# INVESTIGATION OF THE GENETIC CAUSE OF HEARING LOSS IN NEWFOUNDLAND FAMILIES

by © Cindy Penney

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# Abstract

The purpose of this study was to determine the genetic cause of hearing loss in a seven-generation Newfoundland family. Twenty-nine family members were recruited segregating autosomal dominant hearing loss. Genome-wide SNP genotyping and linkage analysis showed significant linkage (LOD=4.77) to chromosome 13q34. The region contained 26 genes and a known deafness locus (*DFNA33*). Exome sequencing identified 13 variants of interest within the linked region, but only 3 co-segregated with hearing loss: *F10* c.865+26C>T, *ADPRHL1* c.380-17C>A and c.380-16T>G. All three were absent from 81 population controls, yet the *ADPRHL1* c.380-17C>A and c.380-16T>G were identified in two other probands with hearing loss. All three were predicted to affect splicing of nearby exons, however cDNA analysis of *ADPRHL1* showed no effect.

*F10* c.865+26C>T, *ADPRHL1* c.380-17C>A and c.380-16T>G are rare, co-segregate with hearing loss, and are possibly pathogenic. Conversely, they may help form a disease haplotype and exist in linkage disequilibrium with the causal mutation. However, the putative *ADPRHL1* variants have been found in multiple families with hearing loss and further investigations are necessary to elucidate their effect.

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# List of Abbreviations and Symbols

| | |
|---|---|
| **A** | Adenine |
| **ABI** | Applied Biosystems |
| **ACD** | Acid Citrate Dextrose |
| **ADP** | Adenosine Diphosphate |
| **ADPRHL1** | Adenosine Diphosphate Ribosylhydrolase Like 1 |
| **ARH** | Adenosine Diphosphate Ribosylhydrolase |
| **ARH1** | Adenosine Diphosphate Ribosylhydrolase 1 |
| **ARH2** | Adenosine Diphosphate Ribosylhydrolase 2 |
| **ARH3** | Adenosine Diphosphate Ribosylhydrolase 3 |
| **BLSNHL** | Bilateral Sensorineural Hearing Loss |
| **C** | Cytosine |
| **cDNA** | Complementary Deoxyribonucleic Acid |
| **CNV** | Copy Number Variation |
| **COCH** | Coagulation Factor C Homolog |
| **dB** | Decibels |
| **DFNA** | Autosomal Dominant Deafness Loci |
| **dH2O** | De-ionized Water |
| **DNA** | Deoxyribonucleic Acid |
| **dNTP** | Dideoxynucleotide Triphosphate |
| **EBV** | Epstein–Barr Virus |

| | |
|---|---|
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ESE** | Exonic Splicing Enhancer |
| **ExAC** | Exome Aggregation Consortium |
| **F10** | Coagulation Factor X |
| **FOXL1** | Forkhead Box L1 |
| **G** | Guanine |
| **gDNA** | Genomic Deoxyribonucleic Acid |
| **GJB2** | Gap Junction, Beta 2 |
| **GJB6** | Gap Junction, Beta 6 |
| **HNPCC** | Hereditary Non-Polyposis Colon Cancer |
| **Hz** | Hertz |
| **ISP** | Ion Sphere Particles |
| **KCNQ4** | Potassium Voltage-Gated Channel 4 |
| **L** | Litre |
| **MAF** | Minor Allele Frequency |
| **$MgCl_2$** | Magnesium Chloride |
| **mM** | Millimolar |
| **MSH2** | MutS Homolog 2 |
| **Mt** | Mutant |
| **MT-RNR1** | Mitochondrially Encoded 12S RNA |
| **mRNA** | Messenger Ribonucleic Acid |
| **NCBI** | National Center for Biotechnology |
| **ng** | Nanogram |

| | |
|---|---|
| **NGS** | Next-Generation Sequencing |
| **NL** | Newfoundland and Labrador |
| **NMD** | Nonsense-Mediated Decay |
| **NSHL** | Nonsyndromic Hearing Loss |
| **OMIM** | Online Mendelian Inheritance of Man |
| **PARP1** | Poly (Adenosine Diphosphate-Ribose) Polymerase 1 |
| **PCDH15** | Protocadherin-15 |
| **PCR** | Polymerase Chain Reaction |
| **PID** | Patient Identification |
| **pM** | Picomolar |
| **RNA** | Ribonucleic Acid |
| **RPMI** | Roswell Park Memorial Institute |
| **rcf** | Relative Centrifugal Force |
| **SMPX** | Small Muscle Protein, X-linked |
| **SNHL** | Sensorineural Hearing Loss |
| **SNP** | Single Nucleotide Polymorphism |
| **T** | Thymine |
| **Taq** | Thermus aquaticus DNA Polymerase |
| **TBE** | Tris/Borate/EDTA Buffer |
| **TCAG** | The Centre for Applied Genomics |
| **TECTA** | Tectorin Alpha |
| **TMPRSS3** | Transmembrane Protease 3, Serine 3 |
| **U** | Uracil |

| | |
|---|---|
| **WFS1** | Wolframin Syndrome 1 (wolframin) |
| **Wt** | Wild-type |
| **µL** | Microlitre |
| **µM** | Micromolar |
| **UV** | Ultraviolet |

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# Chapter 1 Introduction

## Genetic Variation and the Human Genome

DNA or deoxyribonucleic acid contains the molecular instructions necessary for human life. Our DNA encodes approximately 20,000-25,000 genes and contains 2.85 billion nucleotides (International Human Genome Sequencing Consortium, 2004). The base sequence of DNA is composed of adenine (A), guanine (G), cytosine (C), and thymine (T), and provides the instructions for making proteins. A permanent change in the DNA sequence is called a variant. Variants can be sporadic (spontaneous) or inherited and there are several different types, ranging from small substitutions, insertions and deletions, to large chromosomal rearrangements.

More than 10,000,000 variants have been identified in the human genome (Lek et al., 2016). These genetic variations alter the DNA sequence and can have an effect on the transcribed mRNA (messenger ribonucleic acid) and consequently the translated amino acid sequence of the protein. Thankfully, most of these variants have little to no impact and are considered benign but some are pathogenic and lead to disease.

A variant's impact on phenotype is influenced by its sequence location. The variant position is important because only 1.2% of the genome is translated into proteins (International Human Genome Sequencing Consortium, 2004). Therefore, if the variant occurs in a protein-coding exon (expressed sequence) it is more likely to have an effect on protein function than if it occurs in an intron (intervening sequence). Not surprisingly,

85% of pathogenic mutations are located in the exome (Majewski, Schwartzentruber, Lalonde, Montpetit, & Jabado, 2011).

It is important to note that on average the exome contains a variant every 8 base pairs (bp), so not all exonic variants are pathogenic (Lek et al., 2016). Evolutionary conservation of the amino acid across multiple species is a good indicator that it plays an important role in protein function and that changes could have a devastating effect. Bioinformatics software like SIFT (**S**orting **I**ntolerant **F**rom **T**olerant) examine the degree of conservation of amino acid residues and predict the effect of a change (http://sift.jcvi.org/). Another aspect to consider is the actual amino acid substitution. Programs like Polyphen-2 (**Poly**morphism **Phen**otyping v**2**) attempt to predict the possible impact of an amino acid substitution on the structure and function of a protein (http://genetics.bwh.harvard.edu/pph2/). Biochemical differences between the amino acids, such as charged versus uncharged side chains, can cause a loss of function. Furthermore, some genetic variations can lead to stop codons and premature truncation of the protein.

Intronic variants can also be pathogenic. Part of mRNA processing is the removal of introns and ligation of exons through RNA splicing (Wang et al., 2015). This process is dependent on the recognition of conserved, intronic sequences like the splice donor and acceptor sites which denote the boundary between intron and exon (Chen & Manley, 2009). RNA splicing is further aided by nearby exonic splicing enhancers (ESEs) that encourage recognition of splice sites by aiding in the recruitment of splicing proteins (Chen & Manley, 2009). Variants that occur within the intron-exon boundary or splicing enhancer sites can affect mRNA processing and the resultant protein.

Mutations at splice sites can inhibit the recognition of the intron-exon boundary, resulting in exon loss, intron inclusion or creation of alternative splice sites (cryptic splice sites) (**Figure 1.1**) (Ward & Cooper, 2010). Altered transcripts may create truncated proteins, contain incorrect amino acids or be targeted for nonsense-mediated decay (NMD). Overall, splicing mutations can result in a loss of function, reduce the amount of active protein or create an aberrant protein with dominant negative effects. Geneticists are able to empirically evaluate the effect of splice site mutations by examining mRNA transcripts. mRNA can be extracted from cells, reverse transcribed into cDNA (complementary DNA) and amplified by polymerase chain reaction (PCR) to evaluate splicing changes.

Several software programs have been developed that can predict splicing changes. Programs like GeneSplicer (http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml), Human Splicing Finder (http://www.umd.be/HSF) and MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) examine the variant's effect on recognition of the intron-exon junction, the removal of introns and splicing together of exons.

Researchers use a variety of molecular techniques to search for genetic variations, however the gold standard has been Sanger sequencing since its development in the mid-1970s. Sanger sequencing reads short sequences of DNA and can identify single nucleotide polymorphisms (SNPs) or small insertions or deletions. Despite the pivotal role it has played in genetic research, Sanger sequencing has a disadvantage in that it can

only effectively read sequences of less than 1,000 bp, meaning several reactions would be necessary to analyze a single gene making the process long and costly.

With thousands of possible disease genes, researchers needed a way to narrow the search region from the entire genome to a small number of candidate genes. For that reason, genome-wide SNP genotyping and linkage analysis became the basis for disease gene localization. Genotyped SNPs act as genetic markers for a particular locus or position on a chromosome creating a genetic map. By comparing the genotypes of affected and unaffected family members it was possible to see which SNPs were co-segregating with a particular phenotype. The region surrounding the SNPs was assigned a statistical measurement of linkage called a LOD (logarithm of odds) score. As the LOD score increased linkage was more likely, but as it decreased the gene and trait were less likely to be linked. Within Mendelian families, LOD scores greater than or equal to 3 are statistically significant. A couple of disadvantages of this method are that it requires multiple family members from different generations to be effective. It also requires careful phenotyping to ensure the correct disease status is assigned.

The powerful combination of linkage analysis and Sanger sequencing has been extremely effective in identifying disease-causing mutations. Ahmed and colleagues used the duo in 2004 to identify *TMPRSS3* mutations in a Newfoundland family (Ahmed et al., 2004). However, the methods are not foolproof. Misdiagnosis or the presence of phenocopies (samples that have a similar phenotype, but do not share the genotype) can greatly reduce the LOD score of a disease gene location (Abdelfatah, McComiskey et al., 2013).

Today, with access to next-generation sequencing (NGS) technology, whole exome sequencing and targeted-gene panels have become more commonplace. Exome sequencing involves large-scale multiplex reactions, interrogating thousands of exons and genes all at once. It has provided researchers with a quick and cost-effective way to sequence all the coding regions of the genome that harbor the majority of pathogenic mutations. Despite the amazing capabilities of exome sequencing, it is unable to detect large insertions/deletions and will often miss deep intronic mutations that can occur in regulatory regions. Furthermore, the presence of false-positives means variants identified by this method must still be confirmed or validated by a different method like Sanger sequencing. Additionally, NGS requires sophisticated analysis software, computing capacity and storage to overcome the large volumes of data generated.

Over the past few years, international collaborations have used NGS data to form multiple population and disease-specific genetic databases, which confer information about the frequency and pathogenicity of genetic variations. Databases like ClinVar (http://www.ncbi.nlm.nih.gov/clinvar) and OMIM (http://www.omim.org) help researchers and clinicians document and share their knowledge of the association between genetics and disease, while databases like the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/), Exome Variant Server (EVS) (http://evs.gs.washington.edu/EVS), 1000 Genomes (http://browser.1000genomes.org), and dbSNP (http://www.ncbi.nlm.nih.gov/snp) share variant frequency data. These sources help in the analysis of variants and indicate how common it is in the general 'healthy' population. Rare variants, i.e. those with a minor allele frequency (MAF) of less

than 1%, are more likely to be concerning since they have not been passed-on and allowed to accumulate in the population.

Most researchers will rely on information from multiple databases and bioinformatics tools to interpret the pathogenicity of a variant. For example, Alamut (Interactive Biosoftware V 2.7.1) integrates multiple public databases like ExAC and ClinVar with prediction tools like SIFT and PolyPhen.

In the last 150 years we have amassed a great volume of genetics data and seen a rapid advancement in computing capacity. For example, the first human genome took thirteen years to sequence whereas today it would only take one week (National Human Genome Institute, 2014). It is exciting to imagine what will be possible in the next few years and how it will increase our understanding of the relationship between human genetics and disease. As researchers better understand the mechanism of disease, they can implement preventative measures and develop new therapeutic targets.

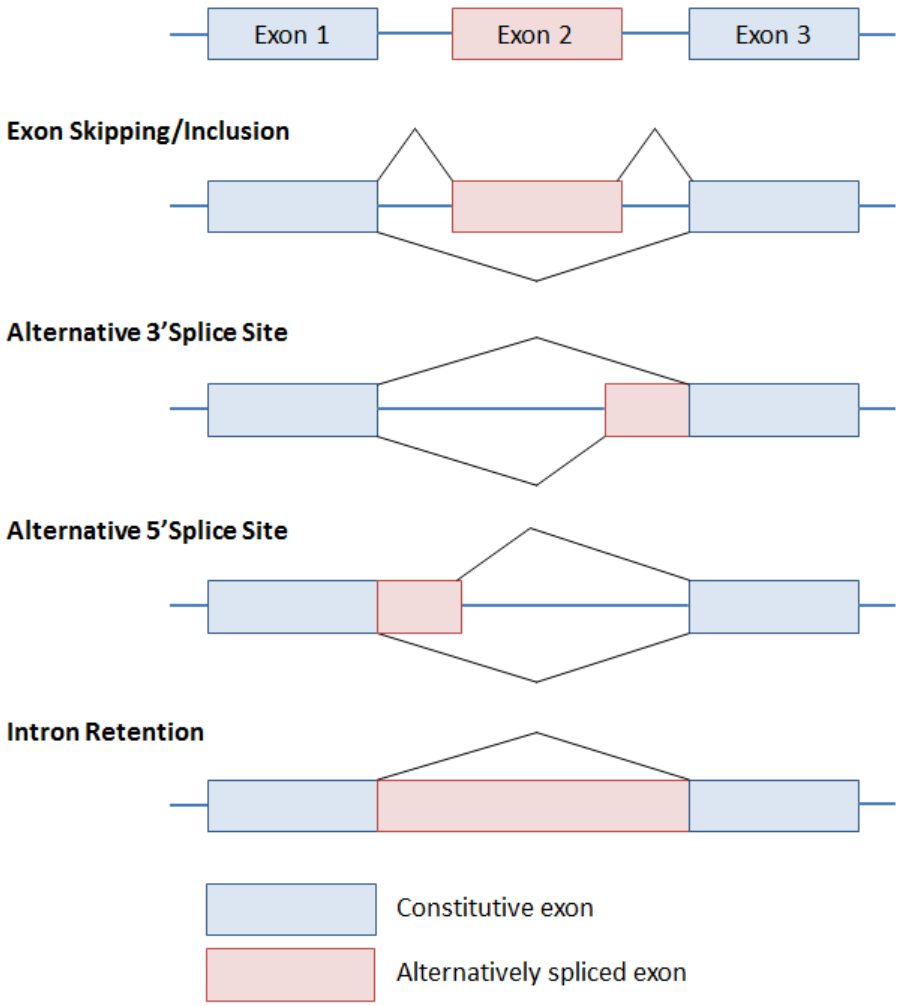


**Figure 1.1 Mechanism of Alternative Splicing**

mRNA transcripts can undergo constitutive (shown in blue) or alternative (shown in pink) exon splicing. Constitutive splicing simply removes all introns and ligates remaining exons. However, genetic variations at natural splice sites can cause alternative splicing resulting in exon skipping or inclusion, the creation of alternative splice sites or intron retention.

## Auditory Function and Hearing Loss

Hearing is one of the five major senses and is an important part of everyday life. Audition is the process of hearing and begins with sound waves or vibrations in the air that stimulate the auditory system. In essence, the human ear acts as an energy transducer and the ability to hear is dependent on mechanotransduction - the conversion of mechanical stimuli into electrical impulses, which the brain can then interpret. The human auditory system is very versatile and is able to process frequencies ranging from 20 – 20,000 hertz (Hz) and detect minor changes in frequency and intensity (Seikel, King, & Drumright, 2010).

The human ear has an external, middle and inner component, each playing a role in the perception of sound (**Figure 1.2)**. The structure of the external ear includes the auricle and ear canal. It collects sound waves, acting as a funnel and directs them toward the middle ear. The structure of the middle ear includes: the tympanic membrane (ear drum), tympanic cavity, Eustachian tube, and the ossicular chain (the bones of the middle ear: the malleus, incus, and stapes). The middle ear transmits sound waves from the external to inner ear. Sound waves strike against the tympanic membrane and send vibrations through the ossicular chain and into the inner ear. The organ of the inner ear involved in hearing is the cochlea. The cochlea converts the mechanical energy of sound waves into electrical impulses that are then relayed by the 8$^{th}$ cranial nerve (vestibulocochlear nerve) to the brain stem and auditory cortex in the temporal lobes of the brain for processing (Ervin, 2014).

The cochlea is a complex structure and a critical component of the auditory apparatus (**Figure 1.3**). The cochlea has three fluid-filled chambers: two outer chambers filled with perilymph and an inside chamber filled with endolymph. Lining the inside chamber is the basilar membrane and the Organ of Corti. The Organ of Corti is the primary sensory organ of the cochlea and is lined with thousands of hair-like projections called cilia (commonly referred to as hair cells), which facilitate the mechanotransduction process (**Figure 1.4**). At the apical surface of hair cells are bundles of mechanically sensitive stereocilia, organized into staircases of decreasing height. At the tips of stereocilia are cation-selective mechanotransduction channels, which open upon deflection by mechanical stimuli and facilitate an electrochemical response (Muller, 2008). They are connected to nerve fibers which relay sensory information to the brain (Ervin, 2014).

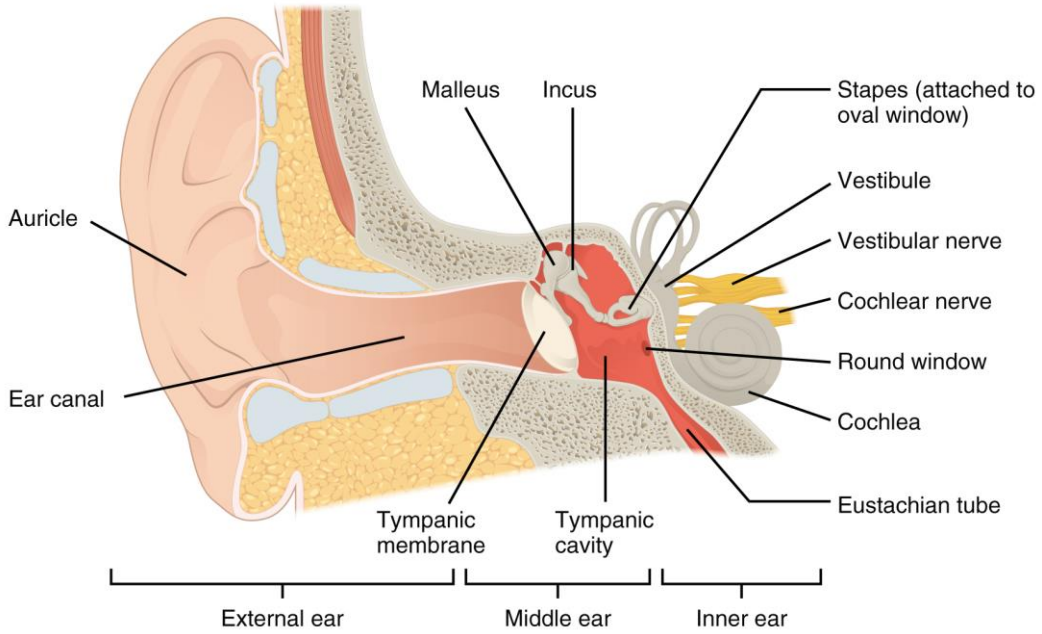


**Figure 1.2 Basic Structure of the Human External, Middle and Inner ear.**

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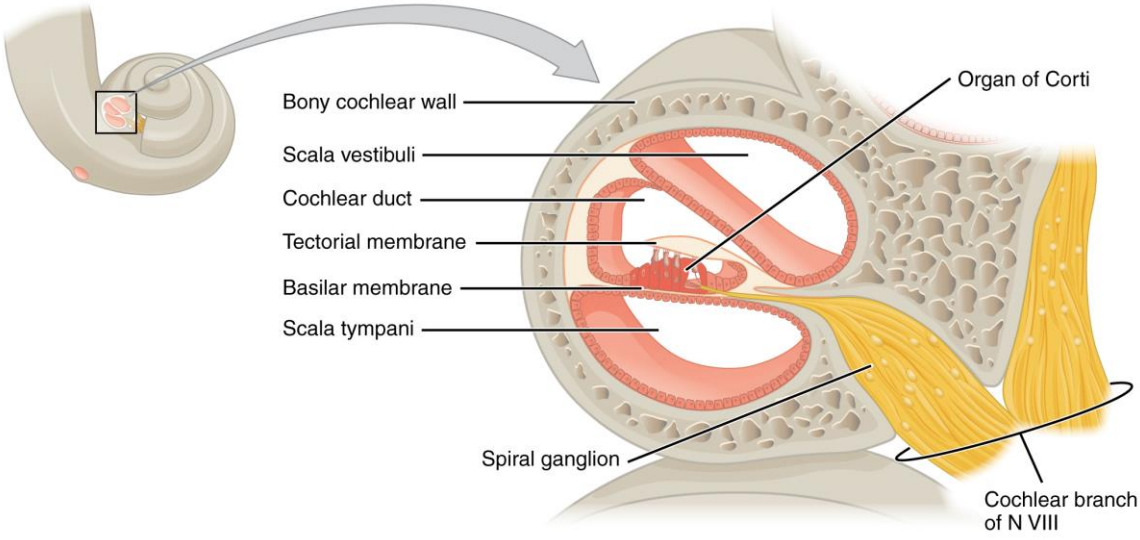


**Figure 1.3 Detailed Structure of the Human Inner Ear**

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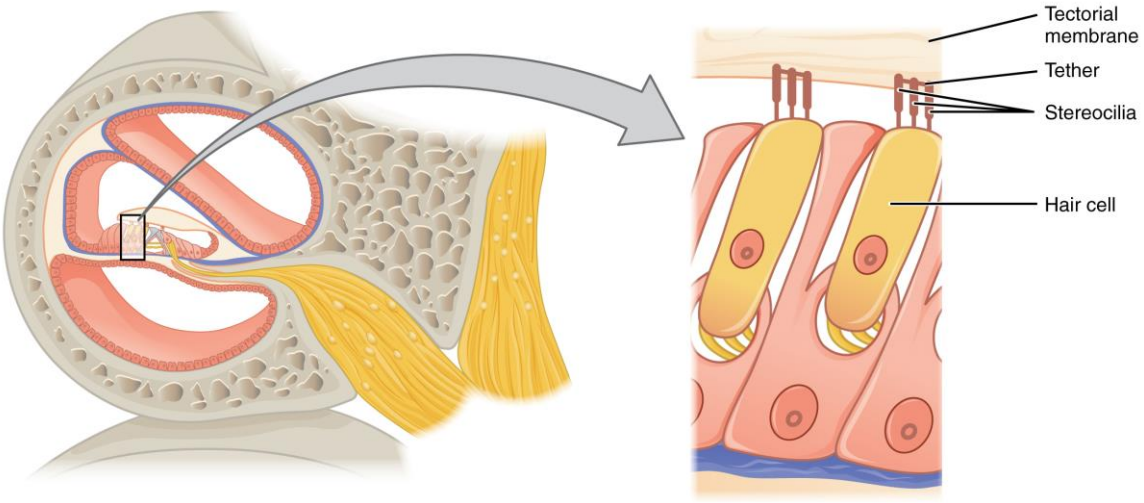


**Figure 1.4 Structure of a Human Hair Cell of the Inner Ear**

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Hearing loss is the most common sensory defect and affects more than 870,000 Canadians aged 15 and older (Bizier, Contreras, & Walpole, 2016). Additionally, 360 million people worldwide have hearing loss and approximately 32 million of these are children (World Health Organization, 2017a). The practice of audiology encompasses the identification, differential diagnosis and the treatment of hearing loss and balance disorders. Audiologists rely on a number of objective and behavioral exams to assess a person's auditory function. Part of that assessment is thinking of sound as both a physical phenomenon and a psychological experience.

Sound is generated by vibrations that travel through the air as pressure waves. Each wave consists of a region of compression, where air molecules are pushed together, and refraction, where they pull apart. A single compression and refraction is called a cycle and the number of cycles per second is the wave frequency. A single frequency is defined as a pure-tone and is measured in hertz (Hz). Another characteristics of sound waves is amplitude. "Loud sounds" apply a greater force to air molecules, leading to greater compression/refraction, increasing the intensity and amplitude of the waves. The amplitude is measured in decibels (dB) and is expressed on a logarithmic scale (Martin & Clarke, 2014).

Audiologists use their knowledge of the physical characteristics of sound such as pure-tone measurements to test auditory function. However, other features like sound separation, perception and interpretation of sound, and the ability to distinguish unique sounds from background noise are also important. Audiological exams can be behavioral, and are influenced by patient response such as pure-tone audiometry and speech

audiometry, or objective like Auditory Brainstem Response (ABR), otoacoustic emissions (OAEs) and immittance audiometry.

Pure-tone audiometry assesses a patient's hearing sensitivity. Although humans are capable of perceiving frequencies between 20 to 20,000 Hz, audiologists will often test between 125 and 8000 Hz - the frequencies most important to human speech and everyday activities. To measure air conduction (AC) thresholds, pure-tones are played through head phones at increasing decibel levels (to a maximum level) until a patient indicates the sound was heard. The audiologist is trying to determine a person's auditory threshold or the level at which a sound can only be perceived 50% of the time and is barely audible. For this reason, testing is usually done in a sound-isolated chamber since noise can mask and shift a person's hearing threshold. The minimum detectable intensity (referred to as hearing sensitivity) is measured in both the left and right ear independently and plotted on a graph (audiogram) and expressed as the number of decibels above or below the average normal threshold (defined as 0 dB HL).

An air conduction (AC) audiogram shows the hearing sensitivity for pure-tones and can be used to show sensorineural hearing loss. The frequency is measured along the X-axis and the intensity along the Y-axis. Within the audiogram, AC thresholds for the left ear are marked using X's and for the right ear using O's. These can be compared to normal hearing thresholds, which are less than 20 dB HL across all frequencies (125-8000Hz) (**Figure 1.5**).

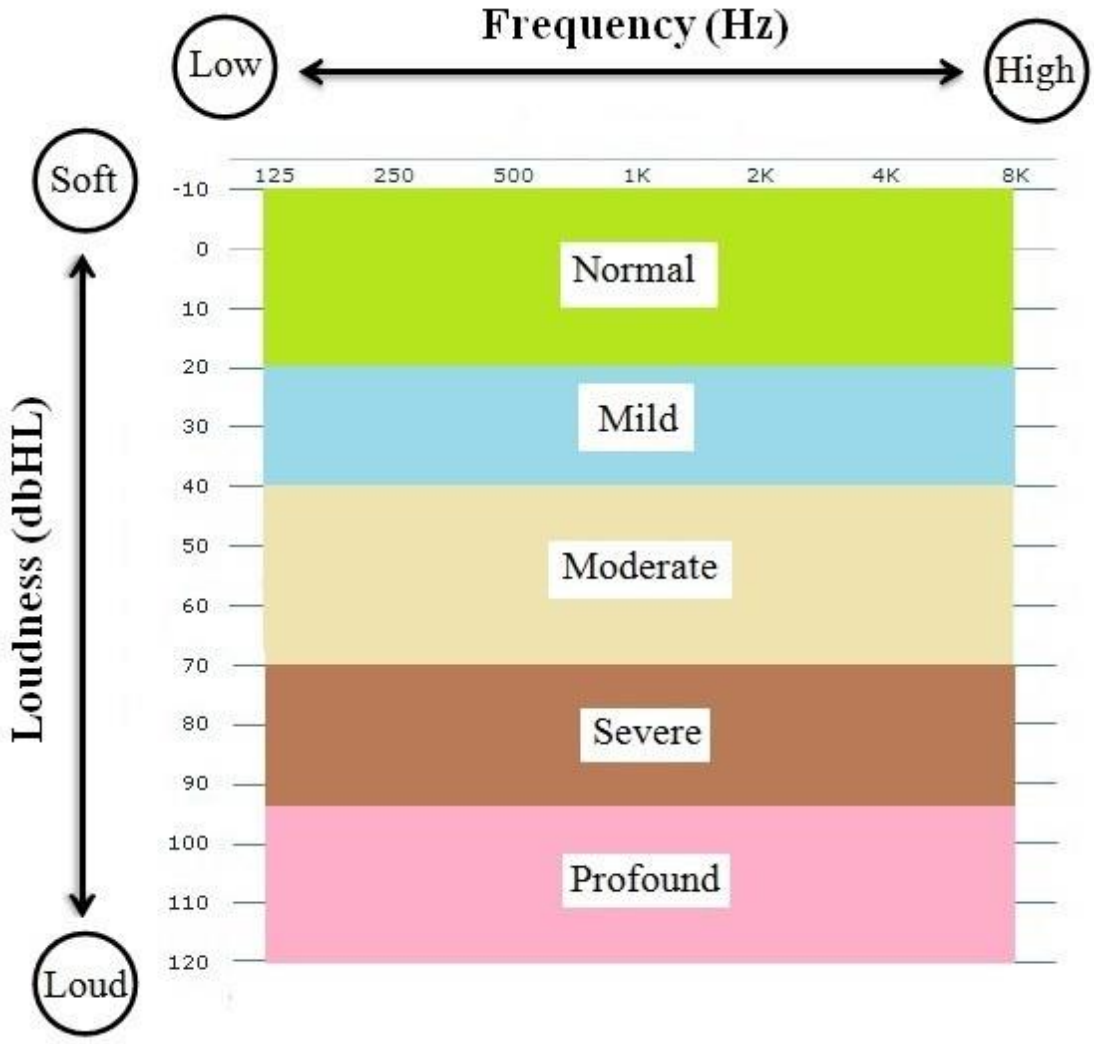


**Figure 1.5 Audiogram Depicting the Characterization of Hearing Loss Severity**

Hearing thresholds that are less than 20 dB HL are within normal hearing limits. Mild hearing loss has hearing thresholds between 20-40 dB HL. Moderate hearing loss has hearing thresholds between 41-70 db HL. Severe hearing loss has hearing thresholds between 41-95 db HL. Profound hearing loss has hearing thresholds in the excess of 95 db HL.

Speech audiometry measures a patient's speech-recognition threshold. It uses two syllable words like "baseball" to assess the lowest level at which speech can be understood. Words that contain high frequency sibilants like "s" "f" and "h" are more difficult to understand than low frequency sibilants like "m" "d" and "b". Speech audiometry provides a method of comparing speech comprehension to pure-tone thresholds. Poor correlation between the two may suggest a neural component to the hearing loss.

Auditory evoked responses potentials (AEPs) are physiological measures of auditory function and can be used to estimate hearing sensitivity in infants and those unable to participate in behavioral audiometric tests. AEPs are derived from the cochlear hair cells and auditory nerve. For example, the Auditory Brainstem Response (ABR) is one type of AEP that is used to measure signals originating in the $8^{th}$ cranial nerve and the brainstem, and provide insight into neural integrity. Additional tests, such as otoacoustic emissions (OAEs) and immittance audiometry that evaluate the inner and middle ear functions respectively, can aide in the differential diagnosis of hearing loss.

Assessments of the patient's height, craniofacial and musculoskeletal features, neurological function, external ear abnormalities and their hair and skin may also be included (Toriello & Smith, 2013). These examinations help to rule out syndromic hearing loss that occurs with symptoms affecting other parts of the body. For example, Usher syndrome, type 1F (OMIM 602083) is a disorder characterized by both vision loss and hearing loss. Wolfram syndrome (OMIM 222300) affects multiple systems and is characterized by having diabetes mellitus, diabetes insipidus, optic atrophy and deafness.

In Canada, hearing loss can be diagnosed at birth through the newborn hearing screening program. If hearing loss develops during childhood it can be diagnosed by a public health nurse before entry into school. However, if hearing loss develops later in adulthood it may be diagnosed through a workplace screening test. Sometimes if the impairment is severe enough, patients may self-refer for assessment. One of the problems in diagnosing hearing loss is that it can progress slowly and may be masked as many people may use visual cues and speech reading to adapt. Families with multiple cases of hearing loss may have trouble recognizing their hearing thresholds as abnormal. Therefore, it is critical to obtain up-to-date audiological tests and not rely on self-report.

Hearing loss phenotypes can be described by type, onset, progression, severity, configuration and the presence of other auditory dysfunctions. Guidelines have been established for describing hearing loss phenotypes based upon audiological data and are called the GENDEAF recommendations (**Appendix A**)

There are four different types of hearing loss: conductive, sensorineural, mixed and central auditory dysfunction. Conductive hearing loss is caused by abnormalities of the outer or middle ear, whereas sensorineural hearing loss is caused by abnormalities of the inner ear or auditory nerve. Mixed hearing loss is a combination of both and has conductive and sensorineural components. Meanwhile, central auditory dysfunction is associated with the central nervous system and affects the transmission and processing of auditory information (Smith, Shearer, Hildebrand, & Van Camp, 2014). Hearing loss can also present with other auditory dysfunctions such as tinnitus (described as a “ringing” in the ears) or vestibular dysfunction like vertigo (Van Camp & Smith, 2014).

The age of onset of hearing loss can be prelingual or post-lingual. Prelingual hearing loss occurs prior to speech development, typically before the age of 3. Post-lingual hearing loss occurs after the development of speech (Smith et al., 2014).

The severity of hearing loss can be mild, moderate, severe or profound (**Figure 1.5**). For example, a person with mild hearing loss would have difficulty hearing and understanding soft speech like a whisper. A person with moderate hearing loss would have difficulty hearing regular speech, while a person with severe hearing loss may only hear very loud speech or environmental sounds like a truck siren. Lastly, a person with profound hearing loss may only perceive loud sounds as vibrations (World Health Organization, 2017b)

The configuration of the hearing loss can be low, mid or high frequency. High frequency configurations can be further described by their slopes as flat, gentle or steep (**Figure 1.6).** Either one ear (unilateral) or both ears (bilateral) may be affected. If both are affected the hearing loss can be symmetric with similar hearing loss in both ears or asymmetric (Van Camp & Smith, 2014). Over time, the hearing loss may progress and become more severe, highlighting the importance of longitudinal data, or it may remain stable. To assist in these classifications, the GENDEAF recommendations provide calculations to determine the severity, configuration, and progression of hearing loss in the participants (**Appendix A**).

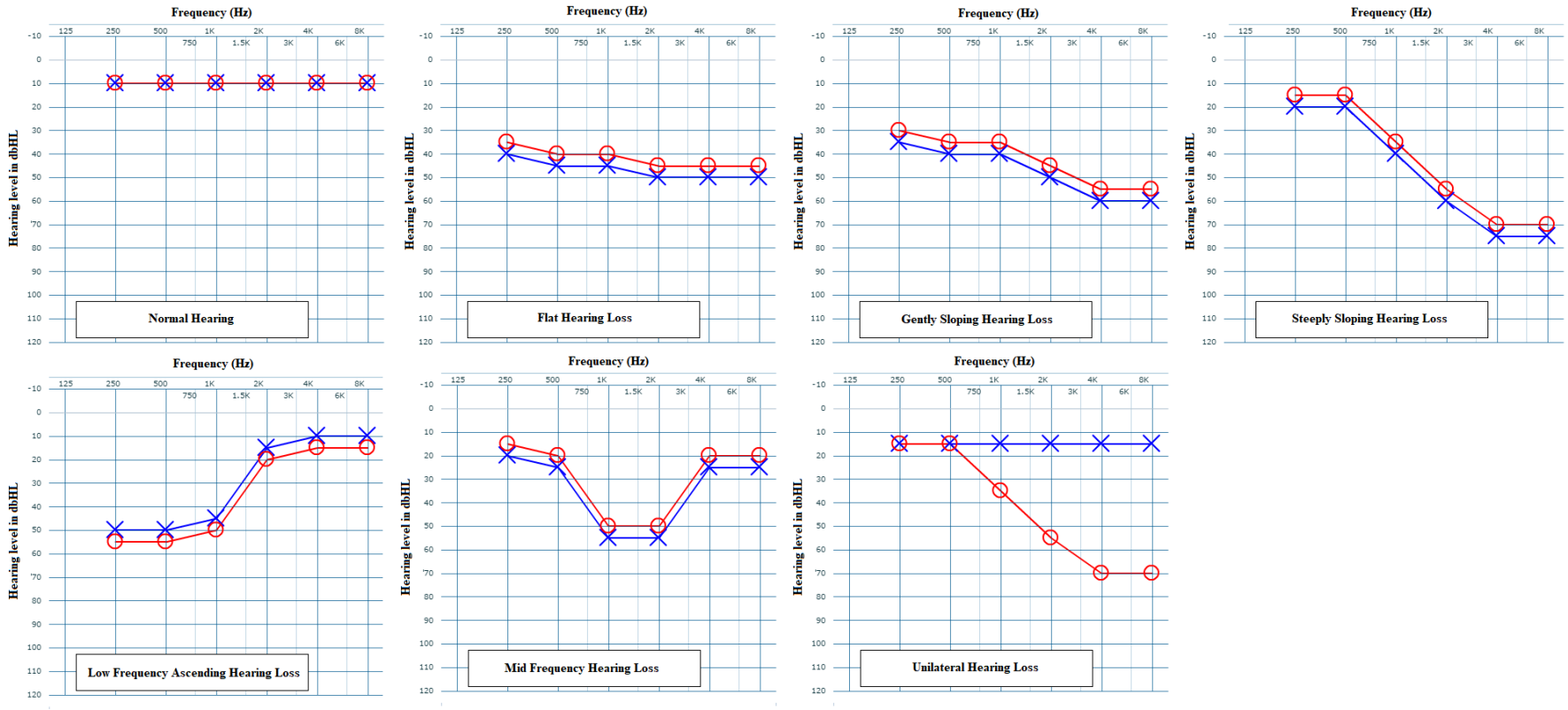


**Figure 1.6 Audiograms Depicting Normal Hearing and Examples of Hearing Loss.**

The red lines indicate the hearing thresholds of the right ear and blue lines the left ear.

The top left audiogram shows normal hearing, then to the right and below are examples of different configurations of hearing loss: flat, gently sloping, steeply sloping, low-frequency ascending (reverse slope), mid frequency (cookie-bite) and unilateral. The audiograms were generated using AudGen (http://audsim.com/audgen/) an online audiogram graphics program.

Hearing loss is clinically and genetically heterogeneous with a variety of etiologies including genetics, ototoxic medications, noise exposure and aging (Bramhall, Kallman, Verrall, & Street, 2008). Hearing loss can be a monogenic disease caused by a single-gene mutation, or multifactorial with both genetic and environmental factors affecting the phenotype. However, a study of school-aged children in the United States found that up to 60% of prelingual or early-onset hearing loss was due to genetic factors (Marazita et al., 1993).

Hereditary hearing loss can be syndromic presenting with symptoms involving other organ systems, or nonsyndromic having no other associated medical disorders (Smith et al., 2014). Nearly 30% of hereditary hearing loss is syndromic and has a recognizable pattern like Usher or Wolfram syndrome. The more predominant form, accounting for 70% of cases, is nonsyndromic hearing loss (NSHL). Researchers have identified more than 158 nonsyndromic deafness loci and greater than 96 deafness-associated genes (http://hereditaryhearingloss.org, 2017).

Nonsyndromic hearing loss is inherited in an autosomal recessive (80%), autosomal dominant (15%), sex-linked (1%) or matrilineal (1%) pattern (Toriello & Smith, 2013). The mode of inheritance is most likely autosomal dominant if each generation is affected and both genders are equally likely to have hearing impairment. Affected family members are typically heterozygotes (inherited one copy of a mutation) and have a 50% chance of passing the deleterious allele onto their offspring. Nonetheless, knowing the genotype is not enough to predict the phenotype. Variable expression and reduced penetrance also play a role. Variable expression refers to the potential for symptoms to differ among people with the same genotype. For example, the age of onset

and level of severity of hearing loss may vary. Penetrance refers to the probability that a genotype will produce a given phenotype. In the case of incomplete penetrance, not all family members with a pathogenic allele will have hearing loss. Other genetic factors and environmental factors (infection, acoustic trauma, and ototoxic drugs), referred to as modifiers can play a role in the hearing loss phenotype. Often the contribution of modifiers is difficult to distinguish from the inherited phenotype. However, understanding the relationship between the underlying genetic cause and potential modifiers is essential to predicting the ultimate phenotype. Furthermore, a hearing loss phenotype can have of combination of etiologies like age-related hearing loss (presbycusis) that occurs gradually as we age and noise-induced hearing loss that is caused by long-term exposure to sounds that are too loud for too long, causing damage to the sensory hair cells.

The correct classification of hearing loss is critical to the study of autosomal dominant families. Variable expression, incomplete penetrance and the presence of phenocopies hamper efforts to study autosomal dominant hearing loss. Through careful analysis of audiological records, team audiologists work to establish the onset, progression, and severity of the family's hereditary hearing loss to distinguish it from other unrelated etiologies.

The mode of inheritance is most likely autosomal recessive if there are only a few hearing-impaired family members. Autosomal recessive disorders require two mutant alleles to be inherited for a phenotype to be expressed and so heterozygotes (carriers) are unaffected. Assuming complete penetrance, parents who are carriers have a 25% chance of having a child who is affected, and as a result typically several generations often pass before two carriers have an affected child.

The mode of inheritance is most likely X-linked if the distribution of males and females with hearing loss is uneven. This occurs because males have an X and Y chromosome whereas females have two X chromosomes. Since males receive their Y chromosome from their father, X-linked inheritance is not possible between father and son. Assuming complete penetrance and dominant X-linked inheritance, an affected father will have all affected daughters but none of his sons will be affected. If the disorder is X-linked recessive an affected mother will have all affected sons but her daughters will only be carriers.

The mode of inheritance is most likely mitochondrial if hearing loss appears to be transmitted maternally. Mitochondria are small organelles in the cytoplasm that are responsible for producing energy in the cell and are transmitted maternally through the ova. Mitochondria have their own DNA (mtDNA), which is 16,569 bp in length (NCBI Reference Sequence: NC_012920.1). An important feature of mitochondrial genetics is heteroplasmy and homoplasmy. Depending on the cell type, each cell has hundreds to thousands of mitochondria. Homoplasmy means all the copies of the mitochondrial genome are the same. Heteroplasmy means there is a mixture of mitochondrial genotypes. The amount of mutant mtDNA becomes important because there is a threshold at which normal mitochondria can compensate for those that are mutated. Mitochondrial mutations affect the energy production of cells and often have a greater impact on cell types with high energy demands like skeletal muscle, heart, eye, ear, and brain resulting in muscle weakness, nervous system disorders, visual problems, hearing loss, and dementia (Keats, Popper, & Fay, 2002).

## The Newfoundland Population and Genetic Research

Over the past 30 years the Newfoundland population has become a focus of human hereditary disorder research. As a founder population it has an advantage in identifying disease genes linked to rare monogenic disorders over more heterogeneous (ethnically diverse) populations. Compared to other founder populations, Newfoundland is relatively young (<20 generations) making it an ideal population for the study of the genetic etiologies of many diseases (e.g. Mendelian and complex).

Viking settlers arrived in Newfoundland around 1000 AD but abandoned their settlements. Europeans then rediscovered the island in 1497 when an Italian explorer named Giovanni Caboto (John Cabot) sailed to Newfoundland. The first English colony was established in 1610. Newfoundland's population continued to grow as settlers immigrated to the island, drawn by the fishing industry. The majority of immigrants were Protestant settlers from Southwest England and Roman Catholic settlers from Southern Ireland (Mannion, 1977).

Over the past 400 years Newfoundland and Labrador's population has increased to 528,817. The majority of residents live in rural communities spread over Newfoundland and Labrador's (NL) vast territory of 370,510.76 square kilometers and nearly 29,000 kilometers of coastline (Statistics Canada, 2012). Geographic and religious segregation has led to the formation of genetic isolates with high inbreeding coefficients. Studies by Bear and colleagues in 1987 and 1988 found a high degree of genetic homogeneity (Bear et al., 1987; Bear et al., 1988). A recent study found the Newfoundland population resembles the British population but can be divided into three

clusters of religious or ethnic origin: Protestant English, Roman Catholic Irish and North American aboriginals (Zhai et al., 2015).

Several genetic disorders and causative mutations are more predominant in the Newfoundland population than in its ancestral European population. For example, Bardet-Biedl syndrome is 10 times more common in the Newfoundland population than admixed Caucasian populations of northern European ancestry (Green et al., 1989; O'Dea et al., 1996). Also, Hereditary Non-Polyposis Colon Cancer (HNPCC) is caused by a single mutation in *MSH2* in 50% of cases of HNPCC in Newfoundland, while it accounts for only 8% of English HNPCC cases (Spirio et al., 1999).

Founder populations like Newfoundland have a unique advantage in mapping and identifying rare disease genes (Kristiansson, Naukkarinen, & Peltonen, 2008). The limited number of founders means disease alleles will have often arisen from a single ancestral chromosome. Furthermore, Newfoundland families are large with deep genealogies and participation from multiple generations. Many of the papers published from other parts of the world are based upon probands or trios (child, mother and father), rather than whole families. There is an undeniable advantage in being able to test co-segregation of mutations and phenotype in a large family versus relying on a single person. By examining the genetic differences between affected and unaffected family members, who share much of their DNA sequence, geneticists are better able to narrow-in on the true causative mutations.

Newfoundland has been a rich resource for genetic discoveries, especially in regards to hereditary hearing loss. The Newfoundland Hereditary Hearing Loss project began with Dr. Elizabeth Ives, who helped establish the Newfoundland Provincial

Genetics Program and was the first professor of Genetics at MUN's Faculty of Medicine (Gray, 2002). In the late 1980s, Dr. Catherine Neville-Smith, the Medical Health Officer of the Central Health Unit, approached the Genetics program about the high frequency of deafness in the Gaultois area. A subsequent visit to the Newfoundland School for the Deaf by Dr. Ives and Dr. Neville-Smith provided further insight into the high occurrence of severe childhood deafness on the South Coast of Newfoundland. These collaborative efforts between the research team, local health authorities and the Newfoundland School for the Deaf was the foundation of the Newfoundland Hereditary Hearing Loss study

In 1999, Dr. Terry-Lynn Young, a PhD candidate within the Discipline of Genetics joined the Newfoundland Hereditary Hearing Loss Project. Since its launch, more than 170 families with varying degrees and types of hearing impairment have been recruited, first through the School for the Deaf (since closed), then as a result of a public campaign through ENT (Ear Nose and Throat) clinics, Provincial Medical Genetics, Provincial Audiology, CHHA (Canadian Hard of Hearing Association) and self-referrals. Detailed medical histories were collected, including previous audiograms, and if possible a current hearing assessment was completed. Further interviews generated family pedigrees, developed family histories and lead to the recruitment of other family members. Having additional members from multiple generations provided researchers with the ability to distinguish the truly pathogenic mutations from the many thousands of variants of unknown significance (VUS).

The pedigrees were created in Progeny (http://www.progenygenetics.com/). The specialized software provided a visual representation of each family pedigree. It also served as a database to store patient and family history information. At MUN the current

configuration is a web-based interface that is stored, administered, and maintained by the Centre for Health Informatics and Analytics (CHIA).

The Hereditary Hearing Loss Project's early scientific approach relied on molecular genetics techniques like genome-wide SNP genotyping and linkage analysis, followed by cascade gene sequencing to solve the first hearing loss families. Later, with the advent of NGS technology, techniques like whole exome sequencing played a larger role in mutation identification.

One of the first families to be solved was 'Family C', a six-generation family from the South Coast of Newfoundland with an apparent autosomal dominant form of low frequency hearing loss. Over several years of clinical work more than 300 family members were recruited, many affected with low frequency, sensorineural hearing loss. Employing an autosomal dominant mode of inheritance and genome-wide SNP genotyping and linkage analysis, Dr. Young and her team identified a region associated with the hearing loss phenotype on chromosome (chr)4p16 (LOD=11.58). This region overlapped with three previously mapped deafness loci: *DFNA38*, *DFNA14*, and *DFNA6*. Sanger sequencing of *WFS1* yielded 11 variants: 5 polymorphisms and 6 novel variants. Only one co-segregated with hearing loss: *WFS1* c.2146G>A, p.Ala716Thr resulting in the substitution of a conserved amino acid. Furthermore, it was absent from 150 controls. Typically homozygous mutations in *WFS1* cause Wolfram syndrome and this was the first association of isolated hearing loss and a single heterozygous mutation in *WFS1* (T. L. Young et al., 2001).

In 2004, another large kindred (Family B) from the South Coast of Newfoundland with hereditary hearing loss was solved. However, this family had a different clinical

presentation and segregated with apparent autosomal recessive, pre-lingual, severe to profound sensorineural hearing loss. Linkage analysis revealed *DFNB8/B10* was linked to hearing loss in Family B. Subsequent sequencing of *TMPRSS3*, a deafness gene residing within the mapped locus, identified two deletion mutations: *TMPRSS3* c.207delC and c.782+3delGAG. Careful segregation analysis showed that both mutant alleles co-segregated with hearing loss in Family B, where the majority of affected relatives inherited two copies of c.207delC and two affected relatives were compound heterozygotes (Ahmed et al., 2004).

In 2009, another large family (Family A) from the South Coast of Newfoundland with hereditary hearing loss was solved. Like Family B, this family presented with apparent autosomal recessive, pre-lingual, profound sensorineural hearing loss. Due to their close geographic location, they were initially screened for the three pathogenic mutations identified in Families B and C, but were negative. Therefore, further studies were needed. Genome-wide SNP genotyping and linkage analysis mapped the hearing loss to chr10q21-22 (LOD=4.0). The linked region included a previously mapped deafness locus, *DFNB23,* and a candidate gene, *PCDH15,* within this region on chromosome 10q. Sequencing of *PCDH15* revealed 33 sequence variants. However, only the *PCDH15* c.1583T>A, p.V528D mutation was also absent from the population controls. The novel mutation within *PCDH15* affected a highly conserved amino acid and was predicted to be deleterious (Doucette et al., 2009).

The Southern Coast of Newfoundland is geographically isolated with limited road access and ferry services, leading to the enrichment of rare pathogenic mutations in the communities. So far, four different mutations causing hearing loss on the Southern Coast

of Newfoundland have been identified by the Young lab: *WFS1* c.2146G>A, *TMPRSS3* c.207delC and c.782+3delGAG, and *PCDH15* c.1583T>A.

Autosomal recessive and dominant patterns of hearing loss are not the only types observed in our collection of Newfoundland families. In 2012, Family 2024 presented with the hallmarks of X-linked hearing loss such as absence of male-to-male transmission and an earlier-onset, more severe phenotype in males compared to females (Abdelfatah, Merner et al., 2013). Because this is an X-linked condition, only genes on the X chromosome were examined. Haplotype analysis of informative markers residing on the X chromosome on a subset of the family members narrowed the region of interest to 48 candidate genes, 13 of which were sequenced based on known mRNA expression, protein function, and deafness-association. Sequencing yielded the novel deletion *SMPX* c.99delC, which caused a frameshift and premature stop codon p.Arg34GlufxX47. The mutation co-segregated with hearing loss in Family 2024. Subsequent screening of the Newfoundland deafness probands showed a second family, Family 2196, which harbored the deletion and pattern of hearing loss. Haplotype analysis further demonstrated the same mutation resided on a shared haplotype across the two families, suggesting a common ancestor.

As more families were recruited to the deafness research study, a targeted mutation-screening protocol was developed and used as a ‘first pass’ for all new probands recruited to the study. Each affected proband (the first family member recruited) was screened for the most common hearing loss mutations, such as *GJB2* c.35delG and a large deletion in *GJB6*, followed by previously reported Newfoundland deafness mutations that included *WFS1* c.2146G>A, *TMPRSS3* c.207delC and c.782+3delGAG, *PCDH15*

c.1583T>A, and *SMPX* c.99delC. Probands whose hearing loss could not be attributed to these known deafness mutations were subsequently categorized based upon the configuration of their hearing phenotype or audioprofile.

In 2012, this clinical work became the foundation of the Audioprofile study. Eighty-two probands were selected and grouped based upon the configuration of their hearing loss: low, mid or high. Audiograms with similar profiles were grouped and submitted to AudioGene (http://audiogene.eng.uiowa.edu/), an online program that uses audiometric data such as frequency affected, hearing loss in decibels, and age to identify gene-specific types of hearing loss. For this next phase of the study, genomic DNA from deafness probands underwent full gene sequencing for each gene predicted. For example, probands with low frequency hearing loss were tested for mutations in *WFS1, POU4F3,* and *DIAPH*. Those with mid frequency loss were Sanger sequenced for *TECTA* and *COL11A2*. Lastly those with high frequency loss were sequenced for *KCNQ4, COCH, MYO6, GJB3,* and *TMC1*. Variants identified through sequencing were checked against prediction programs to assess pathogenicity, tested for co-segregation with hearing loss in affected families, then screened in ethnically-matched population controls to determine the frequency in the Newfoundland population. Part of this work was completed by Ms. Jessica Squires, a Master's student who deduced the cause of hearing loss in two families with *MT-RNR1* m.1555A>G mutations (Squires, 2015) and Mr. David McComiskey, a Master's student who solved a family with a *COCH* c.151 C>T mutation (McComiskey, 2010).

In another example, the investigation of Family 2071 also began with AudioGene (Abdelfatah et al., 2013). In 2013, familial audiograms were submitted to AudioGene,

which linked them to known high-frequency deafness genes like *COCH, KCNQ4*, and *TMC1*. Candidate gene sequencing of *KCNQ4* revealed a possible pathogenic mutation at c.806_808delCCT, p.S269del, although only half of the deaf-relatives (10/23) harbored the mutation. Furthermore, linkage analysis of the region surrounding the mutation had negative LOD scores suggesting the region was not associated with hearing loss in Family 2071. It was also absent in the unaffected relatives and 90 ethnically-matched population controls. However, the mutation did not co-segregate with hearing loss on the left-hand side of the pedigree.

Perhaps there existed two or more genetic factors contributing to the phenotype in this large multigenerational family, with *KCNQ4* c.806_808delCCT being the cause of hearing loss on one side of Family 2071. As a result the study changed focus to the potential phenotypic differences between the two halves of the pedigree. Close examination of familial audioprofiles showed that *KCNQ4* deletion carriers had bilateral, sloping sensorineural hearing loss while non-carriers had variable symmetry and configuration. As confirmation, the linkage analysis was repeated excluding members with differing audioprofiles and resulted in a statistically significant LOD score of 3.3. This cemented the theory that there were at least two factors contributing to hearing loss in Family 2071, with *KCNQ4* c.806_808delCCT being the cause of hearing loss in half of the family.

Another family, Family 2010, was highlighted during the Audioprofile study because of its unique audiometric configuration. The characteristic pattern of normal low-frequency thresholds that steeply slope to severe bilateral, sensorineural hearing loss was unusual. In 2017, the Young lab took an extended field trip to revisit the family. With the

new blood samples and pedigree data collected, a whole exome sequencing strategy was carried out. A PhD candidate, Mr. Justin Pater identified a homozygous mutation in *CLDN14* c.488C>T, p.Ala163Val. Three unrelated families (Family 2033, 2072, and 2075) were later shown to also harbor the exact same *CLDN14* c.488C>T mutation. Genealogical studies and haplotype analysis across the four unrelated families showed a shared 1.4 Mb (megabase pairs) haplotype on chromosome 21 (*DFNB29*), suggesting common ancestry. Subsequent interviews by a genetic counselor would connect families 2010, 2033, and 2075 to a single founding couple six generations back. Interestingly, targeted sequencing of 175 population controls would indicate that the frequency of *CLDN14* c.488C>T is much higher in the Newfoundland population than worldwide (1% versus 0.02564%), signifying a possible founder effect (Pater et al., 2017)

The Hereditary Hearing Loss Project also focused on specific conditions like otosclerosis. Otosclerosis is a disease of the bones of the middle and inner ear causing conductive or mixed hearing loss. In 2014, Dr. Nelly Abdelfatah's PhD research in the Young lab identified the first otosclerosis gene, *FOXL1* (T. Young, Abdelfatah, & Griffin, 2014). Through a combination of linkage analysis and candidate gene sequencing she identified a novel 15 bp deletion, *FOXL1* c.976_990del, in all affected members of Family 2081, and thus the first ever identified otosclerosis gene.

Genetic factors have made a significant impact on hearing loss in the Newfoundland population. Over the past two decades, the Newfoundland Hearing Loss Project has reported 12 different mutations in 11 genes that cause hearing loss (**Table 1.1**). Contained within the Newfoundland population are mutations like *GJB2* c.35delG and a large 342kb deletion in *GJB6* which have been described frequently in the

literature. However, there are also novel mutations in known hearing loss genes like *WFS1* (T. L. Young et al., 2001) and *PCDH15* (Doucette et al., 2009). Most interestingly, there are mutations in genes that have never been associated with hearing loss, such as *FOXL1* c.976_990del (T. Young, Abdelfatah, & Griffin, 2014). It is likely that the Newfoundland population will be key to discovering more deafness loci, genes and pathogenic mutations.

As more deafness-associated mutations were identified (**Table 1.1**), the Newfoundland deafness probands were screened in the hopes of solving other families. The proband of Family 2070 was screened, but his results were negative, suggesting a novel genetic etiology in the Newfoundland population. Since Family 2070 had participation from multiple generations and several affected family members it was an ideal candidate for further study and so the investigation of Family 2070 became the focus of this thesis (**Figure 1.7**).

**Table 1.1 Reported Newfoundland Deafness Mutations to Date November 11th, 2017**

| Gene | Accession Number | Mutation DNA Level | Mutation Protein Level | Type | Inheritance Pattern | Number of Newfoundland Families | References |
|---|---|---|---|---|---|---|---|
| *MT-RNR1* | NC_012920 | m.1555A>G | - | Substitution | Mitochondrial | 2 | (Prezant et al., 1993) |
| *GJB2* | NM_004004 | c.35delG | p.Gln10Hisfs*11 | Deletion | Autosomal Recessive | 8 | (Carrasquillo, Zlotogora, Barges, & Chakravarti, 1997) |
| *COCH* | NM_004086 | c.151C>T | p.Prp51Ser | Substitution | Autosomal Dominant | 1 | (de Kok et al., 1999) |
| *WFS1* | NM_006005 | c.2146G>A | p.Ala716Thr | Substitution | Autosomal Dominant | 1 | (T. L. Young et al., 2001) |
| *TMPRSS3* | NM_024022 | c.208delC | p.His70Thrfs*19 | Deletion | Autosomal Recessive | 1 | (Wattenhofer et al., 2002) |
| *GJB6* | NM_006783 | Del (*GJB6*- D13S1830) | - | Large Deletion | Autosomal Recessive | 4 | (del Castillo et al., 2002) |
| *TMPRSS3* | NM_024022 | c.782+3delGAG | - | Deletion | Autosomal Recessive | 2 | (Ahmed et al., 2004) |
| *PCDH15* | NM_033056 | c.1583T>A | p.Val528Asp | Substitution | Autosomal Recessive | 1 | (Doucette et al., 2009) |
| *KCNQ4* | NM_004700 | c.806_808delCCT | p.Ser269del | Deletion | Autosomal Dominant | 1 | (Abdelfatah et al., 2013) |
| *SMPX* | NM_014332 | c.99delC | p.Arg34Glufs*47 | Deletion | X-Linked | 2 | (Abdelfatah et al., 2013) |
| *FOXL1* | NM_005250 | c.976_990delGGGATCCCCTTCCTC | p.Gly326_Leu330del | Deletion | Autosomal Dominant | 1 | (T. Young et al., 2014) |
| *CLDN14* | NM_012130 | c. 488C>T | p. A163V | Substitution | Autosomal Recessive | 4 | (Pater et al., 2017) |

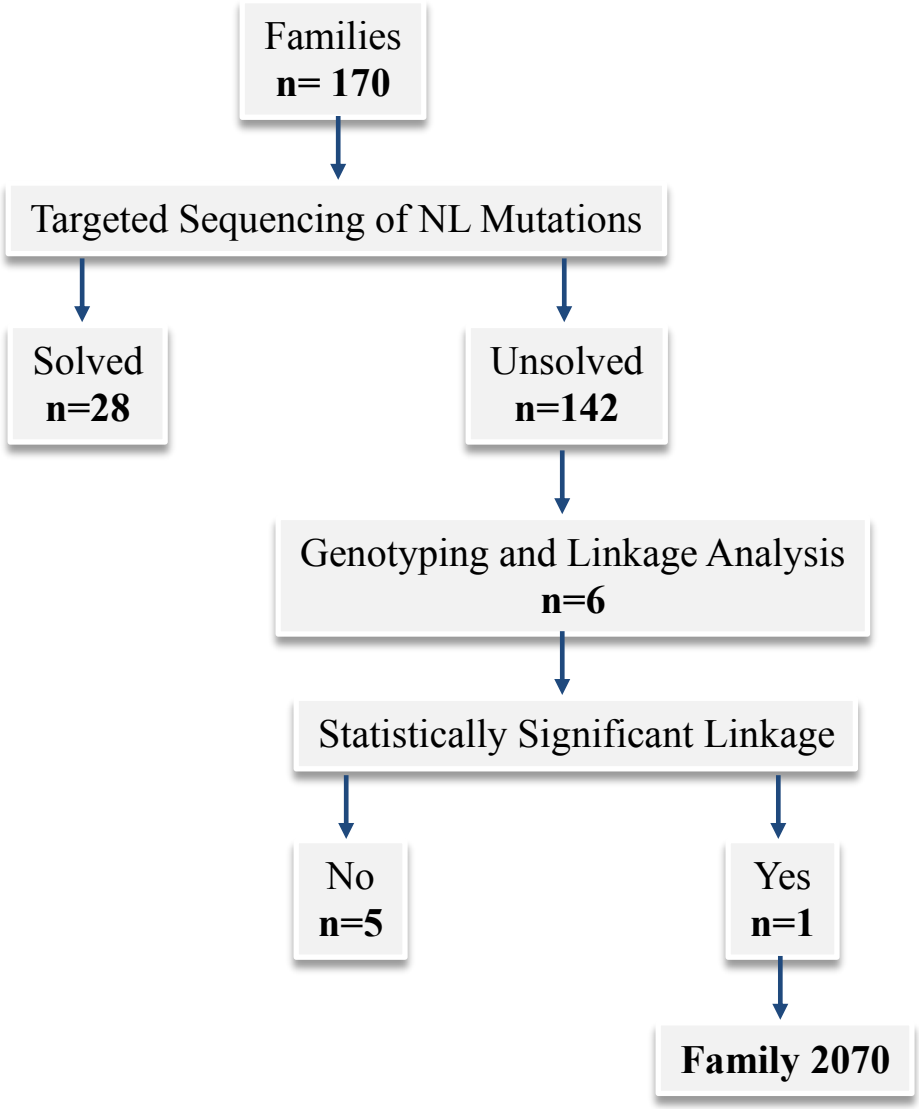


**Figure 1.7 Flowchart Outlining the Newfoundland Hereditary Hearing Loss Study**

Since 1988, the Newfoundland Hereditary Hearing Loss study has recruited 170 families. Each family was screened for 12 mutations known to cause hearing loss in the Newfoundland population and 28 families have been solved, while 142 remain unsolved. In 2012, 6 families were selected for further analysis because of high participation and clear inheritance patterns. They underwent genotyping and linkage analysis at The Centre for Applied Genomics in Toronto and only one family yielded a statistically significant LOD score, Family 2070, the focus of this thesis project.

# Chapter 2 Materials and Methods

## Patient Recruitment

Family 2070 is a seven-generation family from Western Newfoundland with apparent autosomal dominant hearing loss (**Figure 2.1)**. In 2004, the proband of Family 2070 was referred to the Provincial Medical Genetics Program because of his strong family history of hearing loss. Shortly thereafter, the clinical director recruited him to the hereditary hearing loss study. The Human Investigation Committee (HIC) (Research Ethics Board, of St. John's, NL, CANADA) (#01.186) approved the project.

Recruitment was conducted by a research assistant or team audiologist and included obtaining patient consent to the Hereditary Hearing Loss project (**Appendix B**), completing an interview using a medical information questionnaire (**Appendix C**), obtaining consent for release of audiological data (**Appendix D**) and a request for blood or saliva sample (**Appendix E**). If possible, the patient's hearing was assessed during the interview.

Data collected during the interviews was used to create a pedigree in Progeny. The pedigree showed the affected status of each recruited family member and was used to determine the mode of inheritance.

The team's audiologist conducted prospective auditory exams, specifically pure-tone, speech and immittance audiometry. They also examined previous hearing tests conducted by hospitals and private clinics that had been collected to determine the likely age of onset, audiometric configuration and progression of hearing loss in Family 2070.

The hearing loss phenotypes were then recorded following the GENDEAF recommendations. Overall, twenty-four family members were accessed by the team's audiologist or provided consent for their audiological records (**Figure 2.2** and **Figure 2.3**).

Venous blood samples were collected at local clinics and sent to the research laboratory in St. John's for DNA extraction. Genomic DNA was extracted from venous blood samples through a salting out procedure (Miller, Dykes, & Polesky, 1988) or from saliva following the Oragene DNA ethanol precipitation protocol (http://www.dnagenotek.com/US/products/prepITL2P.html). In total, twenty-eight family members provided DNA (**Figure 2.2** and **Figure 2.3**). Another three family members provided additional blood samples that were taken for cell line generation (Patient Identification (PID) IV-2, IV-11, and IV-15) to be used in functional studies (**Figure 2.2**).

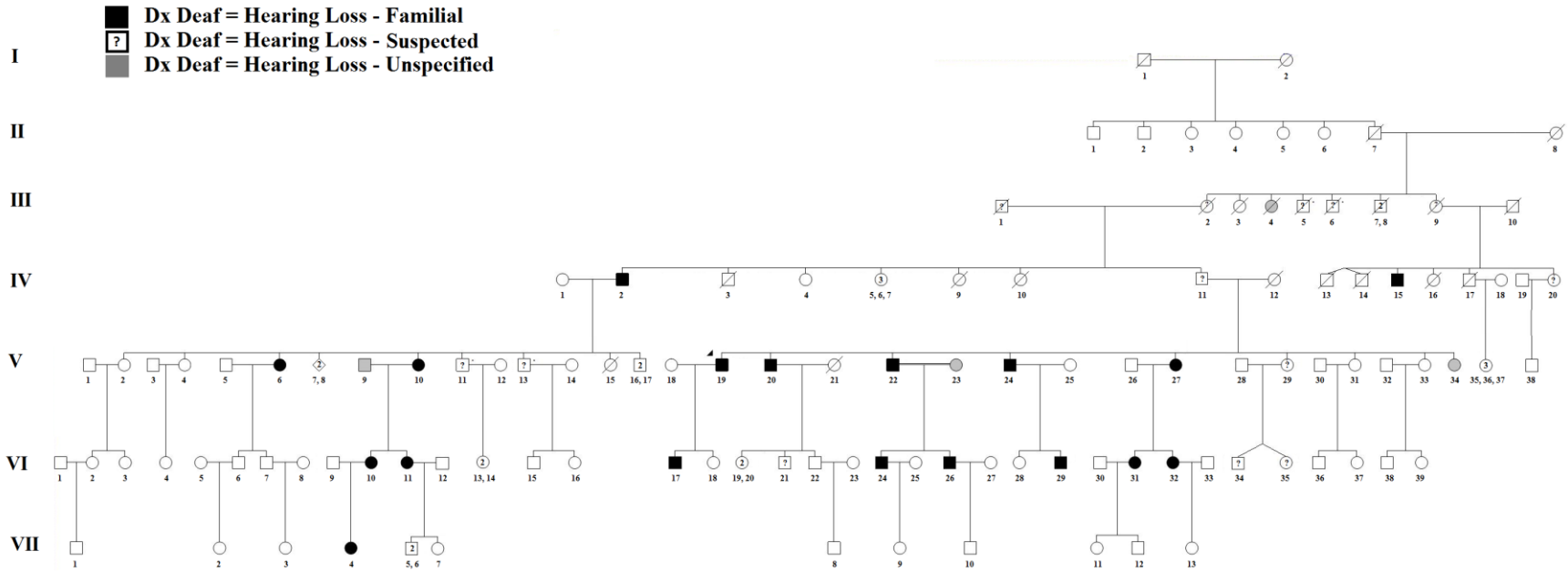


**Figure 2.1 Pedigree of Family 2070.**

The proband is identified by a shaded triangle in the upper left corner. A black-shaded symbol indicates the family member has confirmed hearing loss, audiological evidence suggests the loss is similar to other family members and is most likely inherited. A symbol with a question mark indicates the family member has suspected hearing loss, but no audiological evidence. A grey-shaded symbol indicates the family member has confirmed hearing loss, but audiological evidence suggests that the loss is distinct from other family members and is a phenocopy.

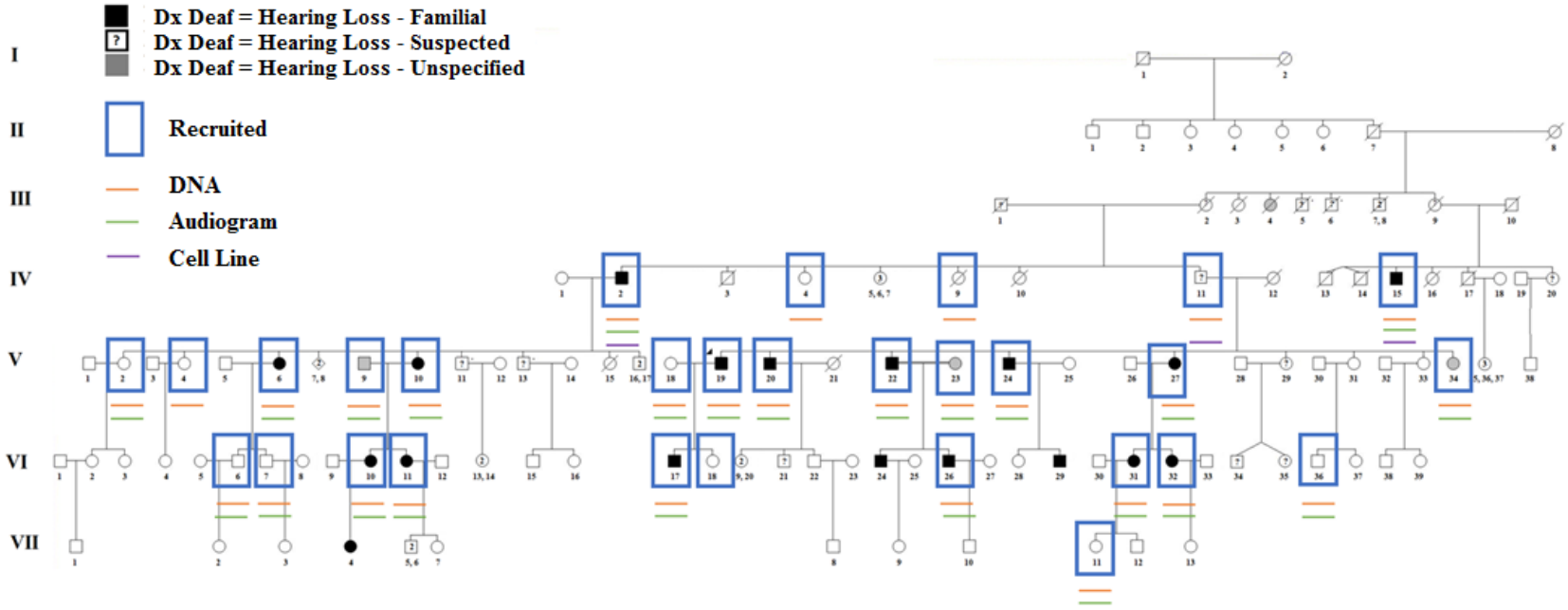


**Figure 2.2 Pedigree of Family 2070 with Recruitment Data**

A blue box surrounding a family member indicates the person has been recruited to the hearing loss project. An orange line underneath the person indicates that DNA has been collected, a green line indicates audiological data has been collected, and a purple line indicates that a cell line was established.

The proband is identified by a shaded triangle in the upper left corner. A black-shaded symbol indicates the family member has confirmed hearing loss, audiological evidence suggests the loss is similar to other family members and is most likely inherited. A symbol with a question mark indicates the family member has suspected hearing loss, but no audiological evidence. A grey-shaded symbol indicates the family member has confirmed hearing loss, but audiological evidence suggests that the loss is distinct from other family members and is a phenocopy.

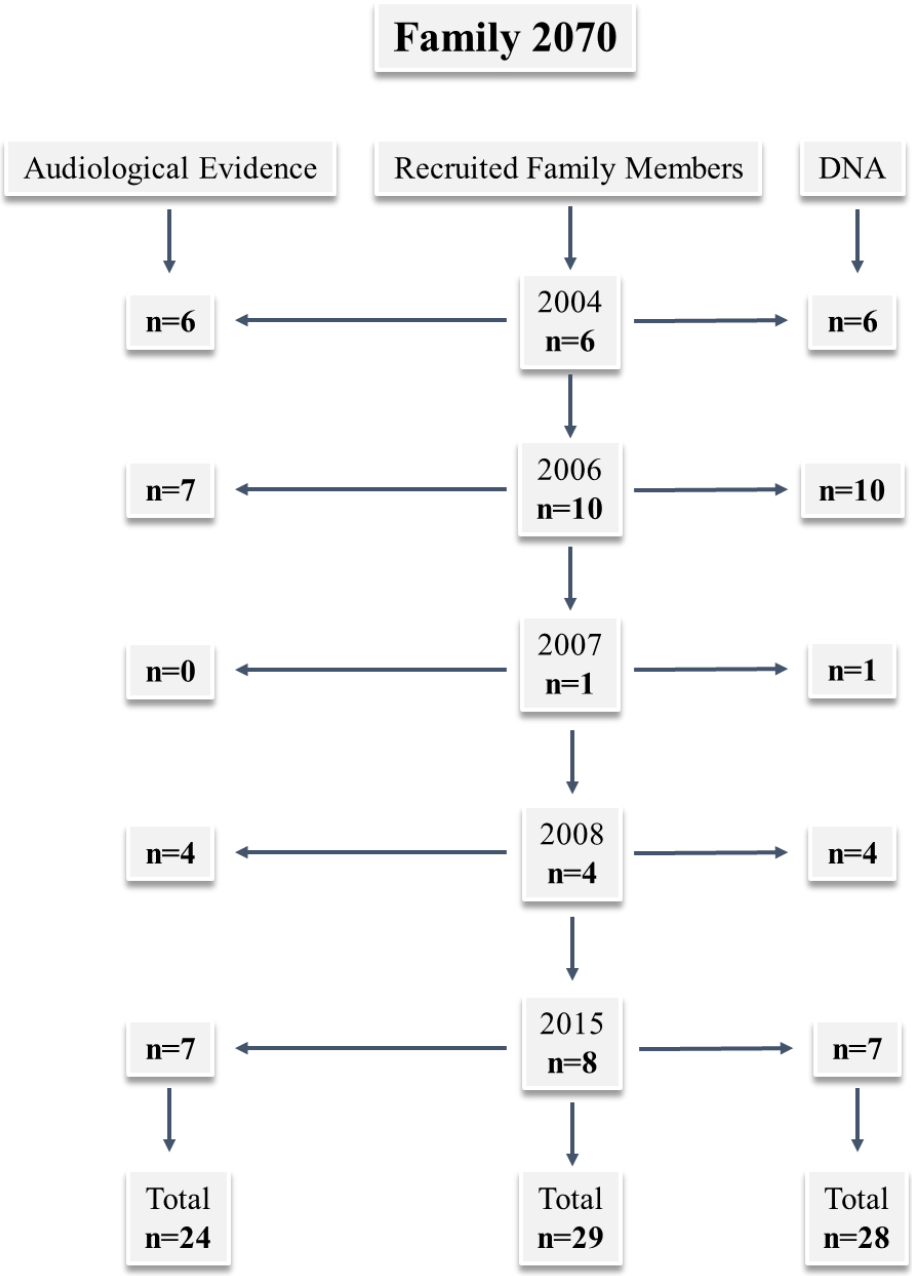


**Figure 2.3 Family 2070 Recruitment Timeline**

The timeline shows the number of family members recruited each year, the number that had an audiological assessment or provided audiological records, and the number that provided a DNA sample.

## Experimental Design

The investigation into the genetic cause of hearing loss in Family 2070 used several molecular genetics techniques, including: genome-wide SNP genotyping and linkage analysis, NGS, bidirectional Sanger sequencing and cDNA analysis. Using a combination of traditional and modern technology the genetic region of interest was narrowed to a small locus and the variants contained within were quickly and efficiently examined.

## Genome-Wide SNP Genotyping and Linkage Analysis of Family 2070

Genome-wide SNP genotyping and linkage analysis was used to identify small regions of the genome shared by members with hearing loss that were absent in those with normal hearing. A PhD candidate in our lab selected twenty family members: eleven with confirmed hearing loss, two with suspected hearing loss and seven with normal hearing. Their DNA samples were sent to The Center for Applied Genomics (TCAG) in Toronto for genome-wide SNP genotyping and linkage analysis (**Appendix F**).

## Next-Generation Sequencing of Family 2070

NGS was used to identify and filter exome variants present in two family members: PID V-10 (affected) and V-34 (unaffected). The Life Technologies Ion Torrent™ Exome Sequencing solution was used for library construction, template preparation and enrichment, sequencing and data analysis.

## Next-Generation Sequencing Protocol

### Library Construction

The exome libraries were constructed by following the Ion AmpliSeq™ Exome RDY Library Preparation User Guide (Publication Number MAN0010084). A copy of the protocol is available at https://www.thermofisher.com/ca/en/home.html. Libraries were constructed using 200 ng of genomic DNA (gDNA). To amplify the exome target regions the Ion AmpliSeq™ Exome RDY kit and an Eppendorf AG 22331 Hamburg thermal cycler were used. To distinguish between the libraries, they were barcoded using Ion Xpress™ Barcode Adapters. The libraries were then purified using the Agencourt AMPure XP kit (Beckman Coulter) and quantified using the Ion Library Quantification Kit (Cat. no. 4468802) and Life Technologies ViiA™7 System real-time PCR instrument.

**Template Preparation and Enrichment**

The template-positive Ion Sphere™ particles (ISPs) were prepared and enriched, following the Ion PI™ Template OT2 200 kit v3 User Guide (publication number MAN0009133). A copy of the protocol is available at https://www.thermofisher.com/ca/en/home.html. The Ion OneTouch™ 2 System and the Ion PI™ Template OT2 200 kit v3 were used to prepare template-positive Ion Sphere™ Particles (ISPs) by emulsion PCR. Next the Ion OneTouch™ ES instrument and the Dynabeads® MyOne™ Streptavidin C1 Magnetic Beads were used to enrich the template-positive ISPs for sequencing on the Ion Proton™ system. Finally, the percentage of template ISPs were measured using the Qubit® 2.0 Fluorometer and the Ion Sphere™ Quality Control kit.

**Sequencing**

The exome libraries were sequenced following the Ion PI™ Sequencing 200 Kit V3 User Guide (publication number MAN0009136). A copy of the protocol is available at https://www.thermofisher.com/ca/en/home.html. The Torrent Suite™ Software was used to plan and monitor the sequencing instrument runs and resultant data. The Ion PI™ Sequencing 200 Kit V3 was then used to clean and initialize the Ion Proton™ Sequencer. Finally, the template-positive ISPs were prepared for sequencing, loaded onto two calibrated Ion PI™ Chip V2 (Cat. no. 4485413) and then sequenced.

**Data Analysis**

The Torrent Suite™ Software and Torrent Server were used to analyze and store the sequencing data from the Ion Proton™ system. Next the variant dataset was filtered using Microsoft Excel to contain only variants within the linked region on chromosome 13, genomic positions 110,708,368-114,312,000 and further filtered to contain only variants with a MAF of less than 1%. The remaining variants were sent to the next phase for validation**.**

## Sanger Sequencing

Bidirectional Sanger sequencing was an important technique employed throughout the project. Below is a description of its multiple uses and the procedure that was followed:

1) **Validation of NGS Variant Dataset in Family 2070**

After exome sequencing, 13 potentially pathogenic variants remained. To verify the NGS base calls, Sanger sequencing was used to screen PID V-10 and V-34 for the 13 variants. This allowed for the validation of the variant dataset by a different method.

2) **Assessment of Co-segregation with Hearing Loss in Family 2070**

Following validation of the variant dataset, only 3/13 variants were verified. To test co-segregation of the 3 variants with hearing loss in Family 2070, Sanger sequencing was used to screen the remaining 26 family members.

3) **Assessment of Population Frequency**

Subsequently, all 3 variants co-segregated with hearing loss. Based upon the previous filtering criteria, all 3 are rare and have a MAF of less than 1% (ExAC Browser), but it is possible they are more common in the Newfoundland population. To determine the variant frequencies in the Newfoundland population, Sanger sequencing was used to screen more than 80 ethnically-matched population controls.

### 4) Identification of Additional Families

Consequently, all 3 variants were absent from the Newfoundland population controls. To search for more evidence regarding the pathogenicity of the 3 variants, Sanger sequencing was used to screen a cohort of 65 Newfoundland deafness probands. As a founder population, it is possible that multiple Newfoundland families will share the same pathogenic mutation and additional affected carriers will lend evidence to the pathogencity of the variant or allow for co-segregation analysis with additional families.

### 5) cDNA Analysis

Since the variants identified were intronic, they could have an effect on gene splicing and therefore resultant protein structure and function. Using polymerase chain reactions (PCRs), gel electrophoresis, and Sanger sequencing, the cDNA of *ADPRHL1* variant carriers and wild-type controls was analyzed.

## Sanger Sequencing Protocol

Bidirectional Sanger sequencing involved three steps: PCR and electrophoresis, preparation for ABI cycle sequencing and automated sequencing using the ABI 3130 XL and ABI 3500 XL genetic analyzers.

### Polymerase Chain Reaction and Electrophoresis

The region surrounding each variant was amplified by PCR using the master mix in **Appendix G** and primers in **Appendix H.** The master mix was vortexed and centrifuged and then 19 µL was aliquoted into each reaction well of a PCR plate, followed by 1.0 µL of gDNA (10 ng/µL). The PCR plate was sealed using adhesive film, labeled, balanced and centrifuged then placed in a GeneAmp PCR system 9700 for amplification using the thermocycler program in **Appendix I**.

The PCR products (3 µL) were electrophoresed using a 1% agarose gel (0.5 g of agarose and 50 mL of 1X TBE Buffer) containing 1.5 µL of Invitrogen SYBR Safe DNA Gel stain and visualized under UV light using the KODAK Gel Logic 100 Imaging System.

### Preparation for ABI Cycle Sequencing

The PCR products that were successfully amplified were purified using Sephacryl S 300 (GE Healthcare). The Sephacryl S 300 was re-suspended and 300 µL was added to a Millipore Multiscreen HTS plate with a waste plate aligned underneath. Then the plate was balanced and centrifuged at 1811 rcf for 5 min and the flow-through was discarded. Next, the Multiscreen plate was positioned over a clean PCR plate, the remaining PCR products were aliquoted into the wells containing Sephacryl and then balanced and

centrifuged at 1811 rcf for another 5 min. Lastly, the purified PCR products were collected in the flow-through and used as template for the ABI cycle sequencing reactions.

Two separate master mixes, one forward and one reverse, were prepared for ABI cycle sequencing reactions, according to the master mix in **Appendix J**. Then 19 µL of master mix was added to each well in a 96-well reaction plate followed by 1 µL of purified PCR product in the appropriate wells. The reaction plate was sealed using a silicone mat, balanced and centrifuged, then loaded onto a thermal cycler programed with **Appendix K**.

The ABI cycle sequencing reaction products were purified through ethanol precipitation. First the products were centrifuged, then 5 µL of 125 mM EDTA was added to each well followed by 65 µL of 95% ethanol. The samples were incubated for at least 15 min at ambient temperature in the dark and then centrifuged for 30 min at 3000 rcf. To decant the ethanol, the plate was inverted over a sink and paper towels were placed beneath to catch the excess ethanol mixture. Then 150 µL of 70% ethanol was added, the plate was covered with a silicone mat, and then balanced and centrifuged at 3000 rcf for 15 min. The ethanol removal procedure was repeated. Then to ensure all ethanol was removed, the plate was placed inverted with paper towels underneath in the centrifuge and spun at 8 rcf for 2 min. The plate was dried in the dark for at least 15 min at ambient temperature.

Lastly, 15 µL of Hi Di formamide (Applied Biosystems) was added to each reaction well and the plate was vortexed, balanced and centrifuged briefly, then placed on

a thermal cycler and denatured at 94 °C for 2 min. The plate was again balanced and centrifuged then loaded onto the DNA analyzer.

**Automated Sequencing Using the ABI 3130 and ABI 3500**

Two DNA analyzers were used for automated sequencing, ABI 3130 XL Genetic Analyzer and ABI 3500 XL Genetic Analyzer. Variants were identified in the resultant sequences using Mutation Surveyor software (Version 4.0.9, SoftGenetics) and then their pathogenicity was assessed using a mutation interpretation program called Alamut (Interactive Biosoftware V 2.7.1).

## cDNA Analysis of Family 2070

As mentioned previously, the 3 putative variants could have an effect on gene splicing. Using PCR, gel electrophoresis and Sanger sequencing, the cDNA of *ADPRHL1* mutation carriers and wild-type controls was analyzed. However, since *F10* was not significantly expressed in B-lymphocytes it was not possible to analyze the cDNA of *F10* mutation carriers.

## cDNA Analysis Protocol

### Isolation, Culture, and Storage of Cell Pellets

Whole blood was collected from three family members and six population controls in ACD (anticoagulant) containing tubes. B-lymphocytes were extracted, cultured and transformed to create Epstein Barr virus (EBV)-immortalized cell lines. The cell lines were than stored in liquid nitrogen (performed by a research assistant).

The frozen B-cell aliquots were re-suspended in RPMI (Roswell Park Memorial Institute) medium, cultured, harvested as a cell pellet and stored at -80 °C (performed by a research assistant).

### Extraction of RNA from Cell Pellets

Total RNA was isolated from the cell pellets using TRIzol Reagent (Life Technologies) following the protocols outlined in MAN0001271. Any remaining DNA was digested using TURBO DNA-free™ kit (ThermoFisher), following the protocols outlined in the user guide, publication number 1907M, revision G. A copy of the protocol is available at https://www.thermofisher.com/ca/en/home.html.

**Complementary DNA Synthesis**

From total RNA, poly $(A)^+$ selected RNA was used to synthesize cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen) following the protocols outlined in MAN0001346. A copy of the protocol is available at https://www.thermofisher.com/ca/en/home.html.

**Polymerase Chain Reaction and Gel Electrophoresis of Target cDNA**

To determine if *ADPRHL1* c.380-17C>A and c.380-16T>G caused alternative mRNA splicing, the region between exon 2-4 was amplified by PCR. The methods described previously for PCR were used, except 2 µL of cDNA was used as template instead of 1 µL of gDNA to amplify the region surrounding *ADPRHL1* c.380-17C>A and c.380-16T>G. The primer sequences are described in **Appendix L**.

The PCR product (10 µL) was electrophoresed using a 1.5% agarose gel (0.75 g of agarose and 50 mL of 1X TBE Buffer) containing 1.5 µL of Invitrogen SYBR Safe DNA Gel stain and visualized under UV light using the Syngene U:Genius imaging system.

**Preparation for ABI Cycle Sequencing**

The methods previously described for purification and ABI cycle sequencing were followed for the PCR products of *ADPRHL1* exon-2-4.

**Automated Sequencing Using the ABI 3130 and ABI 3500**

The methods previously described for sequencing were followed for the PCR products of *ADPRHL1* Ex-2-4.

# Chapter 3 Results

## Hearing Loss in Family 2070

Family 2070 is a large seven-generation family with bilateral, sensorineural hearing loss (BLSNHL). The estimated age of onset among family members is variable, ranging from birth to early adulthood. It begins as a high frequency loss, producing a sloping profile. Over time the loss progresses to affect all frequencies resulting in profound hearing loss.

Since early audiometric data was limited, the estimated age of onset was based upon self-report and factors like estimated age of hearing aid use. The onset of hearing loss in Family 2070 is variable (**Table 3.1**). All affected family members describe having hearing loss before age thirty but some describe hearing loss at birth (PID V-19), others during childhood (PID VI-24) and some in early adulthood (PID VI-31).

The severity of hearing loss in Family 2070 is also variable. For instance, PID V-19 had severe to profound hearing loss at age 34 whereas PID V-6 had moderate hearing loss at age 58 (**Table 3.1**). Despite this variability, similar phenotypes were observed among some family members; the proband (PID V-19), his son (PID V-17) and his uncle (PID IV-2) all have severe to profound hearing loss (**Figure 3.1**).

Three family members PID V-9, V-23 and V-34 had hearing loss that was attributed to other factors (**Appendix M**). It should be noted that PID V-9 and V-34 did not meet the GENDEAF criteria for hearing loss (their hearing was within normal limits) but upon examining their audiograms a mild loss was observed at specific frequencies.

Also, despite being married-into Family 2070, the phenotype of PID V-9 and V-23 was important because they have affected children and it was necessary to compare their phenotype to their children's. The phenotype in these cases differed from their children in severity and configuration, suggesting that factors like age or noise exposure may have affected their hearing. For example, according to the GENDEAF recommendations, PID V-9 has normal hearing and his daughters (PID VI-10 and VI-11) have moderate to severe hearing loss. PID V-23 differs in symmetry from her sons (PID VI-24 and VI-26) and has a unilateral loss. PID V-34 has normal hearing at 58 years of age, while her siblings have severe to profound hearing loss.

The mode of inheritance of hearing loss in Family 2070 is most likely autosomal dominant. Hearing loss is inherited vertically through the pedigree and there are a large number of affected children. For example, in **Figure 2.1**: 55% (5/9) of PID IV-11's children have hearing loss. Furthermore, PID V-10, V-22, and V-27 have 100% (2/2) affected children. Inheritance is not X-linked or mitochondrial because there are instances of male-to-male transmission in the family, as shown in **Figure 2.1** for PID V-19 to VI-17. Moreover, the inheritance is not likely to be autosomal recessive because there are multiple affected family members in each generation.

**Table 3.1 Phenotype of Inherited Hearing Loss in Family 2070**

Related audiograms are shown in **Appendices O, P, and Q**. The patient identification (PID) number indicates the family member's position within the pedigree. The following categories were assessed using the GENDEAF recommendations. The estimated age of onset was based upon audiological evidence or patient recall. The age of recent assessment indicated the age in years of the most recent audiological exam. Bilateral symmetry indicated if both ears were affected similarly. Severity indicated the level of hearing loss. Configuration described the slope of the hearing loss and the overall change in hearing thresholds across frequencies.

| PID Number | Estimated Age of Onset (Years) | Age of Recent Assessment (Years) | Bilateral Symmetry | Severity | Configuration |
|---|---|---|---|---|---|
| IV-2 | Uncertain | 75 | Yes | Profound | Gently Sloping |
| IV-15 | 1-10 | 70 | Yes | Profound | Flat |
| V-6 | 11-30 | 58 | Yes | Moderate | Steeply Sloping |
| V-10 | 11-30 | 57 | Yes | Severe | Gently Sloping |
| V-19 | 0 | 34 | No | Severe | Flat |
| V-20 | Uncertain | 53 | Yes | Profound | Gently Sloping |
| V-22 | Uncertain | 55 | Yes | Profound | Flat |
| V-24 | Uncertain | 43 | Yes | Profound | Flat |
| V-27 | 11-30 | 57 | Yes | Severe | Flat |
| VI-10 | 1-10 | 26 | Yes | Severe | Flat |
| VI-11 | Uncertain | 39 | Yes | Moderate | Steeply Sloping |
| VI-17 | 0 | 16 | Yes | Severe | Flat |
| VI-24 | 1-10 | 14 | Yes | Mild | Steeply Sloping |
| VI-26 | Uncertain | 17 | Yes | Moderate | Gently Sloping |
| VI-31 | Uncertain | 27 | Yes | Mild | Steeply Sloping |
| VI-32 | Uncertain | 26 | Yes | Normal | Gently Sloping |

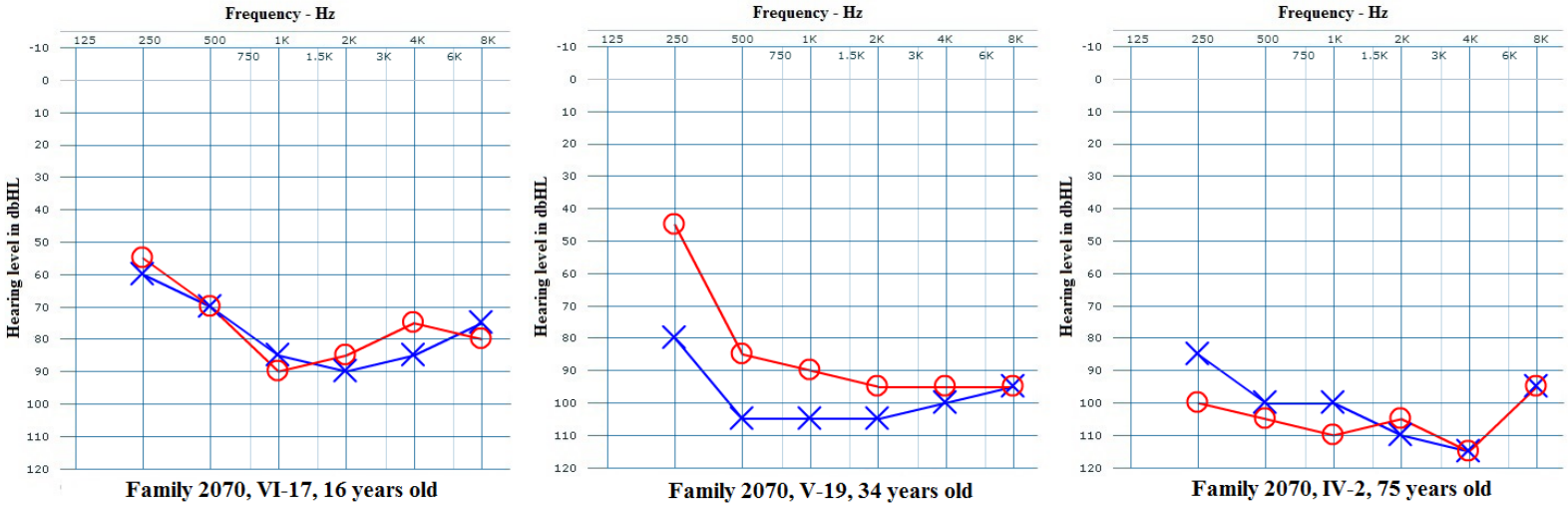


**Figure 3.1 Audiograms from Family 2070**

Audiograms of family members with bilateral, severe to profound, gently sloping to flat sensorineural hearing loss. The red lines indicate the hearing thresholds of the right ear and blue lines the left ear.

## Hearing Loss in Additional Families

Variants that co-segregated with hearing loss in Family 2070 were screened in 65 Newfoundland deafness probands. Two additional families were identified that shared the *ADPRHL1* variants: Family 2110 and 2143. Both have only one family member recruited to the hearing loss project. The phenotype of the probands are described below, along with potential inheritance patterns based upon interview data.

### Hearing Loss in Family 2110

Family 2110 is a four-generation family from the North East Avalon Peninsula, with a history of severe hearing loss. Since 2007, only the proband, PID III-1, has been recruited (**Table 3.2**). It was difficult to determine the role genetics played in her hearing loss since she had a history of ear infections, only recent audiograms were available and at 78 years old there is likely an age-related component to her hearing loss. Her latest audiogram showed bilateral, steeply sloping, moderate sensorineural hearing loss (**Figure 3.2**). These moderate losses in the mid and high frequencies would have a significant effect on her ability to understand regular speech. Interestingly, the hearing loss configuration and severity were similar to PID V-6 from Family 2070 (**Table 3.1**).

Without audiological records from other family members it is challenging to determine the mode of inheritance. Based upon interview data from the proband of Family 2110, the most likely mode of inheritance is autosomal dominant **(Figure 3.3)**. Several of her siblings 91% (10/11) were described as having hearing loss and reported using hearing aids. These individuals (PID V-2, V-3, V-4, V-5, V-7, V-8, V-10, V-11,

and V-12) have been designated as having suspected hearing loss and their phenotype was indicated on the pedigree using a "?", as there was no audiological evidence to confirm their status. The hearing loss appeared to be inherited vertically through the pedigree. The father of the proband, PID II-1, and many of his children have suspected hearing loss. Furthermore, both PID III-8 and her child PID IV-1 have suspected hearing loss. With so many potentially affected family members the mode of inheritance is unlikely to be autosomal recessive. It is also unlikely to be mitochondrial or X-linked because there is possible male-to-male transmission of hearing loss, PID II-1 to III-4.

**Hearing Loss in Family 2143**

Family 2143 is a three-generation family from Central Newfoundland with a history of hearing loss. Since 2008, only the proband, PID III-1, has been recruited (**Table 3.2**). He was first assessed for hearing loss at age 28**.** At this age a conductive and sensorineural component were present, resulting in a mixed type of hearing loss. His most recent assessment at age 41 did not show a conductive component; instead a bilateral, flat mild sensorineural loss (**Figure 3.2**). Also, despite the GENDEAF recommendations describing his loss as being flat, at individual frequencies his audible thresholds are quite different. For example, his right ear audible thresholds jump between 40-60 dB HL. It can be seen that the configuration of the audiogram is irregular and lacks the bilateral symmetry seen in Family 2070. Furthermore, conductive hearing loss was not observed in Family 2070.

As in the case of Family 2110, without audiological records from other family members it is challenging to assess the potential mode of inheritance of hearing loss.

Subsequently the mode of inheritance in Family 2143 cannot be accurately determined (**Figure 3.3**). Based upon interview data from the proband of Family 2143, both of his parents (PID II-1 and II-2) had suspected hearing loss. The vertical pattern is suggestive of autosomal dominant inheritance. However, since the proband has no affected siblings the pattern could also be autosomal recessive. It should be noted that the other family members have not been recruited, so it is possible there are more cases of hearing loss in the family that were not reported during the interview. Without knowing which parent is transmitting the mutant allele it is impossible to rule out X-linked or mitochondrial inheritance.

**Table 3.2 Phenotype of Hearing Loss in Families 2110 and 2143**

Screening of 65 probands for *F10* c.865+26C>T, *ADPRHL1* c.380-17C>A and c.380-16T>G, identified two additional families (2143 and 2110) that harbored the *ADPRHL1* variants.

Related audiograms are shown in **Figure 3.2**. The PID number indicates the family member's position within the pedigree. The following categories were assessed using the GENDEAF recommendations. The estimated age of onset was based upon audiological evidence or patient recall. The age of recent assessment indicated the age in years of the most recent audiological exam. Bilateral symmetry indicated if both ears were affected similarly. Severity indicated the level of hearing loss. Configuration described the slope of the hearing loss and the overall change in hearing thresholds across frequencies.

| Family/PID | Estimated Age of Onset (Years) | Age of Recent Assessment (Years) | Bilateral Symmetry | Severity | Configuration |
|---|---|---|---|---|---|
| 2110 III-1 | 1-10 | 78 | Yes | Moderate | Steeply Sloping |
| 2143 III-1 | 11-30 | 41 | No | Mild | Flat |

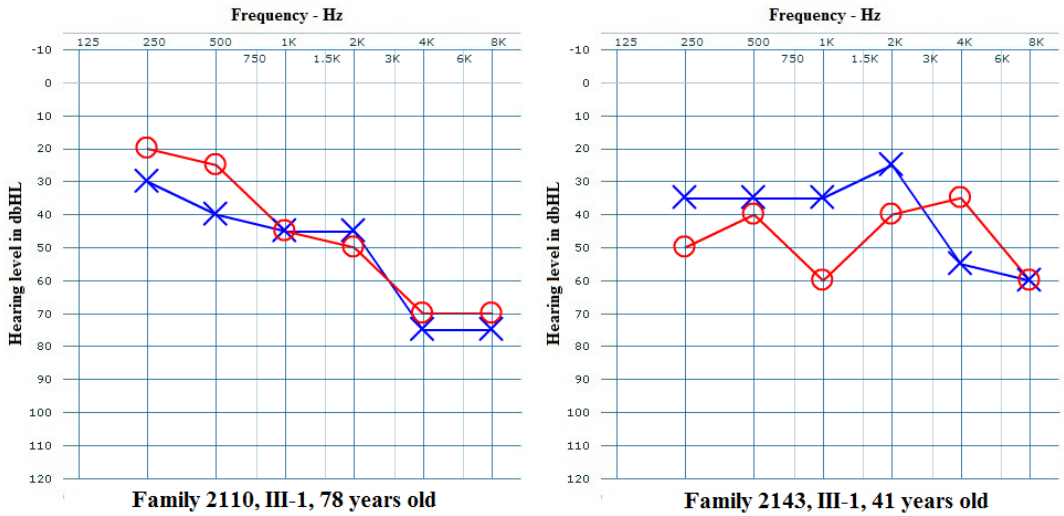


**Figure 3.2 Audiograms from Families 2110 and 2143**

Screening of 65 probands for *F10* c.865+26C>T, *ADPRHL1* c.380-17C>A and c.380-16T>G, identified two additional families (2143 and 2110) that harbored the *ADPRHL1* variants.

Audiogram of the proband of Family 2110 with bilateral, moderate, steeply sloping sensorineural hearing loss. Audiogram of the proband of Family 2143 with bilateral, mild, flat sensorineural hearing loss. The red lines indicate the hearing thresholds of the right ear and blue lines the left ear.

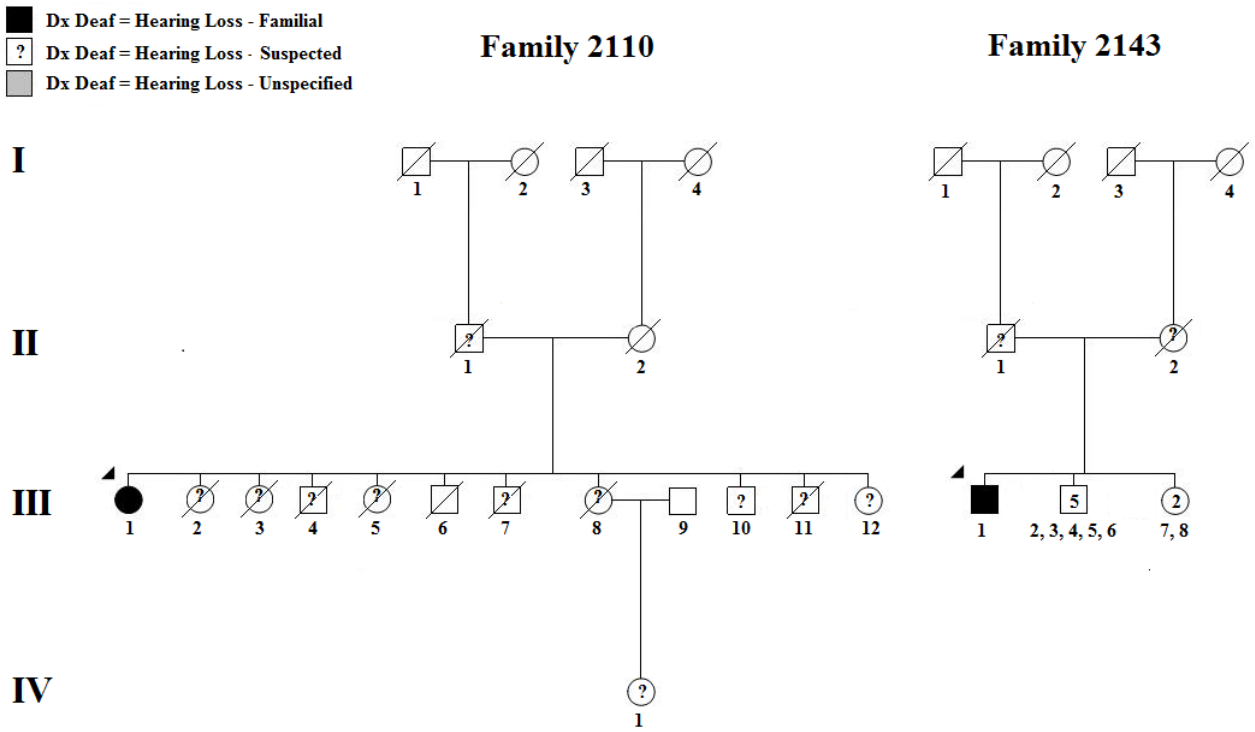


**Figure 3.3 Pedigrees of Families 2110 and 2143**

Screening of 65 probands for *F10* c.865+26C>T, *ADPRHL1* c.380-17C>A and c.380-16T>G, identified two additional families (2143 and 2110) that harbored the *ADPRHL1* variants.

The proband is identified by a shaded triangle in the upper left corner. A black-shaded symbol indicates the family member has confirmed hearing loss, audiological evidence suggests the loss is similar to other family members and is most likely inherited. A symbol with a question mark indicates the family member has suspected hearing loss, but no audiological evidence. A grey-shaded symbol indicates the family member has confirmed hearing loss, but audiological evidence suggests that the loss is distinct from other family members and is a phenocopy.

## Results of Genome-Wide SNP Genotyping and Linkage Analysis of Family 2070

The genome-wide SNP genotyping data was analyzed under a dominant model, using two-point and multipoint linkage analyses, with the results of both approaches indicating the presence of a linked region on chromosome 13.

### Multipoint linkage analysis

Analysis 1 included all thirteen family members with hearing loss and indicated the presence of three linked regions on two chromosomes, with a LOD score >1 (**Appendix F**). As mentioned previously, a positive LOD score suggests linkage between a trait (hearing loss) and a genetic region. Most noteworthy were the adjacent linked regions on chromosome 13, which obtained a statistically significant LOD score of 3.2, the maximum possible for Family 2070 using this model.

Analysis 2 included eleven family members with confirmed hearing loss and indicated the same three regions and an additional one on chromosome 5, with a LOD score >1 (**Appendix F**). Interestingly, the regions on chromosome 13 again obtained a LOD score of 3.2.

### Two-point linkage analysis

Analysis 1 included all thirteen family members with hearing loss and indicated the presence of eight linked regions on four chromosomes, with a LOD score >1.5 (**Appendix F**). However, the only region to achieve a maximum LOD score was the

linked region on chromosome 13, which obtained a LOD score of 4.77, the maximum possible for Family 2070, under this model. The results of Analysis 2 (not shown) were nearly identical to Analysis 1.

Strikingly, both multipoint and two-point analyses obtained maximal LOD scores for regions on chromosome 13 and overlapped between genomic positions 110,708,368 – 114,312,000. The critical region spanned approximately 3.6 Mb and encompassed 26 genes. These results strongly suggest the presence of a linked region on chromosome 13q34.

## Results of Next-Generation Sequencing of Family 2070

### Library construction

The exome libraries of PID V-10 and V-34 were quantified using the Ion Library TaqMan™ quantification kit. The expected yields were between 100-500pM and PID V-10 had a library yield of 380pM, while PID V-34 had 366pM. Both libraries had yields within the expected range for the Ion AmpliSeq™ Exome kit.

### Template preparation and enrichment

The exome libraries of PID V-10 and V-34 were used to generate template-positive Ion PI™ Ion Sphere™ Particles. The optimal percentage of templated ISPs, prior to enrichment is 10-30%. PID V-10 had 27% template-positive ISPs, while PID V-34 had 33%. A percentage of templated ISPs >30 is associated with a multi-templated ISPs and therefore mixed reads. However, the recommended optimal range is not intended to be pass-fail criteria, so the PID V-34 sample was used for sequencing.

### Sequencing

The Ion AmpliSeq Ready-to-Use panel design had 95.69% coverage of coding regions. Within the linked region, coverage was 98.74%, with more than half of the genes (16/26) being 100% covered (**Appendix R**). The total number of reads generated by the exomes of PID V-10 and V-34 was 69,182,853 and 73,821,872 respectively, well within the expected yield at 60-80,000,000 reads. Moreover, the average number of reads per amplicon was high at 205.4 and 185.9. The percentage of amplicons with at least 20

reads was 96.24% and 96.76% respectively. Furthermore, the percentage of amplicons with at least 1 read was 99.70% and 99.65%. Within the linked region, PID V-10's percentage of amplicons with at least 1 read was 92.78%, with half of the genes (13/26) being fully covered at 100% (**Appendix S**). Despite the high coverage of most genes, *SOX1* performed poorly in both exomes, at 14.3% coverage.

**Data analysis**

The Torrent Variant Server identified 58,341 variants in PID V-10 and 55,383 in PID V-34. To narrow the results, variants within the linked region on chromosome 13 were selected, reducing the number of variants to 132 and 128 for PID V-10 and V-34 respectively. Since the mode of inheritance was presumed autosomal dominant, only the variants found exclusively in the affected sample PID V-10 were considered, reducing the number of variants to 55. Finally, since pathogenic variants are more likely to be rare, only variants with a MAF of less than 1% were considered, reducing the number of variants to 13 (**Table 3.3**).

**Table 3.3 Thirteen Variants which Passed Filtering of Exome Variant Datasets**

| Gene | Accession Number | Genomic Region | Variant |
|---|---|---|---|
| *COL4A2* | NM_001846.2 | Intron 17 | c.1012-22T>A |
| | | Intron 20 | c.1339+17A>G |
| | | Exon 42 | c.3895_3896insCG |
| *ING1* | NM_005537.5 | 5'UTR | c.-2569C>A |
| *ARHGEF7* | NM_001113511.1 | Intron 13 | c.1335+31del |
| | | Intron 15 | c.1570-19G>T<br>c.1570-21G>T |
| *MCF2L* | NM_001112732.1 | Intron 10 | c.1117-80A>C<br>c.1117-81G>A |
| *F10* | NM_000504.3 | Intron 7 | c.865+26C>T |
| *PCID2* | NM_001127203.1 | Intron 9 | c.685+24C>T |
| *ADPRHL1* | NM_138430.3 | Intron 3 | c.380-16T>G<br>c.380-17C>A |

## Results of Sanger Sequencing

### Results of Validation of NGS Variant Dataset from Family 2070

After filtering, only thirteen variants remained and were bi-directionally Sanger sequenced (**Table 3.3**). Most of the filtered variants (10/13) were not confirmed through Sanger sequencing and so did not validate. However, 3/13 were validated: *F10* c.865+26C>T (**Figure 3.4)** and *ADPRHL1* c.380-16T>G and c.380-17C>A (**Figure 3.5**).

The variant *F10* c.865+26C>T was a known, intronic variant (rs183118165), with a MAF of 0.24% (ExAC Browser). ALAMUT predicted it could eliminate an ESE site at c.865+22 and affect splicing of exon 7.

The variants *ADPRHL1* c.380-16T>G and c.380-17C>A were also known, intronic variants (rs369286904 and rs373227737), with a MAF of 0.54% (ExAC Browser). ALAMUT predicted *ADPRHL1* c.380-16T>G, alone, would have a -2.2% effect on the downstream acceptor site and could create an ESE site at c.380-19. While *ADPRHL1* c.380-17C>A, alone, would have a -5.2% on the same acceptor site. When assessed together, they had a combined -11.6% effect. However, ALAMUT relies on multiple splicing prediction programs, including MaxEnt, which predicted a much larger negative effect. It predicted *ADPRHL1* c.380-16T>G and c.380-17C>A together would have a -33.9% effect on the downstream acceptor site and also create an alternative splice site at c.380-15.

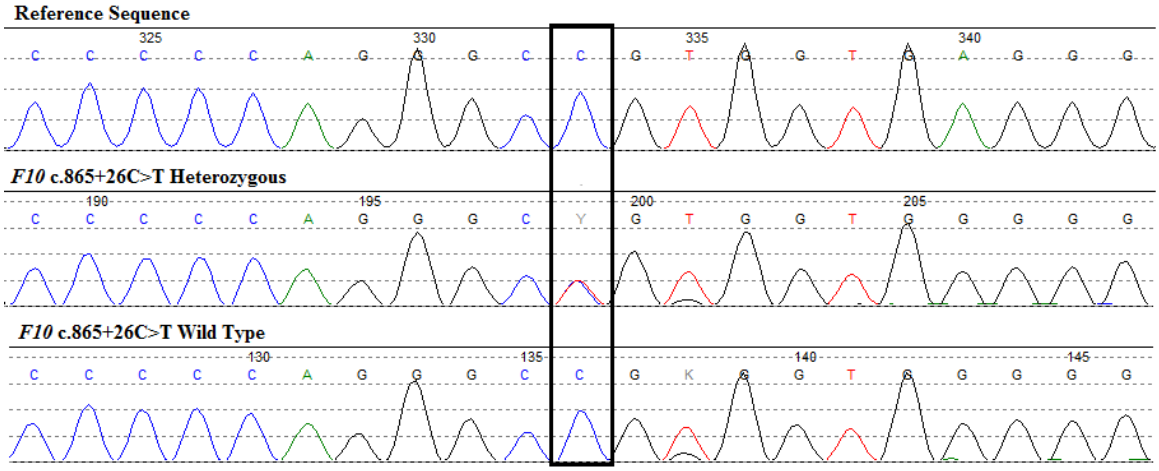


**Figure 3.4 Electropherograms of Heterozygous *F10* c.865+26C>T Variant**

The top trace is from the reference sequence (obtained from NCBI NM_000504.3); the middle trace is from PID V-19; and the bottom trace is from the PID V-2. The black box shows the position of the variant.

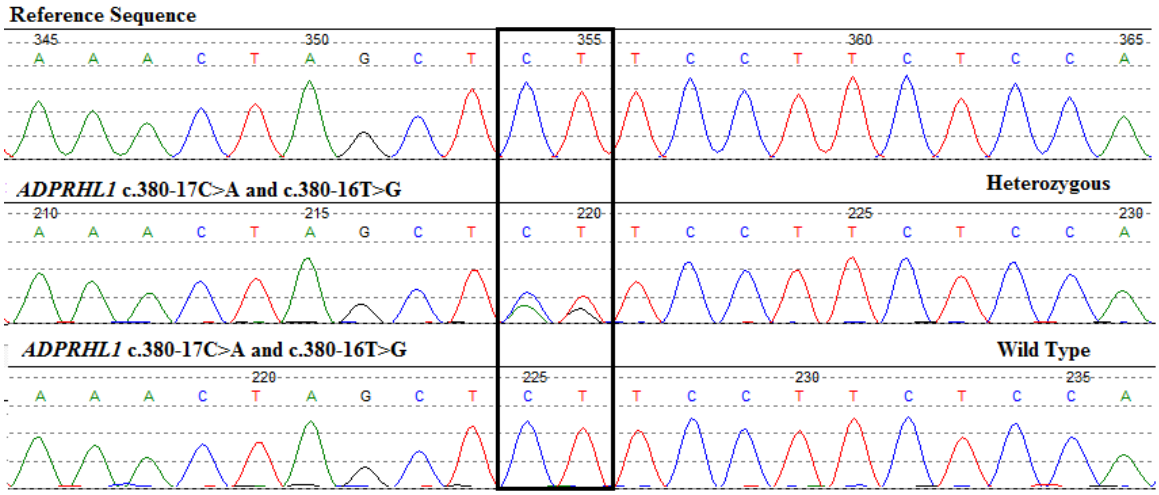


**Figure 3.5 Electropherograms of Heterozygous *ADPRHL1* c.380-17C>A and c.380-16T>G Variants**

The top trace is from the reference sequence (obtained from NCBI NM_138430.3); the middle trace is from PID IV-15; and the bottom trace is from PID V-9. The black box shows the positions of the variants.

**Results of Assessment of Co-Segregation with Hearing Loss in Family 2070**

Twenty-eight family members were screened for the 3 validated NGS variants, *F10* c.865+26C>T and *ADPRHL1* c.380-16T>G and c.380-17C>A, through bi-directional Sanger sequencing.

*F10* c.865+26C>T co-segregated with hearing loss in Family 2070 (**Figure 3.6**). On the left side of the family pedigree, PID IV-2, his children PID V-6 and V-10 and his grandchildren PID VI-10 and VI-11 all have hearing loss and are heterozygotes for the *F10* c.865+26C>T. On the right side of the pedigree, PID IV-11, his children PID V-19, V-20, V-22, V-24, V-27 and his grandchildren PID V-17, V-26, V-31 and V-32 all have hearing loss and are heterozygotes. Additionally, PID IV-15, the cousin of PID IV-2 and IV-11, also has hearing loss and is a heterozygote. Meanwhile, 9 family members with normal hearing from four generations were wild-type for *F10* c.865+26C>T. Additionally, 3 family members (PID V-34, V-9, and V-23) had hearing loss, which could be attributed to other causes and were phenotypically different from the rest of the family, were also wild type.

Similarly, both *ADPRHL1* c.380-16T>G and c.380-17C>A co-segregated with hearing loss in Family 2070 (**Figure 3.6**). In summary, all three variants co-segregated with hearing loss in Family 2070 (**Figure 3.7**), suggesting the inheritance of a disease haplotype whereby the variants are in linkage disequilibrium with each other and are typically inherited together.

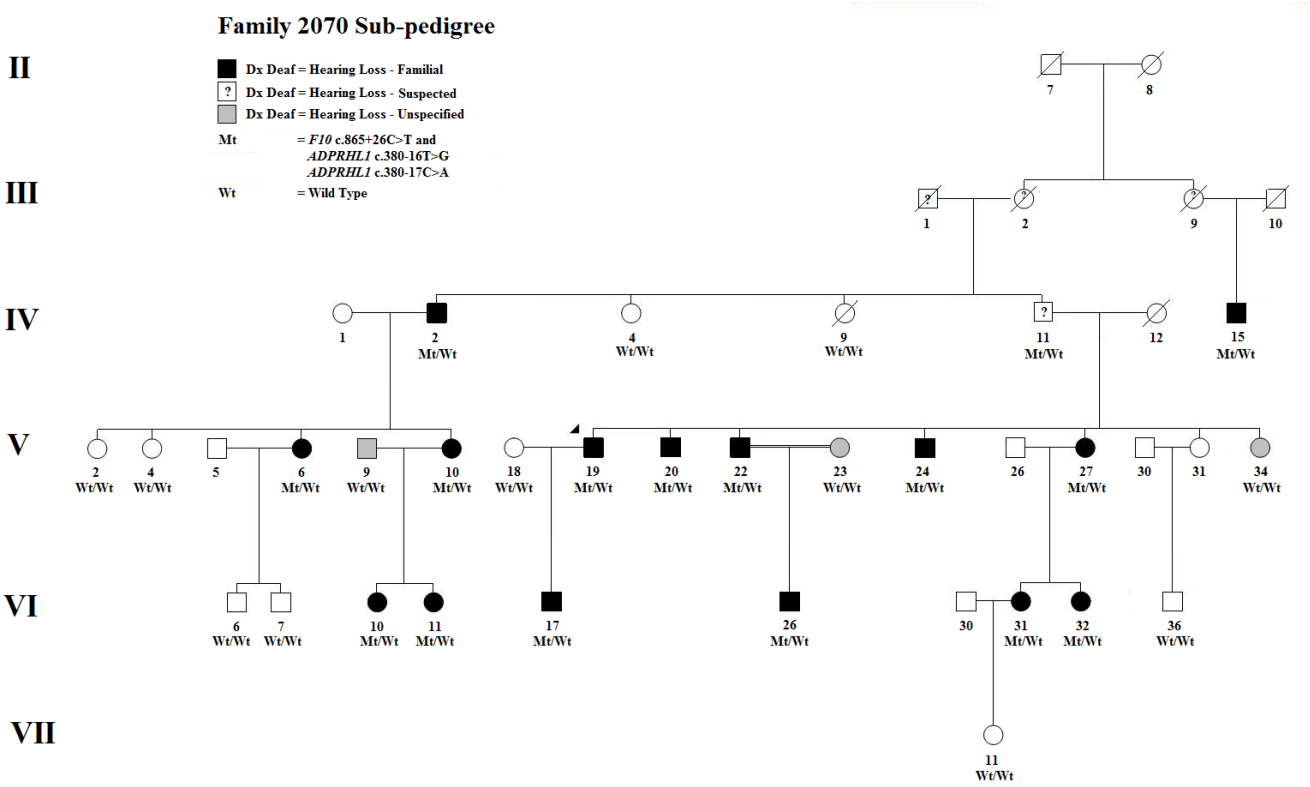


**Figure 3.6 Sub-Pedigree of Family 2070 and Variant Status**

*F10* c.865+26C>T and *ADPRHL1* c.380-16T>G and c.380-17C>A genotypes are shown as Wt for wild-type and Mt for mutant. All three variants were inherited together and never observed separately. The proband is identified by a shaded triangle in the upper left corner. A black-shaded symbol indicates the family member has confirmed hearing loss, audiological evidence suggests the loss is similar to other family members and is most likely inherited. A symbol with a question mark indicates the family member has suspected hearing loss, but no audiological evidence. A grey-shaded symbol indicates the family member has confirmed hearing loss, but audiological evidence suggests that the loss is distinct from other family members and is a phenocopy.

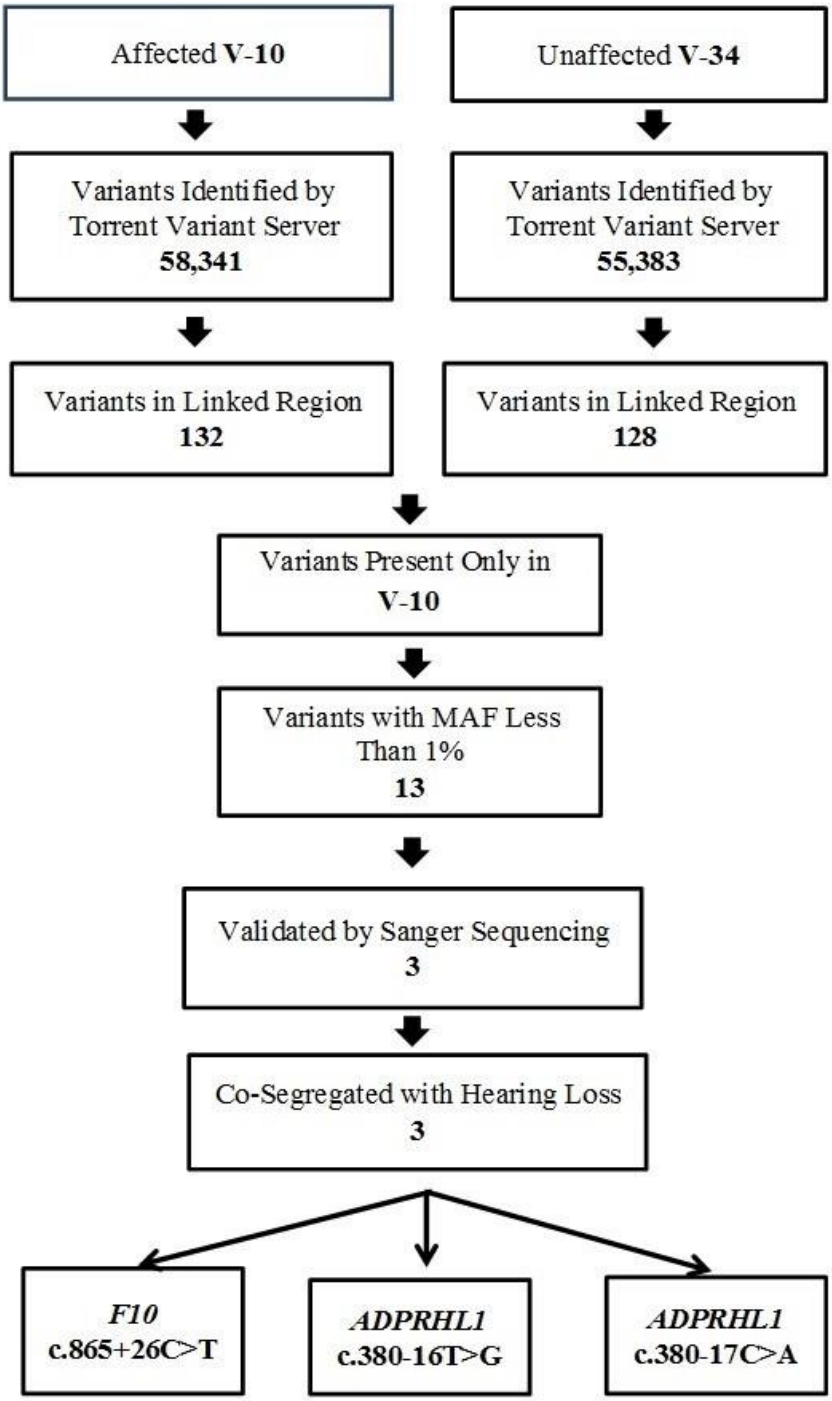


**Figure 3.7 Workflow of Exome Data Filtering, Validation, and Analysis**

**Results of Assessment of Population Frequency**

*F10* c.865+26C>T was screened in 81 ethnically-matched population controls and was absent in all samples. Likewise, *ADPRHL1* c.380-16T>G and c.380-17C>A were screened in 88 ethnically-matched population controls and were also absent from all samples. All three variants were absent in at least 162 alleles, suggesting they are rare in the Newfoundland population.

**Results of Identification of Additional Families**

All three variants were then screened in 65 probands of Newfoundland families with hearing loss. *F10* c.865+26C>T was not found in any of the Newfoundland deafness probands. However, *ADPRHL1* c.380-16T>G and c.380-17C>A were both found in two probands, from Families 2110 and 2143, who were compound heterozygotes, meaning *ADPRHL1* c.380-16T>G and c.380-17C>A has been found in three Newfoundland families with hearing loss.

## Results of cDNA Analysis of Family 2070

To determine if *ADPRHL1* c.380-17C>A and c.380-16T>G caused alternative splicing of its mRNA, the region between exon 2-4 was examined. **Figure 3.8** shows the gel electrophoresis image of the PCR products of *ADPHRL1* exon 2-4. Family 2070 (PID IV-2, IV-11, and IV-15) alongside the wild-type controls produced a band at the expected amplicon size of 390 bp. A second band, suggesting the creation of a cryptic splice site or loss of exon 3, was not observed.

ALAMUT's prediction programs suggested *ADPRHL1* c.380-17C>A and c.380-16T>G could create an alternative splice site at c.380-15. An additional 15 bp would cause a frameshift that would not be differentiated from the 390 bp normal product on an agarose gel. As a result, the PCR products of *ADPRHL1* exon 2-4 were then bidirectionally Sanger sequenced. **Figure 3.9** shows the splice site sequence between *ADPRHL1* exon 2 and 3 CAATGAAAAAG/GCTCAGGGTTT and no insertions or deletions were observed. This suggests that *ADPRHL1* c.380-17C>A and c.380-16T>G do not create an alternative splice site.

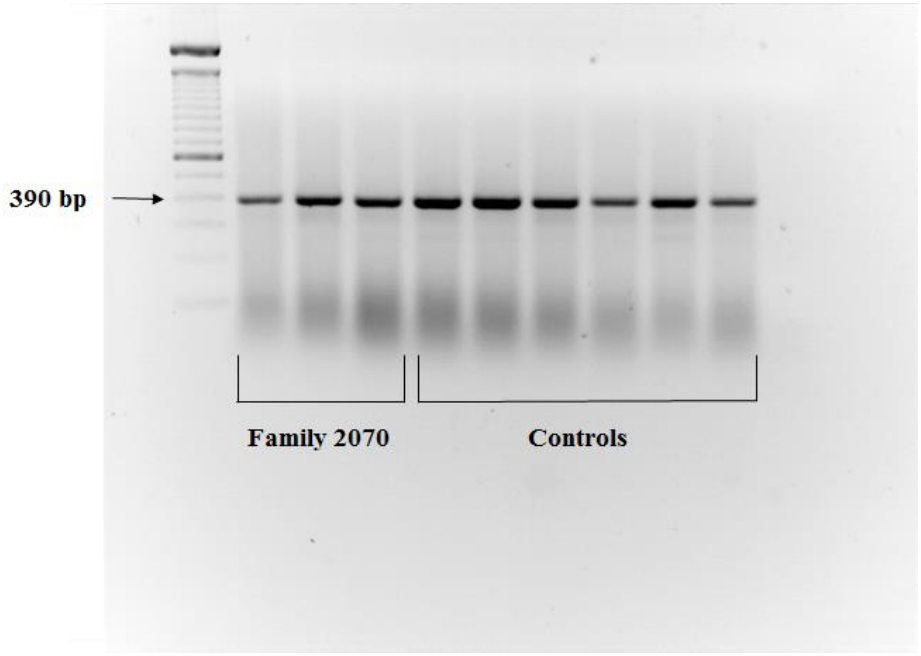


**Figure 3.8 Gel Electrophoresis Image of the PCR Products of *ADPRHL1* cDNA**

cDNA from PID IV-2, IV-11, and IV-15 from Family 2070 was amplified and examined. The expected product size of *ADPRHL1* exon 2-4 was 390 bp and was observed in both *ADPRHL1* c.380-17C>A and c.380-16T>G carriers and controls. No additional bands or significant product loss was observed.

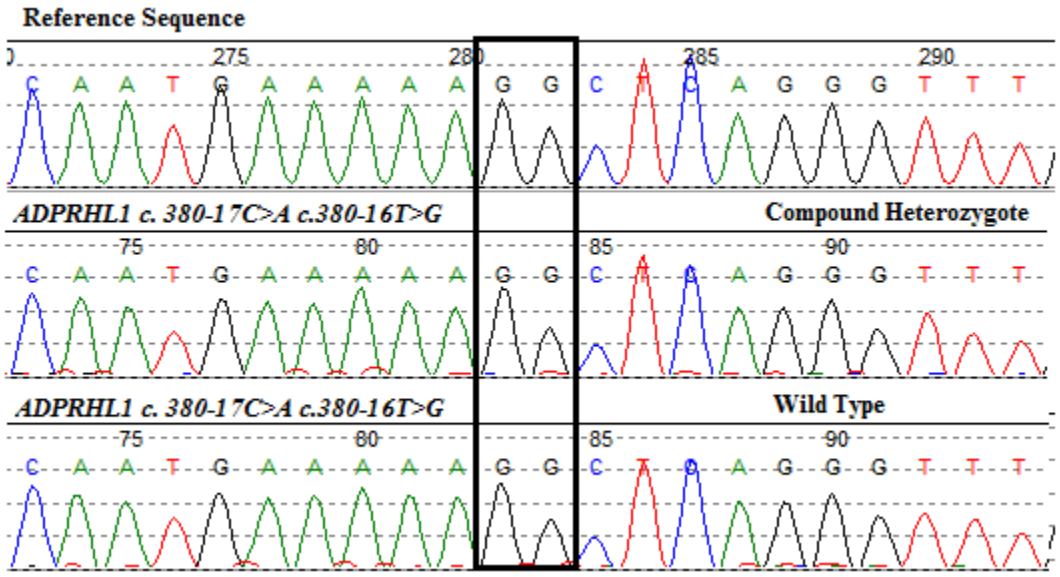


**Figure 3.9 Electropherograms Showing the Splicing of mRNA in *ADPRHL1***

The splice site between exon 2 and 3 is shown in the black box. The top trace is from the reference sequence (obtained from NCBI NM_138430.3); the middle trace is from a compound heterozygote; and the bottom trace is from a control.

# Chapter 4 Discussion

The purpose of this thesis project was to investigate the genetic cause of hearing loss in a Newfoundland family. Through a mix of old and new molecular genetics techniques three potentially pathogenic variants were identified which could either individually or in conjunction cause hearing loss.

The first method employed was genome-wide SNP genotyping and linkage analysis. The goal was to identify specific chromosomal regions associated with hearing loss. It relied upon a statistical test called a LOD (logarithm of odds) score to determine the likelihood that hearing loss and a genetic region were linked. Because positive LOD scores favored linkage, while negative scores suggested absence of linkage, any region with a LOD score greater than 1 was included. The regions that obtained the highest scores were focused upon, especially those greater than 3.3, the threshold for genome-wide significance. A single region on chromosome 13q34 achieved statistical significance and had a LOD score of 4.77. At a LOD score of 4.77, the odds are nearly 60,000 to 1 that the region is linked to hearing loss.

The region on chromosome 13q34 was identified through two-point linkage analysis. As an additional measure the analysis was performed including and excluding two family members with suspected hearing loss. The results of the two analyses were nearly identical suggesting the exclusion of the two family members with suspected hearing loss did not strongly influence the results. Knowing that both family members

with suspected hearing loss have at least one child with confirmed hearing loss, under a dominant model there is a high probability the parents were affected as well.

During the study, multipoint linkage analyses using a map of multiple markers were also employed. Interestingly, multipoint linkage analysis identified an overlapping region on chromosome 13, with a LOD score of 3.22 that was associated with hearing loss. Both two-point and multipoint linkage analyses have their advantages. Two-point analysis was more flexible and allowed inclusion of genotype data from all family members. Multipoint analysis is generally more precise than two-point, but was limited by computational constraints, as the number of markers and family members increased.

Since two-point and multipoint approaches both strongly indicated the presence of a linked region at 13q34, this was the best place to focus the search for the genetic cause of hearing loss in Family 2070. The region encompassed 26 genes, none of which had been associated with hearing loss. Interestingly this region overlapped with a previously identified deafness locus *DFNA33*. It was first identified in a German family with autosomal dominant nonsyndromic hearing loss and mapped to chromosome 13q34-qter, with a LOD score of 3.28 (Bonsch et al., 2009).

Since both the German and Newfoundland families shared a deafness locus, it is possible they share a genetic etiology. Remarkably, both families were phenotypically similar as both had apparent autosomal dominant, early onset, progressive, bilateral, sensorineural hearing loss. They also shared similar audiogram configurations with family members having a sloping profile progressing to a flat loss later in life. However, the estimated age of onset was later in the German family compared to the Newfoundland family (Family 2070). The German phenotype was based upon audiological evidence

from 7 family members with hearing loss, however the youngest affected family member was 29 years old, suggesting an ascertainment bias that may have artificially raised the age of onset. Similarly in Family 2070, the use of self-report to estimate age of onset and a lack of early audiometric data for many individuals could have accounted for onset variability. It is also possible that phenotypic differences observed could be caused by environmental or genetic variation. Overall, the shared deafness locus and phenotype similarities between the German and Newfoundland families led to a focus on chromosome 13q34.

The second method used was whole exome sequencing. It identified 13 variants within the linked region on chromosome 13q34. Only 3 out of 13 were validated by Sanger sequencing: *F10* c.865+26C>T and *ADPRHL1* c.380-17C>A and c.380-16T>G. The 23% concordance between whole exome sequencing and Sanger sequencing was much lower than expected (Hamilton et al., 2016). The discrepancy could be explained by variant filtering criteria. Setting minimum read depths, genotype quality and flanking base scores could remove many of these variants from the final dataset. Some of the false positives could also have been caused by homopolymer stretches (i.e. a string of T's) resulting in polymerase slippage during library preparation. The filtering aired on the side of caution and included all variants rather than remove a potentially removing a pathogenic variant.

All three validated variants co-segregated with hearing loss in Family 2070: *F10* c.865+26C>T and *ADPRHL1* c.380-17C>A and c.380-16T>G. Since they were located in close proximity to one another it suggests the inheritance of a disease haplotype whereby the variants are in linkage disequilibrium with each other and are typically inherited

together. All three were rare, intronic variants that were absent in the Newfoundland population controls. Additionally, *F10* c.865+26C>T and *ADPRHL1* c.380-16T>G and c.380-17C>A were predicted to affect splicing of their respective genes.

*F10* c.865+26C>T was predicted to affect splicing of *F10* mRNA by eliminating an ESE site. Lost ESE sites can result in aberrant splicing and cause hearing loss. For example, *TECTA* c.5331G>A. is thought to cause the loss of an ESE, resulting in the loss of exon 16 and deletion of 37 amino acids from the α-tectorin protein and causing autosomal dominant, mid-frequency/flat hearing loss (Collin et al., 2008). Since *F10* is not highly expressed in B-lymphocytes, we were unable to analyze the cDNA sequence and assess aberrant splicing (Expression Atlas - European Bioinformatics Institute).

Mutations in *F10* have not been associated with hearing loss. *F10,* also known as coagulation factor X, encodes a vitamin K-dependent factor that converts prothrombin into thrombin, as part of the blood coagulation cascade (National Center for Biotechnology Information, 2015b). Mutations in *F10* can cause factor X deficiency, an autosomal recessive, hemorrhagic disorder characterized by prolonged and abnormal bleeding (Millar et al., 2000). The severity is variable and reflective of the activity level of factor X present in the blood plasma, with heterozygotes often being asymptomatic carriers (Brown & Kouides, 2008). This is concurrent with the lack of presentation of bleeding disorders in Family 2070, since they are heterozygotes.

The most common symptoms of factor X deficiency are epistaxis, menorrhagia, and hematuria (OMIM, 2015). In more severe cases, mutations in *F10* have been associated with intracranial hemorrhage and sudden hearing loss (Herrmann et al., 2005). Similarly, mutations in coagulation factor II (F2) have been associated with sudden

hearing loss due to impaired cochlear blood circulation (Mercier et al., 1999). However, hearing loss in Family 2070 is progressive, not sudden. Furthermore, intracranial bleeding is often associated with other neurological abnormalities or even death.

Hearing loss in Family 2070 is not likely to be caused by loss-of-function of F10. The carrier rate of *F10* mutations is thought to be 1 in 500 and often they are asymptomatic (Brown & Kouides, 2008). Furthermore, it would be expected that homozygotes of *F10* mutations would also have severe hearing loss as a part of their phenotype and only sudden hearing loss has been documented in rare cases.

*ADPRHL1* c.380-16T>G and c.380-17C>A were screened in 65 probands and were identified in two other families with hearing loss, Families 2143 and 2110. The inheritance patterns, like Family 2070, were autosomal dominant. The audiogram configuration of the proband of 2110 was also very similar to members of 2070, suggesting a shared genetic etiology. However, the audiogram configuration of the proband of 2143 was different than the other two families. Therefore, to firmly establish the phenotypes of Families 2143 and 2110, longitudinal audiogram data from multiple family members will be needed.

*ADPRHL1* c.380-16T>G and c.380-17C>A are designated in dbSNP as two independent variants, however the proper nomenclature could also be *ADPRHL1* c.380-17_380-16delinsAG, presenting them as one mutation event. Within ExAC browser they have nearly identical minor allele frequencies 0.005369 and 0.005364 and the small difference could be due to sample size, suggesting these variants always occur together. Their pairing was also observed in Family 2070, whereas both variants co-segregated together and were never seen independently. This is suggestive that *ADPRHL1* c.380-

16T>G and c.380-17C>A are in cis, meaning they are travelling on the same chromosome. This is important since cis-acting variants can have a combined negative effect that is greater than it would be acting alone.

*ADPRHL1* c.380-16T>G and c.380-17C>A were predicted to have an effect on splicing. Combined, they may create a cryptic splice site at c.380-15 while reducing preferential splicing of the natural splice site at c.380. If the cryptic splice site was active it would add 15 bp to the mutant mRNA sequence causing a frameshift and resulting in a truncated protein. Abnormal protein could have disastrous effects resulting in either gain-of-function or dominant-negative activity. The results of cDNA analysis did not suggest that *ADPRHL1* c.380-16T>G and c.380-17C>A created a cryptic splice site. Amplified cDNA appeared normal on an agarose gel and Sanger sequencing confirmed the lack of a cryptic splice site in the affected samples. Overall, the *ADPRHL1* cDNA from Family 2070 and the controls were all normal, suggesting c.380-16T>G and c.380-17C>A do not affect splicing of *ADPRHL1*.

*ADPRHL1* c.380-16T>G and c.380-17C>A may alter pre-mRNA splicing, producing a mutant mRNA that is being destroyed. Furthermore, the reduction in normal *ADPRHL1* mRNA may not be visible using standard PCR and gel electrophoresis. Quantification of cDNA would be necessary instead of visual inspection. Therefore, the techniques used in this study may not detect the mutant *ADPRHL1* mRNA.

*ADPRHL1* c.380-16T>G and c.380-17C>A may also alter splicing, but the aberrant transcript is not expressed within B-lymphocytes. *ADPRHL1* is expressed throughout the human body (Expression Atlas - European Bioinformatics Institute) and there are two isoforms NM_138430 and NM_199162. The first isoform, NM_138430

encodes the longer isoform, while the second isoform NM_199162 has a shorter N-terminus compared to isoform 1. Potentially, *ADPRHL1* c.380-16T>G and c.380-17C>A may affect splicing of transcripts expressed within the cochlea, rather than those in B-lymphocytes. As a result, the method used to test for abnormal splicing in this thesis may miss aberrant expression in the auditory system where it would have the greatest impact on hearing.

Mutations in *ADPRHL1* have not been previously associated with hearing loss. However, a recent study suggests *Adprhl1* is expressed in mouse inner ear, postnatal hair cells during development (Scheffer, Shen, Corey, & Chen, 2015). Known deafness genes like *Tmc1* and *Myo7a* were also found in postnatal hair cells in this study. The mouse model has been widely used to model human hereditary hearing disorders (https://www.jax.org/research-and-faculty/tools/hereditary-hearing-impairment).

*ADPRHL1*, also known as adenosine diphosphate (ADP)-ribosylhydrolase like 1, encodes an enzyme that is thought to be involved in the ADP-ribosylation cycle that regulates protein function (National Center for Biotechnology Information, 2015a). *ADPRHL1* is a member of the ADP ribosylhydrolases gene family, called ARHs. The ARH gene family consists of three genes: *ARH1*, *ARH2* (*ADPRHL1*), and *ARH3*. All three share sequence homology and bind ADP-ribose groups (Mashimo, Kato, & Moss, 2014) ARH1 targets mono-ADP ribosylated proteins and is involved in tumorigenesis, while ARH3 targets poly-ADP-ribosylated proteins and suppresses PARP1 (Poly ADP-ribose polymerase 1)-mediated cell death. The exact function of ARH2 is not known, but it may serve a similar function ARH1 or ARH3 (Mashimo et al., 2014).

Neither ARH gene has been associated with hearing loss, but *Parp1* has been linked to sensorineural hearing loss in mice. Parp1 regulates inflammation within cochlear cells and *Parp1* mutant mice are less likely to have noise-induced hearing loss (Shi & Nuttall, 2007). Since Arh3 suppresses the effect of Parp1, mutations in *Arh3* would lead to less regulation of Parp1 and increased sensitivity to noise, through Parp1-mediated cochlear hair cell death. If Arh2 (Adprhl1) shares a similar function to Arh3, then its loss could have a similar effect.

**Limitations of the study**

Twenty-nine family members were recruited to this study and most had audiological assessments. However, 5/29 did not and their phenotype was based upon interview data rather than audiological evidence. Also, some family members had only one audiological assessment so longitudinal data about onset or progression were unavailable. Unless a family is being closely monitored, hearing loss may not be identified until there is significant loss present meaning age of onset and progression cannot be accurately determined. For example, Family 2070's PID VI-31 and VI-32 did not self-identify as having hearing loss, but a loss was noticed during their audiological assessment, as part of recruitment to the study. It was mild and for PID VI-32 did not meet the GENDEAF requirements for hearing loss, but would need to be closely monitored for progression.

In recent years with the development of the newborn hearing screening tests children with hearing loss are being identified earlier and more closely monitored with regular hearing screening tests. In the past, children may not be identified until they attended school. Furthermore, families living in rural communities may not have had access to audiological testing. This would mean critical audiological data showing hearing loss development and progression is missing.

The phenotypes of Families 2143 and 2110 were also not established because of limited audiological data. For these families only a single person was recruited which meant possible modes of inheritance were based upon phenotype reports given by the proband, not direct audiological assessments. In the case of Family 2110, the proband was 78 at the time of recruitment, and many of her relatives were deceased. One of the

difficulties in this type of research is that following recruitment, many months or years may pass before any progress is made on a family. It can then be difficult to reconnect with the family and recruit more family members. At times there is no interest from the family to participate. In the case of hearing loss, some family members may fear participation in a hearing loss study may affect worker's compensation claims for noise-related hearing loss.

The genome-wide SNP genotyping and linkage analysis strongly indicated a linked region on chromosome 13. However, multipoint linkage analysis identified two other linked regions. Similarly, two-point linkage analysis identified seven other linked regions. On the other hand, all other loci had much lower LOD scores than the region on chromosome 13 (4.77).

Exome sequencing was used to identify variants within the linked region on chromosome 13q34. Some of the limitations of exome sequencing are the inability to detect large insertions/deletions or deep intronic mutations as only 5 bp of exon padding is guaranteed. The panel design is also missing 1.26% of coding regions within 13q34, possibly due to the lack of available primers in GC rich or repetitive regions. Additionally, 7.22% of amplicons within the designed region were not sequenced. Since the primers are based upon *in silico* data, some have low specificity, leading to off-target reads and poor sequencing results. In particular, *SOX1* had poor coverage, as both PID V-10 and V-34 had similar missing regions the loss was most likely due to poorly performing primers. Overall, these missing regions may contain pathogenic mutations not identified by this study and would require further investigation.

Bioinformatics tools like Alamut were used to assess the pathogenicity of identified variants. For example, Alamut predicted the alteration of a splice site, in *ADPRHL1* variant carriers. However, these predictions are based upon *in silico* rather than empirical results. To be confident in *ADPRHL1* c.380-16T>G and c.380-17C>A effect on mRNA splicing, it would be necessary to examine the resultant protein within a laboratory setting.

**Strengths of the study**

Dr. Terry-Lynn Young's laboratory has a history of excellence in hereditary hearing loss research and over the past two decades has identified of the genetic cause of hearing loss in 28 Newfoundland families. This work includes 7 publications, reporting 12 different mutations, in 11 genes now associated with deafness. Described below are several factors that have influenced the success of the Newfoundland Hereditary Hearing Loss Project and have bolstered the efforts of this thesis.

Newfoundland is an ideal study population for human hereditary disease research. As a founder population, it has an advantage in mapping and identifying rare disease genes. The limited number of founders means disease alleles will have often arisen from a single ancestral chromosome. As evidenced by the *CLDN14* c.488C>T mutation, which resided on a shared 1.4 Mb disease-associated haplotype, in four Newfoundland families with hearing loss. Additionally through the collection of deep genealogies, three of these families were connected to a single founding couple six-generations ago.

The success of the Newfoundland Hereditary Hearing Loss Project not only lies in its study population, but also the variety of skill sets held by its team members. The efforts of Dr. Young and her students are complemented by a team of research assistants that perform complex laboratory experiments and an audiologist to assist in recruitment and phenotyping.

Within Family 2070 the recruitment level was high, with 29 family members, from 4 generations participating in the study. Nearly all of the participants (24/29) were assessed by the team's audiologist or provided consent for audiological records. Access to longitudinal data, enabled the estimation of onset and progression of hearing loss.

Furthermore, by recruiting both affected and unaffected family members, it was possible to test the co-segregation of potential mutations, *F10* c.865+26C>T, *ADPRHL1* c.380-16T>G and c.380-17C>A, with hearing loss.

Together these factors lend strength to this research project and build evidence that will hopefully lead to the discovery of the genetic cause of hearing loss in Family 2070.

**Future Directions**

The next step for this study would be to recruit additional family members from Family 2070 and also Families 2110 and 2143 to test co-segregation of hearing loss with *ADPRHL1* c.380-16T>G and c.380-17C>A.

Furthermore, splicing of *ADPRHL1* could be examined more closely, using quantitative real-time PCR to test for a reduction in wild-type *ADPRHL1* mRNA suggesting the production of aberrant mRNA.

If analysis of *ADPRHL1* determines the variants are benign, the regions missing from the exome panel design and those poorly covered by the sequencing run could be investigated. A custom next-generation sequencing panel could be designed to cover the missing regions or they could be examined by Sanger sequencing. Additionally, a custom microarray panel could be designed to investigate the 26 genes for exon duplications or deletions.

# Chapter 5 Summary

The goal of this thesis was to determine the genetic cause of hearing loss in a large, seven-generation Newfoundland family. Following recruitment, the proband was screened for 12 reported Newfoundland deafness mutations but none were identified, suggesting the cause of hearing loss in Family 2070 was novel in the Newfoundland population. Using genome-wide SNP genotyping and linkage analysis the focus was narrowed to a single genetic locus on chromosome 13q34. The region overlapped with a previously identified deafness locus in a German family with a similar phenotype. From there, NGS was used to identify all variants within the coding region, filtering the data to just 13 variants in the region of interest. Only 3/13 variants were validated by Sanger sequencing and all co-segregated with hearing loss. Overall, 3 potentially pathogenic variants were identified.

The most likely genetic cause of hearing loss in Families 2070, 2143, and 2110 is *ADPRHL1* c.380-16T>G and c.380-17C>A. The mutations were predicted to have a negative effect on splicing of *ADPRHL1* pre-mRNA, through the loss of an ESE and creation of a cryptic splice site. Since *Adprhl1* is expressed in mice cochleae, it likely has a role in the development, maintenance, or function of the mouse auditory system. Furthermore, mice have been used to model human hearing loss so it is possible *ADPRHL1* also plays a role in the human auditory system.

With recruitment of additional family members from Family 2070, 2143, and 2110 it will be possible to test co-segregation of hearing loss across all three families. Quantitative analysis of *ADPRHL1* cDNA would also provide a mechanism for variant splicing effect and pathogenicity. The identification of a causal mutation will hopefully provide future directions for clinical interventions and genetic counseling.

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# Appendix

## Appendix A GENDEAF Recommendations

1) Type of hearing loss
    a. Conductive: Normal bone conduction thresholds (< 20 dB HL) and an air bone gap > 15 dB HL averaged over 500, 1000 and 2000 Hz
    b. Sensorineural: Air conduction thresholds less than 20 dB HL and an air bone gap of < 15 dB HL averaged over 500, 1000 and 2000 Hz
    c. Mixed: Bone conduction thresholds > 20 dB HL and an air bone gap > 15 dB HL averaged over 500, 1000 and 2000 Hz

2) Severity of hearing loss
    a. Mild: 20-40 dB HL
    b. Moderate: 41-70 dB HL
    c. Severe: 71-95 dB HL
    d. Profound: > 95 dB HL

3) Audiometric configuration
    a. Low frequency ascending: > 15 dB HL from the worst low frequency thresholds to the higher frequencies
    b. Mid frequency "U-shaped": >15 dB difference between the worst mid frequency thresholds and the low and high frequencies
    c. High frequency gently sloping: 15-29 dB HL difference between the average of 500-1000 Hz and 4000-8000 Hz
    d. High frequency steeply sloping: > 30 dB HL difference between the average of 500-1000 Hz and 4000-8000 Hz
    e. Flat: < 15 dB HL difference between the average thresholds of 250-500 Hz, 1000-2000 Hz and 4000-8000 Hz

4) Frequency ranges
    a. Low Frequency: < 500 Hz
    b. Mid Frequency: 500 - 2000 Hz
    c. High Frequency: 2000 Hz - 8000 Hz

5) Unilateral or Bilateral
    a. Asymmetric: > 10 dB HL difference between at least two frequencies, otherwise it is symmetric

6) Age of onset
    a. Congenital (At birth)
    b. Birth to 10 years
    c. 11 to 30 years
    d. 31 to 50 years
    e. > 50 years
    f. Uncertain

7) Progression
    a. Progressive: >15 dB HL average loss over 500 Hz, 1000 Hz and 2000 Hz within a 10 year period, otherwise hearing loss is stable

8) Other auditory system dysfunction
    a. Tinnitus: Present or Absent
    b. Vestibular function: Normal or Abnormal

## Appendix B Consent Form for Hearing Loss Project



Health Research Ethics Authority

**CHECKLIST**

***This checklist is to be completed and submitted with this consent form.***
***It is to be removed from the final version of the consent document.***

- ☐ Most recent version of consent template (July 2011) has been used
- ☐ Footer includes consent version, study name, line for patient initials
- ☐ Font size no less than 12 [except for footer]
- ☐ Left justification of text
- ☐ Grade 9 or lower reading level. Assessed reading level is: _____
- ☐ Accepted definitions for specialized terms used where applicable
- ☐ Plain language principles used for study specific wording – no jargon, no acronyms, short words, short sentences, active voice and, where appropriate, bulleted lists

**Standard, required wording (in bold type) has been used in the following sections:**

| | Yes | No |
|---|---|---|
| Introduction | ☐ | ☐ |
| Benefits (Q6) | ☐ | ☐ |
| Liability Statement (Q7) | ☐ | ☐ |
| Privacy and confidentiality (Q8) | ☐ | ☐ |
| Questions or problem (Q9) | ☐ | ☐ |
| Signature page | ☐ | ☐ |
| Signature page for minor/assenting participants if applicable | ☐ | ☐ |

**If you have answered No to any of the above, please give the rationale for these changes below:**

TCPS2 guidelines provide a list of the information required for informed consent. Please refer to TCPS2, Chapter 3, available at: http://www.pre.ethics.gc.ca/eng/policy-politique/initiatives/tcps2-eptc2/chapter3-chapitre3/.

The HREB Policy Manual provides detailed information on specific consent issues including: consent to research in emergency health situations; the use of substitute decision makers; assent for children; research involving special populations (children, cognitively impaired); managing consent in situations of difficult power relationships; and community consent to research involving Aboriginal communities. Please refer to the HREB Policy Manual on the HREA website: www.hrea.ca

**Faculty of Medicine, Schools of Nursing and Pharmacy of Memorial University of Newfoundland; Health Care Corporation, St. John's; Newfoundland Cancer Treatment and Research Foundation**

## Consent to Take Part in Research

**TITLE**: The Genetics of Hereditary Deafness in Newfoundland

**INVESTIGATOR(S)**: Drs. Terry Young, Lesley Turner, Bridget Fernandez, Tony Batten, Tracy Stockley, Susan Stanton, Kathy Hodgkinson and Anne Griffin

***SPONSOR***: Not Applicable

You have been invited to take part in a research study. Taking part in this study is voluntary. It is up to you to decide whether to be in the study or not. You can decide not to take part in the study. If you decide to take part, you are free to leave at any time. This will not affect your usual health care

Before you decide, you need to understand what the study is for, what risks you might take and what benefits you might receive. This consent form explains the study.

Please read this carefully. Take as much time as you like. If you like, take it home to think about for a while. Mark anything you do not understand, or want explained better. After you have read it, please ask questions about anything that is not clear.

The researchers will:

- discuss the study with you
- answer your questions
- keep confidential any information which could identify you personally
- be available during the study to deal with problems and answer questions

1. **Introduction/Background:**
   Some forms of deafness are hereditary. That means that they are inherited in families as a result of an altered gene. A gene is a piece of genetic material (DNA) which is passed from parents to children. If we can identify the genes that are altered in each family, it would allow us to better understand the process of hearing. We might also learn what goes wrong in some forms of deafness and how it might be treated.

2. **Purpose of study:**
   Our goal is to identify the genes involved in hereditary deafness in families.

3. **Description of the study procedures:**
If you agree to take part in this study, you will be asked to:

- Tell us about your hearing and the hearing of other members of your family, and other related aspects of your health.
- Have your hearing tested by a registered audiologist.
- Have a blood sample drawn for DNA testing.
- Complete a hearing loss questionnaire.

We might also want to review your medical records related to your deafness.

4. **Length of time:**

If you take part in this study, the interview will last 30-60 minutes including the blood sampling. The hearing test will take about 30 minutes. It will be arranged at a time that is convenient for you. The research may take us several years but you will not have to be involved again. We will keep you informed of our findings.

5. **Possible risks and discomforts:**

The only discomfort is that of giving a blood sample.

6. **Benefits:**

It is not known whether this study will benefit you.

7. **Liability statement:**

Signing this form gives us your consent to be in this study. It tells us that you understand the information about the research study. When you sign this form, you do not give up your legal rights. Researchers or agencies involved in this research study still have their legal and professional responsibilities.

8. **What about my privacy and confidentiality?**

Protecting your privacy is an important part of this study. Every effort to protect your privacy will be made. However it cannot be guaranteed. For example we may be required by law to allow access to research records. A copy of this consent will be put in your health record. If you agree, your family doctor will be told that you are taking part in this study.

When you sign this consent form you give us permission to

- Collect information from you
- Collect information from your health record
- Share information with the people conducting the study

- Share information with the people responsible for protecting your safety

**Access to records**
The members of the research team will see health and study records that identify you by name. Other people may need to look at your health records and the study records that identify you by name. This might include the research ethics board. You may ask to see the list of these people. They can look at your records only when supervised by a member of the research team.

**Use of your study information**
The research team will collect and use only the information they need for this research study.

This information will include your
(The following is a sample list of what should be included here and must be modified to ensure it includes all of the information collected in the study)

- date of birth
- sex
- family history
- medical conditions
- medications
- the results of tests and procedures you had before and during the study
- information from study interviews and questionnaires

Your name and contact information will be kept secure by the research team in Newfoundland and Labrador. It will not be shared with others without your permission. Your name will not appear in any report or article published as a result of this study.

Information collected for this study will kept for five years

If you decide to withdraw from the study, the information collected up to that time will continue to be used by the research team. It may not be removed. This information will only be used for the purposes of this study.

After your part in this study ends, we may continue to review your health records to check that the information we collected is correct.

Information collected and used by the research team will be stored (*name the appropriate location)*
Dr Terry-Lynn Young is the person responsible for keeping the information collected and secure. The information will be held within the discipline of genetics at MUN

**Your access to records**
You may ask the researcher to see the information that has been collected about you.

**9. Questions or problems:**

If you have any questions about taking part in this study, you can meet with the investigator who is in charge of the study at this institution. That person is:

**Dr Terry-Lynn Young Phone 709 777 6100**

Or you can talk to someone who is not involved with the study at all, but can advise you on your rights as a participant in a research study. This person can be reached through:
Ethics Office
Health Research Ethics Authority
709-777-6974 or by email at info@hrea.ca

**10. Declaration of financial interest, if applicable - Not applicable**

After signing this consent you will be given a copy.

**Signature Page**

**Study title**: Non-syndromic deafness in Newfoundland – Family ascertainment and gene identification

**Name of principal investigator**:
Dr Terry-Lynn Young

**To be filled out and signed by the participant:**

| | Please check as appropriate: | |
|---|---|---|
| I have read the consent | Yes { } | No { } |
| I have had the opportunity to ask questions/to discuss this study. | Yes { } | No { } |
| I have received satisfactory answers to all of my questions. | Yes { } | No { } |
| I have received enough information about the study. | Yes { } | No { } |
| I have spoken to Dr Hodgkinson or Anne Griffin and she has answered my questions | Yes { } | No { } |
| I understand that I am free to withdraw from the study<br>• at any time<br>• without having to give a reason<br>• without affecting my future care | Yes { } | No { } |
| I understand that it is my choice to be in the study and that I may not benefit. | Yes { } | No { } |
| I understand how my privacy is protected and my records kept confidential | Yes { } | No { } |
| I agree that the study doctor or investigator may read the parts of my hospital records which are relevant to the study. | Yes { } | No { } |
| I agree to take part in this study. | Yes { } | No { } |

| | | |
|---|---|---|
| Signature of participant | Name printed | Year Month Day |
| *Signature of person authorized as Substitute decision maker, if applicable* | *Name printed* | *Year Month Day* |
| *Signature of witness (if applicable)* | *Name printed* | *Year Month Day* |

**To be signed by the investigator or person obtaining consent**

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study and that he or she has freely chosen to be in the study.

| | | |
|---|---|---|
| Signature of investigator | Name printed | Year Month Day |

Telephone number: _____

## Signature Page for Parent/Guardian

**Study title**: Non-syndromic deafness in Newfoundland – Family ascertainment and gene identification

**Name of principal investigator**:
Dr Terry-Lynn Young

**To be filled out and signed by the parent/guardian:**

| | Please check as appropriate: |
|---|---|
| I have read the consent [and information sheet]. | Yes { } No { } |
| I have had the opportunity to ask questions/to discuss this study. | Yes { } No { } |
| I have received satisfactory answers to all of my questions. | Yes { } No { } |
| I have received enough information about the study. | Yes { } No { } |
| I have spoken to Dr Hodgkinson or Anne Griffin and she has answered my questions | Yes { } No { } |
| I understand that I am free to withdraw my child/ward from the study<br>• at any time<br>• without having to give a reason<br>• without affecting future care | Yes { } No { } |
| I understand that it is my choice for child/ward to be in the study and that he/she may not benefit. | Yes { } No { } |
| I agree that the study doctor, the study sponsor or a regulatory agency may read the parts of my child/ward's hospital records relevant to the study. | Yes { } No { } |
| I understand how my child/ward's privacy is protected and records kept confidential | Yes { } No { } |
| I agree that my child/ward's family doctor can be notified of participation in this study | Yes { } No { } NA { } |

I consent for my child/ward _____ to take part in this study.

Print Name

| | | |
|---|---|---|
| Signature of parent/guardian | Name printed | Year Month Day |
| Signature of person conducting the consent discussion | Name printed | Year Month Day |
| Signature of witness **[If applicable]** | Name printed | Year Month Day |

**To be signed by the investigator:**

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the parent/guardian fully understands what is involved in being in the study, any potential risks of the study and that he or she has freely chosen for the child/ward to be in the study.

| | | |
|---|---|---|
| Signature of investigator | Name Printed | Year Month Day |

*To be signed by the minor participant*

**Study title**: Non-syndromic deafness in Newfoundland – Family ascertainment and gene identification

**Name of principal investigator**:
Dr Terry-Lynn Young

**Assent of minor participant:**

**I understand the purpose of this research**
**I understand that it is my decision to take part in this study. I can stop taking part if I chose.**
**I understand that taking part in this research may not help me.**
**I understand that there is a risk that I might have side effects from the study drug.**

I agree that I will take part in this study

Signature of minor participant Year Month Day

Name printed Age

**Appendix C Hearing Loss Study Medical Information Questionnaire**

# Newfoundland and Labrador
# Hearing Loss Study
## Medical Information Questionnaire

Adapted from:

THE HARVARD CENTRE
FOR HEREDITARY HEARING LOSS

1. Your Name_____Date of Birth_____

   Address_____

   Home Phone_____Work Phone_____

   E-mail Address (if you have one) _____

2. To your knowledge, are your parents related, even distantly? ☐ Yes ☐ No ☐ Don't Know

   (This may sound like a strange question, but in a genetic study, we ask it of everyone)

3. Have you ever visited any of the following doctors?

   - An ENT (Ear, Nose and Throat) Doctor? ☐ Yes ☐ No ☐ Don't Know

     _____

   - An Audiologist? (Person performing hearing tests) ☐ Yes ☐ No ☐ Don't Know

     _____

   - An Eye Doctor? (Ophthalmologist) ☐ Yes ☐ No ☐ Don't Know

     _____

   - A Genetics Doctor? (Geneticist) ☐ Yes ☐ No ☐ Don't Know

     _____

   - A doctor who treats diseases of the nervous system? (Neurologist)
     ☐ Yes ☐ No ☐ Don't Know

     _____

   - A Heart Doctor? (Cardiologist) ☐ Yes ☐ No ☐ Don't Know

     _____

4. Have you ever been admitted to hospital? If yes, please give name of hospital and approximate date(s) of admisison.

   _____

   _____

   _____

   _____

5. Left Ear: normal less than normal

Right ear: normal less than normal

6. Left Ear: nothing hearing aid cochlear implant other _____

Right Ear: nothing hearing aid cochlear implant other _____

7. Were you born with hearing loss?.....☐ Yes ☐ No
If yes, tick affected ear.....☐ Right ☐ Left
If no, when did it start?.....☐ During Childhood ☐ During Teen Years ☐ During Adulthood

8. Did your hearing loss begin during or soon after: <u>If yes, tick affected ear:</u>

- being pregnant.....☐ Yes ☐ No ☐ Right ☐ Left
- an airplane flight.....☐ Yes ☐ No ☐ Right ☐ Left
- scuba diving.....☐ Yes ☐ No ☐ Right ☐ Left
- intravenous antibiotic treatment....☐ Yes ☐ No ☐ Right ☐ Left
- chemotherapy for cancer...☐ Yes ☐ No ☐ Right ☐ Left
- a severe infection, such as meningitis?...☐ Yes ☐ No ☐ Right ☐ Left
- exposure to a **sudden** loud noise....☐ Yes ☐ No ☐ Right ☐ Left
- **prolonged** exposure to loud noise..☐ Yes ☐ No ☐ Right ☐ Left
- an ear infection.....☐ Yes ☐ No (If yes, tick affected ear..☐ Right ☐ Left)
- ear surgery (including insertion of T-tubes)..☐ Yes ☐ No
(If yes, tick affected ear..☐ Right ☐ Left)
- injury to the head or the ear....☐ Yes ☐ No (If yes, tick affected ear..☐ Right ☐ Left)

30. Stable (little or no change over years).....☐ Right ☐ Left ☐ N/A (Not Applicable)

31. Fluctuating (sometimes better, sometimes worse).....☐ Right ☐ Left ☐ N/A

32. Slowly progressing (getting worse over years).....☐ Right ☐ Left ☐ N/A

33. Rapidly progressing (getting worse over weeks/months).....☐ Right ☐ Left ☐ N/A

34. Sudden hearing loss .....☐ Right ☐ Left ☐ N/A

35. Did the patient's mother have German measles (Rubella), CMV, toxoplasmosis or any other infections during pregnancy?..... ☐ Yes ☐ No ☐ Don't Know
If yes, which infections?_____

36. Did/does the patient's mother have diabetes?.....☐ Yes ☐ No ☐ Don't Know
If yes, at what age was diabetes diagnosed? _____

37. Did the patient's mother have any other illnesses during pregnancy?..☐ Yes ☐ No ☐ Don't Know
If yes, which illnesses? What time during the pregnancy did they occur?
_____

38. Did the patient's mother have any exposure to medication or drugs during pregnancy?.....☐ Yes ☐ No ☐ Don't Know
If yes, which substances? When during the pregnancy were they taken?
_____

39. Did the patient's mother smoke during pregnancy?.....☐ Yes ☐ No ☐ Don't Know
If yes, how much and how often_____

40. Did the patient's mother receive any vaccinations during pregnancy?...☐ Yes ☐ No ☐ Don't Know
If yes, which vaccinations? When during pregnancy were they received?
_____

41. Was the patient born prematurely?.....☐ Yes ☐ No ☐ Don't Know
If yes, how early?_____

42. At birth, did the patient require: If so, for what reason?

- ☐ any special or intensive care as a newborn _____
- ☐ antibiotic treatment _____
- ☐ help with breathing, eg. ventilator _____
- ☐ prolonged hospitalization _____
- ☐ light therapy (phototherapy) as a newborn _____

43. Did the patient have any other serious illnesses at birth?..... ☐ Yes ☐ No ☐ Don't Know
If yes, what other illnesses?_____

| | | | | |
|---|---|---|---|---|
| 44. | Scarlet fever..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 45. | Measles or German measles...(circle which one)..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 46. | Mumps..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 47. | Meningitis (brain infection)..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 48. | Tuberculosis (TB))..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 49. | Repeated or chronic ear drainage..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 50. | Cyst (cholesteatoma) of middle ear..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 51. | Mastoiditis..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 52. | Ear Surgery (specify)_____..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 53. | Exposure to very loud noises..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 54. | Spinning, dizziness, lightheadedness, or unsteadiness.... | ☐ Yes | ☐ No | ☐ Don't Know |
| 55. | Drop attacks (sudden fainting spells)..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 56. | Tinnitus (ringing, buzzing or other sounds in the ear(s))..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 57. | Unusual marks, skin tags or other abnormalities of the ears or ear lobes..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 58. | Unusual shape to the head or facial features..... ..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 59. | White skin patches..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 60. | Brown skin patches..... | ☐ Yes | ☐ No | ☐ Don't Know |
| 61. | Red skin patches..... | ☐ Yes | ☐ No | ☐ Don't Know |

62. White patch of hair on the head.....☐ Yes ☐ No ☐ Don't Know
Age when this appeared:_____)

63. Premature graying of hair before age 30.....☐ Yes ☐ No ☐ Don't Know
(Not just at the temples)

64. Jerky eye movements (nystagmus).....☐ Yes ☐ No ☐ Don't Know

65. White part of the eye showing a pale blue color.....☐ Yes ☐ No ☐ Don't Know

66. Colored part of the eye showing two different colors in the same eye (eg. Left eye both blue and brown) .....☐ Yes ☐ No ☐ Don't Know

67. Colored part of the eye being different in each eye (eg. Left eye blue, right eye brown).....☐ Yes ☐ No ☐ Don't Know

68. Brilliant blue of the colored parts of the eyes.....☐ Yes ☐ No ☐ Don't Know

69. Night blindness.....☐ Yes ☐ No ☐ Don't Know

70. Color blindness.....☐ Yes ☐ No ☐ Don't Know

71. Severe progressive nearsightedness (able to see near objects more clearly than distant ones without glasses).....☐ Yes ☐ No ☐ Don't Know

72. Cloudy vision (cataracts).....☐ Yes ☐ No ☐ Don't Know

73. Decreased peripheral vision.....☐ Yes ☐ No ☐ Don't Know
(ability to see objects and movement outside of the direct line of vision)

74. Absent or deformed tear ducts.....☐ Yes ☐ No ☐ Don't Know

75. Kidney problems.....☐ Yes ☐ No ☐ Don't Know

76. Diabetes mellitus ("sugar diabetes").....☐ Yes ☐ No ☐ Don't Know

77. Thyroid problems (goiter, underactive, overactive).....☐ Yes ☐ No ☐ Don't Know

78. Impairment of smell, taste, or touch.....☐ Yes ☐ No ☐ Don't Know

79. Learning impairment.....☐ Yes ☐ No ☐ Don't Know

80 Is there anything else which you think we should know about the patient's medical history? _____

_____

_____

_____

_____

81. Hearing test?.....☐ Yes ☐ No ☐ Don't Know

If yes, please give dates and locations if you know them:
Date(s)_____
Place where test was done_____

82. Vestibular Testing (Balance Testing)
(ENG, Rotary Chair, Posturography)?.....☐ Yes ☐ No ☐ Don't Know

If yes, please give dates and locations if you know them:
Date(s)_____
Place where test was done_____

83. CT Scan/MRI scan of the head or ear?.....☐ Yes ☐ No ☐ Don't Know

If yes, please give dates and locations if you know them:
Date(s)_____
Place where test was done_____

**Appendix D Consent for Release of Audiological Records**

## REQUEST FOR AUDIOLOGICAL RECORDS

AUTHORIZATION FOR RELEASE OF INFORMATION

| |
|---|
| Please have or next of kin complete all the information below. Please sign and send back in the envelope provided. |

I _____hereby consent to the release of information to the Hereditary Deafness Study, from the records of:

Name: _____ MCP#: _____

Address: _____ Date of Birth: _____

_____

Signature: _____ Date: _____

Witness: _____ Date: _____

Name and location of audiologist: _____

Please check if you agree to share medical records with:

_____ Family members interested in genetic counseling/genetic testing

_____ [Write name(s) of specific individual(s)]

---

Hereditary Deafness Study Use: NLMGP: _____

Patient Name: _____

Principal Investigator: Terry-Lynn Young

*Please return a copy of this form with the medical records to:*

Anne Griffin
Hearing Loss Project
Family Ascertainment and Gene Identification
Memorial University of Newfoundland, Faculty of Medicine
7 Pinsent Drive, Grand Falls-Windsor, NL, A2A 2S8
Phone: 709-489-0569 or toll-free: 1-888-498-3880

## Appendix E Lab Requisition Form

**LAB REQUISITION FORM**

THE GENERAL HOSPITAL
THE HEALTH SCIENCES CENTRE, ST. JOHN'S, NL

**Genetics/Genomics Laboratory, HSC 5315**
**Faculty of Medicine,**
**Discipline of Genetics, MUN**

***Lab Use Only***

Record # _____

DNA # _____

REQUESTED BY:

_____

PHONE #

COLLECTED BY:

DATE AND TIME
SPECIMEN COLLECTED:

PATIENT STATUS: AFFECTED [ ]
UNAFFECTED [ ]
UNKNOWN [ ]

FIRST NAME _____

LAST NAME _____

MAIDEN NAME _____

D.O.B._____/_____/_____
Year Month Day

MCP _____

DIAGNOSES _____

FAMILY # _____ SEX _____

ETHNICITY _____

ADDRESS _____

PROVINCE _____

POSTAL CODE _____

Home community of patient's Father _____

Home community of patient's Mother _____

Check appropriate box:

***Sample for: DNA banking*** ☐ ***3 tubes EDTA lavender (5ml tubes)***

***Cell line*** ☐ ***1 ACD tube yellow***

**INSTRUCTIONS FOR LABORATORY**:

1. Please label all tubes with name and D.O.B (or MCP) of patient
2. Keep blood samples at room temperature.

**Either: 3a. Drop off at HSC 5315 or call Jim, Tammy or Dante at ext. 6041 or**
**3b. Send by SAMEDAY courier 1-888-999-4104 to the address below:**

Genetics/Genomics Laboratory
c/o Lab Office
Health Sciences Centre
300 Prince Philip Drive
St. John's, NL A1B 3V6
Telephone: 1-709-777-6041

May 17, 2004 (HIC # 01.186)

**Lab Office**
**Please call Jim Houston, Tammy Benteau or Dante Galutira at 777-6041 for immediate pick-up**

# Appendix F Linkage Analysis Report for Family 2070

## Linkage analysis in deafness pedigree 2070

*Data providers:* Terry Lynn Young (tlyoung@mun.ca), Nelly Abdelfatah (nellya@mun.ca), Lance Doucette (lanced@mun.ca), Tammy Benteau (tbenteau@mun.ca)
*Report prepared by:* Nicole Roslin (nroslin@sickkids.ca)
*Last modified:* 24 October 2013

### Data summary
*Chip:* Human610-Quadv1_B.bpm
*Number of markers passing QC:* 573,708
*Number of markers used in linkage analysis:* 17,407
*Family:* 2070 (20 genotyped samples)

### Introduction
Twenty individuals from family 2070, from Newfoundland and Labrador, were genotyped using the Human610-Quad chip from Illumina. The genotypes were of excellent quality, and were consistent with the pedigree structure and genders provided for this family; see the document "*Analysis of SNP genotypes from the Illumina Human610-Quad panel in AMGGI Deafness pedigrees*," dated 22 May 2013, for details.

### Samples, pedigree and phenotype
Family 2070 is a multigenerational pedigree with many individuals with deafness (Figure 1). The hearing loss was early onset that progressed to profound deafness. Eleven individuals had deafness that was confirmed by audiogram analysis, and another two had unconfirmed deafness. The analysis was run twice, once including all 13 individuals known to have deafness, and once including just the 11 individuals with confirmed deafness.

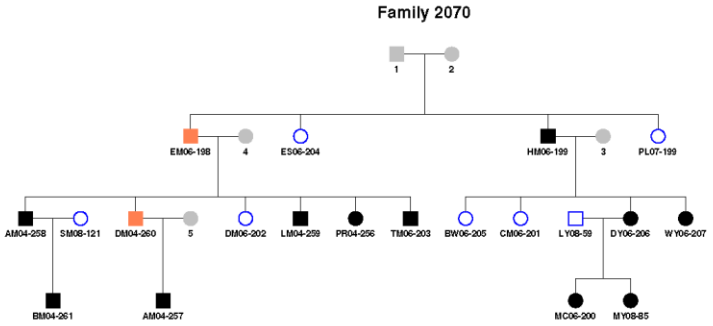


**Figure 1. Family 2070. White symbols outlined in blue are unaffected for hearing loss, grey symbols have unknown phenotype, black symbols have deafness that has been confirmed by audiogram analysis, and orange symbols have unconfirmed deafness. Symbols with hyphenated labels have been genotyped.**

The genetic model to be used in the linkage analysis requires the specification of a disease allele frequency. In linkage analysis, this frequency is used to estimate of the probability that a founder carries the disease allele. Generally, the frequency is derived from the prevalence of the disease in the population. However, statistics are not available regarding the prevalence of hearing loss in Newfoundland and Labrador. Statistics from the Canadian population indicates hearing loss is found in approximately 5% of the adult population. However, there are many different types of hearing loss, with different causes, so the prevalence of the particular type of hearing loss observed in this family will be much less frequent. Therefore, a range of disease allele frequencies was used, with an upper bound corresponding to 5% prevalence.

**Reduced pedigree**

The complete pedigree, including all genotyped members and individuals required to link the pedigree, was composed of 25 individuals. This pedigree is too large to be analyzed using Merlin, which performs exact multipoint analysis. Therefore, the pedigree was reduced to a size that could be handled by Merlin. Five individuals who would provide the least amount of linkage information were removed: the unaffected individuals PL07-199, ES06-204, DM06-202, BW06-205 and CM06-201. The resulting pedigree is shown in Figure 2, and will be referred to as the reduced pedigree in this report.

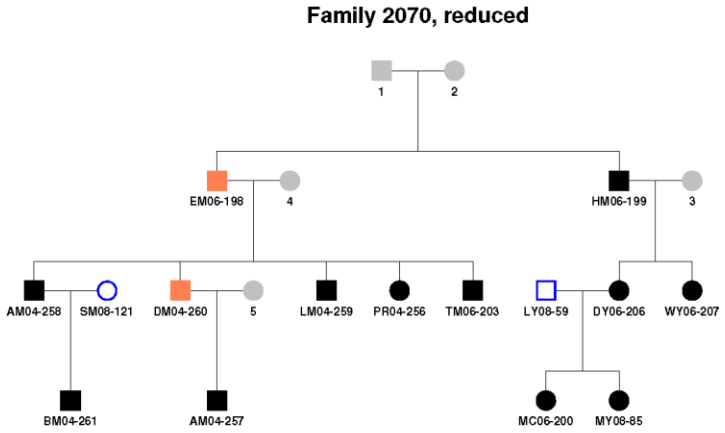


**Figure 2. A reduced version of family 2070. Five unaffected individuals were removed so that exact multipoint linkage could be performed. The symbol legend is the same as in Figure 1.**

*Simulations*

Simulations were performed under the alternate hypothesis of linkage to determine the maximum possible LOD score for the pedigree, under ideal conditions. These conditions included an autosomal disease-causing locus that was perfectly linked to the marker locus, a marker locus that provided perfect information regarding the

segregation of alleles in the pedigree, and an analysis model that was correctly specified. It was also assumed that the pedigree structure and affection status were correct. Genotypes for pedigrees identical to the one in Figure 2 were simulated under dominant and recessive models with a range of disease allele frequencies and penetrances using SLINK 3.02 (Schaffer, et al., 2011), and analyzed under the same model using Merlin 1.1.2 (Abecasis, et al., 2002). The phenocopy rate was fixed at 0.2% for all models. The maximum LOD score obtained from the analysis of 1000 simulated pedigrees was declared the maximum LOD score for any particular model.

For the dominant model, disease allele frequencies of 0.025, 0.005 and 0.0025 were used, corresponding to prevalences of 5%, 1% and 0.5%, respectively, under a fully penetrant model. For the recessive model, the disease allele frequencies used were 0.22, 0.1 and 0.07 (corresponding to the same prevalences). For both dominant and recessive models, simulations were performed using penetrances of 50, 60, 70, 80, 90, 95 and 99%.

For analysis 1, including all 13 individuals with deafness, the maximum simulated LOD score was 3.22, under a dominant model with a disease allele frequency of 0.0025 (Figure 3). Dominant models had consistently higher LOD scores than recessive models.

For analysis 2, including 11 individuals with deafness, the maximum simulated LOD score was also 3.22, under a dominant model with disease allele frequency of 0.0025. The trends were essentially identical to those in analysis 1 (results not shown).

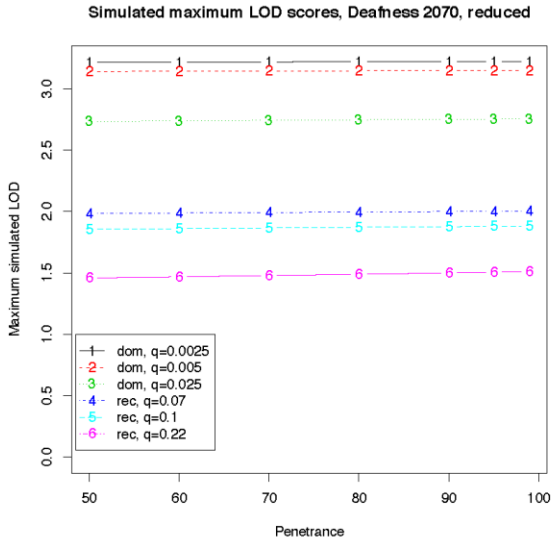


**Figure 3. Simulated maximum LOD scores under the alternative hypothesis of linkage, under a range of genetic models, for analysis 1. Five unaffected individuals were removed from the pedigree to reduce the overall size, as in Figure 2. dom = dominant, rec = recessive, q = disease allele frequency.**

*Multipoint linkage analysis*

SNP filtering

Starting with a set of 572,708 high quality SNPs from the Illumina 610Quad genotyping chip, markers were removed in order to end up with a smaller set suitable for linkage analysis. First, SNPs on chromosomes other than 1 to 22 and X were removed (mitochondrial, Y, XY and unplaced markers). Next, markers that had alleles ambiguous for strand information (A/T and G/C variants) were removed, in order to facilitate strand matching with HapMap data (Altshuler, et al., 2010). Markers that were monomorphic in all genotyped samples were also removed. At this point, approximately 550,000 markers remained. Markers that were present in both this set and the CEU and TSI samples (of western European origin) from HapMap were retained, matching on marker name. Out of this set of about 525,000 markers, SNPs with minor allele frequency >0.45 were kept, which should select for markers with the most linkage information. In order to avoid inconsistencies between the genotypes and the genetic map, only markers with unique locations on the genetic map were extracted. Arbitrarily, for a group of markers at the same genetic position, the marker with the lowest physical position was retained. Since linkage disequilibrium (LD) between the SNPs can arbitrarily inflate multipoint LOD scores (Huang, et al., 2004), the set of markers was pruned for LD between pairs of markers: only SNPs with pairwise $r^2<0.1$ were kept. These steps resulted in a set of 17,407 SNPs across the genome that was suitable for linkage analysis. For this set, the average intermarker distance was 0.26 cM, or 197 kb.

Multipoint linkage analysis

Linkage analysis was performed using Merlin 1.1.2 (Abecasis, et al., 2002) using the observed genotypes. Multipoint linkage analysis was performed under a dominant model with a disease allele frequency ($q$) of 0.0025 and penetrances of 0.2, 99 and 99% for 0, 1, 2 copies of the disease-causing allele, respectively. These parameters correspond to a disease prevalence of approximately 0.5%, and formed the model with the largest simulated maximum LOD score for this family (out of the models tested). Due to the large number of individuals, the pedigree in Figure 1 needed to be reduced in order to be analyzed. Five unaffected individuals (PL07-199, ES06-204, DM06-202, BW06-205 and CM06-201) were removed. Although these individuals contain information for linkage, they do not provide as much information as affected individuals. The reduced pedigree that was used is shown in Figure 2.

For analysis 1 (13 individuals affected with hearing loss, 5 unaffected individuals removed), the largest observed LOD score was 3.22, on chromosome 13 (Table 1, Figure 4, black line). Note that there are two peaks on chromosome 13 that are approximately 5 cM apart. There was also a peak on chromosome 4 with a LOD of 1.07. Using a threshold of LOD >1, the linked regions spanned approximately 37 cM; this represents 1% of the genome, assuming a total genetic length of 36M. The average information content across the genome was 0.99.

For analysis 2 (11 individuals affected with hearing loss, 5 unaffected individuals removed), the largest observed LOD score was 3.22 on chromosome 13 (Table 2). The results were nearly identical between analysis 1 and analysis 2

(Figure 4, red line). The main difference was that there was a peak with LOD = 1.07 on chromosome 5 in analysis 2; this peak had a maximum LOD of 0.64 in analysis 1. However, in both analyses, the peaks on chromosome 13 dominated the results.

Based on the genetic model, autosomal dominant with nearly full penetrance, it should be expected that the results of analyses 1 and 2 are nearly identical. Given this model, and the fact that both individuals with unconfirmed deafness have at least one child with confirmed deafness, there is a high probability that both individuals are also affected.

**Table 1. Regions with maximum observed LOD scores >1 for analysis 1 (5 unaffected individuals removed), under a dominant model with 99% penetrance and a disease allele frequency of 0.0025. A region was defined as the 1-LOD support interval around the maximum. An asterisk following the SNP name indicates that the marker was either the first or last analyzed marker on the chromosome, and so the actual boundary of the linked region is not known. cM = centiMorgans (provided by Illumina), bp = base pairs (GRCh37).**

| Chr | Max LOD | Start | | | End | | |
|---|---|---|---|---|---|---|---|
| | | SNP | cM | bp | SNP | cM | bp |
| 4 | 1.07 | rs4861512 | 183.73 | 183,391,730 | rs11727845 | 189.64 | 185,287,351 |
| 13 | 3.21 | rs2297319 | 92.48 | 99,361,959 | rs7988924 | 94.56 | 100,588,210 |
| 13 | 3.22 | rs2065391 | 100.18 | 104,373,191 | rs9525300* | 129.45 | 114,960,232 |

**Table 2. Regions with maximum observed LOD scores >1 for analysis 2 (5 unaffected individuals removed), under a dominant model with 99% penetrance and a disease allele frequency of 0.0025. See the caption for Table 1 for definitions.**

| Chr | Max LOD | Start | | | End | | |
|---|---|---|---|---|---|---|---|
| | | SNP | cM | bp | SNP | cM | bp |
| 4 | 1.06 | rs4861512 | 183.73 | 183,39,1730 | rs11727845 | 189.64 | 185,287,351 |
| 5 | 1.07 | rs753279 | 140.10 | 140,023,818 | rs4868254 | 188.51 | 172,732,331 |
| 13 | 3.21 | rs2297319 | 92.48 | 99,361,959 | rs7988924 | 94.56 | 100,588,210 |
| 13 | 3.22 | rs2065391 | 100.18 | 104,373,191 | rs9525300* | 129.45 | 114,960,232 |

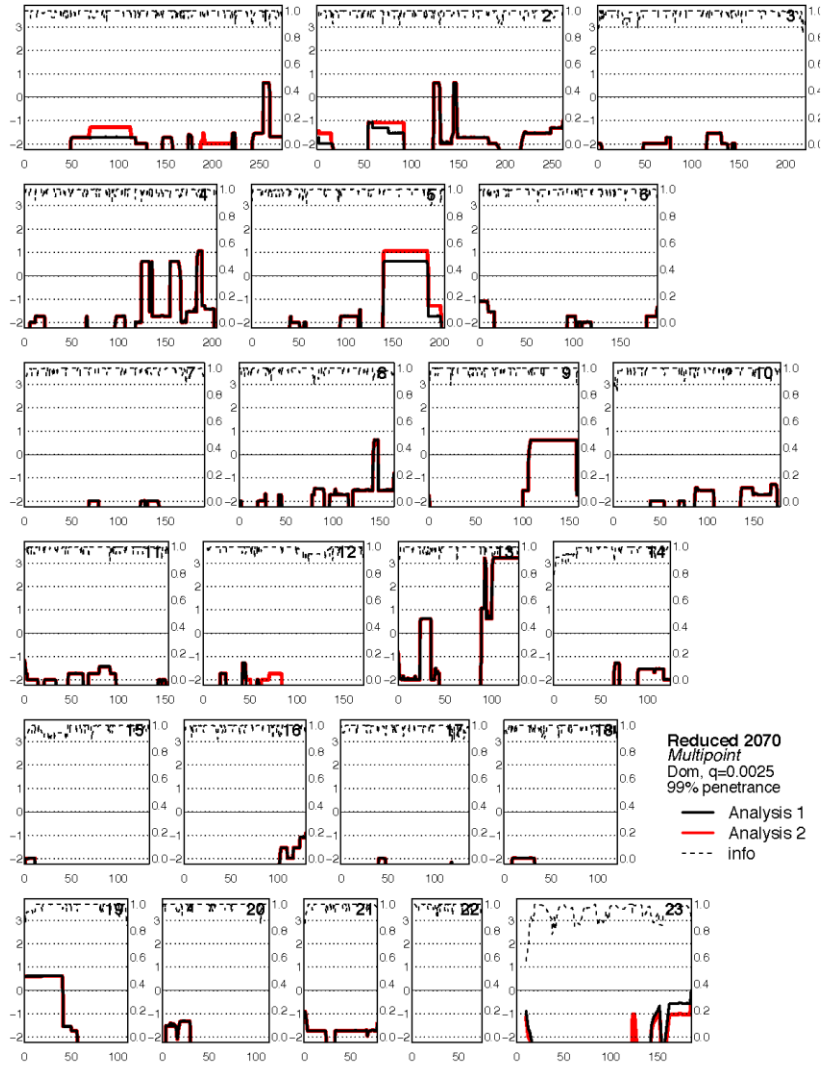


**Figure 4. Multipoint LOD scores (solid lines) and information content (dashed line) for the family shown in Figure 2, under a dominant model with 99% penetrance and $q$=0.0025 for analysis 1 (black line) and analysis 2 (red line). LOD scores use the vertical axis on the left, and information content uses the vertical axis on the right. Only LOD >−2 are shown.**

## Full pedigree

It was not possible to perform exact multipoint linkage analysis on the pedigree in Figure 1, due to computational limitations. In order to extract information from the complete pedigree, two options are available: either perform approximate multipoint analysis, or perform exact singlepoint analysis. It was decided to perform singlepoint analysis, since the re-addition of the five samples back into the pedigree did not increase the complexity (ie, did not add inbreeding loops), it just increased the pedigree size. The resulting analysis will run quickly, but will suffer from a loss of information.

*Simulations*

Simulations were performed under the alternative hypothesis for the full pedigree shown in Figure 1. The same genetic models as in the reduced pedigree were used, and the same assumptions apply. Genotypes were simulated using SLINK, as before, however this time the simulations were analyzed using Superlink 1.7 (Fishelson M, 2004). For analysis 1 (13 affected individuals), the maximum simulated LOD score was 4.77, under a dominant model with 99% penetrance and q=0.0025. Similar results were seen for analysis 2 (11 affected individuals), where the maximum simulated LOD score was also 4.77, under the same model (results not shown).

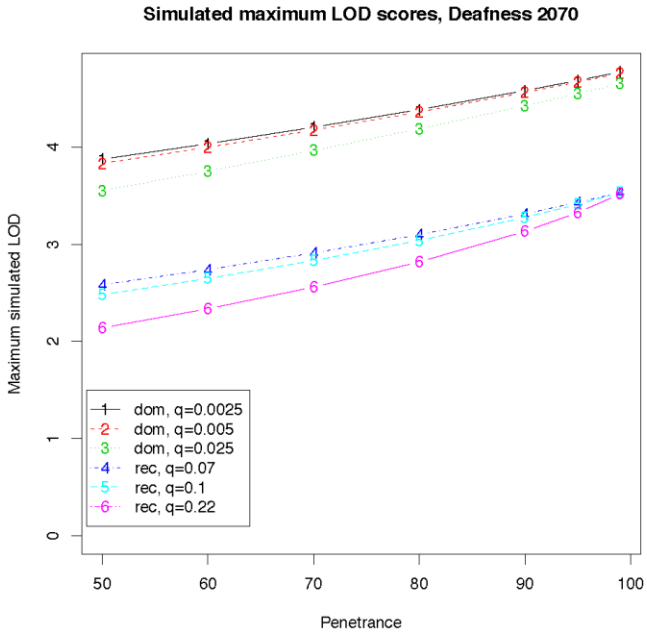


**Figure 5. Simulated maximum LOD scores under the alternative hypothesis of linkage, under a range of genetic models, for analysis 1. The full pedigree shown in Figure 1 was used. dom = dominant, rec = recessive, q = disease allele frequency.**

*Singlepoint linkage analysis*

In order to obtain as much information as possible, windows of five SNPs were examined. For each window, singlepoint linkage was performed at the location of the middle SNP, while the remaining four SNPs were used only for descent information. This approach allowed the genome to be analyzed in approximately 24h. The same genetic model as in the analysis of the reduced family was used: dominant, with q=0.0026 and 99% penetrance. The analysis was performed using Superlink 1.7 (Fishelson M, 2004); this program does not report the information content. Also, the same set of 17,407 SNPs were used in the analysis.

For analysis 1, the maximum LOD score was 4.77, on chromosome 13 (Table 3). This was equal to the maximum LOD score obtained by simulations, indicating that the approach used here was able to extract the full information out of the biallelic markers, at least in this location. Although the 1-LOD support interval may not be the most appropriate way to define a linked region, due to the nature of singlepoint analysis, the 1-LOD support interval extends from rs872484 (117.51 cM) to rs9324254 (128.64 cM) - near the end of the chromosome. This interval is narrower than the one from the multipoint analysis on the reduced pedigree. The next highest LOD score was 2.54, on chromosome 1. Two other chromosomes had LOD >1.5 (chromosomes 5 and 19). Note that the LOD score on chromosome 13 exceeds the threshold for significant linkage proposed by Lander and Kruglyak (1995). Chromosome 4, which had a maximum LOD score of 1.07 in the multipoint analysis, did not achieve LOD >1.5 in the singlepoint analysis. Also, a region on chromosome 1 had LOD >2 in the singlepoint analysis, but had negative LOD scores in the multipoint analysis.

For analysis 2, the results were largely similar. The maximum LOD score was 4.76, also on chromosome 13 (results not shown).

**Table 3. Regions with maximum observed LOD >1.5 in the singlepoint analysis of the full pedigree, analysis 1, under a dominant model with 99% penetrance and a disease allele frequency of 0.0025.**

| Chr | Max | Start | | | End | | |
|---|---|---|---|---|---|---|---|
| | LOD | SNP | cM | bp | SNP | cM | bp |
| 1 | 2.54 | rs591979 | 85.54 | 61,368,955 | rs9629017 | 87.62 | 62,083,960 |
| 1 | 2.14 | rs6593523 | 101.44 | 76,486,908 | rs1360878 | 101.82 | 76,749,088 |
| 1 | 1.87 | rs1325278 | 109.17 | 85,400,182 | rs817485 | 109.42 | 85,573,095 |
| 5 | 1.56 | rs253604 | 161.03 | 155,960,089 | rs6892282 | 163.77 | 159,360,485 |
| 5 | 1.55 | rs11954477 | 167.38 | 163,374,345 | rs253537 | 169.34 | 164,600,485 |
| 13 | 4.77 | rs872484 | 117.51 | 110,708,368 | rs9324254 | 128.64 | 114,312,000 |
| 19 | 1.83 | rs4527136 | 25.72 | 8,186,519 | rs2042300 | 26.79 | 8,580,602 |
| 19 | 1.83 | rs2060260 | 38.32 | 15,704,783 | rs1558139 | 38.55 | 15,997,564 |

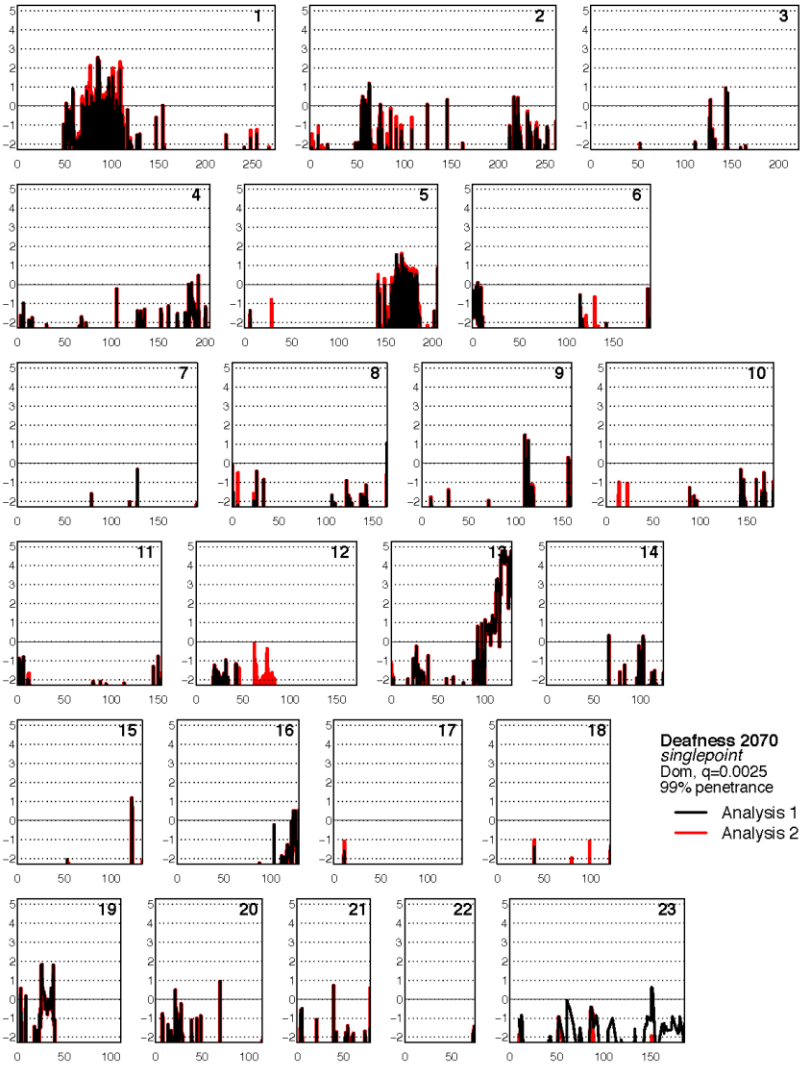


**Figure 6. Singlepoint LOD scores for the full pedigree under a dominant model with 99% penetrance and *q*=0.0025. The black curve represents the score obtained for analysis 1 (13 affecteds), and the red curve represents the scores for analysis 2 (11 affecteds).**

9

## Summary

Multipoint linkage analysis was performed on family 2070, under a dominant model with a disease allele frequency of 0.0025 and a penetrance of 99%, corresponding to a prevalence of 0.5%. Due to the size of the pedigree, two approaches were used to perform the linkage analysis. Multipoint analysis was performed by excluding 5 unaffected individuals. In analysis 1, including all 13 individuals known to have deafness, three regions on two chromosomes attained a LOD score >1, spanning approximately 37 cM (13.7 Mb). Two adjacent regions on chromosome 13 had a LOD of 3.2, the maximum possible for this family and model. In analysis 2, including 11 individuals with deafness confirmed by audiogram analysis, the same three regions had LOD >1, plus one additional region on chromosome 5. Again, two adjacent regions on chromosome 13 had a LOD of 3.2. Singlepoint analysis was also performed on the complete pedigree, using 4 flanking markers for additional information. The largest LOD score was 4.77, observed on chromosome 13 (4.76 for analysis 2). Both singlepoint and multipoint approaches strongly indicate the presence of a linked region at the end of chromosome 13. The two individuals whose deafness was not confirmed by audiogram analysis (EM06-198 and DM04-260) did not appear to strongly influence the linkage results.

## References


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**Appendix G Polymerase Chain Reaction Master Mix.**
Volumes are for one reaction

| Reagent | Volume (µL) |
|---|---|
| Betaine | 5.0 |
| 10x Buffer + $MgCl_2$ | 2.0 |
| dNTP's (10mM) | 0.4 |
| DNA Polymerase | 0.08 |
| $dH_2O$ | 9.92 |
| Primer Reverse | 0.8 |
| Primer Forward | 0.8 |
| DNA Template | 1.0 |
| **Total** | **20.0** |

**Appendix H Primer Sequences for Validation of NGS Dataset**

| Gene | Accession Number | Exon/ Intron | Variant | Primer Sequence | Amplicon Size (bp) |
|---|---|---|---|---|---|
| *COL4A2* | NM_001846.2 | In 17 | c.1012-22T>A | F: CTGGCATTCGACAAGGATTT<br>R: GAATGTGTATGCGATGCTGAA | 380 |
| | | In 20 | c.1339+17A>G | F: CTGCAGGTGAATGCTGTTTG<br>R: TACAGGGCTTCAGCTTCCAT | 389 |
| | | Ex 42 | c.3895_3896insCG | F: AACATAGACAAAGTCATTCCATGC<br>R: TCTCCTGGGAGACCAAACAC | 290 |
| *ING1* | NM_005537.5 | 5'UTR | c.-2569C>A | F: ACCGGGTGTCTGCATACTGT<br>R: AGCCCAATCACGATGAAGAG | 483 |
| *ARHGEF7* | NM_001113511.1 | In 13 | c.1335+31del | F: GACACTTTAATGCTGAGGGAAA<br>R: CCACCATTGTTCTGTTTTGC | 500 |
| | | In 15 | c.1570-19G>T<br>c.1570-21G>T | F: CCTTGTCGCCAGTTGAGTTT<br>R: CCTTCTGAAATACGCAAGCA | 396 |
| *MCF2L* | NM_001112732.1 | In 11 | c.1117-80A>C<br>c.1117-81G>A | F: GTCCCTGCAGACGGTCAAT<br>R: CGCAGAGAACTGGTCACAGA | 350 |
| *F10* | NM_000504.3 | In 7 | c.865+26C>T | F: TCTCAGTCAGGCAACACCTG<br>R: CTCTTCCCCTTCCTCTGCTT | 359 |
| *PCID2* | NM_001127203.1 | In 9 | c.685+24C>T | F: TTTGCCACCTGTTTTTGTTG<br>R: CACTTCAGAGACCCAGTATGGA | 400 |
| *ADPRHL1* | NM_138430.3 | In 3 | c.380-16T>G<br>c.380-17C>A | F: AGCATATCTCCAGGCCATCA<br>R: AGATTTCCAGCCCAGTGTCC | 588 |

Primers were designed using a web-based tool called primer3 version 0.4.0 (http://primer3.sourceforge.net).

**Appendix I Polymerase Chain Reaction Thermocycler Program**

| Number of Cycles | Temperature (ºC) | Time (Minutes) | Description |
|---|---|---|---|
| 1 | 94.0 | 5:00 | Denaturation |
| 5 | 94.0 | 0:30 | Denaturation |
| | 64.0 | 0:30 | Primer Annealing |
| | 72.0 | 0:30 | Primer Extension |
| 30 | 94.0 | 0:30 | Denaturation |
| | 54.0 | 0:30 | Primer Annealing |
| | 72.0 | 0:30 | Primer Extension |
| 1 | 72.0 | 7:00 | Primer Extension |
| | 4.0 | ∞ | Holding |

**Appendix J ABI Cycle Sequencing Master Mix**
Volumes are for one reaction

| Reagent | Volume (µL) |
|---|---|
| BigDye® Terminator v3.1 cycle sequencing enzyme | 0.5 |
| ABI 5X sequencing buffer | 2.0 |
| Forward or Reverse Primer(10pm/µL) | 0.32 |
| PCR grade dH2O | 16.18 |
| Purified PCR product | 1.0 |
| **Total** | **20.0** |

**Appendix K ABI Cycle Sequencing Thermocycler Program**

| Number of Cycles | Temperature (ºC) | Time (Minutes) | Description |
|---|---|---|---|
| 1 | 96.0 | 1:00 | Denaturation |
| | 96.0 | 0:10 | Denaturation |
| 25 | 50.0 | 0:05 | Primer Annealing |
| | 60.0 | 4:00 | Primer Extension |
| 1 | 4.0 | ∞ | Holding |

**Appendix L Primer Sequences for cDNA Analysis of *ADPRHL1***

| Gene | Accession Number | Exon/ Intron | Variant | Primer Sequence | Amplicon Size (bp) |
|---|---|---|---|---|---|
| *ADPRHL1* | NM_138430.3 | Ex 2-4 | c.380-16T>G<br>c.380-17C>A | F: CGGGAGATGGTGAGATGCTA<br>R:GGTCTTCCTGCAGTACTCTTCT | 390 |

Primers were designed using a web-based tool called primer3 version 0.4.0 (http://primer3.sourceforge.net).

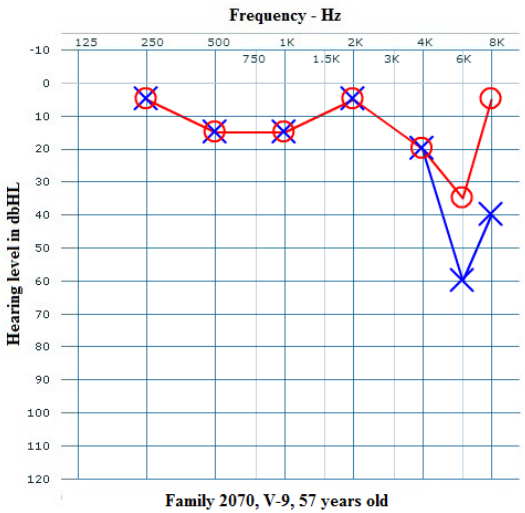
**Appendix M Phenotype of Unspecified Hearing Loss in Family 2070**

Related audiograms are shown in **Appendix N**. The PID number indicates the family member's position within the pedigree. The following categories were assessed using the GENDEAF guidelines. The age of onset was based upon audiological evidence or patient recall. The age of recent assessment indicated the age in years of the most recent audiological exam. Bilateral symmetry indicated if both ears were affected similarly. Severity indicated the level of hearing loss. Configuration described the slope of the hearing loss and the overall change in hearing thresholds across frequencies.

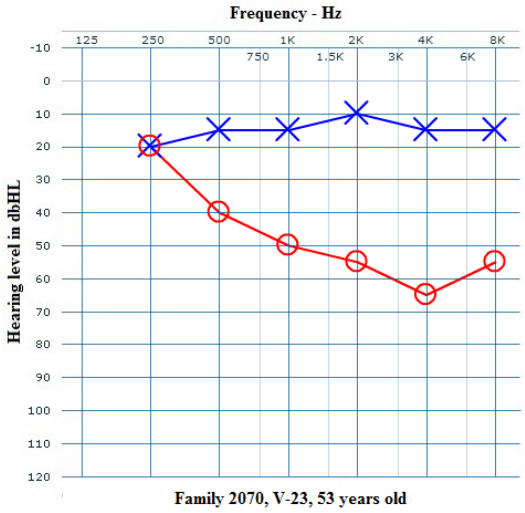
| PID | Age of Onset (Years) | Age of Recent Assessment (Years) | Bilateral Symmetry | Severity | Configuration |
|---|---|---|---|---|---|
| V-9 | Uncertain | 57 | Yes | Normal | Flat |
| V-23 | Uncertain | 53 | No | Moderate | Gently Sloping |
| V-34 | Uncertain | 58 | Yes | Normal | Gently Sloping |

## Appendix N Audiograms of Unspecified Hearing Loss in Family 2070

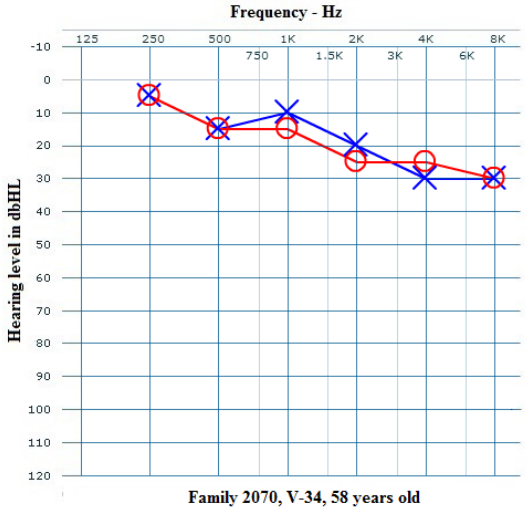
Audiograms are described in **Appendix M**. The red lines indicate the hearing thresholds of the right ear and blue lines the left ear



Family 2070, V-9, 57 years old



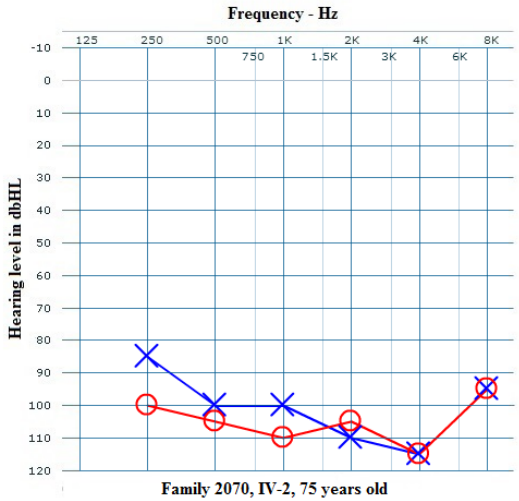
Family 2070, V-23, 53 years old



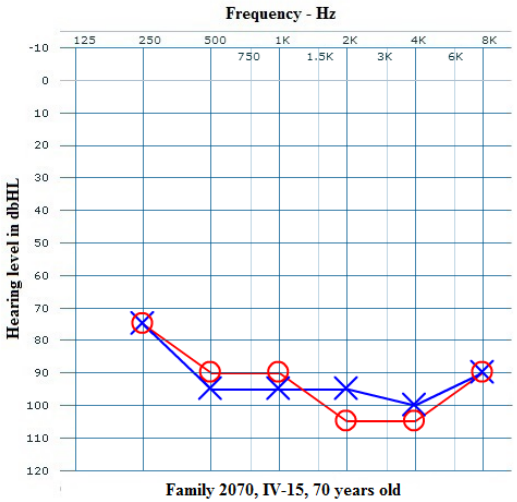
Family 2070, V-34, 58 years old

## Appendix O Audiograms of Inherited Hearing Loss in 4th Generation of Family 2070

Audiograms are described in **Table 3.1**. The red lines indicate the hearing thresholds of the right ear and blue lines the left ear



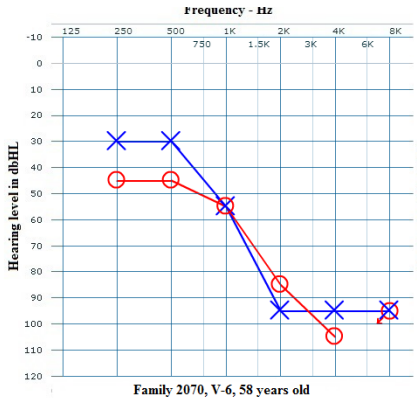

Family 2070, IV-2, 75 years old



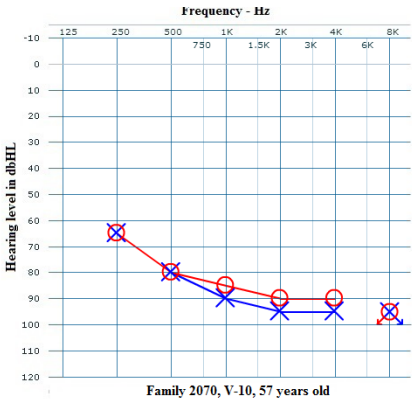

Family 2070, IV-15, 70 years old

## Appendix P Audiograms of Inherited Hearing Loss in 5th Generation of Family 2070

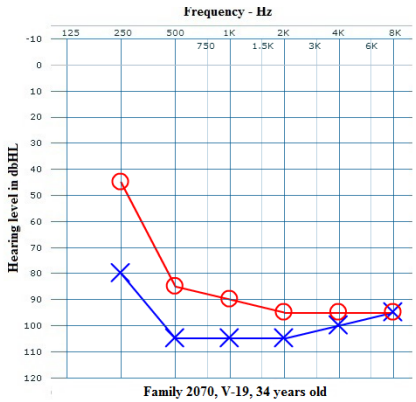
Audiograms are described in **Table 3.1**. The red lines indicate the hearing thresholds of the right ear and blue lines the left ear



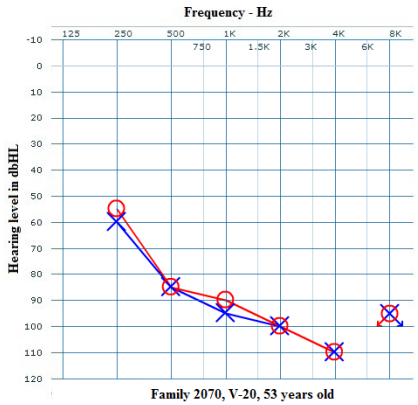
Family 2070, V-6, 58 years old



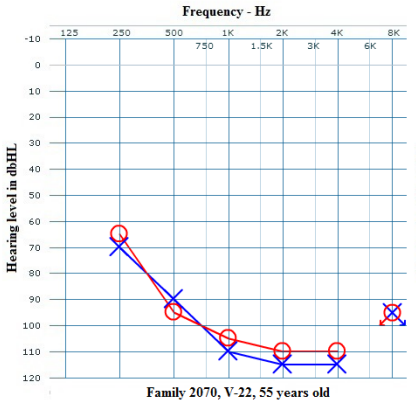
Family 2070, V-10, 57 years old



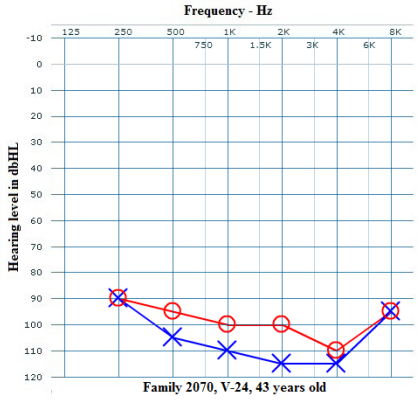
Family 2070, V-19, 34 years old



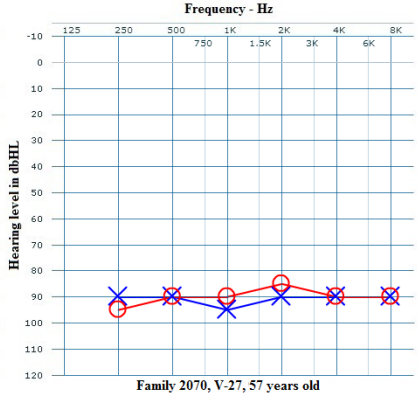
Family 2070, V-20, 53 years old



Family 2070, V-22, 55 years old



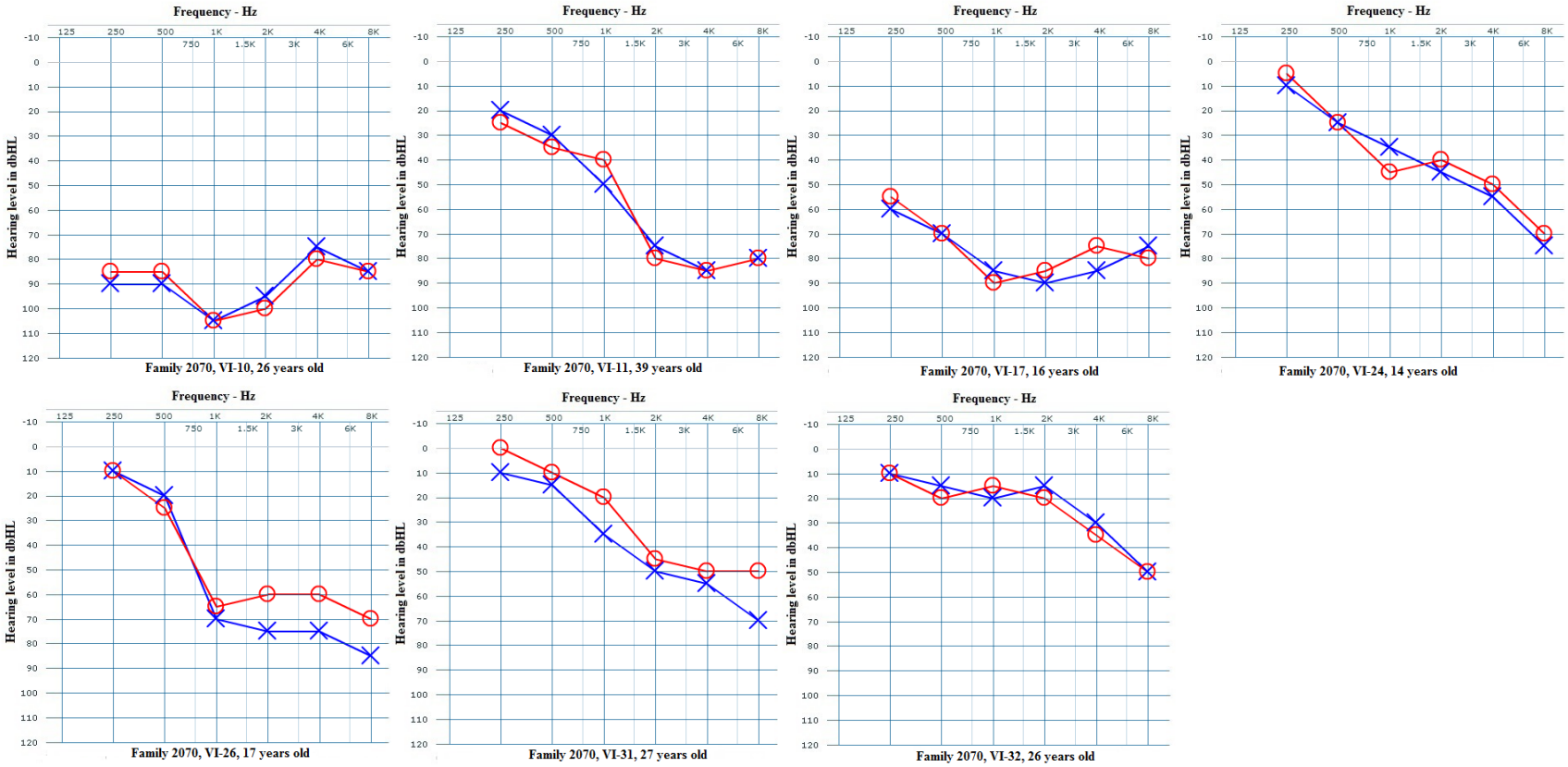
Family 2070, V-24, 43 years old



Family 2070, V-27, 57 years old

## Appendix Q Audiograms of Inherited Hearing Loss in 6th Generation of Family 2070

Audiograms are described in **Table 3.1**. The red lines indicate the hearing thresholds of the right ear and blue lines the left ear



## Appendix R Coverage of Exome Sequencing Panel Design in Linked Region

| Gene | Percentage Covered | Target (bp) | Missing (bp) | Missing Genomic Regions |
|---|---|---|---|---|
| *COL4A1* | 99.7% | 5,010 | 17 | Chr13:110853785-110853801 |
| *COL4A2* | 99.9% | 5,139 | 3 | Chr13:111109690-111109692 |
| *RAB20* | 100% | 705 | 0 | - |
| *CARKD* | 100% | 1,426 | 0 | - |
| *CARS2* | 100% | 1,695 | 0 | - |
| *ING1* | 100% | 2,042 | 0 | - |
| *ANKRD10* | 100% | 1,263 | 0 | - |
| *ARHGEF7* | 97.3% | 2,889 | 78 | Chr13:111811374-111811436 |
| *TEX29* | 100% | 456 | 0 | - |
| *SOX1* | 87.2% | 1,176 | 150 | Chr13:112722584-112722719<br>Chr13:112722923-112722929<br>Chr13:112723142-112723148 |
| *SPACA7* | 100% | 588 | 0 | - |
| *TUBGCP3* | 99.6% | 2,724 | 11 | Chr13:113242219-113242229 |
| *ATP11A* | 100% | 3,654 | 0 | - |
| *MCF2L* | 99.8% | 3,451 | 7 | Chr13:113751118-113751124 |
| *F7* | 99.5% | 1,401 | 7 | Chr13:113765158-113765164 |
| *F10* | 100% | 1,467 | 0 | - |
| *PROZ* | 100% | 1,269 | 0 | - |
| *PCID2* | 100% | 1,200 | 0 | - |
| *CUL4A* | 89.5% | 2,348 | 247 | Chr13:113863942-113864072<br>Chr13:113914921-113915036 |
| *LAMP1* | 95.1% | 1,254 | 61 | Chr13:113951750-113951810 |
| *GRTP1* | 100% | 1,011 | 0 | - |
| *ADPRHL1* | 100% | 1,198 | 0 | - |
| *DCUN1D2* | 99.6% | 780 | 3 | Chr13:114144982-114144984 |
| *TMCO3* | 100% | 2,034 | 0 | - |
| *TFDP1* | 100% | 1,233 | 0 | - |
| *ATP4B* | 100% | 876 | 0 | - |

**Appendix S Coverage of Exome Sequencing Run of V-10 in Linked Region**

| Gene | Percentage Covered | Total Number of Amplicons | Number of Amplicons Missing | Missing Genomic Regions |
|---|---|---|---|---|
| *COL4A1* | 96.4% | 56 | 2 | Chr13:110959096-11095913<br>Chr13:110959302-110959492 |
| *COL4A2* | 100.0% | 58 | 0 | - |
| *RAB20* | 100.0% | 5 | 0 | - |
| *CARKD* | 92.3% | 13 | 1 | Chr13:111268085-111268274 |
| *CARS2* | 95.0% | 20 | 1 | Chr13:111358249-111358455 |
| *ING1* | 92.3% | 13 | 1 | Chr13:111366597-111366787 |
| *ANKRD10* | 90.0% | 10 | 1 | Chr13:111567256-111567468 |
| *ARHGEF7* | 100.0% | 26 | 0 | - |
| *TEX29* | 80.0% | 5 | 1 | Chr13: 111973199-111973418 |
| *SOX1* | 14.3% | 7 | 6 | Chr13:112721905-112722109<br>Chr13:112721986-112722172<br>Chr13:112722318-112722511<br>Chr13:112722372-112722583<br>Chr13:112722720-112722922<br>Chr13:112722930-112723141 |
| *SPACA7* | 100.0% | 7 | 0 | - |
| *TUBGCP3* | 100.0% | 28 | 0 | - |
| *ATP11A* | 97.3% | 37 | 1 | Chr13:113344666-113344871 |
| *MCF2L* | 97.4% | 39 | 1 | Chr13:113751125-113751320 |
| *F7* | 100.0% | 13 | 0 | - |
| *F10* | 100.0% | 13 | 0 | - |
| *PROZ* | 84.6% | 13 | 2 | Chr13:113817158-113817376<br>Chr13:113824606-113824789 |
| *PCID2* | 100.0% | 17 | 0 | - |
| *CUL4A* | 91.7% | 24 | 2 | Chr13:113864073-113864282<br>Chr13:113864096-113864306 |
| *LAMP1* | 100.0% | 10 | 0 | - |
| *GRTP1* | 90.0% | 10 | 1 | Chr13:114018201-114018395 |
| *ADPRHL1* | 100.0% | 11 | 0 | - |
| *DCUN1D2* | 100.0% | 8 | 0 | - |
| *TMCO3* | 100.0% | 20 | 0 | - |
| *TFDP1* | 100.0% | 14 | 0 | - |
| *ATP4B* | 90.9% | 11 | 1 | Chr13:114312359-114312555 |