Process Review of *GJB6* Reflex Testing in Individuals with 0 or 1 *GJB2* Pathogenic Variants and Non-Syndromic Hearing Loss

THESIS

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Abstract

The gene most commonly implicated in autosomal recessive non-syndromic hearing loss (NSHL) is GJB2. Homozygous deletions within the gene GJB6, as well as a heterozygous GJB2 mutation with one GJB6 deletion, can also lead to NSHL. The American College of Medical Genetics and Genomics (ACMG) and European Molecular Genetics Quality Network (EMQN) both recommend that genetic testing in hearing loss begin with analysis of GJB2 and GJB6 prior to panel testing that includes additional genes. At Nationwide Children's Hospital (NCH) the testing protocol in apparently NSHL is to sequence GJB2 with reflex to GJB6 deletion analysis. However, some previous studies and the testing experience at NCH indicate that GJB6 deletions may not be as prevalent as previously thought. In order to determine the most effective testing approach in NSHL, the testing strategies and the average costs of each was determined from public databases. The frequency and distribution of GJB2 mutations and GJB6 deletions in a population of NSHL cases from NCH was elucidated and compared to the frequency of GJB6 deletions in various control populations. Thirty-three different GJB2 sequencing test options from 23 different CLIA-certified laboratories within the United States were analyzed to reveal the average cost of GJB2 sequencing with reflex GJB6 deletion analysis was \$802.50. The average cost of panels including GJB2, GJB6 and additional genes was \$2660. Six hundred five GJB2 sequencing and 528 GJB6 deletion

analysis records representing 607 unique cases from April 2009 to April 2016 at NCH were analyzed. Seventy-six total cases were provided with a genetic diagnosis for their hearing loss from GJB2 sequencing and/or GJB6 deletion analysis representing an overall diagnostic rate of 12.5% (76/607). Only one instance of the 309 kb GJB6 deletion (frequency of 0.16%; 1/607) was found in a case also carrying a likely pathogenic GJB2 variant. The frequency of GJB6 deletions within 10612 microarrays (hearing loss was not noted as an indication for testing) and the frequency of *GJB6* deletions within with Database of Genomic Variants (DGV) was determined. Seven of the 10612 microarrays (0.067%; 7/10612) were found to carry a deletion within or spanning a portion of *GJB6*. Three studies outlined in the DGV found 37 out of 53952 total individuals (0.069%) carried a deletion within GJB6. The GJB6 deletion was found at similar frequencies in cases with hearing loss at NCH and in various control populations. These results indicate that GJB6 deletion analysis is a low yield testing option that may not be cost effective. The overall diagnostic rate of cases at NCH was also lower than found in previous literature. Based upon the data generated from this study, we recommend panel genetic testing which includes GJB2, GJB6 and additional genes be recommended as a first line test in NSHL.

Dedication

This document is dedicated to my husband, parents, and all other friends and family

members.

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CHAPTER 1: INTRODUCTION

Hearing loss is the most common sensory deficit, affecting one in every 500-1000 newborns (Paz-y-Miño, Beaty, López-Cortés, & Proaño, 2014; Hoefsloot, Roux, & Bitner-Glindzicz, 2013). About 30% of hearing loss cases are syndromic in nature, meaning that hearing loss may be accompanied by additional clinical features such as vision/renal/endocrine changes or other health concerns; the remaining 70% of hearing loss cases are non-syndromic (Hoefsloot, Roux, & Bitner-Glindzicz, 2013). NSHL is an isolated case of hearing loss in an otherwise healthy individual that may be present from birth or develop over time. Environmental factors such as rubella, cytomegalovirus (CMV) infection, and ototoxic medications can also lead to hearing loss but are typically not associated with a genetic basis (Korver et al, 2017). Most genetic forms of NSHL are inherited in an autosomal recessive manner (80%), while about 20% of cases demonstrate autosomal dominant inheritance, 1% of cases exhibit X-linked, and 1% display mitochondrial inheritance (Cascella et al., 2016). Over 100 genes are known to cause NSHL and the majority of these genes encode connexin transmembrane proteins (Tayoun et al., 2015; Martínez, Acuña, Figueroa, Maripillan, & Nicholson, 2009). Connexins are gap junction proteins expressed primarily in the inner ear, as well as the brain, skin, nail beds and hair follicles and play a role in the transport of potassium ions.

1

Connexin Structure and Function

Gap junction proteins such as connexin connect adjacent cells to one another by creating an intercellular channel between the cells' cytoplasms. This channel has a pore size of about 10-15 Å, which is larger than other ion channels like K^+ and Ca^{++} , and enables intercellular communication (Wingard & Zhao, 2015). Each intercellular channel is formed from two hemichannels known as connexons, which in turn are made up of six subunits known as connexins (Harris, 2001). The pore size of gap junctions allows ions, as well as molecules up to ~ 1.5 kDa and cell signaling molecules to pass through (Harris, 2001). Gap junction proteins are expressed in vertebrates and invertebrates. In vertebrates, these gap junction proteins are encoded primarily by connexin genes, which lead to greater than 20 connexin isoforms (Willecke et al., 2002). By far, the most common connexin isoform implicated in NSHL is GJB2 (OMIM: 121011), which encodes the protein gap junction protein beta-2, more commonly known as connexin 26. GJB2 mutations are causative in approximately fifty percent (50%) of all NSHL cases (Rabionet et al., 2000). GJB2 was initially discovered in 1997 and immunocytochemical studies have demonstrated that connexin 26 is active in the cochlea beginning in the 22nd week of gestation and recycles potassium ions within the inner ear (Moreira, da Silva, Lopez & Mantovani, 2015). Another common connexin isoform implicated in NSHL is GJB6 (OMIM: 604418), or connexin 30. Gap junction protein beta-6 (GJB6) mutations, typically deletions, can be inherited with a GJB2 mutation, leading to autosomal recessive NSHL.

The exact role of connexin 26 (Cx26) and connexin 30 (Cx30) in the inner ear is

not well understood. Previous studies have shown that Cx26 and Cx30 are widely expressed in the cochlea, specifically in the connective and epithelial tissues (Wingard & Zhao, 2015). Many other connexin proteins are also expressed in the cochlea, but Cx26and Cx30 are the most proliferative. Within the cochlea, gap junction proteins are expressed in supporting cells in the organ of Corti, spiral ligament, spiral limbus, stria vascularis and other structures (Wingard & Zhao, 2015). It is also known that Cx26 is permeable to anionic molecules like IP₃, cAMP, and ATP which are important for cell signaling while Cx30 is impermeable to anionic molecules. Therefore, it is expected that Cx26 plays some role in cell signaling (Wingard & Zhao, 2015). There are two independent gap junction networks within the inner ear. According to Wingard & Zhao in describing Kikuchi and colleague's research these include "the epithelial gap junctional network between supporting cells in the auditory sensory epithelium in the organ of Corti and the connective tissue gap junctional network between the connective tissue cells in the cochlear lateral wall" (Wingard & Zhao, 2015; Kikuchi et al., 1995). Connexin is not expressed in the hair cells though, nor in connections between supporting cells and outer hair cells. There are many hypotheses about the function of gap junction proteins within the cochlea. Some of these include establishing correct ion levels, intercellular signaling, K+ recycling, energy generation like of endocochlear potential, maintenance and creation of the electrochemical environment, epithelial repair, providing aid in the development of other cells expressed in the cochlea, and cochlear amplification (Wingard & Zhao, 2015). While the exact role of gap junction proteins has not been elucidated, it can be expected that mutations in these genes would impact cochlear function. Clinical genetic testing for

both of these genes is widely available and utilized when attempting to discover a genetic basis for NSHL.

GJB2 Gene

GJB2-Related Syndromic Hearing Loss

Syndromic conditions inherited in an autosomal dominant fashion, such as

- Vohwinkel syndrome: associated with hearing loss, abnormal skin patches, and the development of tight bands of fibrous tissue leading to amputation, generally of the fingers or toes, typically in early childhood.
 Three *GJB2* missense mutations have been linked to this condition (Lee & White, 2009)
- Hystrix-like ichthyosis with deafness (HID): characterized by ichthyosis and profound deafness and has been linked to a single *GJB2* variant, p.Asp50Asn. This variant causes the channels to leak ions, leading to decreased efficiency and increased apoptosis of cells within the inner ear and skin (Lee & White, 2009)
- Keratitis-ichthyosis-deafness syndrome (KID): The p.Asp50Asn mutation is also the most common mutation in individuals with KID. KID is associated with keratitis, ichthyosis, and deafness as the name suggests and is caused by eight other less common *GJB2* mutations, all of which are missense. Because of the similarities between KID and HID, many researchers classify these as a single condition, KID/HID (Lee & White, 2009).

- Bart-Pumphrey syndrome: linked to two missense *GJB2* variants,
 p.Gly59Ser and p.Asn54Lys which disrupt the normal function of *GJB2* (Lee & White, 2009). The phenotype of Bart-Pumphrey is characterized
 by palmoplantar keratoderma, leukonychia, hearing loss, and knuckle pads
- Palmoplantar keratoderma with deafness: presents with thickened skin on the palms of the hands and soles of the feet as well as deafness and has been linked to nine different missense mutations of *GJB2*, affecting connexin 26 function (Lee & White, 2009)

The mutations leading to syndromic forms of hearing loss have not been described in individuals with NSHL. Nearly all of the mutations in *GJB2* leading to syndromic hearing loss with skin disorders appear to be clustered within the N-terminus and first extracellular domain. Interestingly, the *GJB2* mutations leading to skin conditions are all missense, leading to a single amino acid change, while NSHL mutations of *GJB2* tend to be loss of function and can be missense, splice site variants, or deletions, among other types. Lee & White, in their paper entitled "Connexin-26 mutations in deafness and skin disease" have postulated that the mutations leading to skin disease as well as deafness are dominant gain of function while NSHL mutations are typically loss of function (Lee & White, 2009). This postulation is based on the observation that complete loss of connexin 26 function is associated with hearing impairment only, meaning that functional Cx26 is unnecessary for human skin homeostasis (Lee & White, 2009).

GJB2-Related NSHL

NSHL loci are named according to their inheritance pattern and the order in which they were discovered. Deafness is shortened to DFN, autosomal dominant is A, autosomal recessive is B, and X-linked is referred to as X. DFNB1 (OMIM: 220290), or autosomal recessive hearing impairment includes the GJB2, cis-regulatory elements that may alter the expression of the GJB2 protein, and GJB6. The majority of GJB2 variants are consistent with the DFNB1 phenotype, which includes congenital sensorineural hearing loss that is typically non-progressive and may be mild to profound (Smith & Jones, 2016). However, some GJB2 variants fall under the DFNA3 umbrella, characterized by autosomal dominant inheritance. DFNA3 includes dominant-negative pathogenic mutations in GJB2 or GJB6. The DFNA3 phenotype is characterized by highfrequency sensorineural hearing impairment that is typically progressive, may be pre-or post-lingual, and ranges from mild to profound (Smith, Ranum & Camp, 2014). Additionally, individuals with DFNA3 mutations tend to have a positive family history, usually an affected parent, given the fact that DFNA3 is inherited in an autosomal dominant fashion. Dominant mutations in GJB2 can be associated with NSHL (DFNA3) or syndromic conditions that tend to affect the skin as well as hearing.

Currently more than 100 different variants in *GJB2* have been described. These variants can lead to a wide array of phenotypic effects; they also have varying patterns of inheritance, and are classified with differing levels of pathogenicity. The carrier frequency of each *GJB2* variant within the general population also varies widely. For some common *GJB2* variants the carrier frequency has been shown to be as high as 1-3%

(Kenneson, Braun, Boyle, 2002). It is also important to note that many of the phenotypes caused by these variants can be complicated by such factors as reduced penetrance and variable expressivity. For example, a variant may affect the expression of gap junctions at the cell surface and could be classified as likely pathogenic with an autosomal recessive pattern of inheritance but also exhibit reduced penetrance. These varieties have long complicated the classification of *GJB2* variants and the decision as to whether the full cause of NSHL has been detected, or if further genetic testing for additional variants is warranted.

The most common *GJB2* variant in Caucasians leading to autosomal recessive sensorineural deafness is c.35delG (at position 35 there is a deletion of a guanine base pair, G, leading to a shift in the reading frame, also known as a frameshift mutation) with a frequency ranging from 28%-63% depending on country of origin (Gasparini et al., 2000). A study completed by Gasparini and colleagues looked at 3270 random controls from 17 European countries to determine carrier frequency of the c.35delG variant. The carrier frequency of this variant was 1 in 35 in southern Europe, and 1 in 79 in central and northern Europe. Altogether, the overall carrier frequency of the c.35delG variant in Europe was found to be 1 in 51. Additionally, this study also found the c.35delG variant to be present in 5 of 376 Jewish subjects of varying origins (Gasparini et al., 2000).

The prevalence of mutations other than c.35delG in *GJB2* is also highly related to ethnicity. Certain mutations may be rare in those of European descent but much more common in another ethnic population. Additionally, mutations in the DFNB1 locus (*GJB2 & GJB6*) may be so rare in some populations that it is more worthwhile to test

other genes. For example, in China, *GJB2*, *SLC26A4*, and *mtDNA12SrRNA* are the three most common genes associated with NSHL, indicating that testing that only includes *GJB2* sequencing and *GJB6* deletion analysis may not be comprehensive enough in these individuals (Ma et al., 2016; Zhang et al., 2016). In Morocco, *GJB2* plays a key role in NSHL (Bakhchane et al., 2016). In Africans from Cameroon, South Africa, São Tomé, and Príncipe however, there is limited clinical utility for testing *GJB2*, *GJB6* and *GJA1* (Caroça et al., 2016; Wonkam et al., 2015). In the Ashkenazi Jewish population, c.167delT is the most common *GJB2* mutation (carrier frequency of 7.5%) (Snoeckx et al., 2005). In the Taiwanese, p.Val37IIe is most common mutation (carrier frequency of 11.6%), and in the Japanese, c.235delC is the most common mutation (carrier frequency of 1-2%) (Snoeckx et al., 2005). When considering genetic testing in NSHL, it is important to consider the ethnic background of a patient and what genes are most often implicated in NSHL cases of others from this ethnicity.

While the majority of pathogenic variants affect the process of connexin 26 gap junctions being transcribed, translated, and transported to the cell surface, other variants can have very different pathogenic effects. Some variants can exhibit dominant negative effects by disrupting wild type *GJB2* allele expression. The p.Met34Thr variant leads to correct expression and movement, but inhibits the gap junction from forming efficient channels (Skerrett, Di, Kasperek, Kelsell, & Nicholson, 2004). An additional variant, p.Val84Leu, allows the gap junction to be permeable to ions but not to smaller molecules like inositol triphosphate (IP₃) (Beltramello, Piazza, Bukauskas, Pozzan, & Mammano, 2005). Finally, variants like p.Gly45Glu and p.Arg75Trp can affect hemichannel activity (two hemichannels form one gap junction) in the presence of Ca⁺⁺ or reduce gap junction plaque formation respectively (Stong, Chang, Ahmad, & Lin, 2006; Kamiya et al., 2014). Clearly, *GJB2* exhibits a wide complexity of variants with many possible mutational effects. Additionally, the phenotypic effects of these variants can range from profound congenital hearing loss to mild hearing loss that may progressively worsen throughout childhood and adulthood.

A large study of 1531 people from 16 different countries completed by Snoeckx and colleagues in 2005 helped determine genotype-phenotype correlations of several variants in GJB2 which are still useful in variant interpretation today. This study looked at 83 different mutations which were classified as either truncating (36) or non-truncating (47). 153 different genotypes were found and were separated as homozygous truncating/truncating (T/T), homozygous nontruncating/nontruncating (NT/NT), and compound heterozygous nontruncating/truncating (NT/T) (Snoeckx et al., 2005). This study determined that homozygous truncating/truncating mutations are associated with significantly more severe hearing loss than that observed in individuals with homozygous nontruncating/nontruncating mutations. The median hearing impairment of c.35delG/p.Arg143Trp was 105 dB and 108 dB for individuals with c.35delG and the common deletion in GJB6 (D13S1830). Additionally, c.35delG homozygotes were found to have a more severe phenotype (more severe hearing loss) than 48 different genotypes (Snoeckx et al., 2005). Two genotypes (c.35delG/p.Arg143Trp and c.35delG/GJB6-D13S1830) were associated with a significantly more severe phenotype than c.35delG homozygotes. Three common GJB2 mutations were also found to be associated with mild to moderate hearing loss (median of 25-40 dB): p.Leu90Pro, p.Met34Thr and p.Val37Ile (Snoeckx et al., 2005). The GeneReviews (Smith & Jones, 2016) for DFNB1 has adapted the genotype-phenotype data published by Snoeckx and colleagues into a table which includes the percentage of cases with varying levels of hearing impairment compared to the genotype present (Smith & Jones, 2016). Below is a redesigned table (Table 1) that presents the information available on GeneReviews initially published by Snoeckx and colleagues (Smith & Jones, 2016; Snoeckx et al., 2005).

		Number of Subjects With Level of Hearing Loss			
Type of Genotype	Number of Snoeckx's Subjects	Profound	Severe	Moderate	Mild
Nontruncating/Nontruncating	34	0	3	8	23
Truncating/Nontruncating	147	31	12	46	58
Truncating/Truncating	1176	777	277	110	12

Table 1: Genotype Class and Level of Hearing Loss in 1357 Subjects With Biallelic Pathogenic *GJB2* Variants (Snoeckx et al., 2005)

GJB6 Gene

GJB6-Related Syndromic Hearing Loss

Just as mutations in GJB2 can lead to syndromic hearing loss, some GJB6

mutations cause Clouston syndrome. Clouston syndrome, also known as hidrotic

ectodermal dysplasia 2, is an autosomal dominant condition characterized by alopecia, nail dystrophy, and hyperkeratosis on the palms of the hands and soles of the feet (Yang et al., 2016). Clouston syndrome can also rarely be associated with eye abnormalities, sensorineural hearing loss, eccrine poromatosis (multiple benign tumors of a major type of sweat gland known as eccrine expressed throughout the skin but most dense on the palms and soles) and oral leukoplakia. The four most common mutations in GJB6 that can lead to a diagnosis of Clouston syndrome include p.Val37Glu, p.Asp50Asn, p.Gly11Arg, and p.Ala88Val (Yang et al., 2016). Previous studies have also indicated that a mutation in GJB2 (p.Arg127His) together with a missense mutation in GJA1 (p.Val41Leu) can lead to Clouston syndrome as well as mutations in *GJB6* (p.Asn14Ser) together with a mutation in GJB2 (p.Phe191Leu) (Yang et al., 2016). Clearly, there is significant phenotypic variability when it comes to mutations within GJB2 and GJB6, which makes the interpretations of variants within these genes that much more difficult. The GJB6 mutation (p.Gly11Arg) has been listed in ClinVar as being associated with Clouston syndrome, but one submitter suggested it may play a role in NSHL as well. The GJB2 mutation (p.Arg127His) is considered a benign or likely benign variant when interpreted in a case of NSHL. Associations with these variants in NSHL alone are uncommon but possible, adding yet another layer of complexity to variant interpretation. GJB6-Related NSHL

As discussed previously, some *GJB2* and *GJB6* variants can be associated with DFNA3. For example, single mutations in *GJB6* can act as a dominant mutation (such as the missense mutation, p.Thr5Met), or create a dominant negative effect that impairs the

development of wild type connexin 26 and 30 (like the p.Ala40Val and p.Ile248Val mutations) (Wingard & Zhao, 2015).

Two large deletions in *GJB6* have been previously described as pathogenic and include a 309 kb deletion, del(*GJB6*-D13S1830) (the most common, previously described as a 342kb deletion) and a 232 kb deletion del(*GJB6*-D13S1854) (less common). Cases where an individual carries one pathogenic *GJB2* variant and one *GJB6* deletion are a well-known cause of digenically inherited NSHL (Zaidieh et al., 2015). No association between the *GJB6* deletions and specific *GJB2* mutations in digenically inherited NSHL has been noted in the literature. Previous research has also postulated that while both deletions truncate *GJB6*, it is the disruption of *GJB2* expression by deletion of *GJB2* cisregulatory elements that is disease-causing (Tayoun et al., 2015). Homozygous deletions of *GJB6* can also cause NSHL (del Castillo et al., 2002).

In a study looking at *GJB2* and *GJB6* variants in Spanish populations, homozygous *GJB6* deletions were found with a frequency of 0.2% or less (del Castillo et al., 2003). Historically, 50% of subjects with autosomal recessive, prelingual NSHL will be found to have a *GJB2* variant. This is then complicated by the finding that 10-50% of those subjects only carry one *GJB2* variant (del Castillo et al., 2003). One study completed on a Spanish population of subjects carrying one *GJB2* variant found that 50% of these individuals carried the *GJB6* deletion del(*GJB6*-D13S1830) as a second causative mutation leading to their hearing loss. The detection rate for the GJB6 deletion in this study was 50%, making it the second most common variant leading to NSHL in Spain after the c.35delG variant in *GJB2* (del Castillo et al., 2002). Based on the findings from this study, further investigations were conducted to determine the frequency of the *GJB6* deletion in individuals with NSHL in nine countries (one of which was the United States) (del Castillo et al., 2003). In the United States, it was found that as high as 15.9% of *GJB2* heterozygotes carried an additional *GJB6* deletion and in all of the countries studied, less than 0.5% were homozygous for *GJB6* deletions (del Castillo et al., 2003). Since this study's completion, many other analyses and studies have been conducted, showing varying results and an expected carrier frequency of 1% for *GJB6* deletions (Zaidieh et al., 2015; Batissoco et al., 2009; Pandya et al., 2003).

There are several research studies describing concurrent screening for *GJB2* mutations and *GJB6* deletions (Miño et al., 2014; Zaidieh et al., 2015). In the two studies cited above, they detected none and 1 case with a deletion out of 111 and 41 subjects, respectively. The study by Miño and colleagues was conducted on an Ecuadorian Mestizo population while the study by Zaidieh and colleagues was conducted in a population of Syrians. As outlined in the previous paragraph, del Castillo's major studies into *GJB6* deletions was conducted primarily on a Spanish population with some investigations performed in the United States and elsewhere. From these previous research studies, there appears to be a wide range in the frequency of *GJB6* deletions depending on the population being studied. For instance, in del Castillo's 2002 and 2003 articles, 50% of Spanish individuals with one *GJB2* variant possessed a deletion in *GJB6* (del Castillo et al., 2002), while only as high as 15.9% of American individuals with one *GJB2* variant also carried a deletion in *GJB6* (del Castillo et al., 2003). This is quite a

difference when thinking about the overall frequency of *GJB6* deletions in the two populations.

Genetic Testing for NSHL

The history of genetic testing for hearing loss goes back to the late 1990s when GJB2 was found to be a major contributor to congenital severe to profound sporadic autosomal recessive NSHL (Jayawardena, Shearer & Smith, 2015). GJB2 has a single coding exon, and therefore sequencing of this gene could be completed quickly and relatively cheaply. In the 2000s as additional genes related to hearing loss were discovered the testing paradigm shifted from GJB2-only to sequential Sanger sequencing of genes suspected based on patient phenotype following normal GJB2 testing (Jayawardena, Shearer & Smith, 2015). A missense mutation in GJB6 was linked to a family with autosomal dominant hearing loss (p.Thr5Met) in a study completed in 1999, but screening for GJB6 deletions did not begin until after del Castillo and colleagues' landmark study published in 2002 (Grifa et al., 1999; del Castillo et al., 2002). More recently, Next Generation Sequencing (NGS)-panel testing has become widely available and utilized in clinical care. Many panels that only include genes related to hearing loss are available or can be customized in order to screen many genes related to the testing indication at once, while also attempting to limit incidental findings not specifically related to the phenotype of hearing loss. NGS panels allow full coverage of a limited number of genes, providing a complete picture of any mutations that may or may not be present. Many panels also offer sequencing as well as deletion/duplication analysis of most, if not all, of the genes on a specific panel. NGS technologies can also cast a wider

net in genetic testing, with whole exome (WES) and whole genome sequencing (WGS) now clinically available. WES and WGS are helpful for finding variants across many known hearing loss-related genes, as well as variants in genes that have not been previously described as playing a role a hearing loss, potentially sparking future research. While WES and WGS are broad testing options, the rate of incidental findings unrelated to the testing indication and variants of uncertain significance is high. Additionally, WES/WGS may not cover all areas of the exome/genome completely and there is still the chance that no genetic cause for an individual's NSHL will be found, as certain types of mutations cannot be detected (large deletions/duplications, aneuploidy, translocations/inversions, triplet repeat disorders) and not all detected mutations are well understood.

There is a wealth of research available concerning testing methodology in nonsyndromic and syndromic hearing loss (Brownstein et al., 2011; Choi et al., 2013; Schrauwen et al., 2013; Shearer et al., 2014; Shearer & Smith, 2015; Yang, Wei, Chai, Li & Wu, 2013). One such study published in 2015 is a review of 20 prior published studies that utilized comprehensive genetic testing via massively parallel sequencing (MPS) in individuals with hearing loss (Shearer & Smith, 2015). These studies included 603 patients with hearing loss of unknown cause and 426 controls. All of the studies reviewed were from the previous 5 years. Sixteen of the studies focused on individuals with unknown causes of hearing loss that had not been previously tested, and sequenced between 34 and 246 genes; currently there are 84 genes that have been related to NSHL (Shearer & Smith, 2015). Studies that sequenced greater than 84 genes (up to 246),

included genes that were relatively new discoveries that may play a role in NSHL, as well as genes linked to certain forms of syndromic hearing loss. Thirteen of these 16 studies pre-screened patients for common mutations (like GJB2) but they do not state if these studies also pre-screened for GJB6 deletions, and altogether the average diagnostic rate of panel testing was 41% regardless of ethnicity, with 31% of the total studies evaluating for copy number variations (Shearer & Smith, 2015). Another study by Shearer and colleagues utilized targeted genomic enrichment and MPS via the OtoSCOPE panel developed at the University of Iowa (the authors' home institution) to sequence all genes known (at that time) to cause NSHL (Shearer et al., 2014). Their study sequenced 66 and 89 genes (a new version of the OtoSCOPE was released in the course of this study) linked to NSHL in 686 patients and evaluated for copy number variations (CNVs) and found that 38.9% had a genetic cause determined for their hearing loss and of those, 18.7% implicated a CNV within a known deafness gene (Shearer et al., 2014). This study specifically found a CNV within GJB6 (a deletion of at least 140-150 kb and up to 309 kb) in 4 of the 686 patients tested. This study clearly demonstrates that CNVs can play a significant role in NSHL and should be screened for in comprehensive genetic testing methodologies, but CNVs within GJB6 have a low prevalence in this study, and appear to be less significant than other CNVs.

Another study by Sloan-Heggen and colleagues executed comprehensive genetic testing for hearing loss via targeted genomic enrichment and MPS via the OtoSCOPE panel in a multiethnic cohort of 1119 individuals with hearing loss (no patients were excluded based on their phenotype, previous testing, or inheritance) (Sloan-Heggen et al.,

2016). This study identified a genetic cause for the hearing loss in 39% (440) of the patients tested, with 49% being missense variants, 18% small deletions or insertions, 18% large CNVs, 6% splice site changes, 8% nonsense variants, and <1% promoter variants (Sloan-Heggen et al., 2016). As a whole, NSHL can be caused by variants in over 90 genes; this study represents the largest patient cohort (to date) tested comprehensively for hearing loss and accounts for a good proportion of causative variants. The diagnostic rate varied by ethnicity (Middle Eastern patients had the highest rate at 72%) and symmetry of the hearing loss (only 1% for cases of unilateral hearing loss) (Sloan-Heggen et al., 2016). In some Middle Eastern cultures, the practice of marrying relatives is more common, which can increase the coefficient of inbreeding and lead to higher carrier frequencies of variants. As the coefficient of inbreeding increases with successive generations, the likelihood of an autosomal recessive condition (such as AR NSHL) due to two identical variants increases. In this testing protocol, 5900 variants were found which were classified according to ACMG guidelines; 14% were reported as pathogenic or likely pathogenic while 82% were reported as variants of uncertain significance (Sloan-Heggen et al., 2016). Several factors increased the chances of finding a genetic cause (diagnostic rate): age of onset (highest at 44% for congenital), physical exam findings (or other clinical abnormalities) decreased diagnostic rate because such cases were more likely to be syndromic), laterality of hearing loss (bilateral much more likely to find a cause-44%), and the inheritance pattern (dominant had highest rate at 50%followed by 41% for recessive and 37% for no family history). If a person had a dominant family history, congenital symmetric (bilateral) hearing loss onset, and a

normal physical exam, their diagnostic rate would jump to 67% (Sloan-Heggen et al., 2016). This study further clarified the diagnostic rate by ethnicity. Caucasians had a diagnostic rate of 38%, Middle Eastern was 72%, Asian was 63% and African Americans had the lowest diagnostic rate at 26%, which is consistent with previous study results outlined below. Multiple studies looking at the genetic basis of NSHL in Africans from Cameroon, South Africa, São Tomé, and Príncipe demonstrated that there is limited clinical utility for testing GJB2, GJB6 and GJA1, given the lack of pathogenic variants detected in these genes (Caroça et al., 2016; Wonkam et al., 2015). Additionally, none of the African American patients studied in Sloan-Heggen and colleague's study were diagnosed with hearing loss related to GJB2 variants (Sloan-Heggen et al., 2016). 72% of all the diagnoses (regardless of inheritance pattern) made in the study could be attributed to 10 genes, with GJB2 (22%), STRC (16%), SLC26A4 (7%) and TECTA (5%) being the most common (Sloan-Heggen et al., 2016). 25.3% of all autosomal recessive diagnoses could be attributed to GJB2 (GJB6 deletions were not noted in this study), which is much lower than the commonly cited 50%. Only 1.6% of autosomal dominant diagnoses were due to mutations in GJB2. The most commonly implicated gene in autosomal dominant hearing loss was TECTA (23.8%). SLC26A4 variants were all associated with severe to profound hearing loss, while GJB2 was most common in severe to profound loss at 20% and STRC and GJB2 variants were most common in mild to moderate hearing loss with 30% and 25% of cases respectively (Sloan-Heggen et al., 2016). In Middle Eastern and Asian patients GJB2 variants were more common than STRC variants, but these variants were equally likely in Caucasians and Hispanics. 9 different genes were found to carry

CNVs (*GJB6* was not found to carry any CNVs in this study) that were causative in 88 patients (accounting for 20% of all diagnoses). Missense variants were most common in dominant inheritance (85%) while variants causing null alleles (indels, nonsense, CNVs, splice variants) were most common in recessive inheritance (Sloan-Heggen et al., 2016). The authors suggest that the low diagnostic rate in African Americans, Native Americans, Bahamans and Africans may be due to a 'discovery gap' of causative genes (Sloan-Heggen et al., 2016). 22% of all the diagnoses made were due to *GJB2* variants. The authors concluded with a statement about why deafness panels may be preferable to WES, "a focused deafness-specific panel continues to offer the advantages of better coverage of targeted regions, greater facility to detect multiple variant types (including CNVs and complicated genomic arrangements), substantially lower costs, higher throughput, simpler bioinformatics analysis, and focused testing" (Sloan-Heggen et al., 2016).

The United States has a great deal of ethnic diversity and therefore the prevalence of the *GJB6* deletions also varies widely. Previous studies by del Castillo and colleagues have found differing allele frequencies for the *GJB6* deletion depending on the population being studied (del Castillo et al., 2002; del Castillo et al., 2003). The *GJB6* deletion was much more prevalent in Madrid, Spain, France and Israel (31.6% to 71.4%) than in Italy, Belgium, and the United States (0% to 15.9%) (del Castillo et al., 2003). This study did not record the ethnicity of the subjects though, which complicates interpretations in the United States and elsewhere. Given that the United States may have a lower rate of *GJB6* deletions when compared to other countries, it would be worthwhile to continue research in this field while also taking into account different testing methodologies prior to making recommendations regarding *GJB6* reflex deletion testing. According to best practice guidelines from the European Molecular Genetics Quality Network (EMQN), it is recommended that laboratories conducting molecular testing in NSHL perform sequence analysis of *GJB2* as well as testing for the two most common deletions in *GJB6* (Hoefsloot, Roux, & Glindzicz, 2013). These practice guidelines state that individuals with zero or one *GJB2* variant should be tested for deletions in *GJB6*.

The American College of Medical Genetics and Genomics (ACMG) is a professional organization of individuals, including researchers and healthcare providers, in the field of genetics that guide the field through the creation of clinical practice guidelines, laboratory standards, and relevant databases. ACMG published guidelines for the clinical evaluation and diagnostic approach to hearing loss in 2014 (Alford et al., 2014). These guidelines were created to help improve diagnostic yield, reduce incidental findings and reduce costs by recommending a tiered approach to the management and genetic testing in hearing loss (Jayawardena, Shearer & Smith, 2015). These guidelines discuss the sometimes difficult process of distinguishing syndromic from non-syndromic hearing loss. For example, some syndromic forms of hearing loss may present with subtle features early on that may not be distinguished and conditions often exhibit variable expressivity and variable age of onset (Alford et al., 2014). Conditions like Usher syndrome and Pendred syndrome can easily be mistaken as NSHL in early childhood given that their non-auditory features may be understated initially. In order to determine if hearing loss is syndromic or non-syndromic, there are many additional non-genetic

tests and screenings that can be considered. These include CMV culture or PCR, CT scan, MRI, ophthalmology consult, electrocardiography, urinalysis, thyroid hormone levels and renal ultrasound just to name a few. Unfortunately, these tests have a low diagnostic yield and can be quite costly (Alford et al., 2014; Jayawardena, Shearer & Smith, 2015). In the end, genetic testing is often utilized in conjunction with other screening and testing modalities to determine a specific cause for an individual's hearing loss diagnosis.

There is a wide array of genetic testing options available for hearing loss. One such option involves sequencing GJB2 as the first line of testing, given that 50% of autosomal recessive NSHL is due to mutations in this gene. However, this is highly contingent upon ethnicity and the remaining 50% of cases are due to a wide array of additional genes. Another option is comprehensive genetic testing, which involves the use of Next Generation Sequencing (NGS) panels which are able to sequence many genes at one time. Comprehensive genetic testing for NSHL can be interpreted in many different ways, but often includes sequencing and deletion/duplication analysis of GJB2, GJB6, and a number of additional genes associated with the clinical phenotype on a panel together. These panels can be customized and many laboratories offer panels of genes related to hearing loss. In some cases, NGS panel genetic testing can help direct future clinical management, and determine which costly non-genetic tests and screenings mentioned above are necessary. For example, if panel testing picks up two causative mutations in TECTA (NSHL), no additional screening is necessary, but two variants in SLC26A4 (Pendred syndrome) would prompt a CT scan to evaluate the vestibular

aqueduct as Pendred syndrome is associated with dilation of the vestibular aqueduct and potentially cochlear hypoplasia (Jayawardena, Shearer & Smith, 2015). These two findings together are known as Mondini malformation. The final testing guidelines from ACMG ultimately recommend a full evaluation of medical, birth and family histories, followed by a tiered genetic testing approach beginning with testing of the DFNB1 locus (provided another etiology or syndromic cause is not suspected). However, ACMG does not define what type of testing at the DFNB1 locus should be pursued (sequencing, deletion and duplication analysis, common mutation analysis, etc.). If testing at the DFNB1 locus is inconclusive, NGS technologies like panels of hearing loss genes, whole exome sequencing, or whole genome sequencing can be considered (Alford et al., 2014). The guidelines also stress understanding the genes included in specific panels, their disease associations, coverage (some panels may use disease-targeted exon capture which may not detect all types of mutations), sensitivity, likelihood of incidental findings and types of mutations detected as well as costs as these are important considerations in further testing. Finally, the guidelines also recognize that the costs of NGS technologies are decreasing so rapidly (these guidelines were published in 2014) that NGS testing may be the most cost effective and appropriate initial test in some cases (Alford et al., 2014).

The ACMG guidelines also stress the need for culturally and linguistically sensitive genetic counseling. Genetic counselors and other genetic specialists are important in the care of individuals and families with hearing loss as they provide explanations of molecular diagnoses, recurrence risks, psychosocial counseling to dispel negative emotions (such as guilt, anxiety, anger, etc.), and provide referrals for additional concerns in the family history (like a history of cancer) (Alford et al., 2014). Individuals within the Deaf and hard of hearing community often view their hearing loss as a positive or neutral trait while the medical and hearing community often see it as pathological; this discrepancy can limit Deaf and hard of hearing individual's access to genetic services (Alford et al., 2014). However, previous studies indicate that when accurate information about genetic services is provided, interest grows and individuals with hearing loss report increased self-identity and understanding as well as increased cultural/group identity as a result of genetic testing (Baldwin, Boudreault, Fox, Sinsheimer & Palmer, 2012; Burton, Winthrow, Arnos, Kalfoglou & Pandya, 2006).

Research into the efficacy of *GJB6* reflex testing may inform future recommendations for genetic testing in NSHL. Currently, the testing capabilities for NSHL create a wealth of choices for providers. Providers can look to the ACMG and EMQN guidelines as a baseline recommendation, but since the arena of available testing options and the costs versus benefits of each option can be difficult to determine, providers may still struggle to determine the appropriate testing methodology for patients. The costs of NGS-based tests continues to decrease and it may be a matter of time before a panel is a cheaper starting point than *GJB2* sequencing followed by *GJB6* deletion analysis. This study aims to outline current laboratory genetic testing processes for *GJB2* and *GJB6* analysis (reflex, stand-alone, panel), calculate the incidence of *GJB6* deletions in individuals with NSHL who had genetic testing performed at Nationwide Children's Hospital (NCH) and the *GJB6* deletion carrier incidence within a population of individuals not known to have hearing loss. All of this information will then be utilized to make recommendations regarding testing methodology in NSHL. At NCH, testing begins with GJB2 sequencing followed by reflex analysis for the common 309 kb deletion in GJB6. However, the laboratory at NCH has seen very low frequencies of the GJB6 deletion, and began to question if this was the most effective testing methodology. Additionally, GJB6 deletions have been detected through microarray analyses in cases not known to have hearing loss as a clinical indication for testing. These findings together led to the development of this study. Ultimately, this research could prove to be quite timely and beneficial to genetic testing laboratories and society at large by encouraging the development of testing recommendations and improving test utilization management (TUM). TUM aims to increase the value of genetic testing by preventing the ordering of unnecessary tests in some populations while encouraging appropriate, needed testing in others (Baird, 2015). As NGS panel testing continues to grow and become cheaper, it is important to reevaluate sequential testing (GJB2 followed by GJB6) versus initial NGS panel testing. The technologies associated with NGS continue to improve at a rapid pace, which means that research in areas involving genetic testing (like NSHL) must continue to ensure we are providing the best care possible. By considering the frequency of GJB6 deletions in individuals that had microarrays performed at NCH combined with frequencies of GJB6 deletions from the Database of Genomic Variants, we can determine how common these deletions are in the general population and if it is cost-effective to screen for these deletions as a standard reflex test. Researching testing results for NSHL compared to the general population will help demonstrate the most effective testing strategies in cases of hearing loss. This research will be particularly valuable to

individuals affected with hearing loss undergoing genetic testing but hopes to have far reaching effects in clinical genetic testing processes, recommendations, and TUM.
CHAPTER 2: MATERIALS AND METHODS

IRB Approval

This study was submitted to the Institutional Review Board (IRB) at Nationwide Children's Hospital (NCH) (IRB16-00845) and was determined to be an improvement project that did not meet the definition of research according to the federal regulation [45 CFR 46.102(d)]. Therefore, the application for review was withdrawn. Reciprocity agreement was granted by The Ohio State University. This study involved review of publicly available data, and retrospective analysis of de-identified records at Nationwide Children's Hospital (NCH).

Research Design

Aim 1: Identify current laboratory practices in genetic testing of GJB2 and GJB6

Current industry practices regarding genetic testing strategies for NSHL in the United States were discerned by interrogating information posted on The Genetic Testing Registry, Gene Tests, and individual laboratory websites. The Genetic Testing Registry (https://www.ncbi.nlm.nih.gov/gtr/) is housed within the National Center for Biotechnology Information's (NCBI) website; NCBI is a United States governmentfunded resource facilitating reference to multiple public databases related to molecular biology data. The Genetic Testing Registry (GTR) is a hub of voluntarily submitted data from individual laboratories about their available genetic testing options, testing methodology, validity, credentialing and certifications, and laboratory contact

information. The submitted data is not verified by the National Institute of Health (NIH), and the NIH makes no endorsements of the available tests. GeneTests (https://www.genetests.org/) is owned by BioReference Laboratories and features over 600 international laboratories in its Laboratory Directory and pulls links to various genetic testing resources through a general search bar. Both GeneTests and GTR allow for organization of search results by disorder, gene, test, laboratory, and clinic. These databases serve as a useful tool for gathering basic genetic testing information and linking out to each laboratory's direct website and contact personnel but do have a risk of being misleading or incorrect as information is not independently verified. The labs currently conducting GJB2 and GJB6 mutation analysis were noted, as well as their testing methodologies (reflex testing offered/performed for GJB6 and in what circumstances). All information was gathered through the aforementioned websites and also through direct contact with laboratories. Only laboratories located in the United States with CLIA-certification were included. CLIA stands for the Clinical Laboratory Improvement Amendments which are federal standards passed in 1988 that all laboratories within the United States offering clinical testing in humans are held to. Many of the laboratories analyzed from GTR and GeneTests also hold College of American Pathologists (CAP) Laboratory Accreditation Program certification as well which are even more stringent standards that laboratories can choose to follow. In addition, the methods of GJB6 analysis each laboratory utilized was documented, and whether testing was offered as a stand-alone test, a reflex test following GJB2 analysis, both, or as part of a comprehensive panel that included additional genes. Finally, the average cost of each

testing option was obtained. The research completed in Aim 1 aided in determining current industry practices in genetic testing for NSHL, specifically for *GJB2* and *GJB6*, so comparison of other laboratory testing practices could be made with the current testing strategy employed at Nationwide Children's Hospital (NCH) Molecular Genetics Laboratory.

Aim 2: Examine the frequency and distribution of GJB2 variants and the 309 kb deletion in GJB6 in proband samples from NCH referred for clinical GJB2 and GJB6 testing

For Aim 2, a retrospective review of records at NCH for information on the spectrum of *GJB2* variants was conducted. Only the results of clinical *GJB2* sequencing and reflex *GJB6* deletion analysis performed from April 2009 through April 2016 were analyzed. In this time frame there were 615 *GJB2* sequencing tests and 530 *GJB6* deletion analyses, representing 617 unique individuals. A few records (9 *GJB2* tests and 2 *GJB6* tests representing 9 unique cases) were for familial variant testing (a relative was previously found to carry a mutation) and these cases were excluded from all analyses. One *GJB2* sequencing case in which no variants were detected was also excluded. This case was noted to have had a *GJB6* deletion detected on a previously performed microarray and *GJB2* sequencing was ordered as a follow-up to this result. This case was not noted to have hearing loss as an indication for either test (microarray or *GJB2* sequencing) and was therefore excluded from analysis. Of the remaining records, 79 were for *GJB2* sequencing only, 2 were for *GJB6* deletion analysis only, and 526 were for both *GJB2* sequencing and *GJB6* deletion analysis. Therefore, there were 605 total *GJB2*

sequencing tests analyzed and 528 *GJB6* deletion analyses representing 607 total unique individuals.

In review of *GJB2* sequencing and *GJB6* deletion analysis records, only the specific variant detected and the total number of cases was recorded, and the frequency of each *GJB2* variant was determined; all other data was de-identified or removed. Each record was provided with a unique 4-digit code for organizational purposes and records with both a *GJB2* and *GJB6* result for a single individual were provided the same code. This study spans 7 years of records and over time resources and strategies for variant classification have changed significantly, meaning that variants could have been interpreted differently from 2009 to 2016 and are therefore not comparable. In order to create a standardized set of genomic results for the study, all variants were researched to determine phenotypic associations and expected pattern of inheritance before being reinterpreted according to the most recent ACMG variant interpretation guidelines (Richards et al., 2015).

Each of the *GJB2* variants were classified according to the most recent ACMG variant interpretation guidelines and information from HGMD, dbSNP, ClinVar, 1000 Genomes, Exome Aggregation Consortium (ExAC), Ensembl, Exome Variant Server, the Connexin Deafness homepage, and the literature were synthesized together for the most accurate classifications (Richards et al., 2015). In cases of conflicting evidence, variant classification was decided as a committee and when enough evidence could not be gathered to fully classify a variant as benign or pathogenic, it was classified as a VUS. Some *GJB2* variants were not found in multiple databases and therefore were classified

as a VUS due to lack of evidence. Examples of these types of variants include c.559G>A (p.Glu187Lys), c.-3248A>G, c.-3224C>A, c.-3190G>A (IVS1+12G>A), c.-3161G>A, c.681*3, and c.409A>C. None of these variants were found in HGMD and many could not be found in any of the databases but had a single mention in the literature. It is possible that evidence does exist for some of these variants but that evidence is not publicly available at this time. Not all laboratories chose to share their variant classifications and even fewer provided a rational for their interpretation in databases like ClinVar.

No personal health information was recorded for the cases from NCH and patients did not have their variants reclassified clinically. This was partly due to the fact that all records were de-identified prior to their use and clinical variant reclassification was outside the scope of this research. Per Nationwide Children's Hospital protocols, cases with 0 or 1 pathogenic *GJB2* variants associated with autosomal recessive inheritance were expected to reflex to *GJB6* deletion analysis. It is important to note that at Nationwide Children's Hospital, only the 309 kb deletion in *GJB6* is screened for. In the review of *GJB6* deletion analysis results, the frequency of deletions was determined and compared to frequencies quoted in the literature. The frequency and distribution of *GJB2* variants as well as the 309 kb deletion in *GJB6* records from NCH determined the diagnostic rate at NCH so that it could be compared to those seen in the literature.

Aim 3: Calculate the frequency of GJB6 deletions from various control populations

In Aim 2 the frequency of *GJB6* deletions in patients with NSHL was calculated. Part of the research question is how effective is *GJB6* reflex testing in finding the cause

of NSHL. This was addressed in Aim 3 in part by determining how frequently the same GJB6 deletions are observed in individuals within various control populations. This was accomplished by reviewing the Database of Genomic Variants (DGV) for the frequency of GJB6 deletions and conducting a retrospective review of testing results from NCH microarray analyses conducted from April 2009 through April 2016 of individuals who carried a deletion within or spanning a portion of the two known common deletions of GJB6. These deletions include the 309 kilobase (kb) (historically thought to be 342 kb) deletion present at g.20797176-21100550 (GRCh37/hg19), and the 232 kb deletion present at g.20802713-21034768 (GRCh37/hg19). These deletions span intron 2 of GJB6, microRNA 4499 (MIR4499), up to intron 4 of CRYL1. The range of error due to probe placement was 626 base pairs (bp) centromerically and 32.11 kb telomerically. Due to the probe coverage and changing platforms of microarrays, it cannot be determined if the detected deletions were exactly the same size as the known deletions. From previous literature outlined in the background section it is known that GJB6 deletions can contribute to NSHL. Therefore, when evaluating results of those affected with NSHL, one expected to see an enrichment of the GJB6 deletion, supporting the argument for reflex testing. Conversely, if GJB6 deletions were not as enriched among the population of those with NSHL compared to those from control populations (frequencies from the DGV and microarrays at NCH); this negatively impacted the rational for reflex GJB6 deletion testing.

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Aim 4: Propose recommendations for NSHL genetic testing based on prior literature and Nationwide Children's Hospital laboratory's data

In Aim 4, results from Aim 1, the records reviewed from NCH, and previously published literature outlined in the background section were synthesized to develop future genetic testing recommendations in the evaluation of NSHL. As previously outlined, the testing protocol utilized at NCH recommends *GJB2* sequencing as a first line test, followed by *GJB6* deletion analysis if 0 or 1 pathogenic variants are found in *GJB2*. In Aim 1, the testing approach for NSHL utilized at many different laboratories was examined. The testing methodologies noted in Aim 1 were compared to the testing protocol utilized at NCH as part of Aim 4. The diagnostic rate at NCH (determined from *GJB2* and *GJB6* records) was compared to diagnostic rates noted within the literature for various testing options. Additionally, the prevalence of *GJB6* deletions within the hearing loss community (in the literature and at NCH), and within control populations (NCH microarrays and the DGV) was also compared. All of these comparisons were then synthesized together to elucidate the most effective testing strategies and propose updated recommendations for how genetic testing in NSHL can be approached.

Recommendations were made based on the determination if *GJB2* sequencing with reflex *GJB6* deletion analysis was the most effective testing strategy (from prevalence of *GJB6* deletions and diagnostic rates at NCH compared to results in the literature) and if not, what is a more effective testing strategy (from previous literature results).

CHAPTER 3: RESULTS

Aim 1: Identify current laboratory practices in genetic testing of GJB2 and GJB6

Through the utilization of the GTR and GeneTests 23 different laboratories offering 33 different *GJB2*-only testing options were identified. The average cost to sequence *GJB2* alone was \$602.44 [\$300-\$1500], while the average cost for deletion/duplication analysis of *GJB2* alone was \$994.94 [\$400-\$2184.71]. The costs of sequencing and deletion/duplication analysis varied widely across laboratories and were further informed by the type of analysis being performed (i.e. single-site mutation analysis versus select exon scanning versus full gene analysis).

Eleven laboratories offered *GJB2* analysis with reflex to *GJB6* deletion analysis (6 labs test for the common 309 kb deletion only and 5 test for both the 309 kb and 232 kb deletion) in the event that the molecular cause of hearing loss was not determined by *GJB2* alone. The average cost of *GJB2* sequencing only with reflex *GJB6* deletion analysis is \$802.50 [\$500-\$1500]. Some laboratories also offer sequential reflex testing for *GJB2*, *GJB6* and mitochondrial analysis. Seventeen laboratories offer 20 different tests for non-reflex, *GJB6* only analysis (some labs offer multiple tests). Ten of these tests were deletion analysis only (average cost: \$437.77 [\$200-\$690]), and 8 were sequencing tests (sequencing only or sequencing and deletion/duplication analysis

together) (average cost: \$517.90 [\$396-\$657.70]). The remaining two tests were a familial variant analysis and sequence analysis of exon 3 only.

There are a variety of testing options available as a panel. Each of these panels include both *GJB2* and *GJB6*, as well as a number of other genes. These panels range from just *GJB2* with *GJB6* and 1 to 3 mitochondrial variant detection or full mitochondrial analysis to panels with 20-50 additional genes (after *GJB2* and *GJB6*), to whole exome and whole genome analysis. The average costs of panel testing ranges from \$800 to well over \$1000 depending on the number of genes included and type of analysis performed. For panels of 21 to 152 genes the average cost is \$2660 [\$1650-\$3800].

Aim 2: Examine the frequency and distribution of GJB2 variants and the 309 kb deletion in GJB6 in proband samples from NCH referred for clinical GJB2 and GJB6 testing

Altogether, there were 607 unique cases that underwent testing. In total, 605 *GJB2* sequencing records and 528 *GJB6* deletion analysis records were reviewed. Forty-four different *GJB2* variants were detected in 605 *GJB2* sequencing results. Each of these was classified according to the most current ACMG variant classification guidelines (Richards et al., 2015). Twenty-four pathogenic or likely pathogenic *GJB2* variants were found (4%; 24/605) as well as 13 variants of uncertain significance (VUS) (2.1%; 13/605) and 7 likely benign variants (representing known polymorphisms) (1.2%; 7/605). These variants and their allele frequencies are shown in Table 2. Case results were organized into 7 different categories depending on the number and type of *GJB2* and *GJB6* variants found. These categories are depicted in Table 3. The total number of cases

that were provided with a genetic diagnosis (or likely genetic diagnosis) based on *GJB2* sequencing and/or *GJB6* deletion analysis was determined to be 76. These diagnoses consist of 75 cases with two pathogenic or likely pathogenic variants in *GJB2* (Category 1) and 1 case with a likely pathogenic variant in *GJB2* and 1 *GJB6* deletion (Category 3). Overall, this represents a diagnostic rate of 12.5% (76/607).

	Allele	Likely	Allele		Allele	Known	Allele
Pathogenic	Frequency	Pathogenic	Frequency	VUS	Frequency	Polymorphisms	Frequency
c23+1G>A (IVS1+1G>A)	1	c.95G>T	1	c3248A>G	1	c22-12C>T (c34C>T)	1
c24A>C (c22-2A>C)	1	c.101T>C	40	c3224C>A	2	c15C>T	9
c.35delG	88	c.109G>A	25	c3190G>A	3	c.79G>A	10
c.71G>A	3	c.208C>G	1	c3161G>A	1	c.249C>G	2
c.167delT	2	c.246C>G	2	c45C>A	4	c.341A>G	3
c.169C>T	1	c.298C>T	1	c6T>A	5	c.380G>A	1
c.223C>T	1	c.416G>A	1	c.681*3	1	c.457G>A	4
c.229T>C	1	c.563A>G	1	c.200A>G	1		
c.231G>A	3			c.409A>C	1		
c.235delC	4			c.478G>A	3		
c.269T>C	11			c.487A>G	1		
c.299_300delAT	1			c.499G>A	1		
c.313_326delAAGTTCATCAAGGG	4			c.559G>A	1		
c.334_335delAA	1						
c.370C>T	1						
c.427C>T	4						

Table 2: Classification of All GJB2 Variants

Category Name	Category Description	Number of Cases
	2 pathogenic or likely pathogenic variants in GJB2, GJB6	
Category 1	deletion analysis not warranted	75
	2 variants found in GJB2 where one variant is a VUS and the	
Category 2	other is a VUS, pathogenic, or likely pathogenic	1
	1 pathogenic or likely pathogenic variant in GJB2, GJB6 deletion	
Category 3	analysis that is positive, negative, or not performed	49
	1 VUS in GJB2, GJB6 deletion analysis that is positive, negative,	
Category 4	or not performed	24
	No VUS, pathogenic, or likely pathogenic variants in GJB2, 1	
Category 5	GJB6 deletion present	0
	No VUS, pathogenic, or likely pathogenic variants in GJB2, 2	
Category 6	GJB6 deletions present	0
	No VUS, pathogenic, or likely pathogenic variants in GJB2, GJB6	
Category 7	deletion analysis negative or not performed	458

Table 3: Organizational Categories of GJB2 and GJB6 Testing Records

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All of the variants found in cases from Category 1 (75 total) as well as the frequencies of these variants are outlined in Table 4. Of note, two cases in this category reflexed to *GJB6* deletion analysis (0.33%; 2/607). Per NCH protocol, reflex *GJB6* analysis is typically only performed if 0 or 1 pathogenic or likely pathogenic variant are found in *GJB2*. One case carried the *GJB2* variants c.35delG (pathogenic) and c.101T>C (likely pathogenic) (*GJB6* deletion analysis was negative), and one case carried the likely pathogenic *GJB2* variants c.109G>A and c.101T>C (*GJB6* deletion analysis was negative).

Homozygotes		Compound Heterozygotes					
Variant	Cases	Variants	Cases	Variants	Cases		
c.35delG	21	c.35delG, c.101T>C	8	c.35delG, c.1-23+1G>A	1		
c.109G>A	9	c.35delG, c.269T>C	6	c.35delG, c.229T>C	1		
c.101T>C	4	c.35delG, c.109G>A	3	c.35delG, c.334_335delAA	1		
c.71G>A	1	c.35delG, c.313_326del	3	c.35delG, c.370C>T	1		
c.231G>A	1	c.35delG, c.167delT	2	c.35delG; c.563A>G	1		
c.235delC	1	c.35delG, c.246C>G	2	c.71G>A, c.231G>A	1		
c.427C>T	1	c.101T>C, c.109G>A	2	c.109G>A, c.269T>C	1		
		c.235delC, c.427C>T	2	c.298C>T, c.416G>A	1		
		c.35delG, c.169C>T	1				

Table 4: (Category 1) 2 Pathogenic or Likely Pathogenic Variants in *GJB2* (75 Total Cases)

Category 2 is composed of cases with at least two variants in GJB2, 1 variant

must be a VUS and the second may be a VUS, pathogenic or likely pathogenic variant.

Only one case fit into this category. The GJB2 variants in this case include a c.235delC

(pathogenic) variant, a c.559G>A (VUS) finding, as well as a c.79G>A (likely benign) finding. No *GJB6* deletion analysis was performed for this case.

In Category 3, 49 cases were found to carry 1 pathogenic or likely pathogenic variant in *GJB2* and had *GJB6* deletion analysis which was positive, negative, or not performed. These cases and their respective variants are outlined in Table 5. Three of the 49 cases in this category did not reflex to *GJB6* deletion analysis. The remaining 43 cases had *GJB6* deletion analysis performed and 1 *GJB6* deletion was found. This case carried the likely pathogenic variant c.101T>C in *GJB2* as well as the 309 kb *GJB6* deletion, providing a likely genetic cause for their hearing loss.

Variant	GJB6 Deletion	Number of Cases
c.101T>C	Positive	1
c.35delG	Not performed	2
c.223C>T	Not performed	1
c.101T>C	Negative	21
c.35delG	Negative	14
c.269T>C	Negative	4
c24A>C (c22-2A>C)	Negative	1
c.95G>T	Negative	1
c.109G>A	Negative	1
c.208C>G	Negative	1
c.299_300delAT	Negative	1
c.313_326del	Negative	1
Total number of cases:	4	9

Table 5: (Category 3) 1 Pathogenic or Likely Pathogenic *GJB2* Variant, Reflex to *GJB6* Deletion Analysis

In Category 4, 24 cases were found to carry 1 VUS in *GJB2* and had *GJB6* deletion analysis which was positive, negative or not performed. The previously described case with one pathogenic, one VUS, and one likely benign variant in *GJB2* was not included in this category, as it meets criteria for inclusion under Category 2 and each case is only delineated to one specific category to prevent cases from being duplicated. All 24 cases had *GJB6* deletion analysis performed and no deletions were detected. All of these cases and their variants are outlined in Table 6.

GJB2 Variant	GJB6 Deletion	Number of Cases
c6T>A	Negative	5
c45C>A	Negative	4
c3190G>A	Negative	3
c.478G>A	Negative	3
c3224C>A	Negative	2
c3248A>G	Negative	1
c3161G>A	Negative	1
c24A>C	Negative	1
c.200A>G	Negative	1
c.409A>C	Negative	1
c.487A>G	Negative	1
c.499G>A	Negative	1
c.681*3	Negative	1
Total number of cases:		24

Table 6: (Category 4) 1 GJB2 VUS, Reflex to GJB6 Deletion Analysis

In Category 5, no cases were found to have a VUS, pathogenic or likely

pathogenic variant in GJB2 but carried 1 GJB6 deletion. In Category 6, no cases were

found to carry zero VUS, pathogenic, or likely pathogenic variants in *GJB2* with 2 *GJB6* deletions. In Category 7, 458 total cases did not carry any VUS, pathogenic, or likely pathogenic variants in *GJB2*, nor did they carry a deletion in *GJB6*. However, 27 of these cases did carry 1 or more likely benign *GJB2* variants. These likely benign variants are outlined in Table 7. Of note, 1 case with the likely benign *GJB2* variants c.79G>A and c.341A>G did not reflex to *GJB6* deletion analysis. Additionally, of the 458 total cases, two were for *GJB6* deletion analysis only (both tested negative for the 309 kb deletion).

Variants	GJB6 Deletion Analysis	Number of Cases	
c15C>T	Negative	9	
c.380G>A	Negative	4	
c.457G>A	Negative	4	
c.79G>A homozygote	Negative	3	
c.249C>G	Negative	2	
c22-12C>T (c34C>T)	Negative	1	
c.79G>A; c.380G>A	Negative	1	
c.79G>A; c.341A>G	Negative	2	
c.79G>A; c.341A>G	Not performed	1	
Total number of cases:	27		

Table 7: Cases with Likely Benign GJB2 Variant(s), Reflex to GJB6 Deletion Analysis

Out of the 607 unique cases that underwent *GJB2* sequencing and/or *GJB6* deletion analysis, the *GJB6* deletion was only found in 1 case. Overall, this gives a frequency of 0.16% (1/607) for the *GJB6* deletion within this hearing loss population from NCH.

Some GJB2 variants had evidence to suggest a benign or pathogenic classification but they did not strictly meet the ACMG guidelines to be classified as such and were therefore interpreted as a VUS, or there was conflicting evidence leading to a VUS interpretation. For example, c.-6T > A is a VUS by the criteria, but multiple laboratories in ClinVar claim it is benign (SCV000061471.4, SCV000219516.2, SCV000331239.2, SCV000383054.2 all version 2015), it is present in approximately 1% of African American control alleles and has been described multiple times in the literature as a novel variant or likely polymorphism (Tang et al., 2006; Shan et al., 2010; Gasmelseed et al., 2004). Given these lines of supporting evidence, c.-6T>A is a likely benign variant but there are not enough ACMG criteria being met and therefore this variant is classified as a VUS. Another VUS in a very similar situation is c.478G>A (p.Gly160Ser). This variant is reported as likely benign by multiple databases and has been described in the literature as a polymorphism and also as a VUS, but the criteria is not being met to classify this as anything other than a VUS. In order for a variant to be classified as likely benign, one strong and one supporting or two supporting criteria must be met (Richards et al., 2015). The variant c.478G>A is currently only meeting 1 supporting criteria (BP6: "reputable database reports variant as benign but without evidence to independently evaluate") (Richards et al., 2015). When the requisite number of criteria are not met, or there are conflicting criteria (pathogenic and benign criteria) met, a variant is classified as a VUS. In order to be classified as benign, a variant must meet 1 stand alone, 2 strong, or 1 strong and 3 or more supporting criteria.

The *GJB2* variant c.380G>A (p.Arg127His) meets criteria to be classified as a likely benign variant given the prevalence of the variant meeting BS1 criteria (17.5% of Asian Indians) but the literature evidence is conflicting¹ (Richards et al., 2015). One functional study of this variant shows that this variant does not affect channel formation and is likely a polymorphism (Thönnissen et al., 2002), but another demonstrates this variant results in defective Cx26 gap junctions that have a greatly reduced transfer of neurobiotin (Wang et al., 2003). None of the databases support pathogenicity of this variant though, and it remains a likely benign variant.

One of the most common *GJB2* variants found in this study was c.35delG. As the most common *GJB2* variant in individuals of Caucasian ethnicity, c.35delG is a well-supported pathogenic variant (Gasparini et al., 2000; Paz-y-Miño, Beaty, López-Cortés, Proaño, 2014; Bakhchane et al., 2016). In the analysis of 606 *GJB2* sequencing results, c.35delG was found in 67 unique individuals (11%; 67/606).

Another *GJB2* variant found frequently in the cases from NCH is c.101T>C (p.Met34Thr; also known as M34T). C.101T>C is a common variant, especially in those of European ancestry as evidenced by the prevalence noted in 1000 Genomes, and the Exome Aggregation Consortium (ExAC). All of these databases provide frequencies of variants in unaffected controls. In 1000 Genomes, c.101T>C is found in 1% of all alleles,

¹ BS1 Strong: "allele frequency is greater than expected for disorder [For genetically heterogenous, autosomal dominant, and fully penetrant disorders, the allele frequency is equivalent to 2 times the allele frequency of the most common pathogenic mutation in the gene of interest. [Pathogenic allele frequency is determined by available normal population databases with >1000 alleles. In the event there is no available frequency for extremely rare dominant disorders (incidence ≤1:1000), then a frequency of 0.1% can be utilized if supported by known or predicted disease incidence.]" Genetics in Medicine, ACMG Laboratory Quality Assurance Committee (2015). Standards and Guidelines For the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards et al., 2015), 405-424.

and 2% of European alleles (1000 Genomes Project Consortium, 2015). In ExAC, c.101T>C is noted in 1032 of 121354 total alleles (0.008504 allele frequency), 815 of 66686 European (non-Finnish) alleles (0.01222 allele frequency), and 147 of 6614 European Finnish alleles (0.02223 allele frequency) (Lek et al., 2016). C.101T>C has been interpreted in ClinVar as likely benign (SVC000383040.2, version 2015), VUS (SVC000193154.1, version 2015), likely pathogenic (SVC000383043.2, version 2015), and pathogenic (SVC000321726.4, version 2015). C.101T>C has been observed in patients with NSHL with reduced penetrance and leads to less severe hearing loss when observed with a second pathogenic *GJB2* variant (Snoeckx et al., 2005; Kenna et al., 2010). Individuals who are homozygous for c.101T>C have been found to have mild to moderate NSHL (Löppönen et al., 2012). This variant was determined to be likely pathogenic in this study as it met one strong and one moderate criteria in the ACMG guidelines (Richards et al., 2015).

- Strong criteria PS3 was met "well established in vitro or in vivo functional studies supportive of a deleterious effect" given the mildly deleterious effect observed by Thönnissen and colleagues (Thönnissen et al., 2002).
- The moderate criteria PM5 "novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before [can also be used if pathogenic missense variant seen in same residue in highly analogous protein(s) (e.g. KRAS/NRAS/HRAS]" was met given that the variants M34I, M34L, M34R, and M34V have been reported in association with hearing loss in the Human Gene Mutation Database (Stenson et al., 2014).

Aim 3: Calculate the frequency of GJB6 deletions from various control populations

During the aforementioned timeframe, approximately 10,970 microarrays were performed. Microarrays performed on tissues, prenatal samples, and parental samples were excluded from our analysis (360 arrays), meaning that 10,612 microarrays were ultimately reviewed. It is important to note that these numbers are approximate, as there may be cancelled microarrays that were not actually run or confirmation samples that could not be excluded. 7 total microarrays (0.067%; 7/10612) were found to contain a deletion within or spanning a portion of the GJB6 deletions. None of these microarrays were noted to have hearing loss as a clinical indication for testing on their requisition forms. Of note, cases 2, 3, and 4 had the same deletions and cases 5, 6, and 7 also had the same deletion (different from the deletion noted in cases 2, 3, and 4). These cases are outlined in Table 8. Additionally, the case outlined in Aim 2 that was previously found to carry a deletion in GJB6 on a microarray and had follow-up sequencing of GJB2 is included among these 7 cases (this case was excluded from GJB2 analyses). Case 6 or 7 (identical deletions found in the same year) represent this case. Pictorial representations of the 3 types of deletions were obtained from The University of California Santa Cruz (UCSC) Genome Browser and are included in Appendix A, B, and C.

Case #	Year	Genome Location	Minimum Size	Probes Lost	Start Gap	End Gap	Platform	Genome Assembly Used at Time of Testing
	2045	HG19 chr13:20,796,453-		20				
1	2015	21,097,971	301.52 KD	29	4.48 KD Cen	32.11 KD TEI	GGXChip + SNP V1.0 4-piex- CGH	UCSC 2009 ng19
		HG19 chr13:20,797,139-						
2	2013	21,097,971	300.83 kb	28	626 bp Cen	32.11 kb Tel	GGXChip + SNP v1.0 4-plex- CGH	UCSC 2009 hg19
		HG19 chr13:20,797,139-						
3	2015	21,097,971	300.83 kb	28	626 bp Cen	32.11 kb Tel	GGXChip + SNP v1.0 4-plex- CGH	UCSC 2009 hg19
		HG19 chr13:20,797,139-						
4	2015	21,097,971	300.83 kb	28	626 bp Cen	32.11 kb Tel	GGXChip + SNP v1.0 4-plex- CGH	UCSC 2009 hg19
		HG19 chr13:20,797,516-						
5	2010	21,103,016	305.50 kb	45	5.65 kb Cen	10.17 kb Tel	NimbleGen CGX-3 v.1 0 3-plex	UCSC 2006 hg18
		HG19 chr13:20,797,516-						
6	2013	21,103,016	305.50 kb	45	5.65 kb Cen	10.17 kb Tel	NimbleGen CGX-3 v.1 0 3-plex	UCSC 2006 hg18
		HG19 chr13:20,797,516-						
7	2013	21,103,016	305.50 kb	45	5.65 kb Cen	10.17 kb Tel	NimbleGen CGX-3 v.1 0 3-plex	UCSC 2006 hg18

 Table 8: Microarray Deletions Spanning GJB6 Detected From April 2009 Through April 2016

The Database of Genomic Variants (DGV) was also referenced to provide additional information on the frequency of *GJB6* deletions in control populations (populations without noted hearing loss). In the DGV, there were 3 studies with deletions that were roughly the same size and location of the 309 and 232 kb deletions of GJB6. The first study by Cooper and colleagues studied copy number variations in individuals with developmental delay and found 14 cases out of 15767 total cases (0.089%) and 12 controls out of 8329 total controls (0.14%) that carried a similar deletion (Cooper et al., 2011). Altogether, 26 out of 24096 total individuals (0.12%) in this study carried a similar deletion spanning GJB6. A second study by Pinto and colleagues found 6 out of 771 individuals (0.78%) with a similar sized deletion (Pinto et al., 2007). The third study by Coe and colleagues found 5 similar deletions out of 29085 (0.017%) individuals (Coe et al., 2014). By combining all 3 studies, the total sample size is equal to 53952 and 37 of these carry a deletion spanning all or a portion of GJB6, which indicates an overall frequency of 0.069% (37/53952) within these varying populations not noted to have hearing loss.

CHAPTER 4: DISCUSSION

The results of Aim 1 indicate that laboratories approach genetic testing for NSHL in a variety of ways. There were 14 panel test options which include 21 to 152 additional genes with an average cost of \$2660. The average cost of reflex testing (GJB2 sequencing followed by GJB6 deletion analysis automatically if the cause of hearing loss is not determined by GJB2 alone) is \$802.50. Conversely, the cost of sequential testing (GJB2 sequencing ordered first with a separate order for GJB6 deletion analysis if needed) would average out to \$1040.21 total with GJB2 sequencing averaging at \$602.44 and GJB6 deletion analysis averaging at \$437.77. This indicates that reflex testing and sequential testing options are both cheaper than panel testing and are more cost-effective, but only if the individual's hearing loss is due to mutations in GJB2 and/or GJB6. If GJB2 and GJB6 testing does not identify a molecular cause of the hearing loss in an individual though, the next option would be some type of panel testing, increasing the total cost of finding a molecular diagnosis. Additionally, each of the available panel tests reviewed included both GJB2 and GJB6 with additional genes, so these two genes would be screened again if the panel was not customized. The EMQN and ACMG guidelines both recommend testing the DFNB1 locus (GJB2 & GJB6) prior to pursuing wider testing such as a panel option (Alford et al., 2014; Hoefsloot, Roux, & Glindzicz, 2013). These are simply guidelines meant to assist providers in the care of individuals with hearing loss though, and do not represent strict testing strategies that must be followed.

Laboratories implement and offer a variety of testing approaches, which enables providers to freely choose between focused and more comprehensive testing as their first line, and providers can also choose to pursue testing from multiple labs at one time. An example would be a provider ordering GJB2 sequencing with reflex GJB6 deletion analysis from one lab and then ordering a panel from another laboratory as follow-up or at the same time. This testing approach may not be the most cost effective though, and may not represent effective test utilization management as two genes are being tested twice (GJB2 and GJB6) and more healthcare dollars are being spent for such testing. It is important to consider the cost of each testing option and which option is most likely to provide a molecular diagnosis based on the clinical information available for the patient. The EMQN and ACMG guidelines are also open to interpretation as they do not state the exact type of testing that should be done in GJB2 and GJB6 as a first line analysis. In addition, ACMG guidelines state that panel testing may be most effective as a first line test in certain cases and the cost-effectiveness of panel testing versus focused testing (GJB2 and GJB6) will need to be continually assessed (Alford et al., 2014).

Aim 2 clearly delineated the frequency and distribution of *GJB2* variants as well as the 309 kb deletion of *GJB6*. Seventy-five of the 607 total cases were provided a molecular diagnosis of NSHL through *GJB2* sequencing alone, representing a diagnostic rate of 12.4% (75/607). However, prior literature has shown that *GJB2* mutations are causative in up to 50% of cases of NSHL (Rabionet et al., 2000). It is unclear why our diagnostic rate is so much lower than that quoted in the literature. One possibility is that our cases include some with syndromic hearing loss or some of an ethnic background most consistent with a different molecular basis for NSHL. It is also possible that cases could have been referred for testing when only unilateral hearing loss was present, which reduces the diagnostic rate. Sloan-Heggen and colleagues found that the diagnostic rate with panel testing in someone with unilateral hearing loss dropped to 1% (Sloan-Heggen et al., 2016). Literature describing panel tests of many genes associated with hearing loss have had diagnostic rates of 41%, 38.9%, and 39% respectively (Shearer & Smith, 2015; Shearer et al., 2014; Sloan-Heggen et al., 2016). Additionally, a previous study found that up to 15.9% of individuals in the United States with one pathogenic variant in GJB2 will carry a deletion in GJB6 (del Castillo et al., 2003). Within our study, only one case was found to carry one pathogenic variant in GJB2 with one deletion in GJB6 (frequency of 0.16%). Clearly, the GJB6 deletion was much less common in our study and our diagnostic rate is lower than rates found in the literature. The lack of GJB6 deletion detection indicates that GJB6 deletions are very uncommon in hearing loss cases at our institution, and it may be prudent to reconsider GJB6 deletion analysis as an automatic reflex test. One reason the diagnostic rate for panel testing is much higher than the diagnostic rate found in our study may be because more genes were being analyzed, but this alone does not fully explain the discrepancy. Additionally, there are multiple factors that can impact diagnostic rates such as laterality of hearing loss, age of onset, presence or absence of other clinical features, ethnicity, and apparent inheritance pattern (Sloan-Heggen et al., 2016). It has previously been shown that individuals with bilateral, congenital, isolated (no other clinical features), and apparently dominant hearing loss, have a diagnostic rate as high as 67% while those with unilateral hearing loss only have a

diagnostic rate of 1% (Sloan-Heggen et al., 2016). Since our study did not note the clinical history of each of the cases being tested, it is possible that our data includes cases with unilateral hearing loss, or later onset hearing loss, or hearing loss that was accompanied by additional clinical features (more likely syndromic). All of these factors would have decreased the diagnostic rate.

As noted previously, two cases in which two pathogenic or likely pathogenic GJB2 variants were found to have reflexed to GJB6 deletion analysis, which would not have been expected by NCH protocol. One case carried c.35delG (pathogenic) and c.101T>C (likely pathogenic) and the other case carried c.101T>C and c.109G>A (likely pathogenic). Because all of the records viewed from NCH were de-identified it cannot be determined if these variants were previously classified as a VUS or likely benign in the past. However, this would explain why reflex GJB6 deletion analysis was performed in these cases. There were also 3 cases in which 1 pathogenic or likely pathogenic variant were found in GJB2 but reflex GJB6 deletion analysis was not performed. These cases carried one copy of the GJB2 variant c.35delG (2 cases) or c.223C>T (1 case). The GJB2 variant c.223C>T is associated with autosomal dominant hearing loss, explaining why reflex GJB6 deletion analysis was not performed. However, the GJB2 variant c.35delG is associated with recessive inheritance, indicating that reflex GJB6 deletion analysis was not performed for another reason. The lack of GJB6 testing in these 2 cases could be due to the ordering provider requesting that reflex analysis not be performed or these cases were unlabeled familial cases where only GJB2 sequencing was necessary. There was also one case with two likely benign variants that did not reflex to GJB6 deletion

analysis. This case may have only had testing for *GJB2* ordered or *GJB6* analysis could have been cancelled due to new clinical features or history information that pointed to another condition. Lastly there were also two *GJB6* analyses performed with no previously completed *GJB2* sequencing. These cases could represent testing that was completed before or after the sampled time frame (before April 2009 or after April 2016), mislabeled familial cases, or cases in which *GJB6* deletion analysis alone was requested by a care provider.

As previously outlined, c.35delG was found in 67 unique cases out of 605 total *GJB2* sequencing results for a prevalence of 11% (67/605). This prevalence is higher than any other variant found in records from NCH. Given the high prevalence of c.35delG in Caucasians and several other ethnicities as outlined in previous literature, it is not surprising to find that c.35delG is the most common mutation found in cases at NCH as well (Hashemi, Ashraf, Saboori, Azarpira, Darai, 2012; Paz-y-Miño, Beaty, López-Cortés, Proaño, 2014; Bakhchane et al., 2016).

Aim 3 calculated the frequency of *GJB6* deletions from the DGV and from microarray cases at NCH not noted to have hearing loss as an indication for study. Both of these groups found very low frequencies of the *GJB6* deletions at 0.069% and 0.067% respectively. These results, combined with the absence of the 309 kb deletion in all but one case with hearing loss, indicate that *GJB6* deletions are just as likely in control populations as populations with hearing loss tested at NCH. Given this finding one might conclude that screening for *GJB6* deletions is a low yield testing option that is less cost effective than other, more comprehensive testing strategies. These findings are quite different from del Castillo and colleagues' study which found the 309 kb *GJB6* deletion in 50% of Spanish cases with 1 pathogenic *GJB2* variant and up to 15.9% of cases in the United States with 1 pathogenic *GJB2* variant (del Castillo et al., 2002; del Castillo et al., 2003). Although, it should be noted that subsequent studies have found much lower frequencies of the *GJB6* deletion in those with hearing loss, with 0% (n=111) cases and 2.4% (n=41) carrying a deletion (Miño et al., 2014; Zaidieh et al., 2015). Our results are much more in line with these later studies. Additionally, the United States is an area of such ethnic diversity that the frequency of all variants, including the *GJB6* deletions, will vary from institution to institution.

The data evaluated from NCH's laboratory testing of *GJB2* and *GJB6* indicate that *GJB6* deletions are a rare cause of NSHL in this population (only 1 *GJB6* deletion was detected in 7 years of testing), and are uncommon in those from control populations as well. As previously stated, these similar frequencies in case and control populations could indicate that *GJB6* deletion analysis is a less cost effective, lower yield testing option when compared to more comprehensive testing options. Given the results of our study and a review of previous research, recommendations for genetic testing in hearing loss were developed.

All patients should ultimately be given the choice of what type of testing (if any) they would prefer initially after having a full discussion of the benefits, risks, and limitations of each testing option. However, there are some clinical scenarios where *GJB2* sequencing as a first line test may be most effective and could be pursued prior to broader testing options. In cases where a patient is worried about the increased potential

for variants of uncertain significance with broad testing (such as a panel) or familial *GJB2* cases, *GJB2* sequencing is appropriate as a first line test. In cases of NSHL in an individual from an ethnic background where *GJB2* mutations are a common cause of hearing loss (such as European), where the hearing loss is congenital and bilateral, inheritance appears to be autosomal dominant or recessive, and the most common syndromic conditions have been effectively ruled out, *GJB2* sequencing could also be considered as a first line test. These common syndromic conditions can be ruled out through a combination of non-genetic testing and screening as well as the age of the patient (additional clinical features are expected by a certain age in many conditions). *GJB2* sequencing would be an effective first line test in cases where the individual or family is highly concerned about cost as well. From our study results, panel testing for hearing loss has a significantly higher average cost than *GJB2* sequencing alone or in conjunction with reflex *GJB6* deletion analysis.

If *GJB2* sequencing is pursued as a first line test and zero or one variant is found, we put forth the recommendation that a panel option of *GJB6* and several additional genes should be considered as a reflex testing option. The number of additional genes can be customized based on the comfort level of the patient and care provider. This recommendation comes from the fact that *GJB6* deletions were found to have a low prevalence both in cases of NSHL at NCH and in various control populations, suggesting that *GJB6* deletion analysis is a low yield testing option. Additionally, there is conflicting literature regarding the prevalence of *GJB6* deletions and in a study by del Castillo and colleagues the prevalence in the United States was only as high as 15.9% (del Castillo et al., 2003). This prevalence is lower than the overall diagnostic rates (39-41%) previously noted in the literature for panel testing options, suggesting that panel genetic testing may have a higher yield (Shearer & Smith, 2015; Sloan-Heggen et al., 2016). GJB6 deletion analysis should only be considered as a reflex testing option if one pathogenic GJB2 variant is found and GJB6 deletion analysis is expressly desired by the patient or provider. It may also be considered if there is literature to suggest that GJB6 deletions (in conjunction with a pathogenic GJB2 variant) play a highly significant role in hearing loss cases from the same or similar ethnic background. An example of such an ethnic background would be Spanish or Israeli, where 50% and 71.4% of individuals with one pathogenic GJB2 variant carried a GJB6 deletion as a second causative mutation (del Castillo et al., 2002; del Castillo et al., 2003). If GJB2 sequencing with reflex GJB6 deletion analysis is performed and the molecular basis of an individual's hearing loss is not determined, panel testing that includes additional genes related to hearing loss can be considered. The number of genes included on such a panel can be determined by the patient and care provider.

Outside of these initial considerations however, we put forth the recommendation that comprehensive genetic testing, which includes *GJB2*, *GJB6*, and several other genes associated with hearing loss should be pursued as a first line test in most cases of hearing loss. The number of additional genes included on such a panel, the rate of variants of uncertain significance, the specific cost and the particular laboratory ordered from should be tailored to the needs and preferences of the patient and their care provider. If an individual does not fall into one of the above scenarios where *GJB2* sequencing (with or

without reflex *GJB6* deletion analysis) should be considered as a first line test, panel genetic testing should be considered the first line of testing. This recommendation for panel testing was crafted based on multiple points of data, the first of which being that previous literature has found higher diagnostic rates (39-41%) for panel testing compared to the rate in our study (12.5%) when utilizing *GJB2* sequencing with reflex *GJB6* deletion analysis (Shearer & Smith, 2015; Sloan-Heggen et al., 2016). Additionally, a panel option of multiple genes can more easily distinguish between syndromic and non-syndromic hearing loss; a task that can be difficult in the clinic setting given variable expressivity and the age-dependent onset of symptoms in some syndromic conditions (Alford et al., 2014). In some cases, comprehensive genetic testing (such as a panel) can help direct future clinical management, which ultimately saves healthcare dollars, and the overall cost of NGS technologies are continually decreasing, making them a more accessible testing option (Alford et al., 2014).

If panel testing is negative, or if the full cause of an individual's hearing loss is not determined (VUS, autosomal recessive with only one variant) broader testing such as such as whole exome sequencing could be considered. The risks and limitations of WES need to be considered and balanced before ordering such testing though. A limitation would be that WES is not able to detect all types of mutations such as translocations, large deletions/duplications, or triplet repeat disorders. The risk of finding a VUS, and the overall cost of WES is higher. The benefits of WES (potentially providing a diagnosis in the family, directing future clinical care) should outweigh the risks and limitations before moving forward with such testing. Another testing option after a negative hearing loss panel would be a larger hearing loss panel (more genes) but this is likely not cost effective given overlapping genes and the combined cost of multiple panels versus whole exome sequencing.

As previous studies have outlined, there are a variety of factors that can increase or decrease the likelihood of finding a genetic basis for an individual's hearing loss (Shearer & Smith, 2015; Sloan-Heggen et al., 2016). Diagnostic rates vary based on ethnicity first and foremost. Certain genes are more likely to carry a mutation in hearing loss cases of certain ethnicities such as GJB2, SLC26A4, and 12S rRNA in Chinese, GJB2 in those of European, Spanish, and Moroccan descent, and unknown genes in those of African ancestry (Ma et al., 2016; Bakhchane et al., 2016; Bosch et al., 2014; Caroça et al., 2016). In addition, while there are mutations that are commonly found in certain genes, the majority of mutations related to NSHL are quite rare and are private mutations found in an individual or family (Atik et al., 2015). Given the ethnic prevalence of certain NSHL mutations, ethnicity should be a consideration when deciding how to proceed with genetic testing. For instance, patients of Chinese and African ancestry may benefit from comprehensive genetic testing as a first line test as other genes (SLC26A4, 12S rRNA, unknown genes) are known to contribute to a considerable percent of hearing loss cases in individuals from these ethnic backgrounds and could therefore be more cost effective than GJB2/GJB6 reflex testing. GJB2 sequencing with reflex to GJB6 deletion analysis would not be the most effective first line test as GJB6 deletions are quite uncommon in our study and in previous literature (Miño et al., 2014; Zaidieh et al., 2015) and genes outside of GJB2 play a significant role in hearing loss in these ethnic backgrounds.

Another factor that affects the diagnostic rate of genetic testing for NSHL is whether the hearing loss is truly non-syndromic or if it may be syndromic in nature. The Online Mendelian Inheritance in Man (OMIM) lists over 400 syndromes that have been related to hearing loss (Atik et al., 2015). Some of these syndromic hearing loss conditions are easily mistaken as non-syndromic especially at younger ages (like Usher syndrome and Pendred syndrome) and genes that cause NSHL can also cause syndromic hearing loss depending on the mutation (Atik et al., 2015). In some cases, comprehensive genetic testing can save healthcare dollars by directing future clinical management and preventing unnecessary screening by determining the exact condition a patient has before pursuing costly screening and non-genetic tests (Sloan-Heggen et al., 2016). In previous studies utilizing comprehensive genetic testing, the diagnostic rate was as high as 41% (Shearer & Smith, 2015) and 39% (Sloan-Heggen et al., 2016). In this study of just GJB2 sequencing and GJB6 deletion analysis, there was a diagnostic rate of 12.5%. This is considerably lower than comprehensive genetic testing but this must be considered in conjunction with the cost of testing. When considering comprehensive genetic testing it is important to make sure that the genes included are relevant to the patient's condition and that testing is truly comprehensive by providing full sequencing (not select exon capture), deletion/duplication analysis, and CNV detection (increasing recognized as a cause of hearing loss). Each patient is different and the clinical and family history should play a significant role in determining the most cost effective testing methodology.

Study Limitations

Some of the limitations of this study stem from the fact that a limited data set with no additional information regarding the case undergoing testing, aside from the gene and the specific variant found, was used. Without additional information about a particular case, such as the individual's ethnicity, it is impossible to fully compare the GJB2 mutation and GJB6 deletion rates from NCH to those published in the literature. The lack of case information also prevented the determination of the reasoning behind certain decisions such as not conducting reflex testing as expected and how the classification of GJB2 variants has changed over the past few years. Additionally, information about the case's family and clinical history is not available with de-identified results, which would have been helpful in interpreting results and the breakdown of cases in this study. Another limitation of this study is that we cannot determine for certain if the microarray cases screened in Aim 3 were truly noted not to have hearing loss. Each microarray case was ordered by a particular care provider and it is possible that hearing loss was not noted in the paperwork as an indication for study even if it was a clinical feature. In the microarray cases, it is also possible that the individual tested could have developed hearing loss later in life, or were simply carriers for a GJB6 deletion. A limitation is that NCH only screens for the 309 kb deletion of GJB6. While recognized as the most common GJB6 deletion, there is also a 232 kb deletion that can occur and act as a second mutation in NSHL. This means that some of the cases tested at NCH could potentially carry a deletion in GJB6 that was not detected. Finally, another study limitation is the fact that a cost-benefit analysis was not conducted. A cost-benefit analysis would have

incorporated the average costs found in Aim 1 with the review of results completed in Aim 2 and 3 to make recommendations regarding the most cost-effective testing methodology.

Future Studies

Future studies looking into testing methodology in NSHL should aim to maintain greater access to records so that aspects of clinical history and/or ethnicity can be used to help clarify variants and make statements on the broader frequencies of variants given particular background information. Additionally, future studies should utilize multiple testing locations to compare and contrast results in order to best adapt testing methodology to the particular population. Future studies could also compare the detection rates in GJB2 sequencing with GJB6 deletion analysis versus various panel testing options. Studies that take into account the varying detection rates in differing ethnicities, laterality of hearing loss, age of onset, and presence or absence of additional clinical features are also helpful for continual assessment and improvement of testing methodologies. Studies should also utilize a cost-benefit analysis when determining the most cost effective testing methodologies. The cost of testing plays a significant role in the testing practices utilized by providers and should therefore be regularly assessed. Another potential area of study is the use of whole exome or whole genome sequencing in the assessment of hearing loss. As WES and WGS become more commonplace, their role within the realm of determining a molecular cause for hearing loss will be important to study, as well as the benefits and limitations of such broad testing. As more research is

completed in various aspects of NSHL, we will continue to improve diagnostic accuracies while also utilizing the most cost-effective strategies in genetic testing.

Conclusions

Our study results indicate that the average cost of *GJB2* sequencing with reflex GJB6 deletion analysis (\$802.50) is cheaper than the average cost of panel testing (\$2660), but GJB2 and GJB6 testing at NCH has a low diagnostic rate of 12.5% (76/607). In addition, the frequency of GJB6 deletions was only 0.16% (1/607) in cases at NCH and between 0.067% and 0.069% in various control populations not noted to have hearing loss. These results indicate that GJB6 deletions are relatively rare in the included cases, and are equally likely in those with NSHL and within various control populations. Given these results, we pose the recommendation that reflex GJB6 deletion analysis only be ordered under certain circumstances. We recommend panel genetic testing (which includes GJB2, GJB6 and a number of additional genes) be offered as a first line test in most cases of hearing loss in order to maximize cost-effectiveness. However, GJB2 sequencing may be appropriate as a first line test in certain circumstances. When panel testing does not determine a molecular cause for hearing loss either as a first line or follow-up test, broader testing such as whole exome sequencing can be considered if testing has the potential to alter medical management. Ultimately, genetic testing in hearing loss should strive to maintain effective test utilization management. The most cost-effective testing method for each patient needs to be determined so that laboratories provide access to testing to patients that need it most, while limiting unnecessary spending and maximizing benefit which is a major goal of test utilization management.
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APPENDIX A: CASE 1 DELETION

Below is a screenshot from the University of California Santa Cruz (UCSC) Genome Browser of the deletion found in case 1 of the microarray data (*http://genome.ucsc.edu*). The genome assembly GRCh37/hg19 was used. This deletion spanned chr13:20,796,453-21,097,971.



APPENDIX B: CASES 2, 3, & 4 DELETION

Below is a screenshot from UCSC Genome Browser of the deletion found in cases 2, 3, and 4 of the microarray data (*http://genome.ucsc.edu*). The genome assembly GRCh37/hg19 was used. This deletion spanned chr13:20,797,139-21,097,971.



APPENDIX C: CASES 5, 6, & 7 DELETION

Below is a screenshot from UCSC Genome Browser of the deletion found in cases 5, 6, and 7 of the microarray data (*http://genome.ucsc.edu*). The genome assembly GRCh37/hg19 was used. This deletion spanned chr13:20,797,516-21,103,016.

