic effects. Indeed, patients who receive the same dose of cisplatin may have very different drug responses, and these differences have not yet been accounted for. Additional factors which have been explored for their role in ototoxicity include patient age, sex, the presence of pre-existing hearing loss or exposure to noise, reduced renal function, anaemia, a protein-poor diet, and genetic risk loci (Lautermann et al, 1995; Bokemeyer et al, 1998; De Jongh et al, 2003; Li et al, 2004; Mukherjea and Rybak, 2011; Yancey et al, 2012), but their contribution to drug-induced hearing loss remains inconclusive.

Current clinical practice involves monitoring patient hearing throughout treatment and reacting to any impairment accordingly. This approach is limited to the detection of hearing loss and is unable to prevent it. However, considering the progressive and permanent nature of cisplatin-induced hearing loss, prevention of this ADR is preferable in order to better preserve patient quality-of-life. Understanding the role of intrinsic risk factors in ototoxicity may enable the identification of high-risk patients prior to the initiation of chemotherapy, and minimise this morbidity. The objective of the present investigation is to assess the influence of clinical and demographic variables on hearing sensitivity changes in response to cisplatin chemotherapy in SA cancer patients.

2.2 Methods

2.2.1 Patients

Between 2012 and 2015, cisplatin-receiving cancer patients were recruited into a molecular investigation of cisplatin-induced ototoxicity (Chapters 3 and 4), for which ethical approval was obtained from the Human Research Ethics Committee (HREC), University of Cape Town (UCT) (HREC reference no.: 389/2012 and 220/2014) (Appendix A). Recruitment was

conducted by a registered research nurse from the Division of Human Genetics, UCT, after obtaining written informed consent from each potential participant (Appendix B, Appendix C, Appendix D). This prospective cohort was supplemented with retrospective patient data to form a cross-sectional study of the patients who attended GSH for cisplatin-based chemotherapy between January 2006 and April 2015. All patients who were managed by the Department of Radiation Oncology, GSH, and referred to the Audiology Clinic, UCT/GSH, were potentially eligible. Only those who had sufficient audiometric data were included in the analysis. Audiometric requirements were the presence of both baseline and follow-up audiometric tests, so that hearing function before and after treatment could be assessed. Approval for the review of patient records was obtained from GSH (Appendix E) as well as the HREC (Appendix A).

In addition to audiometric data, information relating to the treatment, such as cancer diagnosis, individual and cumulative cisplatin doses, and the use of cranial irradiation was recorded for each patient when available. Demographic data included patient age at the start of treatment, sex and ethnicity. For patients attending GSH between 2012 and 2015 (n = 217), ethnicity was self-reported. For those identified retrospectively (n = 78), ethnicity was assigned according to race and linguistic group. The patients in the study were Caucasian, indigenous (Xhosa-speaking) African, Indian, or of mixed ancestry.

2.2.2 Treatment details

In each treatment cycle for testicular, ovarian and cervical (reproductive) cancers, cisplatin was administered in a low dose (25-60 mg/m^2) every day for three days, together with 160 mg/m^2 etoposide and 15 000 IU bleomycin. The treatment of all the other cancer types consisted of a single high dose (> 60 mg/m^2) of cisplatin per cycle, administered in a bolus, together with 8 mg ondansetron, 8 mg dexamethasone, 1 000 mg/m^2 5-FU, and 500 ml mannitol. Patients were pre-treated by saline hydration 24 h prior to each cycle of chemotherapy.

2.2.3 Audiometric monitoring

Patient hearing thresholds were determined at frequencies between 0.25 kHz and 8.0 kHz using a GSI 61 audiometer (Grason-Stadler, Eden Prairie, MN USA) before each cycle of treatment. Hearing thresholds ≥ 25 dB at any test frequency in the baseline audiogram were recorded as pre-existing hearing loss. Ototoxicity was defined and graded using the Chang ototoxicity grading scale, in which grade 0 indicates that hearing remained unchanged during

treatment, and grade 1A (≥ 40 dB change in threshold at frequencies ≥ 6.0 kHz) upwards indicates a treatment-induced decrease in hearing sensitivity (Chang and Chinosornvatana, 2010). Grade 2A (≥ 40 dB change in threshold at ≥ 4.0 kHz) and greater hearing loss was regarded as clinically significant (Chang and Chinosornvatana, 2010). To assess the impact of treatment on the patients' hearing, changes in sensitivity threshold were calculated at each test frequency for the worst affected ear.

2.2.4 Statistical analyses

Associations between categorical variables and ototoxicity were assessed using Chi-squared tests, or Fisher's exact tests when any expected values were less than 5. Differences in the distribution of numerical variables between groups were analysed with Mann-Whitney U tests and Kruskal-Wallis tests. In all the analyses, any missing data was excluded from these tests. The ability of demographic and clinical factors to predict ototoxicity was analysed using binary forward logistic regression. Kaplan-Meier survival analysis was used to assess the time to develop hearing loss, in which the onset of ototoxicity was regarded as an event. The time of event-free survival was calculated as the number of days between the first audiogram and the date at which ototoxicity was first noted. Final dates on which no hearing loss was detected were censored in the analysis. All statistical analyses were performed using the IBM® SPSS® software version 22.0.0.0 (IBM® Corp., Armonk, NY USA), where p-values < 0.05 were considered statistically significant.

2.3 Results

2.3.1 Patient cohort

A total of 430 potentially eligible patients received cisplatin treatment at GSH during the study period, of whom 295 met the audiometric inclusion criteria (Table 2.1). The 295 patients were predominantly (70.8%) male, and the primary cancer site recorded was the head and neck regions. Using the Chang ototoxicity grading scale, the incidence of hearing impairment in these patients was 39.7%. While patient age and gender did not show any significant associations with hearing loss, ethnicity did show significant differences. In particular, the indigenous Africans were significantly over-represented in Grades > 0 (OR: 2.52, 95% CI: 1.43; 4.43). However, the anatomical site of cancer also differed significantly between the two groups, with reproductive cancer patients at a reduced risk of ototoxicity (OR: 0.34, 95% CI: 0.15; 0.76), and head and neck cancer patients at an increased risk (OR: 1.84, 95% CI: 1.14; 2.97).

Table 2.1: Characteristics of eligible patients

Variable	Grade 0 (n = 178)	Grade > 0 (n = 117)	p-value
Age (years)			0.999[a]
Median	48.00	48.00	
Range (min; max)	14; 74	14; 75	
Sex (n,%)			0.071[b]
Male	133 (74.7%)	76 (65.0%)	
Female	45 (25.3%)	41 (35.0%)	
Ethnicity (n,%)			**0.005**[c]
Caucasian	35 (68.6%)	16 (31.4%)	0.156[b]
Mixed ancestry	109 (63.0%)	64 (37.0%)	0.176[b]
Black African	27 (42.2%)	37 (57.8%)	**0.001**[b]
Indian	3 (100.0%)	0	0.276[c]
Unknown*	4	0	
Cancer diagnosis (n,%)			**0.017**[b]
Head and neck	77 (53.5%)	67 (46.5%)	**0.013**[b]
Lymphoma	31 (66.0%)	16 (34.0%)	0.413[b]
Osteosarcoma	9 (47.4%)	10 (52.6%)	0.221[b]
Reproductive	32 (80.0%)	8 (20.0%)	**0.007**[b]
Other	25 (67.6%)	12 (32.4%)	0.354[b]
Unknown*	4	5	
Individual dose (mg/m^2)			**0.002**[a]
Median	80.81	100.00	
Range	19.38; 197.00	34.10; 200.00	
Cumulative dose (mg/m^2)			**< 0.001**[a]
Median	190.64	272.73	
Range	47.62; 500.00	79.70; 524.16	
Cranial irradiation (Gy)			0.573[a]
Median dose	55.00	35.15	
Range (min; max)	5; 67.2	10; 68	

* Excluded from analysis; [a] Mann-Whitney U test; [b] Chi-squared test; [c] Fisher exact test
max, maximum; *min*, minimum; *n*, number of individuals

2.3.2 Association of clinical characteristics with ototoxicity

Both the individual and cumulative cisplatin doses were significantly higher in patients who developed ototoxicity during treatment (Table 2.1). This dosage data associated with the anatomical site of the cancer, and there were significant differences in ethnicity distribution between the cancer diagnoses (Table 2.2). Caucasian patients were over-represented and black African patients were under-represented in the reproductive cancer group (Chi-squared test, p = 0.001 and p = 0.011, respectively). Additionally, no significant differences in cisplatin dosage were detected between the head and neck cancers, lymphomas and osteosarcomas (Kruskal-Wallis test, p = 0.060). Although 48.2% of the head and neck cancer patients received cranial irradiation as part of their treatment, only a small proportion (37.0%) of these received their RT before or during cisplatin exposure. In these patients, the use of RT did not associate with ototoxicity (Table 2.1).

Table 2.2: Analysis of variables according to cancer diagnosis

	Head and neck	Lymphoma	Osteosarcoma	Reproductive	Other	p-value
Individual dose (mg/m^2)						**< 0.001**[a]
Median	100.00	100.00	100.00	35.00	77.06	
Range	35.00; 200.00	95.24; 180.00	100.00; 103.23	19.38; 100.53	55.32; 136.00	
Cumulative dose (mg/m^2)						**0.025**[a]
Median	200.00	200.00	297.73	211.76	160.00	
Range	68.97; 481.77	95.24; 400.00	192.60; 500.00	47.62; 524.16	59.41; 511.56	
Ethnicity (n,%)						**0.006**[b]
Cau	25 (17.4%)	7 (14.9%)	1 (5.3%)	14 (37.8%)	3 (8.1%)	**0.009**[b]
MA	88 (61.1%)	29 (61.7%)	12 (63.2%)	20 (54.1%)	21 (56.8%)	0.923[c]
BA	31 (21.5%)	10 (21.3%)	6 (31.6%)	2 (5.4%)	12 (32.4%)	**0.031**[b]
Ind	0 (0.0%)	1 (2.1%)	0 (0.0%)	1 (2.7%)	1 (2.7%)	0.125[b]
Sex (n,%)						**0.001**[c]
Males	111 (77.1%)	27 (57.4%)	10 (52.6%)	35 (87.5%)	22 (57.9%)	
Females	33 (22.9%)	20 (42.6%)	9 (47.4%)	5 (12.5%)	16 (42.1%)	

[a] Kruskal-Wallis test; [b] Fisher's exact test; [c] Chi-squared test
BA, Black African; *Cau*, Caucasian; *Ind*, Indian; *MA*, mixed ancestry; *n*, number of individuals

2.3.3 Association of demographic variables with ototoxicity

Because head and neck cancers, lymphomas and osteosarcomas were treated using similar dosage regimens, the analysis of further covariates was restricted to this group of patients (n = 210), where the ototoxicity incidence rate was 44.8%. Again, the ethnicity of the patient was significantly associated with hearing impairment (Table 2.3). Indigenous Africans were significantly skewed towards the development of ototoxicity (OR: 2.43, 95% CI: 1.25; 4.73) as well as Grade ≥ 2A ototoxicity (OR: 2.46, 95% CI: 1.15; 5.26). Pre-existing hearing loss, age at the initiation of treatment, and patient sex did not associate with ototoxicity.

Table 2.3: Demographics of head and neck cancer, lymphoma and osteosarcoma patients

	Grade 0	Grade > 0	p-value	Grade < 2A	Grade ≥ 2A	p-value
Age (years)			0.169[a]			0.534[a]
Median	50.00	48.00		49.00	47.50	
Range	14; 74	14; 75		14; 74	20; 75	
Sex (n,%)			0.323[b]			0.274[b]
Males	85 (%)	63 (%)		124 (83.8%)	24 (16.2%)	
Females	31 (50.0%)	31 (50.0%)		48 (77.4%)	14 (22.6%)	
Pre-existing HL (n,%)			0.065[b]			0.233[b]
None	44 (48.4%)	47 (51.6%)		71 (78.0%)	20 (22.0%)	
Present	71 (61.2%)	45 (38.8%)		98 (84.5%)	18 (15.5%)	
ND*	1	2		3	0	
Ethnicity (n,%)			**0.037**[c]			**0.010**[c]
Cau	20 (60.6%)	13 (39.4%)	0.498[b]	32 (97.0%)	1 (3.0%)	**0.014**[b]
MA	77 (59.7%)	52 (40.3%)	0.102[b]	106 (82.2%)	23 (17.8%)	0.888[b]
BA	18 (38.3%)	29 (61.7%)	**0.008**[b]	33 (70.2%)	14 (29.8%)	**0.018**[b]
Ind	1 (100.0%)	0 (0.0%)	1.000[c]	1 (100.0%)	0 (0.0%)	1.000[c]

* Not included in analysis; [a] Mann-Whitney U test; [b] Chi-squared test; [c] Fisher's exact test
BA, Black African; *Cau*, Caucasian; *HL*, hearing loss; *Ind*, Indian; *MA*, mixed ancestry; *n*, number of individuals; *ND*, not determinable

The different ethnic groups displayed significant differences in threshold shifts due to cisplatin treatment (Figure 2.1). In all patients, the higher frequencies (6.0-8.0 kHz) were subject to greater changes as a result of treatment; however, indigenous Africans tended to experience the greatest shifts in hearing, and Caucasians the lowest. These differences were maintained between 3.0 kHz and 8.0 kHz (Table 2.4). There were also significant differences in the time to develop ototoxicity in the sub-population groups (Figure 2.2). Indigenous African patients developed hearing loss in a median time of 48 days, which was a significantly shorter time compared to the other patient sub-populations (Log-rank test, p = 0.001). Caucasian and mixed ancestry patients developed ototoxicity in median periods of 85 and 72.5 days, respectively.

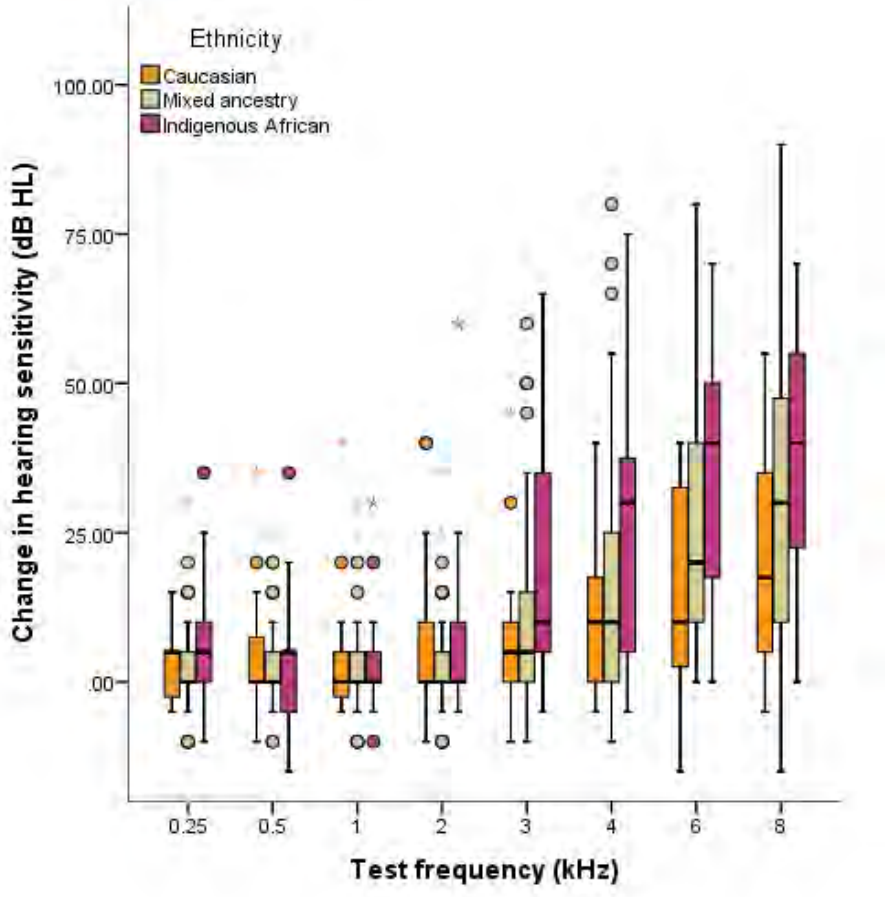


Figure 2.1: Average threshold changes at each test frequency in head and neck cancer, osteosarcoma and lymphoma patients of different ethnicities. Orange, purple and grey represent the Caucasians, indigenous Africans and mixed ancestry patients, respectively. The distributions of hearing sensitivity changes are represented by box-and-whisker plots, in which the boxes delineate the upper and lower quartiles, and the whiskers indicate the minimum and maximum values. Lines within the boxes are the median values. Statistical outliers are indicated by circles (1.5-3.0 interquartile ranges from the whisker) and stars (> 3.0 interquartile ranges from the whisker). Hearing sensitivity changes are measured on the y-axis, in which a greater value indicates more hearing impairment. The test frequency, in kHz, is represented on the x-axis.

Table 2.4: Median threshold shifts per test frequency in the different ethnic groups

Test frequency (kHz)	Caucasian	Mixed ancestry	Indigenous African	p-value[a]
0.25	5.00	0.00	5.00	0.221
0.5	0.00	0.00	5.00	0.969
1.0	0.00	0.00	0.00	0.408
2.0	0.00	0.00	0.00	0.678
3.0	5.00	5.00	10.00	**0.037**
4.0	10.00	10.00	30.00	**0.023**
6.0	10.00	20.00	40.00	**0.001**
8.0	17.50	30.00	40.00	**0.007**

[a] Determined by Kruskal-Wallis test

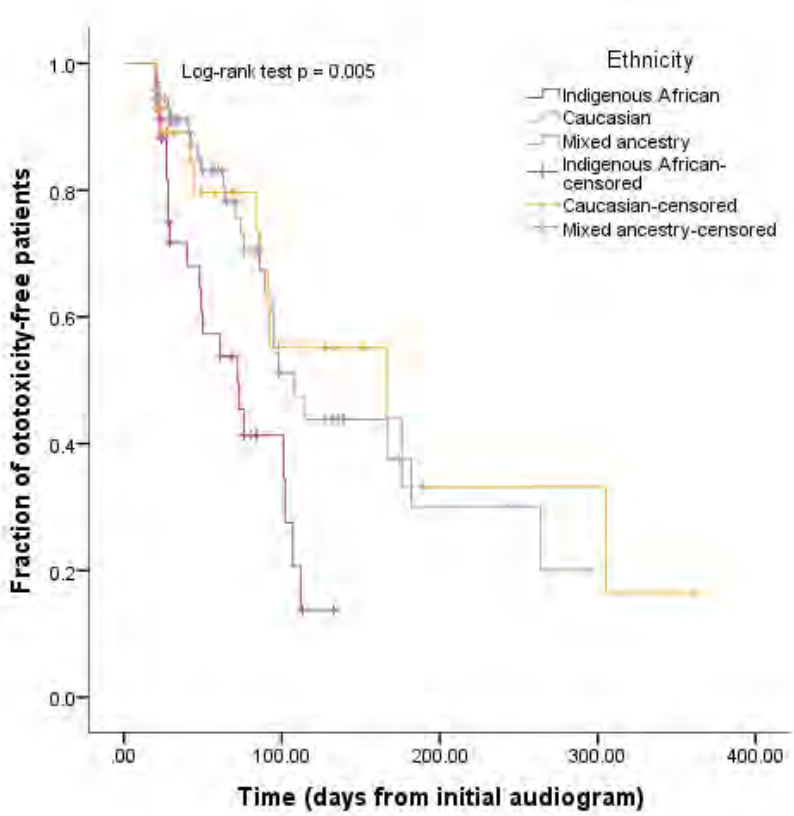


Figure 2.2: Kaplan-Meier survival analysis plot of ototoxicity-free survival between the different ethnic groups in head and neck cancer, lymphoma and osteosarcoma patients. Start dates were the date of first exposure to cisplatin, and end points were taken as the date at which ototoxicity was first observed in the patient. The time between these two dates, in days, is on the x-axis. The fraction of patients without ototoxicity is on the y-axis. Patients who did not develop Grade > 0 ototoxicity were censored at the date of their last recorded audiogram (crossed lines).

2.3.4 Statistical modelling of cisplatin-induced ototoxicity

The ability of cumulative dose, individual dose and ethnicity to predict ototoxicity was modelled using forward logistic regression. When investigating the head and neck, lymphoma and osteosarcoma patients, each of the covariates was retained as a significant

predictor of hearing loss (cumulative cisplatin dose: $p < 0.001$; African ethnicity: $p = 0.012$; individual cisplatin dose: $p = 0.041$). In comparison, only ethnicity was identified as a predictor of clinically relevant (Grade ≥ 2A) ototoxicity using forward logistic regression ($p = 0.006$). Cumulative dose ($p = 0.171$) and individual dose ($p = 0.170$) could not predict Grade ≥ 2A ototoxicity. Similar results were obtained when considering all the patients, regardless of cancer diagnosis: forward logistic regression identified the cumulative dose of cisplatin ($p < 0.001$), as well as black African ethnicity ($p = 0.005$) and individual dose ($p = 0.003$) as predictors of ototoxicity. Regarding clinically significant ototoxicity, ethnicity was the most significant predictor of hearing impairment ($p = 0.001$); however, when analysing the total cohort, both the cumulative dose ($p = 0.014$) and the individual dose ($p = 0.042$) were identified as predictors as well.

2.4 Discussion

Ototoxicity is a severe, potentially disabling ADR which can affect a large proportion of cisplatin-receiving cancer patients, and can alter treatment outcomes due to quality-of-life considerations. The characterisation of risk factors is therefore of interest, as this could enable the *a priori* identification of patients who are more susceptible to ototoxicity, and play a role in the future personalisation of treatment regimens. The only conclusive risk factor which has been identified to date, however, is a high cumulative dose of cisplatin (Bokemeyer et al, 1998; Li et al, 2004), which was a predictor of ototoxicity in this study. In addition, this investigation has described a previously unreported association between geographic ethnicity and ototoxicity.

Due to significant differences in the distribution of ethnicity between the cancer diagnoses, patients diagnosed with head and neck cancers, lymphomas and osteosarcomas were focussed on in order to reduce the effect of possible confounding clinical factors. This is because it was evident that different modalities of treatment presented different risks of ototoxicity to patients. In particular, those with cancers of the reproductive system (i.e. testes, cervix and ovaries) were at a 2.9-fold reduced risk of hearing impairment, possibly due to the lower dose of cisplatin administered in the treatment of these cancers (median dose: 35.00 mg/m^2). The data also suggested that osteosarcoma, lymphoma, and head and neck cancer patients were exposed to similar treatment regimens, and there was no significant difference in dosage between these regimens (median dose: 100.00 mg/m^2). In this group of patients, the ethnicity of the patient remained in significant association with ototoxicity, with indigenous Africans at a 2.4 times greater risk of hearing impairment. These patients were also 2.5 times more susceptible to the development of ototoxicity in Grades

2A, 2B and 3, the grades of hearing loss at which clinical interventions such as hearing aids are usually required (Chang and Chinosornvatana, 2010).

Analysis of audiograms from indigenous African patients indicated significantly greater cisplatin-induced hearing loss across a wide range of frequencies; all test frequencies between 3.0 kHz and 8.0 kHz were subject to greater shifts due to treatment. These results were also observed in the total cohort of patients (Appendix F). Notably, audiometric thresholds in the speech frequencies (0.5-4.0 kHz) were more likely to be affected in indigenous Africans, while hearing impairment tended to be limited to the high frequencies (4.0-8.0 kHz) in the Caucasian and mixed ancestry patients. This indicates that ototoxicity tends to be more severe in indigenous Africans, and has a greater potential to affect quality-of-life. In addition, this patient population developed hearing loss at a significantly faster rate. In the United States of America (USA), it has been reported that individuals of African ancestry usually maintain better hearing across all ages compared to Caucasians (Agrawal et al, 2008; Lin et al, 2011). Melanin is present in the cochlea, at a density which is proportional to pigmentation of the skin, and may play a role in otoprotection and antioxidant function (Murillo-Cuesta et al, 2010; Xiong et al, 2011). Pigmented animals have displayed more resistance to aminoglycoside ototoxicity than albino animals (Wu et al, 2001), although the protective effects of melanin are equivocal. Melanin has been demonstrated to bind drugs such as aminoglycosides and anthracyclines, which may potentiate their toxic effects (Svensson et al, 2003). Although this may explain the increased incidence of hearing loss observed amongst the black African individuals in this cohort of cisplatin-receiving patients, melanin has not shown any affinity for cisplatin *in vitro* (Svensson et al, 2003).

No other demographic variables associated with ototoxicity in this group of patients. While young paediatric patients may be more susceptible to the ototoxic effects of cisplatin (Li et al, 2004), there is currently no suggestion that the effect of young age is maintained in adults (Oldenburg et al, 2007), or even other paediatric populations (Bertolini et al, 2004; Ross et al, 2009). It has been reported that male patients are at a greater risk of hearing impairment in response to chemotherapy than female patients (Yancey et al, 2012), although similarly this association has not been replicated (De Jongh et al, 2003; Bertolini et al, 2004; Li et al, 2004). In contrast, few human correlational investigations into cisplatin-induced ototoxicity have considered the role of ethnicity. Those which have did not report any association with the condition (Li et al, 2004; Pussegoda et al, 2013; Yang et al, 2013). This is likely due to the high proportion (> 75%) of Caucasian patients included in these analyses; it is unclear what proportion of the remainder consisted of indigenous Africans. Of the patients with an

assigned ethnicity in this study, a total of 84.3% were non-Caucasian, of whom 26.6% were indigenous Africans.

It should be noted that information regarding co-medications and co-morbidities was not available in this investigation, and therefore the role of these potential covariates in ototoxicity cannot be ascertained. Additionally, data regarding the use of cranial irradiation amongst head and neck cancer patients was limited, and has been identified as a potential risk factor, particularly at doses exceeding 48 Gy (Chen et al, 2006). While this may explain why head and neck cancer patients seemed more likely to develop ototoxicity in this study, the majority (63.0%) of patients who received RT to the head and neck regions did so after cisplatin therapy, and therefore after audiological monitoring had ceased. There was no indication that the use of RT during treatment increased the risk of hearing loss. The concurrent administration of RT and cisplatin has failed to associate with ototoxicity in other investigations (Ilveskoski et al, 1996; Knight et al, 2005; Ross et al, 2009; Choeyprasert et al, 2013) and may be due to the dose of radiation used: in this study, the average dose (median dose: 45 Gy) was less than the reported threshold of 48 Gy (Chen et al, 2006).

A previous epidemiological investigation focussing on the prevalence of ototoxicity in a similar patient cohort reported an ototoxicity incidence of 55.1% (Whitehorn et al, 2014a). In the present study, however, 39.7% of all patients were affected. Although 188 more patients were included in this study compared to the previous, the difference should rather be attributed to the different definitions of ototoxicity employed in the analyses. The Chang ototoxicity grading scale is a modification of the Brock criteria which are widely used in studies of ototoxicity (Brock et al, 1991), and accounts for the fact that ototoxicity initially affects the higher ranges of hearing before progressing into the lower frequencies (Chang and Chinosornvatana, 2010). It is therefore possible that non-ototoxic shifts in hearing were included in the 55.1% originally reported, as the less stringent ASHA criteria of ototoxicity were used (ASHA, 1994). When analysing the head and neck cancer, lymphoma and osteosarcoma patients with the Chang grading scale, an ototoxicity incidence of 44.8% was observed, although 61.7% of indigenous Africans developed hearing impairment at these doses. These results suggest that the indigenous African population of SA may be a sub-group of patients which is more at risk of cisplatin's ototoxic effects.

2.5 Conclusion

The increased susceptibility of indigenous Africans to hearing loss in this investigation indicates a new potential risk group of patients. Since all patients were attending the same

hospital and were subjected to a similar standard of care, the discrepancy between the ethnicities cannot be attributed to differences in healthcare. Previous studies have identified genetic variants which may modify the risk of ototoxicity in Caucasian cancer patients (Oldenburg et al, 2007; Riedemann et al, 2008; Caronia et al, 2009; Ross et al, 2009; Pussegoda et al, 2013; Spracklen et al, 2014; Lanvers-Kaminsky et al, 2015). It is therefore probable that the increased susceptibility of the indigenous African population may be due to unique underlying genetic influences. Recent population genomic studies have shown that relatively few populations, even those in Africa, have remained free of admixture (Lachance et al, 2012; Pickrell et al, 2014). Consequently, detailed genomic studies of these sub-populations may be valuable in elucidating the contribution of genetics to ototoxicity.

Genetic variations in the pathways which metabolise cisplatin and modulate cell response after exposure to the drug may be involved, as might variants affecting the normal functioning of the inner ear. Although a previous investigation of the SA population identified novel modifiers of cisplatin-induced ototoxicity in the gene *OTOS*, these results were limited to Caucasian and mixed ancestry patients, and could not explain hearing loss in indigenous Africans (Spracklen et al, 2014). Further analysis of this genetically unique population may therefore reveal new genes in association with ototoxicity, contributing to our knowledge of this complex condition and how it occurs.

3. Investigation of candidate variants and their role in cisplatin-induced ototoxicity

3.1 Introduction

Platinum-based chemotherapy is prevalently employed in the treatment of various soft-tissue neoplasias. Cisplatin exerts its cytotoxic properties through the induction of apoptotic pathways in cancer cells, and it is thought that cell death is primarily triggered by damage to DNA (Wang and Lippard, 2005). Other molecular targets such as proteins and RNA have been suggested, as nucleus-independent pathways may also contribute to cell death (Akaboshi et al, 1992; Neault et al, 2001; Yu et al, 2008). Although the incorporation of cisplatin into chemotherapy regimens has proved to be a largely successful strategy in the management of cancer (McKeage, 1995; Einhorn, 2002), the effective use of the drug may be compromised when these apoptotic pathways are activated in noncancerous cells in the body, leading to the development of severe ADRs such as ototoxicity, nephrotoxicity, and neurotoxicity in the patient (McKeage, 1995).

Ototoxicity, or drug-induced hearing loss, is a potentially disabling reaction to cisplatin. It occurs when the drug accumulates in and damages cells of the inner ear, resulting in hearing loss which typically affects the higher frequencies of hearing (6-8 kHz) before progressing into the lower frequencies (0.5-4 kHz) (Kopelman et al, 1988; de Andrade et al, 2013). Ototoxicity is a prevalent adverse reaction to cisplatin chemotherapy, causing permanent hearing impairment in between 25% and 100% of patients treated with the drug (Kopelman et al, 1988; Bokemeyer et al, 1998; De Jongh et al, 2003; Knight et al, 2005; Kushner et al, 2006; Coradini et al, 2007; Arora et al, 2009; Yancey et al, 2012; Choeyprasert et al, 2013; Yang et al, 2013; Peleva et al, 2014). The reported incidences of ototoxicity vary depending on the sensitivity of hearing tests employed in the analyses, as well as the audiometric definition of ototoxicity used. Indeed, there are as yet no universally accepted audiological criteria for drug-induced hearing loss, which may complicate the comparison of different study populations.

There are numerous ototoxicity classifications and scales, including the ASHA guidelines (ASHA, 1994), the Brock criteria (Brock et al, 1991), the Chang ototoxicity grading scale (Chang and Chinosornvatana, 2010), and the Common Terminology Criteria for Adverse Events (CTCAE) published by the National Cancer Institute (NCI) (NCI, 2010). Two more recently proposed criteria are the International Society of Paediatric Oncology Boston (SIOP) ototoxicity grading scale (Brock et al, 2012), and the TUNE criteria, which were developed specifically for use in adult patients (Theunissen et al, 2014). These scales (Table 3.1) differ

both in terms of how the test frequencies are ranked in order of significance, as well as which threshold changes are considered markers of drug-induced hearing loss. For example, the ASHA and CTCAE criteria do not place any emphasis on lower or higher frequencies in the audiogram, whereas the other grading scales do account for the fact that ototoxicity initially affects higher frequencies of hearing (> 6 kHz); the higher grades of the Chang, Brock, SIOP and TUNE scales all reflect progression of hearing impairment to the lower frequencies of hearing. Nevertheless, the CTCAE are still largely used in studies of ototoxicity and have demonstrated superiority over the Brock criteria, which may underestimate hearing loss incidence due to the high threshold change it requires (Landier et al, 2014). The ASHA, Brock, Chang, SIOP and CTCAE ototoxicity grading scales have all shown a degree of concordance with one another in their abilities to detect hearing loss (Bass et al, 2014; Landier et al, 2014). However, no data on the comparability of the TUNE grading system is available as yet.

Table 3.1: Different grading scales of ototoxicity

Scale	Grade 1[a]	Grade 2	Grade 3	Grade 4
ASHA	≥ 20 dB at any freq or ≥ 10 dB at any two adjacent freqs	-	-	-
Brock	≥ 40 dB at 8 kHz	≥ 40 dB at 4 kHz and above	≥ 40 dB at 2 kHz and above	≥ 40 dB at 1 kHz and above
Chang	1A: ≥ 40 dB at 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 any freq < 4 kHz	≥ 40 dB at 2 or 3 kHz and above	≥ 40 dB at 1 kHz and above
CTCAE	≥ 15 and ≤ 25 dB averaged at any two adjacent freqs	> 25 dB averaged at any two adjacent freqs	> 25 dB averaged at any three adjacent freqs	> 80 dB at 2 kHz and above
SIOP	> 20 dB at 6 or 8 kHz	> 20 dB at 4 kHz and above	> 20 dB at 2 or 3 kHz and above	> 40 dB at 2 kHz and above
TUNE	1A: ≥ 10 dB at 8 kHz or above 1B: ≥ 10 dB at 1 to 4 kHz	2A: ≥ 20 dB at 8 kHz or above 2B: ≥ 20 dB at 1 to 4 kHz	≥ 35 dB at 1 to 4 kHz	≥ 70 dB at 1 to 4 kHz

[a] Grade 0 (no hearing loss) omitted from all scales for clarity

ASHA, American Speech-Language-Hearing Association; *CTCAE*, Common Terminology Criteria for Adverse Events; *dB*, decibels; *freq*, frequency; *kHz*, kilohertz; *SIOP*, International Society of Paediatric Oncology Boston

Regardless of the audiometric criteria, there is much inter-individual variability in the development of hearing impairment amongst patients. One risk factor of ototoxicity which has been consistently and independently identified is the cumulative dose of cisplatin, in which patients exposed to greater total doses of the drug are more likely to develop significant hearing loss (Bokemeyer et al, 1998; Bertolini et al, 2004; Li et al, 2004;

Whitehorn et al, 2014b). Predictive predisposing factors would be more effective, however, if they were not related to the treatment plan itself, and are inherent to the patient. Non-clinical risk factors which have been suggested to date include patient gender, age extremes, pre-existing audiological markers such as hearing loss and prior noise exposure, and impaired renal function. Unfortunately, none of these variables have been able to explain variability in patient response with much success (Bokemeyer et al, 1998; De Jongh et al, 2003; Li et al, 2004; Chen et al, 2006; Yancey et al, 2012; Choeyprasert et al, 2013; Kirkim et al, 2014).

A potential source of inter-individual variability in drug response is the patient genome. There has been increasing focus on the pharmacogenetics of cisplatin-induced ototoxicity, and several genetic polymorphisms have been reported in association with an altered risk of hearing impairment as a result of treatment (Rybak et al, 2007). Many of these results could not be replicated in independent investigations, indicating that they may be false positives or population-specific. Although the heritability of cisplatin sensitivity has been demonstrated at the cellular level (Dolan et al, 2004; Shukla et al, 2008), it is evident that pharmacogenetic studies into cisplatin response have so far been unable to identify reliable predictors of ototoxicity. While it can be expected that the effect sizes of genetic associations may decrease in replication studies (Boddy, 2013), genetic variants in *TPMT*, *XPC*, *GSTP1*, and *LRP2* failed to associate with ototoxicity in populations alternative to those in which they were originally reported (Riedemann et al, 2008; Ross et al, 2009; Choeyprasert et al, 2013; Whitehorn et al, 2014b; Yang et al, 2013; Hagleitner et al, 2014; Lanvers-Kaminsky et al, 2014). This reduces confidence in the role those markers play in the development of cisplatin-induced ototoxicity. With the exception of *GSTP1* and *OTOS*, none of the genes which have been investigated so far have a known function in the pathway whereby cisplatin induces ototoxicity (Oldenburg et al, 2007; Spracklen et al, 2014). For example, *TPMT*, *COMT*, *ABCC3*, *ABCB5* and *DPYD* were identified through a screen of all ADME-related genes (Ross et al, 2009; Pussegoda, 2012; Pussegoda et al, 2013), while *LRP2* was chosen as a candidate by similarity, since its encoded product is a transporter of another class of ototoxic drugs, the aminoglycosides (Riedemann et al, 2007).

In order to explain the pharmacogenetics of cisplatin-induced ototoxicity, new genes need to be investigated as potential sources of variability in drug response. Given the inconsistencies of the aforementioned genetic association studies, future candidate genes may be better chosen based on knowledge of the pathway by which cisplatin induces its ototoxicity, with an emphasis on functional variants. The aim of this investigation is to consider the role of new candidate genes in the development of cisplatin-induced ototoxicity in SA cancer patients.

3.2 Methods

3.2.1 Research design

This project is the continuation of an investigation, initially started in 2012, into the pharmacogenetics of cisplatin-induced ototoxicity in adult SA cancer patients (HREC reference no.: 389/2012). It is a collaborative study involving the Division of Audiology (UCT/GSH) and the Department of Radiation Oncology (GSH), which previously identified two potential genetic modifiers of ototoxicity in the genes *OTOS* and *COMT* (Spracklen et al, 2014; Whitehorn et al, 2014b).

The present research will investigate new potential candidate genes that may modify patient susceptibility to hearing loss. This is a longitudinal investigation in which the participants are all exposed to cisplatin, and audiometric data that is collected throughout treatment is used to assess the development of ototoxicity.

3.2.2 Patient recruitment

Ethical approval for this study was obtained from the HREC (HREC reference no.: 220/2014; Appendix A), and patients who attended the Audiology Clinic and the Department of Radiation Oncology at GSH were recruited between 2012 and 2015. Potential participants were approached prospectively or retrospectively by a registered research nurse from the Division of Human Genetics, UCT, if the patients met the inclusion criteria of the study. Prospective recruitment involved contacting eligible patients in the wards when they were admitted for treatment, while previous GSH patients were identified retrospectively by compiling a list of those who met the inclusion criteria according to the hospital records.

3.2.2.1 Inclusion and exclusion criteria

Eligible patients were those whose primary diagnosis was cancer of the soft tissues, for which they were receiving cisplatin-based chemotherapy at GSH. Additional inclusion criteria were the presence of a baseline hearing test prior to treatment and at least one post-treatment audiometric examination, so that the audiological response to cisplatin could be measured. Patients were ineligible if they did not have any recorded audiometric tests before or during treatment, as there would be insufficient data to determine the development of hearing impairment. Patients were also excluded if they were in the terminal stages of their diseases, did not consent to participate, or did not understand the project and its

implications. Patients with baseline hearing loss were included in the study, although its presence was noted as a possible confounding factor.

3.2.2.2 Collection of data, biological samples, and informed consent

Written informed consent was obtained from each patient during the recruitment process (Appendix B), as well as basic demographic information such as sex, date of birth, and self-reported ethnicity. Additionally, a biological sample of either blood or saliva was acquired from each participant for use in molecular investigations (Appendix C). Informed consent was requested for access to patient folders (Appendix D), from which clinical information relating to cisplatin treatment, including the cancer diagnosis, drug dosages, cycle information and the results of audiometric tests were obtained. Approval for the retrieval of patient folders was obtained from GSH (Appendix E). The medical records were also reviewed for other relevant information such as the use of cranial irradiation during treatment, the development of tinnitus and vestibular problems, as well as whether the treatment regimen was altered due to an adverse reaction to cisplatin.

3.2.2.3 Audiometric analysis

Audiometry tests were conducted on patients at the Audiology Clinic, GSH, before each cycle of cisplatin treatment. A GSI 61 audiometer (Grason-Stadler, Eden Prairie, MN USA) was used to determine hearing thresholds by pure-tone audiometry, in which air and bone conduction thresholds were measured in each ear at frequencies between 0.25 kHz and 8.0 kHz.

Pre-treatment audiograms were analysed to determine patient baseline hearing thresholds, including the existence of any pre-existing hearing loss. This was defined as a hearing threshold of ≥ 25 dB at any frequency in the audiogram and classified as high-frequency, conductive, or noise-induced, according to the type of hearing loss presented. Changes in hearing during treatment were assessed using the follow-up audiometric data. Audiograms obtained before each subsequent cisplatin cycle were compared to the baseline audiograms to determine hearing threshold changes during treatment, relative to their baseline. Significant hearing loss was defined and graded using the Chang ototoxicity grading scale (Chang and Chinosornvatana, 2010), the ASHA guidelines (ASHA, 1994), CTCAE version 4.03 (NCI, 2010), and TUNE (Theunissen et al, 2014) definitions of ototoxicity.

3.2.3 DNA extraction and quality control

Genomic DNA was extracted from the patients' biological samples and archived at -4°C within the Division of Human Genetics, UCT. DNA was isolated from peripheral blood lymphocytes using a modified salting out method (Miller et al, 1988) (Appendix G), while the ORAgene (DNA Genotek Inc., Ontario, Canada) or Norgen Biotek Saliva DNA Collection, Preservation and Isolation (Norgen Biotek Corp., Ontario, Canada) kits were used for the isolation of DNA from peripheral salivary endothelial cells, as following the manufacturers' recommendations (Appendix G).

DNA extracts were quantified using a NanoDrop® 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE USA) and diluted to working concentrations of 100 ng/µl. The integrity of the DNA samples was interrogated by agarose gel electrophoresis on 1% (w/v) agarose gels (Appendix H) which were stained with 1X SYBR® Safe DNA gel stain (Life Technologies™, Carlsbad, CA USA).

3.2.4 Candidate selection and the amplification of target DNA regions

Candidate genes and variants were selected based on the literature. Genes which have a reported role in the cellular response to cisplatin were prioritised, as variation within those genes may be more likely to influence patient responses to treatment. In order to focus on variants with possible functional effects, those with prior associations with modified disease susceptibility were selected for analysis in the cohort. Although SNPs within genes encoding drug metabolising enzymes such as *TPMT* and *GSTP1* were previously investigated and found to have no role in ototoxicity in the SA population (Whitehorn et al, 2014b), two variants which had not been investigated in this population are *ABCC3* c.4509A>G (rs1051640) and *ABCB5* c.1290+1532G>T (rs10950831), and were included as variants of interest in the investigation.

The polymerase chain reaction (PCR) was used to amplify genomic regions which contained the variants of interest. The target SNPs were *SLC22A2* c.808T>G (rs316019), *nuclear factor, erythroid 2-like 2* (*NFE2L2*) g.177265309C>A (rs6721961), *EIF3A* c.3527-704C>T (rs10787899) and g.119081149A>G (rs3824830), *CDH13* g.82609046A>T (rs11646213), *ABCC3* rs1051640 and *ABCB5* rs10950831. PCR primers were synthesised by Integrated DNA Technologies (IDT) (IDT Inc., Coralville, IA USA) (Table 3.2) and resuspended in 1X Tris/EDTA (TE) buffer (Appendix H) following the manufacturer's instructions. The optimum PCR conditions such as annealing temperature (T_a) and primer concentration were

established in singleplex for each primer pair, as well as in multiplex. The T_a was optimised by performing PCR over a gradient of increasing annealing temperatures between 50°C and 60°C while other reaction conditions remained constant.

Table 3.2: Properties of the primers used for amplification of target regions

Target gene	Variant	Primer no.	S/A	Primer sequence (5`-3`)	Expected product size (bp)
ABCB5	rs10950831	1	S	gttgactctacatccaggtctt	792
		2	A	agtctgaggttgctctaatgat	
ABCC3	rs1051640	3	S	tgagcaagtacccagaagag	196
		4	A	ttaggcaagtccagcatc	
CDH13	rs11646213	5	S	tgtgacattcggaagaga	583
		6	A	ggagtaggcaagcactac	
EIF3A	rs10787899	7	S	cacgcagatttcagcatagt	983
		8	A	gggtaacagtatagcagattc	
EIF3A	rs3824830	9	S	cagttcacaggctcttca	258
		10	A	atgattgtctctcacggttg	
NFE2L2	rs6721961	11	S	aagagttgtttgcgaaggtc	479
		12	A	tttggtgggaagaggttc	
SLC22A2	rs316019	13	S	aatgggtctggagagtga	263
		14	A	ggagaacagtggggattttt	

A, antisense primer; *ABCB5*, ATP-binding cassette, sub-family B, member 5; *ABCC3*, ATP-binding cassette, sub-family C, member 3; *bp*, base pairs; *CDH13*, cadherin 13; *EIF3A*, eukaryotic translation initiation factor 3, subunit A; *NFE2L2*, nuclear factor erythroid 2-like 2; *no.*, number; *S*, sense primer; *SLC22A2*, solute carrier family 22, member 2

3.2.4.1 PCR conditions

Amplification of the target regions was performed in three multiplex PCRs (Table 3.3). Each reaction was performed in a final volume of 25 µl and contained 150 ng DNA template, 1X Colourless GoTaq® reaction buffer (Promega, Madison, WI USA), 0.24 mM dNTPs (Bioline, London, UK), 0.75 units GoTaq® DNA polymerase (Promega), and between 0.20 and 0.52 µM of each primer (Table 3.3). To check for contamination of the PCR reagents, a no-template control was included for each reaction, which contained water instead of DNA.

The reactions were performed in a Bio-Rad T100™ thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA USA). Thermal cycling conditions included an initial denaturation step of 94°C for 3 mins, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at the optimised T_a (Table 3.3) for 30 s, and extension at 72°C for 1 min. A final extension step of 72°C for 10 mins was also included. PCR products were visualised by agarose gel

electrophoresis on 2.5% (w/v) agarose gels stained with 1X SYBR® Safe DNA gel stain (Life Technologies™). GeneRuler™ 100 bp Plus DNA ladder (Thermo Scientific, Waltham, MA USA) was included as a size standard in each electrophoresis experiment (Appendix H).

Table 3.3: Optimised multiplex PCR conditions

	Target gene	Variant	Primer pair	Primer concentration (µM)
PCR 1 (T_a = 56°C)				
	ABCB5	rs10950831	1/2	0.40
	EIF3A	rs3824830	9/10	0.40
PCR 2 (T_a = 56°C)				
	ABCC3	rs1051640	3/4	0.40
	CDH13	rs11646213	5/6	0.52
	EIF3A	rs10787899	7/8	0.52
PCR 3 (T_a = 55°C)				
	NFE2L2	rs6721961	11/12	0.40
	SLC22A2	rs316019	13/14	0.40

ABCB5, ATP-binding cassette, sub-family B, member 5; *ABCC3*, ATP-binding cassette, sub-family C, member 3; *bp*, base pairs; *CDH13*, cadherin 13; *EIF3A*, eukaryotic translation initiation factor 3, subunit A; *NFE2L2*, nuclear factor erythroid 2-like 2; *PCR*, polymerase chain reaction; *SLC22A2*, solute carrier family 22, member 2; T_a, annealing temperature

3.2.4.2 Purification of PCR products

PCR products were prepared for use in downstream molecular applications using alkaline phosphatase (AP) and exonuclease I (ExoI) purification. Each purification reaction contained 1.5 units FastAP™ (Thermo Scientific) and 4 units ExoI (Thermo Scientific) in a final volume of 25 µl, and was conducted in a Bio-Rad T100™ thermal cycler (Bio-Rad Laboratories Inc.). The reaction conditions consisted of a 37°C incubation period of 1 h, followed by a 75°C step for 15 mins.

3.2.5 SNaPshot® genotyping

The variants of interest were genotyped using the SNaPshot® multiplex kit (Life Technologies™). Primers for the single nucleotide extension sequencing reactions were synthesised by IDT (Table 3.4). Each reaction contained a total of 5 µl purified PCR product, 2 µl reaction mix and 1 µl primer mix, to a total volume of 10 µl. The reaction conditions consisted of 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 30 s, conducted in a GeneAmp® 9700 thermal cycler (Applied Biosystems, Austin, TX USA).

Table 3.4: Primers for SNaPshot® genotyping

Target gene	Variant	SNaPshot® primer (5`-3`)	S/A	Length (bp)	Expected product size (bp)
ABCB5	rs10950831	aaatgatgttaatatcctttatagtctgtatcacg	S	35	36
ABCC3	rs1051640	cctggtcctggacaaaggagtagtagctga	S	30	31
CDH13	rs11646213	aaagcatacagaaaacatatctttgaaagaagttgc	S	36	37
EIF3A	rs10787899	ttggatatgaagggcttgaaaa	S	22	23
EIF3A	rs3824830	gattcccaggctgga	S	15	16
NFE2L2	rs6721961	gggagatgtggacagc	A	16	17
SLC22A2	rs316019	gagcaagaagaagaagttgggcagag	A	26	27

A, antisense strand; *ABCB5*, ATP-binding cassette, sub-family B, member 5; *ABCC3*, ATP-binding cassette, sub-family C, member 3; *bp*, base pairs; *CDH13*, cadherin 13; *EIF3A*, eukaryotic translation initiation factor 3A; *NFE2L2*, nuclear factor erythroid 2-like 2; *S*, sense strand; *SLC22A2*, solute carrier family 22, member 2

3.2.5.1 Visualisation and analysis of SNaPshot® reaction products

The reaction products were purified through the addition of 1 unit of FastAP™ (Thermo Scientific) to each. Purification reactions were incubated at 37°C for 1 h, after which they were heated to 75°C for 15 mins, and 95°C for 5 mins in a GeneAmp® 9700 thermal cycler (Applied Biosystems).

In preparation for capillary electrophoresis, 5 µl of the purified SNaPshot® product was mixed with 4.7 µl Hi-Di™ formamide (Life Technologies™) and 0.3 µl GeneScan™ 120 LIZ® size standard (Life Technologies™) in a MicroAmp® 96-well reaction plate (Life Technologies™). The products were heated to 95°C for 5 mins in a Hybaid touchdown thermal cycler (The Scientific Group, Johannesburg, SA) before separation and visualisation by capillary electrophoresis on an ABI PRISM® 3130*xl* genetic analyser (Applied Biosystems), using the 3130*xl* Genetic Analyser Data Collection software version 3.1 (Applied Biosystems). SNaPshot® genotyping results were viewed and analysed using the GeneMapper® software version 4.1 (Applied Biosystems), with the default SNaPshot® analysis method selected. For each SNP of interest, the colour peak indicated the genotype at that position, with red labelling thymine, green labelling adenine, blue labelling guanine, and black labelling cytosine.

3.2.6 Validation of genotyping

The results of SNaPshot® genotyping were validated by direct cycle sequencing of PCR products. At least five percent of the genotype calls of each gene variant were confirmed in this manner.

3.2.6.1 Direct cycle sequencing reaction conditions

PCR products were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing kit (Life Technologies™). Sequencing reactions included 2 µl purified PCR product, 1X sequencing buffer, 0.25X Terminator Mix, and 2 µM primer, to a total volume of 10 µl. The reactions were conducted in a GeneAmp® 9700 thermal cycler (Applied Biosystems), and thermal cycling conditions consisted of initial denaturation at 98°C for 5 mins, followed by 30 cycles of denaturation at 96°C for 30 s, annealing at 50°C for 15 s and extension at 60°C for 4 mins.

Sequencing products were purified by ethanol precipitation: 2.2 volumes of 100% ethanol (Sigma-Aldrich®, St Louis, MO USA) and 0.36 M sodium acetate (pH 5.21) (Merck Chemicals, Darmstadt, Germany) were added to each sequencing reaction. The reactions were then incubated at -22°C overnight and centrifuged at 10,000 rpm for 10 mins in a Centrifuge 5415D (Eppendorf AG, Hamburg, Germany), after which 30 µl of 70% ethanol was added to each precipitate. These were centrifuged again at 10,000 rpm for 10 mins, and the supernatant discarded. Once dry, the pellet was resuspended in 10 µl sdH_2O (Adcock Ingram, Johannesburg, SA).

To visualise the sequencing products, 5 µl of the resuspended reaction was mixed with 8 µl Hi-Di™ formamide (Life Technologies™) in a MicroAmp® 96-well reaction plate (Life Technologies™). Sequencing products were denatured at 95°C for 5 mins in a Hybaid touchdown thermal cycler (The Scientific Group), before separation and visualisation on an ABI PRISM® 3130*xl* Genetic Analyser (Applied Biosystems). The 3130*xl* genetic analyser data collection software version 3.1 (Applied Biosystems) was used for capture of the sequencing reaction output.

3.2.6.2 Analysis of sequencing products

The FinchTV software version 1.4.0 (http://www.geospiza.com/Products/finchtv.shtml; accessed 10.07.2014) was used to visualise and edit electropherograms. This involved removal of indistinct regions at the edges of the electropherograms, and confirming each base call in the remaining sequence. The edited sequences were aligned to a reference sequence downloaded from the Ensembl database (http://www.ensembl.org/index.html, accessed 30.03.2014, accession numbers: *ABCB5*, ESNG00000004846; *ABCC3*, ESNG00000108846; *CDH13*, ESNG00000140945; *EIF3A*, ESNG00000107581; *NFE2L2*, ESNG00000116044; *SLC22A2*, ESNG00000112499) using the ClustalW multiple sequence

alignment tool in the BioEdit Sequence Alignment Editor version 7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html; accessed 10.07.2014). The alignment was analysed at the position of the variant, to see if the genotyping call matched that of the sequence.

3.2.7 Statistical analysis of data

Observed genotypes were analysed for deviation from those expected under Hardy-Weinberg Equilibrium (HWE) using Chi-squared analysis (www.tufts.edu/~mcourt01/.../Court%20lab%20-%20HW%20calculator.xls, accessed 29.05.2013). All other statistical analyses were performed using the IBM® SPSS® Statistics programme, version 22.0.0.0 (IBM® Corp.). Categorical variables such as patient ethnicity, sex, cancer diagnosis, and genotypic data were analysed individually for their associations with cisplatin-induced ototoxicity by Chi-squared tests, or Fisher's exact tests when expected frequencies were less than 5. The distribution of numerical variables, which included patient age and dosage information, was examined using the Shapiro-Wilk test. Since all the numerical data was not normally distributed, Mann-Whitney U tests were applied to the data. The variables were also tested for their ability to predict ototoxicity using conditional forward logistic regression, in which all the data was included as potential covariates, and the development of ototoxicity was a binary dependent variable. Nondirectional binary logistic regression was used to adjust the observed allele frequency differences between patient groups for the identified non-genetic confounders. In all the analyses, p-values < 0.05 were considered significant. In the analysis of genetic data, this value was adjusted to 0.007 by Bonferroni correction to account for the seven SNPs analysed.

Linkage disequilibrium (LD) between variants was tested for those which are located on the same chromosome. The online version of SHEsis (http://analysis2.bio-x.cn/myAnalysis.php; accessed 22.08.2015) was used to calculate and compare pair-loci r^2 and D` values for each of the ethnic groups represented in this study (i.e. Black African, Caucasian, mixed ancestry, and Indian). For variants in LD, haplotypes were treated as categorical variables and tested for their associations with ototoxicity using the statistical approaches described above.

3.3 Results

3.3.1 Patient cohort

During the study period, a total of 349 cancer patients were recruited from GSH. Of these, 222 patients met the inclusion criteria and were considered for molecular investigations (Table 3.5). The remaining 127 patients were excluded mainly for audiological reasons: 22 patients did not undergo any audiological testing during treatment, 58 received only a baseline examination with no follow-up audiometry performed, and three had audiological examinations during treatment but without any measure of pre-treatment hearing levels. Two patients who did meet the audiometric inclusion criteria were not included due to unreliable or unclear baseline audiometric readings, and six patients did not receive any cisplatin treatment. The hospital folders of 35 patients could not be accessed during the study period, and one patient withdrew from the study.

Table 3.5: Characteristics of the patient cohort used in molecular investigations

Demographic characteristics	
Age (years)	
Median	48
Range (min; max)	14; 75
Sex	
Male	158 (71.2%)
Female	64 (28.8%)
Ethnicity	
Black African	46 (20.7%)
Caucasian	38 (17.1%)
Mixed Ancestry	135 (60.8%)
Indian	3 (1.4%)
Baseline hearing impairment	
High-frequency	80 (36.0%)
Conductive	30 (13.5%)
Noise-induced	6 (2.7%)
Mixed	2 (0.9%)
None	104 (46.8%)
Clinical characteristics	
Anatomical site of cancer	
Head/Neck	112 (50.5%)
Oesophagus	10 (4.5%)
Testes, ovaries	33 (14.7%)
Stomach	11 (5.0%)
Osteosarcoma	13 (5.9%)
Lymphoma	31 (14.0%)
Other	10 (4.5%)
Unknown	2 (0.9%)
Cumulative dose (mg/m^2)	
Median	200.00
Range (min; max)	50.00; 629.21
Individual dose (mg/m^2)	
Median	90.91
Range (min; max)	17.98; 107.80
Duration of audiometric follow-up (days)	
Median	56.50
Range (min; max)	20.00; 1225.00

max, maximum; *min*, minimum

The cohort of 222 patients consisted of predominantly (71.2%) male individuals, and the primary ethnic group was Cape mixed ancestry (60.8%). Pre-existing hearing loss was observed in approximately half (53.1%) of the cohort: the most common form of hearing impairment was that limited to the high frequencies; conductive and noise-induced hearing losses were less commonly observed. Patients were also classified according to the anatomical site of cancer, and the most frequent cancer diagnoses were those of the head and neck (50.5%), testes and ovaries (14.7%) and lymphomas (14.0%). Cancers grouped under “other” included cancer of the bladder (n = 3), anus (n = 1), breasts (n = 1), skin (n = 1), thymus gland (n = 1), and mixed cancers (n = 3).

3.3.2 Incidence of ototoxicity in the cohort

Four different audiometric definitions of ototoxicity were included in this investigation, yielding ototoxicity incidence rates which ranged between 39.2% and 74.8% (Figure 3.1). While the development of ototoxicity is already a binary variable in the ASHA guidelines, the grading scales used in the Chang, CTCAE and TUNE criteria were dichotomised by comparing grade 0 to the other grades. Using this approach, demographic and clinical variables were analysed for possible hearing loss risk factors.

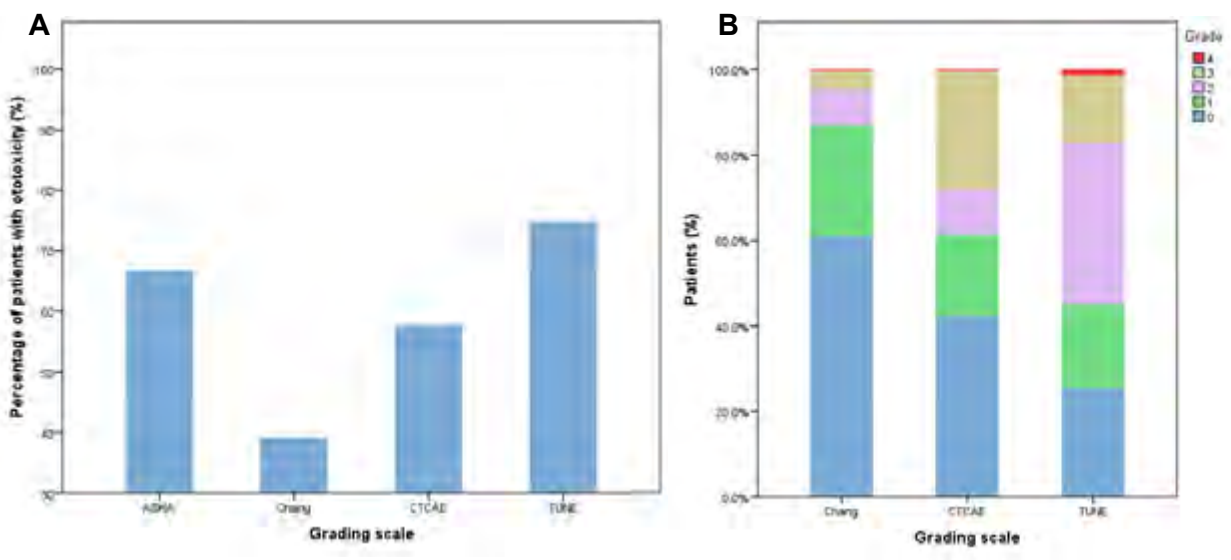


Figure 3.1: The incidences of hearing loss according to each of the ototoxicity definitions used (A), as well as the relative distributions of ototoxicity grades 0-4 in the Chang, CTCAE and TUNE grading scales (B).

Although the clinical and demographic risk factors of ototoxicity have been discussed in detail previously (Chapter 2), similar analyses were performed using the different ototoxicity definitions in the prospective cohort (Table 3.6). Clinical risk factors such as the cancer diagnosis and dosage information associated with ototoxicity despite the criteria used, while the demographic factors age, ethnicity and pre-existing hearing loss correlated less consistently at the level of $\alpha = 0.05$ (Table 3.6). Patients who developed ASHA- and TUNE-level ototoxicity were significantly older than those who did not, and individuals presenting with baseline hearing impairment were more likely to develop ototoxicity according to all the grading scales used, with the exception of the Chang grading scale. The gender of the patient, however, did not associate with an altered risk of ototoxicity for any of the definitions included (Table 3.6).

Table 3.6: Demographic and clinical characteristics of patients with and without ototoxicity

	No ototoxicity	Ototoxicity	p-value
Median age (years)			
ASHA	43.0	49.0	**0.009**[a]
Chang	48.0	48.0	0.543[a]
CTCAE	43.5	49.0	0.053[a]
TUNE	42.5	48.0	**0.026**[a]
Male sex			
ASHA	53 (71.6%)	105 (70.9%)	0.917[b]
Chang	102 (75.6%)	56 (64.4%)	0.072[b]
CTCAE	69 (73.4%)	89 (69.5%)	0.529[b]
TUNE	42 (75.0%)	116 (69.9%)	0.464[b]
Black African ethnicity			
ASHA	11 (14.9%)	35 (23.6%)	0.128[b]
Chang	18 (13.3%)	28 (32.2%)	**0.001**[b]
CTCAE	12 (12.8%)	34 (26.6%)	**0.012**[b]
TUNE	7 (12.5%)	39 (23.5%)	0.079[b]
Baseline hearing impairment			
ASHA	31 (41.9%)	87 (58.8%)	**0.017**[b]
Chang	70 (51.9%)	48 (55.2%)	0.628[b]
CTCAE	40 (42.6%)	78 (60.9%)	**0.007**[b]
TUNE	23 (41.1%)	95 (57.2%)	**0.036**[b]
Head/neck, oesophageal, osteosarcoma and lymphoma cancers			
ASHA	43 (58.1%)	123 (83.1%)	**< 0.001**[b]
Chang	91 (67.4%)	75 (86.2%)	**0.002**[b]
CTCAE	56 (59.6%)	110 (85.9%)	**< 0.001**[b]
TUNE	32 (57.1%)	134 (80.7%)	**< 0.001**[b]
Reproductive cancers			
ASHA	21 (28.3%)	12 (8.1%)	**< 0.001**[b]
Chang	29 (21.5%)	4 (4.6%)	**0.001**[b]
CTCAE	26 (27.7%)	7 (7.4%)	**< 0.001**[b]
TUNE	17 (30.4%)	16 (9.6%)	**< 0.001**[b]
Median cumulative dose			
ASHA	200.00	202.15	0.117[a]
Chang	198.48	230.78	**0.002**[a]
CTCAE	198.48	208.97	**0.013**[a]
TUNE	180.51	206.25	**0.030**[a]
Median individual dose			
ASHA	78.88	97.56	**0.002**[a]
Chang	79.65	98.84	**< 0.001**[a]
CTCAE	79.19	98.04	**< 0.001**[a]
TUNE	76.83	96.59	**0.003**[a]

[a] Mann-Whitney U test; [b] Chi-squared test

ASHA, American Speech-Language-Hearing Association; *CTCAE*, Common Terminology Criteria for Adverse Events

3.3.3 Candidate gene study

To explore potential genetic modifiers of cisplatin-induced ototoxicity, seven SNPs were genotyped in the patient cohort. Although 222 patients had audiometric phenotypes, genotyping experiments were conducted on 214 samples, because six samples had insufficient DNA, and two samples failed consistently during the analyses. One genotype could not be obtained due to lack of amplification at the *ABCB5* rs1051640 locus; this genotype was estimated to be T/G based on the ethnicity of the patient. All genotype frequencies were in accordance with HWE (Table 3.7).

Table 3.7: Observed genotype frequencies for each variant analysed

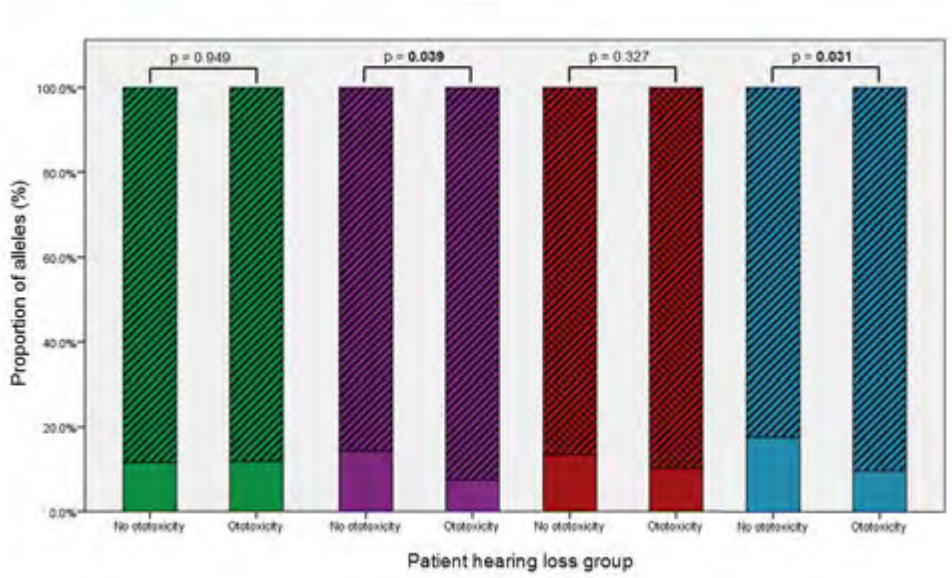
Variant	Genotype counts	Minor allele frequency	Hardy-Weinberg p-value
ABCB5 rs10950831	T/T: 60 T/G: 102 G/G: 52	0.48	0.507
ABCC3 rs1051640	A/A: 147 A/G: 60 G/G: 7	0.17	0.773
CDH13 rs11646213	A/A: 101 A/T: 87 T/T: 26	0.32	0.285
EIF3A rs10787899	C/C: 81 C/T: 92 T/T: 41	0.41	0.111
EIF3A rs3824830	A/A: 108 A/G: 83 G/G: 23	0.30	0.248
NFE2L2 rs6721961	C/C: 184 C/A: 29 A/A: 1	0.07	0.901
SLC22A2 rs316019	G/G: 168 G/T: 43 T/T: 3	0.11	0.895

ABCB5, ATP-binding cassette, sub-family B, member 5; *ABCC3*, ATP-binding cassette, sub-family C, member 3; *CDH13*, cadherin 13; *EIF3A*, eukaryotic translation initiation factor 3, subunit A; *NFE2L2*, nuclear factor erythroid 2-like 2; *SLC22A2*, solute carrier family 22, member 2

3.3.3.1 Single-site analysis

The association of each of the SNPs with ototoxicity was explored in all patients with available clinical data (n = 196) without stratification (Appendix I). At the level of $\alpha = 0.05$, the variant *SLC22A2* rs316019 demonstrated potential protective effects against Chang- and TUNE-level ototoxicity, while *NFE2L2* rs6721961 was similarly associated with reduced rates of ASHA- and CTCAE-level hearing impairment (Figure 3.2 and Appendix I).

A



B

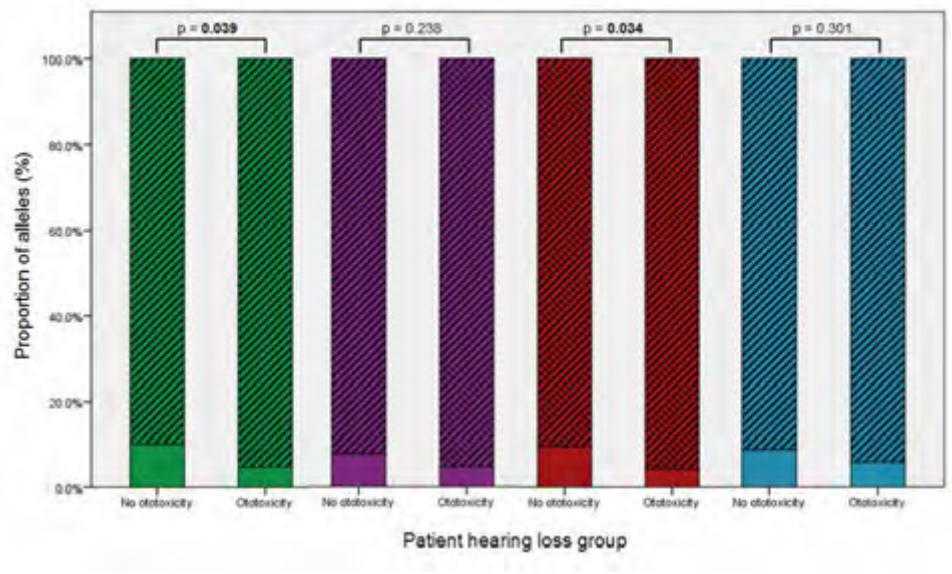


Figure 3.2: Presence of the *SLC22A2* rs316019 (A) and *NFE2L2* rs6721961 (B) variant alleles in the different ototoxicity classes. Green: ASHA; purple: Chang; red: CTCAE; blue: TUNE. Bars with diagonal markings indicate the reference allele, and unmarked bars represent the variant alleles of each SNP.

Because the total dose of cisplatin is a well-demonstrated risk factor of ototoxicity, patients who were exposed to below-average cumulative doses (median dose: 200 mg/m^2) were excluded in order to better identify SNPs with protective effects (Tables 3.8 – 3.11). When considering patients with cumulative doses greater than or equal to 200 mg/m^2 (n = 113), the variant *NFE2L2* rs6721961 was associated with ototoxicity regardless of the audiometric

criteria used (Tables 3.8 – 3.11). In all cases, the alternative A-allele associated with a reduced risk of ototoxicity. The association with ASHA- and CTCAE-grade ototoxicity retained significance after correction for multiple testing. However, the protective effects of the *SLC22A2* rs316019 T-allele were not maintained, even at the pre-correction p-value of 0.05 (Tables 3.9 and 3.11).

Table 3.8: Association of variants with ASHA-grade ototoxicity in patients exposed to cumulative cisplatin doses ≥ 200 mg/m^2

Variant	No ototoxicity	Ototoxicity	p-value	Odds ratio (95% CI)
ABCB5 rs10950831			0.204[a]	
T/T	6 (16.2%)	24 (31.6%)	0.083[a]	0.419 (0.154; 1.139)
T/G	21 (56.8%)	33 (43.4%)	0.183[a]	1.710 (0.774; 3.779)
G/G	10 (27.0%)	19 (25.0%)	0.817[a]	1.111 (0.455; 2.712)
T-allele	33 (44.6%)	81 (53.3%)	0.220[a]	0.706 (0.404; 1.233)
G-allele	41 (55.4%)	71 (46.7%)		
ABCC3 rs1051640			0.205[b]	
A/A	30 (81.1%)	51 (67.1%)	0.122[a]	2.101 (0.811; 5.442)
A/G	6 (16.2%)	24 (31.6%)	0.083[a]	0.419 (0.154; 1.139)
G/G	1 (2.7%)	1 (1.3%)	0.550[b]	2.083 (0.127; 34.265)
A-allele	66 (89.2%)	126 (82.9%)	0.214[a]	1.702 (0.730; 3.969)
G-allele	8 (10.8%)	26 (17.1%)		
CDH13 rs11646213			0.749[b]	
A/A	20 (54.1%)	35 (46.1%)	0.425[a]	1.378 (0.627; 3.032)
A/T	13 (35.1%)	32 (42.1%)	0.478[a]	0.745 (0.330; 1.681)
T/T	4 (10.8%)	9 (11.8%)	1.000[b]	0.902 (0.259; 3.148)
A-allele	53 (71.6%)	102 (67.1%)	0.492[a]	1.237 (0.673; 2.273)
T-allele	21 (28.4%)	50 (32.9%)		
EIF3A rs10787899			0.928[a]	
C/C	16 (43.2%)	30 (39.5%)	0.286[a]	0.650 (0.295; 1.435)
C/T	14 (37.8%)	31 (40.8%)	0.764[a]	0.884 (0.394; 1.980)
T/T	7 (18.9%)	15 (19.7%)	0.918[a]	0.949 (0.350; 2.574)
C-allele	46 (62.2%)	91 (59.9%)	0.740[a]	1.101 (0.622; 1.949)
T-allele	28 (37.8%)	61 (40.1%)		
EIF3A rs3824830			0.762[b]	
A/A	18 (48.6%)	41 (53.9%)	0.597[a]	0.809 (0.368; 1.776)
A/G	16 (43.2%)	27 (35.5%)	0.428[a]	1.383 (0.620; 3.084)
G/G	3 (8.1%)	8 (10.5%)	1.000[b]	0.750 (0.187; 3.009)
A-allele	52 (70.3%)	109 (71.7%)	0.822[a]	0.932 (0.506; 1.717)
G-allele	22 (29.7%)	43 (28.3%)		
NFE2L2 rs6721961			**0.002**[b]	
C/C	28 (75.7%)	73 (96.1%)	**0.002**[b]	0.128 (0.032; 0.507)
A/C	8 (21.6%)	3 (3.9%)	**0.005**[b]	6.713 (1.664; 27.083)
A/A	1 (2.7%)	0 (0.0%)	0.327[b]	-
C-allele	64 (86.5%)	149 (98.0%)	**0.001**[b]	0.129 (0.034; 0.484)
A-allele	10 (13.5%)	3 (2.0%)		
SLC22A2 rs316019			0.252[b]	
G/G	30 (81.1%)	58 (76.3%)	0.567[a]	1.330 (0.500; 3.537)
G/T	6 (16.2%)	18 (23.7%)	0.362[a]	0.624 (0.225; 1.732)
T/T	1 (2.7%)	0 (0.0%)	0.327[b]	-
G-allele	66 (89.2%)	134 (88.2%)	0.820[a]	1.108 (0.458; 2.681)
T-allele	8 (10.8%)	18 (11.8%)		

[a] Chi-squared test; [b] Fisher's exact test

ABCB5, ATP-binding cassette, sub-family B, member 5; *ABCC3*, ATP-binding cassette, sub-family C, member 3; *CDH13*, cadherin 13; *CI*, confidence interval; *EIF3A*, eukaryotic translation initiation factor 3, subunit A; *NFE2L2*, nuclear factor erythroid 2-like 2; *SLC22A2*, solute carrier family 22, member 2

Table 3.9: Association of variants with Chang-grade ototoxicity in patients exposed to cumulative cisplatin doses ≥ 200 mg/m^2

Variant	No ototoxicity	Ototoxicity	p-value	Odds ratio (95% CI)
ABCB5 rs10950831			0.621[a]	
T/T	14 (23.0%)	16 (30.8%)	0.348[a]	0.670 (0.290; 1.550)
T/G	30 (49.2%)	24 (46.2%)	0.748[a]	1.129 (0.538; 2.369)
G/G	17 (27.9%)	12 (23.1%)	0.561[a]	1.288 (0.548; 3.025)
T-allele	58 (47.5%)	56 (53.8%)	0. 354[a]	0.777 (0.460; 1.312)
G-allele	64 (52.5%)	48 (46.2%)		
ABCC3 rs1051640			0.835[b]	
A/A	45 (73.8%)	36 (69.2%)	0.593[a]	1.250 (0.551; 2.838)
A/G	15 (24.6%)	15 (28.8%)	0.610[a]	0.804 (0.349; 1.856)
G/G	1 (1.6%)	1 (1.9%)	1.000[b]	0.850 (0.052; 13.934)
A-allele	105 (86.1%)	87 (83.7%)	0.613[a]	1.207 (0.582; 2.504)
G-allele	17 (13.9%)	17 (16.3%)		
CDH13 rs11646213			0.872[a]	
A/A	31 (50.8%)	24 (46.2%)	0.621[a]	1.206 (0.575; 2.530)
A/T	23 (37.7%)	22 (42.3%)	0.618[a]	0.825 (0.388; 1.757)
T/T	7 (11.5%)	6 (11.5%)	0.992[a]	0.994 (0.312; 3.168)
A-allele	85 (69.7%)	70 (67.3%)	0.703[a]	1.116 (0.636; 1.959)
T-allele	37 (30.3%)	34 (32.7%)		
EIF3A rs10787899			0.861[a]	
C/C	24 (39.3%)	22 (42.3%)	0.749[a]	0.885 (0.417; 1.878)
C/T	24 (39.3%)	21 (40.4%)	0.910[a]	0.958 (0.450; 2.038)
T/T	13 (21.3%)	9 (17.3%)	0.592[a]	1.294 (0.503; 3.327)
C-allele	72 (59.0%)	65 (62.5%)	0.593[a]	0.864 (0.505; 1.477)
T-allele	50 (41.0%)	39 (37.5%)		
EIF3A rs3824830			0.527[a]	
A/A	29 (47.5%)	30 (57.7%)	0.282[a]	0.665 (0.315; 1.400)
A/G	26 (42.6%)	17 (32.7%)	0.279[a]	1.529 (0.708; 3.304)
G/G	6 (9.8%)	5 (9.6%)	0.969[a]	1.025 (0.294; 3.576)
A-allele	84 (68.9%)	77 (74.0%)	0.391[a]	0.775 (0.433; 1.387)
G-allele	38 (31.1%)	27 (26.0%)		
NFE2L2 rs6721961			0.092[b]	
C/C	51 (83.6%)	50 (96.2%)	**0.031**[a]	0.204 (0.043; 0.978)
A/C	9 (14.8%)	2 (3.8%)	0.051[a]	4.327 (0.891; 21.019)
A/A	1 (1.6%)	0 (0.0%)	1.000[b]	-
C-allele	111 (91.0%)	102 (98.1%)	**0.022**[a]	0.198 (0.043; 0.914)
A-allele	11 (9.0%)	2 (1.9%)		
SLC22A2 rs316019			0.206[b]	
G/G	44 (72.1%)	44 (84.6%)	0.111[a]	0.471 (0.184; 1.203)
G/T	16 (26.2%)	8 (15.4%)	0.160[a]	1.956 (0.760; 5.031)
T/T	1 (1.6%)	0 (0.0%)	1.000[b]	-
G-allele	104 (85.2%)	96 (92.3%)	0.097[a]	0.481 (0.200; 1.158)
T-allele	18 (14.8%)	8 (7.7%)		

[a] Chi-squared test; [b] Fisher's exact test

ABCB5, ATP-binding cassette, sub-family B, member 5; *ABCC3*, ATP-binding cassette, sub-family C, member 3; *CDH13*, cadherin 13; *CI*, confidence interval; *EIF3A*, eukaryotic translation initiation factor 3, subunit A; *NFE2L2*, nuclear factor erythroid 2-like 2; *SLC22A2*, solute carrier family 22, member 2

The *ABCB5* rs10950831 T-allele did show a weak association with TUNE-defined ototoxicity (Table 3.11). Because one of these genotypes was estimated to be T/G, the statistical tests were repeated using both homozygous alternative genotype calls. While the inclusion of G/G strengthened the association of this variant with hearing loss (Chi-squared test, p = 0.021), changing the genotype to T/T abolished any significance (Chi-squared test, p = 0.074). This is likely because the patient did not develop significant hearing loss according to any of the ototoxicity definitions, and it is therefore more likely to influence analysis were the ototoxicity incidence rate is high, such as with the TUNE grading scale. In order to overcome this, the

patient was excluded and the analyses performed again. The *ABCB5* rs10950831 T-allele remained in association with TUNE ototoxicity (Chi-squared test, p = 0.037, OR: 0.489, 95% CI: 0.248; 0.966). In all the statistical tests, the variant did not show any associations with the other ototoxicity grading scales (data not shown).

Table 3.10: Association of variants with CTCAE-grade ototoxicity in patients exposed to cumulative cisplatin doses ≥ 200 mg/m^2

Variant	No ototoxicity	Ototoxicity	p-value	Odds ratio (95% CI)
ABCB5 rs10950831			0.229[a]	
T/T	8 (17.8%)	22 (32.4%)	0.086[a]	0.452 (0.181; 1.132)
T/G	24 (53.3%)	30 (44.1%)	0.337[a]	1.448 (0.679; 3.084)
G/G	13 (28.9%)	16 (23.5%)	0.523[a]	1.320 (0.562; 3.102)
T-allele	40 (44.4%)	74 (54.4%)	0.142[a]	0.670 (0.392; 1.145)
G-allele	50 (55.6%)	62 (45.6%)		
ABCC3 rs1051640			0.703[b]	
A/A	34 (75.6%)	47 (69.1%)	0.457[a]	1.381 (0.589; 3.239)
A/G	10 (22.2%)	20 (29.4%)	0.397[a]	0.686 (0.286;1.645)
G/G	1 (2.2%)	1 (1.5%)	1.000[b]	1.523 (0.093; 24.985)
A-allele	78 (86.7%)	114 (83.8%)	0.558[a]	1.254 (0.587; 2.682)
G-allele	12 (13.3%)	22 (16.2%)		
CDH13 rs11646213			0.914[a]	
A/A	23 (51.1%)	32 (47.1%)	0.673[a]	1.176 (0.553; 2.500)
A/T	17 (37.8%)	28 (41.2%)	0.718[a]	0.867 (0.401; 1.878)
T/T	5 (11.1%)	8 (11.8%)	0.718[a]	0.867 (0.401; 1.878)
A-allele	63 (70.0%)	92 (67.6%)	0.709[a]	1.116 (0.627; 1.986)
T-allele	27 (30.0%)	44 (32.4%)		
EIF3A rs10787899			0.990[a]	
C/C	18 (40.0%)	28 (41.2%)	0.901[a]	0.952 (0.442; 2.052)
C/T	18 (40.0%)	27 (39.7%)	0.975[a]	1.012 (0.469; 2.185)
T/T	9 (20.0%)	13 (19.1%)	0.908[a]	1.058 (0.410; 2.730)
C-allele	54 (60.0%)	83 (61.0%)	0.877[a]	0.958 (0.556; 1.651)
T-allele	36 (40.0%)	53 (39.0%)		
EIF3A rs3824830			0.326[b]	
A/A	20 (44.4%)	39 (57.4%)	0.179[a]	0.595 (0.278; 1.271)
A/G	21 (46.7%)	22 (32.4%)	0.125[a]	1.830 (0.843; 3.972)
G/G	4 (8.9%)	7 (10.3%)	1.000[b]	0.850 (0.234; 3.091)
A-allele	61 (67.8%)	100 (73.5%)	0.350[a]	0.757 (0.422; 1.357)
G-allele	29 (32.2%)	36 (26.5%)		
NFE2L2 rs6721961			**0.003**[b]	
C/C	35 (77.8%)	66 (97.1%)	**0.002**[a]	0.106 (0.022; 0.511)
A/C	9 (20.0%)	2 (2.9%)	**0.006**[b]	8.250 (1.691; 40.257)
A/A	1 (2.2%)	0 (0.0%)	0.398[b]	-
C-allele	79 (87.8%)	134 (98.5%)	**0.001**[a]	0.107 (0.023; 0.496)
A-allele	11 (12.2%)	2 (1.5%)		
SLC22A2 rs316019			0.608[b]	
G/G	35 (77.8%)	53 (77.9%)	0.984[a]	0.991 (0.400; 2.453)
G/T	9 (20.0%)	15 (22.1%)	0.793[a]	0.883 (0.349; 2.235)
T/T	1 (2.2%)	0 (0.0%)	0.398[b]	-
G-allele	79 (87.8%)	121 (89.0%)	0.783[a]	0.890 (0.389; 2.038)
T-allele	11 (12.2%)	15 (11.0%)		

[a] Chi-squared test; [b] Fisher's exact test

ABCB5, ATP-binding cassette, sub-family B, member 5; *ABCC3*, ATP-binding cassette, sub-family C, member 3; *CDH13*, cadherin 13; *CI*, confidence interval; *EIF3A*, eukaryotic translation initiation factor 3, subunit A; *NFE2L2*, nuclear factor erythroid 2-like 2; *SLC22A2*, solute carrier family 22, member 2

Table 3.11: Association of variants with TUNE-grade ototoxicity in patients exposed to cumulative cisplatin doses ≥ 200 mg/m^2

Variant	No ototoxicity	Ototoxicity	p-value	Odds ratio (95% CI)
ABCB5 rs10950831			0.088[a]	
T/T	2 (8.7%)	28 (31.1%)	**0.030**[a]	0.211 (0.046; 0.962)
T/G	13 (56.5%)	41 (45.6%)	0.347[a]	1.554 (0.617; 3.910)
G/G	8 (34.8%)	21 (23.3%)	0.262[a]	1.752 (0.653; 4.704)
T-allele	17 (37.0%)	97 (53.9%)	**0.040**[a]	0.502 (0.258; 0.977)
G-allele	29 (63.0%)	83 (46.1%)		
ABCC3 rs1051640			0.139[b]	
A/A	19 (82.6%)	62 (68.9%)	0.192[a]	2.145 (0.668; 6.890)
A/G	3 (13.0%)	27 (30.0%)	0.100[a]	0.350 (0.096; 1.277)
G/G	1 (4.3%)	1 (1.1%)	0.367[b]	4.045 (0.243; 67.250)
A-allele	41 (89.1%)	151 (83.9%)	0.375[a]	1.575 (0.574; 4.323)
G-allele	5 (10.9%)	30 (16.1%)		
CDH13 rs11646213			0.428[b]	
A/A	9 (39.1%)	46 (51.1%)	0.305[a]	0.615 (0.242; 1.564)
A/T	10 (43.5%)	35 (38.9%)	0.688[a]	1.209 (0.478; 3.054)
T/T	4 (17.4%)	9 (10.0%)	0.298[b]	1.895 (0.527; 6.810)
A-allele	28 (60.9%)	127 (70.6%)	0.207[a]	0.649 (0.331; 1.273)
T-allele	18 (39.1%)	53 (29.4%)		
EIF3A rs10787899			0.317[b]	
C/C	12 (52.2%)	34 (37.8%)	0.210[a]	1.797 (0.714; 4.520)
C/T	6 (26.1%)	39 (43.3%)	0.132[a]	0.462 (0.166; 1.280)
T/T	5 (21.7%)	17 (18.9%)	0.771[b]	1.193 (0.388; 3.665)
C-allele	30 (65.2%)	107 (59.4%)	0.475[a]	1.279 (0.651; 2.514)
T-allele	16 (34.8%)	73 (40.6%)		
EIF3A rs3824830			0.137[b]	
A/A	9 (39.1%)	50 (55.6%)	0.159[a]	0.514 (0.202; 1.310)
A/G	13 (56.5%)	30 (33.3%)	**0.041**[a]	2.600 (1.022; 6.614)
G/G	1 (4.3%)	10 (11.1%)	0.452[b]	0.333 (0.041; 2.737)
A-allele	31 (67.4%)	130 (72.2%)	0.518[a]	0.795 (0.396; 1.597)
G-allele	15 (32.6%)	50 (27.8%)		
NFE2L2 rs6721961			0.053[b]	
C/C	18 (78.3%)	83 (92.2%)	0.066[b]	0.304 (0.086; 1.066)
A/C	4 (17.4%)	7 (7.8%)	0.230[b]	2.496 (0.663; 9.398)
A/A	1 (4.3%)	0 (0.0%)	0.268[b]	-
C-allele	40 (87.0%)	173 (96.1%)	**0.028**[b]	0.270 (0.086; 0.846)
A-allele	6 (13.0%)	7 (3.9%)		
SLC22A2 rs316019			0.148[b]	
G/G	16 (69.6%)	72 (80.0%)	0.282[a]	0.571 (0.205; 1.597)
G/T	6 (26.1%)	18 (20.0%)	0.571[b]	1.412 (0.487; 4.093)
T/T	1 (4.3%)	0 (0.0%)	0.204[b]	-
G-allele	38 (82.6%)	162 (90.0%)	0.161[a]	0.528 (0.214; 1.304)
T-allele	8 (17.4%)	18 (10.0%)		

[a] Chi-squared test; [b] Fisher's exact test

ABCB5, ATP-binding cassette, sub-family B, member 5; *ABCC3*, ATP-binding cassette, sub-family C, member 3; *CDH13*, cadherin 13; *CI*, confidence interval; *EIF3A*, eukaryotic translation initiation factor 3, subunit A; *NFE2L2*, nuclear factor erythroid 2-like 2; *SLC22A2*, solute carrier family 22, member 2

3.3.3.2 Haplotype analysis

Two of the analysed SNPs were located in the gene *EIF3A*, 42,006 bp apart. The variants *EIF3A* rs10787899 and rs3824830 were noted to be in some degree of LD in the Caucasian and Indian populations (Table 3.12); however, the sample size of the Indians was very limited (n = 3), and this cannot be considered an accurate representation of the amount of LD between these SNPs in this population group. The SNPs were not in any LD in the mixed ancestry and Black African population groups (Table 3.12).

Table 3.12: Analysis of linkage disequilibrium between the variants *EIF3A* rs10787899 and rs3824830 in the different ethnic population groups

Population	r^2 value	D` value
Caucasian	0.520	0.840
Mixed ancestry	0.087	0.547
Black African	0.002	0.125
Indian	1.000	1.000

The role of the rs10787899-rs3824830 haplotype in ototoxicity was explored in the Caucasian and Indian populations. Similar to the single-site analysis of these variants, no significant associations between the *EIF3A* haplotypes and ototoxicity were observed (Table 3.13).

Table 3.13: Associations between the *EIF3A* rs10787899 and rs3824830 haplotypes and ototoxicity in Caucasian and Indian patients

	No ototoxicity	Ototoxicity	p-value	Odds ratio (95% CI)
ASHA			0.616[a]	-
C-G	7 (21.9%)	9 (23.7%)	0.857[a]	0.902 (0.293; 2.774)
C-A	8 (25.0%)	11 (28.9%)	0.711[a]	0.818 (0.282; 2.371)
T-G	12 (37.5%)	9 (23.7%)	0.209[a]	1.933 (0.687; 5.443)
T-A	5 (15.6%)	9 (23.7%)	0.401[a]	0.597 (0.178; 2.006)
Chang			0.539[b]	-
C-G	14 (25.9%)	2 (12.5%)	0.329[b]	2.450 (0.494; 12.156)
C-A	13 (24.1%)	6 (37.5%)	0.343[b]	0.528 (0.161; 1.735)
T-G	10 (18.5%)	4 (25.0%)	0.723[b]	0.682 (0.181; 2.562)
T-A	17 (31.5%)	4 (25.0%)	0.761[b]	1.378 (0.387; 4.903)
CTCAE			0.951[a]	-
C-G	10 (25.0%)	6 (20.0%)	0.622[a]	1.333 (0.424; 4.193)
C-A	10 (25.0%)	9 (30.0%)	0.624[a]	0.778 (0.270; 2.243)
T-G	8 (20.0%)	6 (20.0%)	1.000[a]	1.000 (0.306; 3.266)
T-A	12 (30.0%)	9 (30.0%)	1.000[a]	1.000 (0.356; 2.809)
TUNE			0.361[a]	-
C-G	7 (23.3%)	9 (22.5%)	0.935[a]	1.048 (0.340; 3.230)
C-A	7 (23.3%)	12 (30.0%)	0.535[a]	0.710 (0.240; 2.098)
T-G	4 (13.3%)	10 (25.0%)	0.227[a]	0.462 (0.129; 1.648)
T-A	12 (40.0%)	9 (22.5%)	0.114[a]	2.296 (0.811; 6.505)

[a] Chi-squared test; [b] Fisher's exact test

ASHA, American Speech-Language-Hearing Association; *CI*, confidence interval; *CTCAE*, Common Terminology Criteria for Adverse Events

3.3.3.3 Regression modelling of clinical, demographic and genetic variables

The predictive value of each of the genetic and non-genetic factors was assessed by forward logistic regression. Using the development of ototoxicity as a binary dependent variable, data from the individual site analyses was entered as potential covariates in a step-wise model. This included the cumulative and individual cisplatin doses, the anatomical site of cancer (grouping the head, neck, oesophagus, lymphoma and/or osteosarcoma cancers and those of the testes or cervix into two separate groups), whether the patient was of black African ethnicity, the presence of baseline hearing impairment, as well as patient age, sex,

and genotypic information. Using this approach, various demographic, clinical, and genetic predictors of cisplatin-induced hearing loss were identified for each of the definitions of ototoxicity (Table 3.14).

Table 3.14: Risk factors of ototoxicity identified by forward logistic regression

Ototoxicity scale	Identified risk factor	p-value
ASHA	*NFE2L2* rs6721961 A-allele	0.004
	Cumulative cisplatin dose	0.023
	Individual cisplatin dose	< 0.001
	Black African ethnicity	0.004
Chang	*NFE2L2* rs6721961 A-allele	0.046
	Cumulative cisplatin dose	0.017
	Reproductive cancers	< 0.001
	Black African ethnicity	< 0.001
CTCAE	*NFE2L2* rs6721961 A-allele	0.007
	Baseline hearing impairment	0.020
	Cumulative cisplatin dose	0.008
	Individual cisplatin dose	< 0.001
	Black African ethnicity	0.002
TUNE	Baseline hearing impairment	0.047
	Reproductive cancers	< 0.001
	Black African ethnicity	0.044

ASHA, American Speech-Language-Hearing Association; *CTCAE*, Common Terminology Criteria for Adverse Events; *NFE2L2*, nuclear factor, erythroid 2-like 2

Notably, the only risk factor identified across all the grading scales was black African ethnicity. Cumulative cisplatin dose and the *NFE2L2* rs6721961 A-allele were retained as significant predictors of ASHA-, Chang-, and CTCAE-level ototoxicity (Table 3.14). Regression analysis was also used to adjust the genetic association results for patient ethnicity (Table 3.15). The association between *NFE2L2* rs6721961 and ototoxicity amongst patients receiving total cisplatin doses ≥ 200 mg/m^2 remained significant after this correction, for all the definitions of ototoxicity used. In addition, the *CDH13* rs11646213 variant was significantly associated with TUNE-defined ototoxicity, despite not being identified in the prior analyses (Table 3.11). *ABCB5* rs10950831 was identified again; however, this significance was lost when repeating the analyses with G/G and T/T replacing the estimated T/G genotype (logistic regression, p = 0.177 and p = 0.473, respectively). Exclusion of this patient from the model also abolished the significance (logistic regression, p = 0.265), while the association of *NFE2L2* rs6721961 remained significantly associated with hearing loss (logistic regression, p = 0.005).

Table 3.15: Genetic association results after adjustment for patient ethnicity

Variant	Allele	No ototoxicity	Ototoxicity	p-value	Odds ratio (95% CI)
ASHA					
ABCB5 rs10950831	G	41 (55.4%)	71 (46.7%)	0.324	0.706 (0.404; 1.233)
ABCC3 rs1051640	G	8 (10.8%)	26 (17.1%)	0.330	1.702 (0.730; 3.969)
CDH13 rs11646213	T	21 (28.4%)	50 (32.9%)	0.763	1.237 (0.673; 2.273)
EIF3A rs10787899	T	28 (37.8%)	61 (40.1%)	0.658	1.101 (0.622; 1.949)
EIF3A rs3824830	G	22 (29.7%)	43 (28.3%)	0.551	0.932 (0.506; 1.717)
NFE2L2 rs6721961	A	10 (13.5%)	3 (2.0%)	**0.005**	0.129 (0.034; 0.484)
SLC22A2 rs316019	T	8 (10.8%)	18 (11.8%)	0.964	1.108 (0.458; 2.681)
Chang					
ABCB5 rs10950831	G	64 (52.5%)	48 (46.2%)	0.409	0.777 (0.460; 1.312)
ABCC3 rs1051640	G	17 (13.9%)	17 (16.3%)	0.803	1.207 (0.582; 2.504)
CDH13 rs11646213	T	37 (30.3%)	34 (32.7%)	0.796	1.116 (0.636; 1.959)
EIF3A rs10787899	T	50 (41.0%)	39 (37.5%)	0.178	0.864 (0.505; 1.477)
EIF3A rs3824830	G	38 (31.1%)	27 (26.0%)	0.294	0.775 (0.433; 1.387)
NFE2L2 rs6721961	A	11 (9.0%)	2 (1.9%)	**0.028**	0.198 (0.043; 0.914)
SLC22A2 rs316019	T	18 (14.8%)	8 (7.7%)	**0.038**	0.481 (0.200; 1.158)
CTCAE					
ABCB5 rs10950831	G	50 (55.6%)	62 (45.6%)	0.139	0.670 (0.392; 1.145)
ABCC3 rs1051640	G	12 (13.3%)	22 (16.2%)	0.747	1.254 (0.587; 2.682)
CDH13 rs11646213	T	27 (30.0%)	44 (32.4%)	0.916	1.116 (0.627; 1.986)
EIF3A rs10787899	T	16 (34.8%)	73 (40.6%)	0.303	0.958 (0.556; 1.651)
EIF3A rs3824830	G	29 (32.2%)	36 (26.5%)	0.277	0.757 (0.422; 1.357)
NFE2L2 rs6721961	A	11 (12.2%)	2 (1.5%)	**0.004**	0.107 (0.023; 0.496)
SLC22A2 rs316019	T	11 (12.2%)	15 (11.0%)	0.440	0.890 (0.389; 2.038)
TUNE					
ABCB5 rs10950831	G	29 (63.0%)	83 (46.1%)	**0.040**	0.502 (0.258; 0.977)
ABCC3 rs1051640	G	5 (10.9%)	30 (16.1%)	0.798	1.575 (0.574; 4.323)
CDH13 rs11646213	T	18 (39.1%)	53 (29.4%)	**0.036**	0.649 (0.331; 1.273)
EIF3A rs10787899	T	16 (34.8%)	73 (40.6%)	0.607	1.279 (0.651; 2.514)
EIF3A rs3824830	G	15 (32.6%)	50 (27.8%)	0.309	0.795 (0.396; 1.597)
NFE2L2 rs6721961	A	6 (13.0%)	7 (3.9%)	**0.008**	0.270 (0.086; 0.846)
SLC22A2 rs316019	T	8 (17.4%)	18 (10.0%)	0.112	0.528 (0.214; 1.304)

ABCB5, ATP-binding cassette, sub-family B, member 5; *ABCC3*, ATP-binding cassette, sub-family C, member 3; *ASHA*, American Speech-Language-Hearing Association; *CDH13*, cadherin 13; *CI*, confidence interval; *CTCAE*, Common Terminology Criteria for Adverse Events; *EIF3A*, eukaryotic translation initiation factor 3, subunit A; *NFE2L2*, nuclear factor erythroid 2-like 2; *SLC22A2*, solute carrier family 22, member 2

3.3.3.4 Other markers of treatment response

Although ototoxicity is the primary focus of this investigation, data about other toxic ADRs was collected because cisplatin is also a known nephro- and neurotoxin. Five of the included patients developed significant renal dysfunction during treatment, and five others showed signs of myelosuppresion, neutropaenia, hypokalaemia, hypomagnesaemia and/or hypocalcaemia after cisplatin treatment. Additionally, nine patients self-reported post-chemotherapy tinnitus, which is another effect cisplatin may have in the inner ear. No patients were recorded as symptomatic of peripheral neurotoxicity.

Notably, none of the patients who showed signs of nephrotoxicity had the variant *SLC22A2* rs316019 T-allele, and none of those who developed electrolyte deficiencies or myelosuppresion carried the *NFE2L2* rs6721961 A-allele (Table 3.16). While these

differences did not reach statistical significance (data not shown), no such directionality was observed with any of the other potential risk factor SNPs (Table 3.16).

Table 3.16: Select genotypes for each of the patients who developed other cisplatin-induced ADRs

Patient[a]	Adverse reaction type(s)	***ABCB5*** **rs10950831**	***CDH13*** **rs11646213**	***NFE2L2*** **rs6721961**	***SLC22A2*** **rs316019**
1	Nephrotoxicity	T/G	A/A	C/C	G/G
2	Nephrotoxicity	T/T	A/A	C/C	G/G
3	Nephrotoxicity	G/G	A/A	A/A	G/G
4	Nephrotoxicity	T/G	T/T	C/C	G/G
5	Nephrotoxicity	T/T	A/A	C/C	G/G
6	Neutropaenia	T/G	A/T	C/C	G/G
7	Neutropaenia	T/G	T/T	C/C	G/G
8	Neutropaenia	T/T	A/A	C/C	G/G
9	Nutrient deficiency[b]	G/G	A/A	C/C	G/G
10	Neutropaenia and nutrient deficiency[c]	G/G	T/T	C/C	T/G
11	Tinnitus	T/T	T/T	C/C	G/G
12	Tinnitus	T/G	A/A	A/C	G/G
13	Tinnitus	G/G	A/T	C/C	G/G
14	Tinnitus	NA	A/T	C/C	G/G
15	Tinnitus	G/G	A/A	C/C	T/G
16	Tinnitus	G/G	A/T	C/C	G/G
17	Tinnitus	G/G	A/T	C/C	G/G
18	Tinnitus	T/G	A/T	C/C	G/G
19	Tinnitus	T/G	A/T	C/C	T/G

[a] patients given a numerical identifier which is unrelated to the cisplatin-induced ototoxicity study; [b] hypokalaemia and hypomagnesaemia; [c] hypocalcaemia and hypomagnesaemia
ABCB5, ATP-binding cassette, sub-family B, member 5; *CDH13*, cadherin 13; *NA*, not available; *NFE2L2*, nuclear factor, erythroid 2-like 2; *SLC22A2*, solute carrier family 22, member 2

3.4 Discussion

Cisplatin is a chemotherapeutic which is prevalently administered in the management of cancer. The use of the drug can be limited by the development of permanent hearing loss, which is a common adverse reaction to cisplatin chemotherapy. However, the efficacy of cisplatin as an antineoplastic agent means that it has remained the drug of choice in first-line chemotherapy regimens, despite the risk of ototoxicity as well as other severe ADRs which can impair patient quality-of-life.

The incidence of ototoxicity observed in this cohort varied considerably depending on the audiometric definition of ototoxicity used. The TUNE grading system was the most conservative scale, with 74.8% of all patients displaying signs of hearing loss compared to 39.2% according to the Chang ototoxicity scale. Both of these grading scales prioritise the progressivity of ototoxicity by limiting their higher grades to hearing loss occurring in the lower frequencies. However, the threshold shifts considered significant in the TUNE scale are a quarter that of in the Chang grading scale, which accounts for these very different results. The CTCAE, which are arguably the most prevalently used in studies of cisplatin-

induced ototoxicity, yielded an ototoxicity rate of 57.7%, and the ASHA guidelines gave an incidence of 66.7%. Because the TUNE scale is relatively new, there is a paucity of literature regarding its usefulness and comparability; however, the incidence of ototoxicity in this study did not differ significantly from that reported by Theunissen et al (2014) (Chi-squared test, $p = 0.136$). The observed ototoxicity incidences were significantly higher compared to other study populations, graded using the Chang (Yang et al, 2013; Peleva et al, 2014) and CTCAE (De Jongh et al, 2003; Yang et al, 2013; Hagleitner et al, 2014) grading scales. Equally, however, there are study populations in which the prevalence of hearing loss did not differ significantly from those reported here (Knight et al, 2005; Lanvers-Kaminsky et al, 2015); these inconsistencies are most likely due to inter-study differences regarding chemotherapy regimens, length of audiometric follow-up, and quality of audiological monitoring.

Given the high incidence of ototoxicity, globally as well as amongst SA patients (Whitehorn et al, 2014a), the identification of individuals who are at an altered risk of hearing loss is preferable, so that quality-of-life issues can be considered in the selection of treatment strategies. While the role of various clinical and demographic factors has been discussed previously (Chapter 2), it is demonstrated here that predictive models are invariably improved by the inclusion of patient genotypic information. The SNP *NFE2L2* rs6721961 was identified as a possible predictor of ototoxicity in patients exposed to average or above-average total doses of cisplatin, as it associated with ototoxicity regardless of definition before and after adjusting for patient ethnicity. The encoded protein product of *NFE2L2*, NRF2, is a transcription factor involved in the protection of cells against oxidative and other stresses (Venugopal and Jaiswal, 1998). Under normal cellular conditions, NRF2 is localised to the cytoplasm, where it is bound to and targeted for proteasomal degradation by kelch-like ECH-associated protein 1 (KEAP1) (Suzuki and Yamamoto, 2015). When ROS, electrophiles or xenobiotics accumulate in cells, the activity of KEAP1 is inhibited, allowing NRF2 to stabilise and translocate to the nucleus (Hybertson and Gao, 2014). There, NRF2 binds to antioxidant response elements (AREs), activating the expression of numerous genes involved in cellular protection. AREs are located upstream of genes encoding, for example, haem oxygenase 1 (HO-1), various GST enzymes, peroxiredoxins, glutathione peroxidases, and ferroxidases, as well as drug metabolising enzymes and multidrug resistance transporters (Ishii et al, 2000; Thimmulappa et al, 2002; Vollrath et al, 2006; Malhotra et al, 2010).

Since cisplatin acts in part through the induction of oxidative stress in cells (Clerici et al, 1996), NRF2 may also be involved in the cellular response to cisplatin. Indeed, flunarizine,

an activator of the NRF2/HO-1 pathway, prevented cisplatin-induced apoptosis and reduced the production of pro-inflammatory cytokines in a human organ of Corti cell line (So et al, 2008). Transfection of wild type *NFE2L2* constructs into the cells had a similar effect, while inhibition of *NFE2L2* expression by siRNA attenuated the protective effects of flunarizine (So et al, 2008). The protective role of NRF2 has also been demonstrated in mouse models of nephrotoxicity, where mice without a functional copy of Nrf2 showed greater signs of renal impairment following exposure to cisplatin compared to wild type mice, despite a similar accumulation of cisplatin-DNA adducts in the kidney cells of both experimental and control animals (Aleksunes et al, 2010). Further, various compounds which have been implicated as protectants against cisplatin-induced ototoxicity or nephrotoxicity have been found to exert their effects through the activation of NRF2-related pathways (Jin et al, 2015; Kim et al, 2015a; Kim et al, 2015b; Ma et al, 2015; Sahu et al, 2015); conversely, inhibition of these pathways may sensitise cancerous cells to cisplatin treatment or reverse the development of cisplatin resistance (Shi et al, 2014a; Hou et al, 2015; Lee et al, 2015).

The SNP rs6721961 occurs in the promoter of *NFE2L2*, 617 bp upstream of the transcription start site. The region contains a putative ARE-like transcription factor binding site, through which NRF2 may positively regulate its own expression in a positive feedback loop mechanism (Marzec et al, 2007). Promoter sequences which contain the rs6721961 A-allele were shown to bind NRF2 less efficiently than wild type promoters (Marzec et al, 2007), resulting in an approximately 40% reduction in *NFE2L2* expression (Marczak et al, 2012; Suzuki et al, 2013). In the present investigation, this promoter region variant was associated with protection against ototoxicity. Given that a reduction in *NFE2L2* expression increases cellular sensitivity to cisplatin, this association is in a direction which is opposite to that which might have been expected. Nevertheless, similar protective effects of the *NFE2L2* rs6721961 A-allele have been reported for various conditions which are influenced by oxidative stress, such as infection-induced asthma in children (Ungvári et al, 2012), bronchopulmonary dysplasia (Sampath et al, 2015), reduced responses to vasodilators (Marczak et al, 2012), and Parkinson's disease (von Otter et al, 2010; Todorovic et al, 2015). Todorovic et al (2015) further demonstrated that olfactory cells which contained the alternative A-allele were significantly protected from pesticide-induced cell death, which may indicate that the SNP can play a protective role against toxic insults. Other conditions with which the SNP has been associated include breast and lung cancers (Hartikainen et al, 2012; Suzuki et al, 2013), type 2 diabetes mellitus (Shimoyama et al, 2014; Wang et al, 2015), defective spermatogenesis and poor semen quality (Yu et al, 2012; Yu et al, 2013), acute lung injury (Marzec et al, 2007), and acute respiratory distress syndrome (O'Mahony et al, 2012).

Another gene involved in the cellular antioxidant response is *CDH13*. Although its encoded protein, cadherin-13, primarily functions in the development and functioning of the nervous system, it is also involved in functions such as cell proliferation, migration, survival, and cell cycle progression (Rivero et al, 2013). *CDH13* overexpression in endothelial cells increased their protection against oxidative stress-induced apoptosis, indicating its role as a promoter of cell survival (Joshi et al, 2005; Rivero et al, 2013). Immunohistochemistry experiments demonstrated cadherin-13 expression in multiple cell types in the murine cochlea, including the hair cells, spiral ganglion cells and stria vascularis (Girotto et al, 2014). In accordance with the authors' hypothesis that the gene plays a role in hearing, variation in the gene was associated with sloping high frequency hearing loss in human subjects (Girotto et al, 2014). *CDH13* genetic variation was also identified in association with cisplatin cytotoxicity using a genome-wide linkage approach (Shukla et al, 2008). The SNP *CDH13* rs11646213, which is located upstream of the gene, has been linked to metabolic syndrome and obesity (Fava et al, 2011), hypertension (Org et al, 2009), and early-onset preeclampsia (Wan et al, 2013). While it did show a mild association with TUNE-grade ototoxicity in the present study, rs11646213 did not associate with any of the other ototoxicity scales used after adjustment for patient ethnicity. It is possible that the role of cadherin-13 in hearing may obscure its association with cisplatin-induced hearing loss; indeed, when examining only patients who had normal pre-treatment audiograms, *CDH13* rs11646213 did associate weakly with Chang ototoxicity (data not shown). Notably, however, its association with TUNE ototoxicity was lost in the analysis. While its link to cisplatin-induced hearing impairment may be tenuous, the role of *CDH13* in hearing and cellular protection may warrant further investigation of this gene for genetic markers of treatment response.

In contrast, polymorphisms in *EIF3A* did not associate with ototoxicity in this study, despite a functional link between the gene and the cellular response to cisplatin. Primarily involved in the translation of mRNA into protein products, EIF3A has also been shown to regulate cell growth (Dong et al, 2004), cell cycle progression (Dong et al, 2009), differentiation (Liu et al, 2007), and oncogenesis (Zhang et al, 2007). Levels of *EIF3A* expression may also affect individual responses to chemotherapy, as significantly greater survival rates were reported in cervical and oesophageal cancer patients with higher expression of the gene (Dellas et al, 1998; Chen and Burger, 1999). Similarly, *EIF3A* levels were reportedly correlated with sensitivity to platinum-based and other chemotherapeutic drugs in the treatment of lung cancer (Yin et al, 2011). These results were only observed when investigating DNA-damaging drugs such as cisplatin, and it was further demonstrated that *EIF3A* overexpression suppresses NER pathway activity by regulating the translation of various key NER proteins (Yin et al, 2011). In a pilot investigation in a Chinese lung cancer patient

cohort, the novel non-synonymous variant *EIF3A* rs77382849 was associated with an increased susceptibility to platinum-induced ototoxicity coupled with a reduced rate of nephrotoxicity (Xu et al, 2012b). Because of the SNP's low frequency in non-Asian populations, it was not included in this study. The SNPs *EIF3A* rs3824830 and rs10787899, however, have been associated with an increased risk of breast cancer (Olson et al, 2010), and decreased survival in pancreatic cancer patients (Couch et al, 2010), and may therefore affect expression of the gene, although no studies have yet explored this hypothesis. Neither SNP associated with treatment response in the present investigation, and similar results were recently reported in Chinese ovarian and lung cancer patient populations (Yin et al, 2015; Zhang et al, 2015).

An alternative level at which functional genetic variants may be identified in association with cisplatin-induced ototoxicity is the transport of cisplatin into or out of cells. It was originally thought that cisplatin entered cells purely by passive diffusion, but there is now substantial evidence that the process of cisplatin uptake is actively facilitated by various transporter proteins (Waissbluth and Daniel, 2013). One such transporter is OCT2, which is encoded by the gene *SLC22A2*. Because OCTs are predominantly expressed in the kidney, initial interest concerned their role in nephrotoxicity. Homozygous deletion of *SLC22A2* in mice reduced urinary excretion of cisplatin and conferred protection against cisplatin-induced kidney damage (Filipski et al, 2009). OCT2 is also expressed in the cochlea at a level 48.2 times greater than CTR1, another known transporter of cisplatin (Ciarimboli et al, 2010). Co-administration of cimetidine, an OCT2 substrate, with cisplatin, abolished ototoxicity and significantly reduced nephrotoxicity in mice (Ciarimboli et al, 2010). *SLC22A2* rs316019 is a non-synonymous SNP which results in an altered substrate specificity and reduced transport activity (Kang et al, 2007; Song et al, 2008; Song et al, 2012). Patients with the T-allele had a reduced renal uptake of metformin (Song et al, 2008) and were shown to have significantly fewer signs of renal damage and nephrotoxicity following cisplatin treatment when compared to patients without the SNP (Filipski et al, 2009). More recently, the polymorphism was associated with protection against cisplatin-induced ototoxicity, graded with the CTCAE, in German paediatric and adult patients (Lanvers-Kaminsky et al, 2015). Although the association of variant with hearing loss was replicated here, this was limited to the Chang ototoxicity grading scale, and with a smaller effect size. Incidentally, none of the patients who developed significant renal dysfunction carried the SNP, further illustrating its potential as a protective SNP. This relationship did not reach statistical significance, presumably because of the small number of patients presenting with nephrotoxicity in this investigation (n = 5); however, it is in accordance with previously published data.

Other transporter gene variants which have been associated as modifiers of cisplatin-induced ototoxicity are *ABCC3* rs1051640 and *ABCB5* rs10950831, which were proposed to have an effect on the cellular efflux of cisplatin (Pussegoda, 2012). These polymorphisms were identified through a screen of all known ADME genes, and therefore may not have any direct links with the cellular response to cisplatin. Indeed, neither association was replicated in the present investigation, and although *ABCB5* rs10950831 did associate with a decreased risk of TUNE ototoxicity, this association cannot be considered robust because one of the genotypes was estimated, and the significance could be abolished by changing that single genotype, or excluding that patient from the analyses.

3.5 Conclusion

Pharmacogenetic investigations have demonstrated the influence of patient genetic information in the response to numerous drugs, contributing not only to the understanding of the molecular basis of drug response, but also in developing models for patient risk profiling. It is likely that there are similar genetic modifiers which play a role in variable cisplatin responses. In the case of cisplatin-induced ototoxicity, the ability to identify patients who are at an altered risk of significant hearing loss is preferable, as this would allow the prediction of ototoxicity before treatment and circumvent the need to modify therapy in response to ADRs. It is this difference between predictive and responsive medicine that forms the basis of personalised therapy. The present investigation identified potential modifiers of cisplatin-induced ototoxicity by exploring variation in genes known to play a role in cisplatin response. This approach yielded potential modifiers in *NFE2L2* rs6721961, *SLC22A2* rs316019, and *CDH13* rs11646213. Of these, the variant in *NFE2L2* demonstrated the greatest promise, as inclusion of this genotypic information improved predictive models of ototoxicity according to three commonly used ototoxicity grading scales. It is possible that this promoter region variant influences patient response by affecting the expression levels of *NFE2L2*, a gene important in the cellular response to oxidative stress, although this association should be replicated in an independent patient cohort. However, the functional effects of *SLC22A2* and *CDH13* may warrant further investigation into the role of rs316019 and rs11646213, or other gene variants, in the development of ototoxicity.

4. Whole-exome sequencing to identify potential genetic modifiers of drug response

4.1 Introduction

Cisplatin is a potent anti-cancer drug which is used prevalently in chemotherapy regimens for various tumours, including those of the head and neck, lungs, testes, cervix and ovaries. Although high success rates can be achieved when incorporating cisplatin in treatment plans (Einhorn, 2002), this use can be limited by ADRs such as ototoxicity affecting the patient (McKeage, 1995). Ototoxicity is characterised by progressive, permanent and bilateral high-frequency hearing loss, and can affect between 25% and 100% of patients. In the GSH patient population, an ototoxicity incidence of about 40% was reported using the Chang ototoxicity grading scale, although over 57% of patients developed hearing loss as defined by the CTCAE, which are more prevalently used in studies of ototoxicity (Chapter 3). Since other dose-limiting ADRs such as nephrotoxicity and nausea can be controlled (by pre-hydration and anti-emetics, respectively), ototoxicity remains one of the most prevalent dose-limiting adverse reactions to cisplatin. In comparison to the high ototoxicity incidence, only five patients in the prospective cohort developed significant nephrotoxicity during treatment (Chapter 3).

Due to quality-of-life considerations, research into predictive or preventative measures against ototoxicity is desirable. Numerous otoprotectant molecules have been explored to date, including antioxidants, apoptosis inhibitors, and channel blockers (Rybak et al, 2009); however, the promise of these agents is limited by issues concerning their delivery and neutralising effects on cisplatin (Wimmer et al, 2004). Predictive markers of ototoxicity, in contrast, would enable the identification of at-risk patients before treatment. Currently, the only risk factor which has been reported with any consistency is the cumulative dose of cisplatin, in which patients who are exposed to a higher total dose are more likely to develop significant hearing impairment (Bertolini et al, 2004; Li et al, 2004). In the SA population, patient ethnicity was a better predictor of ototoxicity, although clinical factors such as dose and treatment regimen also play a role (Chapter 2). The relative inability of other factors such as patient age, sex, and pre-existing hearing loss to predict ototoxicity has resulted in an increased interest in the pharmacogenomics of cisplatin-induced hearing impairment (Bokemeyer et al, 1998; De Jongh et al, 2003; Li et al, 2004; Chen et al, 2006; Yancey et al, 2012; Choeyprasert et al, 2013; Kirkim et al, 2014). While numerous genetic risk factors have been suggested, including those in the genes encoding GSTs, methyltransferases and other drug metabolising and transporting genes, there is as yet little consensus as to the role these genetic variants may play in the development of ototoxicity.

Investigations into the pharmacogenomics of cisplatin-induced hearing loss have, to date, been largely candidate gene-based, in which genes are chosen based on prior knowledge of cisplatin and its mechanism of action. These candidate gene studies have yielded potential genetic modifiers in *GSTP1*, *GSTT1*, *GSTM1* (Oldenburg et al, 2007), *XPC* (Caronia et al, 2009), *LRP2* (Riedemann et al, 2008; Choeyprasert et al, 2013) and, in the SA population, *OTOS* (Spracklen et al, 2015). Screening of 220 ADME-related genes in Canadian cancer patients has also identified potential modifiers in *COMT*, *TPMT*, *DPYD*, *ABCC3* and *ABCB5* (Ross et al, 2009; Pussegoda, 2012; Pussegoda et al, 2013). However, many of these studies have yielded results which fail to replicate in subsequent studies, indicating population specificity or false positives. Regardless of the interpretation, this reduces the likelihood that these variants have any biological or functional relevance in the cellular response to cisplatin.

The failings of candidate gene studies may be due to a lack of a clear understanding of the pathway whereby cisplatin induces its ototoxicity, leading to incorrect candidates being chosen, and false positives reported (Boddy, 2013). Work already conducted in the SA population has demonstrated that the candidate gene approach is a viable one, and variants in *OTOS* (Spracklen et al, 2014) and *NFE2L2* (Chapter 3) have been identified as novel predictors of treatment response. However, candidate gene studies depend on knowledge of the pathways resulting in cisplatin-induced ototoxicity, which is limited. Therefore, the study of cisplatin-induced ototoxicity may be benefitted by hypothesis-free approaches such as GWAS and NGS projects. A pilot GWAS of 238 cisplatin-receiving paediatric patients revealed a new potential modifier of ototoxicity in the *ACYP2* gene (Xu et al, 2015). Although the association was replicated in an additional 68 patients in the same investigation, the relatively small sample sizes restrict the clinical utility of this finding (Xu et al, 2015). Furthermore, GWAS is limited to the detection of variants which are already known. NGS approaches, in comparison, allow both common and novel variants to be analysed at the genome scale. The only NGS study into cisplatin response to date investigated whole-exome sequencing (WES) data from 50 urothelial carcinoma patients' tumour cells, which identified *excision repair cross-complementation group 2* (*ERCC2*) somatic mutations as a potential marker of treatment response (Van Allen et al, 2014).

WES is an NGS strategy which targets and sequences the coding regions of the genome only. This greatly reduces the amount of sequence data produced, while focussing on variants which are more likely to have a tangible functional effect. The aim of this pilot investigation is to utilise WES approaches to explore functional variants in a subset of the patient cohort. By investigating the exomes of patients who represent phenotype extremes

(i.e. increased sensitivity to cisplatin's ototoxic effects, or heightened resistance to them), this may yield novel candidate genes for future studies, and provide an insight into potential new mechanisms of ototoxicity and the cellular response to cisplatin.

4.2 Methods

4.2.1 Patients and samples

Patients from the prospective cohort were eligible for WES if they displayed extreme susceptibility to cisplatin's ototoxic effects, or extreme resistance to them. Extremely resistant patients were identified if they developed no hearing loss during treatment despite receiving multiple (≥ 3) cycles of chemotherapy, reaching a cumulative cisplatin dose which was above the average for the cohort (median cumulative dose: 200 mg/m^2). Fifteen patients met these criteria. Conversely, extreme susceptibility was classified as the onset of clinically significant hearing impairment (Chang grade ≥ 2B) after a single cycle of chemotherapy, or the progression of hearing loss to clinical significance over two cycles of treatment; ten such patients were identified. The degree of hearing loss experienced by each patient was defined using ASHA classifications as mild (26 to 39 dB HL), moderate (40 to 54 dB HL), moderately severe (55 to 69 dB HL), severe (70 to 89 dB HL), or profound (≥ 90 dB HL) (Clark, 1981). Since all the patients were obtained from the prospective cohort, the contribution of previously genotyped markers (Whitehorn et al, 2014b; Spracklen et al, 2014) to variable cisplatin response was considered, when available. DNA samples from 11 patients were prepared for exome sequencing by diluting them to solutions of 30 ng/µl in 1X TE buffer (Appendix H). Four DNA samples had a high 260/280 ratio, and a purification step was performed in order to decontaminate the DNA of proteins. This consisted of a repeat of the salting-out DNA extraction protocol (Appendix G).

4.2.2 WES

Targeted sequencing of the exome was performed on each sample by the Central Analytical Facility (CAF) at Stellenbosch University, using the Ion Torrent™ platform. The Ion AmpliSeq™ Exome RDY-IC kit (Life Technologies™) was used for exome capture, and sequencing libraries were constructed using the Ion Chef™ platform (Life Technologies™). The sequences were read on an Ion Proton™ sequencer (Life Technologies™), producing BAM files which were aligned to the *Homo sapiens* reference genome sequence (UCSC version hg19) using the Ion Reporter software on the CAF servers, to generate sequence files in the variant call format (VCF).

4.2.3 Variant annotation

The table_annotate.pl function of ANNOVAR (Wang et al, 2010) was used to annotate variants in the exomes with information regarding the type of variant, the affected gene, and, in the case of non-synonymous variants, the predicted pathogenicity. The annotation databases dbSNP version 138 (snp138), dbNFSP version 2.6 (ljb26_all), 1000 Genomes Project 2014 October version (1000g2014oct), NIH-NBBI 6500 exome database version 2 (esp6500siv2_all), refGene, cytoBand, and genomicSuperDups were downloaded from the ANNOVAR website (http://annovar.openbioinformatics.org; accessed 20.02.2015). Each exome was annotated using chromosome build UCSC hg19 as a reference, which was downloaded as part of the Genome Analysis Toolkit (GATK) resource bundle (https://www.broadinstitute.org/gatk/; accessed 29.01.2015). Twelve pathogenicity predictors were included for the annotation of non-synonymous variants (Table 4.1). These were SIFT, PolyPhen 2 HumDiv, PolyPhen 2 HumVar, LRT, MutationTaster, MutationAssessor, FATHMM, RadialSVM, LR, GERP++, PhyloP, and SiPhy, all of which provide a pathogenicity score which was included in the ANNOVAR annotation. In addition, with the exception of GERP++, PhyloP and SiPhy, a pathogenicity prediction was provided for each, which converted the score into discreet groups to reflect their deleteriousness (Table 4.1).

Table 4.1: LJB23 databases included in ANNOVAR table_annovar.pl annotation

Pathogenicity prediction tool	Score range	Categorical prediction
SIFT	> 0.05	"Tolerated" (T)
	≤ 0.05	"Deleterious" (D)
PolyPhen 2 HumDiv	≤ 0.452	"Benign" (B)
	0.453 – 0.956	"Possibly damaging" (P)
	≥ 0.957	"Probably damaging" (D)
PolyPhen 2 HumVar	≤ 0.446	"Benign" (B)
	0.447 – 0.909	"Possibly damaging" (P)
	≥ 0.909	"Probably damaging" (D)
LRT	-	"Neutral" (N)
		"Deleterious" (D)
		"Unknown" (U)
MutationTaster	-	"Polymorphism_automatic" (P)
		"Polymorphism" (N)
		"Disease_causing_automatic" (A)
		"Disease_causing" (D)
MutationAssessor	-	"Neutral" (N)
		"Low" (L)
		"Medium" (M)
		"High" (H)
FATHMM	< -1.5	"Tolerated" (T)
	> -1.5	"Deleterious" (D)
RadialSVM	-	"Tolerated" (T)
		"Deleterious" (D)
LR	-	"Tolerated" (T)
		"Deleterious" (D)
GERP++, PhyloP, SiPhy	-	Higher scores are more deleterious

Adapted from http://annovar.readthedocs.org/en/latest/user-guide/filter/index.html?highlight=converted%20score (accessed 13.09.2015)

4.2.4 Scoring and filtering of exonic variants

The sequenced exomes were divided into two groups: group A contained the patients resistant to cisplatin-induced ototoxicity, and group B comprised the susceptible patients. To filter the results of exome sequencing, two pipeline approaches, which consisted of variant-level and gene-level analyses, were applied to the sequence data. In both instances, the variants were initially assigned a score out of 8 based on the ANNOVAR annotation output. For non-synonymous single nucleotide variants (SNVs), this score was equal to the number of pathogenicity predictors, not including PolyPhen 2 HumVar, GERP++, PhyloP, or SiPhy, which classified the variant as deleterious, probably damaging or disease causing (Table 4.1). Synonymous and non-frameshift insertions, deletions and substitutions were given a score of 0, while stop-gain, stop-loss and frameshift mutations were scored 8. All the variants with scores ≥ 4 were selected for the variant- and gene-level pipelines. Variants classified as unknown were excluded from any further analysis.

4.2.4.1 Variant-level analysis

In the identification of exonic variants enriched in one group of patients, all variants which were common to both patient groups were excluded. To further this enrichment, all the variants occurring in ≥ 3 patients in either group were investigated for functional links to cisplatin and its ototoxicity. These included the analysis of gene-disease relationships and gene prioritisation using online bioinformatics tools.

4.2.4.1.1 Analysis of gene-disease relationships

Each gene in which an enriched variant was identified was investigated for a potential functional link to cisplatin response by interrogating the diseases with which the gene has been associated. Searches were performed in the Online Mendelian Inheritance in Man® (OMIM®) (http://www.omim.org/; accessed 06.05.2015) and MalaCards (http://www.malacards.org/; accessed 06.05.2015) databases. Genes were considered potentially significant if mutations in that gene have been linked to a disease in an organ or tissue which is known to be a possible non-cancerous target of cisplatin (Table 4.2).

Table 4.2: Off-target organs which can be susceptible to cisplatin-induced ADRs

Adverse drug event	Affected organ or tissue
Ototoxicity	Inner ear (cochlea and/or vestibular apparatus)
Nephrotoxicity	Kidney
Neurotoxicity	(Peripheral) nervous system
Nausea, emesis	Gastrointestinal tract
Electrolyte disturbance	Kidney
Myelosuppression	Bone marrow
Haemolytic anaemia	Erythrocytes

Adapted from McKeage, 1995

4.2.4.1.2 Gene prioritisation

The online gene prioritisation tool VarElect (LifeMap Sciences Inc., CA USA; http://varelect.genecards.org; accessed 14.06.2015) was used to assess the relationship between input genes and the phenotype of interest (in this instance, specified as "cisplatin AND ototoxicity"). VarElect incorporates the GeneCards® human gene database (http://www.genecards.org/), MalaCards human disease database (http://www.malacards.org/), and PathCards pathway unification database (http://pathcards.genecards.org/) to suggest genes which are directly or indirectly associated with the phenotype, providing a score which is used to rank the genes.

4.2.4.2 Gene-level analysis

Similar to the variant-level analysis, genes which were mutated in ≥ 3 patients in one group, and had no variation in the other patient group, were selected for gene-disease relationship analysis (4.2.4.1.1) and prioritisation (4.2.4.1.2). Additional analyses which were performed at the gene level were pathway and panel-based analyses.

4.2.4.2.1 Pathway analysis

All of the genes which were mutated in only one of the patient groups were analysed for the pathways in which they act using ConsensusPathDB (CPDB) (http://consensuspathdb.org/; accessed 27.06.2015). Initially, an over-representation gene set analysis was performed on each set of genes (i.e. the genes enriched in each group), to identify the pathways in which those genes have been implicated. Then, the gene sets for each of the enriched pathways were downloaded from CPDB, and searched against the total dataset to identify pathways which contained more variation in either of the patient groups.

4.2.4.2.2 Panel analysis

Genes which were mutated more than once, and uniquely, in either patient group were interrogated against four separate gene panels (Appendix J). The panels included were a deafness gene panel of 87 genes (http://personalizedmedicine.partners.org/Laboratory-For-Molecular-Medicine/Tests/Hearing-Loss/OtoGenome.aspx; accessed 31.03.2015), an ADME gene panel of 267 genes (http://pharmaadme.org/joomla/index.php?option=com_content &task=view&id=12&Itemid=27; accessed 23.02.2015), a DNA repair gene panel of 177 genes (http://sciencepark.mdanderson.org/labs/wood/dna_repair_genes.html; accessed 14.04.2015), and a custom cisplatin response gene panel, which was generated based on genes frequently associated with cisplatin toxicity or resistance (41 genes).

4.2.5 Statistical analysis of patient demographic and genomic data

Basic statistical tests were performed using the IBM® SPSS® Statistics programme, version 22.0.0.0 (IBM® Corp.). These included descriptive analyses of numerical data between the patient groups, in which the normality of the distribution was first analysed by Shapiro-Wilk test. For normally distributed data, the t-test was employed, and for non-normal datasets, the Mann-Whitney U test was employed. Because of the small sample sizes, Fisher's exact tests were utilised when examining differences in categorical variables between the two groups.

Population structures in the extreme phenotype cohort were compared to the patients' self-reported ethnicities using principal component analysis (PCA). Bi-allelic PCA was performed in R version 3.0 (https://www.r-project.org/; accessed 28.04.2015) using the SNPRelate package version 0.9.19 (http://www.bioconductor.org/packages/release/bioc/html/SNPRelate .html; accessed 18.05.2015). Population controls were all individuals from the 1000 Genomes Project: genomic information of each individual included in the 1000 Genomes Project was downloaded per chromosome (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/ release/2013 0502.html; accessed 29.04.2015) and exomic regions extracted. The experimental WES VCF files were merged with the 1000 Genomes Project exomes using GATK's CombineVariants function. The gdsfmt package (http://www.bioconductor.org/ packages/release/bioc/html/gdsfmt/; accessed 18.05.2015) was used to convert the WES VCF files into genomic data structure files, which is the file format required by SNPRelate.

4.3 Results

4.3.1 Extreme-phenotype cohort

Out of the 25 eligible patients, 11 were chosen for WES: 6 patients developed severe hearing loss over a short time period (Group B), while 5 demonstrated increased resistance to cisplatin's ototoxic effects (Group A) (Table 4.3). These groups differed significantly in terms of the cumulative cisplatin dose (Mann-Whitney U test, $p = 0.004$) and the number of cycles to the end of audiological monitoring (Mann-Whitney U test, $p = 0.004$). The median cumulative dose and number of cycles in Group B was 150.38 mg/m^2 and 1.5 cycles respectively, compared to 338.24 mg/m^2 and 4.0 cycles in Group A. There were no significant differences in age (independent samples t-test, $p = 0.987$) or sex (Fisher's exact test, $p = 0.567$) distributions between the two groups. Group A was comprised of patients of mixed ancestry only, while group B was predominantly (66.7%) black African; this difference approached significance at $\alpha = 0.05$ (Fisher's exact test, $p = 0.061$).

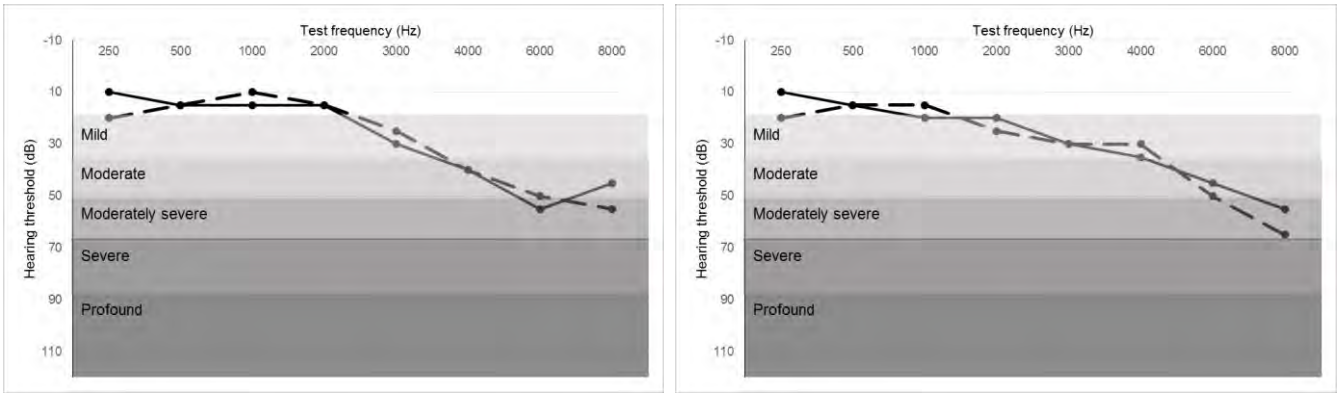
Table 4.3: Characteristics of patients selected for whole-exome sequencing

Patient no.	Ethnicity	Sex	Age	Site of cancer	Cumulative dose (mg/m^2)	Cycles to endpoint[a]	Chang grade
1	MA	M	67	Head/Neck	338.24	3	0
2	MA	F	52	Head/Neck	297.22	4	0
3	MA	F	15	Head/Neck	431.65	6	0
4	MA	F	48	Head/Neck	304.35	3	0
5	MA	M	14	Osteosarcoma	431.21	4	0
6	BA	M	25	Lymphoma	100.00	1	3
7	BA	M	26	Osteosarcoma	202.15	2	3
8	BA	M	47	Head/Neck	200.00	2	3
9	MA	M	44	Lymphoma	100.00	1	2B
10	MA	F	43	Head/Neck	100.75	1	3
11	BA	F	49	Lymphoma	201.12	2	3

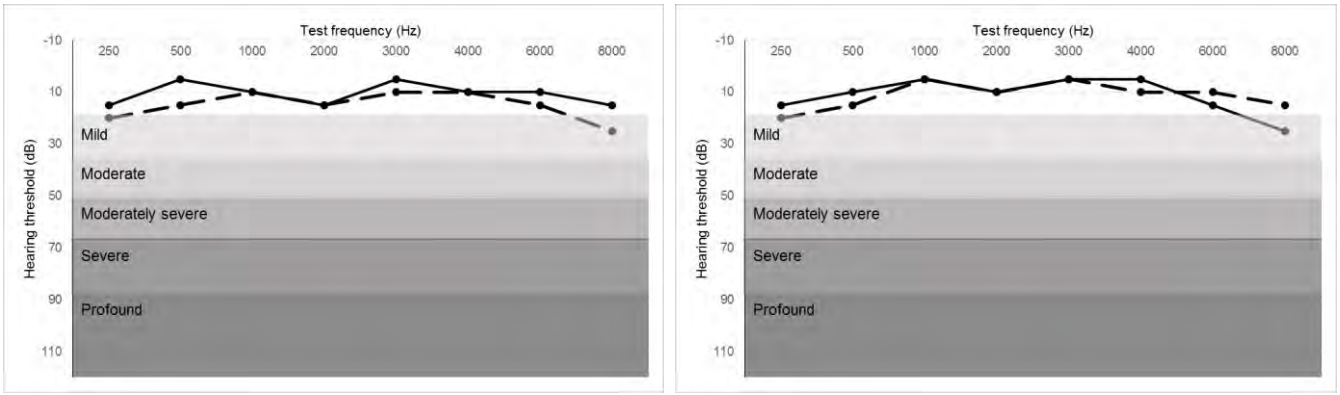
[a] endpoint taken as the end of audiological monitoring
BA, Black African; *F*, female; *M*, male; *MA*, mixed ancestry; *no.*, number

Each patient in Group A received ≥ 3 cycles of cisplatin therapy but maintained unchanged hearing throughout (Figure 4.1). For patients 3 and 4, hearing seemed to improve slightly during treatment (Figure 4.1 C and D), while for others, hearing sensitivities remained similar to the pre-treatment levels. In contrast, the patients in Group B developed significant hearing loss after a single cycle of treatment (Figure 4.2), but three patients received a second cycle of treatment, during which their hearing deteriorated further. Using the ASHA classifications, hearing sensitivities were indicative of moderately severe to severe deafness in all the patients, although patient 11 developed profound deafness in the left ear despite having a normal base-line audiogram (Figure 4.2F).

A



B



C

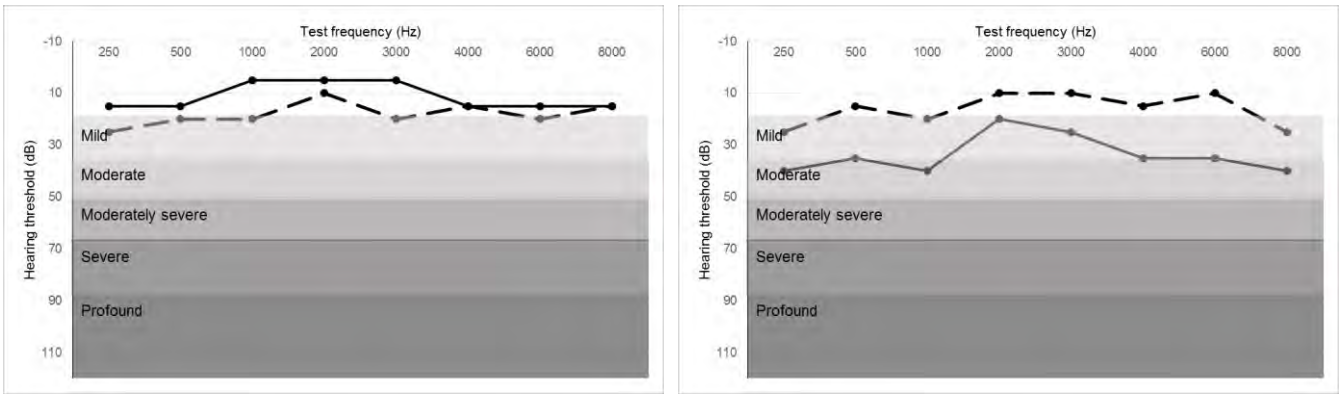
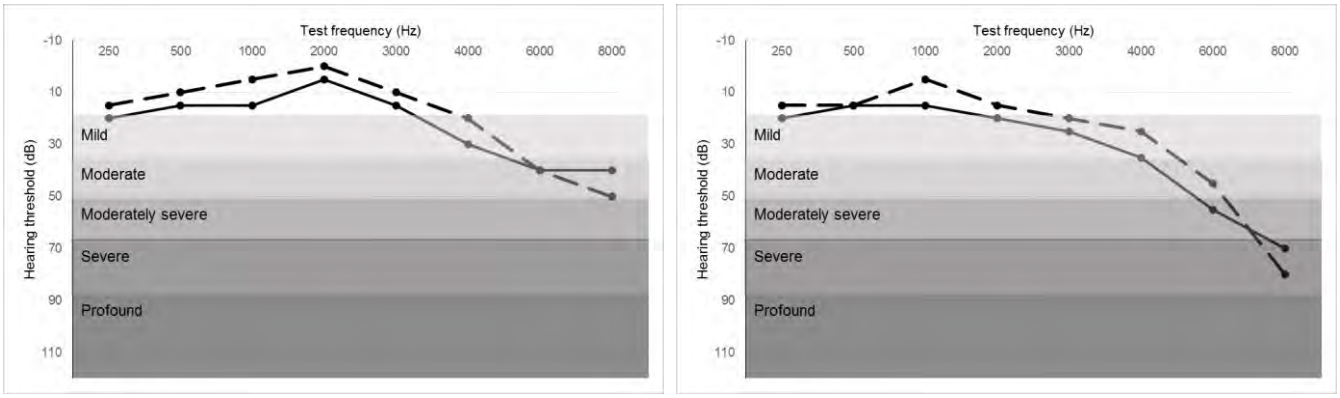


Figure 4.1: Patient audiograms showing hearing levels in each Group A ear before treatment (solid black line) compared to hearing at the last audiogram (dashed black line). For patient 1 (A), patient 4 (D), and patient 5 (E), this was 4 cycles of cisplatin, while for patient 2 (B) and patient 3 (C), this was 5 and 6 cycles, respectively. Audiograms on the left depict hearing levels in the left ear, while those from the right ear are opposite. In each audiogram, the x-axis (top of the graph), designates the test frequency (Hz) and the y-axis indicates the hearing sensitivity at each test frequency. ASHA hearing loss severity classifications are included in the grey boxes for comparison.

Figure 4.1 continued on p80

D



E

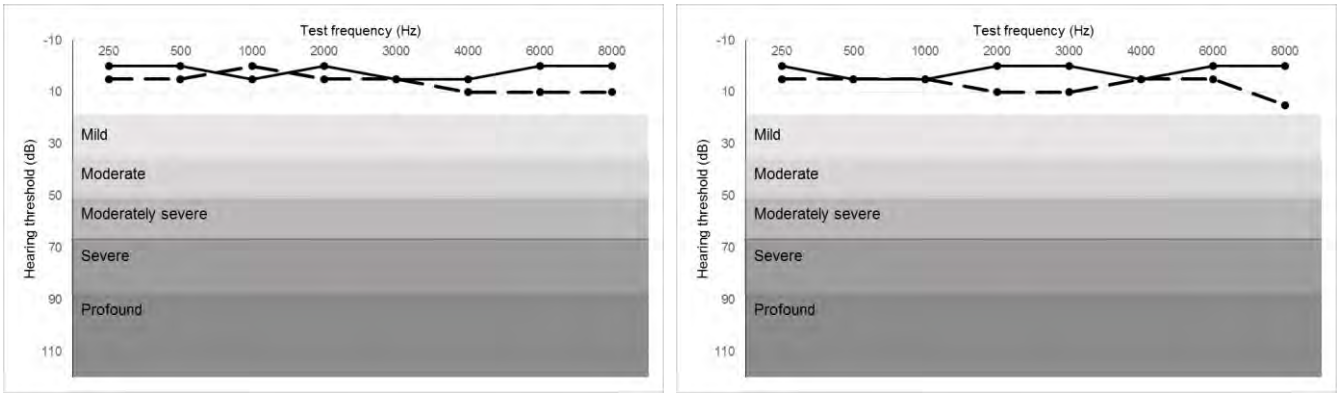
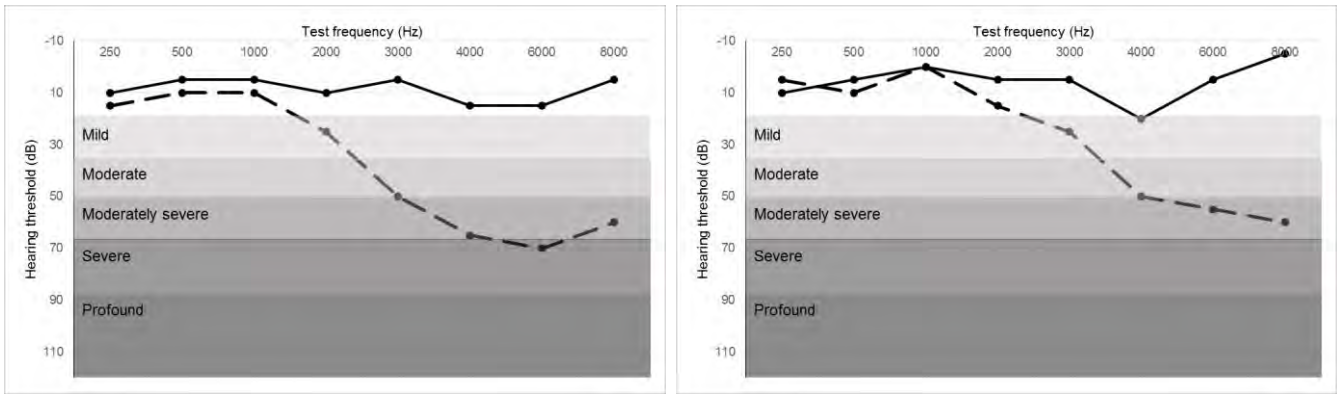


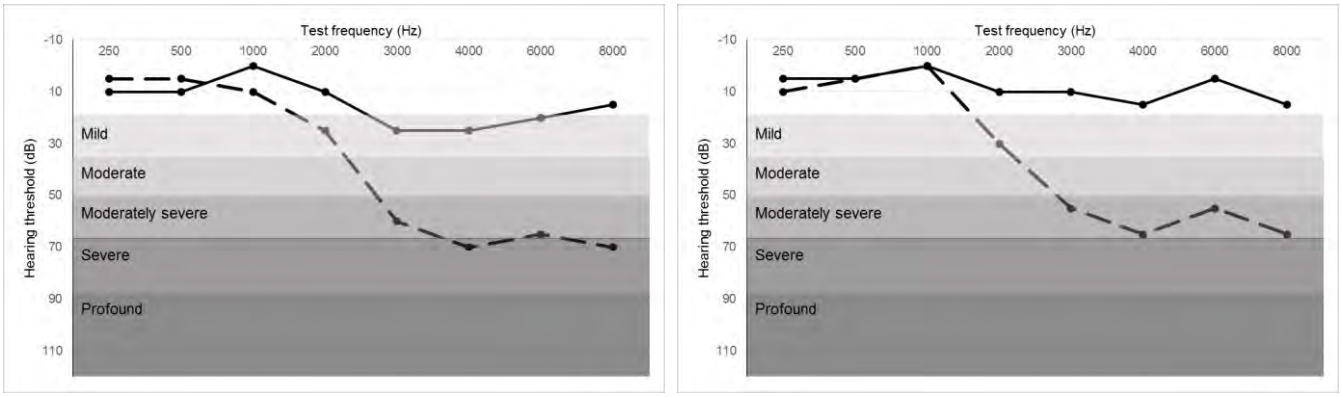
Figure 4.1 (cont.): Patient audiograms showing hearing levels in each Group A ear before treatment (solid black line) compared to hearing at the last audiogram (dashed black line). For patient 1 (A), patient 4 (D), and patient 5 (E), this was 4 cycles of cisplatin, while for patient 2 (B) and patient 3 (C), this was 5 and 6 cycles, respectively. Audiograms on the left depict hearing levels in the left ear, while those from the right ear are opposite. In each audiogram, the x-axis (top of the graph), designates the test frequency (Hz) and the y-axis indicates the hearing sensitivity at each test frequency. ASHA hearing loss severity classifications are included in the grey boxes for comparison.

For each Group B patient, the hearing impairment showed the expected pattern of ototoxicity, in which higher frequencies were affected more than lower frequencies. However, the implication of frequencies < 6.0 kHz in all Group B patients indicates progression and severity of hearing impairment experienced by these patients. With the exception of patient 8, who displayed audiometric signatures typical of sloping high-frequency (age-related) hearing loss (Figure 4.2C), all of the Group B patients had normal pre-treatment audiograms (Figure 4.2). In all cases, the development of hearing impairment resulted in a change in treatment regimen.

A



B



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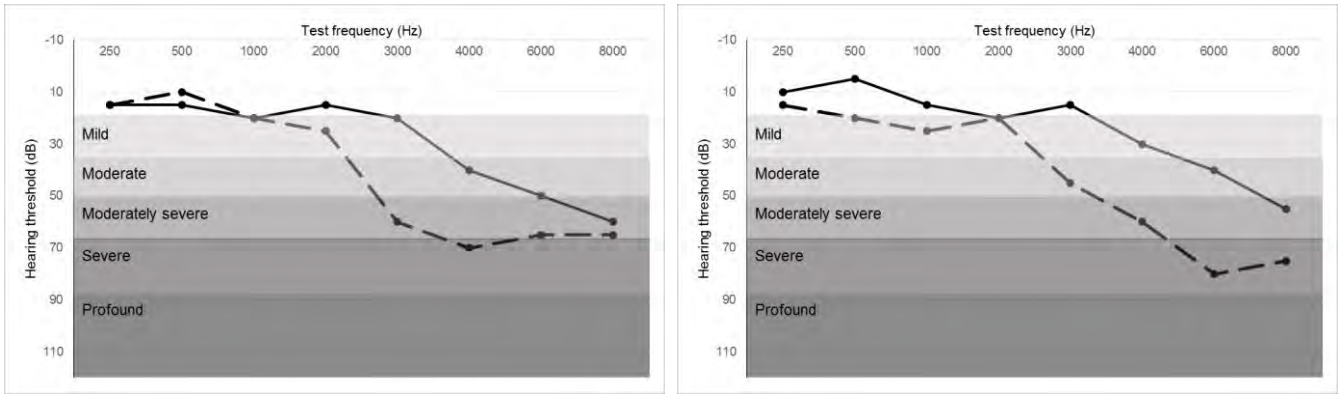
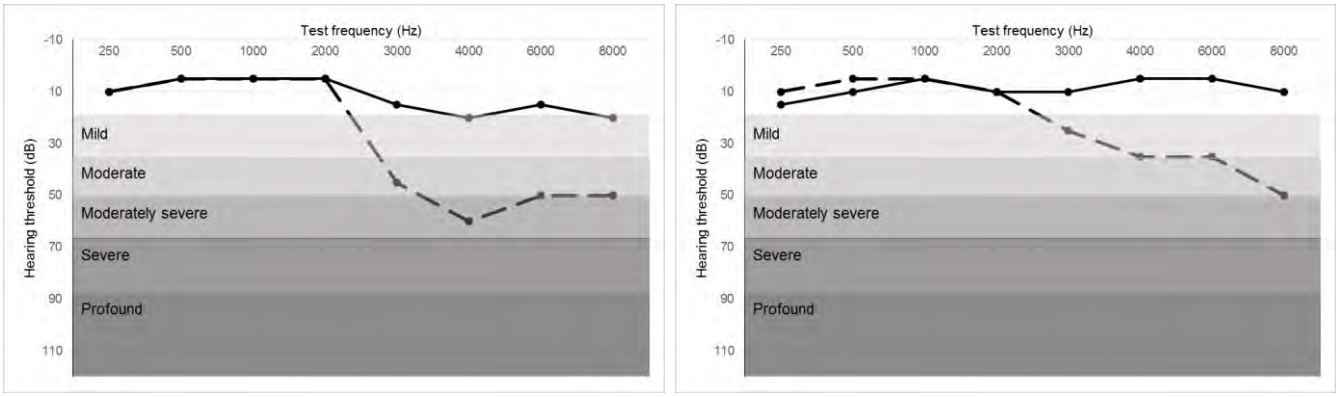


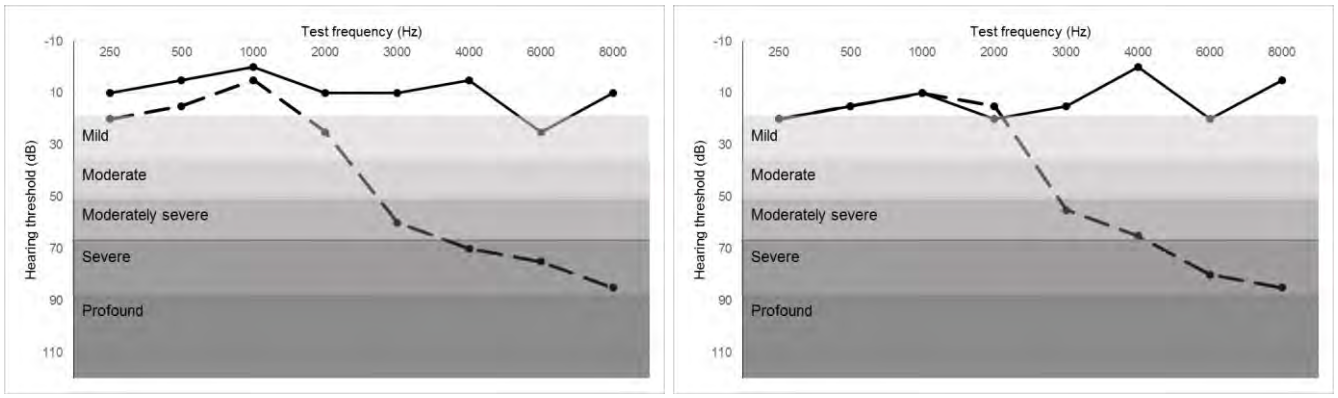
Figure 4.2: Patient audiograms showing hearing levels in each Group B ear before treatment (solid black line) compared to hearing at the last audiogram (dashed black line). For patient 9 (D) and patient 10 (E), this was one cycle of cisplatin, while for patient 6 (A), patient 7 (B), patient 8 (C) and patient 11 (F), this was two cycles. Audiograms on the left depict hearing levels in the left ear, while those from the right ear are opposite. In each audiogram, the x-axis (top of the graph), designates the test frequency (Hz) and the y-axis indicates the hearing sensitivity at each test frequency. ASHA hearing loss severity classifications are included in the grey boxes for comparison.

Figure 4.2 continued on p82

D



E



F

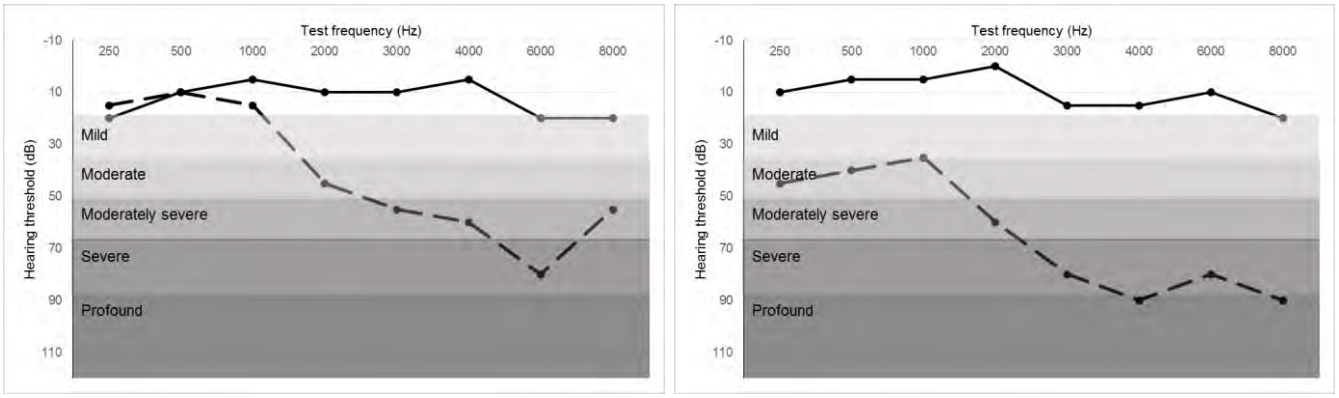


Figure 4.2 (cont.): Patient audiograms showing hearing levels in each Group B ear before treatment (solid black line) compared to hearing at the last audiogram (dashed black line). For patient 9 (D) and patient 10 (E), this was one cycle of cisplatin, while for patient 6 (A), patient 7 (B), patient 8 (C) and patient 11 (F), this was two cycles. Audiograms on the left depict hearing levels in the left ear, while those from the right ear are opposite. In each audiogram, the x-axis (top of the graph), designates the test frequency (Hz) and the y-axis indicates the hearing sensitivity at each test frequency. ASHA hearing loss severity classifications are included in the grey boxes for comparison.

The results of previous genetic investigations were explored for all of the patients in the extreme phenotype cohort, to see if their extreme response could be explained in part by these reported genetic modifiers (Table 4.4). Three patients in the extreme phenotype cohort carried the *NFE2L2* rs6721961 A-allele, two of whom were in Group B. Only one patient carried the *SLC22A2* rs316019 T-allele, and this patient was also in Group B. Both of the *OTOS* variant alleles for rs2291767 and rs77124181 only occurred in Group A patients; however, some of these genotypes were missing.

Table 4.4: Genotypes of previously associated genetic variants in the extreme phenotype cohort

Gene variant	Group A					Group B					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
ABCB5 rs10950831	T/G	T/T	T/G	T/G	T/G	T/T	T/G	T/T	T/T	T/G	T/G
ABCC3 rs1051640	A/A	A/G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	G/G	A/G
CDH13 rs11646213	A/A	A/A	A/A	A/A	A/T	A/A	A/A	T/T	A/T	A/T	A/A
COMT rs4646316	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/T	C/C	C/C	-
COMT rs4818	C/C	C/C	C/C	G/G	C/C	C/C	C/G	C/G	G/G	C/C	-
COMT rs9332377	C/C	C/C	C/T	C/C	C/C	C/C	C/T	T/T	T/T	C/C	-
EIF3A rs10787899	T/T	C/T	C/C	C/C	C/T	T/T	C/C	C/T	T/T	C/T	C/C
EIF3A rs3824830	T/T	C/T	C/T	T/T	T/T	T/T	C/C	C/T	T/T	C/T	T/T
GSTP1 rs1695	A/G	A/G	A/G	A/G	A/G	A/G	A/A	A/G	A/A	A/G	-
LRP2 rs2075252	A/G	A/G	G/G	G/G	A/G	G/G	G/G	A/G	G/G	G/G	-
LRP2 rs2228171	G/G	G/A	G/A	G/G	G/A	G/G	G/G	G/A	G/G	G/A	-
NFE2L2 rs6721961	C/C	C/C	C/C	C/C	C/A	C/C	C/C	C/A	C/C	C/A	C/C
OTOS rs2291767	A/A	A/A	A/G	-	-	A/A	A/A	A/A	A/A	A/A	-
OTOS rs77124181	C/G	C/C	C/G	-	-	C/C	C/C	C/C	C/C	C/C	-
SLC22A2 rs316019	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/T	G/G	G/G
TPMT rs1142345	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	-
TPMT rs1800460	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	-
TPMT rs12201199	T/A	T/A	T/T	T/A	T/T	T/A	T/T	T/A	T/T	T/T	-
XPC rs2228001	C/A	C/A	C/C	C/A	C/A	C/A	C/A	A/A	A/A	C/A	-

ABCB5, ATP-binding cassette, sub-family B, member 5; *ABCC3*, ATP-binding cassette, sub-family C, member 3; *CDH13*, cadherin 13; *COMT*, catechol O-methyltransferase; *EIF3A*, eukaryotic translation initiation factor 3, subunit A; *GSTP1*, glutathione S-transferase; *LRP2*, low density lipoprotein receptor-related protein 2; *NFE2L2*, nuclear factor, erythroid 2-like 2; *OTOS*, otospiralin; *SLC22A2*, solute carrier family 22, member 2; *TPMT*, thiopurine S-methyltransferase; *XPC*, xeroderma pigmentosum, complementation group C

4.3.2 WES

Exomic regions of DNA were successfully captured and sequenced in all 11 patients, with a median coverage of 113X (Table 4.5). Target coverage was higher in Group A compared to Group B (median coverage 135X and 99X, respectively; Mann-Whitney U test $p < 0.001$). Similarly, the median Phred-based quality scores in Group A were significantly greater than in Group B (358.46 and 296.77 respectively; Mann-Whitney U test $p < 0.001$). The mean mutation rate was 4.3 mutations/Mb in both patient groups; the distribution of mean mutation rates between the two groups was statistically similar (independent samples t-test $p = 0.771$).

Table 4.5: Depth of coverage and quality scores obtained from WES

Patient no.	Median DP	Min DP	Max DP	Median quality score	Min quality score	Max quality score	Group
1	227.00	4.00	2740.00	604.62	13.00	3904.15	A
2	113.00	6.00	2284.00	317.98	10.00	3853.70	A
3	156.00	6.00	1730.00	431.60	15.04	3886.63	A
4	98.00	5.00	1603.00	280.08	10.00	3898.10	A
5	119.00	5.00	1541.00	348.40	10.00	3861.25	A
6	121.00	6.00	1271.00	344.54	10.00	3911.17	B
7	100.00	5.00	1226.00	300.25	12.00	3853.65	B
8	73.00	6.00	838.00	219.49	15.00	3832.87	B
9	72.00	5.00	973.00	219.20	15.04	3853.15	B
10	118.00	5.00	2524.00	357.24	13.00	3873.98	B
11	135.00	6.00	1547.00	377.81	10.00	3853.83	B
Overall	113.00	-	-	334.82	-	-	-

DP, read depth; *no.*, number; *max*, maximum; *min*, minimum

The sequencing files were annotated using the table_annovar.pl function of ANNOVAR. According to the annotation output, a total of 257,831 variants were identified in the patient exomes, the majority (96.09%) of which were synonymous or non-synonymous SNVs (Figure 4.3A). The remaining 3.91% of the variants consisted of frameshift and non-frameshift insertions, deletions, and substitutions, stop-gain and stop-loss mutations, or variants with unknown consequences. The ratio of the different mutation types appeared similar between patients and between groups (Figure 4.3B).

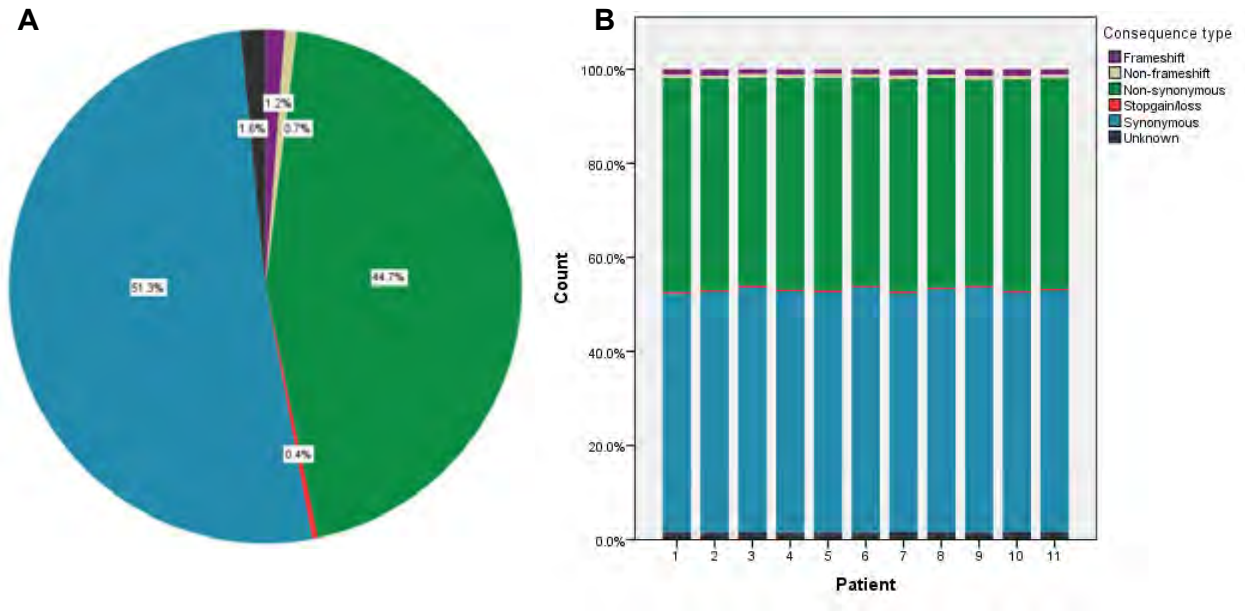


Figure 4.3: Proportion of the different variant consequences in the patient exomes, according to the ANNOVAR annotation output, overall (A), and by patient (B). Patients 1-5 experienced no hearing loss during treatment (Group A), while patients 6-11 developed severe ototoxicity (Group B). Frameshift and non-frameshift mutations include insertions, deletions and substitutions.

Variants were classified as novel, or previously unreported, if they were not assigned a dbSNP identifier in the ANNOVAR output. Of the 257,831 coding region variants, 5.05% were considered novel by this definition. A higher proportion of novel mutations was observed in those variants which resulted in a loss of protein function, including insertions, deletions, substitutions and stop-gain or stop-loss mutations (Figure 4.5A). This was particularly true of the frameshift mutations, 82.0% of which were not included in the dbSNP database. The difference in distribution of novelty was statistically significant (Chi-squared test, $p < 0.001$).

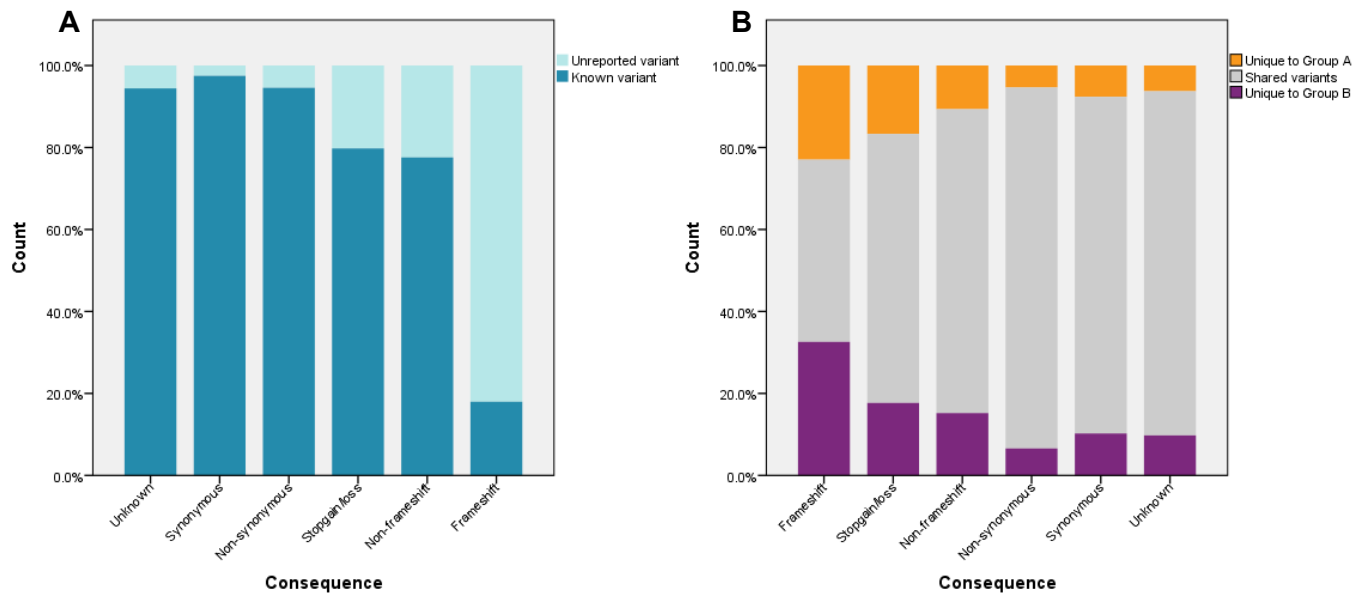


Figure 4.5: Proportion of novel mutations (A) and shared variants (B) for each consequence type in all 11 patient exomes. Frameshift and non-frameshift mutations include insertions, deletions and substitutions.

When considering the commonality of the variants, the loss of function variants were less likely to be shared between the patient groups, as a higher proportion of frameshift insertions, deletions and substitutions, and stop-gain or stop-loss mutations was unique to either patient group (Figure 4.5B). Only 44.5% of all frameshift variants, and 65.6% of stop-gain or stop-loss variants were shared between Groups A and B, compared to 88.1% of non-synonymous and 82.1% of synonymous SNVs. This may be because the higher proportion of novel variants was observed in these mutation types, and these may have been limited to a single patient, in which case they would have been identified as not shared in the analysis.

4.3.2.1 Principal component analysis (PCA)

The population structure of the extreme phenotype cohort was examined by PCA, in which genomic data from the 1000 Genomes Project was compared to the 11 patient exomes. A total of 86,672 exonic SNPs were extracted from the 1000 Genomes data, for all individuals, and merged with the experimental cohort, giving 2,515 samples for PCA. In the analysis, 1,757 non-autosomal SNPs were excluded, as were 777 monomorphic or missing SNPs, leaving 79,138 variants for PCA (Figure 4.6).

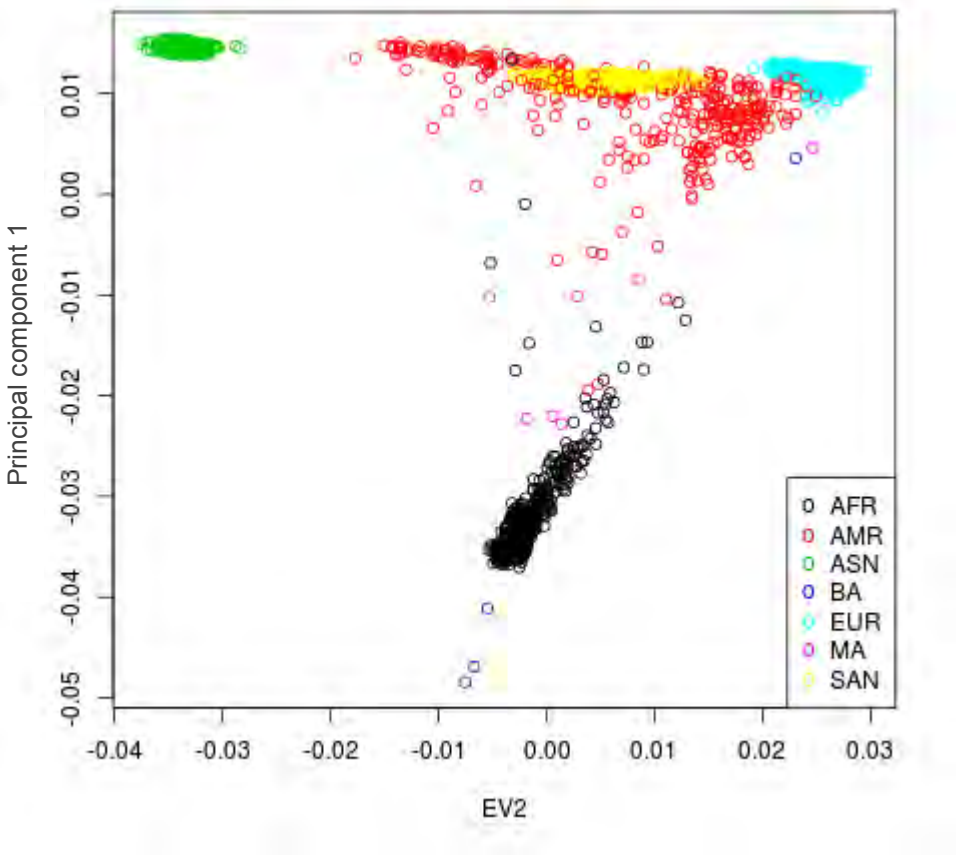


Figure 4.6: Principal component analysis of the extreme phenotype cohort population structure in comparison to 1000 Genomes reference samples. Plotted are the first two principal components from the SNPRelate output, using 79,138 bi-allelic, autosomal SNPs from 2,515 individuals. Each circle represents an individual in the analysis: black circles indicate individuals from the 1000 Genomes African (AFR) populations, red circles indicate those from the 1000 Genomes admixed American (AMR) population, green circles indicate those from the 1000 Genomes East Asian (ASN) population, cyan circles indicate those from the 1000 Genomes European (EUR) populations, and yellow circles indicate those from the 1000 Genomes South Asian (SAN) populations. Dark blue and pink circles represent patients from the extreme phenotype patient cohort, indicating individuals of black African (BA) and mixed (MA) self-reported ancestries, respectively.

The first two principal components distinguished three of the black African patients from the other populations; these clustered nearer to the 1000 Genomes African (AFR) population

(Figure 4.6). The other self-reported black African in the extreme phenotype cohort was located nearer to the 1000 Genomes European (EUR) and admixed American (AMR) populations, and one of the mixed ancestry patients from the experimental cohort. The other mixed ancestry patients also demonstrated more complex ethnicity: three patients clustered relatively closely to the AFR population, while the remaining three were located between this population and the AMR group (Figure 4.6). When considering the second and third principal components, a similar distribution of individuals was observed (Figure 4.7). However, the mixed ancestry patients were more clustered between the AFR and 1000 Genomes South Asian (SAN) populations. In fact, one of the mixed ancestry patients clustered with the SAN population in this plot.

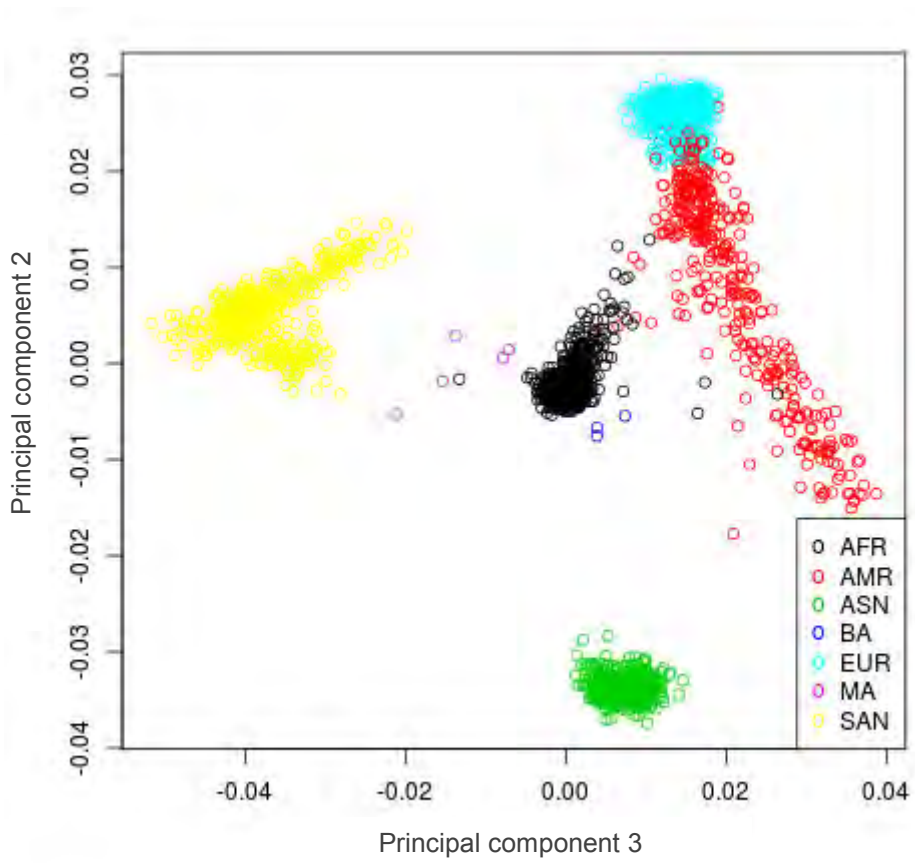


Figure 4.7: Principal component analysis of the extreme phenotype cohort population structure in comparison to 1000 Genomes reference samples. Plotted are the second and third principal components from the SNPRelate output, using 79,138 bi-allelic, autosomal SNPs from 2,515 individuals. Each circle represents an individual in the analysis: black circles indicate individuals from the 1000 Genomes African (AFR) populations, red circles indicate those from the 1000 Genomes admixed American (AMR) population, green circles indicate those from the 1000 Genomes East Asian (ASN) population, cyan circles indicate those from the 1000 Genomes European (EUR) populations, and yellow circles indicate those from the 1000 Genomes South Asian (SAN) populations. Dark blue and pink circles represent patients from the extreme phenotype patient cohort, indicating individuals of black African (BA) and mixed (MA) self-reported ancestries, respectively.

4.3.2.2 Filtering of variants

All the exonic variants were scored according to their functional annotation. Out of the 257,831 variants, 7,632 had a score of at least 4 and were included in the filtering pipelines (Figure 4.8). In the variant-level analysis, 4,429 variants were identified as unique to either patient group (2,030 in Group A and 2,399 in Group B), 44 of which occurred in at least three patients (Table 4.6). According to the gene-level analysis, 2,589 genes were mutated uniquely in either group of patients (1,198 in Group A and 1,391 in Group B); 47 of these genes had variants scoring at least 4 in three or more patients in that group (Figure 4.8).

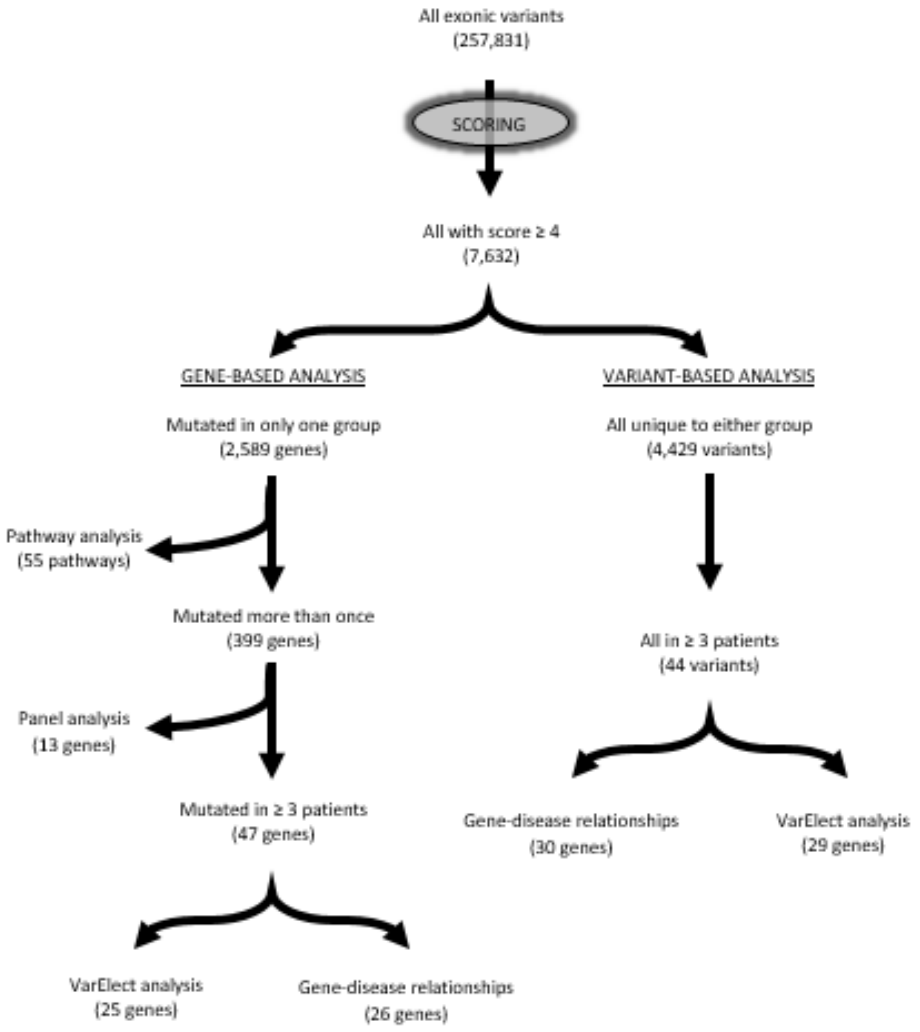


Figure 4.8: Summary of the filtering pipelines applied to the WES data. Variants were scored according to their consequence type: synonymous, non-frameshift and unknown variants were scored 0, frameshift, stop-gain and stop-loss variants were scored 8, and non-synonymous SNVs received a score which was equal to the number of pathogenicity predictors which classified that variant as deleterious.

Table 4.6: Genetic variants enriched in Group A or B

Gene	Hg19 coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patients	Group	MAF	MAF (Afr)
APC2	19:1457105	C	A	Stop-gain	8	-	203.67	40.85	6,8,11	B	-	-
BORA	13:73317740	G	GGTGA	**Frameshift insertion**	8	rs10648315	47.67	215.97	6,8,11	B	-	-
C3	19:6712379	T	TGG	Frameshift insertion	8	-	12	59.51	6,10,11	B	-	-
CHAF1A	19:4422644	G	GA	Frameshift insertion	8	-	11.67	29.45	9,10,11	B	-	-
CREB3L2	7:137593054	C	CG	Frameshift insertion	8	-	29.67	60.70	9,10,11	B	-	-
CYFIP1	15:22993154	T	TC	Frameshift insertion	8	-	14	87.42	9,10,11	B	-	-
DMGDH	5:78351635	GA	G	Frameshift deletion	8	-	37.33	133.46	6,8,11	B	-	-
DNAH14	1:225239490	T	TTA	**Frameshift insertion**	8	-	13	111.77	1,2,3	A	-	-
DYX1C1	15:55722882	C	A	Stop-gain	8	rs57809907	190	343.66	2,3,4	A	0.16	0.49
EIF5B	2:99980798	T	TA	**Frameshift insertion**	8	-	35.67	257.28	6,10,11	B	-	-
FCRL6	1:159785370	T	C	**Stop-loss**	8	rs4301626	145.67	693.21	6,9,10	B	0.18	0.40
FREM2	13:39430313	A	AT	**Frameshift insertion**	8	-	14	81.52	6,8,11	B	-	-
GP6	19:55526103	G	GCAGA	Frameshift insertion	8	rs138680589	137.67	196.59	6,8,10	B	0.06	0.08
ING1	13:111368163	CT	C	Frameshift deletion	8	-	76.33	743.42	8,9,11	B	-	-
KIAA1429	8:95547097	G	A,GA	Stop-gain/insertion	8	-	16.67	31.58	1,4,5	A	-	-
LRRC10B	11:61277300	CCG	C	Frameshift deletion	8	-	83.33	349.47	8,10,11	B	-	-
MUC5B	11:1269867	G	GC	Frameshift insertion	8	-	30.67	140.50	7,9,10	B	-	-
MXD4	4:2252968	C	CG	**Frameshift insertion**	8	-	59.67	497.29	6,8,11	B	-	-
NID2	14:52481916	C	CT	**Frameshift insertion**	8	-	24.33	818.33	2,3,4	A	-	-
OR52H1	11:5566550	GA	G	Frameshift deletion	8	rs374926660	341	674.58	1,3,5	A	0.01	0.02
PDE4DIP	1:144873957	CT	C	Frameshift deletion	8	rs11341221	251	359.70	6,8,11	B	-	-
PER1	17:8045707	GCC	GC	Frameshift deletion	8	-	33.67	220.58	8,9,10	B	-	-
RELA	11:65423198	C	CG,CGG	Frameshift insertion	8	-	16.33	114.30	3,4,5	A	-	-
REXO1	19:1827083	G	GGC	Frameshift insertion	8	-	12	31.80	1,4,5	A	-	-
SF3A2	19:2248449	T	TC	**Frameshift insertion**	8	-	15.67	110.28	6,8,10	B	-	-
SLC6A18	5:1225702	GA	G	Frameshift deletion	8	-	361.67	2890.97	1,4,5	A	-	-
SPTA1	1:158655035	CG	C	Frameshift deletion	8	-	145.67	1391.27	7,8,11	B	-	-
ZFYVE19	15:41099899	T	TGGGGC	Frameshift insertion	8	rs142730574	25.67	42.29	7,8,11	B	0.34	0.39
ZNF80	3:113955187	A	C	Stop-gain	8	rs3732781	157.20	404.91	6,7,9-11	B	0.19	0.03
BCKDHA	19:41929001	G	A	Non-synonymous	7	rs375957868	151	377.58	2,3,4	A	-	-
EP300	22:41574697	T	C	**Non-synonymous**	6	rs200876858	9.67	63.00	6,7,11	B	< 0.01	< 0.01
AQP7	9:33385689	C	G	Non-synonymous	5	-	226	100.27	1,2,3	A	-	-
CUBN	10:17171176	C	T	Non-synonymous	5	rs12259370	28.33	55.11	6,8,11	B	0.03	0.11
FOXO3	6:108882487	A	T	Non-synonymous	5	-	384	1559.17	8,9,11	B	-	-
KRT4	12:53207619	C	A	Non-synonymous	5	-	32.67	105.40	2,3,4	A	-	-
SDHC	1:161332153	A	C	**Non-synonymous**	5	-	9.33	82.70	1,2,3	A	-	-

[a] consequence types in bold are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant

Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

Table 4.6 continued on p90

Table 4.6: Genetic variants enriched in Group A or B

Gene	Hg19 coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patients	Group	MAF	MAF (Afr)
COL9A1	6:70944257	T	C	Non-synonymous	4	rs6910140	149.33	293.18	1,4,5	A	0.07	0.11
CTBP2	10:126715436	C	T	Non-synonymous	4	rs3781411	39.75	116.38	6-8,11	B	0.12	0.11
DGKQ	4:954951	A	C	**Non-synonymous**	4	-	9	86.63	6,8,10	B	-	-
DYSF	2:71788979	G	A	Non-synonymous	4	-	195.67	246.79	3,4,5	A	-	-
DYTN	2:207559599	T	C	Non-synonymous	4	rs16838593	122	251.16	9,10,11	B	0.07	0.28
FOXH1	8:145700346	T	A	Non-synonymous	4	rs112028242	74	180.88	6,8,9	B	0.02	0.10
OR4B1	11:48239071	C	A	Non-synonymous	4	rs12292056	328.67	446.02	1,3,4	A	0.1	0.22
TJP1	15:30008977	T	G	Non-synonymous	4	rs2291166	144.67	249.53	3,4,5	A	0.04	0.01

[a] consequence types in bold are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant
Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

4.3.2.2.1 Variant-based filtering

Of the 44 variants identified as enriched in Group A or B (Table 4.6), 30 had known gene-disease relationships according to OMIM® or MalaCards (Appendix K), and 29 were identified as indirectly related to the phenotype according to VarElect (Table 4.7). Five of the variants occurred in genes associated with diseases which affect cisplatin-sensitive organs (Table 4.8). In Group A, these included a novel non-synonymous variant in *AQP7*, and *DYX1C1* rs57809907. The Group B variants were novel frameshift insertions in *FREM2* and *C3*, and the non-synonymous SNP *CUBN* rs12259370. Of these, *AQP7*, *C3* and *CUBN* were indicated as indirectly associated with the phenotype, through their associations with genes involved in cisplatin response and/or ototoxicity (Table 4.7).

Comparative genomic analysis of the novel non-synonymous SNVs indicated that the *AQP7* variant occurs at a highly conserved region of the genome, as did the SNV in *FOXO3* (Figure 4.9). Similar results were observed for the other variants (data not shown).

Table 4.7: VarElect prioritisation of genes containing the enriched variants

Gene	Implicating gene(s)	Score
RELA	*MAPK8*, *CASP3*, *LRP2*, *GSTP1*, *CAT*	26.21
EP300	*MAPK8*, *CASP3*, *TPMT*, *COMT*, *LRP2*	25.97
FOXO3	*MAPK8*, *CASP3*, *CAT*, *GSTP1*, *LRP2*	25.86
CREB3L2	*MAPK8*, *CASP3*, *COMT*, *LRP2*, *GSTP1*	25.64
APC2	*MAPK8*, *CASP3*, *LRP2*, *GSTP1*, *ABCB1*	25.59
TJP1	*MAPK8*, *CASP3*, *LRP2*, *NTF3*, *BDNF*	25.58
CTBP2	*MAPK8*, *CASP3*, *GSTP1*, *CAT*, *NTF3*	25.57
C3	*MAPK8*, *CASP3*, *LRP2*, *NTRK2*, *BDNF*	25.54
SDHC	*MAPK8*, *CASP3*, *TPMT*, *COMT*, *LRP2*	25.52
PER1	*MAPK8*, *CASP3*, *COMT*, *GSTP1*, *GSTM3*	25.51
COL9A1	*MAPK8*, *CASP3*, *LRP2*, *NTF3*, *NTRK2*	25.50
DGKQ	*MAPK8*, *CASP3*, *TPMT*, *COMT*, *LRP2*	25.49
FOXH1	*MAPK8*, *CASP3*, *LRP2*, *NTF3*, *NTRK2*	25.48
CUBN	*LRP2*, *TPMT*, *COMT*, *GSTP1*, *CAT*	25.43
CYFIP1	*MAPK8*, *CASP3*, *NTF3*, *BDNF*, *NTRK2*	25.39
AQP7	*MAPK8*, *CASP3*, *ABCB1*, *NTF3*, *BDNF*	25.32
BCKDHA	*TPMT*, *COMT*, *LRP2*, *GSTP1*, *CAT*	25.19
DMGDH	*TPMT*, *COMT*, *LRP2*, *GSTP1*, *CAT*	25.18
OR52H1	*MAPK8*, *CASP3*, *LRP2*, *NTRK2*	24.58
OR4B1	*MAPK8*, *CASP3*, *LRP2*, *NTRK2*	24.58
MUC5B	*MAPK8*, *TGFA*, *EIF2AK3*	21.66
NID2	*CASP3*, *LRP2*	19.67
SLC6A18	*ABCB1*, *SLC12A1*	18.28
KRT4	*MAPK8*	13.29
GP6	*MAPK8*	13.27
SPTA1	*MAPK8*	13.27
BORA	*GSTP1*	12.84
REXO1	*MT-RNR1*	11.84
EIF5B	*EIF2AK3*	11.30

Unrelated genes: *DNAH14*, *DYSF*, *DYX1C1*, *KIAA1429*, *CHAF1A*, *DYTN*, *FCRL6*, *FREM2*, *ING1*, *LRRC10B*, *MXD4*, *PDE4DIP*, *SF3A2*, *ZFYVE19*, *ZNF80*

Table 4.8: Select gene-disease relationships of genes enriched in the variant-level pipeline

Gene	OMIM® disorder(s)	MalaCards disease(s)
AQP7	Glycerol quantitative trait locus	Constipation Endolymphatic hydrops Insulin resistance Ménière's disease Morbid obesity
C3	C3 deficiency Haemolytic uremic syndrome, atypical, susceptibility to, 5 Macular degeneration, age-related, 9	Age related macular degeneration Arteriolosclerosis Atypical haemolytic-uraemic syndrome C3 deficiency Capillary leak syndrome Dense deposit disease Haemolytic uraemic syndrome, atypical 5 Retinal drusen
CUBN	Megaloblastic anaemia-1, Finnish type Megaloblastic anaemia-1, Norwegian type	Dent's disease Megaloblastic anaemia Megaloblastic anaemia-1, Finnish type Megaloblastic anaemia-1, Norwegian type Nephropathic cystinosis
DYX1C1	Ciliary dyskinesia, primary, 25 Dyslexia, susceptibility to, 1	Articulation disorder Ciliary dyskinesia, primary, 11 Ciliary dyskinesia, primary, 25 Dyslexia 1
FREM2	Fraser syndrome	Fraser syndrome Unilateral renal agenesis

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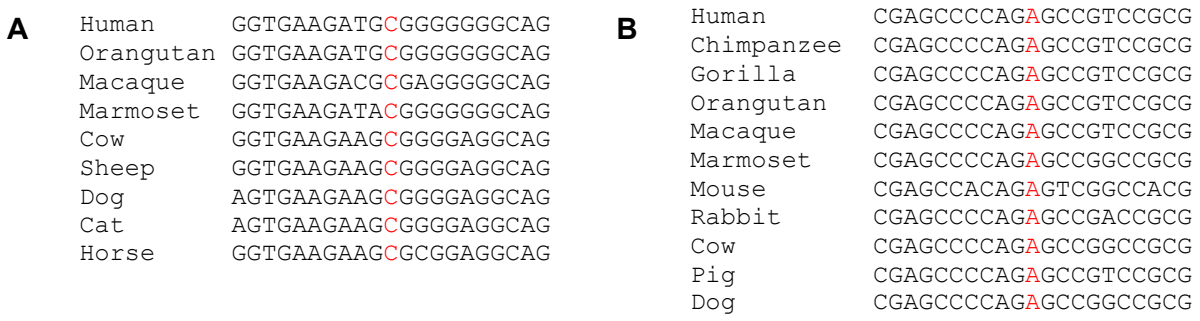


Figure 4.9: Comparative alignment of mammalian genomic regions to the human hg19 genome construct at positions *AQP7* 9:33385679-33385699 (A) and *FOXO3* 6:108882477-108882497 (B), using the Ensembl genome browser (http://ensembl.org; accessed 16.09.2015). Red bases indicate the nucleotides which are variant in the WES data. Mammalian genomes not included did not align at the specified positions.

4.3.2.2.2 Gene-based filtering

In the gene-level pipeline, 47 genes were identified as mutated in ≥ 3 patients in only one of the groups (Tables 4.9 and 4.10). Of these, 24 genes were already identified in the variant-level investigation (Table 4.6), so the analyses were limited to the remaining 23 genes. Only one additional gene, *WFS1*, was identified through the gene-disease relationship analysis (Appendix K), which was associated with Wolfram syndrome, and 11 genes were indirectly associated with the phenotype (Table 4.11).

Table 4.9: Genes uniquely mutated in at least three Group A patients

Gene	Hg19 coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patient(s)	MAF	MAF (Afr)
CDHR5	11:618744	T	TC	Frameshift insertion	8	-	6	24.25	2	-	-
	11:618998	GC	G	Frameshift deletion	8	-	93.5	342.80	1,5	-	-
DNAH14	1:225239490	T	TTA	**Frameshift insertion**	8	-	13	111.77	1,2,3	-	-
	1:225586762	A	AAC	Frameshift insertion	8	-	17	17.30	4	-	-
	1:225490866	TAA	TA	Frameshift deletion	8	-	108	661.45	2	-	-
	1:225239461	A	AAT	**Frameshift insertion**	8	-	9	56.32	1	-	-
DYX1C1	15:55722882	C	A	Stop-gain	8	rs57809907	190	343.66	2,3,4	0.16	0.49
KIAA1429	8:95547097	C	A,GA	Stop-gain/insertion	8	-	16.67	31.58	1,4,5	-	-
RELA	11:65423198	C	CG,CGG	Frameshift insertion	8	-	16.33	114.30	3,4,5	-	-
	11:65423191	G	GA	Frameshift insertion	8	-	19	54.88	3	-	-
SRPK3	X:153046673	C	G	Stop-gain	8	-	7	15.78	2	-	-
	X:153049534	CA	C	**Frameshift deletion**	8	-	260	2355.48	3	-	-
	X:153050572	T	C	Non-synonymous	4	-	46	34.85	1	-	-
TRPM2	21:45798980	C	A	Stop-gain	8	-	173	404.36	3	-	-
	21:45846577	C	T	Non-synonymous	4	rs113563173	90.5	191.48	4,5	< 0.01	< 0.01
TUBA3E	2:130949410	TG	A	**Frameshift deletion**	8	-	7	62.00	1,3	-	-
	2:130951583	CT	T	**Frameshift deletion**	8	-	129	1203.13	4	-	-
BCKDHA	19:41929001	G	A	Non-synonymous	7	rs375957868	151	377.58	2,3,4	-	-
AQP7	9:33385689	C	G	Non-synonymous	5	-	226	100.27	1,2,3	-	-
SDHC	1:161332153	A	C	**Non-synonymous**	5	-	9.33	82.70	1,2,3	-	-
ASPM	1:197072752	C	T	Non-synonymous	4	rs112230218	54	81.32	3	0.01	0.03
	1:197091296	C	TAA	**Frameshift insertion**	8	-	6	49.05	2	-	-
	1:197091297	C	A	Non-synonymous	6	-	71	16.36	1	-	-
C4orf33	4:130027802	C	T	Non-synonymous	4	rs113988895	38	36.61	1	< 0.01	< 0.01
	4:130030763	C	T	Stop-gain	8	rs10009430	120	300.35	2,3	0.01	0.06
COL19A1	6:70639431	C	T	Non-synonymous	4	rs150284256	126	320.17	2	< 0.01	< 0.01
	6:70672731	T	G	Non-synonymous	4	rs111265263	6	16.08	3	< 0.01	0.01
	6:70873265	C	G	Non-synonymous	6	rs61743753	126.5	251.24	1,5	0.01	0.06

[a] consequence types in bold are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant

Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

Table 4.10: Genes uniquely mutated in at least three Group B patients

Gene	Hg19 coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patient(s)	MAF	MAF (Afr)
BORA	13:73317740	G	GGTGA	**Frameshift insertion**	8	rs10648315	47.67	215.97	6,8,11	-	-
C3	19:6712379	T	TGG	Frameshift insertion	8	-	12	59.51	6,10,11	-	-
CACNA1F	X:49063205	C	CG	Frameshift insertion	8	-	27	73.62	6	-	-
	X:49065838	GGC	G,GG	Frameshift deletion	8	-	7	29.58	8	-	-
	X:49082499	C	T	Non-synonymous	5	rs34162630	102	280.71	10	0.01	< 0.01
CHAF1A	19:4422644	G	GA	Frameshift insertion	8	-	11.67	29.45	9,10,11	-	-
CREB3L2	7:137593054	C	CG	Frameshift insertion	8	-	29.67	60.70	9,10,11	-	-
	7:137593056	C	CG	Frameshift insertion	8	-	27	156.23	10	-	-
CYFIP1	15:22993154	T	TC	Frameshift insertion	8	-	14	87.42	9,10,11	-	-
	15:22993112	T	G	Non-synonymous	4	-	6	15.96	9	-	-
	15:22993147	C	CG	Frameshift insertion	8	-	8	32.66	9	-	-
EIF5B	2:99980798	T	TA	**Frameshift insertion**	8	-	35.67	257.28	6,10,11	-	-
ERBB2	17:37879687	CGG	CG	Frameshift deletion	8	-	84	645.69	11	-	-
	17:37879687	CG	C,CGT	Deletion/insertion	8	-	71	90.35	6	-	-
	17:37866378	AGGGG	AGGG	Frameshift deletion	8	-	10	52.89	8	-	-
	17:37879688	G	GT	Frameshift insertion	8	-	62	47.17	8	-	-
	17:37864584	A	C	Non-synonymous	6	rs61737968	297	598.59	6	< 0.01	0.01
FAM171B	2:187559057	C	CAACA	Frameshift insertion	8	-	96	396.48	6	-	-
	2:187559066	CA	C,CAGC	Insertion/deletion	8	-	52	40.04	9	-	-
	2:187615884	C	T	Non-synonymous	4	rs79964543	70	99.79	11	0.01	0.05
FCRL6	1:159785370	T	C	**Stop-loss**	8	rs4301626	145.67	693.21	6,9,10	0.18	0.40
	1:159785413	C	T	Stop-gain	8	rs61823162	216	332.76	7	0.08	0.01
ING1	13:111368163	CT	C	Frameshift deletion	8	-	76.33	743.42	8,9,11	-	-
KRTAP5-2	11:1619179	CAG[24]CCA	A	Frameshift deletion	8	-	52.5	33.45	6,11	-	-
	11:1619441	A	T	Frameshift insertion	8	-	18	20.52	8	-	-
LRRC10B	11:61277300	CCG	C	Frameshift deletion	8	-	83.33	349.47	8,10,11	-	-
OR4Q3	14:20215772	T	A	Stop-gain	8	-	192	33.22	11	-	-
	14:20215994	CAT[9]GCT	C	Frameshift deletion	8	-	104	241.47	6	-	-
	14:20216064	C	T	Stop-gain	8	rs148505982	103	136.29	7,9	0.02	0.07
PCSK1N	X:48690747	GGC	G,GG	**Frameshift deletion**	8		57	487.66	11	-	-
	X:48690748	GC	G	**Frameshift deletion**	8	-	16	143.66	6,9	-	-
PER1	17:8045707	GCC	GC	Frameshift deletion	8	-	33.67	220.58	8,9,10	-	-
	17:8053361	G	A	Non-synonymous	4	rs139241830	99	105.20	9	-	-
SF3A2	19:2248449	T	TC	**Frameshift insertion**	8	-	15.67	110.28	6,8,10	-	-
SLC22A1	6:160543122	GT	G	Frameshift deletion	8	-	178	500.44	6,10	-	-
	6:160557643	C	T	Non-synonymous	7	rs2282143	170	594.11	9	0.07	0.08

[a] consequence types in bold are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant
Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

Table 4.10 continued on p95

Table 4.10 (cont.): Genes uniquely mutated in at least three Group B patients

Gene	Hg19 coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patient(s)	MAF	MAF (Afr)
SLC25A45	11:65144441	T	TGTGCA	Frameshift insertion	8	-	19	21.61	9	-	-
	11:65144444	A	T	Non-synonymous	5	-	49.5	34.47	6,10	-	-
SPTA1	1:158655035	CG	C	Frameshift deletion	8	-	145.67	1391.27	7,8,11	-	-
TPST2	22:26937471	CCG	C,CC	Frameshift deletion	8	-	173	182.9	9,11	-	-
	22:26937466	GCCCCCCG	GCCCCC(G)	Deletion/substitution	8	-	91	119.83	8	-	-
ZFYVE19	15:41099899	T	TGGGGC	Frameshift insertion	8	rs142730574	25.67	42.29	7,8,11	0.34	0.39
ZNF180	19:44981589	GC	G,GCG(A)	Deletion/insertion	8	-	22	202.03	11	-	-
	19:44981544	T	TC,TTC	Frameshift insertion	8	-	13	62.17	6,9	-	-
	19:44981590	C	CG	Frameshift insertion	8	-	10	72.04	9	-	-
EP300	22:41574697	T	C	**Non-synonymous**	6	rs200876858	9.67	63.00	6,7,11	< 0.01	< 0.01
CNGB1	16:57996915	G	T	Non-synonymous	5	-	6	24.08	8	-	-
	16:57935470	C	T	Non-synonymous	6	rs79889567	152.5	362.52	6,11	0.02	0.07
FOXO3	6:108882487	A	T	Non-synonymous	5	-	384	1559.17	8,9,11	-	-
	6:108882490	C	G	Non-synonymous	6	-	770	621.80	11	-	-
	6:108985494	G	GC	Frameshift insertion	8	-	6	33.38	6	-	-
AATK	17:79093210	C	T	Non-synonymous	4	-	11	19.90	7	-	-
	17:79093835	A	ACG	Frameshift insertion	8	-	19.5	110.80	10,11	-	-
DYTN	2:207559599	T	C	Non-synonymous	4	rs16838593	122	251.16	9,10,11	0.07	0.28
	2:207564562	T	A	Non-synonymous	5	rs112735024	110	232.73	8	< 0.01	0.01
FBXO32	8:124553244	A	G	**Non-synonymous**	4	-	9	66.74	10	-	-
	8:124516917	C	T	Non-synonymous	4	rs150551215	30	68.16	8,9	0.01	0.03
FOXH1	8:145700346	T	A	Non-synonymous	4	rs112028242	74	180.88	6,8,9	0.02	0.10
KRT79	12:53216989	G	A	**Non-synonymous**	4	rs17688627	116.5	488.75	7,10	0.10	0.01
	12:53225406	C	T	Non-synonymous	5	rs75633465	103	257.45	8	0.01	0.06
TBC1D21	15:74178447	T	C	Non-synonymous	4	rs140701676	101	238.83	8,11	< 0.01	0.01
	15:74177406	G	A	Non-synonymous	4	rs146169837	43	142.27	6	-	-
WFS1	4:6304133	G	A	Non-synonymous	4	rs71532874	170	360.70	7	< 0.01	< 0.01
	4:6302502	T	C	Non-synonymous	5	-	25	46.46	8	-	-
	4:6304118	G	A	Non-synonymous	4	rs3821945	80	198.01	9	0.01	0.01

[a] consequence types in bold are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant

Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

Table 4.11: VarElect prioritisation of the enriched genes not identified in variant-level pipeline

Gene	Implicating gene(s)	Score
ERBB2	*MAPK8*, *CASP3*, *COMT*, *GSTP1*, *LRP2*	26.27
CACNA1F	*MAPK8*, *CASP3*, *COMT*, *LRP2*, *NTF3*	25.77
SLC22A1	*ABCB1*, *COMT*, *TPMT*, *LRP2*, *GSTP1*	25.51
CNGB1	*MAPK8*, *CASP3*, *LRP2*, *NTF3*, *GSR*	25.51
COL19A1	*MAPK8*, *CASP3*, *NTF3*, *BDNF*, *NTRK2*	25.34
WFS1	*MAPK8*, *GSTP1*, *CAT*, *ABCB1*, *NTRK2*	25.21
OR4Q3	*MAPK8*, *CASP3*, *LRP2*, *NTRK2*	24.58
TRPM2	*ABCB1*, *NTF3*, *BDNF*, *NTRK2*, *SLC12A1*	24.22
FBXO32	*MAPK8*, *CAT*	19.33
TUBA3E	*MAPK8*, *NOX3*	16.12
KRT79	*GSR*	12.56

Unrelated: *ASPM*, *C4orf33*, *CDHR5*, *SRPK3*, *AATK*, *FAM171B*, *KRTAP5-2*, *PCSK1N*, *SLC25A45*, *TBC1D21*, *TPST2*

4.3.2.2.2.1 Panel-based gene-level analysis

The deafness, ADME, DNA repair and cisplatin response gene panels were applied to all the genes which contained variants scoring ≥ 4 in more than one patient in either group (Figure 4.8). Two genes were identified in the deafness panel, *COCH* and *WFS1*, which are associated with non-syndromic dominant hearing loss and Wolfram syndrome, respectively (Table 4.12). *WFS1* was previously identified in the gene-level analysis, as three Group B patients had variants in this gene (Table 4.10), while two other patients in this group had variation in *COCH*.

In the ADME gene analysis, two transporter genes were identified, *ABCC6* and *SLCO4A1*, of which *ABCC6* is part of the ABC family of multidrug resistance associated proteins (MRPs) (Table 4.12). The variant is a known non-synonymous SNV (rs41278174) and was observed in two of the resistant patients. Other ADME genes were those encoding phase I enzymes, including *DDO*, *PON3* and *NOS3*, and the modifier genes *CFTR* and *NR1I2*. *CFTR* was also included in the cisplatin response panel; two non-synonymous SNVs, rs1800076 and rs121908759, occurred in Group A patients. A novel homozygous frameshift insertion in *NOS3* occurred in two of the Group B patients.

Regarding the DNA repair gene panel, homozygous 4 bp deletions in *POLI* were observed in two of the susceptible patients (Table 4.12). *BIVM-ERCC5* was also included in the cisplatin response panel, and is involved in the NER pathway. Four mutations in three Group B patients occurred in *PER1*. This gene was also identified in the gene- and variant-level analyses, and was implicated here as a checkpoint protein involved in the detection of DNA damage. Similarly, *SLX4*, in which two mutations occurred, is a checkpoint protein and functions in the cellular response to DNA adducts and other lesions.

Table 4.12: Gene panel analysis

Gene	Coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patient(s)	Group	MAF	MAF (Afr)
Deafness-related gene panel												
COCH	14:31355311	T	G	Non-synonymous	7	rs61759484	296	724.06	10	B	-	-
	14:31358897	A	G	Non-synonymous	4	rs17097468	52	211.99	6	B	0.01	0.05
WFS1	4:6304133	G	A	Non-synonymous	4	rs71532874	170	360.70	7	B	< 0.01	< 0.01
	4:6302502	T	C	Non-synonymous	5	-	25	46.46	8	B	-	-
	4:6304118	G	A	Non-synonymous	4	rs3821945	80	198.01	9	B	0.008	0.01
ADME gene panel												
ABCC6	16:16259596	G	A	Non-synonymous	6	rs41278174	257.5	625.49	3,4	A	0.01	< 0.01
CFTR	7:117149147	G	A	Non-synonymous	7	rs1800076	121	225.00	1	A	0.01	< 0.01
	7:117232086	G	A	Non-synonymous	7	rs121908759	102	212.50	2	A	< 0.01	< 0.01
DDO	6:110734521	C	T	Non-synonymous	7	rs147873486	268	565.67	1	A	-	-
	6:110714357	C	T	Non-synonymous	4	rs147072212	148	329.05	3	A	< 0.01	0.01
PON3	7:94996689	T	C	Non-synonymous	4	rs140234471	49	89.39	2,4	A	-	-
	7:94993334	G	T	Non-synonymous	4	rs17883013	8	15.55	10	A	0.01	0.05
SLCO4A1	20:61287937	T	TCG	Frameshift insertion	8	-	54.5	144.94	1,3	A	-	-
	20:61297803	C	T	Non-synonymous	4	rs147153778	371	552.70	9	A	< 0.01	< 0.01
NOS3	7:150696110	A	AG	**Frameshift insertion**	8	-	117.5	696.35	6,11	B	-	-
NR1I2	3:119526203	G	A	Non-synonymous	4	rs12721607	219	468.13	7,11	B	0.01	< 0.01
DNA repair gene panel												
BIVM-ERCC5	13:103527848	A	AC	**Frameshift insertion**	8	-	20	168.40	1	A	-	-
	13:103510736	C	T	Non-synonymous	4	rs56255799	65	125.62	5	A	< 0.01	< 0.01
PER1	17:8045707	GCC	GC	Frameshift deletion	8	-	33.67	220.58	8,9,10	B	-	-
	17:8053361	G	A	Non-synonymous	4	rs139241830	99	105.20	9	B	-	-
POLI	18:51795957	ACGAC	A	**Frameshift deletion**	8	-	176.5	1553.09	6,11	B	-	-
SLX4	16:3647623	G	GGC	Frameshift insertion	8	-	9	20.24	7	B	-	-
	16:3656625	G	A	Non-synonymous	4	rs79842542	166	503.64	11	B	0.06	0.07
Cisplatin response gene panel												
BIVM-ERCC5	13:103527848	A	AC	**Frameshift insertion**	8	-	20	168.40	1	A	-	-
	13:103510736	C	T	Non-synonymous	4	rs56255799	65	125.62	5	A	< 0.01	< 0.01
CFTR	7:117149147	G	A	Non-synonymous	7	rs1800076	121	225.00	1	A	0.01	< 0.01
	7:117232086	G	A	Non-synonymous	7	rs121908759	102	212.50	2	A	-	-

[a] consequence types in bold are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant

Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

Numerous other genes were identified at the individual level when the gene panel analysis was not limited to those genes mutated in more than one patient (Appendix L). For example, variants in five Usher syndrome genes were noticed in three of the patients: *CDH23* and *CIB2* variants in one Group A patient, and *CLRN1*, *DFNB31* and *MYO7A* variants in two Group B patients. Similarly, various high-scoring ABC MRP transporter gene variants were observed in the cohort; most notable was *ABCB6* rs111677240, which was a homozygous non-synonymous SNV which scored 8 in the analysis, and occurred in a Group B patient. Regarding the cisplatin response panel, three patients in the cohort had variation in *NOX3*. The variant *NOX3* rs3749930 occurred in one Group A patient, and *NOX3* rs142268682 was observed in two of the Group B patients. Both of the SNVs were non-synonymous and had a score of 5.

4.3.2.2.2.2 Pathway gene-level analysis

All of the genes which contained one or more variant with a score of at least 4 were interrogated using CPDB to identify pathways over-represented in each of the patient groups. A total of 55 pathways were identified: 26 which were enriched in Group A, and 29 which were enriched in Group B (Appendix M). The full gene set of each of the pathways was downloaded, giving a pathway panel of 2,404 genes, which was then applied to the total dataset (Appendix N). Many of the pathways were similarly mutated in both patient groups (Figure 4.10); however, three were uniquely affected in Group A, and six in Group B (Table 4.13). In particular, variation in the ERBB2 signalling pathway (pathway 25) was observed in five of the six Group B patients, with seven mutations occurring in the genes *ERBB2*, *ERBB3* and *NRG1*. Similarly, the pathway involved in the activation of C3 and C5 (pathway 1) contained variation in four Group B patients, implicating the genes *C2*, *C5*, *CFB* and *C3*, of which *C3* was identified in the previous variant- and gene-level analyses (Tables 4.6 and 4.10). *EP300* is another gene which was also identified in the variant-level pipeline (Table 4.6). This gene was implicated in two pathways involving IFN production (pathway 30) and oestrogen receptor regulation (pathway 38), and three patients contained the homozygous non-synonymous SNV rs200876858.

The pathway which was most affected in Group A was the p53 signalling pathway (pathway 36), which contained frameshift deletions in three of the five patients. Of these, *BCL2* was included in the cisplatin response panel (Appendix J). This gene contained a homozygous frameshift deletion in the patient, although coverage of this locus was low (5X), and the variant would therefore need validation, for example through direct cycle sequencing.

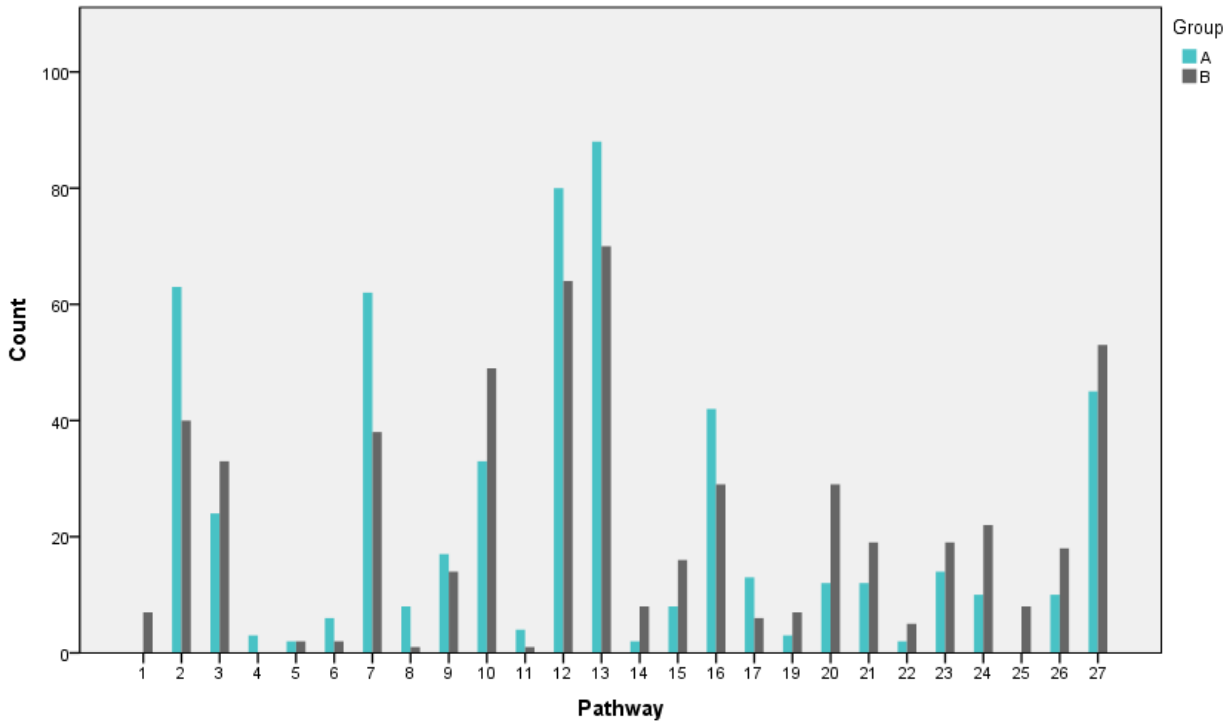
Table 4.13: Details of affected genes identified in the uniquely mutated pathways

Gene	Coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patient(s)	Group	MAF	MAF (Afr)
Activation of C3 and C5												
C2	6:31912522	G	GC	**Frameshift insertion**	8	-	276	2231.56	10	B	-	-
C3	19:6712379	T	TGG	Frameshift insertion	8	-	12	59.51	6,10,11	B	-	-
C5	9:123792679	C	T	Non-synonymous	4	rs112959008	108	279.71	6	B	< 0.01	< 0.01
CFB	6:31914024	T	A	Non-synonymous	4	rs4151667	45	135.87	7	B	0.03	0.01
Aripiprazole metabolic pathway												
CYP2D6	22:42526694	G	A	Non-synonymous	5	rs1065852	225.5	485.09	1,4	A	0.24	0.11
CYP3A43	7:99434077	TA	T	Frameshift deletion	8	rs61469810	43	91.7	4	A	0.11	0.32
GRB7 events in ERBB2 signalling												
ERBB2	17:37879687	CGG	CG	Frameshift deletion	8	-	84	645.69	11	B	-	-
	17:37879687	CG	C,CGT	Deletion/insertion	8	-	71	90.35	6	B	-	-
	17:37866378	AGGGG	AGGG	Frameshift deletion	8	-	10	52.89	8	B	-	-
	17:37879688	G	GT	Frameshift insertion	8	-	62	47.17	8	B	-	-
	17:37864584	A	C	Non-synonymous	6	rs61737968	297	598.59	6	B	< 0.01	0.01
ERBB3	12:56490612	T	G	Non-synonymous	5	-	7	15.63	10	B	-	-
NRG1	8:32611970	G	T	Non-synonymous	4	rs74942016	141	324.22	7	B	0.01	< 0.01
LRR FLII-interacting protein 1 (LRRFIP1) activates type I IFN production												
EP300	22:41574697	T	C	**Non-synonymous**	6	rs200876858	9.67	63.00	6,7,11	B	< 0.01	< 0.01
CREBBP	16:3779222	C	CGC	Frameshift insertion	8	-	6	16.32	11	B	-	-
LRRFIP1	2:238672699	AGGGC	AGGC	Substitution	8	-	29	166.92	8	B	-	-
Opsins												
OPN1MW	X:153459074	C	CT	Frameshift insertion	8	-	6	20.88	2	A	-	-
OPN3	1:241757758	ACTT	AT	**Substitution**	8	-	11	80.4	4	A	-	-
RHO	3:129247728	G	C	Non-synonymous	4	rs149079952	107	391	2	A	< 0.01	0.02
p53 signalling pathway												
CCND1	11:69465965	CCA	C	Frameshift deletion	8	-	10	57.89	2	A	-	-
TP53	17:7579579	CG	C	Frameshift deletion	8	-	20	73.38	4	A	-	-
BCL2	18:60985775	GCCCCCG	GCCCC	**Frameshift deletion**	8	-	5	36.81	4	A	-	-
TIMP3	22:33253290	TCC	TC	Frameshift deletion	8	-	263	1781.98	5	A	-	-
PELP1 modulation of oestrogen receptor activity												
EP300	22:41574697	T	C	**Non-synonymous**	6	rs200876858	9.67	63.00	6,7,11	B	< 0.01	< 0.01
CREBBP	16:3779222	C	CGC	Frameshift insertion	8	-	6	16.32	11	B	-	-
PELP1	17:4607229	TTG	T	Frameshift deletion	8	-	138	36.84	9	B	-	-
Purine deoxyribonucleosides degradation												
ADA	20:43255233	G	A	Non-synonymous	7	rs121908736	14	45.7	8	B	< 0.01	0.01
PNP	14:20943408	G	A	Non-synonymous	4	rs138702206	72	103.52	9	B	0.01	0.02
Purine ribonucleosides degradation to ribose-1-phosphate												
ADA	20:43255233	G	A	Non-synonymous	7	rs121908736	14	45.7	8	B	< 0.01	0.01
PNP	14:20943408	G	A	Non-synonymous	4	rs138702206	72	103.52	9	B	0.01	0.02
PGM2	4:37857332	A	G	Non-synonymous	7	rs139469063	94	283.29	10	B	< 0.01	< 0.01

[a] consequence types in bold are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant

Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

A



B

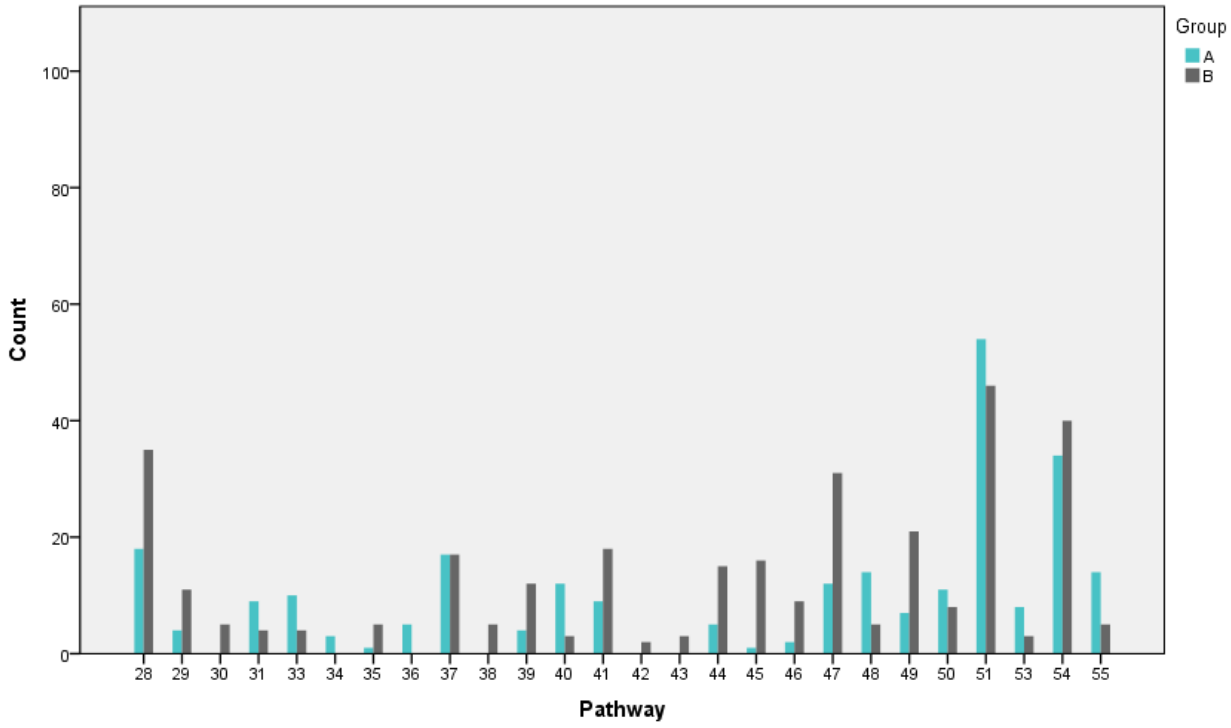


Figure 4.10: Number of variants identified in pathways 1 to 27 (A) and 28 to 55 (B). Blue bars indicate the frequency of variation in Group A, and grey bars indicate variation in Group B patients. Pathways which contained variant counts > 100 (pathways 18, 32 and 52) were excluded. The pathway numerical codings are explained in Appendix M.

4.4 Discussion

Hearing loss is a permanent adverse reaction to cisplatin therapy which can affect treatment outcomes and patient quality-of-life. Although its high prevalence is widely reported (Kopelman et al, 1988; Knight et al, 2005; Kushner et al, 2006; Coradini et al, 2007; Yancey et al, 2012; Choeyprasert et al, 2013; Peleva et al, 2014; Whitehorn et al, 2014a), inter-individual variability in drug response amongst patients has implicated the role of various clinical and non-clinical risk factors in the development of ototoxicity. Patients who are exposed to similar treatment regimens can have very different responses to therapy, and this difference has been demonstrated to have a heritable component (Dolan et al, 2004; Shukla et al, 2008). Consequently, the identification of the genetic factors which play a role in ototoxicity would be beneficial and assist in the future personalisation of treatment plans for cancer.

In this investigation, 11 patients who demonstrated an extreme audiological reaction to cisplatin were selected for WES analysis. Six of these patients developed significant hearing loss after a single cycle of treatment and were included in the extreme susceptibility category (Group B). In contrast, five patients demonstrated an increased resistance to cisplatin's ototoxic effect (Group A). These patients received multiple doses of the drug, reaching total doses above the average for the cohort (median cumulative dose: 200 mg/m^2). Cumulative cisplatin dose is a well characterised risk factor of ototoxicity (Bokemeyer et al, 1998; Bertolini et al, 2004; Li et al, 2004; Whitehorn et al, 2014b), and the high doses these patients were exposed to indicates their resistance to drug-induced hearing loss. Other factors such as patient age and sex were not different between the two groups of patients, and have been shown to have no effect on ototoxicity in SA or other populations (Bokemeyer et al, 1998; De Jongh et al, 2003; Chen et al, 2006; Choeyprasert et al, 2013; Whitehorn et al, 2014a). Because all the patients included in this investigation were head and neck, osteosarcoma or lymphoma cancer patients, they were treated with a similar strategy. The differences in treatment outcome can therefore not be attributed to differences in clinical parameters such as cisplatin infusion rates or method of administration.

Four of the six Group B patients were of black African self-reported ethnicity; indeed, this population has been found to be more susceptible to ototoxicity than SA Caucasian and mixed ancestry patients (Chapter 2). Three of these individuals clustered together in the PCA, as expected, while the fourth clustered more closely with the mixed ancestry and European populations. This indicates that the ancestry of this individual may be more complex than reported. All the other individuals in the investigation, including the five who

demonstrated an increased resistance to cisplatin-induced ototoxicity, were of mixed ancestry; their positioning in the PCA reflected this self-reported complex ethnicity. The indigenous African and Cape mixed ancestry populations are relatively unexplored in pharmacogenomic studies of ototoxicity, and their investigation may yield novel findings. A review of available genotypes for previously associated genetic modifiers of ototoxicity indicated that the majority were unable to predict these patients' reactions to cisplatin treatment. The protective variants *OTOS* rs2291767 and rs77124181 were only observed in patients who displayed resistance to cisplatin's ototoxicity, while the *COMT* rs4646316 variant alleles were limited to the susceptible patient group. These were the only variants which occurred in the direction in which they were originally reported (Ross et al, 2009; Spracklen et al, 2015), indicating that genotypic information from the other modifiers could not explain the extreme phenotypic response demonstrated by these patients. NGS analysis of this cohort of patients may therefore yield new insights into the genetics of cisplatin-induced ototoxicity.

The exonic regions of all 11 patients were successfully captured and sequenced, generating a total of 257,831 coding region variants for the analysis. When applying this exomic data to the cisplatin response panel, which included all the genes which have been previously implicated as modifiers of cisplatin-induced ototoxicity, few noteworthy results were obtained. In fact, no variants were identified in any of the previous modifiers in the filtering pipelines, although other genes which were previously implicated in cisplatin response, such as *cystic fibrosis transmembrane conductance regulator* (*CFTR*) and *NADPH oxidase 3* (*NOX3*), had potentially modifying variants which occurred at low frequencies. The inability of the cisplatin response gene panel to generate conclusive results indicates the need for hypothesis-generating approaches such as WES in the study of the pharmacogenomics of cisplatin's ototoxicity. By targeting coding regions of the genome for NGS, WES is a strategy which allows functional variants to be prioritised in the study of complex traits. In this investigation, variants which were common to both groups of patients were excluded in order to select those variants with a greater possibility of being implicated in individual cisplatin response. Initial analyses indicated that stop-gain, stop-loss and frameshift variants, which are more likely to be functional due to their effects on the protein product, were shared between groups less often than other variants. These variants also presented a higher rate of novelty, however, which increases the possibility of them being false positives. Therefore, variants which occurred in multiple individuals were prioritised in the analysis, although genes which had multiple hits in one group were also included. To further focus on functionally relevant variants, the exomes were also interrogated for gene-disease relationships, pathway over-representations, and representation on various gene panels.

In the gene-disease relationship analysis, all genes which were associated with disorders which affect any of the cisplatin-sensitive organs in the body were considered. Six such genes were identified, of which *aquaporin 7* (*AQP7*) and *Wolfram syndrome 1* (*WFS1*) have their effects in the inner ear. *AQP7* encodes an aquaporin (AQP) which was associated with Ménière's disease and endolymphatic hydrops, two disorders involving dysregulation of endolymph levels in the cochlea and vestibular apparatus. The gene was also identified in the prioritisation analysis through its associations with genes involved in cisplatin response. AQPs are membrane transporters of water which are typically expressed in cells of the kidney, where they regulate body water homeostasis (Kortenoeven and Fenton, 2014). They are also expressed in the inner ear, where they regulate the water content of the endolymph and perilymph (Eckhard et al, 2012). In rats, cisplatin treatment reduced renal protein expression of AQP1, AQP2, and AQP3 (Kishore et al, 2000; Kim et al, 2001; Kang et al, 2004), and differential expression of *AQP1*, *AQP3*, *AQP5*, and *AQP8* has been observed after treating ovarian carcinoma cells with the drug (Yang et al, 2012; Xuejun et al, 2014). Furthermore, the sensitivity of ovarian cancer cells to cisplatin could be increased *in vitro* through incubation with mercuric chloride, an AQP inhibitor (Xuejun et al, 2014), while silencing *AQP5* expression sensitised colon cancer cells to cisplatin treatment (Shi et al, 2014b). Together, these results suggest that the permeability of cells may be important in cisplatin's cytotoxicity. Polymorphisms in *AQP2* and *AQP9* have been associated with the response to platinum-based chemotherapy in 338 Chinese lung cancer patients (Wang et al, 2014a), indicating that AQP genetic variation may play a role in variable treatment outcomes. In this investigation, *AQP7* was identified as a potential modifier of ototoxicity, because a single non-synonymous SNV was observed in three of the patients who were resistant to cisplatin-induced ototoxicity One of the patients, however (patient 1), was observed to develop renal dysfunction during treatment. AQPs have previously been associated with inner ear-related disorders, such as Ménière's disease (Ishiyama et al, 2010), age-related hearing loss (Christensen et al, 2009), and non-syndromic deafness (Li and Verkman, 2001; Nicchia et al, 2011). Although little is known about the function of *AQP7*, the gene is expressed in the cochlea and vestibular apparatus (Huang et al, 2002), and homozygous deletion of the gene caused a renal phenotype similar to cisplatin-induced nephrotoxicity (Sohara et al, 2005). This indicates that *AQP7* may be involved in the mediation of cisplatin-induced inner ear damage, although its precise role in hearing is currently unclear.

In contrast, *WFS1* has been thoroughly implicated in non-syndromic dominant hearing loss (Pennings et al, 2003; Bramhall et al, 2008; Rendtorff et al, 2011; Bai et al, 2014; Häkli et al, 2014) and Wolfram syndrome, an autosomal recessive condition in which subjects often

present progressive high-frequency hearing loss (Haghighi et al, 2013; Lopez de Heredia et al, 2013; Matsunaga et al, 2014). Additionally, the gene was prioritised by VarElect due to its associations with *GSTP1* and catalase (*CAT*), two enzymes which may be involved in the antioxidant response to cisplatin. In this cohort, three different non-synonymous *WFS1* variants were observed in three of the susceptible patients. Due to the link between *WFS1* and hearing, it may be that these variants were predisposing factors. Indeed, the disease phenotype which is the most related to cisplatin-induced ototoxicity is deafness. For this reason, a deafness gene panel was included in the analysis, and incorporated all genes which have been implicated in syndromic and non-syndromic forms of hearing impairment. Similar to the cisplatin response panel, the deafness gene panel did not offer many potential candidate genes beyond *WFS1*. An additional gene was *cochlin* (*COCH*), which encodes an extracellular protein with unclear functions (Bae et al, 2014). Mutations in *COCH* have been associated with autosomal dominant hearing loss (de Kok et al, 1998; Nagy et al, 2004; Tsukada et al, 2015) and it is therefore uncertain if the variants identified here would be functionally relevant. When considering other forms of syndromic hearing loss, variants in five Usher syndrome genes were noted. Usher syndrome is a recessive condition which is characterised by hearing and visual impairment (Mathur and Yang, 2015), and it may be hypothesised that individuals heterozygous for variants in these genes are predisposed to drug-induced hearing loss. Unfortunately, no phenotypic data was available for any of the known variation, and because the variants occurred at an individual basis, the role of the Usher syndrome genes in predisposition to cisplatin-induced ototoxicity is unclear at this stage. A previous study which considered deafness genes failed to find any association between variants in *GJB2*, *SLC26A4*, and the mitochondrial genome, and cisplatin-induced ototoxicity (Knoll et al, 2006); however, no work beyond this pilot investigation has been conducted to date, and the role of deafness genes in ototoxicity cannot be conclusively rejected.

Cisplatin is also known to affect the kidneys, erythrocytes and nervous system (McKeage, 1995). Mutations in the genes *complement component 3* (*C3*), *cubilin* (*CUBN*), *dyslexia susceptibility 1 candidate 1* (*DYX1C1*), and *FRAS1 related extracellular matrix protein 2* (*FREM2*) have been associated with disorders which affect some of these organs or tissues. *FREM2*, for instance, has been implicated in Fraser syndrome, a developmental disorder which can affect the formation of the kidneys and ears (van Haelst et al, 2007). Homozygous 1 bp insertions were observed in this gene in three of the patients who were susceptible to cisplatin-induced ototoxicity. A gene ontology (GO) term associated with *FREM2* is “inner ear development” (GO identifier: 0048839, http://www.ebi.ac.uk; accessed 08.04.2015), and it is possible that variation in the gene may predispose patients to cisplatin ototoxicity at a

developmental level; however, there is currently no data on the role of *FREM2* in drug-induced or any other form of hearing impairment. *DYX1C1* is another gene with a developmental function, playing a role in neuronal migration (Wang et al, 2006; Tammimies et al, 2013). A single variant in *DYX1C1*, rs57809907, occurred in three of the resistant patients. This is a stop-gain SNP which is prevalent in the 1000 Genomes project African populations, and a reported risk factor of dyslexia (Taipale et al, 2003). Dyslexia is a cognitive disorder which primarily affects reading ability, although auditory processing may be affected in some individuals (Christmann et al, 2015). Homozygous deletion of the gene in rats caused both disrupted neuronal migration and deficient auditory processing (Wang et al, 2006; Rosen et al, 2007; Szalkowski et al, 2013), indicating the role of *DYX1C1* in the development of the nervous system. Furthermore, *DYX1C1* was also associated with ciliary dyskinesia according to the gene-disease output (Tarkar et al, 2013). The role of *DYX1C1* in the development and function of ciliated cells has been demonstrated in zebrafish, when loss of the gene resulted in ciliary defects (Chandrasekar et al, 2013; Tarkar et al, 2013). *DYX1C1* gene expression was also detected in murine tracheal cells undergoing differentiation in cell culture (Hoh et al, 2012), and in transcriptomic data from ciliated epithelia found in human tissues including the brain, tracheae, lungs and fallopian tubes (Ivliev et al, 2012). Hair cells of the cochlea possess stereocilia, and it is unknown if these require *DYX1C1* in the same way. Nevertheless, the role of *DYX1C1* in auditory processing and neuronal development indicates it may be a candidate similar to *FREM2*; however, in the case of this gene, the variant was enriched in the patients resistant to ototoxicity. Another possible risk factor was the non-synonymous SNP *CUBN* rs12259370, which occurred in three Group B patients. The gene was identified as a potential risk candidate through its association with megaloblastic anaemia (Tanner et al, 2004), and kidney-related disorders such as Dent's disease and nephropathic cystinosis (Gaide Chevronnay et al, 2014; Raggi et al, 2014). Although the role of the gene in cisplatin response and hearing is so far unknown, *CUBN* scored highly in the VarElect prioritisation analysis, and was associated with *GSTP1* and *CAT*, similar to *DYX1C1*.

The final gene from the gene-disease relationship analysis was *C3*. Associated with dense deposit disease and haemolytic uraemic syndrome (Frémeaux-Bacchi et al, 2008; Martínez-Barricarte et al, 2010), the gene *C3* encodes a component and central regulator of the complement cascade. The complement system is an innate inflammatory immune response which can be induced by three distinct pathways which all converge on the activation of C3 and subsequent activation of C5 (Cook and Botto, 2006). *C3* has been associated with both hearing and cisplatin response, when increased levels of activated C3 were observed in individuals with sudden unilateral deafness (Nordang et al, 1998), and up-regulation of *C3*

expression was reported in models of cisplatin-induced ovarian failure in rats (Li et al, 2013). Further experiments in rats demonstrated that cisplatin exposure increases serum complement activity and, in animals which were unable to produce C5, significantly less nephrotoxicity than wild type rats was observed (Pan et al, 2009). These results indicate that complement proteins may play a role in targeting and clearing cisplatin-damaged cells, and they can have their effects in the inner ear. Here, a novel 2 bp insertion in the gene was identified in three of the Group B patients. Additionally, the genes *C2*, *C5*, and *complement factor B* (*CFB*) also had variation in this group of patients. Together with *C3*, these genes occurred in the pathway involved in the activation of C3 and C5, which further suggests the role of complement deficiencies in predisposition to cisplatin-induced ototoxicity.

The investigation of pathways is another approach to the analysis of WES data which investigates groups of genes, rather than individual genes, for their contribution to a phenotype, on the basis that those genes are all involved in the same process. Pathways which were affected in the susceptible patient group were involved in immune response, signalling, and purine metabolism. Immune response pathways which were potentially compromised in Group B patients were the activation of C3 and C5, which is discussed above, and the production of interferon (IFN). IFNs are cytokines which are involved in the regulation of the immune response and cell proliferation (de Andrea et al, 2002). Cancer cells in which IFN over-expression was forced were sensitised to cisplatin (Mecchia et al, 2000), and those co-treated with IFNs and cisplatin displayed greater apoptosis compared to cells exposed to cisplatin alone (Mecchia et al, 2000; Meister et al, 2007). Variation in the IFN production pathway was observed in four of the susceptible patients, implicating the genes *leucine rich repeat interacting protein 1* (*LRRFIP1*), *CREB binding protein* (*CREBBP*), and *E1A binding protein p300* (*EP300*). *LRRFIP1* encodes a transcription factor which has been associated with cisplatin resistance in various cancer cell lines (Shen et al, 2012), while *EP300* has been implicated in the recognition of and response to DNA damaging agents, and the modulation of apoptosis in cisplatin-treated cells (Van Den Broeck et al, 2012; Wang et al, 2013; Cao et al, 2014). The non-synonymous variant *EP300* rs200876858 occurred in three of the susceptible Group B patients. Although the functional effect of this SNV is unknown, its homozygosity in affected patients, as well as the role of *EP300* in cisplatin response, indicates its potential as a candidate variant.

Other pathways of interest in Group B concerned Erb-B2 receptor tyrosine kinase 2 (ERBB2) signalling and purine degradation. Although very few genes occurred in the purine degradation pathways, cisplatin is a DNA damaging agent which preferentially targets purine deoxyribonucleotides (Plooy et al, 1984; Fichtinger-Schepman et al, 1985), which makes the

role of *adenosine deaminase* (*ADA*), *purine nucleoside phosphorylase* (*PNP*) and *phosphoglucomutase 2* (*PGM2*) somewhat compelling. In particular, ADA may be involved in the response to cisplatin, since its activity and expression increased in rat models of cisplatin-induced nephrotoxicity (Söğüt et al, 2004; Gulec et al, 2006; Cure et al, 2014). The role of ERBB2 (also referred to as HER2) signalling in breast cancer is well documented, as HER2-positive breast cancers can be targeted with the drug trastuzumab (trade name Herceptin) (Molina et al, 2001; Slamon et al, 2001). ERBB2 belongs to a family of epidermal growth factor receptors which are involved in the regulation of cell proliferation and survival, amongst other cellular processes (Seshacharyulu et al, 2012). *ERBB2* was the highest scoring gene in the gene prioritisation analysis, although this was at least partly because of its associations with the genes *COMT* and *LRP2*, which may not be involved in cisplatin-induced ototoxicity (Riedemann et al, 2008; Choeyprasert et al, 2013; Yang et al, 2013; Hagleitner et al, 2014; Lanvers-Kaminsky et al, 2014; Whitehorn et al, 2014b). Nevertheless, expression of *ERBB2* has been associated with the response to cisplatin therapy, for instance in lung (Kuyama et al, 2008; Calikusu et al, 2009), oesophageal (Akamatsu et al, 2003), bladder (Koga et al, 2011; Inoue et al, 2014), cervical (Kim et al, 1999), and head and neck cancers (Shiga et al, 2000), where expression of the gene typically reduces the efficacy of the drug. Notably, ERBB2, ERBB3 and neuregulin 1 (NRG1) proteins are expressed in various cells in the inner ear (Zhang et al, 2002; Stankovic et al, 2004), and it was these components of the pathway which were affected in the Group B patients. The disruption of ERBB2 signalling in mice, through the expression of mutant *erbB2*, *erbB3* and *erbB4* genes, was associated with severe hearing impairment (Zhang et al, 2002; Stankovic et al, 2004). Furthermore, incubation of rat inner ear cells with NRG1 (also referred to as heregulin) could ameliorate the ototoxic effects of the aminoglycoside gentamicin (Zheng et al, 1999), while an ERBB inhibitor proved ototoxic in zebrafish and mouse models (Tang et al, 2015). These findings strongly suggest that deficiencies in ERBB2 signalling may confer susceptibility to cisplatin-induced ototoxicity, although more research is required to confirm this.

The pathway which was most implicated in Group A was the p53 signalling pathway, which contained variation in three of the five resistant patients. This is one of the pathways whereby cisplatin induces apoptosis in affected cells (Müller et al, 1998; Park et al, 2000), as p53 is a transcriptional activator and key regulator of DNA repair, cell cycle arrest and apoptosis in the response to many cellular stresses (Budanov, 2014). A reduction of this pathway's ability to induce apoptosis may be protective against cell death in cisplatin-damaged cells (Guntur et al, 2010; Tang et al, 2013; Yang et al, 2015). In this investigation, a novel 1 bp deletion in *tumour protein P53* (*TP53*), the gene which encodes p53, was only observed in one of the Group A patients; the other variants occurred in the genes *B-cell*

CLL/lymphoma 2 (*BCL2*), *cyclin D1* (*CCND1*), and *TIMP metallopeptidase inhibitor 3* (*TIMP3*), although all of the identified variants were also frameshift deletions. While these may have been cancer predisposing events in these patients, *TIMP3* genetic variation has been associated with survival rates amongst adenocarcinoma patients treated with cisplatin and 5-FU combination therapy (Bashash et al, 2013), while a reduced expression of *BCL2* increased the susceptibility of gastric cancer cells to cisplatin-induced apoptosis (Hu et al, 2014b; Wang et al, 2014b). Similarly, *CCND1* deletion could sensitise tumour cells to cisplatin exposure (Kornmann et al, 1998; Noel et al, 2010), and over-expression of the gene has been associated with resistance to cisplatin treatment (Biliran et al, 2005; Noel et al, 2010).

Because cisplatin is a DNA-damaging agent, one of the panels applied to the WES dataset was a DNA repair gene panel. Genes of interest were *SLX4 structure-specific endonuclease subunit* (*SLX4*), which is involved in the detection of DNA crosslinks, double-strand breaks, and other lesions including those associated with cisplatin exposure (Muñoz et al, 2009; Hodskinson et al, 2014), and *period 1* (*PER1*). *PER1* encodes a period protein which functions in the maintenance of circadian rhythm in mammalian cells, and was identified here through its role as a cell cycle checkpoint protein which may control the recognition of DNA damage (Gery et al, 2006). The circadian rhythm has previously been implicated in chemotherapy response, because of its ubiquitous effects on cellular processes such as drug metabolism, cellular proliferation and the response to genotoxic stress (Kondratov and Antoch, 2007). In lung cancer patients, the timing of cisplatin administration has been associated with the incidence of ADRs such as neutropaenia and gastrointestinal toxicity, although no effect on cisplatin efficacy was noted (Li et al, 2015). This may be due to the role of circadian cycles in DNA repair (Kang and Sancar, 2009). Murine cells had differing NER abilities depending on the time of day: optimal DNA repair capacity was observed during the afternoon and evening (Kang et al, 2009), the time at which cisplatin-induced toxicity was reportedly reduced in the study of lung cancer patients (Li et al, 2015). Accordingly, interference of *aryl hydrocarbon receptor nuclear translocator-like* (*Arntl*, also known as *Bmal1*), another component of the circadian cycle, reduced the expression *Tp53*, and attenuated DNA damage by cisplatin in murine colon cancer cells (Zeng et al, 2010). In the extreme phenotype cohort, a novel 1 bp deletion was detected in three of the susceptible patients. Silencing of *PER1* expression by siRNA in human oral cancer cells increased apoptosis in response to cisplatin treatment, indicating that the gene may have anti-apoptotic effects in response to DNA damage (Sato et al, 2011).

The final gene panel investigated the role of ADME genes in cisplatin-induced ototoxicity. *ABCC6* and *CFTR* are transporter genes which were variant in Group A patients, of which *CFTR* was also included in the cisplatin response panel. CFTR is a chloride ion channel, mutations in which have been associated with cystic fibrosis (Riordan et al, 1989). It also mediates glutathione efflux, and may therefore be involved in the cellular response to oxidative stress (l'Hoste et al, 2010; Duranton et al, 2012). Inhibition of CFTR in renal cells and rat models of nephrotoxicity caused significant protection against apoptosis upon treatment with cisplatin, and reduced ROS accumulation without affecting cisplatin uptake (Rubera et al, 2013). In this cohort, non-synonymous SNVs were found in *CFTR* in two of the protected patients, indicating that these deleterious variants may confer protection through a reduced transport of glutathione out of inner ear cells. Also involved in the oxidative stress response, homozygous 1 bp insertions were observed in *nitric oxide synthase 3* (*NOS3*) in two of the Group B patients. Nitric oxide is a free radical, and promoter region and non-synonymous variation within *NOS3* has been associated with treatment outcomes in breast cancer patients receiving combination chemotherapy, not including cisplatin (Choi et al, 2009). The role of *NOS3* variation in cisplatin response has not yet been reported. However, a reduction in *NOS3* expression was noted when treating cisplatin-sensitive ovarian cancer cells with the drug, while cisplatin-resistant cells were characterised by unchanged *NOS3* levels (Leung et al, 2008). Variation in *NOS3* has been associated with sudden hearing loss (Teranishi et al, 2013), and increased levels of NOS3 in the inner ear were detected after exposure of guinea pigs to noise (Heinrich et al, 2005) and gentamicin (Heinrich et al, 2006). These results indicate that *NOS3* may play a role in the response to trauma in the inner ear.

Other genes of interest which were not identified in any of the panels include *solute carrier family 22, member 1* (*SLC22A1*), *inhibitor of growth family, member 1* (*ING1*), and *V-rel avian reticuloendotheliosis viral oncogene homologue A* (*RELA*). *SLC22A1* encodes a member of the OCT family, OCT1, which is a paralogue of OCT2 (*SLC22A2*). OCT2 variation was reported as protective against cisplatin-induced ototoxicity in the prospective cohort (Chapter 3) as well as a cohort of German cancer patients (Lanvers-Kaminsky et al, 2015). *SLC22A1*, in which two variants were observed in three of the Group B patients, is expressed in mouse cochleae, although at a level 2.2 times less than *SLC22A2* (Ciarimboli et al, 2010). The variants included a 1 bp deletion which occurred in two patients, and the non-synonymous SNP rs2282143. OCT1 is a reported transporter of cisplatin, although its ability to transport the drug is lower when compared to OCT2 (Yonezawa et al, 2006). These mutations may therefore confer susceptibility to ototoxicity through an altered transport of cisplatin in the inner ear. *ING1* was also mutated in three of the Group B patients; in this

instance, the same 1 bp deletion was observed. *ING1* encodes a tumour suppressor which was up-regulated in response to cisplatin-induced DNA damage in glioblastoma cells, and those in which *ING1* expression was reduced by siRNA were more sensitive to cisplatin-induced apoptosis (Tallen et al, 2008). Similarly, expression of *ING1* was shown to prevent oxidative stress-induced apoptosis in a colon cancer cell line (Rotte et al, 2014), which may explain its relation with cisplatin response. *RELA* encodes p65, a component of the nuclear factor-kappaB (NF-κB) transcription factor, which is involved in the regulation of diverse cellular processes such as cell proliferation and survival in response to genotoxic stresses (Wang et al, 2002). NF-κB is activated following the exposure of cells to cisplatin, and its inhibition typically confers increased sensitivity of cells to the drug (Yeh et al, 2004; Venkatraman et al, 2005; He et al, 2010; El-Kady et al, 2011; Hong et al, 2012; Yang et al, 2015). In this investigation, three of the resistant patients carried heterozygous 1 or 2 bp deletions in *RELA*, which may be in a direction opposite to what is expected, given that reduction in NF-κB activity is associated with cytotoxicity (Venkatraman et al, 2005; He et al, 2010; Hong et al, 2012). Nevertheless, the role of NF-κB in cisplatin response indicates its potential as a genetic modifier of ototoxicity.

Although this study has produced various potential genetic modifiers of cisplatin-induced ototoxicity, two key limitations are a lack of validation and the small sample size investigated. The WES data was generated using the Ion Torrent™ NGS platform, which requires validation of genotype calls. Because this has not yet been performed, some of the variants discussed here may be false positives, particularly those with relatively low sequence coverage. Although all of the variants passed CAF's quality control procedures, validation of the variants will have to be conducted on all the potential candidates before applying this information to the rest of the prospective patient cohort. The small number of patients included in this investigation indicates that the patients selected may not represent the total patient population, and that meaningful statistical analyses could not be performed on the data as any statistical tests would likely be underpowered. However, the extreme phenotype cohort described here was utilised for the discovery of new candidate genes rather than the description of statistically significant genetic modifiers of ototoxicity. It is demonstrated here that NGS analysis of a well-phenotyped subset of the patient cohort may yield potential candidate genes, as numerous promising genes were identified, many of which are previously unreported in the context of cisplatin and its ototoxicity.

4.5 Conclusion

In this investigation, an extreme phenotype cohort of cisplatin-receiving cancer patients was described and subjected to WES analysis. Numerous potential modifiers of cisplatin-induced ototoxicity were identified through pipelines which were designed to focus on variants more likely to be functionally linked to cisplatin and its ototoxicity. Promising results were obtained in genes involved in various biological processes, including transmembrane transport (*AQP7*, *CFTR*, *SLC22A1*), development (*DYX1C1*, *FREM2*), hearing (*COCH*, *WFS1*), and the response to DNA damage (*ING1*, *PER1*, *SLX4*). Considering development and hearing, it may be suggested that variation in these genes can confer an altered risk of drug-induced hearing loss at a developmental level, while those genes involved in transport and DNA damage modify at the level of response to cisplatin exposure. Also implicated in cisplatin response were genes involved in immune reactions such as the complement cascade and interferon production, as well as the ERBB2, p53 and NF-κB signalling pathways. These have all been demonstrated to play a role in cisplatin's cytotoxicity before, and variation in these pathways may well confer resistance or susceptibility to cisplatin's ototoxic effects.

Notably, few candidates were obtained when considering known cisplatin response and deafness genes. While this validates the need for hypothesis-generating approaches such as WES, this alone cannot conclusively resolve the role of these genes in cisplatin-induced ototoxicity. Further research is recommended to include an analysis of the genes and pathways identified in this investigation for their role in predisposition to ototoxicity, or protection from it, in the South African patient population.

5. Conclusion and future perspectives

The results reported in this submission indicate the potential of genotypic information to identify cisplatin-receiving cancer patients who are at an altered risk of developing ototoxicity. In particular, the variants *NFE2L2* rs6721961 and *SLC22A2* rs316019 are the most promising genetic modifiers, as both genes have been demonstrated to play a role in the cellular processing of cisplatin, and the protective effects of these SNPs were maintained after correction for patient ethnicity. This study also demonstrates the inability of the variants *EIF3A* rs10787899 and rs3824830, *ABCB5* rs10950831 and *ABCC3* rs1051640 to predict hearing loss in the SA patient population, while *CDH13* rs11646213 was potentially associated with TUNE-defined ototoxicity. Further studies are recommended to validate the role of *NFE2L2*, *SLC22A2* and *CDH13* genetic polymorphisms in cisplatin response and the onset of drug-induced hearing impairment.

The response to cisplatin is a clinically heterogeneous phenotype which is likely influenced by multiple genetic factors (Dolan et al, 2004; Shukla et al, 2008; Boddy, 2013). Due to this polygenic nature, as well as the limited knowledge of cisplatin's mechanism of action, future research should also consider new genes for their role in cisplatin-induced ototoxicity. These investigations would be better aided by genome-scale techniques such as GWAS, WES and whole genome sequencing, which allow novel loci to be considered for their contribution to the phenotype. In this study, a pilot WES investigation of eleven cisplatin-receiving cancer patients is described, in which many potential new loci are identified and may play a role in the development of ototoxicity amongst SA indigenous African and mixed ancestry cancer patients. As such, research aims in the prospective patient cohort should include the validation of these genetic variants, for example through direct cycle sequencing of the relevant loci, and the selection of novel candidates for screening in the rest of the patient cohort. Previous work by our research group reported novel genetic modifiers of cisplatin's ototoxicity in *OTOS* (Spracklen et al, 2014), suggesting that the local patient population may provide an environment in which new genetic modifiers may be identified. This notion is supported by the identification of *NFE2L2* genetic variation in association with hearing loss in this study, as the *NFE2L2* rs6721961 variant has not, to our knowledge, been reported in association with cisplatin-induced ototoxicity before.

An additional consideration of future research should be the long term audiometric follow-up of cisplatin-receiving patients. Because a high proportion of the patients in this study displayed signs of hearing loss immediately following cisplatin exposure (in the retrospective analysis, approximately 40% of patients attending GSH between 2006 and 2015 developed

Chang grade > 0 ototoxicity), the audiological effects of cisplatin may be more pronounced when considering a longer timeframe. If available for a subset of the surviving patient cohort, this data may enable the long term impact of cisplatin treatment on cancer patients to be ascertained. Indeed, platinum-based chemotherapy regimens can have several quality-of-life implications, of which hearing impairment may be severe and potentially disabling. While ototoxicity resulting from other drugs such as loop diuretics can be reversible, the effects of cisplatin on the inner ear appear to be permanent. Therefore, because there are currently no effective methods of preventing this ADR, there is a need for a better means of predicting treatment outcomes in order to avoid patient morbidity. This would be particularly valuable in developing countries such as SA, where the burden of cancer and the economic impacts of ADR development in healthcare systems can be profound.

6. References

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APPENDIX A: Letter of ethical approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
Email: sharetta.thomas@uct.ac.za
Website: http://www.health.uct.ac.za/fhs/research/humanethics/forms

05 June 2014

HREC REF: 220/2014

Prof R Ramesar
Human Genetics
Suite N3.17
Wernher & Beit North Building
IIDMM

Dear Prof Ramesar

PROJECT TITLE: ***INVESTIGATING THE GENETIC BASIS OF CISPLATIN-INDUCED OTOTOXICITY IN ADULT SOUTH AFRICAN PATIENTS - linked to 389/2012***

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th June 2015

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student, Timothy Spracklen is also involved in this study.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the HREC reference no in all your correspondence.

Yours sincerely

T Burgess

pp **PROFESSOR M BLOCKMAN**
CHAIRPERSON, FHS HUMAN ETHICS
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

APPENDIX B: Molecular study information sheet provided to patients during recruitment

Introduction

Hello, I am a researcher in the Division of Human Genetics, here at UCT/Groote Schuur Hospital. I am doing research as part of contributing to improving cancer treatment and invite you to participate in this research project. I would be very grateful for your participation. Before agreeing to participate, it is important that you read the following explanations of the purpose of the study, the study procedures, benefits, risks, discomforts, and precautions as well as your right to withdraw from the study at any time. This information leaflet is to help you to decide if you would like to participate. You should fully understand what is involved before you agree to participate.

Purpose of the study

You have been diagnosed with cancer and your doctor has advised you of the treatment. Like any other treatment, some patients respond well while others do not respond so well. In some instances a treatment of one part of the body may affect another part of the body. In this instance, we know that an anticancer drug such as cisplatin, in some individuals might result in hearing impairment. We stress that this occurs only in some individuals, and we have reason to believe that this might be because of hereditary factors. The aim of the study is to determine the role of what we inherit from our parents in the differences in response to anticancer medication. DNA is what each of us inherits from our parents and this DNA can be extracted from blood. If you decide to participate, you will not be asked to take any additional drugs except what your doctors have prescribed for your condition.

Procedures

If you agree to participate, your medical information including demographic information will be accessed from the clinic files. A qualified nurse will draw 9 ml of blood, or a saliva sample, from you for the extraction of DNA that will be used for genetic analysis. This will be done only once. Possible side effects which may be associated with obtaining a blood sample include pain, bruising, light-headedness and on rare occasions infection. Precautions will be taken to avoid these difficulties. The entire procedure should take less than 5 minutes.

Unforeseen risks and potential benefits

You will continue on your regular medication, but you should contact your prescribing doctor immediately if you experience any side effects. There are no direct benefits for you arising from

participating in this study; however, the findings may help future patients in the choice and doses of their medication.

Rights as a participant in the study: It is up to you to decide whether or not to take part in this study. Your participation is voluntary. If you decide to take part, you are still free to withdraw from the study at any time and without giving reason. This will not affect the standard of care that you receive or the relationship you have with your doctor. You are free to refuse to participate or withdraw your consent at any time. The results of the tests will be made available to you on your request. The results will be strictly confidential and if the data are published your name will not be used. There will be no costs to you from the study and there are no risks attached to this study.

Ethical approval: *This clinical study protocol has been submitted to the University of Cape Town, Human Research Ethics Committee (HREC) and written approval has been granted by that committee.*

Source of additional information: For the duration of the study you are still under the care of your prescribing doctor, who you should contact at any time should you feel that any of your symptoms are causing you problems. For questions relating to the study, you can contact Dr Sameera Dalvie at the Radiation and Oncology Clinic any time or alternatively on the following telephone number: 021 404 4261 or email: S.Dalvie@uct.ac.za.

Prof. Raj S Ramesar (Supervisor) (021) 406 6297	Raj.Ramesar@uct.ac.za
Dr. Lebogang Ramma (Supervisor) (021) 406 6954	Lebogang.Ramma@uct.ac.za
Timothy Spracklen (Researcher) (021) 406 6501	sprtim002@myuct.ac.za
Gameda Benefeld (Nurse)	Gameda.Benefeld@uct.ac.za

University of Cape Town, Faculty of Health Sciences , Human Research Ethics Committee:

A/Prof. Marc Blockman	Marc.Blockman@uct.ac.za

If you agree to participate, please sign the consent form.

Thank you
Professor R Ramesar

APPENDIX C: Informed consent form for access to patient records

RESEARCH FOLDER REVIEW

Informed Consent

Division of Human Genetics
Faculty of Health Sciences
University of Cape Town
Anzio Road, Observatory 7925
Cape Town, South Africa
Telephone: (021) 406 6297
Email: Raj.Ramesar@uct.ac.za
Lebogang.Ramma@uct.ac.za
sprtim002@myuct.ac.za

This is to certify that I, _____, hereby agree to be a participant in this study which has been authorised by the University of Cape Town, and that I have given permission for my patient folder to be reviewed after each visit to the Audiology Outpatients' Department. The study and my participation in it have been clearly explained to me in full by the researcher, and I understand all the explanations given to me. The questions that I asked were answered to my satisfaction, and I understand that I can stop participating in the study at any time if I wish to do so.

_____

Participant's signature

I the undersigned have defined and fully explained the study to the above participant. I have also answered all questions raised by the participant.

_____

Researcher

APPENDIX D: Informed consent for the collection, analysis and storage of patient biological samples

REQUEST FOR MOLECULAR STUDIES

Molecular Laboratory

Division of Human Genetics

IIDMM, Level 3

UCT Medical School,

Observatory 7925

Tel: (021) 406 6425 Fax: (021) 406 6826

Blood should be drawn in 2 plastic EDTA Tubes

(Purple top) +/- 5 ml each using a plastic barrel.

Each tube should be inverted to mix and should be clearly labelled with the patient's code name or number.

Keep blood in fridge at 4°C until able to send to laboratory.

Please DO NOT send specimens on ice or frozen.

Please fill in all the information requested:

Surname: _____ First Name(s):_____

New Family: Yes ☐ No ☐

Family name: _____

Medical Aid: _____Medical Aid No: _____

Sex: M ☐ F ☐ Date of Birth: Year: _____ Month: _____ Day: _____

Ethnic Origin: Black ☐ Indian ☐ Mixed ancestry ☐ Caucasian ☐ Other ☐

Contact Address: _____

Tel: _____ Fax:_____ Cell:_____

Email: _____

Referring Doctor/Sister: _____ Fax:

Hospital or Address: _____ Town: _____ Tel: _____

Blood tissue taken for: Research ☐

Date of follow up: _____

For Laboratory use only:

DNA number: _____ *Vol. Blood/Saliva:* _____*(ml)*

Date Received: Year: _____ *Month:* _____ *Day:* _____

*Computer Index No:*_____

CONSENT FOR COLLECTION, ANALYSIS AND STORAGE OF BIOLOGICAL MATERIAL FOR RESEARCH

1. I, _____ , consent to my/my child's biological material (blood/saliva sample) being used for research aimed at studying the genetic factors that may contribute to cisplatin ototoxicity in cancer patients undergoing chemotherapy treatment, as outlined to me and as per the information sheet handed to me, provided that any information from such research will remain anonymous and that anonymised/non-identifiable data can be deposited in public databases as required for publication in international journals, as per international standards in research.

2 I request that no portion of the sample be stored for later use. ☐ (MARK IF APPLICABLE)

Or

I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE):

(a) possible re-analysis

(b) Research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain anonymous and that anonymised/non-identifiable data can be deposited in public databases as required for publication in international journals, as per international standards in research.

3 I authorise / do not authorise my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the Division of Human Genetics, UCT.

4 I have been informed that:

(a) The analysis procedure is specific to the current study and cannot determine the complete genetic makeup of an individual.

(b) The genetics laboratory is under an obligation to respect medical confidentiality.
(c) Where biological material is used for research purposes, there may be no direct benefit to me.

5 I understand that I may withdraw my consent for any aspect of the above at any time without this affecting my/my child future medical care.

6 ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY:

_____ DATE: _____

Patient signature _____

Witnessed consent _____

APPENDIX E: Letter of GSH approval

Western Cape Government
Health

GROOTE SCHUUR HOSPITAL
Enquiries: Dr Bernadette Eick
E-mail : Bernadette.Schmitz@westerncape.gov.za

Professor R. Ramesar
Human Genetics
Suite 3.14
Wehner & Beit North
IIDMM

E-mail: raj.ramesar@uct.ac.za / SPRTIM002@myuct.ac.za

Dear Professor Ramesar

RESEARCH PROJECT: Pharmacogenetics: Investigating the Genetic Basis of Cisplatin and Streptomycin Induced Ototoxocity in Adult South African Patients

Your recent letter to the hospital refers.

You are hereby granted permission to proceed with your research.

Please note the following:

a) Your research may not interfere with normal patient care
b) Hospital staff may not be asked to assist with the research.
c) No hospital consumables and stationary may be used.
d) **No patient folders may be removed from the premises or be inaccessible.**
e) Please introduce yourself to the person in charge of an area before commencing.
f) Please discuss the study with the Head of Radiation Oncology before commencing.
g) Please provide the research assistant/field worker with a copy of this letter as verification of approval.
h) Confidentiality must be maintained at all times.

I would like to wish you every success with the project.

Yours sincerely

DR BERNADETTE EICK
CHIEF EXECUTIVE OFFICER
Date: 6th July 2014

C.C Mr L. Naidoo
Dr R. Kirsten
Professor R. Abratt

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APPENDIX F: Analysis of threshold changes in all prospective and retrospective patients

Average sensitivity threshold changes were calculated at each test frequency for each patient, irrespective of cancer diagnosis. Similar to when this analysis was limited to patients with head and neck cancer, lymphoma or osteosarcoma, significant differences were observed between the different ethnic groups, where black Africans demonstrated greater hearing loss than Caucasian and mixed ancestry patients (Figure F1). Again, these changes were limited to frequencies 3.0 kHz to 8.0 kHz (Table F1).

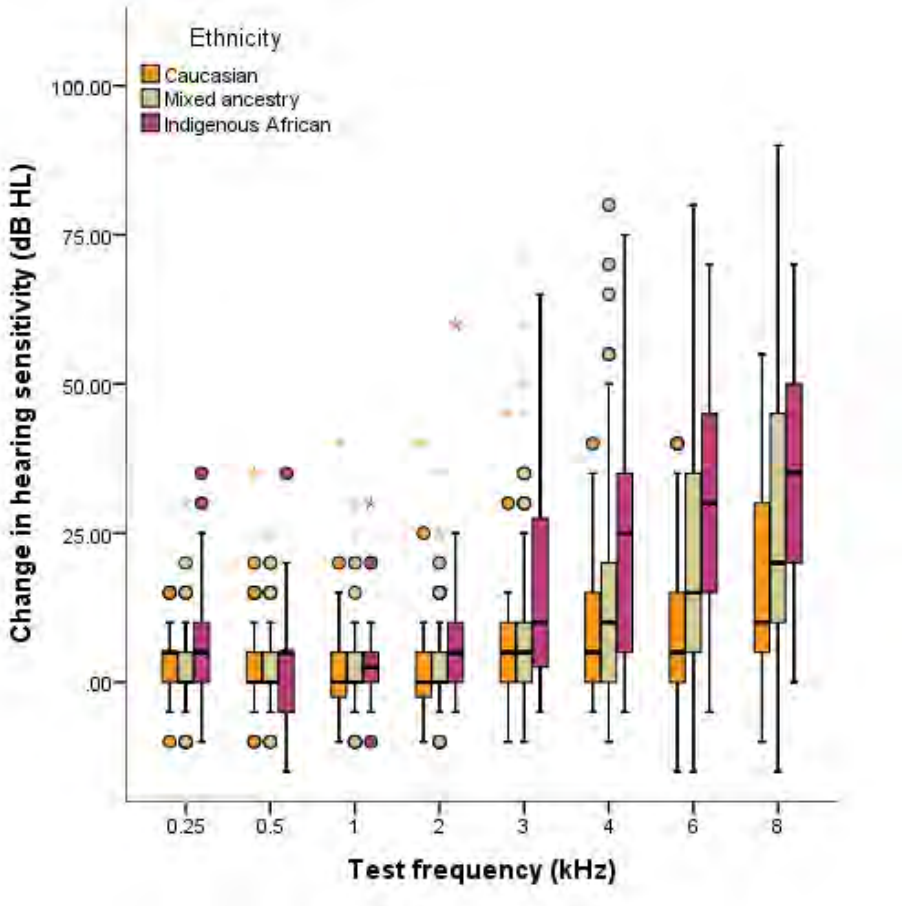


Figure F1: Average threshold changes at each test frequency in the total patient cohort. Orange, purple and grey represent the Caucasians, indigenous Africans and mixed ancestry patients, respectively. The distributions of hearing sensitivity changes are represented by box-and-whisker plots, in which the boxes delineate the upper and lower quartiles, and the whiskers indicate the minimum and maximum values. Lines within the boxes are the median values. Statistical outliers are indicated by circles (1.5-3.0 interquartile ranges from the whisker) and stars (> 3.0 interquartile ranges from the whisker). Hearing sensitivity changes are measured on the y-axis, in which a greater value indicates more hearing impairment. The test frequency, in kHz, is represented on the x-axis.

Table F1: Median threshold shifts per test frequency in the different ethnic groups in the total patient cohort

Test frequency (kHz)	Caucasian	Mixed ancestry	Indigenous African	p-value[a]
0.25	5.00	0.00	5.00	**0.041**
0.5	0.00	0.00	5.00	0.616
1.0	0.00	0.00	2.50	0.161
2.0	0.00	0.00	5.00	0.239
3.0	5.00	5.00	10.00	**0.007**
4.0	5.00	10.00	25.00	**0.002**
6.0	5.00	15.00	30.00	**< 0.001**
8.0	10.00	20.00	35.00	**< 0.001**

[a] Determined by Kruskal-Wallis test

Similar results were observed when focussing on the indigenous African patients: hearing threshold changes between 3.0 kHz and 8.0 kHz were significantly higher in this group of patients than the Caucasian and mixed ancestry patients combined (Table F2). Conversely, Caucasian patients had threshold changes which were, on average, lower than the other principal ethnic groups. This difference was limited to the frequencies 4.0 kHz to 8.0 kHz, however (Table F3).

Table F2: Median threshold shifts per test frequency in indigenous Africans compared to other ethnicity groups

Test frequency (kHz)	Caucasian and mixed ancestry patients	Indigenous African patients	p-value[a]
0.25	0.00	5.00	0.059
0.5	0.00	5.00	0.340
1.0	0.00	2.50	0.056
2.0	0.00	5.00	0.096
3.0	5.00	10.00	**0.003**
4.0	10.00	25.00	**0.001**
6.0	12.50	30.00	**0.001**
8.0	15.00	35.00	**0.001**

[a] Determined by Kruskal-Wallis test

Table F3: Median threshold shifts per test frequency in Caucasians compared to other ethnicity groups

Test frequency (kHz)	Black African and mixed ancestry patients	Caucasian patients	p-value[a]
0.25	0.00	5.00	0.249
0.5	0.00	0.00	0.994
1.0	0.00	0.00	0.634
2.0	0.00	0.00	0.468
3.0	5.00	5.00	0.067
4.0	10.00	5.00	**0.029**
6.0	20.00	5.00	**< 0.001**
8.0	30.00	10.00	**0.001**

[a] Determined by Kruskal-Wallis test

APPENDIX G: DNA extraction protocols

Saliva DNA extraction and purification using ORAgene Kit (DNA Genotek Inc.):

Procedure

1. invert ORAgene saliva tube (supplied with kit) to mix the sample
2. incubate in water bath at 50°C for 1 hour
3. transfer 4 ml sample to a 15 ml tube
4. add 1/25th volume (approximately 180 µl) ORAgene DNA Purifier (supplied with kit) and mix for a few seconds
5. incubate at -20°C for 10 mins
6. centrifuge for 10 mins at 2 400 rpm
7. transfer supernatant to a fresh 15 ml tube
8. add double that volume of absolute ethanol (Sigma-Aldrich®) and mix by inversion
9. centrifuge at 2 400 rpm for 10 mins
10. decant supernatant and resuspend the pellet in 1 ml ice-cold 70% ethanol
11. mix by inversion
12. recover the pellet by centrifuging at 2 400 rpm for 10 mins
13. decant the supernatant and air-dry the pellet for a minimum of 2 hours
14. reconstitute the DNA in 200 µl 1X TE buffer for at least 2 days

Saliva DNA extraction and purification using Norgen Kit (Norgen Biotek Corp.)

Procedure

1. incubate saliva sample in water bath at 55°C for 30 mins
2. mix by inversion
3. transfer sample to a fresh 15 ml tube
4. add 1 600 mg proteinase K and mix
5. incubate at 55°C for 1 hour
6. add an equal volume of room temperature isopropanol
7. invert the tube 10 times
8. centrifuge at 3 500 rcf/g for 10 mins
9. decant supernatant and add 1 ml 70% ethanol to the pellet
10. leave the tube to stand at room temperature for 1 min
11. centrifuge at 3500 rcf/g for 10 mins
12. decant supernatant and air-dry the pellet for a minimum of 30 mins
13. reconstitute the DNA in 500 µl 1X TE buffer for 2 hours at 55°C
14. centrifuge at 5 000 g for 10 mins and transfer the supernatant to a fresh tube

DNA extraction from blood: salting-out method

Solutions

Red blood cell (RBC) lysis solution:
- 8.28 g NAH_4Cl (Sigma-Aldrich®)
- 0.79 g NAH_4HCO_3 (Sigma-Aldrich®)
- 0.5 M EDTA (pH 7.4) (Melford Laboratories Ltd., Ipswich, UK)
- make to final volume of 1 L using sdH_2O and filter sterilise using 0.2 µM micropore filter

Cell lysis solution:
- 25 ml of 1 M Tris-HCl (pH 7.5) (Melford Laboratories Ltd.)
- 16.7 ml of 3 M NaCl (Merck Chemicals)
- 1 ml of 0.5 M EDTA (Melford Laboratories Ltd.)
- make up to 500 ml using sdH_2O

Procedure

1. obtain buffy coat by centrifuging blood sample for 10 mins at 2 000 rpm
2. remove 500 µl buffy and plasma
3. add 900 µl RBC lysis solution and incubate at 37°C for 1 hour
4. centrifuge for 10 mins at 2 500 rpm
5. remove supernatant and add 1 000 µl RBC lysis solution to the pellet
6. centrifuge at 2 500 rpm for 10 mins
7. decant supernatant and resuspend the pellet in residual RBC lysis solution
8. add 300 µl cell lysis solution, 10 µl 20% SDS (Sigma-Aldrich®) and 2 µl Proteinase K (Roche Applied Science)
9. mix well
10. incubate overnight or at room temperature until the solution clears (approximately 2 hours)
11. add 200 µl saturated 6 M NaCl (Merck Chemicals) and vigorously mix by vortexing
12. incubate at 4°C for 5 mins
13. centrifuge at 2500 rpm for 15 mins
14. remove 500 µl supernatant and add to a new tube containing 900 µl ice-cold absolute ethanol (Sigma-Aldrich®)
15. mix by inversion (50X) and centrifuge for 2 mins at 10 000 rpm
16. decant the solution and add 400 µl ice-cold 70% ethanol
17. resuspend the pellet by vortexing
18. recover DNA by centrifuging at 10 000 rpm for 2 mins
19. pour off the ethanol and allow to air-dry for 2 hours
20. resuspend in 100 µl 1X TE for at least 2 days

APPENDIX H: Buffers and reagents

DNA resuspension

10X TE (stock):
- 10 mM Tris (Melford Laboratories Ltd.)
- 1 mM EDTA (Melford Laboratories Ltd.)
- pH 8.0

1X TE (working):
- Made by a 1:10 dilution of stock 10X TE buffer, with sterile distilled water

Agarose gel electrophoresis

Tris/Borate/EDTA (TBE) buffers
10X TBE (stock):
- 0.89 M Tris (Melford Laboratories Ltd.)
- 0.89 M Boric acid (Melford Laboratories Ltd.)
- 0.04 M EDTA (Melford Laboratories Ltd.)
- Made to a total volume of 2 L with sterile distilled water (Adcock Ingram)

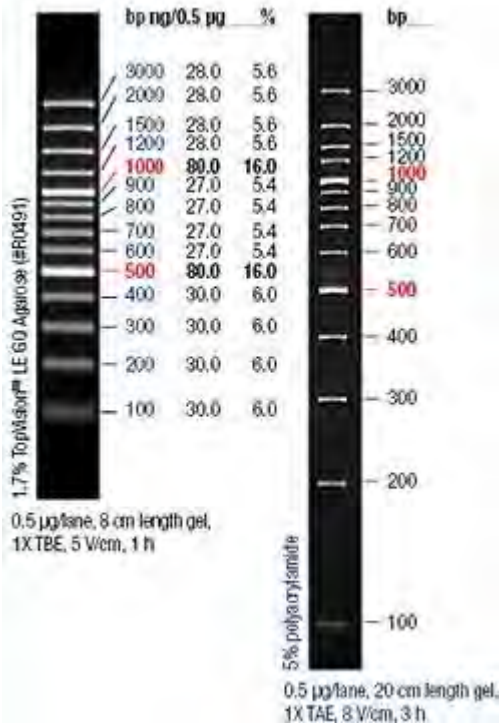
1X TBE (working):
- Made by a 1:10 dilution of stock 10X TBE buffer, with sterile distilled water

Agarose gels
1% agarose gel:
- 0.5 g SeaKem® LE Agarose (Lonza, Basel, Switzerland)
- 50 ml 1X TBE
- 1X SYBR® Safe DNA gel stain (Life Technologies™)

2.5% agarose gel:
- 2.5 g SeaKem® LE Agarose (Lonza)
- 100 ml 1X TBE
- 1X SYBR® Safe DNA gel stain (Life Technologies™)

Molecular weight marker

GeneRuler™ 100 bp Plus DNA ladder:



(Adapted from https://static.fishersci.ca/images/F100438~wl.jpg; accessed 10.09.2015)

Appendix I: Tests of association and regression between genotypic and allelic information and ototoxicity in the total patient cohort

Table I1: Association of variants with ASHA-grade ototoxicity in all patients

Variant	No ototoxicity	Ototoxicity	p-value	Odds ratio (95% CI)	Regression[c]
ABCB5 rs10950831			0.804[a]		
T/T	21 (30.4%)	34 (26.8%)	0.586[a]	1.197 (0.627; 2.283)	
T/G	31 (44.9%)	63 (49.6%)	0.531[a]	0.829 (0.460; 1.492)	-
G/G	17 (24.6%)	30 (23.6%)	0.874[a]	1.057 (0.534; 2.094)	
T-allele	73 (52.9%)	131 (51.6%)	0.802[a]	1.054 (0.696; 1.597)	0.185
G-allele	65 (47.1%)	123 (48.4%)			
ABCC3 rs1051640			0.769[b]		
A/A	48 (69.6%)	89 (70.1%)	0.940[a]	0.976 (0.516; 1.847)	
A/G	18 (26.1%)	35 (27.6%)	0.825[a]	0.928 (0.478; 1.801)	-
G/G	3 (4.3%)	3 (2.4%)	0.427[b]	1.879 (0.369; 9.569)	
A-allele	114 (82.6%)	213 (83.9%)	0.751[a]	0.914 (0.526; 1.589)	0.951
G-allele	24 (17.4%)	41 (16.1%)			
CDH13 rs11646213			0.613[a]		
A/A	35 (50.7%)	56 (44.1%)	0.374[a]	1.305 (0.725; 2.349)	
A/T	25 (36.2%)	55 (43.3%)	0.336[a]	0.744 (0.407; 1.360)	-
T/T	9 (13.0%)	16 (12.6%)	0.929[a]	1.041 (0.434; 2.496)	
A-allele	95 (68.8%)	167 (65.7%)	0.535[a]	1.151 (0.738; 1.794)	0.222
T-allele	43 (31.2%)	87 (34.3%)			
eIF3α rs10787899			0.618[a]		
C/C	23 (33.3%)	51 (40.2%)	0.347[a]	0.745 (0.403; 1.376)	
C/T	31 (44.9%)	53 (41.7%)	0.666[a]	1.139 (0.631; 2.057)	-
T/T	15 (21.7%)	23 (18.1%)	0.539[a]	1.256 (0.606; 2.603)	
C-allele	77 (55.8%)	155 (61.0%)	0.315[a]	0.806 (0.530; 1.227)	0.348
T-allele	61 (44.2%)	99 (39.0%)			
eIF3α rs3824830			0.517[a]		
A/A	35 (50.7%)	66 (52.0%)	0.868[a]	0.951 (0.529; 1.710)	
A/G	29 (42.0%)	46 (36.2%)	0.424[a]	1.277 (0.701; 2.325)	-
G/G	5 (7.2%)	15 (11.8%)	0.313[a]	0.583 (0.203: 1.680)	
A-allele	99 (71.7%)	178 (70.1%)	0.730[a]	1.084 (0.686; 1.713)	0.615
G-allele	39 (28.3%)	76 (29.9%)			
NFE2L2 rs6721961			0.082[b]		
C/C	56 (81.2%)	115 (90.6%)	0.060[a]	0.449 (0.193; 1.049)	
A/C	12 (17.4%)	12 (9.4%)	0.105[a]	2.018 (0.853; 4.772)	-
A/A	1 (1.4%)	0 (0.0%)	0.352[b]	NA	
C-allele	124 (89.9%)	242 (95.3%)	**0.039**[a]	0.439 (0.197; 0.978)	**0.035**
A-allele	14 (10.1%)	12 (4.7%)			
SLC22A2 rs316019			0.392[b]		
G/G	55 (79.7%)	98 (77.2%)	0.681[a]	1.163 (0.567; 2.384)	
G/T	12 (17.4%)	28 (22.0%)	0.440[a]	0.744 (0.351; 1.577)	-
T/T	2 (2.9%)	1 (0.8%)	0.283[b]	3.761 (0.335; 42.242)	
G-allele	122 (88.4%)	224 (88.2%)	0.949[a]	1.021 (0.535; 1.948)	0.902
T-allele	16 (11.6%)	30 (11.8%)			

[a] Chi-square test; [b] Fisher exact test; [c] logistic regression, adjusting associations for patient ethnicity

Table I2: Association of variants with Chang-grade ototoxicity in all patients

Variant	No ototoxicity	Ototoxicity	p-value	Odds ratio (95% CI)	Regression[c]
ABCB5 rs10950831			0.750[a]		
T/T	36 (29.5%)	19 (25.7%)	0.563[a]	1.212 (0.632; 2.323)	
T/G	56 (45.9%)	38 (51.4%)	0.459[a]	0.804 (0.451; 1.433)	-
G/G	30 (24.6%)	17 (23.0%)	0.797[a]	1.093 (0.554; 2.159)	
T-allele	128 (52.5%)	76 (51.4%)	0.831[a]	1.045 (0.695; 1.573)	**0.034**
G-allele	116 (47.5%)	72 (48.6%)			
ABCC3 rs1051640			0.875[b]		
A/A	87 (71.3%)	50 (67.6%)	0.580[a]	1.193 (0.639; 2.229)	
A/G	31 (25.4%)	22 (29.7%)	0.509[a]	0.805 (0.423; 1.533)	-
G/G	4 (3.3%)	2 (2.7%)	1.000[b]	1.220 (0.218; 6.832)	
A-allele	205 (84.0%)	122 (82.4%)	0.683[a]	1.120 (0.650; 1.931)	0.472
G-allele	39 (16.0%)	26 (17.6%)			
CDH13 rs11646213			0.502[a]		
A/A	59 (48.4%)	32 (43.2%)	0.486[a]	1.229 (0.688; 2.198)	
A/T	46 (37.7%)	34 (45.9%)	0.255[a]	0.712 (0.396; 1.279)	-
T/T	17 (13.9%)	8 (10.8%)	0.525[a]	1.336 (0.546; 3.269)	
A-allele	164 (67.2%)	98 (66.2%)	0.839[a]	1.046 (0.678; 1.613)	0.175
T-allele	80 (32.8%)	50 (33.8%)			
eIF3α rs10787899			0.789[a]		
C/C	44 (36.1%)	30 (40.5%)	0.531[a]	0.827 (0.457; 1.497)	
C/T	53 (43.4%)	31 (41.9%)	0.832[a]	1.065 (0.594; 1.911)	-
T/T	25 (20.5%)	13 (17.6%)	0.616[a]	1.209 (0.575; 2.542)	
C-allele	141 (57.8%)	91 (61.5%)	0.470[a]	0.857 (0.565; 1.302)	0.765
T-allele	103 (42.2%)	57 (38.5%)			
eIF3α rs3824830			0.348[a]		
A/A	59 (48.4%)	42 (56.8%)	0.254[a]	0.714 (0.399; 1.276)	
A/G	48 (39.3%)	27 (36.5%)	0.690[a]	1.129 (0.622; 2.050)	-
G/G	15 (12.3%)	5 (6.8%)	0.214[a]	1.935 (0.673; 5.564)	
A-allele	166 (68.0%)	111 (75.0%)	0.142[a]	0.709 (0.448; 1.123)	0.251
G-allele	78 (32.0%)	37 (25.0%)			
NFE2L2 rs6721961			0.614[b]		
C/C	104 (85.2%)	67 (90.5%)	0.281[a]	0.604 (0.239; 1.523)	
A/C	17 (13.9%)	7 (9.5%)	0.354[a]	1.550 (0.610; 3.936)	-
A/A	1 (0.8%)	0 (0.0%)	1.000[b]	NA	
C-allele	225 (92.2%)	141 (95.3%)	0.238[a]	0.588 (0.241; 1.434)	0.144
A-allele	19 (7.8%)	7 (4.7%)			
SLC22A2 rs316019			0.312[b]		
G/G	90 (73.8%)	63 (85.1%)	0.062[a]	0.491 (0.230; 1.047)	
G/T	29 (23.8%)	11 (14.9%)	0.134[a]	1.786 (0.832; 3.835)	-
T/T	3 (2.5%)	0 (0.0%)	0.291[b]	NA	
G-allele	209 (85.7%)	137 (92.6%)	**0.039**[a]	0.479 (0.236; 0.976)	**0.034**
T-allele	35 (14.3%)	11 (7.4%)			

[a] Chi-square test; [b] Fisher exact test; [c] logistic regression, adjusting associations for patient ethnicity

Table I3: Association of variants with CTCAE-grade ototoxicity in all patients

Variant	No ototoxicity	Ototoxicity	p-value	Odds ratio (95% CI)	Regression[c]
ABCB5 rs10950831			0.836[a]		
T/T	25 (28.1%)	30 (28.0%)	0.994[a]	1.003 (0.536; 1.875)	
T/G	41 (46.1%)	53 (49.5%)	0.629[a]	0.870 (0.495; 1.529)	-
G/G	23 (25.8%)	24 (22.4%)	0.577[a]	1.205 (0.625; 2.325)	
T-allele	91 (51.1%)	113 (52.8%)	0.740[a]	0.935 (0.628; 1.392)	0.337
G-allele	87 (48.9%)	101 (47.2%)			
ABCC3 rs1051640			0.960[b]		
A/A	63 (70.8%)	74 (69.2%)	0.805[a]	1.081 (0.585; 1.997)	
A/G	23 (25.8%)	30 (28.0%)	0.731[a]	0.894 (0.474; 1.688)	-
G/G	3 (3.4%)	3 (2.8%)	1.000[b]	1.209 (0.238; 6.145)	
A-allele	149 (83.7%)	178 (83.2%)	0.888[a]	1.039 (0.608; 1.775)	0.681
G-allele	29 (16.3%)	36 (16.8%)			
CDH13 rs11646213			0.737[a]		
A/A	44 (49.4%)	47 (43.9%)	0.441[a]	1.248 (0.710; 2.195)	
A/T	34 (38.2%)	46 (43.0%)	0.497[a]	0.820 (0.462; 1.455)	-
T/T	11 (12.4%)	14 (13.1%)	0.880[a]	0.937 (0.402; 2.181)	
A-allele	122 (68.5%)	140 (65.4%)	0.514[a]	1.152 (0.754; 1.759)	0.181
T-allele	56 (31.5%)	74 (34.6%)			
eIF3α rs10787899			0.890[a]		
C/C	32 (36.0%)	42 (39.3%)	0.635[a]	0.869 (0.486; 1.554)	
C/T	39 (43.8%)	45 (42.1%)	0.804[a]	1.075 (0.609; 1.897)	-
T/T	18 (20.2%)	20 (18.7%)	0.787[a]	1.103 (0.542; 2.243)	
C-allele	103 (57.9%)	129 (60.3%)	0.628[a]	0.905 (0.604; 1.356)	0.612
T-allele	75 (42.1%)	85 (39.7%)			
eIF3α rs3824830			0.488[a]		
A/A	42 (47.2%)	59 (55.1%)	0.268[a]	0.727 (0.414; 1.278)	
A/G	38 (42.7%)	37 (34.6%)	0.244[a]	1.410 (0.790; 2.515)	-
G/G	9 (10.1%)	11 (10.3%)	0.969[a]	0.982 (0.388; 2.487)	
A-allele	122 (68.5%)	155 (72.4%)	0.400[a]	0.829 (0.536; 1.282)	0.375
G-allele	56 (31.5%)	59 (27.6%)			
NFE2L2 rs6721961			0.063[b]		
C/C	73 (82.0%)	98 (91.6%)	**0.046**[a]	0.419 (0.175; 1.001)	
A/C	15 (16.9%)	9 (8.4%)	0.073[a]	2.207 (0.916; 5.320)	-
A/A	1 (1.1%)	0 (0.0%)	0.454[b]	NA	
C-allele	161 (90.4%)	205 (95.8%)	**0.034**[a]	0.416 (0.181; 0.957)	**0.036**
A-allele	17 (9.6%)	9 (4.2%)			
SLC22A2 rs316019			0.205[b]		
G/G	68 (76.4%)	85 (79.4%)	0.609[a]	0.838 (0.426; 1.650)	
G/T	18 (20.2%)	22 (20.6%)	0.954[a]	0.980 (0.487; 1.969)	-
T/T	3 (3.4%)	0 (0.0%)	0.092[b]	NA	
G-allele	154 (86.5%)	192 (89.7%)	0.327[a]	0.735 (0.397; 1.361)	0.383
T-allele	24 (13.5%)	22 (10.3%)			

[a] Chi-square test; [b] Fisher exact test; [c] logistic regression, adjusting associations for patient ethnicity

Table I4: Association of variants with TUNE-grade ototoxicity in all patients

Variant	No ototoxicity	Ototoxicity	p-value	Odds ratio (95% CI)	Regression[c]
ABCB5 rs10950831			0.551[a]		
T/T	14 (27.5%)	41 (28.3%)	0.910[a]	0.960 (0.470; 1.959)	
T/G	22 (43.1%)	72 (49.7%)	0.423[a]	0.769 (0.404; 1.463)	-
G/G	15 (29.4%)	32 (22.1%)	0.291[a]	1.471 (0.717; 3.020)	
T-allele	50 (49.0%)	154 (53.1%)	0.478[a]	0.849 (0.541; 1.334)	0.581
G-allele	52 (51.0%)	136 (46.9%)			
ABCC3 rs1051640			0.366[b]		
A/A	36 (70.6%)	101 (69.7%)	0.901[a]	1.046 (0.520; 2.103)	
A/G	12 (23.5%)	41 (28.3%)	0.512[a]	0.780 (0.372; 1.638)	-
G/G	3 (5.9%)	3 (2.1%)	0.183[b]	2.958 (0.578; 15.151)	
A-allele	84 (82.4%)	243 (83.8%)	0.737[a]	0.903 (0.497; 1.640)	0.882
G-allele	18 (17.6%)	47 (16.2%)			
CDH13 rs11646213			0.731[a]		
A/A	22 (43.1%)	69 (47.6%)	0.584[a]	0.836 (0.439; 1.589)	
A/T	21 (41.2%)	59 (40.7%)	0.951[a]	1.020 (0.533; 1.952)	-
T/T	8 (15.7%)	17 (11.7%)	0.466[a]	1.401 (0.565; 3.475)	
A-allele	65 (63.7%)	197 (67.9%)	0.438[a]	0.829 (0.517; 1.331)	0.879
T-allele	37 (36.3%)	93 (32.1%)			
eIF3α rs10787899			0.237[a]		
C/C	17 (33.3%)	57 (39.3%)	0.449[a]	0.772 (0.395; 1.510)	
C/T	20 (39.2%)	64 (44.1%)	0.541[a]	0.817 (0.426; 1.565)	-
T/T	14 (27.5%)	24 (16.6%)	0.090[a]	1.908 (0.897; 4.059)	
C-allele	54 (52.9%)	178 (61.4%)	0.136[a]	0.708 (0.449; 1.116)	0.242
T-allele	48 (47.1%)	112 (38.6%)			
eIF3α rs3824830			0.232[a]		
A/A	24 (47.1%)	77 (53.1%)	0.458[a]	0.785 (0.414; 1.488)	
A/G	24 (47.1%)	51 (35.2%)	0.133[a]	1.638 (0.858; 3.129)	-
G/G	3 (5.9%)	17 (11.7%)	0.236[a]	0.471 (0.132; 1.678)	
A-allele	72 (70.6%)	205 (70.7%)	0.985[a]	0.995 (0.606; 1.633)	0.712
G-allele	30 (29.4%)	85 (29.3%)			
NFE2L2 rs6721961			0.265[b]		
C/C	43 (84.3%)	128 (88.3%)	0.466[a]	0.714 (0.288; 1.771)	
A/C	7 (13.7%)	17 (11.7%)	0.708[a]	1.198 (0.466; 3.080)	-
A/A	1 (2.0%)	0 (0.0%)	0.260[b]	NA	
C-allele	93 (91.2%)	273 (94.1%)	0.301[a]	0.643 (0.277; 1.493)	0.195
A-allele	9 (8.8%)	17 (5.9%)			
SLC22A2 rs316019			**0.016**[b]		
G/G	36 (70.6%)	117 (80.7%)	0.134[a]	0.574 (0.277; 1.192)	
G/T	12 (23.5%)	28 (19.3%)	0.520[a]	1.286 (0.597; 2.769)	-
T/T	3 (5.9%)	0 (0.0%)	**0.017**[b]	NA	
G-allele	84 (82.4%)	262 (90.3%)	**0.031**[a]	0.499 (0.263; 0.947)	**0.023**
T-allele	18 (17.6%)	28 (9.7%)			

[a] Chi-square test; [b] Fisher exact test; [c] logistic regression, adjusting associations for patient ethnicity

Appendix J: Gene panels used in analysis of WES data

Table J1: Deafness-associated gene panel

Gene symbol	Gene name	Type(s) of deafness
ACTG1	*Actin gamma 1*	Baraitser-Winter syndrome 2 DFNA20
ATP6V1B1	*ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B1*	Renal tubular acidosis with deafness
BSND	*Barttin CLCNK-type chloride channel accessory beta subunit*	Bartter syndrome 4A DFNB
CATSPER2	*Cation channel, sperm associated 2*	Deafness-infertility syndrome
CACNA1D	*Calcium channel, voltage-dependent, L type, alpha 1D subunit*	Sinoatrial node dysfunction and deafness
CCDC50	*Coiled-coil domain containing 50*	DFNA44
CDH23	*Cadherin-related 23*	DFNB12 USH1D, USH1DF
CEACAM16	*Carcinoembryonic antigen-related cell adhesion molecule 16*	DFNA4B
CIB2	*Calcium and integrin binding family member 2*	DFNB48 USH1J
CLDN14	*Claudin 14*	DFNB29
CLPP	*Caseinolytic mitochondrial matrix peptidase proteolytic subunit*	Perrault syndrome 3
CLRN1	*Clarin 1*	USH3A
COCH	*Cochlin*	DFNA9
COL11A2	*Collagen, type XI, alpha 2*	DFNA13, DFNB53 Stickler syndrome 3
DIABLO	*Diablo, IAP-binding mitochondrial protein*	DFNA64
DFNA5	*Deafness, autosomal dominant 5*	DFNA5
DFNB31	*Deafness, autosomal recessive 31*	DFNB31 USH2D
DFNB59	*Deafness, autosomal recessive 59*	DFNB59
DIAPH1	*Diaphanous-related formin 1*	DFNA1
EDN3	*Endothelin 3*	Waardenburg syndrome 4B
EDNRB	*Endothelin receptor type B*	ABCD syndrome Waardenburg syndrome 4A
ESPN	*Espin*	DFNB36
ESRRB	*Oestrogen-related receptor beta*	DFNB35
EYA1	*EYA transcriptional coactivator and phosphatase 1*	Branchiootic syndrome 1 Branchiootorenal syndrome 1 Otofaciocervical syndrome 1
EYA4	*EYA transcriptional coactivator and phosphatase 4*	DFNA10
GIPC3	*GIPC PDZ domain containing family, member 3*	DFNB15
GJB2	*Gap junction protein, beta 2, 26kDa*	Bart-Pumphrey syndrome DFNA3A, DFNB1A Vohwinkel syndrome Keratitis-ichthyosis-deafness syndrome Ichthyosis hystrix-like with deafness syndrome
GJB6	*Gap junction protein, beta 6, 30kDa*	DFNA3B, DFNB1B
GPR98	*Adhesion G protein-coupled receptor V1*	USH2C
GPSM2	*G-protein signalling modulator 2*	Chudley-McCullough syndrome
GRHL2	*Grainyhead-like 2 (Drosophila)*	DFNA28
GRXCR1	*Glutaredoxin, cysteine rich 1*	DFNB25
HARS2	*Histidyl-tRNA synthetase 2, mitochondrial*	Perrault syndrome 2
HSD17B4	*Hydroxysteroid (17-beta) dehydrogenase 4*	Perrault syndrome 1
HGF	*Hepatocyte growth factor (hepapoietin A; scatter factor)*	DFNB39
ILDR1	*Immunoglobulin-like domain containing receptor 1*	DFNB42
KARS	*Lysyl-tRNA synthetase*	DFNB89
KCNE1	*Potassium channel, voltage gated subfamily E regulatory beta subunit 1*	Jervell and Lange-Nielsen syndrome 2
KCNQ1	*Potassium channel, voltage gated KQT-like subfamily Q, member 1*	Jervell and Lange-Nielsen syndrome 1
KCNQ4	*Potassium channel, voltage gated KQT-like subfamily Q, member 4*	DFNA2A

DFNA, deafness, autosomal dominant; *DFNB*, deafness, autosomal recessive; *DFNX*, deafness, X-linked; *USH*, Usher syndrome

Table J1 continued on p153

Table J1 (cont.): Deafness-associated gene panel

Gene symbol	Gene name	Type(s) of deafness
LARS2	*Leucyl-tRNA synthetase 2, mitochondrial*	Perrault syndrome 4
LHFPL5	*Lipoma HMGIC fusion partner-like 5*	DFNB67
LOXHD1	*Lipoxygenase homology domains 1*	DFNB77
LRTOMT	*Leucine rich transmembrane and O-methyltransferase domain containing*	DFNB63
MARVELD2	*MARVEL domain containing 2*	DFNB49
MIR96	*MicroRNA 96*	DFNA50
MITF	*Microphthalmia-associated transcription factor*	Tietz syndrome Waardenburg syndrome 2A Waardenburg syndrome 2, with ocular albinism, autosomal recessive
MSRB3	*Methionine sulfoxide reductase B3*	DFNB74
MTRNR1	*Mitochondrially encoded 12S RNA*	Mitochondrial non-syndromic sensorineural deafness
MT-TS1	*Mitochondrially encoded tRNA serine 1 (UCN)*	Mitochondrial non-syndromic sensorineural deafness
MYH14	*Myosin, heavy chain 14, non-muscle*	DFNA4A
MYH9	*Myosin, heavy chain 9, non-muscle*	Alport syndrome DFNA17 Epstein syndrome Fechtner syndrome
MYO15A	*Myosin XVA*	DFNB3
MYO3A	*Myosin IIIA*	DFNB30
MYO6	*Myosin VI*	DFNA22, DFNB37 Deafness, sensorineural, with hypertrophic cardiomyopathy
MYO7A	*Myosin VIIA*	USH1B DFNA11, DFNB2
OTOA	*Otoancorin*	DFNB22
OTOF	*Otoferlin*	DFNB9
OTOG	*Otogelin*	DFNB18B
OTOGL	*Otogelin-like*	DFNB84B
P2RX2	*Purinergic receptor P2X, ligand gated ion channel, 2*	DFNA41
PAX3	*Paired box 3*	Craniofacial-deafness-hand syndrome Waardenburg syndrome 1 Waardenburg syndrome 3
PCDH15	*Protocadherin-related 15*	DFNB23 USH1F
POU3F4	*POU class 3 homeobox 4*	DFNX2
POU4F3	*POU class 4 homeobox 3*	DFNA15
PRPS1	*Phosphoribosyl pyrophosphate synthetase 1*	ARTS syndrome DFNX1
RDX	*Radixin*	DFNB24
SERPINB6	*Serpin peptidase inhibitor, clade b (ovalbumin), member 6*	DFNB91
SIX1	*SIX homeobox 1*	DFNA23
SLC26A4	*Solute carrier family 26 (anion exchanger), member 4*	DFNB4 Pendred syndrome
SMPX	*Small muscle protein, X-linked*	DFNX4
SOX10	*SRY (Sex Determining Region Y)-box 10*	Waardenburg syndrome 2E Waardenburg syndrome 4C
STRC	*Stereocilin*	Deafness-infertility syndrome DFNB16
SYNE4	*Spectrin repeat containing, nuclear envelope family member 4*	DFNB76
TBC1D24	*TBC1 domain family, member 24*	Deafness, onychodystrophy, osteodystrophy, mental retardation, and seizures syndrome DFNA65, DFNB86
TECTA	*Tectorin alpha*	DFNA12, DFNB21
TIMM8A	*Translocase of inner mitochondrial membrane 8 homologue A (Yeast)*	Jensen syndrome Mohr-Tranebjaerg syndrome
TMC1	*Transmembrane channel-like 1*	DFNA36, DFNB7
TMIE	*Transmembrane inner ear*	DFNB6
TMPRSS3	*Transmembrane protease serine 3*	DFNB8

DFNA, deafness, autosomal dominant; *DFNB*, deafness, autosomal recessive; *DFNX*, deafness, X-linked; *USH*, Usher syndrome

TableJ1 continued on p154

Table J1 (cont.): Deafness-associated gene panel

Gene symbol	Gene name	Type(s) of deafness
TPRN	*Taperin*	DFNB79
TRIOBP	*TRIO and F-actin binding protein*	DFNB28
TSPEAR	*Thrombospondin-type laminin G domain and EAR repeats*	DFNB98
USH1C	*Usher syndrome 1C (autosomal recessive, severe)*	DFNB18A USH1C
USH1G	*Usher syndrome 1G (autosomal recessive)*	USH1G
USH2A	*Usher syndrome 2A (autosomal recessive, mild)*	USH2A
WFS1	*Wolfram syndrome 1 (wolframin)*	DFNA6 Wolfram syndrome 1

DFNA, deafness, autosomal dominant; *DFNB*, deafness, autosomal recessive; *DFNX*, deafness, X-linked; *USH*, Usher syndrome

TableJ2: ADME gene panel

Gene symbol	Gene name	Class
ABCA1	*ATP-binding cassette, sub-family A (ABC1), member 1*	Transporter
ABCA4	*ATP-binding cassette, sub-family A (ABC1), member 4*	Transporter
ABCB11	*ATP-binding cassette, sub-family B (MDR/TAP), member 11*	Transporter
ABCB4	*ATP-binding cassette, sub-family B (MDR/TAP), member 4*	Transporter
ABCB5	*ATP-binding cassette, sub-family B (MDR/TAP), member 5*	Transporter
ABCB6	*ATP-binding cassette, sub-family B (MDR/TAP), member 6*	Transporter
ABCB7	*ATP-binding cassette, sub-family B (MDR/TAP), member 7*	Transporter
ABCB8	*ATP-binding cassette, sub-family B (MDR/TAP), member 8*	Transporter
ABCC1	*ATP-binding cassette, sub-family C (CFTR/MRP), member 1*	Transporter
ABCC10	*ATP-binding cassette, sub-family C (CFTR/MRP), member 10*	Transporter
ABCC11	*ATP-binding cassette, sub-family C (CFTR/MRP), member 11*	Transporter
ABCC12	*ATP-binding cassette, sub-family C (CFTR/MRP), member 12*	Transporter
ABCC13	*ATP-binding cassette, sub-family C (CFTR/MRP), member 13*	Transporter
ABCC3	*ATP-binding cassette, sub-family C (CFTR/MRP), member 3*	Transporter
ABCC4	*ATP-binding cassette, sub-family C (CFTR/MRP), member 4*	Transporter
ABCC5	*ATP-binding cassette, sub-family C (CFTR/MRP), member 5*	Transporter
ABCC6	*ATP-binding cassette, sub-family C (CFTR/MRP), member 6*	Transporter
ABCC8	*ATP-binding cassette, sub-family C (CFTR/MRP), member 8*	Transporter
ABCC9	*ATP-binding cassette, sub-family C (CFTR/MRP), member 9*	Transporter
ABCG1	*ATP-binding cassette, sub-family G (WHITE), member 1*	Transporter
ADH1A	*Alcohol dehydrogenase 1A (class I), alpha polypeptide*	Phase I
ADH1B	*Alcohol dehydrogenase IB (class I), beta polypeptide*	Phase I
ADH1C	*Alcohol dehydrogenase 1C (class I), gamma polypeptide*	Phase I
ADH4	*Alcohol dehydrogenase 4 (class II), pi polypeptide*	Phase I
ADH5	*Alcohol dehydrogenase 5 (class III), chi polypeptide,methionyl aminopeptidase 1*	Phase I
ADH6	*Alcohol dehydrogenase 6 (class V)*	Phase I
ADH7	*Alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide*	Phase I
ADHFE1	*Alcohol dehydrogenase, iron containing, 1*	Phase I
AHR	*Aryl hydrocarbon receptor*	Modifier
ALDH1A1	*Aldehyde dehydrogenase 1 family, member A1*	Phase I
ALDH1A2	*Aldehyde dehydrogenase 1 family, member A2*	Phase I
ALDH1A3	*Aldehyde dehydrogenase 1 family, member A3*	Phase I
ALDH1B1	*Aldehyde dehydrogenase 1 family, member B1*	Phase I
ALDH2	*Aldehyde dehydrogenase 2 family (mitochondrial)*	Phase I
ALDH3A1	*Aldehyde dehydrogenase 3 family, memberA1*	Phase I
ALDH3A2	*Aldehyde dehydrogenase 3 family, member A2*	Phase I
ALDH3B1	*Aldehyde dehydrogenase 3 family, member B1*	Phase I
ALDH3B2	*Aldehyde dehydrogenase 3 family, member B2*	Phase I
ALDH4A1	*Aldehyde dehydrogenase 4 family, member A1*	Phase I
ALDH5A1	*Aldehyde dehydrogenase 5 family, member A1*	Phase I
ALDH6A1	*Aldehyde dehydrogenase 6 family, member A1*	Phase I
ALDH7A1	*Aldehyde dehydrogenase 7 family, member A1*	Phase I
ALDH8A1	*Aldehyde dehydrogenase 8 family, member A1*	Phase I
ALDH9A1	*Aldehyde dehydrogenase 9 family, member A1*	Phase I
AOX1	*Aldehyde oxidase 1*	Phase I
ARNT	*Aryl hydrocarbon receptor nuclear translocator*	Modifier

Table J2 continued on p155

Table J2 (cont.): ADME gene panel

Gene symbol	Gene name	Class
ARSA	*Arylsulfatase A*	Modifier
ATP7A	*ATPase, Cu++ transporting, alpha polypeptide (Menkes syndrome)*	Modifier
ATP7B	*ATPase, Cu++ transporting, beta polypeptide*	Modifier
CAT	*Catalase*	Modifier
CBR1	*Carbonyl reductase 1*	Phase I
CBR3	*Carbonyl reductase 3*	Phase I
CDA	*Cytidine deaminase*	Modifier
CES1	*Carboxylesterase 1 (monocyte/macrophage serine esterase 1)*	Phase I
CES2	*Carboxylesterase 2 (intestine, liver)*	Phase I
CFTR	*Cystic fibrosis transmembrane conductance regulator*	Modifier
CHST1	*Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1*	Phase II
CHST10	*Carbohydrate sulfotransferase 10*	Phase II
CHST11	*Carbohydrate (chondroitin 4) sulfotransferase 11*	Phase II
CHST12	*Carbohydrate (chondroitin 4) sulfotransferase 12*	Phase II
CHST13	*Carbohydrate (chondroitin 4) sulfotransferase 13*	Phase II
CHST2	*Carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2*	Phase II
CHST3	*Carbohydrate (chondroitin 6) sulfotransferase 3*	Phase II
CHST4	*Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 4*	Phase II
CHST5	*Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 5*	Phase II
CHST6	*Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6*	Phase II
CHST7	*Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7*	Phase II
CHST8	*Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 8*	Phase II
CHST9	*Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 9*	Phase II
CYB5R3	*Cytochrome B5 reductase 3*	Phase I
CYP11A1	*Cytochrome P450, family 11, subfamily A, polypeptide 1*	Phase I
CYP11B1	*Cytochrome P450, family 11, subfamily B, polypeptide 1*	Phase I
CYP11B2	*Cytochrome P450, family 11, subfamily B, polypeptide 2*	Phase I
CYP17A1	*Cytochrome P450, family 17, subfamily A, polypeptide 1*	Phase I
CYP19A1	*Cytochrome P450, family 19, subfamily A, polypeptide 1*	Phase I
CYP1B1	*Cytochrome P450, family 1, subfamily B, polypeptide 1*	Phase I
CYP20A1	*Cytochrome P450, family 20, subfamily A, polypeptide 1*	Phase I
CYP20A1	*Cytochrome P450, family 20, subfamily A, polypeptide 1*	Phase I
CYP21A2	*Cytochrome P450, family 21, subfamily A, polypeptide 2*	Phase I
CYP24A1	*Cytochrome P450, family 24, subfamily A, polypeptide 1*	Phase I
CYP26A1	*Cytochrome P450, family 26, subfamily A, polypeptide 1*	Phase I
CYP26C1	*Cytochrome P450, family 26, subfamily C, polypeptide 1*	Phase I
CYP27A1	*Cytochrome P450, family 27, subfamily A, polypeptide 1*	Phase I
CYP27B1	*Cytochrome P450, family 27, subfamily B, polypeptide 1*	Phase I
CYP2A13	*Cytochrome P450, family 2, subfamily A, polypeptide 13*	Phase I
CYP2A7	*Cytochrome P450, family 2, subfamily A, polypeptide 7*	Phase I
CYP2C18	*Cytochrome P450, family 2, subfamily C, polypeptide 18*	Phase I
CYP2D7P1	*Cytochrome P450, family 2, subfamily D, polypeptide 7 pseudogene 1*	Phase I
CYP2F1	*Cytochrome P450, family 2, subfamily F, polypeptide 1*	Phase I
CYP2J2	*Cytochrome P450, family 2, subfamily J, polypeptide 2*	Phase I
CYP2R1	*Cytochrome P450, family 2, subfamily R, polypeptide 1*	Phase I
CYP2S1	*Cytochrome P450, family 2, subfamily S, polypeptide 1*	Phase I
CYP39A1	*Cytochrome P450, family 39, subfamily A, polypeptide 1*	Phase I
CYP3A43	*Cytochrome P450, family 3, subfamily A, polypeptide 43*	Phase I
CYP3A7	*Cytochrome P450, family 3, subfamily A, polypeptide 7*	Phase I
CYP46A1	*Cytochrome P450, family 46, subfamily A, polypeptide 1*	Phase I
CYP4A11	*Cytochrome P450, family 4, subfamily A, polypeptide 11*	Phase I
CYP4B1	*Cytochrome P450, family 4, subfamily B, polypeptide 1*	Phase I
CYP4F11	*Cytochrome P450, family 4, subfamily F, polypeptide 11*	Phase I
CYP4F12	*Cytochrome P450, family 4, subfamily F, polypeptide 12*	Phase I
CYP4F2	*Cytochrome P450, family 4, subfamily F, polypeptide 2*	Phase I
CYP4F3	*Cytochrome P450, family 4, subfamily F, polypeptide 3*	Phase I
CYP4F8	*Cytochrome P450, family 4, subfamily F, polypeptide 8*	Phase I
CYP4Z1	*Cytochrome P450, family 4, subfamily Z, polypeptide 1*	Phase I
CYP51A1	*Cytochrome P450, family 51, subfamily A, polypeptide 1*	Phase I
CYP7A1	*Cytochrome P450, family 7, subfamily A, polypeptide 1*	Phase I

Table J2 continued on p156

Table J2 (cont.): ADME gene panel

Gene symbol	Gene name	Class
CYP7B1	*Cytochrome P450, family 7, subfamily B, polypeptide 1*	Phase I
CYP8B1	*Cytochrome P450, family 8, subfamily B, polypeptide 1*	Phase I
DDO	*D-aspartate oxidase*	Phase I
DHRS1	*Dehydrogenase/reductase (SDR family) member 1*	Phase I
DHRS12	*Dehydrogenase/reductase (SDR family) member 12*	Phase I
DHRS13	*Dehydrogenase/reductase (SDR family) member 13*	Phase I
DHRS2	*Dehydrogenase/reductase (SDR family) member 2*	Phase I
DHRS3	*Dehydrogenase/reductase (SDR family) member 3*	Phase I
DHRS4	*Dehydrogenase/reductase (SDR family) member 4*	Phase I
DHRS4L1	*Dehydrogenase/reductase (SDR family) member 4 like 1*	Phase I
DHRS4L2	*Dehydrogenase/reductase (SDR family) member 4 like 2*	Phase I
DHRS7	*Dehydrogenase/reductase (SDR family) member 7*	Phase I
DHRS7B	*Dehydrogenase/reductase (SDR family) member 7B*	Phase I
DHRS7C	*Dehydrogenase/reductase (SDR family) member 7C*	Phase I
DHRS9	*Dehydrogenase/reductase (SDR family) member 9*	Phase I
DHRSX	*Dehydrogenase/reductase (SDR family) X-linked*	Phase I
DPEP1	*Dipeptidase 1 (renal)*	Phase I
EPHX1	*Epoxide hydrolase 1, microsomal (xenobiotic)*	Phase I
EPHX2	*Epoxide hydrolase 2, cytoplasmic*	Phase I
FMO1	*Flavin containing monooxygenase 1*	Phase I
FMO2	*Flavin containing monooxygenase 2*	Phase I
FMO3	*Flavin containing monooxygenase 3*	Phase I
FMO4	*Flavin containing monooxygenase 4*	Phase I
FMO5	*Flavin containing monooxygenase 5*	Phase I
FMO6P	*Flavin containing monooxygenase 6*	Phase I
GPX1	*Glutathione peroxidase 1*	Phase I
GPX2	*Glutathione peroxidase 2 (gastrointestinal)*	Phase I
GPX3	*Glutathione peroxidase 3 (plasma)*	Phase I
GPX4	*Glutathione peroxidase 4 (phospholipid hydroperoxidase)*	Phase I
GPX5	*Glutathione peroxidase 5 (epididymal androgen-related protein)*	Phase I
GPX6	*Glutathione peroxidase 6 (olfactory)*	Phase I
GPX7	*Glutathione peroxidase 7*	Phase I
GSR	*Glutathione reductase*	Phase I
GSS	*Glutathione synthetase*	Phase I
GSTA1	*Glutathione S-transferase A1*	Phase II
GSTA2	*Glutathione S-transferase A2*	Phase II
GSTA3	*Glutathione S-transferase A3*	Phase II
GSTA4	*Glutathione S-transferase A4*	Phase II
GSTA5	*Glutathione S-transferase A5*	Phase II
GSTCD	*Glutathione S-transferase, C-terminal domain containing*	Phase II
GSTK1	*Glutathione S-transferase kappa 1*	Phase II
GSTM2	*Glutathione S-transferase M2 (muscle), Glutathione S-transferase M4*	Phase II
GSTM3	*Glutathione S-transferase M3 (brain)*	Phase II
GSTM4	*Glutathione S-transferase M4*	Phase II
GSTM5	*Glutathione S-transferase M5*	Phase II
GSTO1	*Glutathione S-transferase omega 1, Glutathione S-transferase omega 2*	Phase II
GSTO2	*Glutathione S-transferase omega 2*	Phase II
GSTT2	*Glutathione S-transferase theta 2*	Phase II
GSTZ1	*Glutathione transferase zeta 1 (maleylacetoacetate isomerase)*	Phase II
HAGH	*Hydroxyacylglutathione hydrolase*	Phase I
HNF4A	*Hepatocyte nuclear factor 4, alpha*	Modifier
HNMT	*Histamine N-methyltransferase*	Phase II
HSD11B1	*Hydroxysteroid (17-beta) Dehydrogenase 11*	Phase I
HSD17B11	*Hydroxysteroid (17-beta) Dehydrogenase 11*	Phase I
HSD17B14	*Hydroxysteroid (17-beta) Dehydrogenase 14*	Phase I
IAPP	*Islet amyloid polypeptide*	Modifier
KCNJ11	*Potassium inwardly-rectifying channel, subfamily J, member 11*	Modifier
LOC728667	*Similar to Dehydrogenase/reductase (SDR family) member 2 isoform 1*	Phase I
LOC731356	*Similar to Dehydrogenase/reductase (SDR family) member 4 like 2*	Phase I
LOC731931	*Similar to Dehydrogenase/reductase (SDR family) member 2 isoform 1*	Phase I

Table J2 continued on p157

Table J2 (cont.): ADME gene panel

Gene symbol	Gene name	Class
MAT1A	*Methionine adenosyltransferase I, alpha*	Modifier
METAP1	*Methionyl aminopeptidase 1*	Phase I
MGST1	*Microsomal glutathione S-transferase 1*	Phase II
MGST2	*Microsomal glutathione S-transferase 2*	Phase II
MGST3	*Microsomal glutathione S-transferase 3*	Phase II
MPO	*Myeloperoxidase*	Modifier
NNMT	*Nicotinamide N-methyltransferase*	Phase II
NOS1	*Nitric oxide synthase 1 (neuronal)*	Phase I
NOS2A	*Nitric oxide synthase 2A (inducible, hepatocytes)*	Phase I
NOS3	*Nitric oxide synthase 3 (endothelial cell)*	Phase I
NR1I2	*Nuclear receptor subfamily 1, group I, member 2*	Modifier
NR1I3	*Nuclear receptor subfamily 1, group I, member 3*	Modifier
PDE3A	*Phosphodiesterase 3A, cGMP-inhibited*	Phase I
PDE3B	*Phosphodiesterase 3B, cGMP-inhibited*	Phase I
PLGLB1	*Plasminogen-like B1*	Phase I
PNMT	*Phenylethanolamine N-methyltransferase*	Phase II
PON1	*Paraoxonase 1*	Phase I
PON2	*Paraoxonase 2*	Phase I
PON3	*Paraoxonase 3*	Phase I
POR	*P450 (cytochrome) oxidoreductase*	Modifier
PPARA	*Peroxisome proliferator-activated receptor alpha*	Modifier
PPARD	*Peroxisome proliferative activated receptor, delta*	Modifier
PPARG	*Peroxisome proliferative activated receptor, gamma*	Modifier
RXRA	*Retinoid X receptor, alpha*	Modifier
SERPINA7	*Serpin peptidase inhibitor, clade A, member 7*	Modifier
SLC10A1	*Solute carrier family 10 (sodium/bile acid cotransporter family), member 1*	Transporter
SLC10A2	*Solute carrier family 10 (sodium/bile acid cotransporter family), member 2*	Transporter
SLC13A1	*Solute carrier family 13 (sodium/sulfate symporters), member 1*	Transporter
SLC13A2	*Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2*	Transporter
SLC13A3	*Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3*	Transporter
SLC15A1	*Solute carrier family 15 (oligopeptide transporter), member 1*	Transporter
SLC16A1	*Solute carrier family 16 (monocarboxylic acid Transporter), member 1*	Transporter
SLC19A1	*Solute carrier family 19 (folate transporter), member 1*	Transporter
SLC22A10	*Solute carrier family 22 (organic anion/cation transporter), member 10*	Transporter
SLC22A11	*Solute carrier family 22 (organic anion/cation transporter), member 11*	Transporter
SLC22A12	*Solute carrier family 22 (organic anion/cation transporter), member 12*	Transporter
SLC22A13	*Solute carrier family 22 (organic cation transporter), member 13*	Transporter
SLC22A14	*Solute carrier family 22 (organic cation transporter), member 14*	Transporter
SLC22A15	*Solute carrier family 22 (organic cation transporter), member 15*	Transporter
SLC22A16	*Solute carrier family 22 (organic cation transporter), member 16*	Transporter
SLC22A17	*Solute carrier family 22 (organic cation transporter), member 17*	Transporter
SLC22A18	*Solute carrier family 22 (organic cation transporter), member 18*	Transporter
SLC22A18AS	*Solute carrier family 22 (organic cation transporter), member 18 antisense*	Transporter
SLC22A3	*Solute carrier family 22 (extraneuronal monoamine transporter), member 3*	Transporter
SLC22A4	*Solute carrier family 22 (organic cation transporter), member 4*	Transporter
SLC22A5	*Solute carrier family 22 (organic cation transporter), member 5*	Transporter
SLC22A7	*Solute carrier family 22 (organic anion transporter), member 7*	Transporter
SLC22A8	*Solute carrier family 22 (organic anion transporter), member 8*	Transporter
SLC22A9	*Solute carrier family 22 (organic anion/cation transporter), member 9*	Transporter
SLC27A1	*Solute carrier family 27 (fatty acid transporter), member 1*	Transporter
SLC28A1	*Solute carrier family 28 (sodium-coupled nucleoside transporter), member 1*	Transporter
SLC28A2	*Solute carrier family 28 (sodium-coupled nucleoside transporter), member 2*	Transporter
SLC28A3	*Solute carrier family 28 (sodium-coupled nucleoside transporter), member 3*	Transporter
SLC29A1	*Solute carrier family 29 (nucleoside Transporter), member 1*	Transporter
SLC29A2	*Solute carrier family 29 (nucleoside Transporter), member 2*	Transporter
SLC2A4	*Solute carrier family 2 (facilitated glucose transporter), member 4*	Transporter
SLC2A5	*Solute carrier family 2 (facilitated glucose/fructose transporter), member 5*	Transporter
SLC5A6	*Solute carrier family 5 (sodium-dependent vitamin transporter)*	Transporter
SLC6A6	*Solute carrier family 6 (neurotransmitter transporter, taurine), member 6*	Transporter
SLC7A5	*Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5*	Transporter

Table J2 continued on p158

Table J2 (cont.): ADME gene panel

Gene symbol	Gene name	Class
SLC7A7	*Solute carrier family 7 (cationic amino acid transporter, y+ system), member 7*	Transporter
SLC7A8	*Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8*	Transporter
SLCO1A2	*Solute carrier organic anion transporter family, member 1A2*	Transporter
SLCO1C1	*Solute carrier organic anion transporter family, member 1C1*	Transporter
SLCO2A1	*Solute carrier organic anion transporter family, member 2A1*	Transporter
SLCO2B1	*Solute carrier organic anion transporter family, member 2B1*	Transporter
SLCO3A1	*Solute carrier organic anion transporter family, member 3A1*	Transporter
SLCO4A1	*Solute carrier organic anion transporter family, member 4A1*	Transporter
SLCO4C1	*Solute carrier organic anion transporter family, member 4C1*	Transporter
SLCO5A1	*Solute carrier organic anion transporter family, member 5A1*	Transporter
SLCO6A1	*Solute carrier organic anion transporter family, member 6A1*	Transporter
SOD1	*Superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))*	Modifier
SOD2	*Superoxide dismutase 2, mitochondrial*	Modifier
SOD3	*Superoxide dismutase 3, extracellular precursor*	Modifier
SULF1	*Sulfatase 1*	Phase I
SULT1A2	*Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2*	Phase II
SULT1A3	*Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3*	Phase II
SULT1B1	*Sulfotransferase family, cytosolic, 1B, member 1*	Phase II
SULT1C1	*Sulfotransferase family, cytosolic, 1C, member 1*	Phase II
SULT1C2	*Sulfotransferase family, cytosolic, 1C, member 2*	Phase II
SULT1E1	*Sulfotransferase family 1E, estrogen-preferring, member 1*	Phase II
SULT2A1	*Sulfotransferase family, cytosolic, 2A, DHEA preferring, member 1*	Phase II
SULT2B1	*Sulfotransferase family, cytosolic, 2B, member 1*	Phase II
SULT4A1	*Sulfotransferase family 4A, member 1*	Phase II
TAP1	*Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)*	Transporter
TAP2	*Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)*	Transporter
UGT1A10	*UDP glucuronosyltransferase 1 family, polypeptide A10*	Phase II
UGT1A3	*UDP glucuronosyltransferase 1 family, polypeptide A3*	Phase II
UGT1A4	*UDP glucuronosyltransferase 1 family, polypeptide A4*	Phase II
UGT1A5	*UDP glucuronosyltransferase 1 family, polypeptide A5*	Phase II
UGT1A6	*UDP glucuronosyltransferase 1 family, polypeptide A6*	Phase II
UGT1A7	*UDP glucuronosyltransferase 1 family, polypeptide A7*	Phase II
UGT1A8	*UDP glucuronosyltransferase 1 family, polypeptide A8*	Phase II
UGT1A9	*UDP glucuronosyltransferase 1 family, polypeptide A9*	Phase II
UGT2A1	*UDP glucuronosyltransferase 2 family, polypeptide A1*	Phase II
UGT2B10	*UDP glucuronosyltransferase 2 family, polypeptide B10*	Phase II
UGT2B11	*UDP glucuronosyltransferase 2 family, polypeptide B11*	Phase II
UGT2B28	*UDP glucuronosyltransferase 2 family, polypeptide B28*	Phase II
UGT2B4	*UDP glucuronosyltransferase 2 family, polypeptide B4*	Phase II
UGT8	*UDP glycosyltransferase 8 (UDP-galactose ceramide galactosyltransferase)*	Phase II
XDH	*Xanthine Dehydrogenase*	Phase I

Table J3: DNA repair gene panel

Gene symbol	Gene name	Function
ALKBH2	*AlkB, alkylation repair homologue 2 (E. Coli)*	Reversal of damage
ALKBH3	*AlkB, alkylation repair homologue 3 (E. Coli)*	Reversal of damage
APEX1	*APEX nuclease 1*	BER
APEX2	*APEX nuclease 2*	BER
APLF	*Aprataxin and PNKP like factor*	BER
APTX	*Aprataxin*	Processing of SS-DNA interruptions
ATM	*ATM serine/threonine kinase*	Sensitivity to DNA damaging agents
ATR	*ATR serine/threonine kinase*	ATM- and PI-3K-like essential kinase
ATRIP	*ATR interacting protein*	ATR-interacting protein
BLM	*Bloom syndrome, RecQ helicase-like*	Sensitivity to DNA damaging agents
BRCA1	*Breast cancer 1, early onset*	Homologous recombination
BRCA2	*Breast cancer 2, early onset*	Repair of DNA crosslinks and adducts
BRIP1	*BRCA1 interacting protein C-terminal helicase 1*	Repair of DNA crosslinks and adducts

BER, base excision repair; *MMR*, mismatch repair; *NER*, nucleotide excision repair; *SS*, sinlge-stranded

Table J3 continued on p159

Table J3 (cont.): DNA repair gene panel

Gene symbol	Gene name	Function
C19orf40	*Chromosome 19 open reading frame 40*	Repair of DNA crosslinks and adducts
C1orf86	*Chromosome 1 open reading frame 86*	Repair of DNA crosslinks and adducts
CCNH	*Cyclin H*	NER
CDK7	*Cyclin-dependent kinase 7*	NER
CETN2	*Centrin, EF-hand protein, 2*	NER
CHAF1A	*Chromatin assembly factor 1, subunit A (P150)*	Chromatin assembly
CHEK1	*Checkpoint kinase 1*	Effector kinase
CHEK2	*Checkpoint kinase 2*	Effector kinase
CLK2	*CDC-like kinase 2*	S-phase check point
DCLRE1A	*DNA cross-link repair 1A*	DNA crosslink repair
DCLRE1B	*DNA cross-link repair 1B*	Related to *SNM1*
DCLRE1C	*DNA cross-link repair 1C*	Non-homologous end-joining
DDB1	*Damage-specific DNA binding protein 1, 127kDa*	NER
DDB2	*Damage-specific DNA binding protein 2, 48kDa*	NER
DMC1	*DNA meiotic recombinase 1*	Homologous recombination
DUT	*Deoxyuridine triphosphatase*	Modulation of nucleotide pools
EME1	*Essential meiotic structure-specific endonuclease 1*	Homologous recombination
EME2	*Essential meiotic structure-specific endonuclease 2*	Homologous recombination
ENDOV	*Endonuclease V*	Nuclease
ERCC1	*Excision repair cross-complementation group 1*	NER
ERCC2	*Excision repair cross-complementation group 2*	NER
ERCC3	*Excision repair cross-complementation group 3*	NER
ERCC4	*Excision repair cross-complementation group 4*	NER
ERCC5	*Excision repair cross-complementation group 5*	NER
ERCC6	*Excision repair cross-complementation group 6*	NER
ERCC8	*Excision repair cross-complementation group 8*	NER
EXO1	*Exonuclease 1*	5` exonuclease
FAN1	*FANCD2/FANCI-associated nuclease 1*	5` nuclease
FANCA	*Fanconi anaemia, complementation group A*	Repair of DNA crosslinks and adducts
FANCB	*Fanconi anaemia, complementation group B*	Repair of DNA crosslinks and adducts
FANCC	*Fanconi anaemia, complementation group C*	Repair of DNA crosslinks and adducts
FANCD2	*Fanconi anaemia, complementation group D2*	Repair of DNA crosslinks and adducts
FANCE	*Fanconi anaemia, complementation group E*	Repair of DNA crosslinks and adducts
FANCF	*Fanconi anaemia, complementation group F*	Repair of DNA crosslinks and adducts
FANCG	*Fanconi anaemia, complementation group G*	Repair of DNA crosslinks and adducts
FANCI	*Fanconi anaemia, complementation group I*	Repair of DNA crosslinks and adducts
FANCL	*Fanconi anaemia, complementation group L*	Repair of DNA crosslinks and adducts
FANCM	*Fanconi anaemia, complementation group M*	Repair of DNA crosslinks and adducts
FEN1	*Flap structure-specific endonuclease 1*	5` nuclease
GEN1	*GEN1 Holliday junction 5` flap endonuclease*	Homologous recombination
GTF2H1	*General transcription factor IIH, polypeptide 1, 62kDa*	NER
GTF2H2	*General transcription factor IIH, polypeptide 2, 44kDa*	NER
GTF2H3	*General transcription factor IIH, polypeptide 3, 34kDa*	NER
GTF2H4	*General transcription factor IIH, polypeptide 4, 52kDa*	NER
GTF2H5	*General transcription factor IIH, polypeptide 5*	NER
H2AFX	*H2A histone family, member X*	Phosphorylated after DNA damage
HELQ	*Helicase, POLQ-like*	DNA helicase
HLTF	*Helicase-like transcription factor*	E3 ubiquitin ligase
HUS1	*HUS1 checkpoint homologue (S. Pombe)*	Sensor of damaged DNA
LIG1	*Ligase I, DNA, ATP-dependent*	NER
LIG3	*Ligase III, DNA, ATP-dependent*	BER
LIG4	*Ligase IV, DNA, ATP-dependent*	Non-homologous end-joining
MAD2L2	*MAD2 mitotic arrest deficient-like 2 (Yeast)*	DNA polymerase catalytic subunit
MBD4	*Methyl-CpG binding domain protein 4*	BER
MDC1	*Mediator Of DNA-damage checkpoint 1*	Mediator of DNA damage checkpoint
MGMT	*O-6-methylguanine-DNA methyltransferase*	Reversal of damage
MLH1	*MutL homologue 1*	MMR
MLH3	*MutL homologue 3*	MMR

BER, base excision repair; *MMR*, mismatch repair; *NER*, nucleotide excision repair; *SS*, sinlge-stranded

Table J3 continued on p160

Table J3 (cont.): DNA repair gene panel

Gene symbol	Gene name	Function
MMS19	*MMS19 nucleotide excision repair homologue (S. Cerevisiae)*	NER
MNAT1	*MNAT CDK-activating kinase assembly factor 1*	NER
MPG	*N-methylpurine-DNA glycosylase*	BER
MPLKIP	*M-phase specific PLK1 interacting protein*	Sensitivity to DNA damaging agents
MRE11A	*MRE11 meiotic recombination 11 homologue A (S. Cerevisiae)*	Homologous recombination
MSH2	*MutS homologue 2*	MMR
MSH3	*MutS homologue 3*	MMR
MSH4	*MutS homologue 4*	MMR
MSH5	*MutS homologue 5*	MMR
MSH6	*MutS homologue 6*	MMR
MUS81	*MUS81 structure-specific endonuclease subunit*	Homologous recombination
MUTYH	*MutY homologue*	BER
NABP2	*Nucleic acid binding protein 2*	SS-DNA binding protein
NBN	*Nibrin*	Homologous recombination
NEIL1	*Nei endonuclease VIII-like 1 (E. Coli)*	BER
NEIL2	*Nei endonuclease VIII-like 2 (E. Coli)*	BER
NEIL3	*Nei endonuclease VIII-like 3 (E. Coli)*	BER
NHEJ1	*Nonhomologous end-joining factor 1*	Non-homologous end-joining
NTHL1	*Nth endonuclease III-like 1 (E. Coli)*	BER
NUDT1	*Nudix (nucleoside diphosphate linked moiety X)-type motif 1*	Modulation of nucleotide pools
OGG1	*8-Oxoguanine DNA glycosylase*	BER
PALB2	*Partner and localiser Of BRCA2*	Repair of DNA crosslinks and adducts
PARP1	*Poly (ADP-ribose) polymerase 1*	Protects strand interruptions
PARP2	*Poly (ADP-ribose) polymerase 2*	Protects strand interruptions
PARP3	*Poly (ADP-ribose) polymerase family, member 3*	Protects strand interruptions
PCNA	*Proliferating cell nuclear antigen*	DNA polymerase catalytic subunit
PER1	*Period circadian clock 1*	S-phase check point
PMS1	*PMS1 postmeiotic segregation increased 1 (S. Cerevisiae)*	MMR
PMS2	*PMS2 postmeiotic segregation increased 2 (S. Cerevisiae)*	MMR
PMS2P3	*Postmeiotic segregation increased 2 pseudogene 3*	MMR
PNKP	*Polynucleotide kinase 3'-phosphatase*	BER
POLB	*Polymerase (DNA directed), beta*	DNA polymerase catalytic subunit
POLD1	*Polymerase (DNA directed), delta 1, catalytic subunit*	DNA polymerase catalytic subunit
POLE	*Polymerase (DNA directed), epsilon, catalytic subunit*	DNA polymerase catalytic subunit
POLG	*Polymerase (DNA directed), gamma*	DNA polymerase catalytic subunit
POLH	*Polymerase (DNA directed), eta*	DNA polymerase catalytic subunit
POLI	*Polymerase (DNA directed), iota*	DNA polymerase catalytic subunit
POLK	*Polymerase (DNA directed), kappa*	DNA polymerase catalytic subunit
POLL	*Polymerase (DNA directed), lambda*	DNA polymerase catalytic subunit
POLM	*Polymerase (DNA directed), mu*	DNA polymerase catalytic subunit
POLN	*Polymerase (DNA directed), nu*	DNA polymerase catalytic subunit
POLQ	*Polymerase (DNA directed), theta*	DNA polymerase catalytic subunit
PRKDC	*Protein kinase, DNA-activated, catalytic polypeptide*	Non-homologous end-joining
PRPF19	*Pre-mRNA processing factor 19*	DNA crosslink repair
RAD1	*RAD1 checkpoint DNA exonuclease*	Sensor of damaged DNA
RAD17	*RAD17 homologue (S. Pombe)*	Sensor of damaged DNA
RAD18	*RAD18 E3 ubiquitin protein ligase*	E3 ubiquitin ligase
RAD23A	*RAD23 homologue A (S. Cerevisiae)*	NER
RAD23B	*RAD23 homologue B (S. Cerevisiae)*	NER
RAD50	*RAD50 homologue (S. Cerevisiae)*	Homologous recombination
RAD51	*RAD51 recombinase*	Homologous recombination
RAD51B	*RAD51 paralogue B*	Homologous recombination
RAD51C	*RAD51 paralogue C*	Repair of DNA crosslinks and adducts
RAD51D	*RAD51 paralogue D*	Homologous recombination
RAD52	*RAD52 homologue (S. Cerevisiae)*	Homologous recombination
RAD54B	*RAD54 homologue B (S. Cerevisiae)*	Homologous recombination
RAD54L	*RAD54-like (S. Cerevisiae)*	Homologous recombination
RAD9A	*RAD9 homologue A (S. Pombe)*	Sensor of damaged DNA

BER, base excision repair; *MMR*, mismatch repair; *NER*, nucleotide excision repair; *SS*, sinlge-stranded

Table J3 continued on p161

Table J3 (cont.): DNA repair gene panel

Gene symbol	Gene name	Function
RDM1	*RAD52 motif containing 1*	Similar to *RAD52*
RECQL	*RecQ helicase-like*	DNA helicase
RECQL4	*RecQ protein-like 4*	Sensitivity to DNA damaging agents
RBBP8	*Retinoblastoma binding protein 8*	Homologous recombination
RECQL5	*RecQ protein-like 5*	DNA helicase
REV1	*REV1, polymerase (DNA directed)*	DNA polymerase catalytic subunit
REV3L	*REV3-like, polymerase (DNA directed), zeta, catalytic subunit*	DNA polymerase catalytic subunit
RIF1	*Replication timing regulatory factor 1*	Suppressor of 5`-end-resection
RNF168	*Ring finger protein 168, E3 ubiquitin protein ligase*	E3 ubiquitin ligase
RNF4	*Ring finger protein 4*	E3 ubiquitin ligase
RNF8	*Ring finger protein 8, E3 ubiquitin protein ligase*	E3 ubiquitin ligase
RPA1	*Replication protein A1, 70kDa*	NER
RPA2	*Replication protein A2, 32kDa*	NER
RPA3	*Replication protein A3, 14kDa*	NER
RPA4	*Replication protein A4, 30kDa*	Similar to *RPA2*
RRM2B	*Ribonucleotide reductase M2 B (TP53 inducible)*	Modulation of nucleotide pools
SETMAR	*SET domain and mariner transposase fusion gene*	Histone methylase and nuclease
SHFM1	*Split hand/foot malformation (ectrodactyly) type 1*	Homologous recombination
SHPRH	*SNF2 histone linker PHD RING helicase, E3 ubiquitin protein ligase*	E3 ubiquitin ligase
SLX1A	*SLX1 structure-specific endonuclease subunit homologue A (S. Cerevisiae)*	Homologous recombination
SLX1B	*SLX1 structure-specific endonuclease subunit homologue B (S. Cerevisiae)*	Homologous recombination
SLX4	*SLX4 structure-specific endonuclease subunit*	Repair of DNA crosslinks and adducts
SMUG1	*Single-strand-selective monofunctional uracil-DNA glycosylase 1*	BER
SPO11	*SPO11 meiotic protein covalently bound to DSB*	Endonuclease
SPRTN	*SprT-like N-terminal domain*	Ubiquitination processing
TDG	*Thymine-DNA glycosylase*	BER
TDP1	*Tyrosyl-DNA phosphodiesterase 1*	Repair of topoisomerase crosslinks
TDP2	*Tyrosyl-DNA phosphodiesterase 2*	Repair of topoisomerase crosslinks
TOPBP1	*Topoisomerase (DNA) II binding protein 1*	DNA damage checkpoint control
TP53	*Tumour protein P53*	Cell cycle regulation
TP53BP1	*Tumour protein P53 binding protein 1*	Chromatin-binding checkpoint protein
TREX1	*Three prime repair exonuclease 1*	3` exonuclease
TREX2	*Three prime repair exonuclease 2*	3` exonuclease
UBE2A	*Ubiquitin-conjugating enzyme E2A*	Ubiquitin conjugation
UBE2B	*Ubiquitin-conjugating enzyme E2B*	Ubiquitin conjugation
UBE2N	*Ubiquitin-conjugating enzyme E2N*	Ubiquitin conjugation
UBE2V2	*Ubiquitin-conjugating enzyme E2 variant 2*	Ubiquitin conjugation
UNG	*Uracil-DNA glycosylase*	BER
UVSSA	*UV-stimulated scaffold protein A*	NER
WRN	*Werner syndrome, RecQ helicase-like*	Sensitivity to DNA damaging agents
XAB2	*XPA binding protein 2*	NER
XPA	*Xeroderma pigmentosum, complementation group A*	NER
XPC	*Xeroderma pigmentosum, complementation group C*	NER
XRCC1	*X-ray repair complementing defective repair in Chinese hamster cells 1*	BER
XRCC2	*X-ray repair complementing defective repair in Chinese hamster cells 2*	Homologous recombination
XRCC3	*X-ray repair complementing defective repair in Chinese hamster cells 3*	Homologous recombination
XRCC4	*X-ray repair complementing defective repair in Chinese hamster cells 4*	Non-homologous end-joining
XRCC5	*X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining)*	Non-homologous end-joining
XRCC6	*X-ray repair complementing defective repair in Chinese hamster cells 6*	Non-homologous end-joining

BER, base excision repair; *MMR*, mismatch repair; *NER*, nucleotide excision repair; *SS*, sinlge-stranded

Table J4: Cisplatin response gene panel

Gene symbol	Gene Name	Nature of association
ABCB1	*ATP-binding cassette, sub-family B, member 1*	Transmembrane drug transporter
ABCC3	*ATP-binding cassette, sub-family C, member 3*	Transmembrane drug transporter
ACYP2	*Acylphosphatase 2, muscle type*	Potential modifier of ototoxicity
ATP7A	*ATPase, Cu++ transporting, alpha polypeptide*	Transmembrane drug transporter
ATP7B	*ATPase, Cu++ transporting, beta polypeptide*	Transmembrane drug transporter
BCL2	*B-cell CLL/lymphoma 2*	Induction of apoptosis
CDH13	*Cadherin 13*	Anti-oxidant response
CFTR	*Cystic fibrosis transmembrane conductance regulator*	Anti-oxidant response
COMT	*Catechol-O-methyltransferase*	Potential modifier of ototoxicity
EIF3A	*Eukaryotic translation initiation factor 3, subunit A*	DNA damage response
ERCC1	*Excision repair cross-complementation group 1*	DNA damage response
ERCC2	*Excision repair cross-complementation group 2*	DNA damage response
ERCC4	*Excision repair cross-complementation group 4*	DNA damage response
ERCC5	*Excision repair cross-complementation group 5*	DNA damage response
ERCC6	*Excision repair cross-complementation group 6*	DNA damage response
GDNF	*Glial cell derived neurotrophic factor*	Potential otoprotectant
GSTA1	*Glutathione S-transferase alpha 1*	Drug detoxification or inactivation
GSTM1	*Glutathione S-transferase mu 1*	Drug detoxification or inactivation
GSTM3	*Glutathione S-transferase mu 3*	Drug detoxification or inactivation
GSTP1	*Glutathione S-transferase pi 1*	Drug detoxification or inactivation
GSTT1	*Glutathione S-transferase theta 1*	Drug detoxification or inactivation
HMOX1	*Haem oxygenase 1*	Anti-oxidant response
LRP2	*Low density lipoprotein receptor-related protein 2*	Transmembrane drug transporter
NFE2L2	*Nuclear factor, erythroid 2-like 2*	Anti-oxidant response
NOX1	*NADPH oxidase 1*	Mediator of oxidative stress
NOX3	*NADPH oxidase 3*	Mediator of oxidative stress
NOX4	*NADPH oxidase 4*	Mediator of oxidative stress
NQO1	*NAD(P)H dehydrogenase, quinone 1*	Anti-oxidant response
NTF3	*Neurotrophin 3*	Potential otoprotectant
OTOS	*Otospiralin*	Potential modifier of ototoxicity
REV1	*REV1, polymerase*	DNA damage response
REV3L	*REV3-like, polymerase, zeta, catalytic subunit*	DNA damage response
SLC31A1	*Solute carrier family 31, member 1*	Transmembrane drug transporter
SLC31A2	*Solute carrier family 31, member 2*	Transmembrane drug transporter
SLC47A1	*Solute carrier family 47, member 1*	Transmembrane drug transporter
TPMT	*Thiopurine S-methyltransferase*	Potential modifier of ototoxicity
TRPA1	*Transient receptor potential cation channel, subfamily A, member 1*	Transmembrane drug transporter
TRPV1	*Transient receptor potential cation channel, subfamily V, member 1*	Transmembrane drug transporter
XIAP	*X-linked inhibitor of apoptosis*	Induction of apoptosis
XPC	*Xeroderma pigmentosum, complementation group C*	DNA damage response
XRCC1	*X-ray repair complementing defective repair in Chinese hamster cells 1*	DNA damage response

Appendix K: Gene-disease relationships for WES data

Table K1: All diseases associated with the genes identified in the variant-level analysis

Gene	OMIM disorder(s)	MalaCards disease(s)
APC2	-	Familial adenomatous polyposis
AQP7	Glycerol QTL	Meniere's disease Endolymphatic hydrops Morbid obesity Constipation Obesity Insulin resistance
BCKDHA	Maple syrup urine disease	Maple syrup urine disease Pseudogout Bacteriuria Urethral syndrome
BORA	-	-
C3	C3 deficiency Haemolytic uremic syndrome, atypical, susceptibility to, 5 Macular degeneration, age-related, 9	Haemolytic uraemic syndrome, atypical 5 C3 deficiency Age-related macular degeneration 9 C3 deficiency, autosomal recessive C3-related atypical haemolytic-uremic syndrome Systemic lupus erythematosus Multiple sclerosis Age related macular degeneration Complement deficiency Lupus erythematosus Hyperapobetalipoproteinaemia Arteriolosclerosis Pneumococcal meningitis Capillary leak syndrome Paediatric systemic lupus erythematosus Dense deposit disease Tuberculosis Retinal drusen Atypical haemolytic-uraemic syndrome Afibrinogenaemia
CHAF1A	-	Cornelia de Lange syndrome
COL9A1	Epiphyseal dysplasia, multiple, 6 Stickler syndrome, type IV	Interstitial keratitis Stickler syndrome, type IV COL9A1-related multiple epiphyseal dysplasia Epiphyseal dysplasia, multiple, 6 COL9A1-related Stickler syndrome Osteoarthritis Lipoid proteinosis Talipes equinovarus Pseudoachondroplasia Stickler syndrome type 1 Stickler syndrome Multiple epiphyseal dysplasia Spinal stenosis Osteochondritis dissecans Multiple epiphyseal dysplasia, dominant Pierre Robin sequence
CREB3L2	-	Myxofibrosarcoma
CTBP2	-	-
CUBN	Megaloblastic anaemia-1, Finnish type Megaloblastic anaemia-1, Norwegian type	Megaloblastic anaemia Megaloblastic anaemia-1, Finnish type Dent's disease Nephropathic cystinosis Megaloblastic anaemia-1, Norwegian type 3 methylglutaconic aciduria type I
CYFIP1	-	Angelman syndrome Fragile X syndrome
DGKQ	-	Dominant optic atrophy
DMGDH	Dimethylglycine dehydrogenase deficiency	Dimethylglycine dehydrogenase deficiency Sarcosinemia
DNAH14	-	-

Table K1 continued on p164

Table K1 (cont.): All diseases associated with the genes identified in the variant-level analysis

Gene	OMIM disorder(s)	MalaCards disease(s)
DYSF	Muscular dystrophy, limb-girdle, type 2B Myopathy, distal, with anterior tibial onset Miyoshi muscular dystrophy 1	Miyoshi muscular dystrophy 1 Miyoshi myopathy Limb-girdle muscular dystrophy Dysferlinopathy Limb-girdle muscular dystrophy, type 2b Myopathy, distal, with anterior tibial onset Congenital myopathy, paradas type Distal muscular dystrophy Muscular dystrophy Rippling muscle disease Sarcoglycanopathies Dystrophinopathies Myopathy
DYTN	-	-
DYX1C1	Dyslexia, susceptibility to, 1 Ciliary dyskinesia, primary, 25	Ciliary dyskinesia, primary, 25 Dyslexia 1 Dyslexia Articulation disorder Ciliary dyskinesia, primary, 11
EIF5B	-	-
EP300	Colorectal cancer, somatic Rubinstein-Taybi syndrome 2	Rubinstein-Taybi syndrome 2 EP300-related Rubinstein-Taybi syndrome Rubinstein-Taybi syndrome Hypoxia Colorectal cancer Acute monocytic leukaemia Monocytic leukaemia Colorectal cancer, somatic Breast cancer
FCRL6	-	Viral pneumonia Chronic lymphocytic leukaemia
FOXH1	-	FOXH1-related holoprosencephaly Ventricular septal defect Septopreoptic holoprosencephaly Midline interhemispheric variant of holoprosencephaly Alobar holoprosencephaly Single median maxillary central incisor Semilobar holoprosencephaly Lobar holoprosencephaly
FOXO3	-	Acute leukaemia Rhabdomyosarcoma Neuroblastoma
FREM2	Fraser syndrome	Unilateral renal agenesis FREM2-related Fraser syndrome Fraser syndrome Cryptophthalamos Macrostomia
GP6	Bleeding disorder, platelet-type, 11	Cervix uteri carcinoma in situ Glycoprotein 1a deficiency Bleeding disorder, platelet-type, 11 Gray platelet syndrome Coronary thrombosis
ING1	Squamous cell carcinoma, head and neck, somatic	Squamous cell carcinoma of the head and neck Squamous cell carcinoma, head and neck, somatic
KIAA1429	-	Oxyphilic adenoma
KRT4	White sponge nevus 1	Hereditary mucosal leukokeratosis Leukoplakia White sponge nevus 1 White sponge nevus of cannon, krt4-related Hymenolepiasis Oral leukoplakia Basaloid squamous cell carcinoma Epidermodysplasia verruciformis Pterygium Oral lichen planus
LRRC10B	-	-

Table K1 continued on p165

Table K1 (cont.): All diseases associated with the genes identified in the variant-level analysis

Gene	OMIM disorder(s)	MalaCards disease(s)
MUC5B	Pulmonary fibrosis, idiopathic, susceptibility to	Idiopathic pulmonary fibrosis Chronic obstructive pulmonary disease Diffuse panbronchiolitis Pulmonary fibrosis Pseudomyxoma peritonei Biliary papillomatosis Pulmonary fibrosis, familial
MXD4	-	-
NID2	-	-
OR4B1	-	-
OR52H1	-	-
PDE4DIP	-	Eosinophilia
PER1	-	Advanced sleep phase syndrome Delayed sleep phase syndrome
RELA	-	Ependymoma Hypersplenism
REXO1	-	-
SDHC	Gastrointestinal stromal tumour Paraganglioma and gastric stromal sarcoma Paragangliomas 3	Paragangliomas 3 SDHC-related paraganglioma and gastric stromal sarcoma Gastrointestinal stromal tumour Carotid body tumour Chondroma Carney triad Pheochromocytoma Hereditary paraganglioma-pheochromocytoma syndromes Paraganglioma Extra-adrenal pheochromocytoma Paragangliomas 4 Paraganglioma and gastric stromal sarcoma Gastrointestinal stromal tumour, somatic Gastrointestinal stromal tumours, familial Familial medullary thyroid carcinoma Cowden syndrome 1 Neural crest tumour Familial renal cell carcinoma Multiple endocrine neoplasia Von Hippel-Lindau disease
SF3A2	-	-
SLC6A18	-	Iminoglycinuria Hyperglycinuria Iminoglycinuria, digenic
SPTA1	Elliptocytosis-2 Pyropoikilocytosis Spherocytosis, type 3	Elliptocytosis 2 Pyropoikilocytosis Common hereditary elliptocytosis SPTA1-related spherocytosis Spherocytosis, type 3 Pyropoikilocytosis hereditary Hereditary elliptocytosis Homozygous hereditary elliptocytosis Hereditary spherocytosis Hypophosphatasia
TJP1	-	Malaria
ZFYVE19	-	-
ZNF80	-	-

Table K2: All diseases associated with the new genes identified in the gene-level analysis

Gene	OMIM disorders	MalaCards diseases
AATK	-	Neuroblastoma
ASPM	Microcephaly 5, primary, autosomal recessive	Microcephaly Primary autosomal recessive microcephaly type 5 Primary autosomal recessive microcephalies and seckel syndrome spectrum disorders Oculocerebrorenal syndrome
C4orf33	-	-
CACNA1F	Aland Island eye disease Cone-rod dystropy, X-linked, 3 Night blindness, congenital stationary (incomplete), 2A, X-linked	Aland island eye disease Eye disease CACNA1F-related X-linked congenital stationary night blindness Night blindness, congenital stationary , 2a, x-linked
CDHR5	-	-
CNGB1	Retinitis pigmentosa 45	Retinitis pigmentosa 45 CNGB1-related retinitis pigmentosa Retinitis pigmentosa Achromatopsia
COL19A1	-	-
ERBB2	Adenocarcinoma of lung, somatic Gastric cancer, somatic Glioblastoma, somatic Ovarian cancer, somatic	Breast scirrhous carcinoma Lipid-rich carcinoma Ductal carcinoma in situ Transitional cell carcinoma
FAM171B	-	-
FBXO32	-	Myopathy of critical illness Acquired Immunodeficiency Syndrome
KRT79	-	-
KRTAP5-2	-	-
OR4Q3	-	-
PCSK1N	-	Amyotrophic lateral sclerosis-parkinsonism/dementia complex 1 Obesity Amyotrophic lateral sclerosis-parkinsonism/dementia complex
SLC22A1	-	Chronic myeloid leukaemia
SLC25A45	-	-
SRPK3	-	-
TBC1D21	-	-
TPST2	-	-
TRPM2	-	-
TUBA3E	-	-
WFS1	?Cataract 41 Deafness, autosomal dominant 6/14/38 Wolfram syndrome Wolfram-like syndrome, autosomal dominant Diabetes mellitus, noninsulin-dependent, association with	Chromosome 4p deletion WFS1-related disorders Cataract 41 Wolfram syndrome

Appendix L: Panel-based analysis of all unique variants scoring ≥ 4

Table L1: Deafness gene panel analysis of all variants with score ≥ 4 in uniquely mutated genes

Gene	Coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patient(s)	Group	MAF	MAF (Afr)
CDH23	10:73567085	G	A	Non-synonymous	6	rs376189742	393	717.97	4	A	-	-
CIB2	15:78401595	A	AT	Frameshift insertion	8	-	17	36.92	4	A	-	-
CLRN1	3:150659515	C	T	Non-synonymous	5	-	80	239.62	6	B	-	-
COCH	14:31355311	T	G	Non-synonymous	7	rs61759484	296	724.06	10	B	-	-
	14:31358897	A	G	Non-synonymous	4	rs17097468	52	211.99	6	B	0.01	0.05
COL11A2	6:33136310	G	T	Non-synonymous	6	rs2229784	76	263.99	10	B	0.06	0.09
DFNB31	9:117168688	C	T	Non-synonymous	4	rs138767834	306	590.02	6	B	< 0.01	< 0.01
ESRRB	14:76957918	A	C	Non-synonymous	5	-	487	631.58	1	A	-	-
GPSM2	1:109465165	ACTTCTT	ACTTC	Frameshift deletion	8	-	33	287.79	3	A	-	-
KCNQ1	11:2608893	C	G	Non-synonymous	5	rs28730756	15	73.80	3	A	0.01	0.02
LOXHD1	18:44109144	C	T	Non-synonymous	4	rs187587197	256	427.69	1	A	< 0.01	< 0.01
MYO3A	10:26434455	G	T	Non-synonymous	6	rs33947968	25	105.31	3	A	0.03	0.01
MYO7A	11:76890902	G	A	Non-synonymous	7	rs371029653	128	507.65	7	B	-	-
PAX3	2:223158963	G	T	Non-synonymous	4	rs376921556	111	326.53	6	B	-	-
TSPEAR	21:46131349	T	TC	Frameshift insertion	8	-	14	23.82	2	A	-	-
WFS1	4:6304133	G	A	Non-synonymous	4	rs71532874	170	360.70	7	B	< 0.01	< 0.01
	4:6302502	T	C	Non-synonymous	5	-	25	46.46	8	B	-	-
	4:6304118	G	A	Non-synonymous	4	rs3821945	80	198.01	9	B	0.008	0.01

[a] consequence types in bold type are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant
Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

Table L2: ADME gene panel analysis of all variants with score ≥ 4 in uniquely mutated genes

Gene	Coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patient(s)	Group	MAF	MAF (Afr)
ABCA1	9:107574883	C	G	Non-synonymous	4	rs147743782	26	39.21	4	A	< 0.01	< 0.01
ABCB11	2:169783728	C	T	Non-synonymous	6	rs1521808	138	329.52	6	B	0.01	0.04
ABCB6	2:220078890	C	T	**Non-synonymous**	8	rs111677240	131	1249.81	6	B	-	-
ABCC10	6:43400685	G	A	Non-synonymous	6	rs140348023	580	936.79	2	A	< 0.01	< 0.01
ABCC12	16:48122456	C	T	Non-synonymous	7	rs77775459	102	241.95	1	A	0.02	0.06
ABCC5	3:183696402	AG	A	Frameshift deletion	8	-	37	166.46	10	B	-	-
ABCC6	16:16259596	G	A	Non-synonymous	6	rs41278174	257.5	625.49	3,4	A	0.01	< 0.01
ABCG1	21:43640145	GAC	G	Frameshift deletion	8	-	78	231.89	8	B	-	-
ADH1B	4:100229017	G	A	Non-synonymous	4	rs2066702	128	234.16	5	A	0.05	0.19
ADH4	4:100062706	G	A	Non-synonymous	4	rs111700010	132	268.48	4	A	< 0.01	< 0.01
ATP7A	X:77268383	G	T	Non-synonymous	6	-	82	40.71	10	B	-	-
CFTR	7:117149147	G	A	Non-synonymous	7	rs1800076	121	225.00	1	A	0.01	< 0.01
	7:117232086	G	A	Non-synonymous	7	rs121908759	102	212.50	2	A	-	-
CHST13	3:126260696	G	C	Non-synonymous	4	-	7	15.78	7	B	-	-
CHST3	10:73767863	GC	G	**Frameshift deletion**	8	-	9	84.69	9	B	-	-
CYP1B1	2:38301588	A	G	Non-synonymous	4	rs112059845	107	240.23	3	A	-	-
CYP11B1	8:143958274	C	G	Non-synonymous	6	-	159	297.07	11	B	-	-
CYP2A7	19:41381646	GA	G	**Frameshift deletion**	8	-	55	522.51	7	B	-	-
CYP2C18	10:96447562	T	A	Stop-gain	8	rs41291550	177	127.97	2	A	0.03	0.08
CYP3A43	7:99434077	TA	T	Frameshift deletion	8	rs61469810	43	91.7	4	A	0.11	0.32
CYP4B1	1:47280746	GAT	G	Frameshift deletion	8	rs3215983	112	189.35	10	B	0.14	0.04
	1:47279898	C	T	Non-synonymous	5	rs45446505	103	153.55	11	B	0.01	0.03
	1:47282755	G	C	Non-synonymous	5	rs59694031	64	101.58	8	B	0.01	0.04
CYP4F11	19:16045140	CT	C	**Frameshift deletion**	8	-	17	159.75	9	B	-	-
DDO	6:110734521	C	T	Non-synonymous	7	rs147873486	268	565.67	1	A	-	-
	6:110714357	C	T	Non-synonymous	4	rs147072212	148	329.05	3	A	< 0.01	0.01
DHRS4	14:24424366	C	CA	**Frameshift insertion**	8	-	299	2565.30	3	A	-	-
DHRS4L2	14:24459477	C	T	Non-synonymous	4	rs61729874	141	252.16	8	B	0.02	0.07
GSTA2	6:52621101	C	T	Non-synonymous	4	rs75013911	52	118.82	11	B	< 0.01	0.01
GSTA3	6:52767205	T	G	Non-synonymous	5	rs1052661	43	171.48	8	B	0.04	0.14
GSTA4	6:52852179	CTTTT	C	Frameshift deletion	8	-	51	78.08	4	A	-	-
HAGH	16:1859304	A	C	Non-synonymous	8	-	45	129.05	7	B	-	-
HSD17B11	4:88258483	T	TC	Frameshift insertion	8	-	14	123.53	10	B	-	-
MPO	17:56356527	T	TA	Frameshift insertion	8	-	27	169.49	2	A	-	-
NOS3	7:150696110	A	AG	**Frameshift insertion**	8	-	117.5	696.35	6,11	B	-	-
NR1I2	3:119526203	G	A	Non-synonymous	4	rs12721607	219	468.13	7,11	B	0.01	< 0.01
PDE3B	11:14666054	CCG	C	Frameshift deletion	8	-	71	24.33	11	B	-	-

[a] consequence types in bold type are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant
Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

Table L2 continued on p169

Table L2 (cont.): ADME gene panel analysis of all variants with score ≥ 4 in uniquely mutated genes

Gene	Coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patient(s)	Group	MAF	MAF (Afr)
PON3	7:94996689	T	C	Non-synonymous	4	rs140234471	49	89.39	2,4	A	-	-
	7:94993334	G	T	Non-synonymous	4	rs17883013	8	15.55	4	A	0.01	0.05
RXRA	9:137320963	A	AC	Frameshift insertion	8	-	14	32.39	7	B	-	-
SERPINA7	X:105280779	C	T	**Non-synonymous**	5	-	143	1366.51	1	A	-	-
SLC15A1	13:99371493	AG	A	**Stop-gain**	8	-	69	602.61	5	A	-	-
SLC22A10	11:63072221	G	GT	Frameshift insertion	8	-	91	25.51	10	B	-	-
	11:63064782	C	T	Stop-gain	8	rs111381363	155	330.70	11	B	< 0.01	< 0.01
	11:63057930	T	A	Non-synonymous	5	-	151	455.05	6	B	-	-
	11:63064875	C	T	Non-synonymous	7	rs200183991	176	285.04	6	B	-	-
	11:63064888	G	A	Non-synonymous	8	rs377529003	178	228.14	6	B	-	-
SLC22A14	3:38347916	G	A	Non-synonymous	6	-	164	363.63	4	A	-	-
SLC22A16	6:110752377	TG	TA	Non-synonymous	8	-	72	377.12	7	B	-	-
SLC2A4	17:7186627	C	CG	Frameshift insertion	8	-	12	16.22	7	B	-	-
SLC7A7	14:23282121	C	A	Non-synonymous	7	-	66	234.57	11	B	-	-
SLC7A8	14:23652038	G	A	Non-synonymous	4	rs149980964	279	304.59	1	A	0.01	< 0.01
SLCO4A1	20:61287937	T	TCG	Frameshift insertion	8	-	54.5	144.94	1,3	A	-	-
	20:61297803	C	T	Non-synonymous	4	rs147153778	371	552.70	3	A	< 0.01	< 0.01
SULT1C2	2:108924881	GA	G	Frameshift deletion	8	rs143858235	189	276.46	6	B	0.01	0.05
TAP2	6:32803106	G	A	Non-synonymous	6	rs138586326	220	420.14	2	A	< 0.01	0.02
UGT2A1/2	4:70462042	C	T	Non-synonymous	5	rs4148301	52	93.55	8	B	0.07	0.06
UGT2B11	4:70066322	G	A	Non-synonymous	4	rs150196832	222	664.94	2	A	< 0.01	0.01

[a] consequence types in bold type are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant

Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

Table L3: DNA repair gene panel analysis of all the unique variants scored at least 4 in the filtering pipeline

Gene	Coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patient(s)	Group	MAF	MAF (Afr)
ATM	11:108143456	C	G	Non-synonymous	4	rs1800057	136	243.76	7	B	0.01	< 0.01
ATR	3:142178118	G	C	Non-synonymous	5	rs33972295	42	72.88	11	B	0.01	0.05
BIVM-ERCC5	13:103527848	A	AC	**Frameshift insertion**	8	-	20	168.40	1	A	-	-
	13:103510736	C	T	Non-synonymous	4	rs56255799	65	125.62	5	A	< 0.01	< 0.01
BLM	15:91346922	C	CA	**Frameshift insertion**	8	-	70	340.49	11	B	-	-
C19orf40	19:33464178	C	T	Non-synonymous	4	-	38	150.99	3	A	-	-
CHAF1A	19:4422644	G	GA	Frameshift insertion	8	-	15	29.45	9,10,11	B	-	-
DCLRE1A	10:115609831	T	A	Stop-gain	8	-	342	37.31	1	A	-	-
FANCC	9:97934359	C	T	Non-synonymous	4	rs1800362	43	72.52	8	B	0.01	0.05
GFT2H1	11:18382239	G	C	Non-synonymous	4	-	27	43.60	1	A	-	-
HELQ	4:84362474	C	T	Non-synonymous	5	rs142206532	51	99.09	5	A	0.01	0.02
MPLKIP	7:40173878	AC	A	**Frameshift deletion**	8	-	6	63.24	2	A	-	-
MSH3	5:79974821	C	T	Non-synonymous	6	rs143211109	37	142.12	4	A	< 0.01	< 0.01
MSH5	6:31725978	C	G	Non-synonymous	6	rs28399976	115	185.48	7	B	0.01	< 0.01
OGG1	3:9796430	A	G	Non-synonymous	4	rs368423806	133	301.54	4	A	-	-
PARP2	14:20815025	CAT	C	Frameshift deletion	8	rs375274966	157	328.20	4	A	-	-
PER1	17:8045707	GCC	GC	Frameshift deletion	8	-	33.67	220.58	8,9,10	B	-	-
	17:8053361	G	A	Non-synonymous	4	rs139241830	99	105.20	9	B	-	-
PMS1	2:190656614	G	C	Non-synonymous	8	rs5742973	96	151.21	3	A	< 0.01	0.01
POLE	12:133245280	A	G	Non-synonymous	4	-	7	15.16	5	A	-	-
POLG	15:89860691	G	A	Non-synonymous	6	rs369544574	337	699.08	1	A	< 0.01	< 0.01
POLI	18:51795957	ACGAC	A	**Frameshift deletion**	8	-	176.5	1553.09	6,11	B	-	-
POLM	7:44113786	TGG	TG(GGT)	Deletion/insertion	8	-	76	506.11	9	B	-	-
POLN	4:2130959	A	G	Non-synonymous	5	-	21	141.43	8	B	-	-
POLQ	3:121263697	A	C	Non-synonymous	4	-	13	29.82	2	A	-	-
RAD23B	9:110084279	A	AC	Frameshift insertion	8	-	263	27.38	1	A	-	-
RAD51B	14:68353913	T	G	Non-synonymous	4	rs33929366	48	56.96	8	B	0.01	0.03
RAD54B	8:95390844	T	TC	Frameshift insertion	8	-	102	24.73	2	A	-	-
RAD54L	1:46739409	C	T	Non-synonymous	6	rs28363240	367	664.35	3	A	< 0.01	0.01
REV1	2:100019495	C	CG	**Frameshift insertion**	8	-	66	399.79	1	A	-	-
SLX4	16:3647623	G	GGC	Frameshift insertion	8	-	9	20.24	7	B	-	-
	16:3656625	G	A	Non-synonymous	4	rs79842542	166	503.64	11	B	0.06	0.07
TOPBP1	3:133356733	G	C	Non-synonymous	4	rs142305736	85	148.46	8	B	< 0.01	< 0.01
TP53	17:7579579	CG	C	Frameshift deletion	8	-	20	73.38	5	A	-	-
TREX1	3:48508608	G	A	Non-synonymous	5	-	28	43.48	5	A	-	-
WRN	8:30924631	G	A	Non-synonymous	7	-	25	48.24	8	B	-	-
XAB2	19:7689247	C	T	Non-synonymous	4	rs61761630	252	663.80	10	B	< 0.01	< 0.01

[a] consequence types in bold type are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant

Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

Table L4: Cisplatin response gene panel analysis of all the unique variants scored at least 4 in the filtering pipeline

Gene	Coordinates	Ref	Var	Consequence[a]	Score	dbSNP ID	DP[b]	Q score[b]	Patient(s)	Group	MAF	MAF (Afr)
ABCC6	16:16259596	G	A	Non-synonymous	6	rs41278174	257.5	625.49	3,4	A	0.01	< 0.01
ATP7A	X:77268383	G	T	Non-synonymous	6	-	82	40.71	10	B	-	-
BCL2	18:60985775			Deletion/substitution	8	-			4	A	-	-
BIVM-ERCC5	13:103527848	A	AC	**Frameshift insertion**	8	-	20	168.40	1	A	-	-
	13:103510736	C	T	Non-synonymous	4	rs56255799	65	125.62	5	A	< 0.01	< 0.01
CFTR	7:117149147	G	A	Non-synonymous	7	rs1800076	121	225.00	1	A	0.01	< 0.01
	7:117232086	G	A	Non-synonymous	7	rs121908759	102	212.50	2	A	-	-
REV1	2:100019495	C	CG	**Frameshift insertion**	8	-	66	399.79	1	A	-	-
TRPA1	8:72984051			Frameshift deletion	8	-			7	B	-	-

[a] consequence types in bold type are homozygous in at least one of the patients; [b] mean read depth and quality scores averaged over all patients with the variant

Afr, 1000 Genomes Project African population; *DP*, read depth; *ID*, identifier; *MAF*, minor allele frequency; *Q score*, quality score; *Ref*, reference allele; *Var*, variant allele

Appendix M: Results of pathway analysis

Table M1: Pathways enriched in Groups A and B

Pathway name	Pathway no.	CPDB set size	No. candidates	Group	p-value
Activation of C3 and C5	1	7	4 (57.1%)	B	0.000794
Amoebiasis - *Homo sapiens* (human)	2	109	16 (14.7%)	A	0.00108
AMPK signalling pathway - *Homo sapiens* (human)	3	124	18 (14.6%)	B	0.00311
Aripiprazole metabolic pathway	4	2	2 (100.0%)	A	0.00385
Assembly of collagen fibrils and other multimeric structures	5	44	8 (18.2%)	A	0.00512
Benzodiazepine pathway, pharmacokinetics	6	11	4 (36.4%)	A	0.00342
Beta1 integrin cell surface interactions	7	66	12 (18.2%)	A	0.00066
Beta-oxidation of pristanoyl-CoA	8	8	4 (50.0%)	A	0.000842
Bile acid biosynthesis	9	53	9 (17.0%)	A	0.00491
cAMP signalling pathway - *Homo sapiens* (human)	10	200	24 (12.0%)	B	0.00965
Codeine metabolism pathway	11	4	3 (75.0%)	A	0.000909
Collagen biosynthesis and modifying enzymes	12	64	11 (17.2%)	A	0.00177
Collagen formation	13	87	16 (18.4%)	A	0.0000767
Complement activation, classical pathway	14	17	5 (29.4%)	B	0.00579
Constitutive androstane receptor pathway	15	32	7 (21.9%)	B	0.00684
Degradation of the extracellular matrix	16	84	14 (16.7%)	A	0.000609
Endochondral ossification	17	64	11 (17.5%)	A	0.00155
Extracellular matrix organisation	18	264	36 (13.7%)	A	0.00000595
Formation of senescence-associated heterochromatin foci (SAHF)	19	17	5 (29.4%)	B	0.00579
FoxO signalling pathway - *Homo sapiens* (human)	20	134	18 (13.5%)	B	0.00716
Glucose metabolism	21	72	12 (16.9%)	B	0.00456
Glucuronidation	22	26	6 (23.1%)	B	0.00921
Glycolysis / gluconeogenesis - *Homo sapiens* (human)	23	67	12 (17.9%)	B	0.00279
Glycolysis and gluconeogenesis	24	67	13 (19.7%)	B	0.000754
GRB7 events in ERBB2 signalling	25	5	3 (60.0%)	B	0.00337
Interaction between L1 and ankyrins	26	29	7 (24.1%)	B	0.00383
Ion channel transport	27	197	28 (14.2%)	B	0.000424
Ion transport by P-type ATPases	28	47	9 (19.1%)	B	0.0058
Lectin induced complement pathway	29	12	5 (41.7%)	B	0.001
LRR FLII-interacting protein 1 (LRRFIP1) activates type I IFN production	30	5	3 (60.0%)	B	0.00337
Mono-unsaturated fatty acid beta-oxidation	31	21	5 (23.8%)	A	0.00804
Olfactory transduction - *Homo sapiens* (human)	32	407	39 (9.6%)	A	0.00426
Omega-6 fatty acid metabolism	33	28	6 (21.4%)	A	0.0065

AMPK, AMP-activated protein kinase; *C3*, complement component 3; *C5*, complement component 5; *cAMP*, cyclic adenosine monophosphate; *CPDB*, ConsensusPathDB; *ERBB2*, Erb-B2 receptor tyrosine kinase; *FLII*, flightless I homologue (Drosophila); *GRB7*, growth factor receptor-bound protein 7; *LRR*, leucine rich repeat; *no.*, number; *PELP1*, proline, glutamate and leucine rich protein 1

Table M1 continued on p173

Table M1 (cont.): Pathways enriched in Groups A and B

Pathway name	Pathway no.	CPDB set size	No. candidates	Group	p-value
Opsins	34	11	4 (36.4%)	A	0.00342
Other glycan degradation - *Homo sapiens* (human)	35	18	5 (27.8%)	A	0.00395
p53 signalling pathway	36	13	4 (30.8%)	A	0.0067
p73 transcription factor network	37	79	11 (14.1%)	A	0.00843
PELP1 modulation of oestrogen receptor activity	38	5	3 (60.0%)	B	0.00337
Pentose phosphate pathway - *Homo sapiens* (human)	39	29	7 (24.1%)	B	0.00383
Peroxisomal lipid metabolism	40	21	7 (33.3%)	A	0.000186
Pregnane X receptor pathway	41	33	7 (21.2%)	B	0.00815
Purine deoxyribonucleosides degradation	42	2	2 (100.0%)	B	0.00522
Purine ribonucleosides degradation to ribose-1-phosphate	43	3	3 (100.0%)	B	0.000376
Pyruvate metabolism - *Homo sapiens* (human)	44	40	9 (22.5%)	B	0.00183
Regulation of androgen receptor activity	45	51	12 (23.5%)	B	0.000214
Regulation of complement cascade	46	24	6 (25.0%)	B	0.00609
Regulation of nuclear SMAD2/3 signalling	47	78	12 (15.6%)	B	0.00882
Retinol metabolism	48	37	8 (21.6%)	A	0.00164
Role of nicotinic acetylcholine receptors in the regulation of apoptosis	49	17	5 (29.4%)	B	0.00579
Saturated fatty acids beta-oxidation	50	25	7 (28.0%)	A	0.000618
Stimuli-sensing channels	51	100	13 (13.0%)	A	0.00873
Transmembrane transport of small molecules	52	579	64 (11.1%)	B	0.000361
Trihydroxycoprostanoyl-CoA beta-oxidation	53	12	4 (33.3%)	A	0.00488
Validated nuclear oestrogen receptor alpha network	54	64	12 (18.8%)	B	0.00186
Vitamin A deficiency	55	37	8 (21.6%)	A	0.00164

AMPK, AMP-activated protein kinase; *C3*, complement component 3; *C5*, complement component 5; *cAMP*, cyclic adenosine monophosphate; *CPDB*, ConsensusPathDB; *ERBB2*, Erb-B2 receptor tyrosine kinase; *FLII*, flightless I homologue (Drosophila); *GRB7*, growth factor receptor-bound protein 7; *LRR*, leucine rich repeat; *no.*, number; *PELP1*, proline, glutamate and leucine rich protein 1

Appendix N: Pathway panels

After identifying the 55 pathways enriched in Group A and Group B, the full gene sets from each pathway were downloaded from CPDB, to construct a pathway panel of genes for application to the total dataset. The gene sets used are provided below.

1. Activation of C3 and C5 (n = 8)

C2	*C4A*	*C4B_2*	*CFB*
C3	*C4B*	*C5*	*LOC101928623*

2. Amoebiasis - *Homo sapiens* (human) (n = 119)

ACTN1	*COL5A1*	*LAMA4*	*PRKCB*
ACTN2	*COL5A2*	*LAMA5*	*PRKCG*
ACTN3	*COL5A3*	*LAMB1*	*PRKX*
ACTN4	*CSF2*	*LAMB2*	*PTK2*
ADCY1	*CTSG*	*LAMB3*	*RAB5A*
ARG1	*CXCL1*	*LAMB4*	*RAB5B*
ARG2	*CXCL8*	*LAMC1*	*RAB5C*
C8A	*FN1*	*LAMC2*	*RAB7A*
C8B	*GNA11*	*LAMC3*	*RAB7B*
C8G	*GNA14*	*MUC2*	*RELA*
C9	*GNA15*	*NFKB1*	*SERPINB1*
CASP3	*GNAL*	*NOS2*	*SERPINB10*
CD14	*GNAQ*	*PIK3CA*	*SERPINB13*
CD1D	*GNAS*	*PIK3CB*	*SERPINB2*
COL11A1	*HSPB1*	*PIK3CD*	*SERPINB3*
COL11A2	*IFNG*	*PIK3CG*	*SERPINB4*
COL1A1	*IL10*	*PIK3R1*	*SERPINB6*
COL1A2	*IL12A*	*PIK3R2*	*SERPINB9*
COL24A1	*IL12B*	*PIK3R3*	*TGFB1*
COL27A1	*IL1B*	*PIK3R5*	*TGFB2*
COL2A1	*IL1R1*	*PLCB1*	*TGFB3*
COL3A1	*IL1R2*	*PLCB2*	*TLR2*
COL4A1	*IL6*	*PLCB3*	*TLR4*
COL4A2	*ITGAM*	*PLCB4*	*TNF*
COL4A3	*ITGB2*	*PRKACA*	*VCL*
COL4A4	*LAMA1*	*PRKACB*	
COL4A5	*LAMA2*	*PRKACG*	
COL4A6	*LAMA3*	*PRKCA*	

3. AMPK signalling pathway - *Homo sapiens* (human) (n = 124)

ACACA	*CPT1B*	*G6PC2*	*PCK1*
ACACB	*CPT1C*	*G6PC3*	*PCK2*
ADIPOQ	*CREB1*	*GYS1*	*PDPK1*
ADIPOR1	*CREB3*	*GYS2*	*PFKFB1*
ADIPOR2	*CREB3L1*	*HMGCR*	*PFKFB2*
ADRA1A	*CREB3L2*	*HNF4A*	*PFKFB3*
AKT1	*CREB3L3*	*IGF1*	*PFKFB4*
AKT1S1	*CREB3L4*	*IGF1R*	*PFKL*
AKT2	*CREB5*	*INS*	*PFKM*
AKT3	*CRTC2*	*INSR*	*PFKP*
CAB39	*EEF2*	*IRS1*	*PIK3CA*
CAB39L	*EEF2K*	*IRS2*	*PIK3CB*
CAMKK1	*EIF4EBP1*	*IRS4*	*PIK3CD*
CAMKK2	*ELAVL1*	*LEP*	*PIK3CG*
CCNA1	*FASN*	*LEPR*	*PIK3R1*
CCNA2	*FBP1*	*LIPE*	*PIK3R2*
CCND1	*FBP2*	*LOC101930123*	*PIK3R3*
CD36	*FOXO1*	*MAP3K7*	*PIK3R5*
CFTR	*FOXO3*	*MLYCD*	*PPARG*
CPT1A	*G6PC*	*MTOR*	*PPARGC1A*

PPP2CA	*PPP2R5A*	*PRKAG3*	*SCD5*
PPP2CB	*PPP2R5B*	*RAB10*	*SIRT1*
PPP2R1A	*PPP2R5C*	*RAB11B*	*SLC2A4*
PPP2R1B	*PPP2R5D*	*RAB14*	*SREBF1*
PPP2R2A	*PPP2R5E*	*RAB2A*	*STK11*
PPP2R2B	*PRKAA1*	*RAB8A*	*STRADA*
PPP2R2C	*PRKAA2*	*RHEB*	*STRADB*
PPP2R2D	*PRKAB1*	*RPS6KB1*	*TBC1D1*
PPP2R3A	*PRKAB2*	*RPS6KB2*	*TSC1*
PPP2R3B	*PRKAG1*	*RPTOR*	*TSC2*
PPP2R3C	*PRKAG2*	*SCD*	*ULK1*

4. Aripiprazole metabolic pathway (n = 2)

CYP2D6	*CYP3A43*

5. Assembly of collagen fibrils and other multimeric structures (n = 44)

COL15A1	*COL15A20*	*COL15A31*	*COL15A42*
COL15A10	*COL15A21*	*COL15A32*	*COL15A43*
COL15A11	*COL15A22*	*COL15A33*	*COL15A44*
COL15A12	*COL15A23*	*COL15A34*	*COL15A5*
COL15A13	*COL15A24*	*COL15A35*	*COL15A6*
COL15A14	*COL15A25*	*COL15A36*	*COL15A7*
COL15A15	*COL15A26*	*COL15A37*	*COL15A8*
COL15A16	*COL15A27*	*COL15A38*	*COL15A9*
COL15A17	*COL15A28*	*COL15A39*	
COL15A18	*COL15A29*	*COL15A4*	
COL15A19	*COL15A3*	*COL15A40*	
COL15A2	*COL15A30*	*COL15A41*	

6. Benzodiazepine pathway, pharmacokinetics (n = 11)

CYP1A2	*CYP3A4*	*UGT1A4*	*UGT2B4*
CYP2C19	*CYP3A5*	*UGT1A9*	*UGT2B7*
CYP2D6	*NAT2*	*UGT2B15*	

7. Beta1 integrin cell surface interactions (n = 69)

CD14	*COL6A3*	*ITGA7*	*LOC102725477*
CD81	*COL7A1*	*ITGA8*	*MDK*
COL11A1	*CSPG4*	*ITGA9*	*NID1*
COL11A2	*F13A1*	*ITGAV*	*NPNT*
COL18A1	*FBN1*	*ITGB1*	*PLAU*
COL1A1	*FGA*	*JAM2*	*PLAUR*
COL1A2	*FGB*	*LAMA1*	*SPP1*
COL2A1	*FGG*	*LAMA2*	*TGFBI*
COL3A1	*FN1*	*LAMA3*	*TGM2*
COL4A1	*IGSF8*	*LAMA4*	*THBS1*
COL4A3	*ITGA1*	*LAMA5*	*THBS2*
COL4A4	*ITGA10*	*LAMB1*	*TNC*
COL4A5	*ITGA11*	*LAMB2*	*VCAM1*
COL4A6	*ITGA2*	*LAMB3*	*VEGFA*
COL5A1	*ITGA3*	*LAMC1*	*VTN*
COL5A2	*ITGA4*	*LAMC2*	
COL6A1	*ITGA5*	*LOC102723826*	
COL6A2	*ITGA6*	*LOC102725336*	

8. Beta-oxidation of pristanoyl-CoA (n = 8)

ACOT8	*AMACR*	*HSD17B4*
ACOX2	*CRAT*	*SCP2*
ACOX3	*CROT*	

9. Bile acid biosynthesis (n = 54)

ABCA1	*ACOT2*	*ACOX1*	*ALDH1B1*
ABCB11	*ACOT4*	*ACOX2*	*ALDH2*
ABCC3	*ACOT6*	*ACOX3*	*ALDH3A1*
ACAA1	*ACOT7*	*ADHFE1*	*ALDH3A2*
ACOT1	*ACOT8*	*AKR1D1*	*ALDH7A1*

ALDH9A1	*HSD3B1*	*SLC36A1*	*SLCO4A1*
BAAT	*HSD3B2*	*SLC38A1*	*SOAT1*
CYP27A1	*LOC101929618*	*SLC38A2*	*SOAT2*
CYP3A4	*SCP2*	*SLC38A4*	*SRD5A1*
CYP7A1	*SLC10A1*	*SLC6A14*	*SRD5A2*
CYP7B1	*SLC10A2*	*SLC6A6*	*SRD5A3*
CYP8B1	*SLC27A2*	*SLCO1A2*	*STARD3*
DBI	*SLC27A5*	*SLCO1B1*	
EHHADH	*SLC32A1*	*SLCO1B3*	

10. cAMP signalling pathway - *Homo sapiens* (human) (n = 200)

ABCC4	*AMH*	*CACNA1S*	*CREB1*
ACOX1	*ARAP3*	*CALM1*	*CREB3*
ACOX3	*ATP1A1*	*CALM2*	*CREB3L1*
ADCY1	*ATP1A2*	*CALM3*	*CREB3L2*
ADCY10	*ATP1A3*	*CALML3*	*CREB3L3*
ADCY2	*ATP1A4*	*CALML5*	*CREB3L4*
ADCY3	*ATP1B1*	*CALML6*	*CREB5*
ADCY4	*ATP1B2*	*CAMK2A*	*CREBBP*
ADCY5	*ATP1B3*	*CAMK2B*	*DRD1*
ADCY6	*ATP1B4*	*CAMK2D*	*DRD2*
ADCY7	*ATP2A2*	*CAMK2G*	*DRD5*
ADCY8	*ATP2B1*	*CAMK4*	*EDNRA*
ADCY9	*ATP2B2*	*CFTR*	*EP300*
ADCYAP1R1	*ATP2B3*	*CHRM1*	*F2R*
ADORA1	*ATP2B4*	*CHRM2*	*FFAR2*
ADORA2A	*BAD*	*CNGA1*	*FOS*
ADRB1	*BDNF*	*CNGA2*	*FSHB*
ADRB2	*BRAF*	*CNGA3*	*FSHR*
AKT1	*CACNA1C*	*CNGA4*	*FXYD1*
AKT2	*CACNA1D*	*CNGB1*	*FXYD2*
AKT3	*CACNA1F*	*CNGB3*	*GABBR1*
GABBR2	*HTR1A*	*PDE3B*	*PTGER3*
GHRL	*HTR1B*	*PDE4A*	*RAC1*
GHSR	*HTR1D*	*PDE4B*	*RAC2*
GIPR	*HTR1E*	*PDE4C*	*RAC3*
GLI1	*HTR1F*	*PDE4D*	*RAF1*
GLI3	*HTR4*	*PIK3CA*	*RAP1A*
GLP1R	*HTR6*	*PIK3CB*	*RAP1B*
GNAI1	*JUN*	*PIK3CD*	*RAPGEF3*
GNAI2	*LIPE*	*PIK3CG*	*RAPGEF4*
GNAI3	*MAP2K1*	*PIK3R1*	*RELA*
GNAS	*MAP2K2*	*PIK3R2*	*RHOA*
GPR119	*MAPK1*	*PIK3R3*	*ROCK1*
GRIA1	*MAPK10*	*PIK3R5*	*ROCK2*
GRIA2	*MAPK3*	*PLCE1*	*RRAS*
GRIA3	*MAPK8*	*PLD1*	*RRAS2*
GRIA4	*MAPK9*	*PLD2*	*RYR2*
GRIN1	*MC2R*	*PLN*	*SLC9A1*
GRIN2A	*MLLT4*	*PPARA*	*SOX9*
GRIN2B	*MYL9*	*PPP1CA*	*SSTR1*
GRIN2C	*NFATC1*	*PPP1CB*	*SSTR2*
GRIN2D	*NFKB1*	*PPP1CC*	*SSTR5*
GRIN3A	*NFKBIA*	*PPP1R12A*	*SUCNR1*
GRIN3B	*NPR1*	*PPP1R1B*	*TIAM1*
HCAR1	*NPY*	*PRKACA*	*TNNI3*
HCAR2	*NPY1R*	*PRKACB*	*TSHR*
HCAR3	*ORAI1*	*PRKACG*	*VAV1*
HCN2	*OXTR*	*PRKX*	*VAV2*
HCN4	*PAK1*	*PTCH1*	*VAV3*
HHIP	*PDE3A*	*PTGER2*	*VIPR2*

11. Codeine metabolism pathway (n = 4)

CYP2D6	*CYP3A4*	*OPRM1*	*UGT2B7*

12. Collagen biosynthesis and modifying enzymes (n = 68)

ADAMTS14
ADAMTS2
ADAMTS3
BMP1
COL10A1
COL11A1
COL11A2
COL12A1
COL13A1
COL14A1
COL15A1
COL16A1
COL17A1
COL18A1
COL19A1
COL1A1
COL1A2
COL20A1
COL21A1
COL22A1
COL23A1
COL24A1
COL25A1
COL26A1
COL27A1
COL28A1
COL2A1
COL3A1
COL4A1
COL4A2
COL4A3
COL4A4
COL4A5
COL4A6
COL5A1
COL5A2
COL5A3
COL6A1
COL6A2
COL6A3
COL6A5
COL6A6
COL7A1
COL8A1
COL8A2
COL9A1
COL9A2
COL9A3
COLGALT1
COLGALT2
CRTAP
GPR162
LEPREL2
LOC102723473
LOC102723826
LOC102725336
P3H1
P3H2
P4HB
PCOLCE
PCOLCE2
PLOD1
PLOD2
PLOD3
PPIB
SERPINH1
TLL1
TLL2

13. Collagen formation (n = 91)

ADAMTS14
ADAMTS2
ADAMTS3
BMP1
CD151
COL10A1
COL11A1
COL11A2
COL12A1
COL13A1
COL14A1
COL15A1
COL16A1
COL17A1
COL18A1
COL19A1
COL1A1
COL1A2
COL20A1
COL21A1
COL22A1
COL23A1
COL24A1
COL25A1
COL26A1
COL27A1
COL28A1
COL2A1
COL3A1
COL4A1
COL4A2
COL4A3
COL4A4
COL4A5
COL4A6
COL5A1
COL5A2
COL5A3
COL6A1
COL6A2
COL6A3
COL6A5
COL6A6
COL7A1
COL8A1
COL8A2
COL9A1
COL9A2
COL9A3
COLGALT1
COLGALT2
CRTAP
CTSB
CTSL
CTSS
CTSV
DST
GPR162
ITGA6
ITGB4
LAMA3
LAMB3
LAMC2
LEPREL2
LOC102723473
LOC102723826
LOC102725336
LOX
LOXL1
LOXL2
LOXL3
LOXL4
MMP1
MMP13
MMP20
MMP3
MMP7
MMP9
P3H1
P3H2
P4HB
PCOLCE
PCOLCE2
PLEC
PLOD1
PLOD2
PLOD3
PPIB
SERPINH1
TLL1
TLL2

14. Complement activation, classical pathway (n = 17)

C1QA
C1QB
C1QC
C1R
C1S
C2
C3
C4A
C4B
C5
C6
C7
C8A
C8B
C9
CD55
MASP1

15. Constitutive androstane receptor pathway (n = 29)

ABCB1
ABCC2
ABCC3
ALAS1
CYP2A6
CYP2B6
CYP2C19
CYP2C9
CYP3A4
CYP4A11
DNAJC7
EHHADH
FOXO1
GSTA2
HSP90AA1
NCOA1
NCOA2
NCOA6
PPARGC1A
PPP2R4
RXRA
SMC1A
SULT1A1
SULT2A1
UGT1A3
UGT1A4
UGT1A6
UGT1A8
UGT1A9

16. Degradation of the extracellular matrix (n = 86)

A2M
ACAN
ADAM10
ADAM15
ADAM17
ADAM8
ADAM9
ADAMTS1
ADAMTS16
ADAMTS18
ADAMTS4
ADAMTS5

ADAMTS8
ADAMTS9
BCAN
BMP1
BSG
CAPN1
CAPNS1
CASP3
CD44
CDH1
CMA1
COL12A1
COL14A1
COL16A1
COL17A1
COL18A1
COL23A1
COL25A1
COL26A1
COL9A1
COL9A2
COL9A3
CTRB1
CTSB
CTSG
CTSK
CTSL
CTSS
CTSV
DCN
ELANE
FURIN
HSPG2
KLK2
KLK7
KLKB1
LAMA3
LAMA5
LAMB1
LAMB3
LAMC1
LAMC2
LOC102723830
LOC102725056
MMP1
MMP10
MMP11
MMP12
MMP13
MMP14
MMP15
MMP16
MMP17
MMP19
MMP2
MMP20
MMP24
MMP25
MMP3
MMP7
MMP8
MMP9
NCSTN
NID1
PLG
PRSS1
PRSS2
PSEN1
SPP1
TIMP1
TIMP2
TLL1
TLL2
TPSAB1

17. Endochondral ossification (n = 64)

ACAN
ADAMTS1
ADAMTS4
ADAMTS5
AKT1
ALPL
BMP6
BMP7
BMPR1A
CAB39
CALM1
CDKN1C
CHST11
COL10A1
COL2A1
CST5
CTSV
DDR2
ENPP1
FGF18
FGF2
FGFR1
FGFR3
FRZB
GH1
GHR
GLI3
HDAC4
HMGCS1
IFT88
IGF1
IGF1R
IGF2
IHH
KIF3A
MEF2C
MGP
MMP13
MMP9
NKX3-2
PLAT
PLAU
PRKACA
PTCH1
PTH
PTH1R
PTHLH
RUNX2
RUNX3
SCIN
SERPINH1
SLC38A2
SOX5
SOX6
SOX9
SPP1
STAT1
STAT5B
TGFB1
TGFB2
THRA
TIMP3
TNAP
VEGFA

18. Extracellular matrix organisation (n = 275)

A2M
ACAN
ACTN1
ADAM10
ADAM15
ADAM17
ADAM8
ADAM9
ADAMTS1
ADAMTS14
ADAMTS16
ADAMTS18
ADAMTS2
ADAMTS3
ADAMTS4
ADAMTS5
ADAMTS8
ADAMTS9
AGRN
ASPN
BCAN
BGN
BMP1
BMP10
BMP2
BMP4
BMP7
BSG
CAPN1
CAPNS1
CASK
CASP3
CD151
CD44
CD47
CDH1
CEACAM1
CEACAM6
CEACAM8
CMA1
COL10A1
COL11A1
COL11A2
COL12A1
COL13A1
COL14A1
COL15A1
COL16A1
COL17A1
COL18A1
COL19A1
COL1A1
COL1A2
COL20A1
COL21A1
COL22A1
COL23A1
COL24A1
COL25A1
COL26A1
COL27A1
COL28A1
COL2A1
COL3A1
COL4A1
COL4A2
COL4A3
COL4A4
COL4A5
COL4A6
COL5A1
COL5A2
COL5A3
COL6A1
COL6A2
COL6A3
COL6A5
COL6A6
COL7A1
COL8A1
COL8A2
COL9A1
COL9A2
COL9A3
COLGALT1
COLGALT2
COMP
CRTAP
CTRB1
CTSB
CTSG
CTSK
CTSL
CTSS
CTSV
DAG1
DCN
DDR1
DDR2
DMD
DMP1
DSPP
DST
EFEMP1

EFEMP2	*ITGB1*	*LTBP3*	*PCOLCE*
ELANE	*ITGB2*	*LTBP4*	*PCOLCE2*
F11R	*ITGB3*	*LUM*	*PDGFA*
FBLN1	*ITGB4*	*MADCAM1*	*PDGFB*
FBLN2	*ITGB5*	*MATN1*	*PECAM1*
FBLN5	*ITGB6*	*MATN3*	*PLEC*
FBN1	*ITGB7*	*MATN4*	*PLG*
FBN2	*ITGB8*	*MFAP1*	*PLOD1*
FBN3	*JAM2*	*MFAP2*	*PLOD2*
FGA	*JAM3*	*MFAP3*	*PLOD3*
FGB	*KDR*	*MFAP4*	*PPIB*
FGF2	*KLK2*	*MFAP5*	*PRKCA*
FGG	*KLK7*	*MIR3656*	*PRSS1*
FMOD	*KLKB1*	*MIR4640*	*PRSS2*
FN1	*LAMA1*	*MMP1*	*PSEN1*
FURIN	*LAMA2*	*MMP10*	*PTPRS*
GDF5	*LAMA3*	*MMP11*	*SDC1*
GPR162	*LAMA4*	*MMP12*	*SDC2*
HAPLN1	*LAMA5*	*MMP13*	*SDC3*
HSPG2	*LAMB1*	*MMP14*	*SDC4*
IBSP	*LAMB2*	*MMP15*	*SERPINE1*
ICAM1	*LAMB3*	*MMP16*	*SERPINH1*
ICAM2	*LAMC1*	*MMP17*	*SPARC*
ICAM3	*LAMC2*	*MMP19*	*SPP1*
ICAM4	*LAMC3*	*MMP2*	*TGFB1*
ITGA1	*LEPREL2*	*MMP20*	*TGFB2*
ITGA10	*LOC100505984*	*MMP24*	*TGFB3*
ITGA11	*LOC101927169*	*MMP25*	*THBS1*
ITGA2	*LOC102723473*	*MMP3*	*TIMP1*
ITGA2B	*LOC102723826*	*MMP7*	*TIMP2*
ITGA3	*LOC102723830*	*MMP8*	*TLL1*
ITGA4	*LOC102724576*	*MMP9*	*TLL2*
ITGA5	*LOC102725056*	*MUSK*	*TNC*
ITGA6	*LOC102725336*	*NCAM1*	*TNN*
ITGA7	*LOC102725477*	*NCAN*	*TNR*
ITGA8	*LOX*	*NCSTN*	*TNXB*
ITGA9	*LOXL1*	*NID1*	*TPSAB1*
ITGAD	*LOXL2*	*NID2*	*TRAPPC4*
ITGAE	*LOXL3*	*NRXN1*	*TTR*
ITGAL	*LOXL4*	*NTN4*	*VCAM1*
ITGAM	*LRP4*	*P3H1*	*VCAN*
ITGAV	*LTBP1*	*P3H2*	*VTN*
ITGAX	*LTBP2*	*P4HB*	

19. Formation of senescence-associated heterochromatin foci (SAHF) (n = 17)

ASF1A	*HIRA*	*HIST1H1E*	*TP53*
CABIN1	*HIST1H1A*	*HMGA1*	*UBN1*
CDKN1A	*HIST1H1B*	*HMGA2*	
EP400	*HIST1H1C*	*LMNB1*	
H1F0	*HIST1H1D*	*RB1*	

20. FoxO signalling pathway - *Homo sapiens* (human) (n = 134)

AGAP2	*CCNB2*	*EGFR*	*GABARAPL1*
AKT1	*CCNB3*	*EP300*	*GABARAPL2*
AKT2	*CCND1*	*FASLG*	*GADD45A*
AKT3	*CCND2*	*FBXO25*	*GADD45B*
ARAF	*CCNG2*	*FBXO32*	*GADD45G*
ATG12	*CDK2*	*FOXG1*	*GRB2*
ATM	*CDKN1A*	*FOXO1*	*GRM1*
BCL2L11	*CDKN1B*	*FOXO3*	*HOMER1*
BCL6	*CDKN2B*	*FOXO4*	*HOMER2*
BNIP3	*CDKN2D*	*FOXO6*	*HOMER3*
BRAF	*CHUK*	*G6PC*	*HRAS*
C8orf44-SGK3	*CREBBP*	*G6PC2*	*IGF1*
CAT	*CSNK1E*	*G6PC3*	*IGF1R*
CCNB1	*EGF*	*GABARAP*	*IKBKB*

IL10
IL6
IL7R
INS
INSR
IRS1
IRS2
IRS4
KLF2
KRAS
LOC400927-
CSNK1E
MAP2K1
MAP2K2
MAPK1
MAPK10
MAPK11
MAPK12
MAPK13
MAPK14
MAPK3
MAPK8
MAPK9
MDM2
NLK
NRAS
PCK1
PCK2
PDPK1
PIK3CA
PIK3CB
PIK3CD
PIK3CG
PIK3R1
PIK3R2
PIK3R3
PIK3R5
PLK1
PLK2
PLK3
PLK4
PRKAA1
PRKAA2
PRKAB1
PRKAB2
PRKAG1
PRKAG2
PRKAG3
PRMT1
PTEN
RAF1
RAG1
RAG2
RBL2
S1PR1
S1PR4
SETD7
SGK1
SGK2
SGK3
SIRT1
SKP2
SLC2A4
SMAD2
SMAD3
SMAD4
SOD2
SOS1
SOS2
STAT3
STK11
STK4
TGFB1
TGFB2
TGFB3
TGFBR1
TGFBR2
TNFSF10
USP7

21. Glucose metabolism (n = 70)

AGL
ALDOA
ALDOB
ALDOC
CALM1
CALM2
CALM3
ENO1
ENO2
ENO3
EPM2A
FBP1
FBP2
GAPDH
GAPDHS
GBE1
GOT1
GOT2
GPI
GYG1
GYG2
GYS1
GYS2
LOC102725405
LOC102725475
LOC642969
MDH1
MDH2
PC
PCK1
PCK2
PFKFB1
PFKFB2
PFKFB3
PFKFB4
PFKL
PFKM
PFKP
PGAM1
PGAM2
PGAM4
PGK1
PGM1
PGM2
PHKA1
PHKA2
PHKB
PHKG1
PHKG2
PKLR
PKM
PPP1R3C
PPP2CA
PPP2CB
PPP2R1A
PPP2R1B
PPP2R5D
PRKACA
PRKACB
PRKACG
PYGB
PYGL
PYGM
SLC25A1
SLC25A10
SLC25A11
SLC25A12
SLC25A13
TPI1
UGP2

22. Glucuronidation (n = 26)

HK1
PGM1
PGM2
PGM3
PGM5
UGDH
UGP2
UGT1A1
UGT1A10
UGT1A3
UGT1A4
UGT1A5
UGT1A6
UGT1A7
UGT1A8
UGT1A9
UGT2A1
UGT2A2
UGT2A3
UGT2B10
UGT2B11
UGT2B15
UGT2B17
UGT2B28
UGT2B4
UGT2B7

23. Glycolysis / gluconeogenesis - *Homo sapiens* (human) (n = 67)

ACSS1
ACSS2
ADH1A
ADH1B
ADH1C
ADH4
ADH5
ADH6
ADH7
ADPGK
AKR1A1
ALDH1A3
ALDH1B1
ALDH2
ALDH3A1
ALDH3A2
ALDH3B1
ALDH3B2
ALDH7A1
ALDH9A1
ALDOA
ALDOB
ALDOC
BPGM
DLAT
DLD
ENO1
ENO2
ENO3
FBP1
FBP2
G6PC
G6PC2
G6PC3
GALM
GAPDH
GAPDHS
GCK
GPI
HK1
HK2
HK3
HKDC1
LDHA
LDHAL6A
LDHAL6B
LDHB
LDHC
MINPP1
PCK1
PCK2
PDHA1
PDHA2
PDHB
PFKL
PFKM

PFKP	*PGAM4*	*PGM1*	*PKM*
PGAM1	*PGK1*	*PGM2*	*TPI1*
PGAM2	*PGK2*	*PKLR*	

24. Glycolysis and gluconeogenesis (n = 70)

ACOT12	*HK1*	*NUP37*	*PDHX*
ACSS1	*LOC102723702*	*NUP43*	*PFKFB1*
ADHFE1	*LOC642969*	*NUP50*	*PFKL*
AKR1A1	*ME1*	*NUP54*	*PFKM*
ALDH1B1	*ME2*	*NUP62*	*PFKP*
ALDH2	*ME3*	*NUP62CL*	*PGAM1*
ALDH3A1	*MIR6787*	*NUP85*	*PGAM4*
ALDH7A1	*NUP107*	*NUP88*	*PGK2*
ALDH9A1	*NUP133*	*NUP93*	*PKM*
ALDOB	*NUP153*	*NUP98*	*RANBP2*
CS	*NUP155*	*NUPL1*	*SLC16A1*
DLAT	*NUP160*	*NUPL2*	*SLC16A3*
DLD	*NUP188*	*PC*	*SLC16A7*
ENO1	*NUP205*	*PCK1*	*SLC16A8*
ENO2	*NUP210*	*PCK2*	*SLC25A1*
ENO3	*NUP210L*	*PDHA1*	*SLC25A10*
G6PC	*NUP214*	*PDHA2*	
GPI	*NUP35*	*PDHB*	

25. GRB7 events in ERBB2 signalling (n = 5)

ERBB2	*GRB7*	*NRG2*
ERBB3	*NRG1*	

26. Interaction between L1 and ankyrins (n = 29)

ANK1	*SCN10A*	*SCN4A*	*SPTB*
ANK2	*SCN11A*	*SCN4B*	*SPTBN1*
ANK3	*SCN1A*	*SCN5A*	*SPTBN2*
KCNQ2	*SCN1B*	*SCN7A*	*SPTBN4*
KCNQ3	*SCN2A*	*SCN8A*	*SPTBN5*
L1CAM	*SCN2B*	*SCN9A*	
NFASC	*SCN3A*	*SPTA1*	
NRCAM	*SCN3B*	*SPTAN1*	

27. Ion channel transport (n = 173)

ANO1	*ATP1A4*	*BEST2*	*FXYD7*
ANO10	*ATP1B1*	*BEST3*	*GABRA1*
ANO2	*ATP1B2*	*BEST4*	*GABRA2*
ANO3	*ATP1B3*	*BSND*	*GABRA3*
ANO4	*ATP2A1*	*C8orf44-SGK3*	*GABRA4*
ANO5	*ATP2A2*	*CLCA1*	*GABRA5*
ANO6	*ATP2A3*	*CLCA2*	*GABRA6*
ANO7	*ATP2B1*	*CLCA3P*	*GABRB1*
ANO8	*ATP2B2*	*CLCA4*	*GABRB2*
ANO9	*ATP2B3*	*CLCN1*	*GABRB3*
ARHGEF9	*ATP2B4*	*CLCN2*	*GABRG2*
ASIC1	*ATP2C1*	*CLCN3*	*GABRG3*
ASIC2	*ATP2C2*	*CLCN4*	*GABRQ*
ASIC3	*ATP4A*	*CLCN5*	*GABRR1*
ASIC4	*ATP4B*	*CLCN6*	*GABRR2*
ASIC5	*ATP7A*	*CLCN7*	*GABRR3*
ATP10A	*ATP7B*	*CLCNKA*	*GLRA1*
ATP10B	*ATP8A1*	*CLCNKB*	*GLRA2*
ATP10D	*ATP8A2*	*CLIC2*	*GLRA3*
ATP11A	*ATP8B1*	*FKBP1B*	*GLRA4*
ATP11B	*ATP8B2*	*FXYD1*	*GLRB*
ATP11C	*ATP8B3*	*FXYD2*	*HTR3A*
ATP12A	*ATP8B4*	*FXYD3*	*HTR3B*
ATP1A1	*ATP9A*	*FXYD4*	*HTR3C*
ATP1A2	*ATP9B*	*FXYD6*	*HTR3D*
ATP1A3	*BEST1*	*FXYD6-FXYD2*	*HTR3E*

LOC102724304
LOC102724915
LOC102725085
LOC102725415
MCOLN1
MCOLN2
MCOLN3
NALCN
NEDD4L
OSTM1
PDZD11
PLN
RAF1
RPS27A
RYR1
RYR2
RYR3
SCNN1A
SCNN1B
SCNN1D
SCNN1G
SGK1
SGK2
SGK3
SLC17A3
SLC9B1
SLC9B2
SLC9C1
SLC9C2
SRI
TPCN1
TPCN2
TRDN
TRPA1
TRPC1
TRPC3
TRPC4
TRPC4AP
TRPC5
TRPC6
TRPC7
TRPM1
TRPM2
TRPM3
TRPM4
TRPM5
TRPM6
TRPM7
TRPM8
TRPV1
TRPV2
TRPV3
TRPV4
TRPV5
TRPV6
TSC22D3
TTYH1
TTYH2
TTYH3
UBA52
UBB
UBC
UNC79
UNC80
WNK1
WNK2
WNK3
WNK4
WWP1

28. Ion transport by P-type ATPases (n = 47)

ATP10A
ATP10B
ATP10D
ATP11A
ATP11B
ATP11C
ATP12A
ATP13A4
ATP13A5
ATP1A1
ATP1A2
ATP1A3
ATP1A4
ATP1B1
ATP1B2
ATP1B3
ATP2A1
ATP2A2
ATP2A3
ATP2B1
ATP2B2
ATP2B3
ATP2B4
ATP2C1
ATP2C2
ATP4A
ATP4B
ATP7A
ATP7B
ATP8A1
ATP8A2
ATP8B1
ATP8B2
ATP8B3
ATP8B4
ATP9A
ATP9B
CUTC
FXYD1
FXYD2
FXYD3
FXYD4
FXYD6
FXYD6-FXYD2
FXYD7
PDZD11
PLN

29. Lectin induced complement pathway (n = 14)

C2
C3
C5
C6
C7
C8A
C8B
C8G
C9
LOC101928623
LOC102725441
MASP1
MASP2
MBL2

30. LRR FLII-interacting protein 1 (LRRFIP1) activates type I IFN production (n = 5)

CREBBP
CTNNB1
EP300
IRF3
LRRFIP1

31. Mono-unsaturated fatty acid beta-oxidation (n = 22)

ACAA1
ACAA2
ACADL
ACADM
ACADSB
ACOX1
ACOX3
ACSL1
ACSL3
ACSL4
ACSL5
ACSL6
DBI
ECHS1
EHHADH
HADH
HADHA
HADHB
HSD17B10
HSD17B4
LOC728637
SLC27A2

32. Olfactory transduction - *Homo sapiens* (human) (n = 408)

ADCY3
ADRBK2
ARRB2
CALM1
CALM2
CALM3
CALML3
CALML5
CALML6
CAMK2A
CAMK2B
CAMK2D
CAMK2G
CLCA1
CLCA2
CLCA4
CNGA3
CNGA4
CNGB1
GNAL
GUCA1A
GUCA1B
GUCA1C
GUCY2D
LOC102723532
LOC102725084
OR10A2
OR10A3
OR10A4
OR10A5
OR10A6
OR10A7
OR10AD1
OR10AG1
OR10C1
OR10G2
OR10G3
OR10G4
OR10G7
OR10G8
OR10G9
OR10H1
OR10H2
OR10H3
OR10H4
OR10H5
OR10J1
OR10J3
OR10J5
OR10K1
OR10K2
OR10P1

OR10Q1
OR10R2
OR10S1
OR10T2
OR10V1
OR10W1
OR10X1
OR10Z1
OR11A1
OR11G2
OR11H1
OR11H12
OR11H4
OR11H6
OR11L1
OR12D2
OR12D3
OR13A1
OR13C2
OR13C3
OR13C4
OR13C5
OR13C8
OR13C9
OR13D1
OR13F1
OR13G1
OR13H1
OR13J1
OR14A16
OR14C36
OR14I1
OR14J1
OR1A1
OR1A2
OR1B1
OR1C1
OR1D2
OR1D5
OR1E1
OR1E2
OR1F1
OR1G1
OR1I1
OR1J1
OR1J2
OR1J4
OR1K1
OR1L1
OR1L3
OR1L4
OR1L6
OR1L8
OR1M1
OR1N1
OR1N2
OR1Q1
OR1S1
OR1S2
OR2A1
OR2A12
OR2A14
OR2A2
OR2A25
OR2A4
OR2A42
OR2A5
OR2A7
OR2AE1
OR2AG1
OR2AG2
OR2AK2
OR2AP1
OR2AT4
OR2B11
OR2B2
OR2B3
OR2B6
OR2C1
OR2C3
OR2D2
OR2D3
OR2F1
OR2F2
OR2G2
OR2G3
OR2G6
OR2H1
OR2H2
OR2J2
OR2J3
OR2K2
OR2L13
OR2L2
OR2L3
OR2L5
OR2L8
OR2M2
OR2M3
OR2M4
OR2M5
OR2M7
OR2S2
OR2T1
OR2T10
OR2T11
OR2T12
OR2T2
OR2T27
OR2T29
OR2T3
OR2T33
OR2T34
OR2T4
OR2T5
OR2T6
OR2T8
OR2V1
OR2V2
OR2W1
OR2W3
OR2Y1
OR2Z1
OR3A1
OR3A2
OR3A3
OR4A15
OR4A16
OR4A47
OR4A5
OR4B1
OR4C11
OR4C12
OR4C13
OR4C15
OR4C16
OR4C3
OR4C45
OR4C46
OR4C6
OR4D1
OR4D10
OR4D11
OR4D2
OR4D5
OR4D6
OR4D9
OR4E2
OR4F15
OR4F16
OR4F17
OR4F21
OR4F29
OR4F3
OR4F4
OR4F5
OR4F6
OR4K1
OR4K13
OR4K14
OR4K15
OR4K17
OR4K2
OR4K5
OR4L1
OR4M1
OR4M2
OR4N2
OR4N4
OR4N5
OR4P4
OR4Q3
OR4S1
OR4S2
OR4X1
OR4X2
OR51A2
OR51A4
OR51A7
OR51B2
OR51B4
OR51B5
OR51B6
OR51D1
OR51E1
OR51E2
OR51F1
OR51F2
OR51G1
OR51G2
OR51I1
OR51I2
OR51L1
OR51M1
OR51Q1
OR51S1
OR51T1
OR51V1
OR52A1
OR52A5
OR52B2
OR52B4
OR52B6
OR52D1
OR52E2
OR52E4
OR52E6
OR52E8
OR52H1
OR52I1
OR52I2
OR52J3
OR52K1
OR52K2
OR52L1
OR52M1
OR52N1
OR52N2
OR52N4
OR52N5
OR52R1
OR52W1
OR56A1
OR56A3
OR56A4
OR56A5
OR56B1
OR56B4
OR5A1
OR5A2
OR5AC2
OR5AK2
OR5AN1
OR5AP2
OR5AR1
OR5AS1
OR5AU1
OR5B12
OR5B17
OR5B2
OR5B21
OR5B3
OR5C1
OR5D13
OR5D14
OR5D16
OR5D18
OR5F1
OR5H1
OR5H14
OR5H2
OR5H6
OR5I1
OR5J2
OR5K1
OR5K2
OR5K3
OR5K4
OR5L1
OR5L2
OR5M1
OR5M10
OR5M11
OR5M3
OR5M8
OR5M9
OR5P2
OR5P3

OR5R1
OR5T1
OR5T2
OR5T3
OR5V1
OR5W2
OR6A2
OR6B1
OR6B2
OR6B3
OR6C1
OR6C2
OR6C3
OR6C4
OR6C6
OR6C65
OR6C68
OR6C70
OR6C74
OR6C75
OR6C76
OR6F1
OR6K2
OR6K3
OR6K6
OR6M1
OR6N1
OR6N2
OR6P1
OR6Q1
OR6S1
OR6T1
OR6V1
OR6X1
OR6Y1
OR7A10
OR7A17
OR7A5
OR7C1
OR7C2
OR7D2
OR7D4
OR7E24
OR7G1
OR7G2
OR7G3
OR8A1
OR8B12
OR8B2
OR8B3
OR8B4
OR8B8
OR8D1
OR8D2
OR8D4
OR8G1
OR8G2
OR8G5
OR8H1
OR8H2
OR8H3
OR8I2
OR8J1
OR8J3
OR8K1
OR8K3
OR8K5
OR8S1
OR8U1
OR8U8
OR8U9
OR9A2
OR9A4
OR9G1
OR9G4
OR9G9
OR9I1
OR9K2
OR9Q1
OR9Q2
PDC
PDE1C
PRKACA
PRKACB
PRKACG
PRKG1
PRKG2
PRKX

33. Omega-6 fatty acid metabolism (n = 31)

ACAA1
ACAA2
ACOX1
ACOX2
ACOX3
ACSL1
ACSL3
ACSL4
ACSL5
ACSL6
DBI
DECR1
DECR2
ECH1
ECHS1
ECI1
ECI2
EHHADH
ELOVL1
FADS1
FADS2
FADS3
HADH
HADHA
HADHB
HSD17B10
HSD17B4
LOC728637
MIR1908
MIR6734
SLC27A2

34. Opsins (n = 11)

LOC101060233
OPN1LW
OPN1MW
OPN1MW2
OPN1SW
OPN3
OPN4
OPN5
RGR
RHO
RRH

35. Other glycan degradation - *Homo sapiens* (human) (n = 18)

AGA
ENGASE
FUCA1
FUCA2
GBA
GBA2
GLB1
HEXA
HEXB
HEXDC
MAN2B1
MAN2B2
MAN2C1
MANBA
NEU1
NEU2
NEU3
NEU4

36. p53 signalling pathway (n = 13)

BCL2
CCND1
CCNE1
CDK2
CDK4
CDKN1A
E2F1
GADD45A
MDM2
PCNA
RB1
TIMP3
TP53

37. p73 transcription factor network (n = 81)

ABL1
ADA
AEN
AFP
BAK1
BAX
BBC3
BCL2L11
BIN1
BRCA2
BUB1
BUB3
CASP2
CCNA2
CCNB1
CCNE2
CDK1
CDK2
CDK6
CDKN1A
CHEK1
CLCA2
DCP1B
DEDD
EP300
FAS
FASN
FBXO45
FLOT2
FOXO3
GATA1
GDF15
GNB2L1
GRAMD4
HAGH
HEY2
HSF1
IL1RAP
IL4R
ITCH
JAG2
JAK1
KAT5
MAPK11
MAPK14
MDM2
MIR3190
MIR3191
MYC
NDUFS2
NEDD4L
NSG1

NTRK1
PEA15
PFDN5
PIN1
PLK1
PLK3
PML
PPAP2A
PRKACB
RAD51
RB1
RCHY1
RELA
RNF43
S100A2
SERPINA1
SERPINE1
SFN
SIRT1
SP1
TP53AIP1
TP53I3
TP63
TP73
TUBA1A
UBE4B
WT1
WWOX
YAP1

38. PELP1 modulation of oestrogen receptor activity (n = 5)

CREBBP
EP300
ESR1
PELP1
SRC

39. Pentose phosphate pathway - *Homo sapiens* (human) (n = 28)

ALDOA
ALDOB
ALDOC
DERA
FBP1
FBP2
G6PD
GPI
H6PD
PFKL
PFKM
PFKP
PGD
PGLS
PGM1
PGM2
PRPS1
PRPS1L1
PRPS2
RBKS
RGN
RPE
RPEL1
RPIA
TALDO1
TKT
TKTL1
TKTL2

40. Peroxisomal lipid metabolism (n = 21)

ABCD1
ACAA1
ACOT8
ACOX1
ACOX2
ACOX3
AGPS
AMACR
CRAT
CROT
FAR1
FAR2
GNPAT
HACL1
HSD17B4
IDH1
MLYCD
PHYH
SCP2
SLC25A17
SLC27A2

41. Pregnane X receptor pathway (n = 32)

ABCB1
ABCC2
ABCC3
ABCC4
CYP2A6
CYP2B6
CYP2C19
CYP2C9
CYP3A4
CYP3A7
CYP4F12
DNAJC7
FOXO1
GSTA2
HSP90AA1
NCOA1
NCOA2
NCOA3
NR1I2
NRIP1
PPARGC1A
PSMC5
RXRA
SLCO1B1
SRC
SRPX2
SULT2A1
UGT1A3
UGT1A4
UGT1A6
UGT1A8
UGT1A9

42. Purine deoxyribonucleosides degradation (n = 2)

ADA
PNP

43. Purine ribonucleosides degradation to ribose-1-phosphate (n = 3)

ADA
PGM2
PNP

44. Pyruvate metabolism - *Homo sapiens* (human) (n = 40)

ACACA
ACACB
ACAT1
ACAT2
ACOT12
ACSS1
ACSS2
ACYP1
ACYP2
ALDH1B1
ALDH2
ALDH3A2
ALDH7A1
ALDH9A1
DLAT
DLD
FH
GLO1
GRHPR
HAGH
HAGHL
LDHA
LDHAL6A
LDHAL6B
LDHB
LDHC
LDHD
MDH1
MDH2
ME1
ME2
ME3
PC
PCK1
PCK2
PDHA1
PDHA2
PDHB
PKLR
PKM

45. Regulation of androgen receptor activity (n = 52)

APPBP2
AR
CARM1
CEBPA
CREBBP
DNAJA1
EGR1
EHMT2

EP300
FOXO1
GNB2L1
GSK3B
HDAC1
HDAC7
HOXB13
HSP90AA1
JUN
KAT2B
KAT5
KAT7
KLK2
KLK3
LOC102724277
MAP2K4
MAP2K6
MAPK14
MAPK8
MDM2
NCOA1
NCOA2
NR0B1
NR2C1
NR2C2
NR3C1
PDE9A
PKN1
POU2F1
REL
RXRA
RXRB
RXRG
SENP1
SIRT1
SMARCA2
SMARCC1
SMARCE1
SPDEF
SRC
SRY
TMPRSS2
TRIM24
ZMIZ2

46. Regulation of complement cascade (n = 25)

C2
C3
C4A
C4B
C4B_2
C4BPA
C4BPB
C5
C6
C7
C8A
C8B
C8G
C9
CD46
CD55
CD59
CFB
CFH
CFHR3
CFI
CR1
LOC101928623
PROS1
VTN

47. Regulation of nuclear SMAD2/3 signalling (n = 79)

AKT1
AR
ATF2
CBFB
CDK2
CDK4
CDKN1A
CDKN2B
CEBPB
CITED1
COL1A2
CREB1
CREBBP
CTBP1
DCP1A
DLX1
EP300
ESR1
FOS
FOXG1
FOXH1
FOXO1
FOXO3
FOXO4
GATA3
GSC
HDAC1
HDAC2
HNF4A
HSPA8
IFNB1
IGHA1
IL10
IL5
IRF7
ITGB5
JUN
KAT2A
KAT2B
LAMC1
MAX
MED15
MEF2C
MYC
MYOD1
NCOA1
NCOA2
NCOR1
NKX2-5
NR3C1
PIAS3
PIAS4
RBBP4
RBBP7
RUNX1
RUNX2
RUNX3
SAP18
SAP30
SERPINE1
SIN3A
SIN3B
SKI
SKIL
SMAD2
SMAD3
SMAD4
SMAD7
SNIP1
SNORD14C
SNORD14D
SP1
SP3
TCF3
TFE3
TGIF1
TGIF2
VDR
ZBTB17

48. Retinol metabolism (n = 37)

ADH1A
ALDH1A1
ALDH1A2
AWAT1
BCO1
CYP26A1
CYP2A13
CYP2A6
CYP2A7
CYP2B6
CYP3A4
CYP3A43
CYP3A5
CYP3A7
CYP3A7-CYP3AP1
DGAT1
DHRS3
DHRS4
DHRS9
DNAJB11
ERP29
HSPA5
HYOU1
LRAT
PDIA2
PDIA4
PDIA6
PNPLA4
PPIB
RDH11
RDH12
RDH16
RDH8
RETSAT
RPE65
SDF2L1
UGT1A1

49. Role of nicotinic acetylcholine receptors in the regulation of apoptosis (n = 17)

AGRN
AKT1
BAD
CHRNB1
CHRNG
FASLG
FOXO3
MUSK
PDPK1
PIK3CA
PIK3R1
PTK2
PTK2B
RAPSN
SRC
TERT
YWHAH

50. Saturated fatty acids beta-oxidation (n = 25)

ACAA1
ACAA2
ACADL
ACADM

ACADS	*ACOX2*	*GCDH*	*MECR*
ACADSB	*ACOX3*	*HADH*	*PECR*
ACAT1	*BAAT*	*HADHA*	*SLC25A20*
ACOT4	*DBI*	*HADHB*	
ACOT8	*ECHS1*	*HSD17B10*	
ACOX1	*EHHADH*	*HSD17B4*	

51. Stimuli-sensing channels (n = 100)

ANO1	*CLCA4*	*SCNN1D*	*TRPM4*
ANO10	*CLCN1*	*SCNN1G*	*TRPM5*
ANO2	*CLCN2*	*SGK1*	*TRPM6*
ANO3	*CLCN3*	*SGK2*	*TRPM7*
ANO4	*CLCN4*	*SGK3*	*TRPM8*
ANO5	*CLCN5*	*SLC17A3*	*TRPV1*
ANO6	*CLCN6*	*SLC9B1*	*TRPV2*
ANO7	*CLCN7*	*SLC9B2*	*TRPV3*
ANO8	*CLCNKA*	*SLC9C1*	*TRPV4*
ANO9	*CLCNKB*	*SLC9C2*	*TRPV5*
ASIC1	*CLIC2*	*SRI*	*TRPV6*
ASIC2	*FKBP1B*	*TPCN1*	*TSC22D3*
ASIC3	*MCOLN1*	*TPCN2*	*TTYH1*
ASIC4	*MCOLN2*	*TRDN*	*TTYH2*
ASIC5	*MCOLN3*	*TRPA1*	*TTYH3*
ASPH	*NALCN*	*TRPC1*	*UBA52*
BEST1	*NEDD4L*	*TRPC3*	*UBB*
BEST2	*OSTM1*	*TRPC4*	*UBC*
BEST3	*RAF1*	*TRPC4AP*	*UNC79*
BEST4	*RPS27A*	*TRPC5*	*UNC80*
BSND	*RYR1*	*TRPC6*	*WNK1*
C8orf44-SGK3	*RYR2*	*TRPC7*	*WNK2*
CLCA1	*RYR3*	*TRPM1*	*WNK3*
CLCA2	*SCNN1A*	*TRPM2*	*WNK4*
CLCA3P	*SCNN1B*	*TRPM3*	*WWP1*

52. Transmembrane transport of small molecules (n = 623)

AAAS	*ABCG2*	*AQP11*	*ATP2A2*
ABCA10	*ABCG4*	*AQP2*	*ATP2A3*
ABCA12	*ABCG5*	*AQP3*	*ATP2B1*
ABCA2	*ABCG8*	*AQP4*	*ATP2B2*
ABCA3	*ADCY1*	*AQP5*	*ATP2B3*
ABCA4	*ADCY2*	*AQP6*	*ATP2B4*
ABCA6	*ADCY3*	*AQP7*	*ATP2C1*
ABCA7	*ADCY4*	*AQP8*	*ATP2C2*
ABCA8	*ADCY5*	*AQP9*	*ATP4A*
ABCA9	*ADCY6*	*ARHGEF9*	*ATP4B*
ABCB1	*ADCY7*	*ARL2*	*ATP6AP1*
ABCB10	*ADCY8*	*ASIC1*	*ATP6V0A1*
ABCB5	*ADCY9*	*ASIC2*	*ATP6V0A2*
ABCB6	*ALB*	*ASIC3*	*ATP6V0A4*
ABCB7	*ANO1*	*ASIC4*	*ATP6V0B*
ABCB8	*ANO10*	*ASIC5*	*ATP6V0C*
ABCC1	*ANO2*	*ATP10A*	*ATP6V0D1*
ABCC10	*ANO3*	*ATP10B*	*ATP6V0D2*
ABCC11	*ANO4*	*ATP10D*	*ATP6V0E1*
ABCC2	*ANO5*	*ATP11A*	*ATP6V0E2*
ABCC3	*ANO6*	*ATP11B*	*ATP6V1A*
ABCC4	*ANO7*	*ATP11C*	*ATP6V1B1*
ABCC5	*ANO8*	*ATP12A*	*ATP6V1B2*
ABCC6	*ANO9*	*ATP1A1*	*ATP6V1C1*
ABCC8	*ANXA2*	*ATP1A2*	*ATP6V1C2*
ABCC9	*APOA1*	*ATP1A3*	*ATP6V1D*
ABCD1	*APOB*	*ATP1A4*	*ATP6V1E1*
ABCD2	*APOD*	*ATP1B1*	*ATP6V1E2*
ABCD3	*APOF*	*ATP1B2*	*ATP6V1F*
ABCD4	*AQP1*	*ATP1B3*	*ATP6V1G1*
ABCG1	*AQP10*	*ATP2A1*	*ATP6V1G2*

ATP6V1G3
ATP6V1H
ATP7A
ATP7B
ATP8A1
ATP8A2
ATP8B1
ATP8B2
ATP8B3
ATP8B4
ATP9A
ATP9B
AVP
AVPR2
BEST1
BEST2
BEST3
BEST4
BSND
C8orf44-SGK3
CERK
CETP
CFTR
CLCA1
CLCA2
CLCA3P
CLCA4
CLCN1
CLCN2
CLCN3
CLCN4
CLCN5
CLCN6
CLCN7
CLCNKA
CLCNKB
CLIC2
COX1
COX11
COX14
COX16
COX18
COX2
COX20
COX3
COX4I1
COX5A
COX5B
COX6A1
COX6B1
COX6C
COX7A2L
COX7B
COX7C
COX8A
CP
CRABP2
CYBRD1
CYCS
CYP7A1
CYP7B1
FABP6
FKBP1B
FLVCR1
FTH1
FTL
FXYD1
FXYD2
FXYD3
FXYD4
FXYD6
FXYD6-FXYD2
FXYD7
G6PC
G6PC2
G6PC3
GABRA1
GABRA2
GABRA3
GABRA4
GABRA5
GABRA6
GABRB1
GABRB2
GABRB3
GABRG2
GABRG3
GABRQ
GABRR1
GABRR2
GABRR3
GCK
GCKR
GLRA1
GLRA2
GLRA3
GLRA4
GLRB
GNAS
GNB1
GNB2
GNB3
GNB4
GNB5
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GNG13
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GNGT2
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HK2
HK3
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HMOX2
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HTR3B
HTR3C
HTR3D
HTR3E
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MCOLN2
MCOLN3
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MIR6787
MIR6824
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NCOA2
NEDD4L
NR1H4
NUP107
NUP133
NUP153
NUP155
NUP160
NUP188
NUP205
NUP210
NUP214
NUP35
NUP37
NUP43
NUP50
NUP54
NUP62
NUP85
NUP88
NUP93
NUP98
NUPL1
NUPL2
OSTM1
PDZD11
PEX19
PEX3
PLAT
PLG
PLN
POM121
PRKACA
PRKACB
PRKACG
PRKAR1A
PRKAR1B
PRKAR2A
PRKAR2B
RAE1
RAF1
RANBP2
RARA
RARB
RARG
RHAG
RHBG
RHCG
RPS27A
RXRA
RXRB
RXRG
RYR1
RYR2
RYR3
S100A10
SCNN1A
SCNN1B
SCNN1D
SCNN1G
SCO1
SCO2
SEH1L
SGK1
SGK2
SGK3
SLC10A2
SLC10A6
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SLC9A9
SLC9B1
SLC9B2
SLC9C1
SLC9C2
SLCO1A2
SLCO1B1
SLCO1B3
SLCO1C1
SLCO2A1
SLCO2B1
SLCO3A1
SLCO4A1
SLCO4C1
SRI
STEAP2
STEAP3
SURF1
TACO1
TBCD
TCIRG1
TF
TFRC
TPCN1
TPCN2
TPR
TRDN
TRPA1
TRPC1
TRPC3
TRPC4
TRPC4AP
TRPC5
TRPC6
TRPC7
TRPM1
TRPM2
TRPM3
TRPM4
TRPM5
TRPM6
TRPM7
TRPM8
TRPV1
TRPV2
TRPV3
TRPV4
TRPV5
TRPV6
TSC22D3
TTYH1
TTYH2
TTYH3
TUBB1
TUBB2A
TUBB2B
TUBB3
TUBB4A
TUBB4B
TUBB6
UBA52
UBB
UBC
UNC79
UNC80
WNK1
WNK2
WNK3
WNK4
WWP1

53. Trihydroxycoprostanoyl-CoA beta-oxidation (n = 12)

ACAA1
ACOX1
ACOX2
ACOX3
AMACR
ECHS1
EHHADH
HADH
HADHA
HADHB
HSD17B10
HSD17B4

54. Validated nuclear oestrogen receptor alpha network (n = 65)

ABCA3
ANP32A
AP1B1
APBB1
ATP5J
AXIN2
BRCA1
C3
CALCOCO1
CCND1
CD82
CEBPB
CHUK
COL18A1
CTSD
DDX17
DDX54
DSCAM
EBAG9
EP300
ESR1
ESR2
GREB1
HDAC1
HDAC4
HSF2
JUN
KLRC3
LCOR
LMO4
MED1
MPG
MTA1
MYC
NCOA1
NCOA2
NCOA3
NCOA7
NCOR1
NCOR2
NDUFV3
NEDD8
NR0B1
NR0B2
NRIP1
PCNA
PDIA2
PGR
PHB2
POU4F1
POU4F2
PRDM15
PRL
SAFB
SET
SETSIP
SMAD4
SOD1
SRA1
STAT5A
TFF1
TRIM59
UBA3
UBE2M
XBP1

55. Vitamin A deficiency (n = 37)

ADH1A
ALDH1A1
ALDH1A2
AWAT1
BCO1
CYP26A1
CYP2A13
CYP2A6
CYP2A7
CYP2B6
CYP3A4
CYP3A43
CYP3A5
CYP3A7
CYP3A7-CYP3AP1
DGAT1
DHRS3
DHRS4
DHRS9
DNAJB11
ERP29
HSPA5
HYOU1
LRAT
PDIA2
PDIA4
PDIA6
PNPLA4
PPIB
RDH11
RDH12
RDH16
RDH8
RETSAT
RPE65
SDF2L1
UGT1A1

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And finally – to my family, for your unwavering support and belief in me,

thank you.

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by Timothy Spracklen

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