WEST AFRICAN CENTER FOR CELL BIOLOGY OF INFECTIOUS PATHOGENS

DEPARTMENT OF BIOCHEMISTRY CELL AND MOLECULAR BIOLOGY

COLLEGE OF BASIC AND APPLIED SCIENCES

UNIVERSITY OF GHANA

THE GENETICS OF CONGENITAL NON-SYNDROMIC HEARING IMPAIRMENT IN GHANA

BY

SAMUEL MAWULI ADADEY

(10395444)

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THE GENETICS OF CONGENITAL NON-SYNDROMIC HEARING IMPAIRMENT IN GHANA

A dissertation submitted to the Board of Graduate Studies, University of Ghana, Legon, Ghana

In partial fulfillment of the requirements for the award of the Doctor of Philosophy degree in Biochemistry

BY

SAMUEL MAWULI ADADEY

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Declaration

Presented in this thesis are studies conducted by me, Samuel Mawuli Adadey, at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana and the Division of Human Genetics, University of Cape Town. The thesis was supervised by Prof. Ambroise Wonkam (University of Cape Town), Prof. Gordon A. Awandare (University of Ghana), Dr. Osbourne Quaye (University of Ghana) and Prof. Geoffrey Amedofu (Kwame Nkrumah University of Science and Technology).

I assert that neither the whole work nor any part of it has been or is being submitted for another degree in this or any other university and that this thesis is my original work (except where acknowledgements indicate otherwise).

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Dedication

I dedicate this work to my parents for their support throughout my education, to my dear wife for standing by me during the study. In addition, I dedicate this study to the deaf community in Ghana.

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List of abbreviations

HI	Hearing impairment
NSHI	Non-syndromic hearing impairment
ARNSHI	Autosomal recessive non-syndromic hearing impairment
AOM	Acute Otitis media
ARNSHI	Autosomal Recessive Non-Syndromic Hearing Impairment
CX26	Connexin 26
ENT	Ear, nose, and throat
KBTH	Korle-Bu Teaching Hospital
KATH	Komfo Anokye Teaching Hospital
GJB2	Gap junction beta two
GJB4	Gap junction beta four
GJB6	Gap junction beta six
GJC3	Gap junction gamma three
GJA1	Gap Junction Protein Alpha one
NHS	Newborn hearing screening
WHO	World Health Organization
MAF	Minor allele frequency
ABR	Auditory brainstem response
ASSR	Auditory steady-state response
C.S.M.	Cerebrospinal meningitis
RFLP	Restriction fragment length polymorphism
PDE	Phosphodiesterase

Outline of thesis

This thesis is organized into seven chapters with the following major components: general introduction (chapter one), review of literature (chapters two and three), methods and results (chapters four to six), and general discussion, conclusion, and recommendations (chapter seven). Parts of the literature review and results of the thesis were published in peer-reviewed journals as listed below.

Literature review

Adadey, S. M., Awandare, G., Amedofu, G. K., & Wonkam, A. (2017). Public health burden of hearing impairment and the promise of genomics and environmental research: a case study in Ghana, Africa. *Omics: a journal of integrative biology*, 21(11), 638-646

Objectives 1 and 2

Adadey, S. M., Manyisa, N., Mnika, K., De Kock, C., Nembaware, V., Quaye, O. Q., ... & Wonkam, A. (2019). GJB2 and GJB6 mutations in non-syndromic childhood hearing impairment in Ghana. *Frontiers in genetics*, 10, 841.

Objective 3

Adadey, S.M.; Tingang Wonkam, E.; Twumasi Aboagye, E.; Quansah, D.; Asante-Poku, A.; Quaye, O.; Amedofu, G.K.; Awandare, G.A. & Wonkam, A. (2020). Enhancing Genetic Medicine: Rapid and Cost-Effective Molecular Diagnosis for a GJB2 Founder Mutation for Hearing Impairment in Ghana. *Genes*, 11, 132.

Objective 4

Adadey, S. M., Esoh, K. K., Quaye, O., Amedofu, G. K., Awandare, G. A., & Wonkam, A. (2020). GJB4 and GJC3 variants in non-syndromic hearing impairment in Ghana. *Experimental Biology and Medicine,* 1535370220931035.

Translational policy document

Samuel M. Adadey, Osbourne Quaye, Geoffrey K. Amedofu, Gordon A. Awandare and Ambroise Wonkam. Screening for GJB2-R143W associated hearing impairment; implications for health policy and practice in Ghana. (Submitted for publication)

Abstract

Background: The partial or total inability of an individual to hear sound is known as hearing impairment (HI). Globally, over 466 million people are living with HI, with the majority of cases from developing countries. Although over 123 genes have been associated with HI, only one gene (*GJB2*) has been studied in Ghana. This thesis therefore sought to investigate variants in *GJB2, GJB4, GJB6,* and *GJC3* genes that are associated with HI in Ghana as well as to investigate other non-genetic causes of HI among Ghanaian children.

Method: Hearing-impaired students from 11 schools for the deaf and Adamorobe, a village in Ghana, were enrolled and categorized as familial or non-familial. Control participants who did not have any known family history of HI were also enrolled. DNA was obtained from the blood samples collected from all the participants in 81 families from the schools for the deaf, 8 families from Adamorobe, and 166 non-familial cases. From the DNA samples, the regions that code for *GJB2*, *GJC3*, and *GJB4* proteins were polymerase chain reaction (PCR) amplified using specific primers, Sanger sequenced and analyzed. The large genomic deletion of the *GJB6* gene (*GJB6*-D3S1830) was investigated using multiplex PCR and confirmed with Sanger sequencing.

A rapid diagnostic test was designed for *GJB2*-p.Arg143Trp (rs80338948) using restriction fragment length polymorphism (RFLP) and NciI restriction enzyme. The test was optimized and validated using Sanger sequencing. Bioinformatic tools and online databases were employed in predicting the clinical significance/pathogenicity of the identified variants in the connexin genes. *In silico* protein modeling techniques were used to model the protein structure for a likely pathogenic *GJB4*-p.Asn119Thr (rs190460237) mutant and wild-type proteins. The ligand-binding properties of the modeled proteins were studied.

Results: The hearing-impaired participants enrolled on the study had severe to profound HI with the majority (68.3%) of them having prelingual HI. Nearly all the prelingual HI cases seemed congenital based on their parent's reports, however, 54% of the hearing-impaired students received the first comprehensive hearing test when they had grown past the age of language development, thus between the ages of 6-11years. Cerebrospinal meningitis (CSM) was found to be the most frequent environmental cause of HI. The genetic analyses revealed that *GJB2*-Arg143Trp accounted for HI in 25.9% of the families studied, and 7.9% of isolated cases. A carrier frequency of 1.4% was estimated from randomly selected hearing controls in Ghana. Seven out of eight families from Adamorobe tested positive for the *GJB2*-Arg143Trp founder mutation. We were the first to report the presence of a *GJB2*-Trp44Ter variant in a hearing-impaired family in Ghana.

To facilitate rapid screening of the variant within the population, a rapid *GJB2*-p.Arg143Trp-Nci-RFLP test was developed and found to be 100% sensitive, with no false positive or false negative observed. The test is highly specific for any variant within the recognition site of the restriction enzymes; however, it cannot differentiate between these variants.

When screening other genes associated with HI, we identified one *GJC3* variant that may not associate with HI. Also identified were seven *GJB4* variants, of which 5 were predicted to be as either benign or synonymous, and the remaining 2 were predicted likely pathogenetic. One of the two variants may not be associated with HI because the variant's homozygous form was observed in both patients and controls. We modelled the protein structure and function of the other likely pathogenic *GJB4* variant (p.Asn119Thr) and found subtle but important alterations in the structure and binding characteristics of the mutated protein compared to the wildtype.

Conclusion: We have obtained many important results through these studies. We have confirmed meningitis as the major cause of environmental HI in Ghana. Variations within the *GJB2* gene account for the most HI cases of genetic origin in Ghana and hence we have identified the need to include *GJB2* gene investigations in the national newborn hearing screening (NHS) program. The *GJB2*- p.Arg143Trp-Nci-RFLP test will therefore be instrumental in this capacity. Finally, based on data presented in this thesis, *GJB4*, *GJB6*, and *GJC3* gene variants were not likely associated with HI in the Ghanaian population.

CHAPTER ONE

1.0 Introduction

Hearing impairment (HI) is a global health problem and known to be one of the most common disabling conditions that severely influences the quality of life of the individuals who are affected. Based on the global prevalence reports of HI increased from about 360 million in 2014 (WHO, 2014) to 466 million in 2019 (WHO, 2019), and expected to further increase to more than 900 million by 2050. The World Health Organization (WHO) has reported a higher number of people living with HI when considering only the developing/middle-low income countries (WHO, 2014, 2019).

About 50% of pre-lingual HI cases are caused by genetic/inheritable factors and meningitis was reported from Cameroon as the major cause of environmental HI and other major environmental causes identified in the Cameroonian study were measles, mumps, and ototoxicity (Wonkam *et al.*, 2013). About one out of three of HI cases recorded in the Cameroonian study had unknown causes of HI which may be due to congenital non-syndromic HI of genetic etiology, which explains about half of all congenital HI (Smith *et al.*, 2005). In Ghana, a study reported noise pollution, presbycusis, meningitis, fever, and Meniere's diseases as the major causes of sensorineural HI, while wax, otitis media, foreign bodies, and accident were attributed to Conductive HI (Amedofu *et al.*, 2006).

The prevalence of HI was mostly reported at different study sites in Ghana, and only one study was nationwide (Adadey *et al.*, 2017). A study from the Komfo Anokye Teaching Hospital (KATH) examined 6,428 patients who reported to the facility with hearing problems and 5,734 (89.9%) of them were diagnosed of HI (Amedofu *et al.*, 2006). The results from another prevalence study at Offinso in the Ashanti Region indicated that 135

out of 600 (23%) study participants were diagnosed of conductive HI (Marfoh, 2011). But another study from the capital city of Ghana, Accra, found that as many as 474 out of 715 (66.3%) patients who reported to the Korle-Bu Teaching Hospital (KBTH) were diagnosed with a hearing problem (Nyarko, 2013).

HI that is not linked to signs and symptoms associated with other body parts is known as non-syndromic hearing impairment (NSHI) (Birkenhäger *et al.*, 2007). The characteristics of NSHI vary among the different types of the disease. It can be unilateral (affecting only one of the ears) or bilateral (affecting both ears) and the degree of HI ranges from a difficulty to understand soft speech (mild) to an inability to perceive very loud sounds (profound) (Venkatesh *et al.*, 2015). Genetically, researchers over time have classified the NSHI in different ways, but the classification based on the pattern of inheritance is the most common: autosomal dominant (20%) or recessive (75 to 80%), X-linked (2-5%), or mitochondria (1%). Over 50% of pre-lingual, non-syndromic HI is genetic, often autosomal recessive (Smith *et al.*, 2005), more than 123 genes have been implicated in genetic cases (Van Camp G & Smith, 2020).

Defective gap junction proteins were reported as the cause of most HI in the developed world (Helzner *et al.*, 2005). Developing countries however lack adequate instrumentation to diagnose genetic HI and hence the difficulty in identifying the genetic causes of the disease. From the time of the first discovery of NSHI gene to the end of the $1,000 Genome project in year 2014, about 91 NSHI genes were elucidated, with identified over 141 loci published in peer-reviewed journals (Vona *et al.*, 2015). The most frequent NSHI genes identified over the year are the gap junction protein genes (Vona *et al.*, 2015).

Gap junction beta 2, 4, 6 or alpha 1 genes (*GJB2*, *GJB4*, *GJB6*, or *GJA1*) code for a family of proteins (connexions) which, oligomerizes to form transmembrane protein channels in vertebrates. These channels are referred to as connexons and they form gap junction channels directly between neighboring cells as intercellular communication pathways (Moore, 1991). Connexons are known to transport potassium ions and small molecules across cells. *GJB2*, *GJB6*, and *GJA1* sequences are highly conserved with their proteins consisting of extracellular and middle cytoplasmic loops, N- and C-terminals separated by four transmembrane domains (Lebeko *et al.*, 2015). Mutations in the connexins genes especially *GJB2* were implicated as the main causes of NSHI in Asian and European populations (Lebeko *et al.*, 2015). The most prevalent mutation in the Middle East and Europe was identified to be 35delG. The variants, 235delC and V37I, were the most predominant mutations in East and South East Asia respectively while W24X mutation was prevalent in India (Chan & Chang, 2014). However, there is practically no contribution of connexin genes among people of African descent, the prevalence of *GJB2*- or *GJB6*-associated NSHI in several African populations (e.g. Cameroon (Bosch *et al.*, 2014b), Kenya (Gasmelseed *et al.*, 2004), and Uganda (Javidnia *et al.*, 2014), Nigeria (Lasisi *et al.*, 2014), and in an African population in South Africa (Bosch *et al.*, 2014b; Kabahuma *et al.*, 2011)) is practically zero. In addition, the prevalence of *GJB2*- or *GJB6*-associated NSHI is also rare among African Americans (Shan *et al.*, 2010). The above evidence suggests that mutations other than *GJB2* or *GJB6* related mutations are responsible for NSHI in Africa and people of African descent. As an exception to the other studies reported among Africans, the founder *GJB2* p.Arg143Trp mutation [minor allele frequency (MAF) 15.1%] in addition to five rare *GJB2* variants were identified in Ghana (Hamelmann et al., 2001), but the involvement of others HI genes have not been studied.

1.1 Problem Statement

The contribution of *GJB2, GJB6* or *GJA1* genes to NSHI impairment is extensively studied in the developed world; however, there is a notable gap in literature on the contribution of *GJB2, GJB6,* or *GJA1* variants to hearing impairment over the wide range of the African populations (Lebeko *et al.*, 2015). The paucity of data generated from sub-Saharan Africa (with a few exceptions from Ghana and Sudan) does however suggest that the majority of patients with HI do not have mutated *GJB2, GJB6,* or *GJA1* genes (Wonkam, 2015). It is therefore important to generate enough data from Africans to explain the contributions of other genes to NSHI. Targeted genomic enrichment (TGE) and next-generation sequencing (NGS), using the OtoSCOPE® platform that includes 110 HI genes, has been confirmed to be an effective means for genetic testing for NSHI (Shearer *et al.*, 2010). Using OtoSCOPE®, in seven out of 10 families (70%) from Cameroon, 12 putative pathogenic variations were found in 6 NSHI genes. The 6 genes were *MYO7A, CHD23, SLC26A4, LOXHD1, STRC,* and *OTOF*. Five of the 12 variants (41.6%) were novel (Lebeko *et al.*, 2016). The absence of variants known to be pathogenic in 30% of families is indicative that novel/new HI genes may be discovered in the African population, using NGS technology such TGE, whole exome, and whole-genome sequencing.

In 2001, a research group from Kumasi Center for Collaborative Research in Tropical Medicine (KCCR) identified novel mutations of *GJB2* (A197S, L79P, R184Q, V178A, I203K, and L214P) as major genetic causes of NSHI in Ghana (Hamelmann et al., 2001). A previous work in Ghana discovered R143W mutation of GJB2 as the cause of congenital NSHI in a village in Ghana (Brobby *et al.*, 1998). Since these examples, there has been no extensive study on the genetic causes of NSHI in Ghana, however, about 123 NHSI genes (Van Camp G & Smith, 2020) and 141 loci were identified in European

populations at the end of the 1,000 Genome project in year 2014 (Vona *et al.*, 2015). In this thesis, I described the investigations of contributions of *GJB2*, *GJB4*, *GJB6* and *GJC3* to NSHI in Ghana.

1.2 Aims and Objectives

Main aim

The work described in this thesis investigated the environmental and genetic causes of NSHI in familial (multiplex families) and non-familial (simplex families) cases from Ghana.

Aim 1. To determine the major environmental causes of familial and non-familial HI in Ghana.

Hypothesis: Meningitis and complicated malaria are major environmental causes of HI among Ghanaian children.

Rationale: Preventable diseases such as otitis media, rubella, malaria, and meningitis are among the frequently reported causes of HI globally (WHO, 2019; Wilson *et al.*, 2017a). Similar to the global trend, infectious diseases are also major causes of HI in Africa (Mulwafu *et al.*, 2016), data from a few African countries including Cameroon (Wonkam *et al.*, 2013), The Gambia (McPherson & Holborow, 1985), Nigeria (Ijaduola, 1982), and Sierra Leone (Wright, 1991) provided evidence of meningitis being the leading cause of environmental HI. Some hospital-based studies in Ghana also identified meningitis as one of the major causes of HI (Amedofu *et al.*, 2006; Brobby, 1988). There is however no comprehensive nationwide study on the major environmental cause of HI in Ghana.

Aim 2. To investigate the contribution of *GJB2* and *GJB6* variants to familial and non-familial HI in Ghana.

Hypothesis: Variations in *GJB2* and not *GJB6* account for most HI cases in Ghana.

Rationale: Over 50% of all congenital HI cases are accounted for by mutations in *GJB2* and *GJIB6* genes among the European, Asian and American populations. In Africa, the contribution of these genes to HI is approximately minimal with a few exceptions from Ghana (Lasisi *et al.*, 2014; Wonkam, 2015; Wonkam *et al.*, 2015). Previous reports from Ghana in 1998 and 2001 identified a founder mutation, p.Arg143Trp in *GJB2* gene which accounted for about 17% of the HI cases observed (Brobby *et al.*, 1998; Hamelmann *et al.*, 2001). This study therefore sought to assess *GJB2*'s contribution to the burden of HI in Ghana, 18 years since it was last studied. In addition to assessing the contribution of *GJB2*, this study was designed to assess the contribution of *GJB6* to NSHI in Ghana since there was no published data on *GJB6* from the Ghanaian population.

Aim 3: To design and validate a cost-effective tool for screening the *GJB2*-p.Arg143Trp mutation.

Hypothesis: A population-based newborn screening HI test can be developed for the *GJB2*-p.Arg143Trp founder mutation by employing restriction fragment length polymorphism (RFLP) techniques.

Rationale: Ghana has a high percentage of *GJB2*-p.Arg143Trp mutation cases (Brobby *et al.*, 1998; Hamelmann *et al.*, 2001). This mutation accounted for over 25% of hereditary HI cases in a nationwide study (Adadey *et al.*, 2019). The majority of screening tools for HI gene variants involve the use of DNA sequencing technologies (Schade *et al.*, 2003; Schrauwen *et al.*, 2013; Tayoun *et al.*, 2016) which are not easily accessible in developing countries. Furthermore, in developing countries, it is difficult to implement sequencing technologies in routine clinical practice. Development of cheaper but effective diagnostic tools for HI in developing countries should consider population-specific gene variants as was done for the *GJB2*-35delG variant in Caucasian populations (Antoniadi *et al.*, 2001; Lucotte *et al.*, 2001). There is a need, therefore, to strongly

consider designing an effective HI screening test for the *GJB2*-p.Arg143Trp variant (the most common HI mutation within the Ghanaian population).

Aim 4: To investigate variations in *GJB4* and *GJC3* genes of *GJB2* and *GJB6* negative Ghanaians.

Hypothesis: Variations in *GJB4* and *GJC3* are linked to HI in *GJB2* and *GJB6* negative hearing-impaired participants.

Rationale: *GJB4* and *GJC3* mutants have been associated with HI, however, their contribution to HI is unclear (Ramchander *et al.*, 2010; Yang *et al.*, 2010). *GJB4* was confirmed to be expressed in rat cochlear (Wang *et al.*, 2010a) suggesting its role in the normal functioning of the ear. The diversity of the African population makes it ideal for discovering new HI variants in *GJB4* and *GJC3* compared to the Asian and Caucasian populations where these genes have been studied. These genes were therefore investigated in the multiplex and simplex hearing-impaired families from Ghana.

CHAPTER TWO

2.0 Literature Review

2.1 The hearing process

2.1.1 A brief description of the hearing process

Hearing is a complex process which involves the conversion of sound waves into electrical signals that are carried to the brain for interpretation. The process starts with sound waves entering the pinna (outer ear) and moves through the ear canal to the tympanic membrane (known as the ear drum). The eardrum/tympanic membrane receives the sound waves and vibrates. The 3 tiny bones (incus, stapes, and malleus) located behind the eardrum pick up the vibrations and amplifies them; these amplified sound vibrations are then transmitted to the cochlear. The cochlear contains the basal membrane, a fluid that ripples upon receiving the vibrations, and on top of this basal membrane are the hair cells which transmit the signal to the auditory nerves in the form of an electrochemical signal. The auditory nerves then send these signals to the brain which interprets them. The ear structure is summarized in Figure 2.1 (NIH, 2015; Willems, 2000).

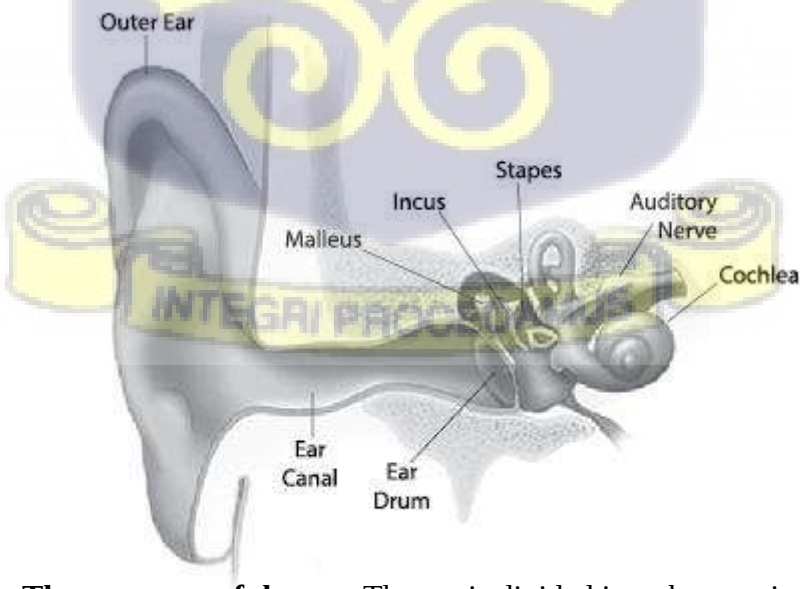

Figure 2.1: The structure of the ear. The ear is divided into three main parts; (1) inner ear which consists of the cochlea, auditory nerves, and semicircular canal, (2) middle ear which is made up of the tiny bones and ear-drum, (3) outer ear which consists of the pinna and auditory canal. Source*: NIH Medical Arts (https://www.nidcd.nih.gov/health/how-do-we-hear)*

2.1.2 Mechanotransduction of sound in the inner ear

The inner ear comprises of the cochlear, hair cells, and neurons actively involved in the transformation of complex mechanical sound into electrochemical signals transmitted to the brain for interpretation. This process is known as the mechanotransduction of sound and is mediated by ion channels in the cells of the inner ear (LeMasurier & Gillespie, 2005; Lotthammer *et al.*, 2020). The hair cells are stimulated by a range of sound waves at specific frequencies and trigger the flow of ions across the cells, altering the electrochemical potential of the hair cells. The alteration in the electrical voltage is transmitted through a series of neural signaling to the acoustic cortex of the brain (Lotthammer *et al.*, 2020). A soft helical spring-like protein known as ankyrin was identified in the mechanical structure of the inner ear. Although the function of ankyrins is not fully known, they are thought to aid sound mechanotransduction in the hair cells (Tang *et al.*, 2020) (Figure 2.2).

Figure 2.2: Ankyrin, a protein found in the inner ear. *This is a cartoon of an inner ear protein ankyrin which is known to participate in the hearing process by aiding in sound mechanotransduction. Source: https://www.ks.uiuc.edu/Research/hearing/*

The hair cells have stereocilia (microvillus-like organelles) that bend in response to vibrations to open mechanical-gated ion channels (Wu *et al.*, 2017). These channels are found at the apex of the stereocilia and allow ionic influx into the hair cells. Structural analysis of the stereocilia further revealed that they are arranged in bundles in an order of increasing height (Figure 2.3) (Wu *et al.*, 2017). The tip of every stereocilia is linked by a well-structed protein known as the tiplink which comprised of cadherins, a transmembrane domain, and ankyrin repeat (Figure 2.3C) (Ge *et al.*, 2018). The cadherins of the tiplink have been implicated in hereditary HI suggesting their essential role in hearing (Jaiganesh *et al.*, 2018).

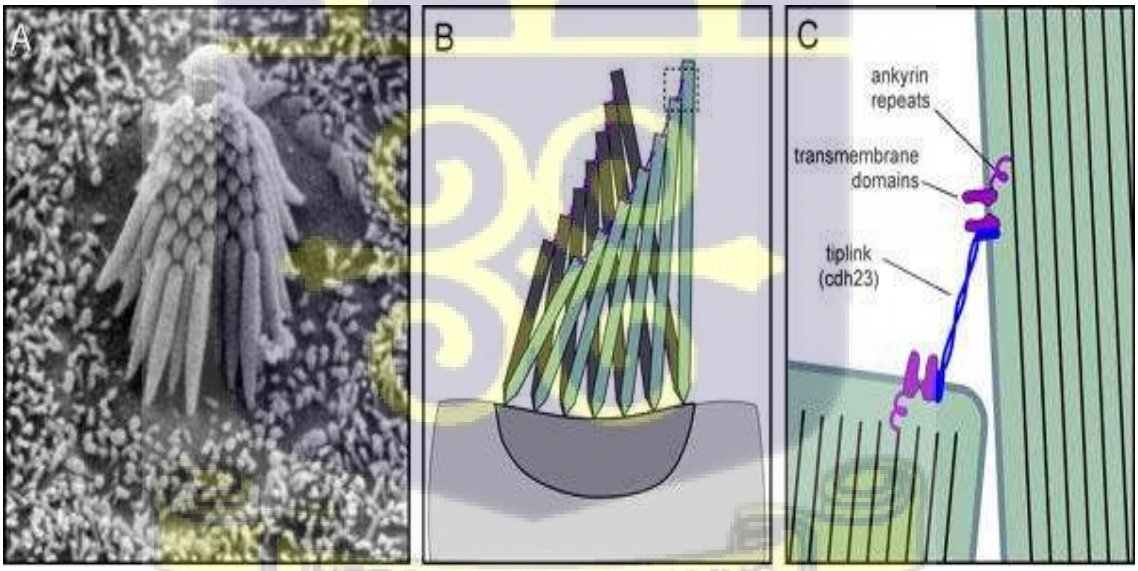

Figure 2.3: Stereocilia of hair cells. *(A) Scanning electron micrograph of the top view of stereocilia. (B) Computational model of stereocilia (C) showing how the stereocilia are linked together by the tiplink protein.*
Source: https://www.ks.uiuc.edu/Research/hearing/

2.2 Hearing impairment

Hearing impairment (HI) is often referred to as hearing loss and is the inability of a person to hear within the "normal" range of hearing (Oxenham, 2018). HI is also defined as a change in the auditory structure/function outside the normal hearing range (Tubbs, 2010).

Based on the World Health Organization (WHO) definitions, HI is when an individual is unable to hear within the normal range of hearing which corresponds to more than 30 and 25 decibels (dB) in the better ear of adults and children less than 14 years old respectively (WHO Media centre, 2014). Hearing ability is known to decline with age and bilateral loss of auditory sensitivity is most prevalent in age related HI. The estimated prevalence of HI associated with age is 30-35% in people who are more than 64 years old, and 75% for people aged over 70 years (Helzner et al., 2005).

HI can be categorized based on the degree of a person's hearing, though the normal range of hearing has been defined in different ways by different researchers across the globe (Awuah, 2012; Burke *et al.*, 2016; Mulwafu *et al.*, 2016). Some studies set a cut-off of 25 dB for normal hearing (Awuah, 2012) while others use the cut-off of 30 dB (Mulwafu *et al.*, 2016). Since hearing declines with age, normal hearing was classified as 0 to 15 dB and 0 to 25 dB in children and adults respectively (Alshuaib *et al.*, 2015). According to WHO, a person who cannot hear below the threshold 25 dB is considered as having HI (WHO, 2019). Mild HI is when an individual only perceives sound within the range of 25 to 40 dB: such a person finds it difficult to hear soft sound and may not understand speech in noisy environments. A person's ability to hear can be considered as moderate hearing loss if he/she only hears sound within 40 to 70 dB. Severe HI is when sound can be heard only at 70 to 90 dB while profound HI is when sound is only perceived at 90 dB and above (WHO, 2020) (Figure 2.4).

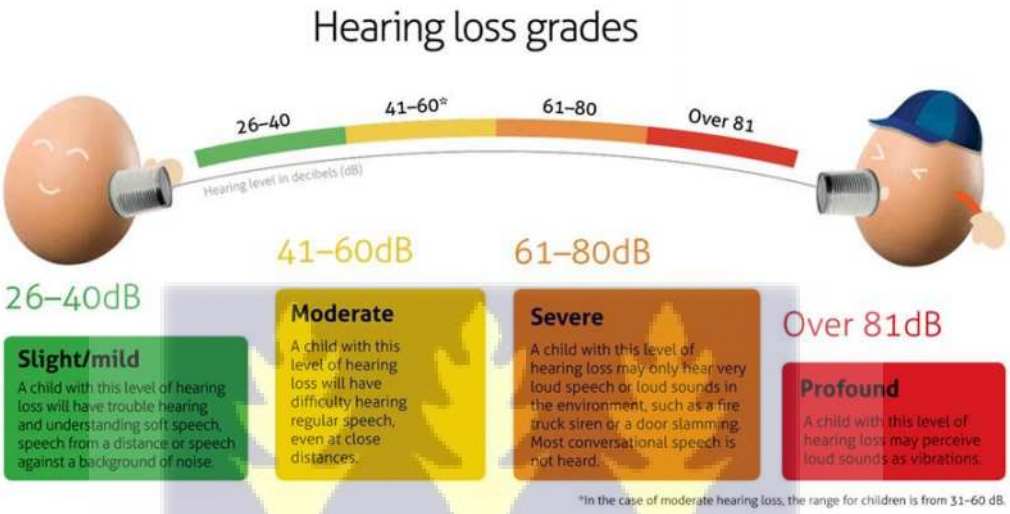

Figure 2.4: Classification of childhood HI based on the degree of hearing. *Hearing less than 25dB in children is considered as normal hearing.*
Source: https://www.who.int/pbd/deafness/hearing_impairment_grades/en/

2.3 Diagnosis of Hearing Impairment

2.3.1 Physiological Examination

Physiological examination can be performed at any age to assess the functional ability of the auditory system. The most common physiological test administered to children is the auditory brainstem response (ABR). ABR evokes and detects the electrophysiological response from the cranial nerves and the auditory brain stem to measure an individual's the hearing sensitivity. One drawback to the ABR hearing test is its inability to measure frequencies lower than 1500Hz (Skoe & Kraus, 2010). Auditory steady-state response (ASSR) works like ABR but uses frequency-specific stimuli, giving it the ability of measuring frequencies as low as 500Hz (Cone-Wesson *et al.*, 2002). Another commonly used physiological test is the evoked otoacoustic emission test, which can assess sound emitting from the cochlear into the auditory carnal. This technique uses a probe connected

to a microphone and a transducer to measure the cochlear hair cell activities over a broad frequency range and is commonly used in newborns (Charaziak & Shera, 2019).

2.3.2 Audiometric Examination

Children less than 6 months old are often examined by behavioral observation audiometry (BOA) (Madell, 2008) and visual reinforcement audiometry (VRA) (Lidén & Kankkunen, 1969): the two types of behavioral audiometry. Both tests require skilled personnel and the test results are prone to error. Accuracy of the test depends on the audiologist and maturity of the patient (Lidén & Kankkunen, 1969; Madell, 2008). Although comparable in performance, pure tone audiometry (PTA) which comprises of air and bone conduction audiometric tests, provides more accurate and reproducible results compared to behavioral audiometry (Ahn *et al.*, 2007). PTA however is subject to the patient's maturity and understating of the procedure. PTA involves the use of an earphone to present octave sound from 250 to 8000Hz with varying intensity measured in decibels. This test can assess speech discrimination and speech reception thresholds. To examine the status of the entire ear, air conduction audiometry which generates sound through earphones is employed. The inner ear function can be examined directly using bone conduction audiometry; in this method, sound is transmitted by a metal vibrator placed on the mastoid bone (forehead), the vibrations produced by the vibrator bypass the external and middle ear thus examining the status of the inner ear. A combination of air and bone conduction is needed for a comprehensive diagnosis of HI (Margolis *et al.*, 2016).

2.4 Types of Hearing Impairment

Based on the etiology of HI, it can be classified as genetic or environmental HI (Willems, 2000) and genetic HI can further be grouped as syndromic or non-syndromic HI. HI that

develops before the age of language development is termed prelingual HI while post-lingual HI develops after language development. Most prelingual HI are congenital (Willems, 2000). The affected site of the auditory system responsible for the impairment is also used to categorize HI as conductive, sensorineural, or mixed.

2.4.1 Conductive Hearing Impairment

For effective auditory function, sound is transmitted through the pinna (outer ear) to the eardrum and then to ossicles in the middle ear. Improper conduction of sound in the middle ear is referred to as conductive HI. (Mcpherson and Swart, 1997; Nyako, 2013), therefore, obstruction, reduction, or altered passage of sound through the outer ear to the eardrum (tympanic membrane) is classed as conductive HI. This may be due to infection, physical blockade by accumulation of wax, or damage to the ear canal, such that sound is not properly transmitted through the ear (Henkel, 2018). In Ghana, this type of hearing disorder occurs more frequently with different types of otitis media in children, though the disorder can however be corrected by surgery (Amedofu et al., 2006).

2.4.2 Sensorineural Hearing Impairment

Sensorineural HI is a heterogenous hearing disorder characterized by injury to the cochlea, cochlear root of the vestibulocochlear nerve or the auditory nerves. Sensorineural HI is known to be the commonest type of congenital HI (Kathryn, 2015). Patients with this type of hearing loss either cannot hear loud sound, or the sound is unclear or muffled. Sensorineural HI cannot be treated through surgery. Middle ear infections, misuse of some drugs, extreme noise, and childbirth complications have been identified as some of the factors that cause sensorineural HI (Smith et al., 2005).

2.4.2 Mixed Hearing Impairment

Mixed HI is the coexistence of both conductive and sensorineural HI in a single ear. This condition may be caused by multiple factors or a single factor and may reduce hearing ability or result in an inability to hear any sound (Amedofu et al., 2006).

2.4.3 Non-Genetic Hearing Impairment

Non-genetic HI is caused by environmental factors that affect the function of any part of the ear. Some of the environmental factors that cause HI are perinatal illnesses such as complicated malaria, meningitis, otitis media, and measles (Willems, 2000). Though the molecular mechanisms of pathogenesis of infection-induced HI are not fully understood, the cochlea and its supporting hair cells have been identified as the major site of injury. Typically in the case of meningitis, the infection often spreads through the cochlear aqueduct and nerves causing injuries which lead to HI (Du *et al.*, 2006). In humans, middle and inner ear infections trigger vigorous inflammatory responses which play key roles in the development HI (Du *et al.*, 2006). Other environmental factors include acoustic or cerebral trauma, application of ototoxic drugs, and complications during childbirth (Willems, 2000).

2.4.4 Genetic Hearing Impairment

The human auditory system is a complex system with many components, hence mutations in genes that control the auditory system could easily induce HI. It has been established for some time that heredity plays a major part in HI, and current estimates are that 50% of all HI are caused by sequence variation in one or more of the many auditory genes. Almost 70% of the known genetic/inheritable HI cases are non-syndromic and may either be familial or non-familial/sporadic (Venkatesh, 2015). HI can result from mutations in one gene (monogenic) or mutations in two or more genes (heterogenous) or a

combination of genetic and environmental factors (multifactorial) (Willems, 2000). Genetic factors account for more than 50% of all congenital HI, 75% of all genetic HI are autosomal recessive, and around 20%, 5%, and less than 1% are autosomal dominant, X-linked, and mitochondrial respectively (Willems, 2000).

2.4.4.1 Syndromic Hearing Impairment

The combination of specific medical anomalies with HI is termed syndromic HI. There are over 400 reported syndromes that are associated with HI (Gettelfinger & Dahl, 2018). Syndromic HI can be grouped as autosomal recessive, dominant, sex-linked or mitochondrial based on the mode of inheritance, and the most frequently reported autosomal dominant syndrome associated with HI is Waardenburg syndrome. However, Usher syndrome is known as most frequently identified syndromic HI (Allen & Goldman, 2018). Other common syndromes associated with HI globally are neurofibromatosis type 2, Jervell and Lange-Nielsen syndrome, Stickler syndrome, branchio-oto-renal syndrome, Pendred syndrome, Refsum disease, Alport syndrome, MELAS, Treacher Collins syndrome, and MERRF (Gettelfinger & Dahl, 2018). In Ghana and Cameroon, Waardenburg's syndrome is was identified as the most prevalent syndrome associated with HI (Adadey *et al.*, 2019; Wonkam *et al.*, 2013). The contributing genes and modes of inheritance of the common syndromes associated with HI are summarized in Table 2.1 below.

2.4.4.2 Non-Syndromic Hearing Impairment (NSHI)

HI that is not related to other medical anomalies/indications is non-syndromic. It can be a partial or complete impairment without signs and symptoms that affect other body parts. The common way NSHI is classified is based on the pattern of inheritance, they are grouped as autosomal dominant, autosomal recessive, X-linked, or mitochondrial. NSHI

can either affect a single ear (unilateral) or both ears (bilateral) with varying characteristics among individuals. The most common form of NSHI is sensorineural, conductive impairment is the least frequent (Smith et al., 2005).

Table 2.1 Mode of inheritance and genes associated with syndromic hearing impairment

Mode of inheritance	Syndrome	Locus/Gene	OMIM number
Autosomal dominant	Neurofibromatosis 2	NF2	607379
	Branchio-oto-renal syndrome	EYA1	601653
		EYA2	601654
		EYA3	601655
	Treacher Collins	SIX1	601205
		SIX5	600963
		TCOF1	606847
		POLR1D	613715
		POLR1C	610060
	Stickler syndrome	STL1/COL2A1	120140
		STL2/COL11A2	120290
		STL3/COL11A1	120280
		STL4/COL9A1	614134
		STL5/COL9A2	614284
	Waardenburg syndrome	PAX3	606597
		MITF	156845
		SNAI2	602150
		EDN3	131242
		EDNRB	131244
		SOX10	602229
Autosomal recessive	Pendred syndrome	PDS/SLC26A4	605646
	Jervell and Lange–Nielsen syndrome	JLNS1/KCNQ1	607542
		JLNS2/KCNE1	176261
	Usher syndrome	USH1B/MYO7A	276903
		USH1C	605242
		USH1D/CDH23	605516
		USH1E	602097
		USH1F/PCDH15	605514
		USH1G/SANS	607696
		USH1H	612632
		USH1J/CIB2	605564
		USH1K	614990
		USH2A	608400
		USH2C/ADGRV1	602851
		USH2D/WHRN	607928

		USH3A/CLRN1	606397
		USH3B/HARS	142810
	Refsum disease	PHYH/PAHX	602026
		PEX7	601757
X-linked dominant	Alport syndrome	COL4A5	303630
		COL4A3	120070
		COL4A4	120131
Mitochondrial	MELAS	MTTL1	590050
	MERRF	MTTK	590060

Abbreviations: MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibers; OMIM, Online Mendelian Inheritance in Man. Source: (Gettelfinger & Dahl, 2018)

The discovery of the first HI genes heralded the identification of many genes involved in congenital HI across the world (Vona et al., 2015), with the aim of improving treatment options, diagnostics, and genetic counselling (Robson, 2006; Zeitler and Lalwani, 2010). Targeted sequencing is the most common molecular method used for investigating HI genes among other methods such as exome sequencing, microarray chips, and SNP genotyping. To date, more than 123 NSHI genes have been reported (Van Camp G & Smith, 2020) with over 141 HI-associated loci published in peer-reviewed journals (Vona et al., 2015). More than 77 of the identified NSHI genes are recessive, 51 and 5 are autosomal dominant and X-linked respectively (Van Camp G & Smith, 2020). There are 3 main classes of NSHI genes based on their loci. The loci are grouped into various groups based on a four-letter naming convention; DFNB (autosomal recessive), and DFNA (autosomal dominant), and DFNX (X-linked). After the four letters, a number is given in order of gene mapping and/or discovery (Shearer *et al.*, 1993).

The NSHI genes most frequently targeted when diagnosing genetic HI are *GJB2*, *GJB6*, *SLC26A4*, and *OTOF*, and the majority of them have been implicated in transport, synaptic, cytoskeleton and ion homeostatic gap junction proteins (Chan and Chang, 2015; Robson, 2006). The connexin genes, *GJB2* and *GJB6* (DFNB1), contribute to over 50% of both autosomal recessive and autosomal dominant NSHI cases in several populations

in the world, with significant exceptions in Africa (Shearer *et al.*, 2010; Wonkam, 2015; Wonkam *et al.*, 2015).

There is an autosomal recessive (DFNB1) and autosomal dominant (DNFA3) colocalization that maps to chromosome 13q12 for both *GJB2* and *GJB6* mutations (Lalwani *et al.*, 1998; Shearer *et al.*, 1993; Wang *et al.*, 2017). Other similar colocalizations include DFNB2 and DFNB11 that map to chromosome 11q13.5 for MYO7A mutations and DFNB21 and DFNA8/12 for TECTA mutations (Shearer *et al.*, 1993).

In most cases, autosomal recessive (DFNB) loci are associated with prelingual HI within the severe to profound HI range with an exception of DFNB8, which manifests as post-lingual and rapidly progresses to HI. Autosomal dominant loci are known to mostly cause post-lingual HI with the exceptions of DFNA3, DFNA8, DFNA12, and DFNA19. X-linked loci (DFNX) on the other hand they can cause both prelingual and post-lingual HI (Shearer *et al.*, 1993; Wang *et al.*, 2017).

2.4.4.3. Tools for investigating genetic hearing impairment

The initial diagnosis of inheritable HI was based on medical and family history in combination with physical and audiological examinations of patients. It was difficult to effectively differentiate between genetic and environmental HI, especially for sporadic/isolated HI. It was also tough to differentiate NSHI from syndromic HI when supplementary tests such as urine analysis, imaging, thyroid functional tests, and electrocardiogram (ECG) were used (Shearer & Smith, 2012). The diagnosis of HI genetics was significantly improved with the development of technology to sequence DNA which aided the first discovery of the HI gene *GJB2*, in 1997 (Kelsell *et al.*, 1997), and aided the understanding of the genetic background of the disease. Ever since over

120 HI genes have been discovered (Van Camp G & Smith, 2020). The heterogeneity of HI has made it difficult to develop a single point of care diagnostic tool for hereditary HI. The early tools designed for screening genetic HI were centered around Sanger sequencing and although useful at the time, it could not offer clinical investigation for all known HI genes (Shearer & Smith, 2012). The above challenge was addressed by developing massive parallel sequencing (MPS)/ next-generation sequencing (NGS) technologies. The MPS can simultaneously sequence billions of DNA and generate extensive genomic data, therefore, favoring a comprehensive screening of HI genes (Shearer & Smith, 2012). Furthermore, MPS has greatly increased the rate of discovery of HI genes. Despite the successes of MPS, heterogeneity of HI genes has made it challenging to develop a comprehensive genetic testing platform for screening HI gene variants. Hence the search for a robust platform for HI genetic testing continues (Shearer *et al.*, 2011).

To date, no HI test has achieved the ideal goal for genetic tests; high sensitivity, specificity, and accuracy. There have been several attempts to develop an ideal test for genetic HI. One of such attempts is the OtoChip™, which was developed on the microarray technology by Harvard University. At the time of its design, the OtoChip™ could investigate 13 deafness genes in about 3 to 4 days (Waldmuller *et al.*, 2008). The microarray technology is known to be time-efficient and not expensive but operationally complicated. The number of nucleotides investigated by microarrays is limited to the physical size of the chip and the tests are not efficient in detecting insertions and deletions (Shearer *et al.*, 2011). The above challenges limit the overall usefulness of microarrays.

At the University of Iowa, a HI tool was also designed using solution-based targeted enrichment and MPS platforms. This tool was called OtoSCOPE® and had the potential of screening more HI genes compared to OtoChip™. It is useful in investigating both

syndromic and non-syndromic HI by targeting about 97% of the coding regions of the human genome (Shearer *et al.*, 2010). One major drawback of the OtoSCOPE® is the inability to resolve the cause of HI in individuals with novel mutations that are not captured on the tool's panel of genes. It is also relatively more expensive compared to OtoChip™ (Shearer *et al.*, 2011).

The available compressive tools for HI gene testing are coupled to sequencing platforms which made them expensive and not feasible for clinical investigations in resource-limited settings. Also, these tools require high-performance computing systems and experts of bioinformatic data analysis, thus providing a disadvantage to its usage (Gu *et al.*, 2015; Shearer *et al.*, 2010; Sloan-Heggen *et al.*, 2016). Therefore, in order to reduce the cost and computational requirements, target gene approaches have been developed for population dominant mutations such as *GJB2*-35delG in Caucasians (Antoniadi *et al.*, 2001; Lucotte *et al.*, 2001). The *GJB2*-35delG variation has not been identified in the Ghana population, hence the cheaper targeted genetic tools for the Caucasians do not apply to clinical investigations in Ghana (Adadey *et al.*, 2019; Brobby *et al.*, 1998; Hamelmann *et al.*, 2001).

2.5. Connexins

Connexins are made of proteins that span the cell membrane and forms hemichannels known as connexons (Srinivas *et al.*, 2019). The connexon consists of a hexamer of connexins, a hexameric pore which connects adjacent cells to form essential gap junctions for cell-to-cell communication (Figure 2.5) (Beyer *et al.*, 1990). Connexins play essential roles in the regulation of cell proliferation, differentiation, and homeostasis through protein-protein interactions (Vinken, 2015). In humans, there are about 21 known

connexins (Beyer & Berthoud, 2018) which were named based on their molecular weight in kilo Daltons (Sáez *et al.*, 2003).

2.5.1. Biosynthesis and biodegradation of connexins

In humans, connexins are expressed in all cell types and are essential for survival and development (Table 2.2). Connexins are highly expressed on the membrane of excitable cells like neurons and cardiomyocytes where they provide electrochemical pathways and transport across the cell (Goodenough & Paul, 2009). The cytoplasmic production of connexins takes place on the endoplasmic reticulum of the cell, and oligomerization of the synthesized connexins is done in the Golgi bodies. The Golgi network transports the synthesized connexins to the cell membrane. Similar or different connexins can oligomerize to form connexons made of similar units (homomeric) or different units (heteromeric) (Martin *et al.*, 2001). Although connexins appear structurally different, they share some basic features: transmembrane domain (M1-M4), extracellular loops (E1 and E2) cytoplasmic loop, N and C terminal domains as illustrated in figure 2.5. The C and the N termini of connexins are in the cytoplasmic region (Leithe *et al.*, 2018).

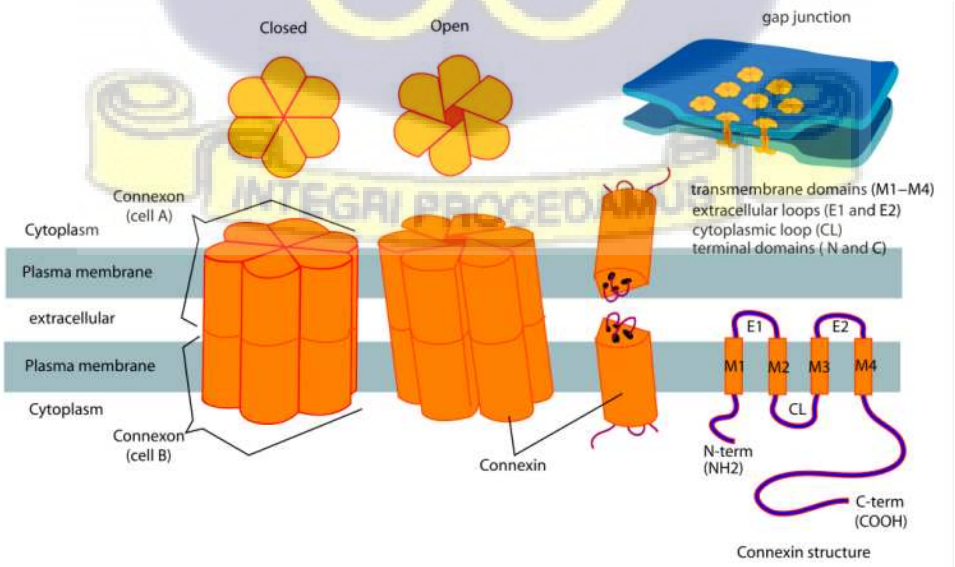

Figure 2.5: The structure of gap junction proteins (connexins). *Connexins expressed in the inner ear form tight gap junctions that allow the movement of ions across the cell to create electrochemical potential and transmit sound-induced signals.*
(Source: https://en.wikipedia.org/wiki/Connexin#/media/File:Connexon_and_connexin_structure.svg)

A proportion of the newly synthesized connexins undergo degradation through an endoplasmic reticulum-associated degradation (ERAD), suggesting a high turnover rate (VanSlyke & Musil, 2002). The number of connexons in the cell membrane is regulated by the cell to control the activity of connexons (Fykerud *et al.*, 2012): adjacent connexons are internalized together into one cell to form a vesicle known as connexosome. The connexosome has three pathways of degradation; direct lysosomal, phagophore mediated, and multivesicular endosome mediated degradations. Lysosomes can directly fuse with the connexosomes to degrade their content (Qin *et al.*, 2003). Phagophore-mediated degradation occurs when the connexosomes are sequestrated by phagophores and subsequently fuse with lysosomes. In the third degradation pathway, the connexosomes are transformed into connexin-enriched multivesicular endosomes. The connexins are organized from the early endosomes into late endosomes which subsequently fuse with lysosomes (Leithe *et al.*, 2009).

2.5.1. Connexins and their associated phenotypes

Animal and human genetic studies have revealed that connexins are expressed in the skin, middle ear, and other parts of the body. Hence connexins are associated with a wide range of phenotypes (Table 2.2) such as HI and skin disorders with erythrokeratodermia variabilis as the commonly associated phenotypes (Evans & Martin, 2002). HI is commonly associated with *GJB1*, *GJB2*, *GJB3*, *GJB6*, *GJC3*, and *GJB2* (Table 2.2), variations in these connexins may result in both syndromic and non-syndromic HI and manifest as partial or total sensorineural HI.

Among the syndromes caused by connexin variants are keratitis-ichthyosis-deafness (KID), Waardenburg, Ushers, and Treacher Collins syndromes. Skin disorders including palmoplantar hyperkeratosis, hydrotic ectodermal dysplasia (hair loss), and erythrokeratodermia variabilis were associated with *GJA1*, *GJB2*, *GJB3*, *GJB4*, *GJB5*,

and *GJB6*. Other connexin associated phenotypes are split-hand/foot malformation/ Syndactyly (*GJA1* and *GJC3*), visual/eye defects (*GJB6, GJD2, GJA3,* and *GJA8*), mental/intellectual disability (*GJB6, GJB1, GJA1,* and *GJC2*), cardiomyopathy/ abnormal heart/blood circulation deficiencies (*GJC3, GJD3, GJA5, GJA1,* and *GJC1*), diabetes (*GJD3*), Olfaction dysfunction (*GJB4*), developmental abnormalities (*GJB5* and *GJB1*), and schizophrenia (*GJA5*) (Evans & Martin, 2002).

Table 2.2: Types of connexins and their phenotypes

Human connexins	Associated phenotype in mice	Associated phenotype in rats	Associated phenotype in human
GJB2 /connexin 26	HI, Keratitis-ichthyosis-deafness (KID) syndrome	Ichthyosis follicularis atrichia photophobia syndrome, NSHI, keratitis-ichthyosis-deafness syndrome	NSHI, palmoplantar hyperkeratosis
GJC3 /connexin 29	Abnormal retinal blood vessel morphology, abnormal auditory brainstem response waveform shape, increased susceptibility to noise-induced HI, increased or absent threshold for auditory brainstem response	Pleomorphic xanthoastrocytoma, NSHI	Speech-language disorder, split-hand/foot malformation, pleomorphic xanthoastrocytoma
GJB6 /connexin 30	Ectodermal dysplasia, Clouston syndrome	Ectodermal dysplasia, visual epilepsy, NSHI, brain hypoxia	NSHI, hydrotic ectodermal dysplasia (hair loss), nail defects, mental deficiency
GJD3 /connexin 31.9	Abnormal impulse conducting system conduction, abnormal atrioventricular node conduction, shortened PQ interval	Diabetes mellitus	
GJB4 /connexin 30.3	Olfaction dysfunction		Erythrokeratoderma variabilis
GJB3 /connexin 31	Transient placental dysmorphogenesis, erythrokeratodermia variabilis,	Erythrokeratodermia Variabilis, Charcot-Marie-Tooth disease dominant intermediate, NSHI	Hearing impairment, erythrokeratoderma variabilis
GJB5 /connexin 31.1	Lethality throughout fetal growth and development	Charcot-Marie-Tooth disease dominant intermediate	Skin disease, neoplasm, attention deficit disorder with hyperactivity, global developmental delay, intellectual disability
GJB1 /connexin 32	Decreased glycogen degradation, increased liver carcinogenesis decreased body weight	Charcot-Marie-Tooth disease type X, syndromic X-linked intellectual disability lubs type, neoplasms, HI	CMTX, X-linked Charcot-Marie-Tooth disease type, X-linked progressive cerebellar ataxia
GJD2 /connexin 36	Visual deficits	Visual defect	Visual defect

GJA4 /connexin 37	Female sterility, Intensive bleeding	Charcot-Marie-Tooth disease dominant intermediate, hypertension, coronary artery disease	Global developmental delay, intellectual disability (formal thought disorder in schizophrenia)
GJA5 /connexin 40	Atrial arrhythmia Dilated cardiomyopathy abnormal heart morphology	Hypertension Schizophrenia Tetralogy of Fallot Wolff-Parkinson-White Syndrome	Schizophrenia, tetralogy of Fallot, Familial atrial fibrillation
GJA1 /connexin 43	Heart malformation and Ventricular arrhythmia	Autistic disorder, neoplasms, erythrokeratodermia variabilis, intellectual disability, oculodentodigital dysplasia, autosomal recessive erythrokeratodermia variabilis, cleft lip.	HI, visceroatrial heteroataxia, syndactyly, hypoplastic left heart syndrome, oculodentodigital dysplasia, erythrokeratodermia variabilis, atrioventricular septal defect
GJC1 /connexin 45	Embryonic growth arrest, abnormal heart development, embryonic lethality prior to tooth bud stage	Hypertension	
GJA3 /connexin 46	Zonular nuclear cataract	Autosomal recessive nonsyndromic deafness, cataract	Congenital cataract, zonular pulverulent cataract
GJC2 /connexin 47	Abnormal motor learning	Neurodegeneration, paraplegia Intellectual disability, Parkinson's disease	Milroy disease, spastic paraplegia, Pelizaeus-Merzbacher disease, intellectual disability
GJA8 /connexin 50	microphthalmia, zonular pulverulant and congenital cataract Cardiovascular system phenotype	Neurodevelopmental disorders, cataract, Schizophrenia, autism spectrum disorder	Zonular pulverulant cataract, schizophrenia

2.5.6. Connexins associated HI

2.5.6.1. Connexin 26 (*GJB2*) associated HI

GJB2 gene is positioned on chromosome 13q:11 and codes for connexin 26 protein which spans the cell membrane four times to form 4 transmembrane domains (M1 to M4). The part of the protein that spans the cell membrane is linked by 2 extracellular and one cytoplasmic loop (Figure 2.5 and 2.6). The initial description of *GJB2*-associated HI in 1997 (Kelsell *et al.*, 1997), paved the way for similar kinds of research to investigate *GJB2* mutations in different populations. Now, *GJB2* is known to be the most frequently associated gene to HI (Chan & Chang, 2014). The inner ear function is highly dependent on the expression of *GJB2* since it is the major connexin that forms gap junctions in the cochlear (Van Camp G & Smith, 2020). Mutations in this gene manifest in a varying range of HI phenotypes from mild to profound HI at birth (congenital) or after spoken language is developed (post-lingual) (Snoeckx *et al.*, 2005), and mostly inherited in an autosomal recessive mode.

Connexin 26 is actively involved in maintaining a high potassium ion (K^+) concentration in the endolymph of the cochlear. Unlike the wild type, the mutant connexins are unable to transport K^+ across the cell upon receiving sound vibrations and ultimately cannot transform the vibrations into neural signals. The inability of the system to transport K^+ across cells via the gap junctions leads to hearing problems (Kathryn, 2015).

Over 200 *GJB2* variants have been identified globally (Table 2.2) and over 50% of these variants are missense and pathogenic variants (Figure 2.6). A fraction of these variants had uncertain pathogenicity and unclear association with HI (Zheng *et al.*, 2015). The global distribution of *GJB2* variants suggests localization of certain mutations to specific populations (Chan & Chang, 2014); while 35delG was common in the European populations, 235delC, V37I, W24X, 167delT, and R143W were common in East Asia

(Fuse *et al.*, 1999), Southeast Asian (Kelley *et al.*, 1998), Indian (Kelsell *et al.*, 1997), Ashkenazim (Zelante *et al.*, 1997), and Ghanaian (Brobby *et al.*, 1998; Hamelmann *et al.*, 2001) populations respectively.

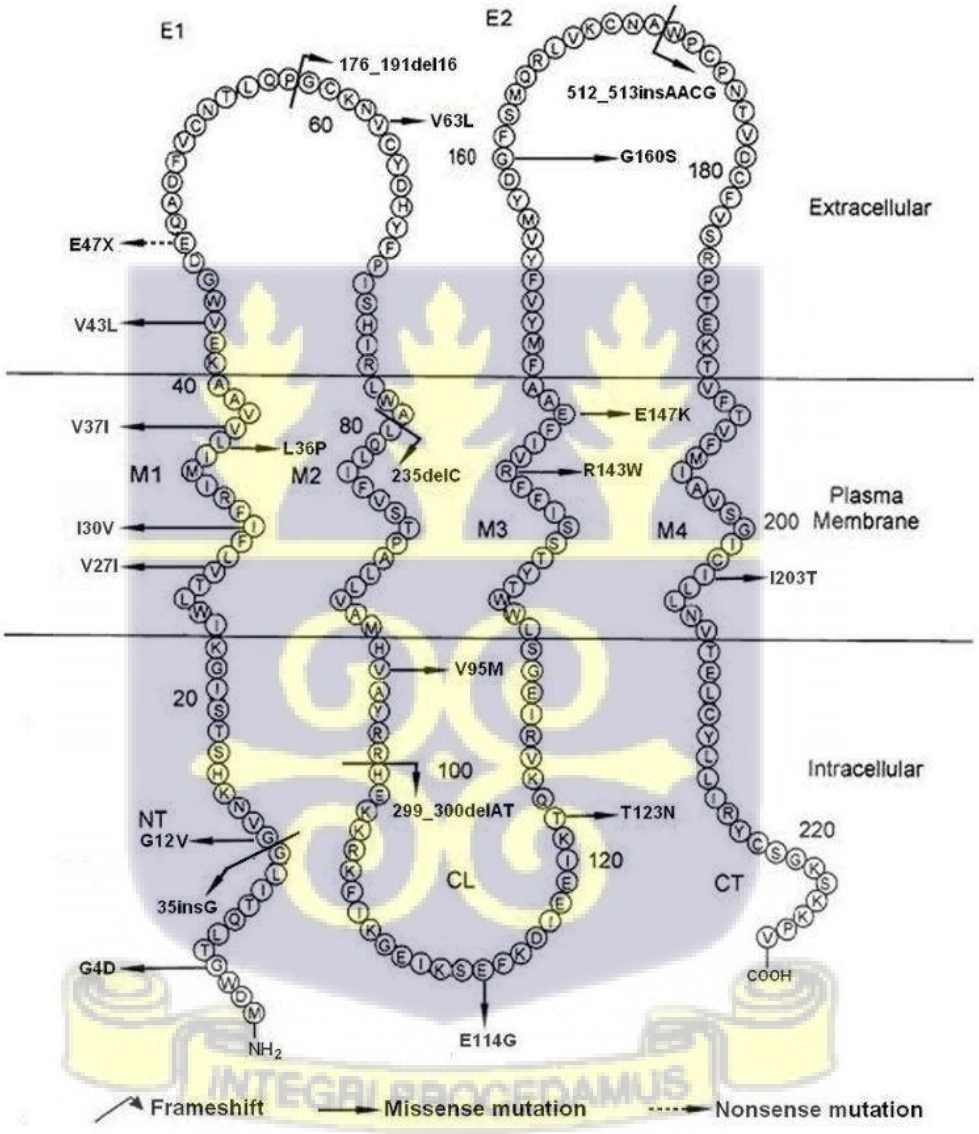

Figure 2.6: Major mutation types in connexin 26. *The diagram shows the amino acid position of connexin 26 variants linked to the HI phenotype.*
Source : Adopted from (Zheng et al., 2015)

The most commonly reported *GJB2* mutant is 35delG, which explains over 50% of inheritable HI in Asia, America, and Europe (Chan & Chang, 2014). Although 35delG variant has a high carrier rate of 1:51 in Europe, Africa has only negligible number of reported cases of the mutation (Chan & Chang, 2014). The high carrier rate of 35delG

in some of the European populations may be due to the tradition of marriage between hearing-impaired persons (Nance *et al.*, 2000). The pathogenicity of the 35delG is caused by the production of a truncated protein which is non-functional and does not allow for the normal inner ear functioning (Denoyelle *et al.*, 1999). Similar to 35delG, other non-sense mutations within the *GJB2* gene (Table 2.2) such as p.E47Ter, and W44Ter also produce truncated non-functonal proteins (Figure 2.7).

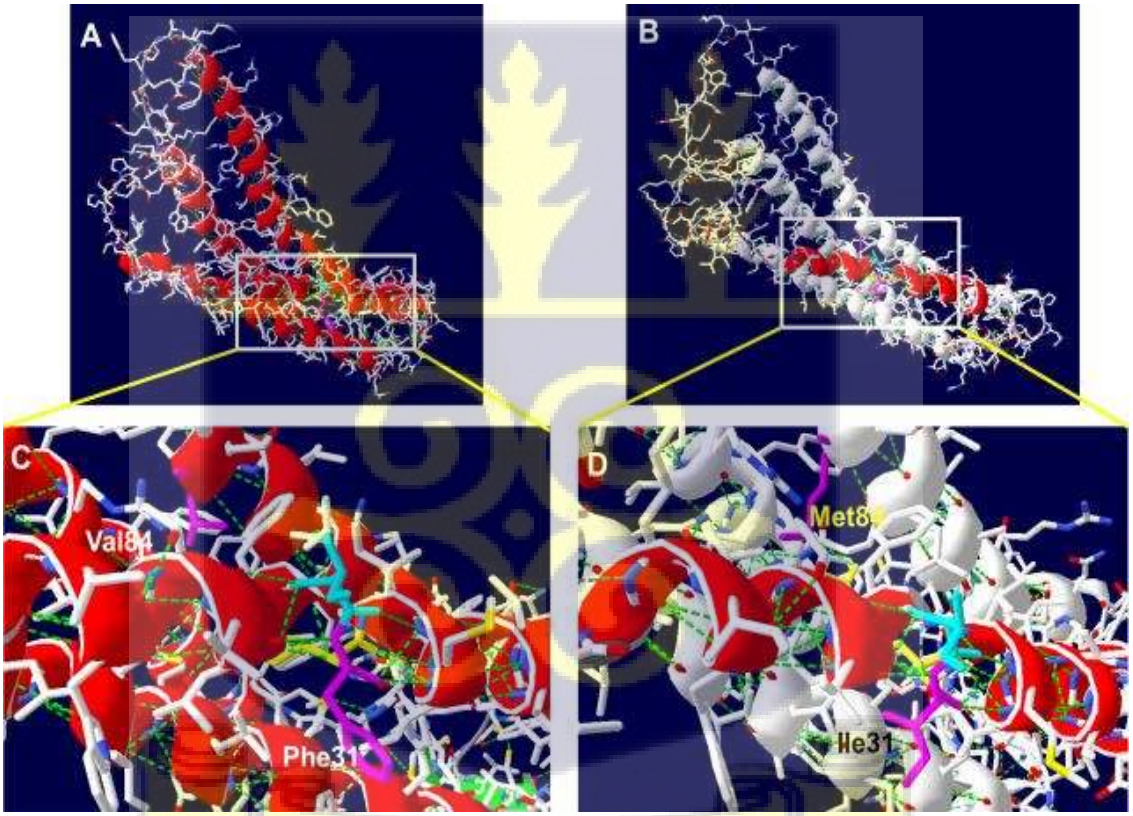

Figure 2.7: Modeling of wildtype and p.W44Ter-truncated *GJB2* proteins. *(A) wild type (B) p.W44Ter GJB2 proteins. The alpha helices are shown in red and the absence of helix as a result of the truncation in white. C and D are zoomed pictures of the white box. The truncated GJB2 proteins are non-functional and may lead to the HI phenotype. Source: (Martínez-Saucedo et al., 2015)*

The first report of p.R143W mutation as a founder mutation was from Adamorobe, a village in Ghana known for its high number of deaf people (Brobby *et al.*, 1998). A nationwide study in 2001 has found the founder mutation in 16% of randomly selected

cases from Ghanaian schools for the deaf (Hamelmann *et al.*, 2001). The p.R143W is not exclusive to Ghana, as studies from China (Gao *et al.*, 2016; Huang *et al.*, 2015), Argentina (Dalamon *et al.*, 2013), Peru (Figueroa-Ildefonso *et al.*, 2019), Turkey (Tekin *et al.*, 2005), Korea (Jung *et al.*, 2017), Japan (Ohtsuka *et al.*, 2003), and USA (Pandya *et al.*, 2003; Wu *et al.*, 2002) also identified the same founder mutation in hearing-impaired patients. The p.R143W mutation located in the cell membrane (Figure 2.6), alters the protein structure and causes a significant change in its function. This mutation is highly penetrant and all affected individuals reported with severe to profound HI (Brobby *et al.*, 1998).

2.5.6.2. Connexin 31 (*GJB3*) associated HI

The mode of the pathogenesis of *GJB3* remains unknown, but it has been shown that the gene is expressed in the cochlea and auditory nerve of mice. This suggests that the *GJB3* protein is involved in the hearing process by ensuring the proper functioning of both the cochlea and auditory nerves (López-Bigas *et al.*, 2001). Also, *GJB3* mutations were seen in recessive and dominant HI among different populations; studies reported mutations such as p.R32W from China (Chen *et al.*, 2018), p.C798T from Austria (Frei *et al.*, 2004), D66del from Spain (López-Bigas *et al.*, 2001), p.A194T from Taiwan (Yang *et al.*, 2010) and p.V27M from Korea (Oh *et al.*, 2013).

2.5.6.3. Connexin 30.3 (*GJB4*) associated HI

Similar to other connexins *GJB4* has been linked to skin disorders such as erythrokeratodermia variabilis, however, its association with HI is uncertain (López-Bigas *et al.*, 2002). *GJB4* knock-down adult mice were found to have normal hearing, but the younger mice were prone to noise-induced deafness. Expression data from mice studies did not report *GJB4* expression in the cochlea (Zheng-Fischhöfer *et al.*, 2007). In

rats however, *GJB4* is expressed in the cochlea implicating it in the functioning of the cochlea (Wang *et al.*, 2010a). Analysis of HI families in Italy identified a frame shift mutation (154del4) in both hearing and deaf participants and p.R103C, p.R124Q, p.R160C, p.C169W and p.E204A mutations in only deaf patients (López-Bigas *et al.*, 2002). In Sudan, hearing-impaired patients were found to have p.E204A change in their *GJB4* gene (Salih *et al.*, 2014). To predict the mutation's effect on the structure of *GJB4* protein, Salih *et al* modeled the wild type and mutant proteins (Figure 2.8). Predictive bioinformatic tools have shown p.E204A mutation to be highly pathogenic and hence may be the causal variant in deaf patients. Although there is not enough evidence to describe the pathogenicity of *GJB4* mutations, *GJB4* is likely associated with HI in humans.

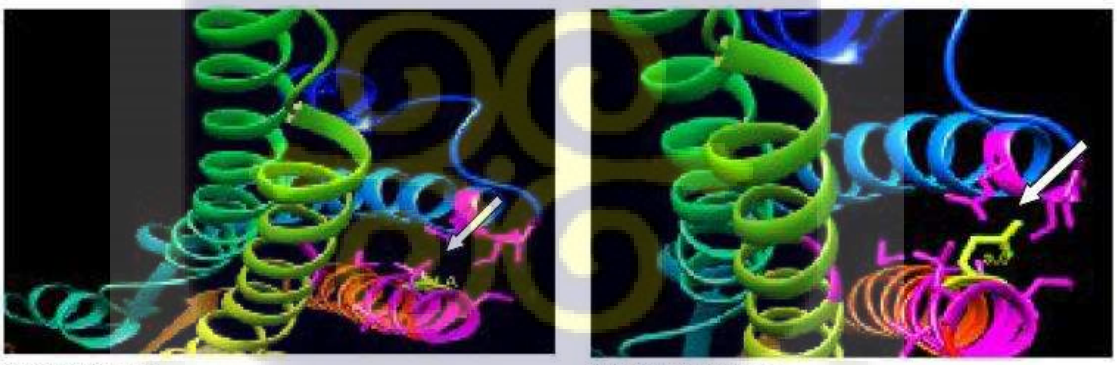

Figure 2.8: *GJB4* protein models. *The protein structure of the wildtype (A) and mutant (B) GJB4 proteins showing the amino acid change at position 204 (white arrow). Source: (Salih et al., 2014)*

2.5.6.4. Connexin 30 (*GJB6*) associated HI

GJB6 is the second commonly associated gene to HI with coding region variants such as p.P70L, p.R32Q, p.E101K, p.E148D, p.Y145H, p.M203V, p.V190A, and p.H124Q reported (Alkowari *et al.*, 2017; Asma *et al.*, 2011; Beck *et al.*, 2015). The commonly reported *GJB6* variant is del(GJB6-D13S1830), which is a large genomic deletion. Although the *GJB6* gene was considered as a HI gene, research has shown that the

variants in the coding region may not be responsible for HI in humans (Ahmad *et al.*, 2007; Rodriguez-Paris & Schrijver, 2009). The evidence mounted included mouse models that had normal hearing despite the total deletion of the *GJB6* coding region. It was however noted that deletion of the trans-acting element of *GJB2* and *GJB6* gene results in the HI phenotype. The large *GJB6* deletions, D13S1830 and D18S1854 span beyond the 5` end of *GJB6* (Figure 2.9) eliminating the trans element which abolishes the expression of *GJB2.* The destruction of the *GJB2* expression in this manner results in deafness (Ahmad *et al.*, 2007; Rodriguez-Paris & Schrijver, 2009). This demonstrates that there is no need to investigate *GJB6* coding region variants in hearing-impaired patients.

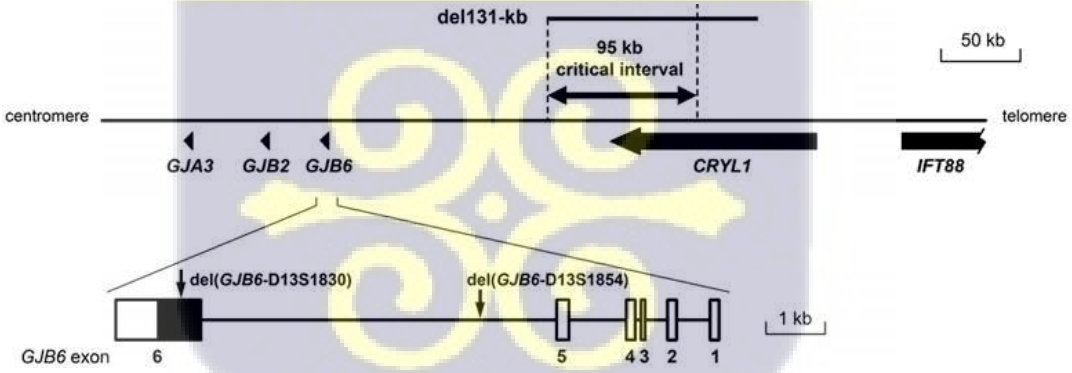

Figure 2.9: Map of chromosome 13 showing the location of *GJA1*, *GJB2*, *GJB6* genes, and *GJB6* deletions (del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854)). *Source: (del Castillo & del Castillo, 2011)*

2.5.6.5. Connexin 29 (*GJC3*) associated HI

GJC3 is located at chromosome 7q22.1 of the human genome and is associated with HI. Animal studies have shown high expression of connexin 29 in the cochlea and its neurons (Tang *et al.*, 2006; Yang *et al.*, 2005). Furthermore, knockout of *GJC3* in animal models resulted in hearing disorders caused by severe loss of myelination, loss of high-frequency sensitivity, and increased risk of noise injury (Tang *et al.*, 2006). Only a few *GJC3* variants, about 6, were globally associated with HI. These variants were reported in Taiwanese [c.43C>G (p.R15G), c.807A>T (p.E269D), c.781+10 C.>G, c.230C>G

(p.T77S), c.525T>G(p.L175L), c.781+15 C>T, 781+62 G>A and c.*+2 T>G] (Wang *et al.*, 2010b; Yang *et al.*, 2005), and Indian [p.I90A (c,569T>A)] (Ramchander *et al.*, 2010) populations.

2.5.6.6. Connexin 43 (*GJA1*) associated HI

GJA1 has been identified as an essential gene for human development, and alterations in this gene can result in autosomal dominant conditions which present with spastic paraplegia, neurodegeneration, craniofacial and limb dysmorphisms, (Wittlieb-Weber *et al.*, 2016). This is mainly because the gene in humans is located on chromosome 6q22-q23, a region of oculodentodigital dysplasia locus (Paznekas *et al.*, 2003). Thus, mutations within the *GJA1* gene predispose to oculodentodigital dysplasia, and are highly associated with conductive HI (Paznekas *et al.*, 2003). *GJA1*-associated HI seems to be common among African Americans (Liu *et al.*, 2001), though it is not a major cause of NSHI in black Africans (Wonkam *et al.*, 2015). The non-synonymous *GJA1* variants, p.L11F and p.V24A were identified in USA (Liu *et al.*, 2001), c.543G>C in Taiwan (Yang *et al.*, 2010), c.717G>A, in Cameroon and c.717G>A, c.758C>T and c.366T>C in South Africa (Bosch *et al.*, 2014b).

2.5.7. Interventions for connexins associated HI

Connexin-associated HI mostly affects hearing in the middle ear and results in permeant loss of hearing. The most appropriate intervention for people with connexin-associated HI is a cochlear implant. In some cases, these patients are given a hearing aid to help them hear the ambiance sound to respond to danger (Yang *et al.*, 2019).

2.5.7.1. Cochlear implant

Over the years, the cochlear implant has proven to effectively restore the hearing of congenital hearing-impaired patients although hearing aids are the widely known intervention given to the deaf. Thus, the cochlear implant remains the most promising

way of treating sensorineural HI especially those caused by mutations in connexin genes (Zhang *et al.*, 2018). During a cochlear implant, an electronic cochlear is inserted under the skin, just behind the ear. This device provides sound perception by sending sound electronically to the brain without going through the damaged cochlear. The implant helps profoundly deaf people to hear and interpret sound which contributes to the development of oral communication (House, 1976).

The effectiveness of the cochlear implant is mostly dependent on the cause of HI and the age of the recipient. Several studies have reported good post-implant outcomes from children with genetic HI (Cullen *et al.*, 2004; Wu *et al.*, 2015). Generally, children with *GJB2* mutations tend to have higher auditory performance, speech production (Yan *et al.*, 2013), word/sentence perception (Sinnathuray *et al.*, 2003), and expressive language (Angeli *et al.*, 2011) compared to children without *GJB2* mutation. Besides, there is no significant difference between the speech recognition of people with *GJB2* mutation who received a cochlear implant and their counterparts without any *GJB2* mutations (Cullen *et al.*, 2004). Therefore, children with congenital HI should be given a cochlear implant before three years to enable them to develop spoken language just as other children (Yan *et al.*, 2013). Research has shown that children who received cochlear implants need about three years post-implantation to develop and display better auditory performance (Wu *et al.*, 2008; Wu *et al.*, 2011).

2.5.7.2. The promises and challenges of gene therapy in HI

The advances in HI gene discovery over the last decade has uncovered several putative targets of therapeutic interest which paved the way for many gene therapy studies. There is a lot to learn from the phase III clinical trial of ocular gene therapy since the eye and cochlear are sensory organs that have major similarities in their physiology (Zhang *et*

al., 2018). Similar to ocular gene therapy (potential treatment for retinal conditions), cochlear gene therapy is a cell-based-therapy and has the potential of restoring normal hearing without a cochlear implant. According to Zhang *et al*, cochlear gene therapy promises to be ultimately less expensive and would offer far more benefits to hearing-impaired patients compared to the cochlear implant.

Appropriate delivery and expression systems are major requirements for exogenous genetic material transfer into mammalian cochlear. Animal studies have shown that non-integrating viral vectors are promising vehicles for efficient delivery and sustainable expression of transgenes in the cochlear (Sacheli *et al.*, 2013). Preclinical gene therapy using congenital HI mouse models has shown rescue of hearing when adeno-associated virus type 1 (AAV1) was used to deliver and express a transporter protein in the hair cell (Akil *et al.*, 2012). A similar gene replacement study also used a viral delivery system to therapeutically replace Kcnq1 gene and restore hearing in mice (Chang *et al.*, 2015). The viral delivery has a reduced risk of undesirable effects since the hair cells and other supporting cells in the cochlear are stable and do not regularly divide (Akil *et al.*, 2012; Roche & Hansen, 2015). There is however a level of risk associated with ectopic expressions in virus-mediated gene therapies when it is place under a strong promoter. The associated risk may range from unwanted immune system reactions to possible infections caused by the virus (Chang *et al.*, 2015; Yu *et al.*, 2014).

Despite of the promises of cochlear gene therapy, its long-term effect remains unknown since the majority of animal studies last for about seven weeks only (Chang *et al.*, 2015; Kim *et al.*, 2016a; Zhang *et al.*, 2018). Lessons from the ocular gene therapy clinical trial revealed that there is a decay of the viral delivered gene after three years (Bainbridge *et al.*, 2015), therefore, there is a need to study extensively, the long-term activity of HI genes in cochlear gene therapy. HI genes are generally large (greater than 5kb) and not

easily carried by viral vectors. This poses the challenge of size limitation and hence only a small number of HI genes can be considered for cochlear gene therapy (Zhang *et al.*, 2018).

In summary, HI is mainly caused by genetic and environmental factors with meningitis as the major environmental cause of HI. Connexins and practically connexin 26, account for over 50% of genetic HI. Connexin 26 is expressed in the inner ear where it forms tight gap junctions to facilitate the formation of electrochemical potential across the cells. The major connexin variant associated with HI is *GJB2*-35delG, a common variant in Europe. Except for Ghana, the contribution of connexin variants to HI in sub-Saharan Africa is approximately zero with many populations yet to be studied. In Ghana, the *GJB2*-R143W variant is the most frequently associated genetic cause of HI. The interventions given to HI patients include cochlear implants, sign-language, and speech therapy. It is important to mention that there are potentials for HI gene therapy.

CHAPTER THREE

3.0. Paper 1: Public health burden of hearing impairment and the promise of genomics and environmental research: A case study in Ghana, Africa

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3.1. Abstract

Hearing impairment (HI) is one of the most disabling conditions of global health concerns and contributes adversely to the social and economic development of a country, if not managed properly. A proper assessment of the nationwide burden and etiology of HI is instrumental in the prevention, treatment, and management of the condition. In this paper we described an expert review of HI in Ghana that determined the present knowledge of the burden of HI and possible causes of the underlying condition. A literature search was conducted in PubMed using the following search strategy: ("hearing loss" OR "hearing impairment" OR deafness) AND Ghana. The literature was scanned until 20 July 2017, with specific inclusion of targeted landmark and background articles on HI. From the search, 18 of out 5869 articles were selected and considered for the review. The results of the search indicated that there were no extensive studies to

determine the national burden of HI in Ghana, however, the few studies assessed suggested that the disease is either acquired or inherited. The number of acquired HI cases was higher in adults than children, women than men, and people working in a noisy rather than quiet environment. Regarding the genetic causes, specific mutations in the *GJB2* gene (R143W, L79P, V178A, R184Q, A197S, I203K, and L214P) were the only identified genetic causes of HI in Ghana, but the other known HI genes were not investigated. There have been some modest efforts to study HI in Ghana, but comprehensive studies on the genetic and environmental etiologies (using the "multi-OMICS" approaches), classification, and burden of HI on Ghana are urgently needed.

3.2. Introduction

Of all the congenital diseases that occur worldwide, hearing impairment (HI) remains the most disabling, with the highest rate for age-standardized disability (Murray *et al.*, 2015). Congenital HI has a global prevalence of about 1 per 1000 live births in developed countries, with a much higher rate in the developing world, as high as 6 per 1000 in sub-Saharan Africa specifically (Olusanya *et al.*, 2014). A child's inability to hear can affect their cognitive development, including for example delayed speech development that eventually leads to their social isolation. Therefore, childhood HI results in underachievement in school and eventual exclusion from the mainstream school programme. Early diagnosis and intervention for children with HI are recommended to maximize their cognitive, social-emotional, speech, and language development (Barnard *et al.*, 2015), but in the absence of widely used new-born screening, the age at diagnosis is usually quite late in Africa for example 3.3 years on average in Cameroon (Wonkam *et al.*, 2013). Approximately 360 million (5.3%) people have HI with 32 million (9%) of these being children (WHO, 2014). According to a report from Stevens et al., the global prevalence of HI as of 2008 was 1.4% in children (5-14 years), 9.8% in females, and

12.2% in males (Stevens *et al.*, 2013) and the most recent study of this kind reported the prevalence of HI to be about 6.8% (Wilson *et al.*, 2017b). HI is most prevalent in South Asia, Asia Pacific, and Sub-Saharan Africa, and other parts of the world. In Africa, 6.8 million (1.9%) people are living with hearing loss defects (WHO, 2014) and environmental factors are reported to be the predominant cause of the disorder (Amedofu *et al.*, 2006). Among the environmental factors, a study in Cameroon identified meningitis as the major cause of HI among other causes such as measles, mumps, and ototoxicity (Wonkam *et al.*, 2013).

HI can be categorized based on the number of ears impaired; unilateral (only one ear affected) or bilateral (both ears affected), and/or the degree of hearing loss, ranging from difficulty to understand soft speech (mild) to inability to hear very loud noises (profound) (Birkenhäger *et al.*, 2007; Matsunaga, 2009; Schrijver, 2004). HI is also classified as conductive, sensorineural, or mixed based on the damaged part of the auditory system (Nyako, 2013).

Conductive HI occurs when there is improper conduction of sound in the middle ear that leads to an inability of the patient to hear faint sounds, or a general reduction in their perceived sound levels (Nyako, 2013). In Ghana, this type of hearing disorder occurs more frequently in all kinds of otitis media in children (Amedofu *et al.*, 2006). Damage to the inner ear (cochlea) or the nerve pathways from the inner ear to the brain results in the condition known as sensorineural HI, the most common type of permanent HI that cannot be treated through surgery. Patients with sensorineural HI cannot hear any sound at all, or the sounds they do hear are unclear or muffled. Middle ear infections, excessive noise, inappropriate use of certain drugs, and problems during childbirth have all identified as some of the factors that cause sensorineural HI (Smith *et al.*, 2005). Mixed

HI is the co-occurrence of both sensorineural and conductive impairments in the same ear. This condition may be caused by multiple factors or a single factor and may result in a reduction in perceived sound levels or inability to hear any sound (Amedofu *et al.*, 2006).

HI that presents before a child develops speech is referred to as pre-lingual HI, which is most commonly congenital (present at birth). In general, children develop speech at one year of age; hence any HI that develops in their first year is pre-lingual. Post-lingual HI, on the other hand, occurs after an individual develops normal speech (Lebeko *et al.*, 2015; Shan *et al.*, 2010). The major causes of pre- and post-lingual HI can be grouped as genetic (inherited) or acquired (caused by environmental factors e.g. an illness or injury). Over 50% of congenital HI is caused by genetic factors with autosomal recessive HI being the most frequent cause of the condition (Lebeko *et al.*, 2015; Shan *et al.*, 2010). Acquired post-lingual HI is usually caused by accident or noise pollution, and the genetic factors that cause post-lingual HI are mostly inherited in the autosomal dominant mode.

The genetic etiology of HI consists of many mutations that occur in genes that control the components of the human auditory system. Several facts about NSHI have been proven over time regardless of population: [1] Half of congenital HI cases have a genetic etiology, of which 70% are non-syndromic (Gorlin *et al.*, 1995; Wonkam *et al.*, 2013); [2] for non-syndromic (NS) HI, 77% of the cases are of autosomal recessive (AR) inheritance, 22% display autosomal dominant (AD) inheritance, ~1% are X-linked, and <1% are mitochondrial (OMIM, 2017); [3] more than 1,000 NSHI genes may remain to be identified based on diseases associated with HI and unique inner ear transcripts (Hertzano & Elkon, 2012). To date, ~170 NSHI loci have been mapped and 98 genes identified (Van Camp G & Smith, 2020). In many populations of European and Asian

descent, pathogenic variants in *GJB2* (connexin 26 gene) and *GJB6* are a major contributor to autosomal recessive NSHI (ARNSHI) (Chan & Chang, 2014), however, the prevalence of *GJB2*- or *GJB6*-related NSHI is practically zero in most sub-Saharan African populations, and little is known about the contribution of other known NSHI genes to HI (Bosch *et al.*, 2014b; Javidnia *et al.*, 2014; Lasisi *et al.*, 2014; Lebeko *et al.*, 2015)

From 1995, when the first HI gene was identified (Vona *et al.*, 2015), many genes involved in congenital HI have been identified across the world (Robson, 2006). Over 141 non-syndromic HI loci have been identified and published in peer-reviewed journals (Vona *et al.*, 2015). The common and most frequently identified NSHI genes used in diagnosing genetic HI are *GJB2*, *GJB6*, *SLC26A4*, and *OTOF*, these genes code for transport, synaptic, cytoskeleton, and ion homeostatic gap junction proteins (Chan & Chang, 2014). The gap junction protein beta- 2, 6, or alpha 1 genes (*GJB2*, *GJB6 or GJA1*) code for a family of proteins (connexins) which, by oligomerization, form transmembrane channels in vertebrates. These channels are referred to as connexons and are intercellular communication pathways, that are made up of gap junction channels formed directly between neighboring cells. A connexon is responsible for transporting potassium ions and some small molecules between cells. *GJB2*, *GJB6*, and *GJA1* sequences are highly conserved: their coded proteins consist of extracellular loops protruding from a middle cytoplasmic loop, and N- and C-terminal cytoplasmic ends are separated by 4 transmembrane domains (Chan & Chang, 2014).

Mutations in the *GJB2* and *GJB6* genes have been implicated as the major causes of NSHI, accounting for up to 50% of cases in populations of European and Asian descent. The most prevalent mutation in Europe and the Middle East was found to be 35delG

(Gasparini *et al.*, 2000; Norouzi *et al.*, 2011). The most prevalent mutations were *GJB2* 235delC and V37I in East and South East Asia respectively while the W24X mutation was most prevalent in India (Chan & Chang, 2014). However, there is a limited contribution from *GJB2* and *GJB6* genes to HI among people of African descent. Indeed, the prevalence of *GJB2*- or *GJB6*-related NSHI in several sub-Saharan populations (e.g. Cameroon in Central Africa (Bosch *et al.*, 2014b), Kenya (Gasmelseed *et al.*, 2004) and Uganda (Javidnia *et al.*, 2014) in East Africa, Nigeria in West Africa (Lasisi *et al.*, 2014), and in African populations in South Africa (Bosch *et al.*, 2014a; Kabahuma *et al.*, 2011) was zero, while *GJB2* C35delG (MAF 6.0%) is more common in Sudan (Gasmelseed *et al.*, 2004). The prevalence of *GJB2*- or GJB6-related NSHI is also rare among African Americans (Morell *et al.*, 1998; Shan *et al.*, 2010).

Although different studies from Ghana have reported on the burden, etiology and genetics of HI, there is currently no extensive review in the subject area that covers the public health burden of the condition. In this paper, we describe a systematic review of published articles on HI in Ghana to determine the reported burden of the condition, etiological agents, and the different types of reported HI. Even though this report is a case study, it is of relevance towards understanding the public health burden, and various genetic and environmental causes of HI in Ghana.

3.3. Methods

A literature search was conducted by the authors from December 2016 to March 2017, covering the literature from 1973 to 2017. We used PubMed (National Library of Medicine), Medline, and Google scholar. Keywords included the individual use or a combination of the following: “hearing loss” OR “hearing impairment” OR “deafness” AND “Ghana”, this strategy was used to search for publications in PubMed and Google

Scholar to obtain a comprehensive but broad review of the literature on the study of HI in Ghana. Additionally, specific expert authors' names active in the field of HI were also used to complement the literature searches. Prior knowledge of research groups working on HI, in Africa generally and Ghana specifically, further facilitated the identification and selection of research articles. Only available full-length articles, in English, were selected. In cases where multiple studies reported a similar result, the most recent report with the most detailed studies was included. The main search was conducted by a Ph.D. student in Human Genetics, reviewed by an expect in medical/human genetics and an ear, nose, and throat (ENT) specialist.

A total of 5869 articles were initially recovered. Successive elimination was performed based on article title and its relevance to the scope of the review (Figure 3.1). The criteria below were used to screen the titles and abstracts of these articles, and 18 were selected for the review.

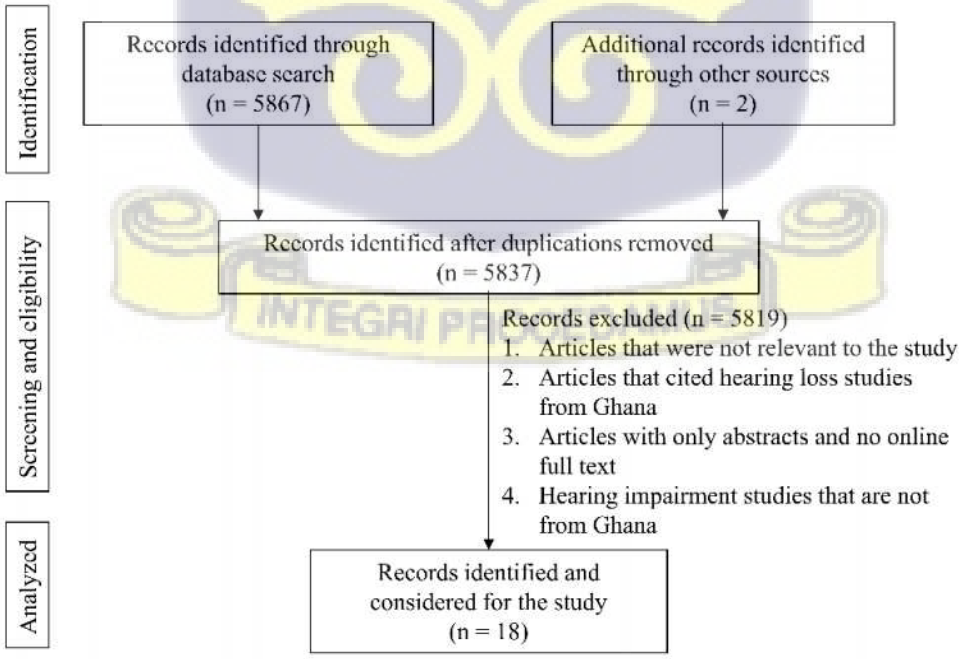

Figure 3.1: Flow diagram for articles selection. *Although it was part of the protocol to exclude articles without full text, no article was excluded based on the availability of full.*

Inclusion criteria: Original research article**,** Report on HI or deafness in Ghana;

Exclusion criteria: HI studies that are not about the Ghanaian population**;** studies that used noise levels to predict the risk of developing HI without measuring the degree of HI of any of the study participants.

3.4. Results

3.4.1. The burden of hearing impairment in Ghana

Many researchers have studied the prevalence of hearing loss in Ghana based on regions of the country (Figure 3.2). Although the majority of these studies were not population based and/or did not have a nationwide coverage, they gave an idea of the burden HI in Ghana. In January 1999, a prevalence study was carried out in a Ghanaian village called Adamarobe that was known to have a high number of HI individuals. This study identified 45 deaf people in 14 families based on their physical examination and family history (Amedofu *et al.*, 1999). Otoscopy and audiometric evaluation from the study also revealed that only seven out of 30 people enrolled in the study had a total loss of hearing while the remaining 23 had residual hearing at the low and middle frequencies. The incidence of HI in this village was calculated as 23.7 per 1000 when a hearing level of greater than 25 dB was used (Amedofu *et al.*, 1999).

A report from Komfo Anokye Teaching hospital (KATH) stated that there is an overall increase in the number of patients (from 3.7% to 15.5%) with hearing loss who visited the hospital between 1999 to 2004 (Amedofu *et al.*, 2006). According to the researchers, the increased number of hearing-impaired patients was due to awareness about the Hearing Assessment Center in Kumasi (Amedofu *et al.*, 2006). Awuah and his co-workers in the year 2006 screened 268 patients suffering from different forms of ear,

nose, and throat (ENT) disease who visited the Komfo Anokye Teaching hospital (KATH). They enrolled 188 of patients of which 51 were diagnosed of acute otitis media (AOM). The prevalence of HI among the AOM patients was calculated as 91.3%; thus 37 out of the 51 patients were further diagnosed of HI (Awuah *et al.*, 2012). In Accra, there was a similar study where 66.3% (474 out of 715) patients who visited the Korle-Bu Teaching Hospital (KBTH) in the year 2013 were screened and diagnosed of HI (Nyako, 2013).

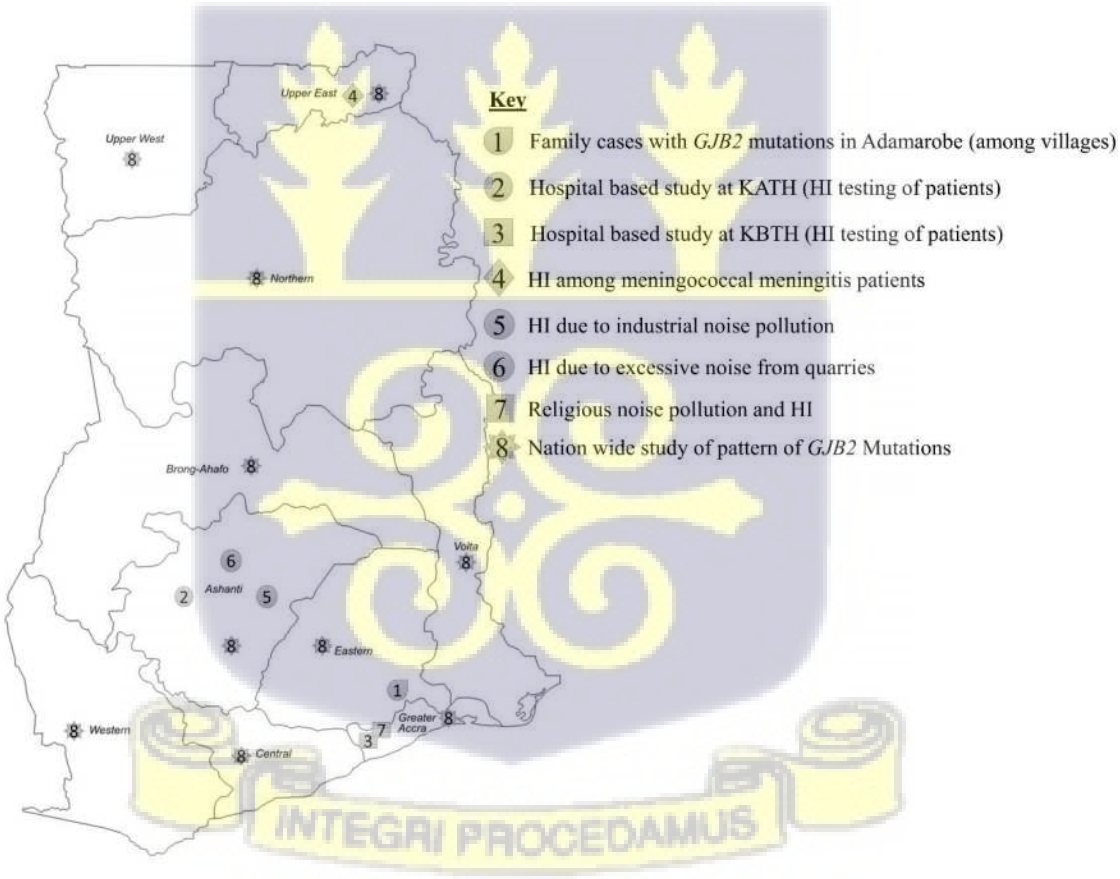

Figure 3.2: Geographical representation of the major studies on hearing impairment from Ghana

The 18 articles we reviewed in this study highlighted a similar pattern of HI across age groups, where younger patients had a mild form of HI and the older patients had moderate to severe HI (Amedofu *et al.*, 2006; Nyako, 2013). The data suggested that HI worsened with increasing age in the Ghanaian population and this finding is consistent with other studies across the globe (Stevens *et al.*, 2013). The age group 60 years and

above visiting KBTH in the year 2013 were reported to have the highest prevalence of HI (Nyako, 2013).

3.4.2. Types of hearing loss identified in Ghana

HI in Ghana was categorized by most researchers as conductive, sensorineural, or mixed. Conductive audiometry tests were performed for 23 people from Adamarobe, a village in Ghana: 17 out of the 23 had hearing loss in the better ear. Three (3) people had moderate sensorineural hearing loss while 14 had mild sensorineural hearing loss (Amedofu *et al.*, 1999). The bone conductive audiometry test, a test used to distinguish between the different types of HI, applied in recent studies has shown that more Ghanaians reported to the hospital with sensorineural HI than the other types of HI (Amedofu *et al.*, 2006; Nyako, 2013). Bilateral HI was predominant among patients with hearing problems, however, some patients had unilateral HI (Nyako, 2013). The most common audiometric configuration of HI was mild HI for both ears (Amedofu *et al.*, 2006; Nyako, 2013).

3.4.3. Some causes of sensorineural HI in Ghana

Factors such as noise, meningococcal meningitis, complicated malaria, presbycusis, mumps and Meniere's disease were identified as the major causes of sensorineural HI (Amedofu *et al.*, 2006). A major sequela of meningococcal meningitis is HI. After a two-year epidemic in northern Ghana, 696 patients who survived the condition were screened for HI. A reduced hearing capacity was reported in 6% of the patients with 1.6% having severe and profound HI in their worse ear (Hodgson *et al.*, 2001).

Recent studies in the field of HI in Ghana tried to identify malaria and sickle cell disease as possible causes of HI in children. The results of a cross-sectional study to determine the role of sickle cell disease in causing HI had only one patient out of 35 sickle cell

affected children who failed an oto-acoustic emissions test. The results, therefore, suggest that early HI does not occur frequently in sickle cell disease (Kegele *et al.*, 2015). Sickle cell disease is not a likely cause of HI or delayed speech in children.

Severe malaria was suggested to influence the function of the inner ear in children, however, the loss of hearing caused by malaria is reversed after treatment. Out of 144 children who had severe malaria, 58 (~40%) failed otoacoustic emission tests, suggesting the development of HI (Schmutzhard *et al.*, 2015).

Noise exposure is another major cause of non-genetic HI in Ghana. The noise generated by gold mines, quarries, mills, and other noisy industrial areas are usually far above normal levels thus predisposing workers to the risk of acquiring HI. Research has proven that the risk of developing HI increases with noise exposure time (Amedofu, 2002). In a surface mining company in 2012, 23% (59 out of 252 workers) were diagnosed with HI (Amedofu, 2002). In a similar study, 818 sawmill, corn mill, and printing press workers were examined for HI. The results of this study showed that 23%, 20% and 7.9% of corn mill, sawmill and printing press workers respectively had evidence of HI. The level and duration of noise produced by the various occupations correlated significantly with development of HI (Boateng & Amedofu, 2004). The contribution of excessive noise from quarries in the Ashanti region of Ghana to the development of HI was evaluated in between April to June 2012, and empirical evidence was found to support the claim that excessive noise generated by the quarries caused 56% (224/400) of the workers to develop a hearing problem. The degree of hearing loss correlated positively with duration of work (Boateng and Amedofu, 2004). Steel/metal workers and communities saturated with a lot of religious noise in Ghana were also reported to be at higher risk of developing HI (Zakpala *et al.*, 2014). Zakpala and his co-workers after, careful examination of noise

generated in Ghana, stated that the night-time noises generated by religious bodies were far higher than the levels recommended by the Environmental Protection Agency of Ghana (Zakpala *et al.*, 2014). Empirical evidence was provided from the quarries (Gyamfi *et al.*, 2016) and market mills (Kitcher *et al.*, 2014) demonstrated that Ghanaians working in these sites are exposed to noise that exceeds tolerable thresholds; hence, the workers at these sites developed noise-induced HI, especially among the elderly and long serving workers.

The false perception of sound in the absence of acoustic stimulation in the environment is known as tinnitus. Tinnitus is associated with age, exposure to noise, ototoxicity, tumor, and damage to the acoustic portion of the eighth cranial nerve. The prevalence of tinnitus in Ghana was estimated to be 19.3% among patients visiting the Komfo Anokye Teaching Hospital (KATH) (Awuah, 2012). The majority of patients studied by Awuah in 2012 had normal hearing, however, patients with mild sensorineural hearing loss had more tinnitus than those with other degrees of hearing loss. Similar observations were made by researchers in Britain (Davis, 1989), USA (Henry, Dennis, & Schechter, 2005; Shargorodsky, Curhan, & Farwell, 2010), Sweden (Widén & Erlandsson, 2004), and other parts of the world.

3.4.4. Genetics of HI in Ghana

Genetic defects are responsible for more than 50% of all pre-lingual, sensorineural HI (Schade *et al.*, 2003). Hearing is mainly affected by the inner ear sensory hair cells, and gene mutations in these cells can result in improper functioning of the cells that may lead to HI at birth or later in life. Common mutations have been found on chromosome 13q11 in the *GJB2* gene that encodes connexin 26 (CX26). CX26 plays an important role in the formation of gap junctions for the intercellular exchange of electrolytes. Globally,

different types of mutations in *GJB2* gene have been identified as a cause of inherited pre-lingual HI; 35delG is the most common mutation, with about 2.5% carrier frequency (Schade *et al.*, 2003). These mutations can be inherited in an autosomal dominant, autosomal recessive, X-linked recessive or mitochondrial inheritance fashion.

In Ghana, the first study to associate HI to genes was published in 1998: Brobby et al. examined several families in a village in the Eastern region of Ghana that had a high prevalence of HI (Brobby *et al.*, 1998). The study screened and sequenced the coding region of the connexon 26 gene of 21 deaf subjects from 11 families. In the connexon 26 gene, the R143W mutation (T was replaced with C) was found at codon 143. A nationwide study was conducted in 2001 to identify *GJB2* mutations responsible for HI in Ghana. Among 365 unrelated individuals examined, 121 mutated chromosomes were identified, and 110 of them were found to carry the previously reported R143W mutation. The other *GJB2* mutations identified were L79P, V178A, R184Q, A197S, I203K, and L214P (Hamelmann *et al.*, 2001). In total, only 63 out of 365 unrelated individuals with evidence for profound congenital sensorineural HI had mutations in the *GJB2* gene. In 2003, Schade et al. also associated mutations in *GJB2* gene to cases of HI from Ghana.

3.5. Discussion

HI research in Ghana has received minimal attention highlighted by the limited number of publications in this field. There is no extensive review from Ghana on the subject of hearing loss; therefore, this review to the best of our knowledge is the first to extensively review the burden of hearing HI in Ghana.

Analysis of the results suggests an inconsistent report of the burden of HI in Ghana (Table 3.1) that does not correlate with the alarmingly high increase of the disease burden globally (Vos *et al.*, 2015). The inconsistency of the disease burden observed may be

due to the hospital-seeking behavior of the patients. In 2012, Awuah reported a high disease burden that was explained by the increased awareness and education on hearing loss after the establishment of the Kumasi hearing assessment center.

Table 3.1: Reports on the burden of hearing impairment in Ghana

Reference	Year of study	Study participants	Study site	Total number of study participants (N)	Participants living with HI (n)
(Awuah, 2012)	1995 to 1998	Patients attending Kumasi Hearing Assessment Centre	Ashanti Region	2207	1987 (90.0%)
(Amedofu *et al.*, 1999)	1999	Villagers in Adamarobe	Eastern Region		Incidence of 23.7/1000
(Brobby *et al.*, 1998)	1998	Families with HI in Adamarobe	Eastern Region	29	Genetics studies
(Amedofu *et al.*, 2006)	1999 to 2004	Patients attending KATH	Ashanti Region	6428	5734 (89.9)
(Hamelmann *et al.*, 2001)	2001	Unrelated individuals	All 10 regions	365	Genetics studies
(Amedofu, 2002)	2002	Workers in a surface gold mining company	Ashanti Region	252	59 (23.4%)
(Boateng & Amedofu, 2004)	2004	Industrial workers	Ashanti Region	818	416 (50.8)
(Awuah *et al.*, 2012)	2005 to 2006	Patients attending KATH	Ashanti Region	268	51 (19.0%)
(Nyako, 2013)	2012	Patients attending KBTH	Greater Accra Region	715	621 (86.9%)
(Gyamfi *et al.*, 2016)	2012	Quarry Workers	Ashanti Region	400	240 (60.0%)
(Kitcher *et al.*, 2014)	2014	Market mill worker	Greater Accra Region	204	32 (0.13%)
(Kegele *et al.*, 2015)	2015	Children with sickle cell disease	Ashanti Region	35	1 (0.03%)

Six out of 18 studies were either social science or environmental science studies that were relevant for this study.

The studies examined in this review reported HI to be prevalent in people working in noisy environments compared to their respective control groups. The industry-based

studies mostly screened for HI among the high-risk populations, and subsequently reported a high prevalence of HI that fluctuated depending on the industry.

In Ghana, the majority of HI studies recruited participants from two major cities (Accra and Kumasi) (Table 3.1); making it difficult to estimate the national prevalence of HI. The studies from Kumasi and Accra suggested that more women report to the hospitals and hearing facility with HI than men, however, only a few of the studies reported the burden of the condition with respect to gender. In addition to the minimal information on gender in the two cities, the results from the study sites cannot be extrapolated to determine the national prevalence of HI based on gender. It is therefore important to assess the burden of HI across all the regions in Ghana in order to determine the true reflection of the disease burden so as to accurately examine the public health impact of the disease and also make an informed decision in terms of national policies.

Mutations in *GJB2*, *GJB6*, and *GJA1* are not the major causes of NSHI among Africans and people of African descent (Lebeko *et al.*, 2015; Wonkam *et al.*, 2015), however, studies that considered HI genes in Ghana were only focused on *GJB2* mutations. Although the study by Hamelmann et al., in 2001 associated *GJB2* mutations to HI in Ghana, only 63 out of 365 unrelated individuals with evidence for profound congenital sensorineural HI had mutations in their *GJB2* gene. This suggests that *GJB2* mutations fully cannot explain the genetic etiology of HI in Ghana; hence, there exists a need to identify other HI genes in Ghanaian patients. We believe that the most promising approach to discover novel HI genes in Africa is through the "OMICS" approach which includes whole (exome) genome analysis on Next Generation Sequencing (NGS) platforms (Lebeko *et al.*, 2015) which are rapidly replacing older techniques.

The most recent approaches to human genomics target ‘‘personalized medicine,’’ ‘‘precision medicine,’’ and ‘‘stratified medicine’’ which separates people into different groups for tailored intervention/treatment (De Andrés *et al.*, 2016; Vos *et al.*, 2016). In order for Ghana, and Africa as a whole, to contribute to the advances in the field of genomics of HI, research on the continent should focus on identification of the major causes, novel genes, and the molecular mechanisms of HI pathogenesis with the aim of developing novel diagnostics and therapeutics for the disease. It is therefore important to employ the “OMICS” approach (genomics, proteomics, transcriptomics, etc.) in HI research on the continent.

3.6. Future directions

The most effective way to control and manage the increasing global burden of HI is to develop an effective diagnosis, treatment, and preventive measures. It has been estimated that over 50% of all HI cases could have been prevented (Wonkam *et al.*, 2013). The following have been identified as future focus areas to effectively manage the disease in Ghana.

1) Identification of the major causes of HI in Ghana: in Ghana, occupational noise and noise pollution are reported as the major causes of HI among adults (Boateng & Amedofu, 2004; Gyamfi *et al.*, 2016; Kitcher *et al.*, 2014) and fever, presbycusis, meningitis and Meniere's diseases were identified at all ages (Amedofu et al., 2006). The major etiology of HI among Ghanaian children is not well established, and therefore more studies must focus on identifying the major causes of HI among the different occupation, sex and age categories.

2) Understanding the mechanism of pathogenesis of HI: to effectively treat and prevent the disease, the molecular mechanisms underlining its pathogenicity must be elucidated.

Over 50% of pre-lingual non-syndromic HI is caused by genetic factors (Wonkam *et al.*, 2013). However, the genetics of HI has not been well studied in Ghana, hence the gap in understanding the contributions of hearing mutant genes to HI. It is also not clear how environmental factors and diseases such as measles cause HI. It is therefore important for future research to focus on elucidating the mechanisms of the pathogenicity of HI in Ghana.

3) Developing effective diagnostics and treatment remedies for HI: future research needs to be focused on developing point-of-care diagnostics for screening newborn babies and rural dwellers for HI. In the field of research to develop global health diagnostics and therapeutics in developing countries, the multi-OMICS technologies described in a recent study (Fang *et al.*, 2016) are useful in the early detection, effective treatment, and management of the disease.

3.7. Caveats of the present review and analysis

The study could not estimate the national prevalence of HI in Ghana because of the limited number of publications on the subject. The retrieved publications from Ghana were mostly hospital-based or industry-based and did not reflect the national burden of the disease.

3.8. Conclusions

The burden of hearing impairment in Ghana was typically studied around the central (Kumasi) and coastal belts (Accra) of the country. A number of these studies were hospital-based, patients who visited the hospital were screened for HI, and the others were industry-based, to determine the role of noise pollution on acquiring HI. There was only a single nationwide study, which identified mutations in the *GJB2* gene that could be responsible for HI. Across the country, the HI cases recorded can be grouped into two

main categories: inherited and acquired HI. The acquired HI is more prevalent in adults than children and female than male. Even though the prevalence of HI in Ghana had been studied at different locations within the country, there is no nationwide study and so the national prevalence of HI remains unknown.

Noise pollution, infectious diseases, and genetic factors were the major causes of HI identified in our study. All the major causes of HI identified could be prevented by (1) the use of the appropriate working apparel for those who work in noisy environments, (2) proper vaccination and treatment of childhood diseases, and (3) identifying HI genes for developing effective diagnostics for neonatal screening for deafness in Ghana. The studies on the genetics of hearing loss in Ghana focused on *GJB2* mutations, hence, there is a need to conduct massive next-generation sequencing of the genomes of HI patients to identify other HI gene mutations.

3.9. Long-term views

There are no extensive studies on the classification, burden, and genetic etiology of HI in Ghana. HI research in the next five years should be aimed at:

1. Completing a national study to determine the prevalence of HI in Ghana and also classify the HI cases in Ghana
2. Generating and analyzing whole-exome genomic data of HI patients to generate a comprehensive list of HI genes and predominant HI gene mutations in Ghana.
3. Studying the molecular mechanism of pathogenicity of HI gene mutations in Ghana.

3.10. Key issues

1. Conductive HI occurs more frequently in children reporting with all kinds of otitis media; sensorineural and bilateral hearing loss were the common types of HI among Ghanaians.

2. Excessive noise, and mutations in *GJB2* gene were identified as the major cause of HI in Ghana.

3. Post-lingual HI progress with age and the degree of exposure to noise.

3.11. Author's Contributions

Conceived and designed the experiments: S.M.A. and A.W. Performed the literature search: S.M.A. and A.W. Analyzed the data: S.M.A., G.A., G.K.A., and A.W. Revised and approved the article: S.M.A., G.A., G.K.A., and A.W.

CHAPTER FOUR

4.0: Paper 2: *GJB2* and *GJB6* mutations in non-syndromic childhood hearing impairment in Ghana

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4.1. Abstract

Our study aimed to investigate *GJB2* (connexin 26) and *GJB6* (connexin 30) mutations associated with non-syndromic childhood hearing impairment (HI) as well as the environmental causes of HI in Ghana. Medical reports of 1104 students attending schools for the deaf were analyzed. Families segregating HI, as well as isolated cases of HI of putative genetic origin were recruited. DNA was extracted from peripheral blood followed by Sanger sequencing of the entire coding region of *GJB2*. Multiplex PCR and Sanger sequencing were used to analyze the prevalence of *GJB6*-D3S1830 deletions. Ninety-seven (97) families segregating HI were identified, with 235 affected individuals, and a total of 166 isolated cases of putative genetic causes, were sampled from 11 schools for the deaf in Ghana. Environmental factors, particularly meningitis, remain a major cause of HI impairment in Ghana. The male/female ratio of the participants in our study was 1.49 and only 59.6% of the patients had their first comprehensive HI test between 6 to 11 years of age. Nearly all the participants had sensorineural HI (99.5%; $n = 639$), and the majority of cases had pre-lingual HI (68.3%, $n = 754$), of which 92.8% were

congenital. Pedigree analysis suggested autosomal recessive inheritance in 96.9% of the familial cases. The *GJB2*-R143W mutation, previously reported as a founder mutation in Ghana, accounted for 25.9% (21/81) in homozygous state in familial cases, and in 7.9% (11/140) of non-familial non-syndromic congenital HI cases, of putative genetic origin. In a control population without HI, we found a prevalence of *GJB2*-R143W carriers of 1.4% (2/145), in a heterozygous state. No *GJB6*-D3S1830 deletion was identified in any of the HI patients. The *GJB2*-R143W mutation accounted for over a quarter of familial non-syndromic HI in Ghana and should be investigated in clinical practice, whereas the large connexin 30 gene deletion (*GJB6*-D3S1830 deletion) does not account for any congenital non-syndromic HI in Ghana. There is a need to employ Next Generation Sequencing approaches and functional genomics studies to identify other genes involved in most families and isolated cases of HI in Ghana.

Key Words: Hearing Impairment; Genetics; *GJB2 and GJB6;* Ghana; Africa

4.2. Introduction

Hearing impairment (HI) is a disabling congenital disease (Neumann *et al.*, 2019), with a high rate of age-standardized years lived with disability (Murray *et al.*, 2015; Vos *et al.*, 2016). Globally, congenital HI has a prevalence of 1.3 people affected in every 1000 (James *et al.*, 2018). It accounts for about 1 per 1000 live births in developed countries, but has a much higher rate of up to 6 per 1000 in sub-Saharan Africa (Olusanya *et al.*, 2014). To improve the cognitive, social, speech, and language development of children living with HI, early diagnosis and intervention is recommended (Barnard *et al.*, 2015), but in the absence of the widely used new-born screening, the age at diagnosis is usually late in Africa, (3.3 years in Cameroon (Wonkam *et al.*, 2013)). In many populations, nearly half of congenital HI cases have a genetic etiology, of which 70% are non-syndromic (Bademci *et al.*, 2016; Sheffield & Smith, 2019) and, among these, nearly

80% of the cases are inherited in the autosomal recessive (AR) mode (Wu *et al.*, 2018; Zhou *et al.*, 2019)). To date, about 98 genes have been identified, in ~170 NSHI loci mapped (Hereditary Hearing Loss Homepage; http://hereditaryhearingloss.org/). Nevertheless, in many populations of European and Asian descent, pathogenic variants in *GJB2* (connexin 26 gene) and *GJB6* are major contributors to autosomal recessive NSHI (ARNSHI) (Chan and Chang, 2014), with the *GJB6*-D13S1830 deletion, identified in up to 9.7% cases studied, as the second biggest genetic etiology of non-syndromic deafness in European populations (del Castillo *et al.*, 2003; del Castillo *et al.*, 2002).

The prevalence of *GJB2*- or *GJB6*-related NSHI is very low in most sub-Saharan African populations (Bosch *et al.*, 2014a; Gasmelseed *et al.*, 2004; Javidnia *et al.*, 2014; Kabahuma *et al.*, 2011; Lasisi *et al.*, 2014). A previous study has shown that a common founder mutation p.R143W (p.Arg143Trp), accounted for about 16.2% of congenital HI in a random sample of Ghanaians affected by hearing loss (Hamelmann *et al.*, 2001). To our knowledge, the contribution of connexin 30 to HI, and the carrier frequency of the *GJB2* mutation in non-affected individuals have not been studied in Ghana (Adadey *et al.*, 2017). In the present study, we aimed to investigate the putative environmental causes of childhood HI, and revisit the contribution of *GJB2*, and to investigate *GJB6* mutations, in carefully selected samples of families segregating HI, samples of isolated cases with putative genetic origin, and samples from our control population of Ghanaian residents not affected by HI.

4.3. Methods

4.3.1. Ethical Approval

The study was performed in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Noguchi Memorial Institute for Medical Research

Institutional Review Board, University of Ghana, Accra (NMIMR-IRB-CPN 006/16-17 revd. 2018), and the University of Cape Town's Human Research Ethics Committee, reference 104/2018. Written informed consent was obtained from all participants, if they were 18 years or older, or from the parents/guardians with verbal assent from children, including permission to publish photographs.

4.3.2. Patients' participants

Hearing impaired patients (97 familial cases and 166 isolated cases) were recruited from 11 schools for the deaf following procedures reported previously in Cameroon (Wonkam *et al.*, 2013), part of which state that individuals with severe HI diagnosed before 15 years of age only should be recorded. For all participants, detailed personal and family histories were obtained, the medical records were reviewed by a medial geneticist and an ENT specialist; relevant data including three-generation pedigree and perinatal history was extracted. If required, a general systemic and otological examination, and audiological evaluation was performed, including pure tone audiometry or an auditory brain stem response test. We followed the recommendation number 02/1 of the *Bureau International d'Audiophonologie (BIAP*), Belgium, to classify the hearing levels (BIAP, 1997; Wonkam *et al.*, 2013). After consultation with the medical geneticist, individuals with syndromic deafness underwent additional assessment, when possible. As previously reported (Wonkam *et al.*, 2013), HI was defined as 1) acquired, when associated with a putative environmental factor such a clinical evidence of meningitis; 2) genetic, when at least two cases were reported in the same family without obvious environmental cause, when consanguinity was present, when dysmorphism or developmental problems in addition to HI where present, or when a well-defined syndrome was clinically suspected; 3) of unknown etiology, if neither an environmental nor a genetic origin were clearly established.

4.3.3. Controls participants

A total of 145 control participants without any personal or familial history of HI were randomly recruited in Ghana, from an apparently healthy subset of a tuberculosis screening study sample.

4.3.4. Molecular methods

Peripheral blood was used for genomic DNA extraction, following instructions from the manufacturer [QIAamp DNA Blood Maxi Kit. ® (Qiagen, USA)], in the Laboratory of the Department of Biochemistry, University of Ghana, Accra, Ghana.

Previously reported, primers for the *GJB2* genes were evaluated using BLAST® and web-based primer analysis software such as the Intergratd DNA Technologies (IDT) OligoAnalyzer (Bosch et al., 2014). The entire coding region of *GJB2* (exon2) was amplified, followed by sequencing using an ABI 3130XL Genetic Analyzer® (Applied Biosystems, Foster City, CA), in the Division of Human Genetics, University of Cape Town, South Africa. The details of the molecular methods used are written in appendix1.

Detection of *GJB6*-D13S1830 was performed using the method and primers described in (del Castillo *et al.*, 2003; del Castillo *et al.*, 2002). The entire coding region of *GJB6* was amplified using the method described by (Chen *et al.*, 2012).

4.3.5. Data analysis

Descriptive statistic and non-parametric tests were used for comparisons. Table and graphs were used to represent the descriptive data collected. The difference between the reported cases of prelingual and post-lingual HI were compared using T-test. The level of significance was set at 5%.

4.4. Results

4.4.1. Sex, age of onset of hearing impairment

A total of 1104 participants were evaluated (Figure 4.1). The male/female ratio was 1.49 (660/444). More than deaf participants (59.6%) had their first comprehensive HI medical test between the ages 6 to 11 years (Figure S4.1A). The median age of the students at the first medical diagnosis was 9 years, within a range of 2 to 22 years. The majority had pre-lingual HI (68.3%, *n* = 754; Figure S4.1B), of which 92.8% were congenital.

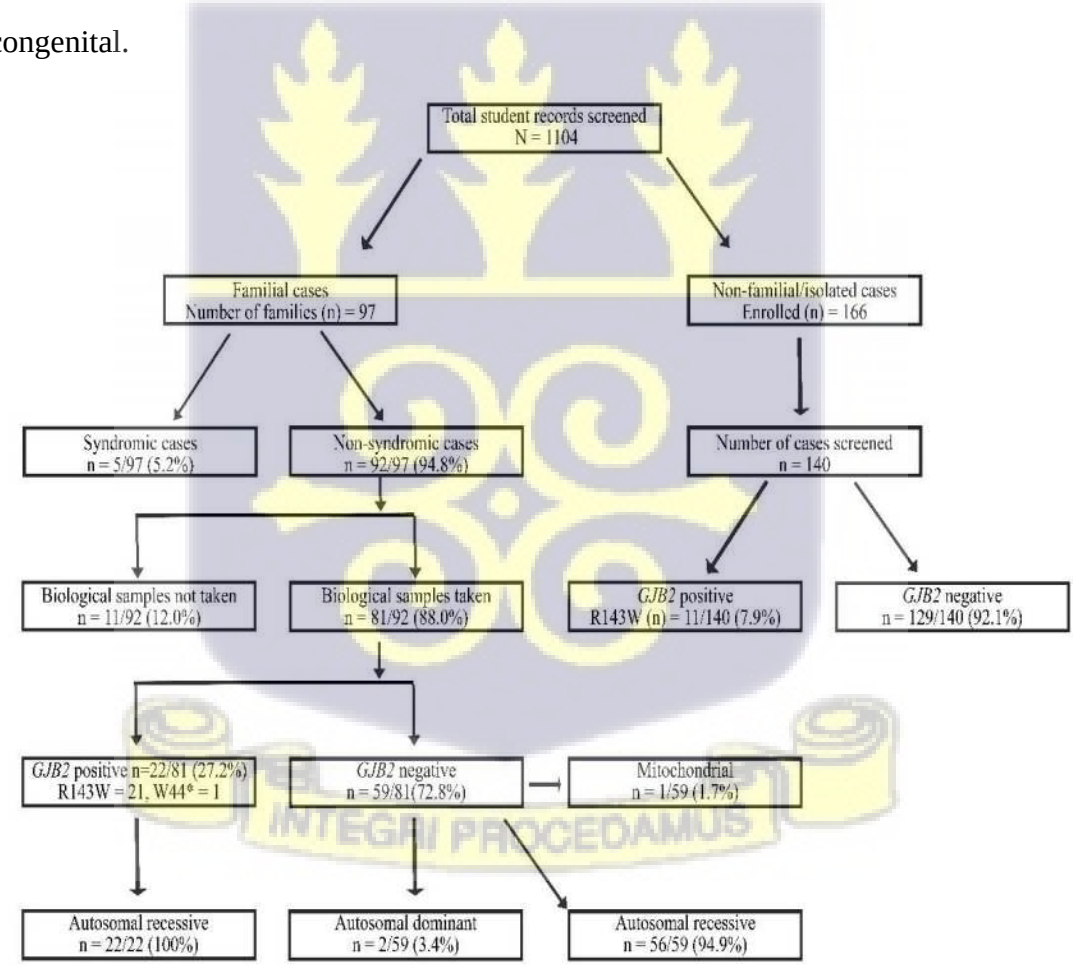

Figure 4.1: Flowchart of the recruitment and molecular analysis of Hearing Impairment cases in Ghana. *GJB2-R143W mutation, previously reported as a founder mutation in Ghana accounted for 27.2% (22/81) of familial, and 7.9% (11/140) of non-familial, non-syndromic congenital HI cases.*

4.4.2. Audiometric characterization of HI

Analysis of the students' medical data indicated that 642 out of the 1104 students had a comprehensive HI test (otoscopic ear examination, pure tone audiometry and/or tympanometry), the characteristics of which are described in Table 4.1. Nearly all the participants had sensorineural HI (99.5%; *n* = 639). Only 1 and 2 students had conductive and mixed HI respectively.

Table 4.1. Age at diagnosis and onset of HI.

Age of onset	**Number of cases, n (%)**
Prelingual (Before 2 years old)	754 (68.3%)
Perilingual (Between 2 and 4 years)	69 (6.3%)
Post-lingual (After 4 years)	281 (25.4%)
Total	1104
Age at first diagnosis	**Number of cases, n (%)**
0-5	157 (14.2%)
6-11	658 (59.6%)
12-17	258 (23.4%)
18-23	31 (2.8%)
Total	1104

4.4.3. Major etiologies of childhood HI in the study population

A flowchart for the cohort is shown in Figure 4.1, and the major causes of HI are displayed in Table 4.2. Convulsion (with undetermined medical cause) was the most common cause of post-lingual HI, followed by cerebrospinal meningitis (C.S.M.). Other diseases, such as cerebral/complicated malaria, otitis media, and mumps, were also reported as causes of post-lingual HI (Figure S4.2). Over 60% of the students had congenital HI of unknown origin (Figure S4.2).

4.4.4. Familial HI with possible patterns of HI inheritance

We identified 97 families segregating HI, in 21.4% of the students. In these families, 50.9% (235/461) of children were living with HI, with an average family size of 6.9. Most of these familial cases were non-syndromic (92/97). The pedigree analysis of the

non-syndromic familial cases suggested autosomal recessive inheritance in 96.7 % (89/92), with only 2 families exhibiting a pattern compatible with non-syndromic autosomal dominant inheritance. One family exhibited a mitochondrial pattern of inheritance.

Table 4.2. Comparison of our results to other studies in developing African countries.

Country	**Gambia**	**Nigeria**	**Sierra Leone**	**Ghana**	**Cameroon**	**Present Study**
Year of publication	**1985**	**1982**	**1998**	**1988**	**2013**	**2018**
Reference	(McPherson & Holborow, 1985)	(Ijaduola, 1982)	(Wright, 1991)	(Brobby, 1988)	(Wonkam *et al.*, 2013)	
Number of patients	**259**	**298**	**354**	**105**	**582**	**1104**
Hereditary	8.1%	13.1%	–	–	14.8%	21.3%
Meningitis	30%	11%	23.9%	8.5%	34.4%	3.9%
Measles	1.9%	13%	4.1%	30%	4.3%	0.9%
Rubella	1.5%	2%	–	3.8%	0.5%	0.2%
Mumps	–	3%	16.7%	3.5%	2.1%	0.5%
Ototoxicity	–	9%	20.8%	–	6%	-
Prematurity	–	–	–	–	0.9%	0.5%
Neonatal jaundice	–	5.7%	–	1.9%	1.4%	0.3%
Head trauma	–	–	–	–	0.3%	1.5%
Other illnesses	–	–	–	–	–	10.8%
Unspecified illness	–	–	–	–	–	6.3%
Unknown	54.4%	41.2%	34.8%	40%	32.6%	53.8%

Table 4.3: *GJB2* mutations among 365 previously studied patients and 97 Ghanaian families with profound sensorineural hearing impairment

		Number of affected individuals			
		Previously reported (Hamelmann *et al.*, 2001)	Our current report		
Nucleotide	Amino acid		Familial cases	Isolated/Non-familial cases	Controls
35 insG	frameshift	1(0.3%)	-	-	-
236T→C	L79P	1(0.3%)	-	-	-
427C→T	R143W	59 (16.2%)	21 (25.9%)	11 (7.9%)	2(1.4%)
533T→C	V178A	2 (0.6%)	-	-	-
551G→A	R184Q	1(0.3%)	-	-	-
589G→T	A197S	1(0.3%)	-	-	-
608TC→AA	I203K	1(0.3%)	-	-	-
641T→C	L214P	1(0.3%)	-	-	-
131G>A	W44*	-	1 (1.2%)	-	-

Waardenburg syndrome, an autosomal dominant condition, was the obvious syndromic and familial condition identified in 5.1% (5/97) of familial cases, with variable expression of heterochromia in affected members (Figure 4.2).

4.4.5. Molecular analysis result of *GJB2* and *GJB6*

A total of 81 families segregating NSHI were molecularly investigated. One individual from each family was sequenced for *GJB2* mutation, and we found a pathogenic mutation in 27.2% (22/81) of individuals with *GJB2*-R143W comprising the majority (21/22) in the homozygous state (Table 4.3); a *GJB2* p.W44* mutation was found in the remaining case in the homozygous state.

Figure 4.2 Probands with both Waardenburg syndrome, that associate variable degree of hearing impairment, and eyes/skin decoloration. *Panels (A) and (C) represent patients expressing the typical bilateral striking blue eyes phenotype of Waardenburg syndrome, while (B) and (D) represent asymmetrical heterochromia, with patients expressing the phenotype in only one eye.*

In non-familial non-syndromic cases, the *GJB2*-R143W mutation was found in 7.9 % (11/140) of patients (Figure 3.1). The control population contained two out of the 145 individuals with the *GJB2*-R143W mutation in a heterozygous state.

No *GJB6*-D3S1830 deletion was identified in the samples screened.

4.5. Discussion

The present report describes the most comprehensive study of the causes of childhood HI in Ghana. Moreover, we have investigated for the first time the prevalence of *GJB2* mutations in a non-HI-affected group of individuals from Ghana.

In this study, we observed HI in more boys than girls (p-value < 0.000), although gender has not been reported as an associated factor that predisposes children to the development of HI (Foerst *et al.*, 2006; Le Roux *et al.*, 2015). This may be due to the fact that more boys enrolled in schools for the deaf compared to girls, especially in resource-limited regions. In many cases, boys with disabilities have more access to formal education compared to girls (Groce, 1997; Nagata, 2003; Rousso, 2015). Although the "female protective model" is not common in HI studies, it has been proposed by some researchers to explain the higher prevalence of genetic disorders in males compared to females (Jacquemont *et al.*, 2014; Werling & Geschwind, 2015). According to this model, females have a higher rate of possible gene disruption but are frequently not associated with genetic disorders, compared to males (Jacquemont *et al.*, 2014).

HI screening aims to detect permanent HI at early developmental ages to facilitate appropriate intervention (Ma *et al.*, 2018; Sarant *et al.*, 2008). There is no universal newborn HI screening program in Ghana, which explains the late diagnosis, as most of the study participants have their first comprehensive hearing test at the school age, 6-9 years, however, parents/guardians of the children included in this study provided information on age of onset of the condition. The late age of diagnosis HI in Ghanaian children is partly tied to the limited number of hearing assessment facilities (Waller *et al.*, 2017). In addition, the majority of HI students we assessed were living in remote rural settlements often with unmarked roads and which limit access to quality health care.

Post-lingual HI in Africa is often caused by environmental factors (Wonkam *et al.*, 2013). Similar to other studies, complicated malaria, cerebrospinal meningitis, and convulsion (with undetermined cause) were identified by our study as major environmental factors that contribute to post-lingual HI in Ghana (Table 4.2). The mechanism by which these pathogens cause HI in the human is not fully understood. In meningogenic HI, the middle ear infection triggers inflammatory responses which is mostly mediated by tumor necrosis factor-alpha (TNF alpha). The pathogens have also been reported to cause damage to the structures of the inner ear which leads to HI (Du *et al.*, 2006). These environmental factors can be prevented by good health care systems as well as preventive health care practices. It is therefore important that governmental

policies are implemented to minimize childhood morbidities to reduce the prevalence of post-lingual HI.

Pre-lingual HI was common in our study population in accordance with other findings (Chibisova *et al.*, 2018). The majority of pre-lingual HI are congenital and are usually caused by genetic factors (Behlouli *et al.*, 2016; Wonkam *et al.*, 2013). Waardenburg syndrome was the most common syndromic HI identified among the congenital cases in line with other African studies (Noubiap *et al.*, 2014).

GJB2 mutations were investigated in Ghana 17 years ago, and the common founder mutation p.R143W (p.Arg143Trp) was identified (Hamelmann *et al.*, 2001). The present study revisited the contribution of *GJB2* mutations to HI in Ghana and confirmed the particularly high proportion of the founder mutation in more than ¼ of families segregating HI. This is much higher than what was previously reported (18%) due to the stringent selection of familial cases we used. There was also a relatively high proportion of *GJB2* mutations among isolated cases of putative genetic origin. This indicates the urgent need to implement *GJB2*-p.R143W testing in patients with HI during routine clinical practices in Ghana. The p.R143W mutation has also been reported in patients with HI in Japan (Kasakura-Kimura *et al.*, 2017; Zheng *et al.*, 2015), South Korea (Kim *et al.*, 2016b), and China (Luo *et al.*, 2017). In addition, we report a variant previously described as "Mayan": a founder *GJB2* nonsense mutation (p.W44*) in a Ghanaian family. The *GJB2* p.W44* mutation is the most common *GJB2* pathogenic variant in Guatemalan deaf populations, and was also reported in Mexico

(Martínez-Saucedo *et al.*, 2015). Ghana is an African exception as most studies in Africa have not identified *GJB2* mutations as a major causes of HI in sub-Saharan African populations (Lebeko *et al.*, 2015; Wonkam, 2015).

This is the first study to investigate the *GJB6*-D13S1830 mutation or *GJB6*-coding region variations in Ghana, and we found no mutation, a result which is in line with other African research (Bosch *et al.*, 2014a; Wonkam *et al.*, 2015). Equally, the *GJB6*-D13S1830 deletion was not found in populations from China (Jiang *et al.*, 2014), India (Padma *et al.*, 2009), Turkey (Tekin *et al.*, 2003), and among African Americans and Caribbean Hispanics (Samanich *et al.*, 2007), therefore, the present data further supports the hypothesis that the *GJB6*-D13S1830 deletion is a founder mutation (del Castillo *et al.*, 2003). These studies highlighted that data is urgently needed for other genetic causes of HI in Ghana (Adadey *et al.*, 2017).

Over a quarter of the familial HI cases were solved and found to have pathogenic *GJB2*-p.R143W variant which shared common haplotype with flanking polymorphisms (Shinagawa *et al.*, 2020). The study also indicates that more than 2/3 of families with HI were unsolved, and are eligible for next-generation sequencing, due to the highly heterogeneous genetic nature of NSHI and the low proportion of families successfully diagnosed using the single-gene approach applied in this study. Future research should either use high-throughput sequencing platforms to investigate known genes (Lebeko *et al.*, 2016; Shearer *et al.*, 2010), or whole-exome sequencing that will allow identification of novel genes (Diaz-Horta *et al.*, 2012). Indeed, based on the

identification of specific inner ear transcripts, it is estimated that more than 1,000 NSHI genes are still to be identified (Hertzano & Elkon, 2012).

To reduce HI incidence in Ghana, policymakers must consider integrating new-born screening for HI into the health care system such that every child is screened for both genetic and acquired HI. Early detection of the condition may lead to early interventions (Copley & Friderichs, 2010) which will eventually reduce the public health impact of this condition.

4.6. Conclusion

Our study has shown that environmental factors remain a major cause of HI in Ghana. The study confirms that Connexin 26 (*GJB2*) mutations are the most common cause of familial non-syndromic HI in Ghana, an exception in sub-Saharan Africa where the observed frequencies mutations in *GJB2* in HI patients is generally not relevant. The *GJB2* p.R143W founder mutation should be considered for implementation in clinical practice, particularly in newborn screening for HI, as it accounted for more than 25% of familial cases and close to 8% of isolated cases of genetic origin. The frequency of the *GJB2* p.R143W founder mutation in the general population without personal and family history of HI was relatively high: 1.4%. The study did not find any *GJB6*-D13S1830 deletions. Future studies should employ whole genome sequencing approaches and functional studies to identify other candidate genes involved in most families and isolated cases of HI in Ghana.

4.7. Author's contribution

Conceived and designed the experiments: GA, GKA, AW. Performed the experiments: SMA, OQ, NM, KM. Patients' recruitment, samples and clinical data collection and processing: SMA, GKA, Analyzed the data: SMA, AW; Contributed reagents/materials/analysis tools: GA, VN, CdK, AW. Wrote the paper: SMA, GA, VN, dK, AW. Revised and approved the manuscript: SMA, OQ, GA, GKA, KM, VN, CdK, NM, AW.

4.8. Supplementary Materials

Table S4.1: Categorization of HI based on degree of HI

		Number of students (%)	
Degree of HI	**Category**	**Left ear**	**Right ear**
Mild-Moderate	30-70 dB	2 (0.31%)	2 (0.31%)
Moderately-Severe	71-90 dB	6 (0.93%)	6 (0.93%)
Severe	91-100 dB	34 (5.30%)	37 (5.76%)
Severe-Profound	101-110 dB	28 (4.36%)	27 (4.21%)
Profound	>120 dB	572 (89.10%)	570 (88.79%)
Total		**642 (100%)**	**642 (100%)**

Table S4.2: Geographical distribution of GJB2 positive families in Ghana

	Number of families (n)

Region/location	*GJB2* positive	*GJB2* negative	Total
Greater Accra	1	5	6
Ashanti	5	10	15
Central	3	1	4
Eastern	7	20	27
Northern	1	2	3
Upper East	2	7	9
Volta	3	8	11
Upper West	0	6	6
Total	**22**	**59**	**81**

Figure S4.1: Onset and time of HI test. *(A) Age of deaf students at the first medical HI test. (B) Onset of HI. Paired T-test was used to compare the mean number of students with pre-lingual (n=754) and post-lingual (n=336) HI from 11 schools for the deaf. There was a significant difference between mean number of people with pre- and post-lingual HI with P value of 0.0001 (t=7.68, df=10).*

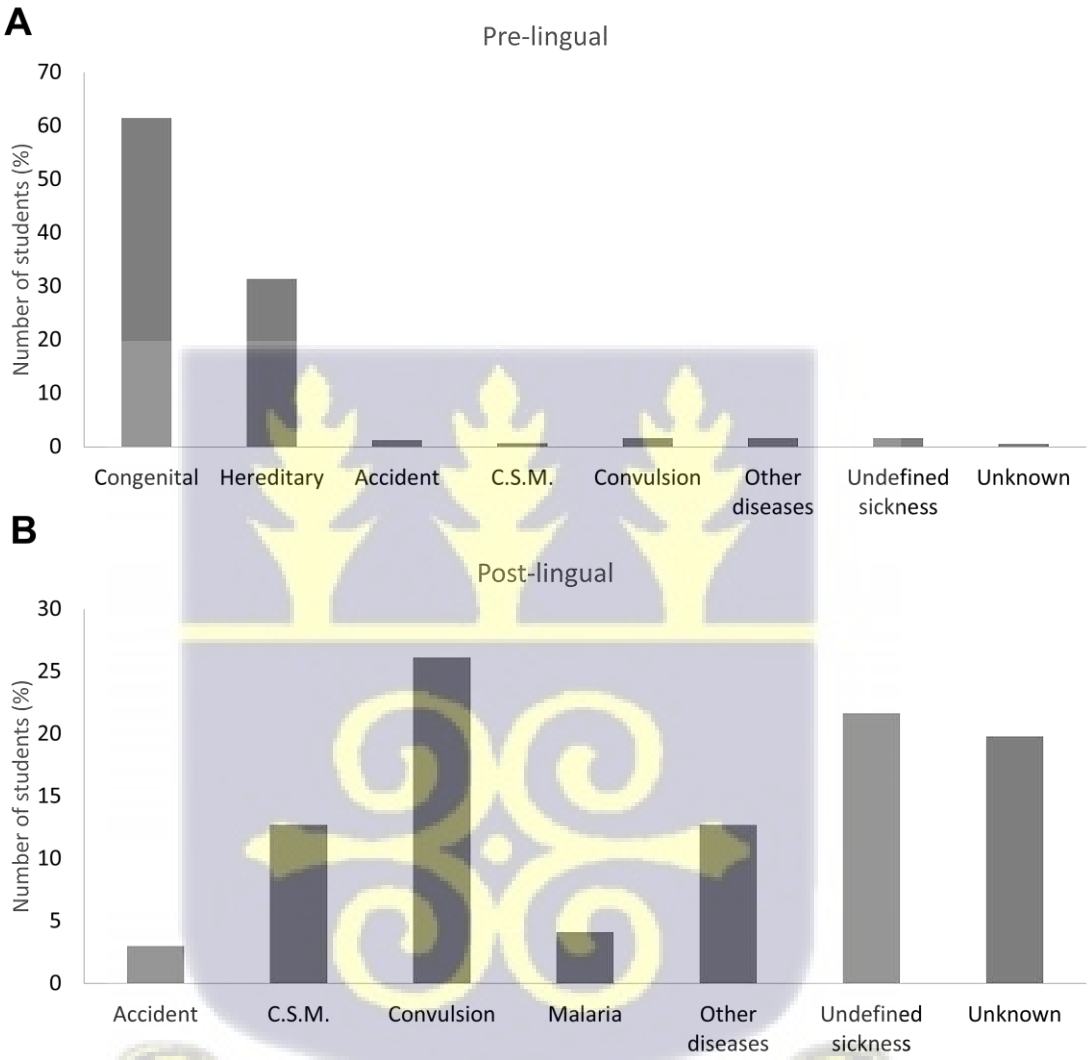

Figure S4.2: Major causes of childhood HI in Ghana. *(A) Major causes of Pre-lingual HI in Ghana. (B) Major causes of post-lingual HI in Ghana. Cerebrospinal meningitis was represented as C.S.M. The cause of HI labelled accident comprises of motor accidents and medical accidents such as wrong medication, childbirth and surgery. Diseases such as boil, anemia, Gilbertese, Jaundice, measles, mumps, Otitis media and rubella were captured as other diseases while undefined sickness consist of individuals who developed the condition due to sickness, but the cause of the sickness was not determined.*

CHAPTER FIVE

5.0. Paper 3: Enhancing Genetic Medicine: Rapid and Cost-Effective Molecular Diagnosis for a *GJB2* Founder Mutation for Hearing Impairment in Ghana

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5.1. Abstract

In Ghana, gap-junction protein β 2 (*GJB2*) variants account for about 25.9% of familial hearing impairment (HI) cases. The *GJB2*-p.Arg143Trp (NM_004004.6:c.427C>T/OMIM: 121011.0009/rs80338948) variant remains the variant most frequently associated with congenital HI in Ghana, but has not yet been investigated in clinical practice. We, therefore, sought to design a rapid and cost-effective test to detect this variant. We sampled 20 hearing-impaired and 10 normal-hearing family members from 8 families segregating autosomal recessive non-syndromic HI. In addition, a total of 111 unrelated isolated individuals with HI were selected, as well as 50 normal-hearing control participants. A restriction fragment length polymorphism (RFLP) test was designed, using the restriction enzyme NciI optimized and validated with Sanger sequencing, for rapid genotyping of the common *GJB2*-p.Arg143Trp variant. All hearing-impaired participants from 7/8 families found to be

were found to be homozygous positive for the *GJB2*-p.Arg143Trp mutation using the NciI-RFLP test, and then was confirmed by Sanger sequencing. Sensitivity of the *GJB2*-p.Arg143Trp NciI-RFLP testing was 100% based on an investigation of 111 individuals with isolated non-syndromic HI that were previously Sanger sequenced. All the 50 control subjects with normal hearing were found to be negative for the variant. Although the test is valuable, it is not 100% specific because it cannot differentiate between other mutations at the recognition site of the restriction enzyme. The *GJB2*-p.Arg143Trp NciI-RFLP-based diagnostic test had a high sensitivity for genotyping the most common *GJB2* pathogenic and founder variant (p.Arg143Trp) within the Ghanaian populations. We recommend the adoption and implementation of this test, for HI-focused genetic clinical genetic investigations, to complement the newborn hearing screening program in Ghana. The present study is a practical case of enhancing genetic medicine in Africa.

5.2. Introduction

Globally, the most prevailing sensorineural disorder is hearing impairment (HI) (Rudman *et al.*, 2017), which accounts for about 466 million people worldwide (WHO, 2019). According to the World Health Organization fact sheet, an estimate of 900 million people will be living with the condition by the year 2050 (WHO, 2019). Over 119 genes (Van Camp G & Smith, 2020) with more than 1000 mutations have been associated with hearing impairment of varied degrees in different populations (Rudman *et al.*, 2017). Gap-junction protein β 2 (*GJB2*) and gap-junction protein β 6 (*GJB6*) are the most common genes associated with HI globally, with high prevalence reported in the European and Asian populations. However, recent data including the use of mouse models have indicated that mutations in the coding region of the *GJB6* gene do not result in HI. The large genomic deletions in *GJB6*, especially *GJB6*-D13S1830, alter a

cis-acting element and subsequently abolish the expression of the *cis-GJB2* allele (Ahmad *et al.*, 2007; Rodriguez-Paris & Schrijver, 2009). Thus, the *GJB6* gene does not contribute to the development of HI but the surrounding sequences consisting of the *cis*-acting elements are responsible for the development of HI (DiStefano *et al.*, 2019b; Rodriguez-Paris & Schrijver, 2009). Nevertheless, in most African populations, *GJB2* and *GJB6* variants are rarely implicated in hearing impairment (Wonkam, 2015; Wonkam *et al.*, 2015) with some *GJB2* cases found in Morocco (Gazzaz *et al.*, 2005; Ratbi *et al.*, 2007), Sudan, and Kenya (Gasmelseed *et al.*, 2004), yet an exceptionally high prevalence is found in Ghana (Adadey *et al.*, 2019; Brobby *et al.*, 1998; Hamelmann *et al.*, 2001). Indeed, in Ghana, a *GJB2* mutation (p.Arg143Trp) in the homozygous state accounts for 25.9% of cases in families segregating non-syndromic HI, as well as 7.9% of non-familial non-syndromic congenital HI cases (Adadey et al., 2019). This Ghanaian exception, in the African context, is predominantly due to a *GJB2* founder mutation (p.Arg143Trp), which was first reported in a village known as "the deaf village", Adamorobe (Brobby *et al.*, 1998). Adamorobe is a village located in the Eastern Region of Ghana and is known to have a high hereditary hearing impairment incidence (Nyst, 2007). As of 2012, 41 people living with deafness were recorded among a population of 3500 in Adamorobe (Kusters, 2012). In this village, both the hearing and the deaf citizens interact and live together in one society.

The exceptionally high proportion of *GJB2* (p.Arg143Trp) variants in Ghana have created the need to develop a simple tool for testing in order to support appropriate informed counselling and planning of appropriate interventions. To develop molecular diagnostic tools for screening non-syndromic HI, there is a need for utilizing population and ethnic-specific genetic markers due to the ethnically diverse nature of HI genes (de Freitas Cordeiro-Silva *et al.*, 2011; Sloan-Heggen *et al.*, 2016). Recent clinical genetic

testing efforts have centered around targeted genomic enrichment and/or massive parallel sequencing (Gu *et al.*, 2015; Shearer *et al.*, 2010; Sloan-Heggen *et al.*, 2016). There are some efforts to develop polymerase chain reaction (PCR)-based diagnostic tools for screening for HI; however, most of these tools are in combination with DNA sequencing technologies (Schade *et al.*, 2003; Schrauwen *et al.*, 2013; Tayoun *et al.*, 2016), which are not easily accessible in low-income countries. To develop cheaper but effective diagnostic tools, mutations specific to populations have been considered, especially in populations where *GJB2* mutations are prevalent. Specific genetic tests have been developed for carrier testing and prenatal diagnoses for *GJB2*-35delG variants in Caucasian populations (Antoniadi *et al.*, 2001; Lucotte *et al.*, 2001). In this study, we sought to design a restriction fragment length polymorphism test for *GJB2*-p.Arg143Trp genotyping in Ghana.

5.3. Materials and Methods

5.3.1. Ethical Approvals

The study was performed in accordance with the Declaration of Helsinki. Ethical approval for the study was obtained from the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB CPN 006/16-17) and the University of Cape Town's Faculty of Health Sciences' Human Research Ethics Committee (HREC 104/2018). Written and signed informed consent was obtained from all participants who were 21 years of age or older, and from parents or guardians in cases of minors, with verbal assent from minors, including permission to publish photographs.

5.3.2. Study Participants

Congenital hearing-impaired patients were recruited from schools for the deaf and from the Adamorobe community following procedures reported previously (Adadey *et al.*, 2019). Briefly, all participants' details, as well as their personal and family histories, were obtained; medical records were reviewed by a medical geneticist and an ear, nose, and throat (ENT) specialist when possible; and relevant data were extracted, including three-generation pedigrees and perinatal histories, using a structured questionnaire to query possible environmental causes of HI. A general systemic and otological examination and audiological evaluation were performed, including a pure tone audiometric test, following the recommendation number 02/1 of the Bureau International d'Audiophonologie (BIAP), Belgium, to classify hearing levels (BIAP, 1997; Wonkam *et al.*, 2013). The audiometric tests were conducted using the KUDUwave portable audiometer (KUDUwave, Johannesburg, South Africa) in a quiet room. In bilateral octaves, the air conduction was performed by presenting sound to through the outer ears at thresholds from 250 H_Z through to 8000 H_Z. In a similar fashion, bone conduction was performed by presenting sound through the cranial bones at thresholds from 250 H_Z through to 4000 H_Z. The pure tone average was determined using thresholds at 500, 1000, 2000, and 4000 H_Z as described previously (Al-Abri *et al.*, 2016; Jonas Brännström & Olsen, 2017).

The study participants were categorized into three groups: (1) deaf community-based familial cases (2) nationwide isolated/non-familial cases (3) control individuals without a personal or family history of HI. The first group had families segregating HI, with at least two affected individuals and with evidence of non-environmental causes. In this group, 30 study participants from 8 families segregating hearing impairment were recruited from the Adamorobe community in the Eastern Region of Ghana. Out of the

30 participants, 20 were hearing-impaired and 10 participants had normal hearing. Apart from the families with putative genetic etiology of hearing loss, an additional family was found to have a putative environmental etiology of the condition and was excluded from the study. The second group had 111 isolated/non-familial cases of unrelated probands with putative genetic causes of hearing impairment and were recruited from 6 schools for the deaf across Ghana. All the affected individuals (familial and isolated cases) considered for the study had congenital non-syndromic HI. The third group (the control group) had 50 normal-hearing participants that were randomly recruited nationwide from the Ghanaian population.

5.3.3. Molecular Analyses

DNA extraction: Venous blood was collected from each participant and DNA was extracted from the blood samples using a QIAamp DNA Blood Maxi Kit (Qiagen, Germantown, MD, USA) in the Laboratory of West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, Accra, Ghana.

Polymerase chain reaction (PCR) and Sanger sequencing: At the Division of Human Genetics, University of Cape Town, specific primers (Table S5.1) were used to amplify the coding regions of *GJB2* (exon 2) and *GJB6*, as described by Bosch et al. in 2014. The annealing and extension temperatures for the PCR were 60 °C and 70 °C for 30 s and 1 min, respectively. The PCR amplicons were Sanger sequenced as described by Bosch et al. (Bosch *et al.*, 2014b) using an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Screening for del(*GJB6*-D13S1830) was performed as previously described (Appendix B), using primers and methods by del Castillo et al. (del Castillo *et al.*, 2002).

Restriction fragment length polymorphism (RFLP) technique: The p.Arg143Trp variant in the *GJB2* gene was investigated using RFLP technique designed as follows. *GJB2*-specific primers (Bosch *et al.*, 2014b) were used to amplify exon 2 of the gene where the p.Arg143Trp variant is located. Carefully selected restriction enzyme NciI (supplied by New England Biolabs Inc., Massachusetts, MA, USA, through Inqaba biotec, Pretoria, South Africa) with the recognition site "CCSGG" was used to digest the PCR amplicons. The gene layout and the *GJB2*-p.Arg143Trp NciI-RFLP design including the cut sites is illustrated in Figure 5.1. The RFLP reaction consisted of 15 µL of the PCR product, 2 µL of 10X buffer, 0.25 µL of NciI enzyme (20,000 units/mL), and 2.75 µL of nuclease-free water. The restriction reaction mixture was incubated overnight at 37 °C. The digested products were resolved on 2% agarose gel for 1.5 h. The accuracy, sensitivity, and specificity of the RFLP test was determined as described by Baratloo et al. (Baratloo *et al.*, 2015) using sequencing as the gold standard.

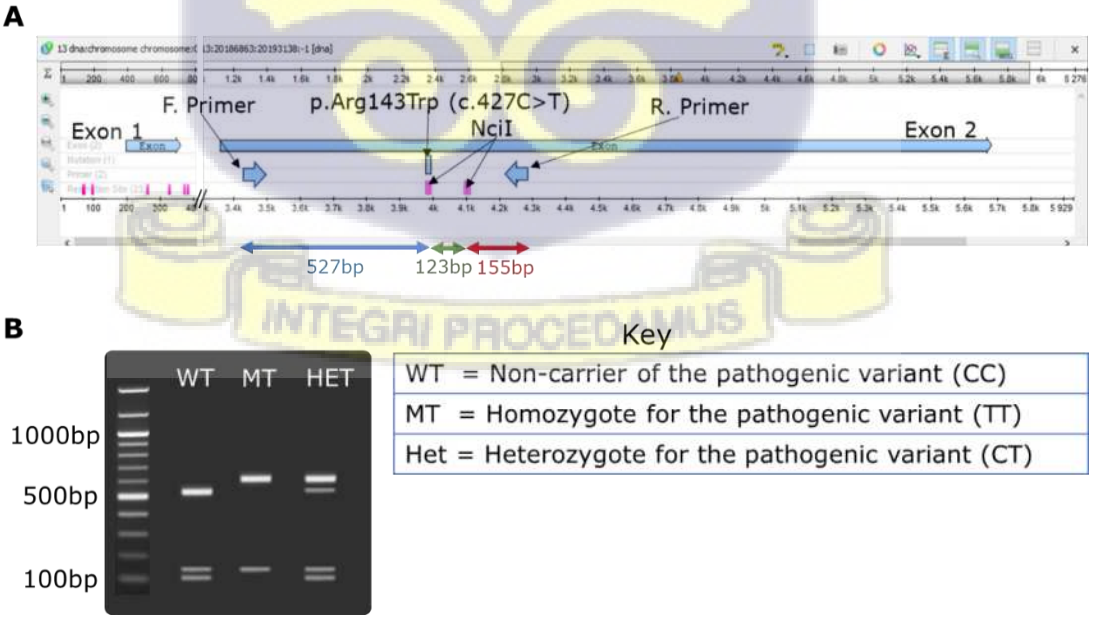

Figure 5.1. NciI restriction fragment polymorphism investigations for gap-junction protein β 2 (*GJB2*)-p.Arg143Trp (c.427C > T rs80338948) variant. *(A) Unipro UGENE (Okonechnikov K, 2012) map of GJB2 exon 2 showing the primer binding sites (F. primer and R. primer) and the restriction sites (CCSGG) for the*

*restriction enzyme NciI and the resulting DNA fragments. (**B**) Expected gel electrophoresis result.*

5.3.4. Data Analysis

Data from the study was captured into Microsoft Excel and analyzed with GraphPad Prism version 6. One-way analysis of variance (ANOVA) was used to determine the differences between the mean hearing measurements (pure-tone average) of the different *GJB2*-p.Arg143Trp genotypes. Tukey's multiple comparisons test was used to compare between the *GJB2*-p.Arg143Trp genotypes. The specificity and sensitivity of the RFLP test were calculated as described by Schrauwen et al. (Schrauwen *et al.*, 2013).

5.4. Results

5.4.1. Selected Families Segregating Hearing Impairment from Adamorobe Village, Ghana

In this study, 8 families from Adamorobe were found to have 2 or more family members living with HI (Figure 5.2), from which 20 congenital deaf and 10 normal hearing family members were identified. Audiological assessment of the participants from Adamorobe revealed that all the hearing-impaired patients had profound sensorineural HI. The unaffected family members without the homozygous mutant (TT) genotype had normal-to-moderate hearing impairment.

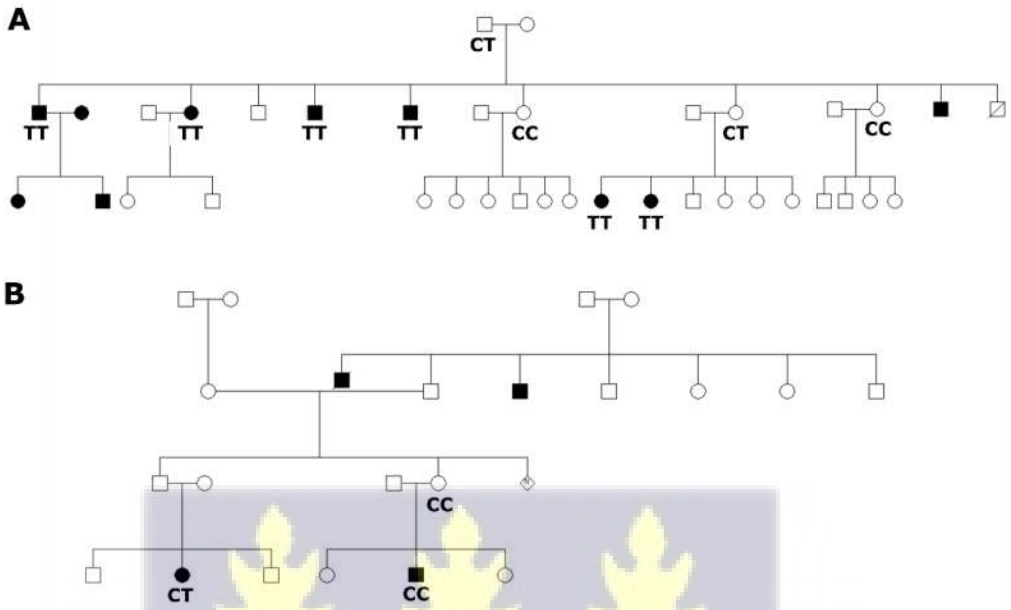

Figure 5.2. Pedigrees and genotypes of familial cases from Adamorobe. ***(A)*** *Representative pedigree of families that segregate GJB2-p.Arg143Trp (c.427C > T rs80338948) variant with hearing impairment. (**B**) Pedigree of a family that did not segregate GJB2-p.Arg143Trp variant with the phenotype. The black shaded square and circles were used to denote hearing-impaired males and females, respectively. The unshaded squares and circles correspond to hearing males and females.*

4.4.2. Restriction Fragment Polymorphism Design for GJB2-p.Arg143Trp

The target region of the *GJB2*-p.Arg143Trp variant was PCR amplified for each participant (Figure S5.1). The NciI restriction enzyme had two restriction sites on the DNA amplified (Figure 5.1A) and cleaves the PCR amplicons of the wildtype (CC genotype) samples to give three products of length 527, 123, and 155 bp. The NciI restriction digest of the homozygous mutant (TT) produced two fragments of the lengths 600 and 155 bp, with the enzyme cutting only once. The heterozygous carriers (CT) yielded four different fragments (600, 527, 123, and 155 bp) (Figure 5.1B). The NciI enzyme cleaved the PCR product in any of the above circumstances; this served as an internal control, and hence an invalid test was when there was no cleavage. The *GJB2*-

p.Arg143Trp NciI-RFLP genotyping results of 20 selected samples from Adamorobe were validated using Sanger sequencing (Figure 5.3).

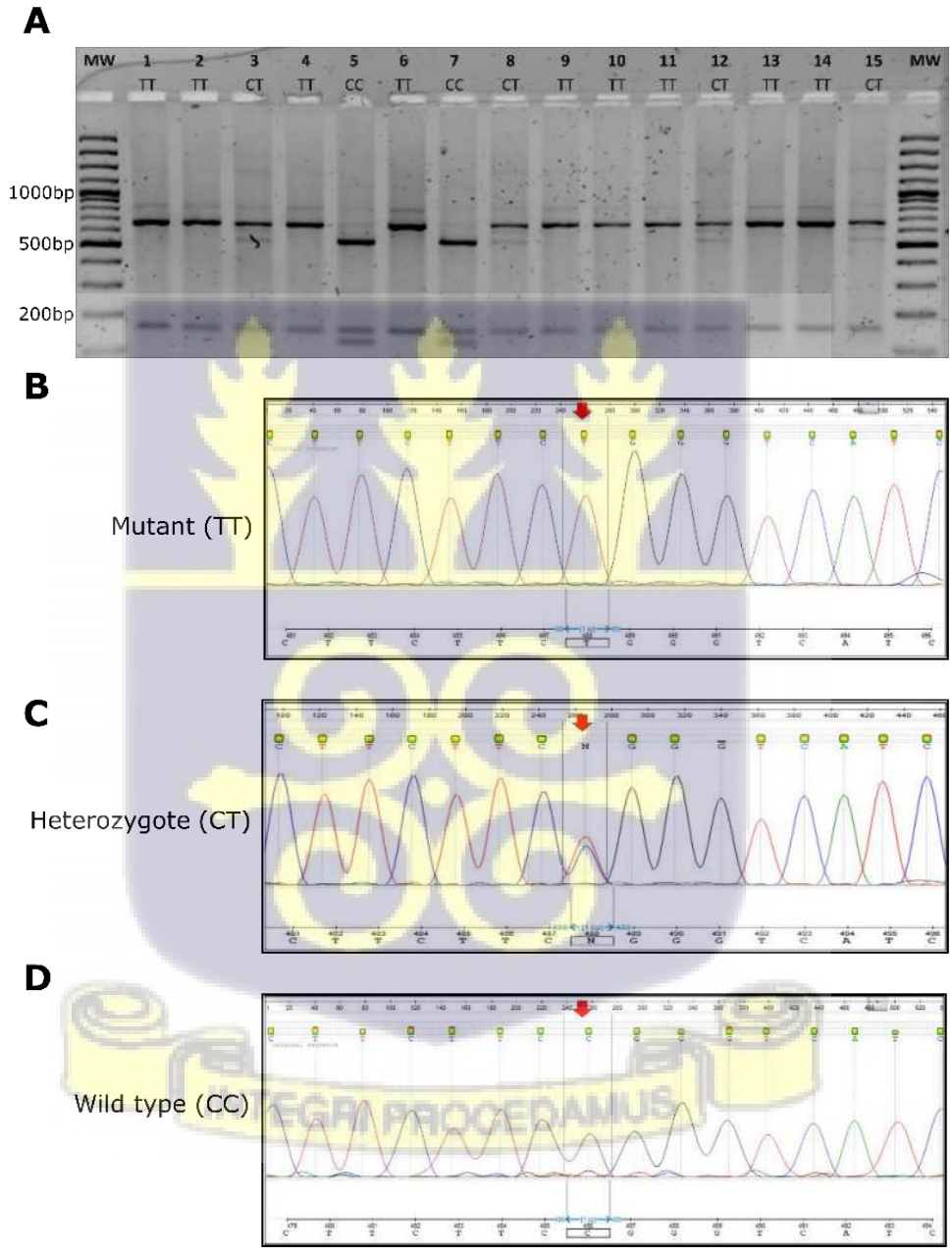

Figure 5.3. *GJB2*-p.Arg143Trp screening. *(**A**) Representative gel of NciI-restriction fragment polymorphism (RFLP) test used to screen samples for GJB2-p.Arg143Trp variant. (**B**–**D**) Representative chromatograms of Sanger sequences validating the p.Arg143Trp NciI-RFLP results.*

5.4.3. *GJB2*-p.Arg143Trp NciI-Restriction Fragment Polymorphism Investigations

The molecular analysis using the *GJB2*-p.Arg143Trp NciI-RFLP test identified 18 out of the 20 hearing-impaired patients, from 7/8 families, to be homozygous for the p.Arg143Trp (TT) variant. In the eighth family were two individuals affected by HI, one was heterozygous (CT) and the other had the CC genotype (Figure 5.2B). In order to exclude *GJB6*-related HI in this family, we examined variants in *GJB6*, and no variant was found. No other participant had a variant in the *GJB6* gene, (n = 20). Seven (7) out of the 10 family members without hearing impairment were heterozygous (CT), thus having the p.Arg143Arg/p.Arg143Trp variant, while the rest had the p.Arg143 variant (Figure 5.3A).

A total of 111 individuals with non-familial isolated non-syndromic HI, whose samples were previously Sanger sequenced for *GJB2* variants (Adadey *et al.*, 2019), were analyzed using the developed *GJB2*-p.Arg143Trp NciI-RFLP test. Table 5.1 illustrates that the *GJB2*-p.Arg143Trp NciI-RFLP test was found to have 100% sensitivity compared to Sanger sequencing as the gold standard. To examine the clinical applicability of the test, 50 control participants with normal hearing were screened and found negative for the *GJB2*-p.Arg143Trp variant.

Table 5.1. Validation of *GJB2*-p.Arg143Trp NciI-restriction fragment polymorphism tests with Sanger sequencing.

Familial Cases from Adamorobe				
		Sanger Sequencing		
	Genotype	TT	CT	CC
GJB2-p.Arg143Trp NciI-RFLP	TT	12	0	0
	CT	0	6	0
	CC	0	0	2
Nation-Wide Isolated/Non-Familial Cases				
		Sanger Sequencing		
	Genotype	TT	CT	CC
GJB2-p.Arg143Trp NciI-RFLP	TT	6	0	0
	CT	0	1	0
	CC	0	0	104

The mutant, heterozygote, and wild type are represented by TT, CT, and CC, respectively.

5.4. Genotype to Phenotype Correlations

On the basis of *GJB2*-p.Arg143Trp genotypic classification of the familial cases from Adamorobe, the pure tone average of homozygous mutant (TT) ranged from 97 to 108 dB with a mean of 105.4 and 107.3 dB in the left and right ears, respectively. The pure tone average range for the heterozygote (CT) was from 17 to 108 dB, with a mean of 43.6 and 40.6 dB in the left and right ears, respectively. The range for the homozygous CC genotype (p.Arg143Arg) was from 18 to 108 dB, with the mean 53 and 46.5 dB in the left and right ears, respectively. There was a statistically significant difference between the audiometric measurements of the TT and CT genotypes in both ears (p-values less than 0.001). Similarly, in both ears, there was a statistically significant difference between the TT and CC genotypes (Figure 5.4).

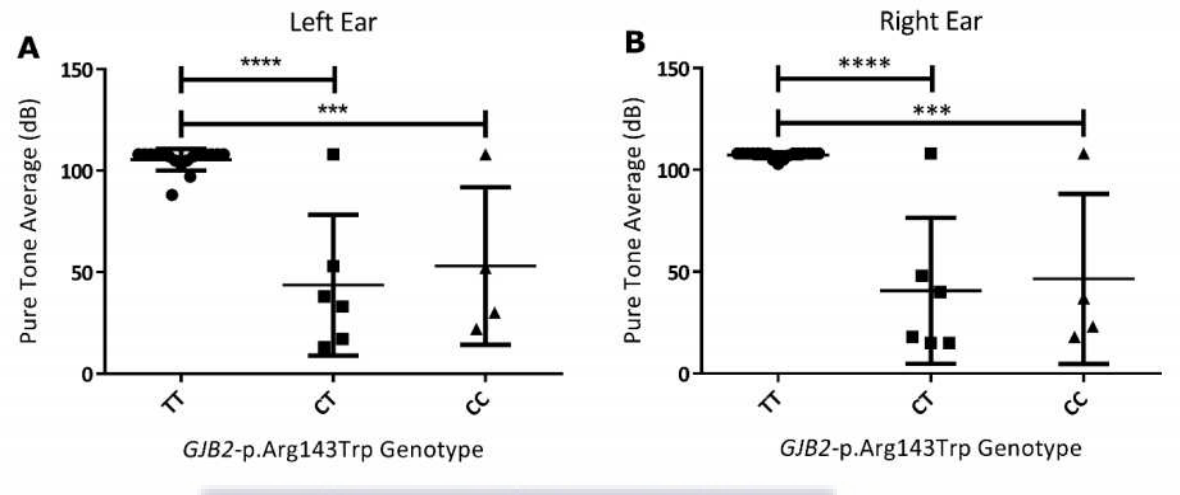

Figure 5.4 Audiological characterization of hearing-impaired participants from the deaf community of Adamorobe. *(**A**) Left ear and (**B**) right ear pure tone average of participants according to their GJB2-p.Arg143Trp genotypes. The age range of the genotypes TT (n = 17), CT (n = 6), and CC (n = 4) were 9 to 80 years, 23 to 66 years, and 11 to 63 years, respectively. p-values less than 0.05 were considered significant. p-values less than 0.0001 and 0.001 are represented by (****) and (***), respectively.*

5.5. Discussion

This study designed a restriction fragment length polymorphism (RFLP) test for effective screening of *GJB2*-p.Arg143Trp (rs80338948). The *GJB2*-p.Arg143Trp variant is a pathogenic point mutation (c.427C > T) in exon 2 of the connexin 26 gene on chromosome 13 (Brobby *et al.*, 1998; Hamelmann *et al.*, 2001). The motivation for an efficient and cost-effective test was the fact that the founder mutation, *GJB2*-p.Arg143Trp, is the most common variant associated with HI in Ghana (Adadey *et al.*, 2019; Brobby *et al.*, 1998; Hamelmann *et al.*, 2001).

The use of next-generation sequencing (NGS) has been proposed as the best tool for the discovery of HI genes (Gao & Dai, 2014), especially in Africa because of the high diversity within the African population (Lebeko *et al.*, 2015; Lebeko *et al.*, 2016). Due to ethical and social challenges, NGS needs to be carefully considered in clinical practice (Pinto *et al.*, 2016). In developing countries, the clinical use of NGS is still a

major challenge because of the associated high cost of the equipment and the computational challenges posed by the approach (Calistri & Palù, 2015; García-García *et al.*, 2020; Yin *et al.*, 2021). However, there were some attempts to develop relatively simple, low-cost, and population-specific screening approaches for some of the major hearing impairment gene mutations (Abe *et al.*, 2018; Brown & Rehm, 2012; Yan *et al.*, 2017).

For the first time, we have designed and tested the effectiveness of RFLP, using the NciI enzyme, to screen for the founder mutation (*GJB2*-p.Arg143Trp) in Ghana. Accuracy, sensitivity, specificity, and predictive values are critical parameters considered for the clinical use of a test (Baratloo *et al.*, 2015; Šimundić, 2009). Our *GJB2*-p.Arg143Trp NciI-RFLP test had good positive and negative predictive values for genotyping of the *GJB2*-p.Arg143Trp variant in the Adamorobe participants from Ghana, and also in a nationwide sample of unrelated affected individuals. However, the test cannot differentiate between other variants within the recognition site of the restriction enzyme; hence, similar results would be obtained for the following pathogenic mutations: p.Phe142Leu (c.426C > A), p.Y142del (c.424_426delTTC), and p.Arg143Gln (c.428G > A). Despite the fact that, to confirm the specific mutation at the enzyme restriction site, Sanger sequencing would be needed, the high prevalence of the *GJB2*-p.Arg143Trp variant within the Ghanaian population makes the NciI-RFLP test relevant. A 100% sensitivity was obtained for the *GJB2*-p.Arg143Trp NciI-RFLP test when compared with the gold standard, Sanger sequencing. Although a single gene test for HI is inefficient for many populations (Yan *et al.*, 2017), the aforementioned qualities of the test would enable it to be used as a first-line diagnosis for HI genetics in the newborn hearing screening program, as well as for prenatal testing. The *GJB2*-p.Arg143Trp NciI-RFLP test would therefore be of great clinical value in Ghana, and

can be used in combination with other approaches as such as micro-array chips, panel sequencing or NGS.

The *GJB2*-p.Arg143Trp NciI-RFLP test identified the founder mutation in the eight (8) Adamorobe families investigated. In all the families, the mutation segregated with the phenotype, and all affected individuals reported a homozygous variant (TT genotype), except in one family where one affected individual was heterozygous (CT) and the other without any variant (CC), suggesting that there are other genes still to be discovered to explain the HI in this family. Similar to a family from Japan (Abe *et al.*, 2018), the heterozygous *GJB2*-p.Arg143Trp variant in the above family did not segregate with the HI phenotype (Figure 2B). Variants in the *GJB6* gene are no longer considered as causes of HI, however, the presence of *GJB6* variants affecting the *cis*-acting element upstream of both *GJB6* and *GJB2* in association with variants in *GJB2* (digenic inheritance) *are now* known to be pathogenic through the modification of *GJB2* expression (DiStefano *et al.*, 2019b; Rodriguez-Paris & Schrijver, 2009). Hence, we sought to exclude any *GJB6* variant that might disrupt the *cis*-acting element. We, therefore, investigated *GJB6* variants in particular; *GJB6*-D13S1830, and no *GJB6* pathogenic variant was identified in this family. Hence, we propose the use of whole-exome sequencing (WES) in future, or targeted panel sequencing, which has been shown to be efficient in Cameroonian families (Lebeko *et al.*, 2015), to further investigate this family, as well as other families that are negative for the *GJB2*-p.Arg143Trp variant in Ghana.

The *GJB2*-p.Arg143Trp variant is known to be associated with profound HI (Abe *et al.*, 2018; Brobby *et al.*, 1998; Hamelmann *et al.*, 2001). The audiometric characterization of the *GJB2*-p.Arg143Trp homozygous individuals showed that they had profound HI. A previous studies by Brobby et al. from the same village indicated that *GJB2*-p.Arg143Trp homozygous individuals express profound HI (Brobby *et al.*, 1998).

Similar to the previous report (Brobby *et al.*, 1998), we found that there was no significant difference between the average hearing levels of the CT (heterozygote for the pathogenic variant) and the CC (non-carrier of the pathogenic variant) genotypes. Our results and previous reports confirmed the autosomal recessive mode of inheritance of *GJB2*-p.Arg143Trp (Adadey *et al.*, 2019; Brobby *et al.*, 1998; Hamelmann *et al.*, 2001).

5.6. Conclusions

We developed a rapid and cost-effective NciI-RFLP test for the *GJB2*-p.Arg143Trp founder mutation in Ghana. The *GJB2*-p.Arg143Trp NciI-RFLP test had 100% sensitivity when compared with Sanger sequencing, the gold standard. We, therefore, propose that testing for the *GJB2*-p.Arg143Trp variant using the NciI-RFLP test should be implemented as part of the newborn hearing screening program in Ghana, a practical case of enhancing genetic medicine in Africa.

5.7. Author Contributions

Conceptualization, A.W., G.A.A., G.K.A., and S.M.A.; methodology, S.M.A., E.T.W., E.T.A., and D.Q.; validation, A.W., G.A.A., G.K.A., and O.Q.; formal analysis, S.M.A., E.T.W., E.T.A., and D.Q.; resources, A.W., G.A.A., G.K.A., A.A.-P., and O.Q.; writing—original draft preparation, S.M.A.; writing—review and editing, S.M.A., E.T.W., E.T.A., D.Q., A.A.-P., O.Q., G.K.A., G.A.A., and A.W.; supervision, A.W., G.A.A., G.K.A., and O.Q.; funding acquisition, A.W. and G.A.A. All authors have read and agreed to the published version of the manuscript.

5.8. Supplementary materials

Table S5.1: Primer sequencing for GJB2 and GJB6 coding region amplification

Gene	Primer	Primer sequence	Product size
GJB2	F4	5’ -GCTTACCCAGACTCAGAGAAG-3’	900
	R1	5’-CTTAATCTAACAACTGGGCAATGC-3’	
GJB6	CDF	5’-TTGGCTTCAGTATGTAATATCACC-3’	990
	CDR	5’-TCATTTACAAACTCTTCAGGCTACAG-3’	

CHAPTER SIX

6.0. Paper 4: *GJB4* and *GJC3* Variants in Non-syndromic Hearing Impairment in Ghana

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6.1. Abstract

The contribution of *GJB4* and *GJC3* gene variants to hearing impairment (HI) in Africa has not yet been studied. Here, we investigated the contribution of these genes to autosomal recessive non-syndromic HI in Ghanaian children. Hearing-impaired children from 141 simplex and 59 multiplex families were enrolled from 11 schools for the deaf in Ghana. The coding regions of *GJB4* and *GJC3* were amplified, sequenced, and analyzed for the study participants previously found to be negative for *GJB2* and *GJB6* variants. Seven *GJB4* and one *GJC3* variants were identified. one out of the seven *GJB4* variants was classified as likely pathogenic while the others were

either benign or synonymous. The likely pathogenic variants (p.Asn119Thr/ rs190460237) were predicted to be associated with HI. We modelled the wild-type and mutant proteins of this variant (p.Asn119Thr) to evaluate the effect of the mutation on protein structure and ligand-binding properties. The mutant and not the wild type had the potential to bind N-Ethyl-5'-Carboxamido Adenosine (DB03719) which was due to a slight structural change that was observed. No clinically relevant variant was identified in the *GJC3* gene. We report for the first time a likely pathogenic *GJB4* variant that may be associated with non-syndromic hearing impairment in Ghana; the finding will add to the body of evidence of the contribution of *GJB4* to hearing impairment cases around the world.

6.1.1. Impact statement

Although connexins are known to be major genetic factors associated with HI, only a few studies have investigated *GJB4* and *GJC3* variants among hearing-impaired patients in Africa. This study is the first to report *GJB4* and *GJC3* variants from an African HI cohort. We have demonstrated that *GJB4* and *GJC3* genes may not contribute significantly to HI in Ghana, hence these genes should not be considered for routine clinical screening in Ghana. However, important to study a larger population to determine the association of *GJB4* and *GJC3* variants with HI.

Keywords: *GJB4, GJC3,* protein modelling, hearing impairment, in silico, virtual screening

6.2. Introduction

Hearing impairment (HI), a disabling congenital disease, is known as one of the major age-standardized disabilities of life globally (Murray *et al.*, 2015; Vos *et al.*, 2015).

According to a World Health Organization (WHO) report in 2019, about 466 million people are estimated to be living with HI (WHO, 2019). A higher prevalence is recorded in sub-Saharan Africa (about 6 out of 1,000 live births) compared to the developed countries (about 1 out of 1,000 live births) (Olusanya *et al.*, 2014). Reports from different populations have shown that about 50% of congenital HI cases are of genetic origin (Olusanya *et al.*, 2014; Wonkam *et al.*, 2013) and about 80% of the genetic cases are non-syndromic (Bademci *et al.*, 2016; Sheffield & Smith, 2019). The majority of all non-syndromic HI cases (nearly 80%) are inherited in the autosomal recessive fashion (Wu *et al.*, 2018; Zhou *et al.*, 2019). HI is genetically highly heterogeneous with over 119 genes identified to date (Van Camp G & Smith, 2020) but the contribution of gene variants to HI has not been equally investigated across global populations, with limited studies from Africa. Hence, there is a great scarcity in the representation of known pathogenic gene variants of African ancestry. As a result, a recent study of pathogenic and likely pathogenic (PLP) autosomal recessive non-syndromic hearing impairment (ARNSHI) variants (selected from the ClinVar and Deafness Variation Databases with their frequencies from gnomAD database), estimated the prevalence of HI due to PLP as 5.2 per 100,000 individuals for Africans/African Americans, compared to a higher prevalence of 96.9 per 100,000 individuals for Ashkenazi Jews (Chakchouk *et al.*, 2019). The knowledge deficit is likely hindering progress in understanding the mechanism of HI in Africans and ultimately affecting the development of therapeutic strategies, genetic diagnoses, prognosis, and genetic counselling (Chakchouk *et al.*, 2019).

Connexin genes are the most frequently reported known HI genes to be associated with HI cases, particularly in populations of European and Asian ancestries (del Castillo *et al.*, 2003; del Castillo *et al.*, 2002). Connexins are a family of gap junction proteins

expressed in almost all human tissues and are involved in intercellular communication (Kelsell *et al.*, 2001; Sabag *et al.*, 2005), and mutations in connexin genes have been implicated in about 28 genetic diseases (Srinivas *et al.*, 2018), with deafness and skin diseases as the most frequently associated condition (Kelsell *et al.*, 2001; Laird *et al.*, 2017). Variations in the gene *GJB2* are most frequently associated with non-syndromic hearing impairment (NSHI) (Karami-Eshkaftaki *et al.*, 2017; Laird *et al.*, 2017). Similar to *GJB2*, *GJB4* and *GJC3* gene variants are associated with skin disorders (Srinivas *et al.*, 2018), however, they are seldom associated with ARNSHI. Associations have been established previously between NSHI and *GJB4* in Iran (Kooshavar *et al.*, 2013; Laleh *et al.*, 2017) and Taiwan (Yang *et al.*, 2010), and between NSHI and *GJC3* in Taiwan (Yang *et al.*, 2010) and India (Ramchander *et al.*, 2010). However, multiple evidence from independent populations is needed for the clinical validity of hearing impairment gene-disease pairs (DiStefano *et al.*, 2019a). Earlier studies investigating *GJB4* mutations among hearing-impaired patients found missense variants such as p.R227W (c.679C>T), p.C169W (c.507C>T) and p.R151S (c.451C>A) (Kooshavar *et al.*, 2013; Laleh *et al.*, 2017; Yang *et al.*, 2010), though the molecular mechanisms of the cause of deafness with respect to these variants were not well elucidated. However, it was suggested that these that these variants may be pathogenic since they were identified among patients and not control participants (Kooshavar *et al.*, 2013; Laleh *et al.*, 2017; Yang *et al.*, 2010). Interestingly, ClinVar and the Rat Genome Database contain *GJB4* variants associated with autosomal non-syndromic deafness (Landrum *et al.*, 2018; Smith *et al.*, 2020), further supporting the pathogenicity of the gene. Moreover, *GJB4* protein was found to be expressed in the cochlea of rats (Wang *et al.*, 2010a). Similar to *GJB4*, some *GJC3* variants (e.g. p.I90A/c.569T>A and c.781 + 62G>A) were reported only among hearing-impaired individuals without any extensive molecular

study on their pathogenicity (Ramchander *et al.*, 2010; Yang *et al.*, 2010). There is therefore the need to interrogate *GJB4* and *GJC3* variants from other populations across the world and to study the molecular mechanisms of pathogenicity of these gene variants.

To date, only *GJB2* and *GJB6* contributions to NSHI have been systematically investigated in Ghana (Adadey *et al.*, 2019; Adadey *et al.*, 2020; Brobby *et al.*, 1998; Hamelmann *et al.*, 2001) and other parts of Africa (Tingang Wonkam *et al.*, 2019; Wonkam *et al.*, 2015). There is no study from Africa on the role of *GJB4* and *GJC3* variants in HI. In this study, we investigated the contribution of *GJB4* and *GJC3* to NSHI in Ghana. We report for the first time, variants in *GJB4* and *GJC3* genes in a Ghanaian HI cohort, and we have used *in silico* protein modelling approaches to explore the possible molecular mechanisms through which a likely pathogenic variant found in *GJB4* could cause deafness.

6.3. Materials and Methods

6.3.1. Ethics consideration

The set of ethical principles of the Declaration of Helsinki were adhered to in this study. Ethical approvals were sought and obtained from two ethics review boards: the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB CPN 006/16-17) and the University of Cape Town's Faculty of Health Sciences' Human Research Ethics Committee (HREC 104/2018). Prior to patient enrolment, the study was explained to each study participant in their native language and informed consent was confirmed by signature.

6.3.2. Study participants

The participants in this study were grouped into 3 categories: 1) isolated/non-familial simplex cases (n=141) living with severe to profound HI with putative genetic cause of deafness; 2) multiplex/familial cases consisting of 59 individuals, each one selected from 59 families who had at least two affected family members with HI (Figure 5.1 and Figure S1); and 3) control participants (n = 47) randomly selected from a general Ghanaian population, with no personal and family history of HI. The medical records of the hearing-impaired students were evaluated to identify families with congenital HI. Both families and isolated cases were compatible with autosomal recessive inheritance, and each hearing-impaired participant was carefully examined and interviewed with a structured questionnaire to eliminate syndromic and environmental causes of HI as described previously (Adadey *et al.*, 2019). All the study participants including the controls had been previously screened and were found to be negative for *GJB2* and *GJB6* gene variants (Figure 1) (Adadey *et al.*, 2019; Adadey *et al.*, 2020).

6.3.3. Genetic Analyses

DNA extraction: At the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, Accra, Ghana, DNA was extracted from the blood samples collected from each participant using [QIAamp DNA Blood Maxi Kit. ® (Qiagen, USA)].

Polymerase chain reaction (PCR) and Sanger Sequencing: The molecular analyses were conducted at the Division of Human Genetics, University of Cape Town. Previously published primers (Kooshavar *et al.*, 2013) specific for *GJB4* exon 2 (F1B4: 5`-TCAATCGCACCAGCATTAAG-3` and R1B4: 5`-GGGGGACCTGTTGATCTTATC-3`) and *GJC3* exon 1 (F1C3: 5`-GCTCCCTCTGAAGGACAGTG-3` and R1C3: 5`-

GGGAGGAGATCATCAGGACA-3`) and *GJC3* exon 2 (F2C3: 5`-TGGGTACGCACTGTGAAAAA-3` and R2C3: 5'-AGCTCCTCCTTGGACAGGAT-3`) were used to amplify the coding regions of *GJB4* and *GJC3*. The PCR amplicons were Sanger sequenced as described by Bosch et al. in 2014 using ABI 3130XL Genetic Analyzer® (Applied Biosystems, Foster City, CA).

6.3.4. Data analysis

The Sanger sequence data was cleaned and analyzed using the FinchTV chromatogram viewer and Unipro UGENE Integrated Bioinformatics Tools (Okonechnikov K, 2012). Odd ratios were calculated to examine how strongly the identified variables are associated with the HI phenotype. We used Fisher's exact test to determine if there is an association between the number of alleles obtained for each variant in different populations. P-values less than 0.05 were considered significant. We used the following online databases, genome browser, and predictive programs to predict the clinical significance of the identified gene variants: VarSome (Kopanos *et al.*, 2019), ClinVar (Landrum *et al.*, 2018), Align GVGD (Align Grantham Variation/Grantham Deviation) (Mathe *et al.*, 2006; Tavtigian *et al.*, 2006), FATHMM version 2.3 (Functional Analysis Through Hidden Markov Models) (Shihab *et al.*, 2013a; Shihab *et al.*, 2013b; Shihab *et al.*, 2014), MutationAssessor release 3 (Reva *et al.*, 2007, 2011), MutationTaster 2020 (Schwarz *et al.*, 2014), MutPred2 (Mutation Prediction 2) (Pejaver *et al.*, 2017), PROVEAN (Protein Variation Effect Analyzer) version 1.1.3 (Choi, 2012; Choi & Chan, 2015; Choi *et al.*, 2012), PolyPhen-2 (Polymorphism Phenotyping V-2) (Adzhubei *et al.*, 2010), SIFT 2019 (Sorting Intolerant From Tolerant) (Kumar *et al.*, 2009; Ng & Henikoff, 2001; Ng & Henikoff, 2002, 2003), EIGEN version 3.1.1 (Ionita-Laza *et al.*, 2016) MPV (pathogenicity of missense variants) (Qi *et al.*, 2018), PrimateAI (Sundaram *et al.*, 2018) and InterVar (Li & Wang, 2017) (Table S5.1).

6.3.5. In silico analysis of c.356A>C (p.Asn119Thr) variant

The forward and reverse "ab1" files (obtained from the ABI 3130XL Genetic Analyzer®) of the sample with the *GJB4* c.356A>C (p.Asn119Thr) variant was trimmed and edited using the SnapGene Viewer v5.0.6 (GSL_Biotech, 2018). The resulting sequence was then saved as a FASTA file which was used to perform a BLASTx search in the non-redundant protein data bank (nrPDB) accessed via the NCBI BLAST web interface. Six hits were obtained from the BLASTx search, of which 4 were human proteins which were retrieved as .pdb files. Only the "A" chain of the PDB hits showed homology with the GJB4 protein, hence, they were the only chains considered for further analysis. The "A" chains were retrieved as PDB files using the "indicate chain" command of PyMOL v1.8.4.0 (DeLano, 2002). For each of the 4 templates (the retrieved "A" chains) the wild type and mutant proteins of the *GJB4* c.356A>C (p.Asn119Thr) variant were modelled.

Modeller v9.0.3 (Webb & Sali, 2017) was used to perform a template-based (TM) modelling of both the wild type and mutant proteins of the *GJB4* c.356A>C (p.Asn119Thr) variant using two strategies; i) single template-based modelling and ii) multiple-template-based modelling (Figure 1). All the scripts used for the modelling were obtained from the Modeller web tutorial and changes were made where necessary.

Single template-based modelling: To identify the best template, the four templates were compared against each other using multiple sequence alignment and phylogenetic tree reconstruction. Pairwise alignment of the best template was performed with both the wild type and mutant proteins of the *GJB4* c.356A>C (p.Asn119Thr) variant from which 50 models were built. The best model was selected based on the lowest DOPE (Discrete Optimized Protein Energy) score (Shen & Sali, 2006).

Multiple template-based modelling: A multiple sequence alignment (MSA) was performed for all the 4 templates followed by pairwise sequence alignment with the wild type and mutant proteins of the *GJB4* c.356A>C (p.Asn119Thr) variant. Similar to the single template-based modelling, 50 models each of the wild type and mutant proteins were built and the top 10 models were selected based on the lowest DOPE score (Shen & Sali, 2006). The best model was selected from the top 10 models based on the Ramachandran plot evaluation (RAMPAGE) and Z-score (ProsA-Web) (Figure 2).

Model Refinement: The best models were run through the Galaxy server's (Ko *et al.*, 2012; Shin *et al.*, 2014) refinement (Refine2) pipeline, which iteratively optimizes the initial structure using global and local operators as loop modelling and hybridization. The top-ranked model based on Galaxy energy, in combination with other parameters, was selected for virtual screening of possible ligands.

Virtual Screening: To assess the possible effect of the *GJB4* c.356A>C (Asn119Thr) variant on the binding property of the protein, virtual screening for ligands was performed using the Galaxy server's Site algorithm. The algorithm predicts binding by comparing the distance between an amino acid residue and a ligand atom with the sum of their van de Waals radii + 0.5angstrom. Binding site residues are considered as those with a smaller difference in distance.

6.4. Results

6.4.1. Molecular analysis of *GJB4* and *GJC3*

To identify *GJB4* and *GJC3* variants that may be associated with HI in Ghana, we investigated hearing-impaired patients identified to be negative for *GJB2* and *GJB6* gene variants, from both multiplex (n = 59/127 affected individuals from 59 unrelated

families) and simplex (n=141) unrelated families segregating ARNSHI (Figures 5.1 and S5.1). These patients were had severe to profound congenital HI and their clinical and demographic data were previously reported (Adadey *et al.,* 2019). The *GJB4* and *GJC3* gene variants were identified in the hearing-impaired patients and were further examined among the control individuals not affected by HI (Table 6.1).

The clinical significance and pathogenicity of the identified variants were predicted using 2 online databases and 12 predictive bioinformatic tools (Table S6.1). The sensitivity, accuracy, and specificity of these predictive tools vary based on the algorithms used (Mahmood *et al.*, 2017). It was, therefore, important to use a combination of predictive tools (Leong *et al.*, 2015). We considered variants that were predicted as likely pathogenic/pathogenic by more than 5 bioinformatic tools as likely pathogenic variants.

6.4.1. Variants in *GJC3*

The molecular, clinical, and pathogenic evaluation of the variants identified in heterozygous state a *GJC3* variant predicted as benign (p.Pro164Ser). Two familial cases were found to be homozygous for the same mutation (Table 6.1).

6.4.2. Variants in *GJB4*

Three *GJB4* synonymous variants (p.Lys123=, p.Arg101=, and p.Thr172=) were identified in all 3 groups of samples. Of the three synonymous variants, p.Lys123= was classified as benign and p.Thr172= as a variant of uncertain significance. The *GJB4* sequence analysis also identified one nonsense and two non-synonymous variants classified as benign (p.Gln80Ter, p.Arg151Ser, and p.Glu204Ala). An additional variant (p.Asn119Thr) was classified as likely pathogenic (Table S6.1). Although the predictive tools suggested that the *GJB4* p.Glu204Ala variant was likely pathogenic,

this was not supported by gene variants-HI correlations since the homozygous form of the variant was identified in both affected hearing-impaired (n = 25) and unaffected hearing control individuals (n = 3) with an odds ratio of 0.81. In addition, the minor allele frequency of the *GJB4* p.Glu204Ala within the global and African populations exceeds the threshold of 0.05, suggesting that it is not disease-causing (Table 6.2).

The *GJB4* p.Asn119Thr variant predicted as likely pathogenic was identified in only one hearing-impaired individual from a simplex family (Figure 6.2A-B). The sample from the participant in whom this variant was found was independently sequenced three times, with each run from a new PCR product. Furthermore, the *GJB4* p.Asn119Thr variant had less than 0.01 allele frequency in the global and African populations, indicating that it is a rare variant (Table 6.2). Since the *GJB4* p.Asn119Thr variant was predicted to be likely pathogenic, we examined it further using protein modeling approaches (Figures 6.3-6.5).

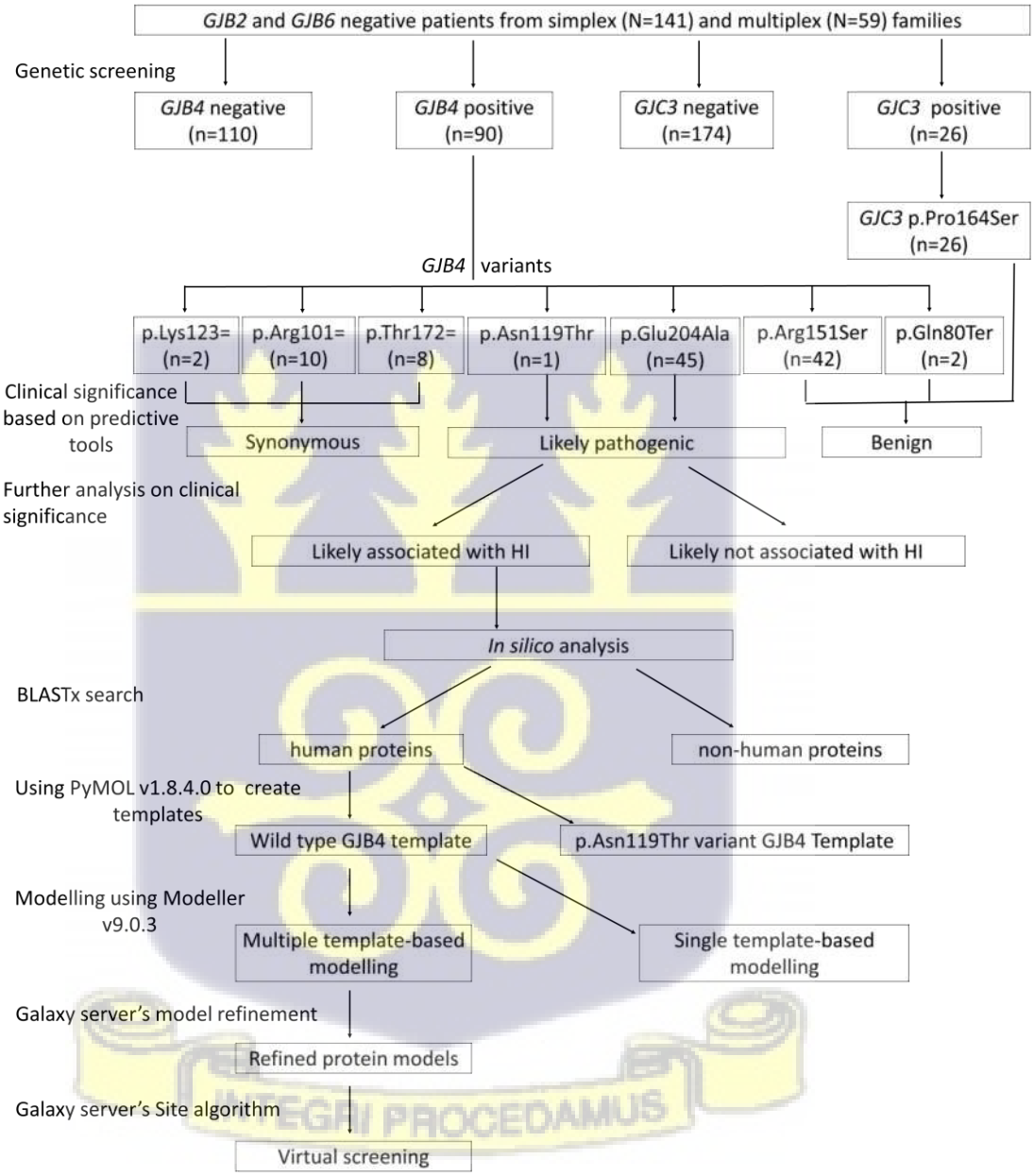


Figure 6.1: Flow chart of genetic screening of patients with *GJC3* and *GJB4* variants, and in silico analysis of *GJB4* c.356A>C (p.Asn119Thr) variant.

Table 6.1: *GJB3* and *GJB4* variants found in hearing-impaired patients and control subjects from Ghana

					Number of participants, n (%)					
Gene	Mutation	Protein Change	Clinical Significance	Geno-types	Multiplex family N = 59	Simplex Family N=141	Total Affected (N=200)	Controls N=47	Odds ratio	P value
GJC3	c.490C>T (rs73405465)	p.Pro164Ser	Benign	GG	50 (84.75%)	124 (87.94%)	174 (87.00%)	41 (87.23%)	-	-
				GA	7 (11.86%)	17 (12.06%)	24 (12.00%)	6 (12.77%)	0.94	0.45
				AA	2 (3.39%)	0	2 (1.00%)	0	-	-
GJB4	c.238C>T (rs114429815)	p.Gln80Ter	Benign	CC	59 (100%)	139 (98.58%)	198 (99.00)	45 (95.74%)	0.11	0.039
				CT	0	1 (0.71%)	1 (0.50%)	2 (4.26%)	-	-
				TT	0	1 (0.71%)	1 (0.50%)	0	-	-
GJB4	c.303C>G (rs138184343)	p.Arg101=	Synonymous	CC	55 (93.22%)	135 (95.74%)	190 (95.00%)	46 (97.87%)	-	-
				CG	1 (1.69%)	4 (2.84%)	5 (2.50%)	1 (2.13%)	1.21	0.43
				GG	3 (5.09%)	2 (1.42)	5 (2.50%)	0	-	-
GJB4	c.356A>C (rs190460237)	p.Asn119Thr	Likely pathogenic	AA	59 (100%)	140 (99.29%)	199 (99.50%)	47 (100.00%)	-	-
				AC	0	0	0	0	-	-
				CC	0	1 (0.71%)	1 (0.50%)	0	-	-
GJB4	c.369G>A (rs142843509)	p.Lys123=	Benign	GG	59 (100%)	139 (98.58%)	198 (99.00%)	46 (97.87%)		-
				GA	0	2 (1.42)	2 (1.00%)	1 (2.13%)	0.46	0.26
				AA	0	0	0	0	-	-
GJB4	c.451C>A (rs78499418)	p.Arg151Ser	Benign	CC	47 (79.66%)	111 (78.72%)	158 (79.00%)	40 (85.11%)		
				CA	8 (13.56%)	18 (12.77%)	26 (13.00%)	3 (6.38%)	2.19	0.11
				AA	4 (6.78%)	12 (8.51)	16 (8.00%)	4 (8.51%)	1.01	0.49
GJB4	c.516T>C (rs111693060)	p.Thr172=	Variant of uncertain significance	TT	56 (94.92%)	136 (96.45%)	192 (96.00%)	46 (97.87%)		
				TC	0	3 (2.13%)	3 (1.50%)	1 (2.13%)	0.72	0.39
				CC	3 (5.08)	2 (1.42%)	5 (2.50%)	0	-	-
GJB4	c.611A>C (rs3738346)	p.Glu204Ala	Benign	AA	49 (83.05%)	106 (75.18%)	155 (77.50%)	38 (80.85%)		
				AC	4(6.78%)	16 (11.35)	20 (10.00%)	6 (12.77%)	0.81	0.34
				CC	6(10.17%)	19 (13.47%)	25 (12.50%)	3 (6.38%)	2.04	0.13

p-values less than 0.05 were considered as significant

Table 6.2: Differential allele frequencies of *GJB4* and *GJC3* variants in the global population

				Our data			Allele frequency (Ensembl)									
Gene	Mutation	rs number	Allele	Cases (n=400)	Control (N=94)	P-value (Cases vs control)	Global	P-value (Our cases vs Global)	Africa	P-value (Our cases vs Africa)	America	P-value (Our cases vs America)	East Asia	P-value (Our cases vs East Asia)	Europe	P-value (Our cases vs Europe)
GJC3	c.490C>T (p.Pro164Ser)	rs73405465	G	0.93	0.94	1.0000	0.98	0.0001	0.94	0.6441	0.99	0.0001	1.00	0.0001	1.00	0.0001
			A	0.07	0.06		0.02		0.06		0.01		0.00		0.00	
GJB4	c.611A>C (p.Glu204Ala)	rs3738346	A	0.83	0.87	0.3548	0.89	0.0001	0.75	0.0023	0.89	0.0022	0.88	0.0075	0.99	0.0001
			C	0.17	0.13		0.11		0.25		0.11		0.12		0.01	
GJB4	c.451C>A (p.Arg151Ser)	rs78499418	C	0.86	0.88	0.6197	0.96	0.0001	0.90	0.0134	0.92	0.0010	0.97	0.0001	1.00	0.0001
			A	0.14	0.12		0.04		0.10		0.08		0.03		0.00	
GJB4	c.516T>C (p.Thr172=)	rs111693060	T	0.97	0.99	0.4863	0.99	0.0001	0.98	0.1931	1.00	0.0001	1.00	0.0001	1.00	0.0001
			C	0.03	0.01		0.01		0.02		0.00		0.00		0.00	
GJB4	c.369G>A (p.Lys123=)	rs142843509	G	0.99	0.99	0.4699	1.00	0.0296	1.00	0.0532	1.00	0.1340	1.00	0.0806	1.00	0.0808
			A	0.01	0.01		<0.01		0.00		<0.01		0.00		0.00	
GJB4	c.356A>C (p.Asn119Thr)	rs190460237	A	0.99	1.00	1.0000	1.00	0.0156	1.00	0.1366	1.00	0.1335	1.00	0.0806	1.00	0.0808
			C	0.01	0.00		<0.01		<0.01		0.00		0.00		0.00	
GJB4	c.303C>G (p.Arg101=)	rs138184343	C	0.96	0.99	0.3282	1.00	0.0001	0.98	0.0302	1.00	0.0001	1.00	0.0001	1.00	0.0001
			G	0.04	0.01		<0.01		0.02		0.00		0.00		0.00	
GJB4	c.238C>T (p.Gln80Ter)	rs114429815	C	0.99	0.98	0.2425	0.99	0.7962	0.96	0.0013	1.00	0.6744	1.00	0.0228	1.00	0.0229
			T	0.01	0.02		0.01		0.04		<0.01		0.00		0.00	

p-values less than 0.05 were considered as significant

6.4.3. Evolutional evaluation of amino acid at position 119 of *GJB4* protein

Since the pathogenetic analysis suggested *GJB4* c.356A>C (p.Asn119Thr) as a likely pathogenic variant, a multiple sequence alignment was performed with *GJB4* protein sequences from different species to investigate the evolutional conservation of the amino acid residue at position 119 of the protein (Figure 5.2). Asparagine (Asn) at position 119 was conserved among all the different species investigated suggesting that the residue is important for the protein's function. It is worth mentioning that some of the amino acid residues around the asparagine 119 were not conserved among some of the species studied.

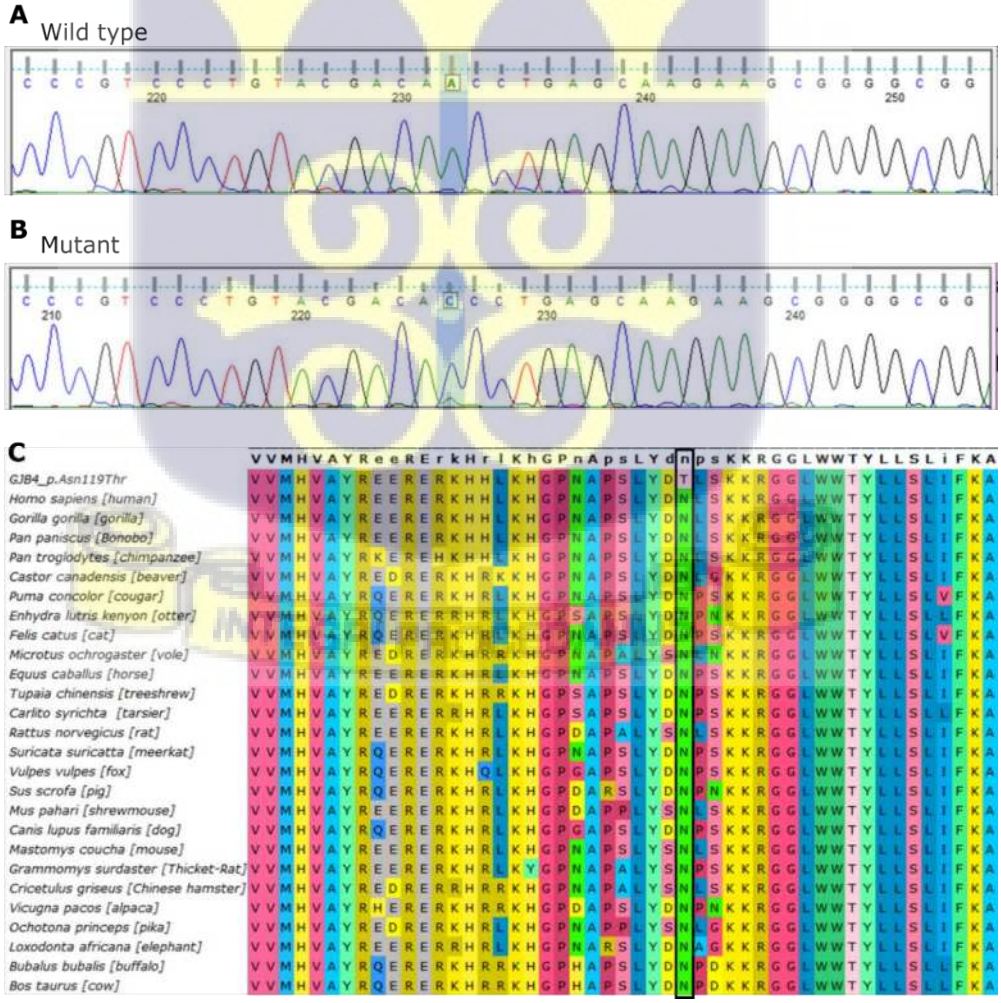


Figure 6.2: Chromatograms and multiple sequence alignment of *GJB4* p.Asn119Thr variant. *Chromatogram of Sanger sequence of (A) wild type and (B) mutant of GJB4 c.356A>C (p.Asn119Thr) variant. The position of the nucleotide change*

is highlighted in blue (C) Multiple sequence alignment of GJB4 protein in different species. Position 119 for the c.356A>C (p.Asn119Thr) variant is boxed.

6.4.4. Modelling of wild type and mutant (c.356A>C (p.Asn119Thr)) GJB4 protein

We examined the possible molecular effect of the change in the conserved amino acid at position 119 of the protein by modelling and comparing the wild type and *GJB4* c.356A>C (p.Asn119Thr) mutant proteins. Good quality models with DOPE scores of ~ -26500 were obtained from the modelling experiment from which the best models were selected. Multiple-template modelling performed better than single-template modelling (Figure 6.1E). Both models were evaluated and found to be within the range of expected values for X-ray crystallography-determined and nuclear magnetic resonance (NMR)-determined proteins. Z-scores of -4.56 and -4.28 were obtained for wild type and mutant (c.356A>C (p.Asn119Thr)) *GJB4* proteins (Figure 6.3A and B) respectively. In addition, more than 98% of the residues were observed to fall within favorable and allowed regions on the Ramachandran plot with highly favorable ProsA Z-scores for both models (Figure 6.3C and D).

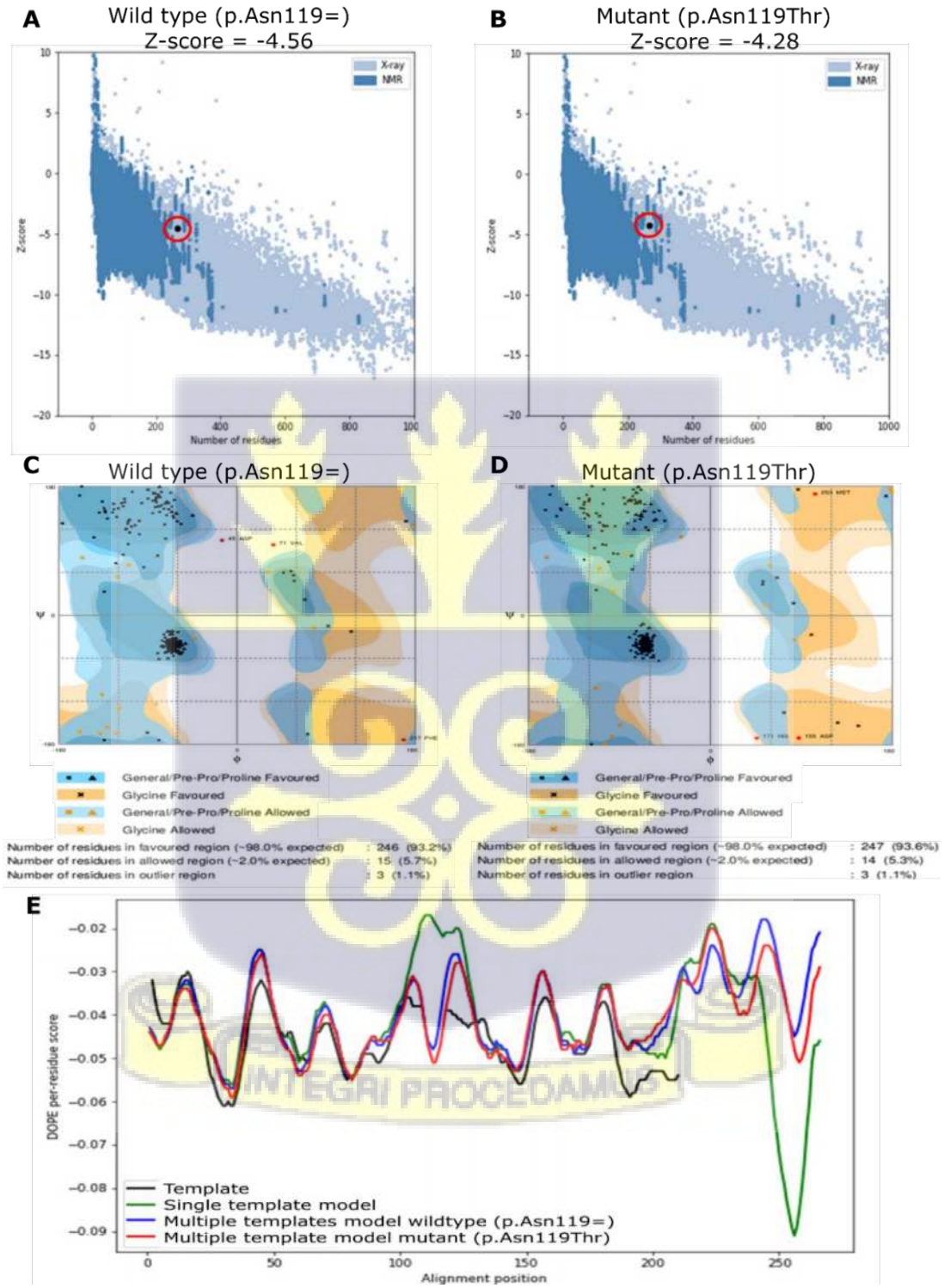


Figure 6.3: Evaluation and validation of *GJB4* protein models. *ProsA web evaluation of (A) wildtype (p.Asn119=) and (B) mutant (p.Asn119Thr) proteins. Ramachandran plot of (C) wildtype (p.Asn119=) and (D) mutant (p.Asn119Thr) proteins. (E) Discrete Optimized Protein Energy (DOPE) profile for wildtype (p.Asn119=) and mutant (p.Asn119Thr) proteins.*

The Galaxy refinement of the wild type and mutant *GJB4* proteins produced 10 models, from which we selected the best-refined (Figure 5.4A and B). The model labelled "MODEL 1" appeared to be the overall best for the wild type, while the model "MODEL 7" appeared as the best-refined for the mutant (Figure 5.4A and B). Figures 5.4C and D show the quality improvement of the selected refined models compared to the unrefined models.

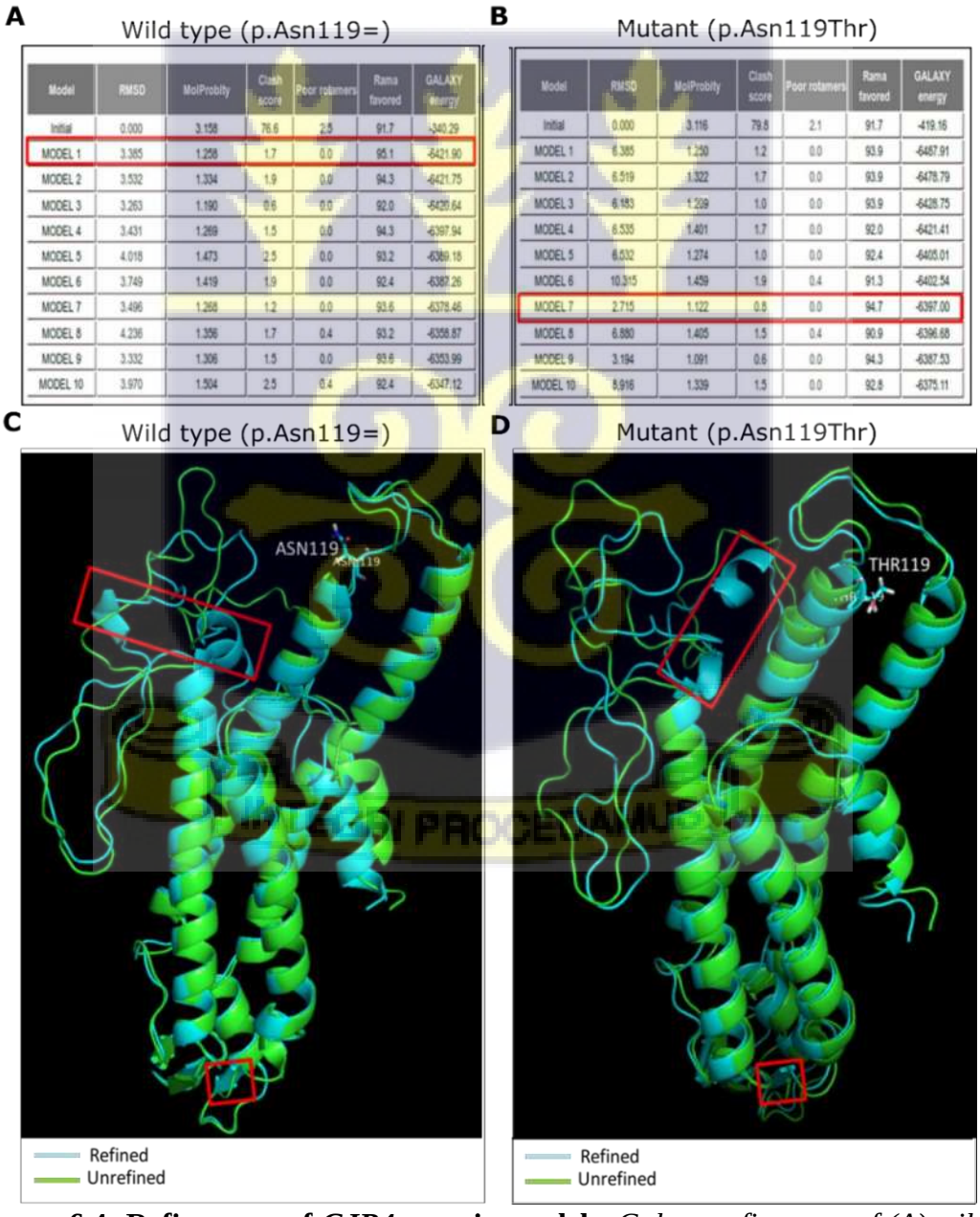


Figure 6.4: Refinement of *GJB4* protein models. *Galaxy refinement of (A) wild type and (B) mutant c.356A>C (p.Asn119Thr)* GJB4 *protein models. The best-ranked models*

are highlighted with red rectangles. Refined and Unrefined models of (C) wild type and (D) mutant GJB4 *c.356A>C (p.Asn119Thr)* GJB4 *protein models.*

The *GJB4* c.356A>C (p.Asn119Thr) mutation slightly modifies the protein structure, which we can observe when the mutant protein is compared with the wild-type protein. On the wild-type protein, asparagine at position 119 forms part of a random coil, however, the same position in the mutant model harboring a threonine residue forms a helix (Figure 6.4). There was, generally, a high degree of conservation of the extracellular E1 and E2 loops, as expected. Refinement further saw the modeling of two short helixes in the C-terminus, in regions of random coil expected for gap junction proteins (Figure 6.5).

6.4.5. Virtual Screening

Connexins are characterized by four transmembrane helices that form the transmembrane pore and extracellular domains; these provide two loops (E1 and E2) that help in cell-cell recognition and docking. These loops are mostly involved in protein-protein interactions, while residues on the alpha-helix transmembrane domains are involved in the process of small molecule shuttling. To the best of our knowledge, the *GJB4* c.356A>C (p.Asn119Thr) mutation (rs190460237) has not been previously reported, hence we modelled the 3D structures of *GJB4* wild type and mutant proteins which revealed subtle but fundamental differences that may have significant implications on the protein function. To assess the possible effect of these differences, we performed virtual screening for ligands using the Galaxy server's Site algorithm. The virtual screening predicted four ligands and their corresponding binding sites for the wild type *GJB4* (1KS, SNT, A8T, and SG8) and five ligands for the mutant *GJB4* c.356A>C (p.Asn119Thr) (NEC, 1KS, SNT, A8T, and SG8) proteins. Although none of the ligands interact with the position 119 residues of both the wild type and mutant models, it appears

that the residue change caused a perturbation in the protein structure which likely modified the conformation of the binding site to alter ligand binding (Figure 6.5).

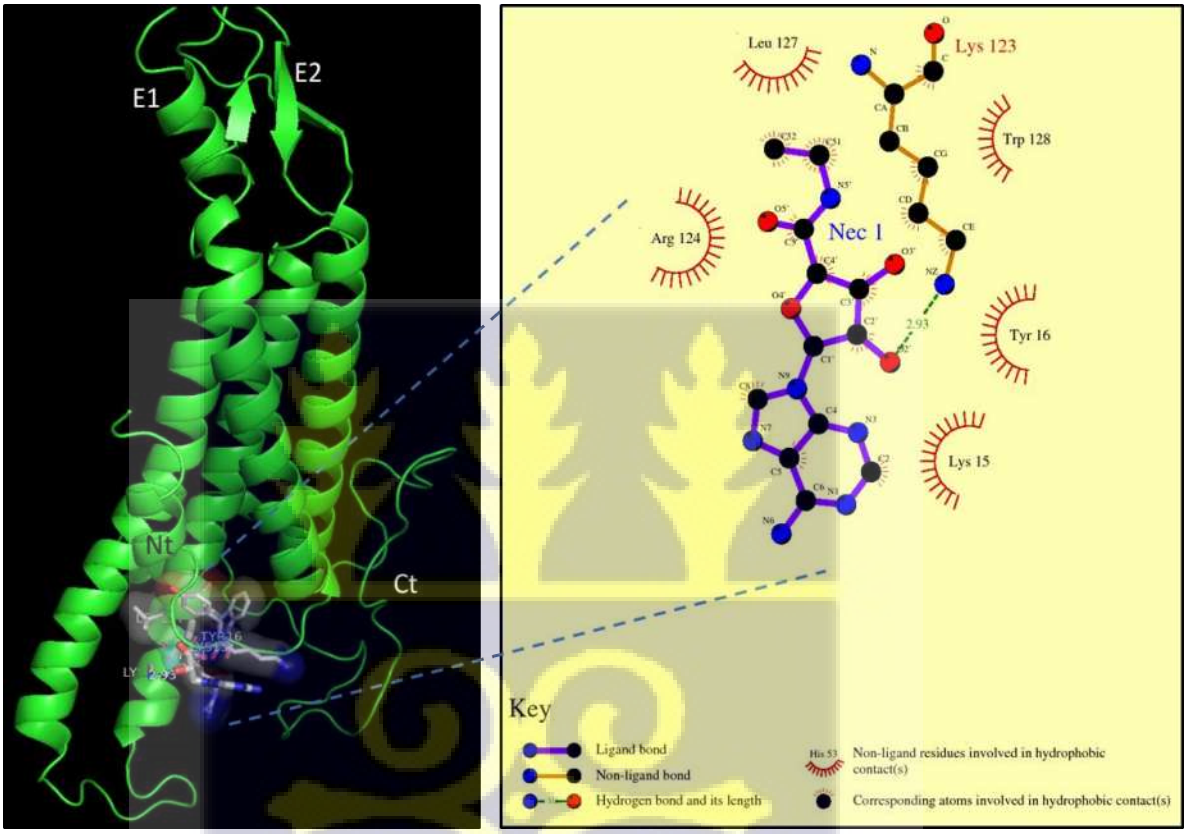


Figure 6.5: *GJB4* mutant protein in complex with NEC. A LigPlus plot shows the interacting residues in detail.

6.5. Discussion

Mutations in connexin genes have been implicated in about 28 genetic diseases, with HI and skin disorders as the predominant cases (Srinivas *et al.*, 2018). Although the *GJC3* gene has been associated with non-syndromic HI with specific pathological alterations in the cochlea (Wingard & Zhao, 2015; Wong *et al.*, 2017), there are limited studies globally and especially from Africa. Unlike other epidermal disease-associated connexins, the role of *GJB4* variants in NSHI is not well elucidated (Lopez-Bigas *et al.*, 2002). To the best of our knowledge, this is the first report on *GJB4* and *GJC3* variants

in African hearing-impaired patients and will add to current knowledge, as well as assist in refining gene-disease pairs and clinical validity curation.

Mouse models created with alterations in the *GJC3* gene indicated that about 50% of homozygous *GJC3* null mice had delayed maturation of hearing thresholds, high-frequency hearing loss, and were vulnerable to noise-induced hearing loss (Bult *et al.*, 2019). An earlier study, however, did not describe any significant difference between the phenotypes (including auditory brainstem response) of the *GJC3* deficient and the wildtype control adult mice (Eiberger *et al.*, 2006). The authors stated that the gene might be functionally associated with other connexins such as connexin 32 and connexin 47 which suggested that it may not be independently associated with the HI phenotype. Our study identified the p.Pro164Ser (c.490C>T/rs73405465) variant in the *GJC3* gene of both hearing-impaired and hearing individuals in Ghana with a 0.94 odds ratio. The missense *GJC3*-p.Pro164Ser variant had a minor allele frequency of 0.064 in African population (Cunningham *et al.*, 2018) which is greater than the threshold of 0.050 used for calling uncommon variants. Considering the odds ratio, minor allele frequency, and occurrence of the variant in controls, the *GJC3*-p.Pro164Ser variant may not be associated with HI. The *GJC3* p.Pro164Ser variant had no record/phenotypic data in ClinVar (Landrum *et al.*, 2018) and Ensembl (Cunningham *et al.*, 2018) and was labelled as benign, non-pathogenic, neutral, or polymorphism by the majority of predictive tools used (Table S1) as well as on the VarSome database (Kopanos *et al.*, 2019), further supporting its non-pathogenicity.

The expression pattern and contribution of *GJB4* to HI remain unclear. A *GJB4* deficient mouse model generated by replacing the coding region of *GJB4* with a lacZ gene did not show any auditory abnormality when assessed by brain stem evoked potentials (Zheng-

Fischhöfer *et al.*, 2007). Interestingly, these mice did not show any skin abnormality, which made it difficult to interpret the role of *GJB4* in humans, however, there have been some studies that investigated and detected *GJB4* gene variants in deaf individuals (Kooshavar *et al.*, 2013; Laleh *et al.*, 2017; Yang *et al.*, 2010). In a rat study, *GJB4* was found to be expressed in rat cochlea, suggesting its role in the hearing process. The present study identified synonymous *GJB4* variants (p.Lys123=, p.Arg101=, and p.Thr172=) in both affected and control samples of which p.Lys123= and p.Thr172= were classified as benign and variant of uncertain significance respectively. But these three variants had no effect on the resultant protein; hence they may not be responsible for HI pathogenesis. We also identified *GJB4* p.Arg151Ser and p.Gln80Ter variants previously predicted to be benign. There was no published data on the *GJB4* p.Gln80Ter variant in hearing HI patients. Similar to our study results, *GJB4* p.Arg151Ser was found in both HI patients and controls in Iran (Kooshavar *et al.*, 2013) suggesting that it may not be associated with the HI phenotype. The variant was associated with skin disorders and found in patients without hearing loss (Alexandrino *et al.*, 2009; Common *et al.*, 2005) hence confirming the above observation.

Similar to our findings, a Spanish study also identified the *GJB4*-p.Glu204Ala in hearing-impaired patients (Lopez-Bigas *et al.*, 2002). Although the majority of bioinformatics tools used in this study predicted the variant as likely pathogenic, we found the variant in both control and affected samples which is consistent with findings from Iran (Kooshavar *et al.*, 2013); our findings suggest that there is no likely association between the *GJB4* p.Glu204Ala variant and HI. The p.Asn119Thr variant may be of clinical significance since it was reported as “likely pathogenic”, according to InterVar and the majority of the predictive tools (Table S1). *GJB4* p.Asn119Thr was predicted to be a variant of uncertain significance by VarSome (Kopanos *et al.*, 2019). According to

the automated clinical interpretation of genetic variants by ACMG/AMP 2015 guideline (Li & Wang, 2017), the variant was found to fall within the categories of PM1, PM2, PP3, and BP1. This implies that the variant is located within a mutational hot spot or a well-established functional domain without benign variation (PM1), and absent from controls in the ESP, 1000Genomes and ExAC databases with extremely low frequency if recessive (PM2). with multiple lines of computational evidence supporting a deleterious effect of the gene product (PP3) (Li & Wang, 2017). A supporting evidence for benign status of a missense variant in a gene which when truncated are known to cause disease (BP1)(Li & Wang, 2017). When the variant was analyzed for "Pathogenic variants Enriched Regions (PER) for genes and gene families" in the PER viewer (Pérez-Palma *et al.*, 2020), it was observed to fall within a region of pathogenic missense burden for both gene family-wise and gene-wise analyses (Figure S2). PER sources disease-associated missense variants from ClinVar and the Human Gene Mutation Database (HGMD), retaining only "pathogenic" and/or "likely pathogenic" variants in ClinVar, and variants with "high confidence" calls in HGMD, all in the GRCh37.p13/hg19 coordinate. Interestingly, *GJB4* p.Asn119Thr (N_119) variant was observed to align with a *GJB2* variant (E_120) which is associated with sensorineural hearing loss (Pérez-Palma *et al.*, 2020). Our study identified the variant in one patient with allele frequency less than 0.01 and none in the control population, but there was not enough evidence to conclude on its pathogenicity.

Analysis of *in silico* protein modelling revealed a striking difference between wildtype and mutant models of the p.Asn119Thr variant. The asparagine at position 119, which is on a cytoplasmic loop, forms random coils in the wild type model, whereas threonine in the same position forms a helix in the mutant model. It appears that the presence of Threonine at this position increases the overall propensity for a helix.

The ligand-binding property of the mutant p.Asn119Thr protein was slightly different from the wild type *GJB4* protein. An extra ligand, N-Ethyl-5'-Carboxamido Adenosine (NEC), was found to bind the *GJB4* p.Asn119Thr mutant protein and not the wild type. NEC (DB03719) is a non-carcinogenic purine nucleoside, a cAMP/cGMP phosphodiesterase (PDE) inhibitor (Knox *et al.*, 2010) that doubles as a human adenosine A (2A) receptor agonist (Lebon *et al.*, 2011). PDE inhibitors are often used in the treatment of erectile dysfunction because of their adenosine A (2A) receptor agonist role. Post-marketing and retrospective clinical trial analysis has shown that these PDE inhibitors have severe side effects such as hearing loss (Huang & Lie, 2013). However, the above observation is inconclusive as there is no direct association established between hearing loss and PDE inhibitors.

6.6. Limitation of the study

The study identified a rare missense variant *GJB4*-p.Asn119Thr, but only in a single hearing-impaired patient which makes it difficult to associate the variant to the hearing impairment phenotype. The pathogenicity of the variant was predicted using *in-silico* predictive tools. Although these tools give a good prediction of the possible clinical effect of the variant which is very useful, they are not as accurate as functional assays; we therefore recommend the use of cell and animal models to confirm the pathogenicity of the *GJB4*-p.Asn119Thr variant.

6.7. Conclusions

In this study, only one possibly pathogenic *GJB4* variant (p.Asn119Thr) was identified in a hearing-impaired patient. The protein modelling and virtual screening identified differences in the protein structure and binding properties of the mutant p.Asn119Thr *GJB4* protein compared to the wild type. There is a need for functional studies and

investigations from larger populations to elucidate the pathogenicity of the variant (*GJB4*-p.Asn119Thr) predicted as "likely pathogenic". We did not identify any *GJC3* variant of clinical significance in the study population. Hence *GJB4* and *GJC3* variants were found not to be significant contributors to non-syndromic autosomal recessive hearing impairment in Ghana. We therefore recommend the used of modern genomic approaches to investigate the associated HI gene variants in the study participants.

6.8. Author's Contributions

Conceptualization, A.W., G.A.A., and S.M.A.; methodology, S.M.A., and K.K.E., validation, A.W., G.A.A., G.K.A., and O.Q.; formal analysis, S.M.A., A.W., and K.K.E.; resources, A.W., G.A.A., G.K.A., and O.Q.; writing—original draft preparation, A.W., S.M.A.; writing—review and editing, S.M.A., K.K.E., O.Q., G.K.A., G.A.A., and A.W.; supervision, A.W., G.A.A., G.K.A., and O.Q.; funding acquisition, A.W. and G.A.A. All authors have read and agreed to the published version of the manuscript.

6.9. Supplementary materials

Table S6.1: In silico prediction of clinical significance/pathogenicity of *GJB4* and *GJC3* variants

	GJC3	*GJB4*						
	c.490C>T (p.Pro164Ser)	c.611A>C (p.Glu204Ala)	c.451C>A (p.Arg151Ser)	c.516T>C (p.Thr172=)	c.369G>A (p.Lys123=)	c.356A>C (p.Asn119Thr)	c.303C>G (p.Arg101=)	c.238C>T (p.Gln80Ter)
Prediction tool	rs73405465	rs3738346	rs78499418	rs111693060	rs142843509	rs190460237	rs138184343	rs114429815
VarSome	Benign	Benign	Benign	Benign	Uncertain Significance	Uncertain Significance	Uncertain Significance	Uncertain Significance
ClinVar	-	Benign	-	-	-	-	-	-
Align GVGD	Non-pathogenic	Pathogenic	Non-pathogenic		-	Pathogenic	-	-
FATHMM	Damaging	Damaging	Damaging	Synonymous	Synonymous	Damaging	Synonymous	Neutral
MutationAssessor	Low	High	Low	-	-	Medium	-	-
MutationTaster	Polymorphism	Likely disease causing	Polymorphism	-	-	Disease causing	-	Disease causing
MutPred2	Non-pathogenic	Pathogenic	Non-pathogenic	-	-	Non-pathogenic	-	-
PROVEAN	Neutral	Deleterious	Neutral	Neutral	Neutral	Deleterious	Neutral	-
PolyPhen-2	Possibly damaging	Damaging	Benign	-	-	Possibly damaging	-	-
SIFT	Tolerated	Deleterious	Tolerated	Tolerated	Tolerated	Tolerated	Tolerated	-
EIGEN	Benign	Pathogenic	Benign			Benign		Pathogenic
MPV	Benign		-			Pathogenic		
PrimateAI	Tolerated	Tolerated	Tolerated			Tolerated		Tolerated
InterVar	Benign	Benign	Benign	Benign	Benign	Likely pathogenic	Benign	Benign

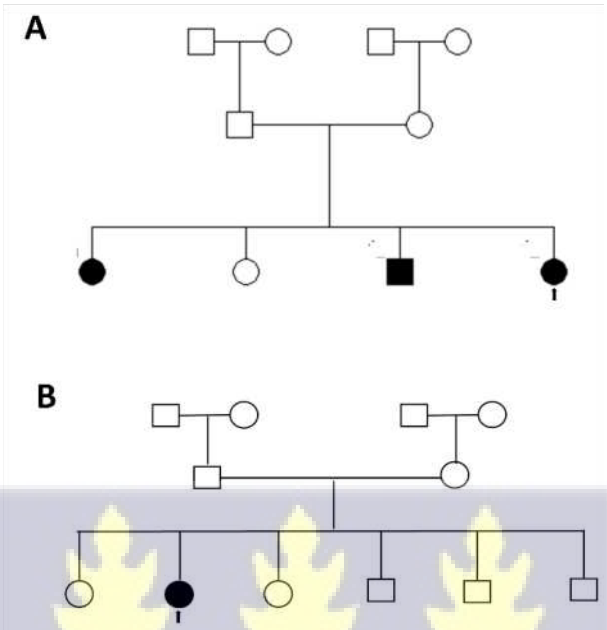


Figure S6.1: Representative pedigree showing *A) multiplex and B) simplex families with hearing impairment The black shaded square and circles were used to denote hearing-impaired males and females, respectively. The unshaded squares and circles correspond to hearing males and females*

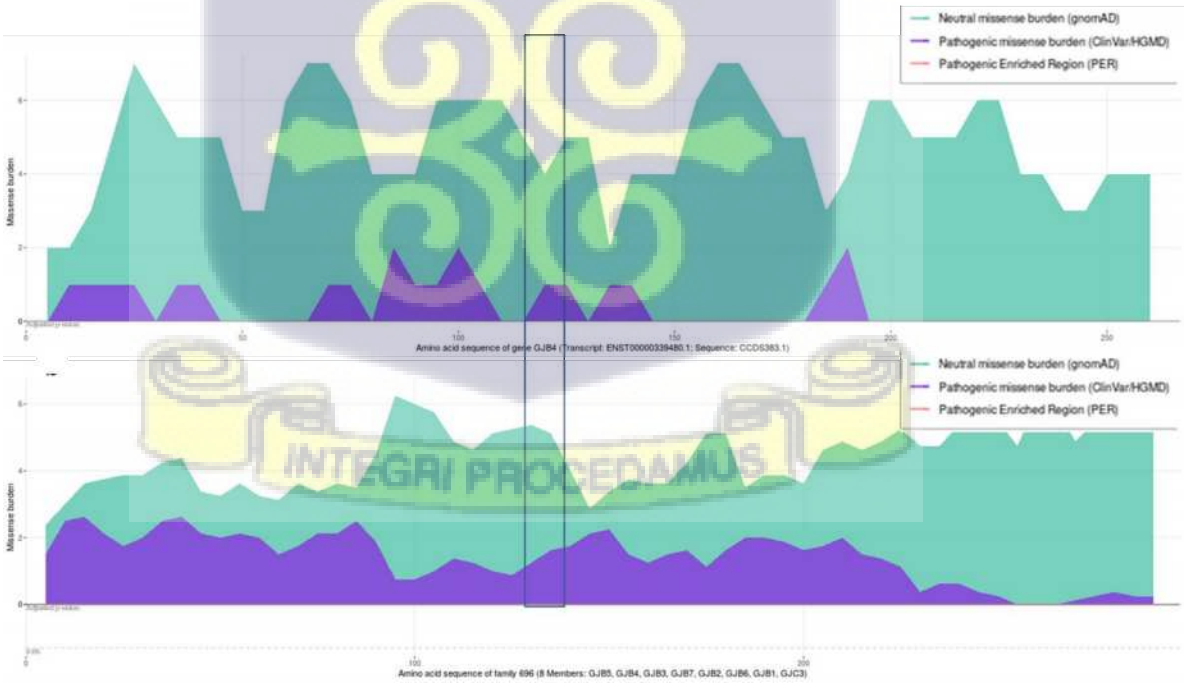


Figure S6.2. Gene-wise and Gene Family-wise PER analysis. *A) Gene-wise (GJB4) PER analysis showing a region of high pathogenic burden harboring the Asn119Thr mutation B) Gene family-wise PER analysis showing a more extensive pathogenic enriched region in the gap junction beta family proteins*

CHAPTER SEVEN

7.0. General discussion

The major etiologies of HI can be broadly classified into two main groups, thus genetic/inheritable and environmental causes (Adadey *et al.*, 2017; WHO, 2019; Wonkam *et al.*, 2013). Meningitis remains as one of the major environmental causes of HI in Ghana (Adadey *et al.*, 2019; Brobby, 1988) and other parts of Africa (Wonkam *et al.*, 2013). In global terms, studies have shown that over 50% of people with HI have possible genetic factors as the cause of their HI and 80% of these are reported as non-syndromic (Wu *et al.*, 2018; Zhou *et al.*, 2019). To date, out of the over 123 genes associated with NSHI (Van Camp G & Smith, 2020), *GJB2* mutations are the most frequently reported (Karami-Eshkaftaki *et al.*, 2017; Laird *et al.*, 2017). Although *GJB2* gene variants have high prevalence among Asians and Caucasians, their contribution to HI in Africa is almost negligible (Wonkam, 2015; Wonkam *et al.*, 2015) with only a few reported cases from Morocco (Gazzaz *et al.*, 2005; Ratbi *et al.*, 2007), Sudan, and Kenya (Gasmelseed *et al.*, 2004) and Ghana (Adadey *et al.*, 2019; Brobby *et al.*, 1998; Hamelmann *et al.*, 2001). This thesis describes the examinations of the major factors responsible for pre-lingual and post-lingual hearing HI and the contribution of *GJB2*, *GJB6*, *GJB4*, and *GJC3* mutations to familial and non-familial HI in Ghana.

Severe to profound sensorineural HI is known to be the most common type of HI among children (Chakrabarti & Ghosh, 2019) and, if not managed early, can delay the lingual, intellectual, and cognitive development (Chakrabarti & Ghosh, 2019). The majority of the study participants enrolled in the studies described in this thesis were found to have severe to profound HI. Many of these participants received a comprehensive test for hearing only when they were about start formal education at the ages of 6 to 11 years. The late HI diagnoses of HI among these children significantly affected their academic

and cognitive performance compared to their able hearing counterparts (Barnard *et al.*, 2015).

Medical reports of the hearing-impaired participants identified cerebrospinal meningitis, convulsion (with undetermined cause), and complicated malaria, among other established environmental factors as the major causes of post-lingual HI in these studies. Although a large proportion of post-lingual HI cases are due to preventable environmental factors (Wonkam *et al.*, 2013), the limited and under-resourced health care facilities in Africa are inadequate to reduce the burden of the condition. A high prevalence of prelingual HI known to be common among HI children was supported by our study (Chibisova *et al.*, 2018). More than half of prelingual HI cases are known to have genetic etiologies (Behlouli *et al.*, 2016; Wonkam *et al.*, 2013), hence, our studies focused on investigating the gene variants associated with HI in Ghanaian populations.

In 1998, the founder variant, *GJB2*-Arg143Trp was reported in Adamorobe, a village in the Eastern Region of Ghana (Brobby *et al.*, 1998). A follow-up nationwide study in 2001 gave a 16% prevalence of the variant (Hamelmann *et al.*, 2001). Our study demonstrated that over a quarter of all familial HI cases (multiplex families) in Ghana are associated to the founder mutation (Adadey *et al.*, 2019). In simplex families, the founder mutation was found in 7.9% of investigated cases and 1.4% in the control populations (Adadey *et al.*, 2019), although the higher prevalence of the *GJB2*-Arg143Trp in the multiplex families compared to the previous report could perhaps be due to the purposive sampling technique employed by our study. This variant is, however, not exclusive to Ghana, as it has also been reported in Japan (Kasakura-Kimura *et al.*, 2017; Zheng *et al.*, 2015), South Korea (Kim *et al.*, 2016b), China (Luo *et al.*, 2017) and Mexico (Martínez-Saucedo *et al.*, 2015). The high prevalence of the founder

mutation in Ghana calls for its clinical investigation in the hearing newborn screening program in Ghana.

GJB2-Arg143Trp is a coding region variant found on the second exon of *GJB2* gene and located with the transmembrane domain of the resultant protein (Mani *et al.*, 2009). The *GJB2*-Arg143Trp gene mutant was identified to be associated with profound HI among Ghanaians (Abe *et al.*, 2018; Brobby *et al.*, 1998; Hamelmann *et al.*, 2001), and the phenotype to genotype correlation of the participants in this study confirmed that patients with the pathogenic TT genotype had profound HI. Carriers of the variants (thus the CT genotype) were found to have similar hearing profile to participants with the non-pathogenic CC genotype. This observation explained the autosomal recessive pattern of inheritance in the families studied.

The high prevalence of the founder mutation *GJB2*-Arg143Trp necessitated the development of a rapid diagnostic tool for easy detection of the gene variant. For the first time, a restriction fragment length polymorphism (RFLP) assay was designed and developed as a rapid screening tool for the founder mutation using the restriction enzyme NciI (Adadey *et al.*, 2020). Previous attempts were made to design rapid tools for screening mutations in HI gene variants (Schade *et al.*, 2003; Schrauwen *et al.*, 2013; Tayoun *et al.*, 2016), however, most of these tests depend on the use of sequencing technologies. Although NGS has been proposed as the most effective tool for screening HI gene variants (Gao & Dai, 2014), it is expensive and not easily adoptable for clinical investigations in developing countries (Calistri & Palù, 2015). To design cost-effective HI screening tools, population-specific gene variants must be considered as described by some studies (Abe *et al.*, 2018; Brown & Rehm, 2012; Yan *et al.*, 2017). The test is very useful within the Ghanaian population due to the high prevalence of *GJB2*-Arg143Trp mutation in Ghana.

The *GJB2*-Arg143Trp NciI test had high accuracy, sensitivity, specificity, and predictive values, attributes of a good diagnostic tool for clinical use (Baratloo *et al.*, 2015; Šimundić, 2009). Although the test was found to be extremely sensitive with respect to Sanger sequencing, its specificity depends on the variations present at the restriction site of the NciI enzyme. Therefore, the test cannot effectively discriminate between variations such as p.Y142del (c.424_426delTTC), p.Phe142Leu (c.426C > A), and p.Arg143Gln (c.428G > A). The above *GJB2* variations would give similar results as the *GJB2* Arg143Trp variant for example.

Further analysis of the *GJB2* gene coding sequence identified a Ghanaian family with a p.Typ44Ter variant. This is the first report of this variant in Ghana, however, it had been previously reported in Mexico, where it was also shown to be a common founder mutation within the deaf population of Guatemala (Martínez-Saucedo *et al.*, 2015), this nonsense mutation leads to the production of a non-functional truncated protein, which explains the molecular mechanism of pathogenesis of the variant (Martínez-Saucedo *et al.*, 2015).

Recent evidence based on mouse models has shown that variants in the region of DNA that codes for GJB6 protein do not result in HI, though large deletions in the *GJB6* gene are in fact associated. This implies that *GJB6* itself has no contribution to the development of HI (Ahmad *et al.*, 2007; Rodriguez-Paris & Schrijver, 2009). Since it is only the large *GJB6* deletions that contribute to the development of HI, the hearing-impaired samples from the study population were screening for *GJB6*-D13S1830 deletion, however none of the samples tested positive for this deletion. Data from Africa has suggested that *GJB6* gene variants may not contribute to HI Africa (Wonkam, 2015; Wonkam *et al.*, 2015) which is consistent with our results and those of the mouse models mentioned above.

Connexin genes including *GJB4* and *GJC3* were found to be linked with several diseases, HI and skin disorders are the commonly associated examples (Srinivas *et al.*, 2018). They are associated with skin disorders, just as all connexins are, though their contribution to HI is unclear. *GJB4* variants have been identified among HI patients from Iran (Kooshavar *et al.*, 2013; Laleh *et al.*, 2017) and Taiwan (Yang *et al.*, 2010) and likewise some studies from Taiwan (Yang *et al.*, 2010) and India (Ramchander *et al.*, 2010) have also identified *GJC3* variants in HI patients. To map the genetic etiologies of HI in the *GJB2 and GJB6* negative samples, we investigated *GJB4* and *GJC3* coding variants.

The only *GJC3* variant identified within the Ghanaian HI cohort was p.Pro164Ser (c.490C>T/rs73405465). *GJC3* knockout mouse models have shown delayed maturation of hearing and were found to be predisposed to noise-induced HI (Bult *et al.*, 2019) suggesting the importance of the gene to the development of normal hearing. The p.Pro164Ser mutant however may not be associated with HI since it was identified in both hearing and deaf participants. In addition, the minor allele frequency of the variant is greater than the threshold of 0.05 in the Ghanaian and African populations (Cunningham *et al.*, 2018). The missense variant also had an odds ratio of 0.94 within the Ghanaian population. The report on VarSome (Kopanos *et al.*, 2019) and other predictive bioinformatic tools used confirmed that p.Pro164Ser mutant may not be responsible for the HI phenotype, therefore, there is a need to conduct animal studies to concluded on its pathogenicity.

GJB4 variants have also been identified in hearing-impaired participants (Kooshavar *et al.*, 2013; Laleh *et al.*, 2017) however their contribution to the etiology of HI remains unknown, and mice deficient of the *GJB4* gene interestingly do not develop HI or any skin disorders (Zheng-Fischhöfer *et al.*, 2007). A study on mouse and human hearing

loss genes showed that some mutant mice with genes associated with HI had normal auditory thresholds (Ingham *et al.*, 2019), but we also know that the *GJB4* gene is expressed in the cochlear of rats, implicating it in auditory functions (Wang *et al.*, 2010a), so the picture provided by the data is currently uncertain.

In the chapter 5 of this thesis, 3 *GJB4* variants that were found to be synonymous variants (p.Lys123=, p.Arg101=, and p.Thr172=) and do not have any effect on the protein structure were identified among both control and hearing-impaired samples, and two variants (p.Arg151Ser and p.Gln80Ter) predicted as benign by bioinformatic tools and online databases were also found in both categories of participants. It was evident that these latter two *GJB4* variants do not associate with the HI phenotype in the study population. A previous study in Iran (Kooshavar *et al.*, 2013) also found the *GJB4* p.Arg151Ser variant in both hearing-impaired and control samples. The variant was however identified in dermatitis patients with normal hearing (Alexandrino *et al.*, 2009; Common *et al.*, 2005).

Further analysis of the coding sequence of the *GJB4* gene identified two non-synonymous variants (p.Glu204Ala and p.Asn119Thr) that ware predicted by bioinformatic tools as "likely pathogenic". Similar to our study, researchers in Spain identified p.Glu204Ala in a cohort of HI patients (Lopez-Bigas *et al.*, 2002) although the *GJB4* p.Glu204Ala variant does not appear to be associated with HI since it was found in hearing-impaired and hearing-participants, both in one of our studies and also in a study from Iran (Kooshavar *et al.*, 2013). In addition to the above variant, we found *GJB4* p.Asn119Thr variant in single deaf patient and not in the controls; this variant had minor allele frequency less than 0.001 in our study population, and also the African and global populations, suggesting that it is a rare variant of clinical importance. Based on the guidelines by automated clinical interpretation of genetic variants by ACMG/AMP

in 2015 (Li & Wang, 2017), the variation p.Asn119Thr is classified as likely pathogenic. This follows from the fact that the variant satisfied the various ACMG/AMP 2015 guideline categories (PM1, PM2, PP2, and BP1) as explained below:

1. PM1: the variant is situated within a hot spot for mutations or at a site well known as a functional domain without variants classified as benign.
2. PM2: absence of the variant from control population in the ESP, ExAC, and 1000Genomes databases or variants with extremely low frequencies
3. PP2: the gene is characterized as having an extremely small number of benign missense variants.
4. BP1: the variant is found in a gene that is known to be pathogenic when truncated.

In addition to the above, the variation in *GJB4* (p.Asn119Thr) was analyzed for "Pathogenic variants Enriched Regions (PER) for genes and gene families" in a PER viewer (Pérez-Palma *et al.*, 2020). The PER analyses revealed that the variant fell within the pathogenic missense zones when it was analyzed using gene family-wise and gene-wise methods. The PER viewer is built on authentic data from ClinVar and the Human Gene Mutation Database (HGMD). To further corroborate the claim that the variant is likely pathogenic, the alignment of *GJB4* p.Asn119Thr identified with a *GJB2* variant (E_120) (Figure S6.2) which is well linked to HI (Pérez-Palma *et al.*, 2020).

To predict the effect of the *GJB4* p.Asn119Thr variant on the protein structure and function, *in silico* methods were used to model the mutant and wildtype proteins. The protein model showed that asparagine at position 119 was at the cytoplasmic region of the protein. Comparison of the wildtype and mutant protein revealed that threonine at position 119 favors the formation of a helix in the mutant protein whereas, in the wildtype protein, asparagine at the same position forms a random coil, the change in amino acid at the 119 position has slightly altered the binding properties of the protein. The binding

assay predicts four common ligands for both the wildtype and the mutant proteins, and an extra ligand was predicted to bind only the mutant and the wildtype protein. This ligand belongs to a class of compounds known as "non-carcinogenic purine nucleoside, a cAMP/cGMP phosphodiesterase (PDE) inhibitors" (Knox *et al.*, 2010). The compound was found to act as adenosine A (2A) receptor agonist in humans (Lebon *et al.*, 2011). It is worth noting that the PDE inhibitors have hearing impairment as one of their associated side effects, however, this is not enough to draw any meaningful conclusion since the PDE inhibitors do not have any direct association with hearing impairment, and the affected patient was found to have congenital hearing loss.

7.1. Limitations of the studies in this thesis

1. The studies in this thesis used targeted sequencing approaches to investigate the genetic causes of HI, which are effective but not comprehensive: the techniques were able to identify HI gene variants in 25.9% of familial cases and 7.9% of isolated cases.
2. The *GJB2*-Arg143Trp-NciI-RFLP test cannot discriminate between variants of *GJB2* located within the recognition site of the restriction enzyme. This impacts on the specific of the RFLP test.
3. From our study population, we found the *GJB4* variant, p.Asn119Thr, in only one affected individual. Although it was predicted as likely pathogenic, there is no biological experimental evidence to confirm the predictions made by the bioinformatic tools.

7.2. Conclusion

This Ph.D. work investigated connexin gene (*GJB2, GJB4, GJB6,* and *GJC3*) variants among hearing-impaired and control participants in Ghana. The genetic analysis of the

GJB2 gene showed that over a quarter of familial HI cases tested positive for the founder mutation Arg143Trp. This implies that the variant can explain the cause of deafness in over 26% of Ghanaian families with two or more affected family members. The high prevalence of the variant in the study population compelled us to investigate its carrier frequency, which we observed to be 1.4% in the randomly selected hearing control participants. Deaf participants with the TT genotype had severe to profound HI. There was however no statistically significant difference between the degree of hearing of the carriers with CT genotype compared to the homozygote CC genotype. For the first time in Ghana, we reported the presence of a *GJB2* Trp44Ter variant in one hearing-impaired family. The two *GJB2* gene variants uncovered in a study in this thesis exhibited an autosomal recessive mode of inheritance.

To reduce the burden of HI in Ghana, a *GJB2*-Arg143Trp-NciI-RFLP test was developed and validated. Using Sanger sequencing as the standard, the *GJB2*-Arg143Trp-NciI-RFLP test was found to be 100% sensitive, although the test is unable to differentiate between *GJB2* variants found within the recognition site of the restriction enzyme. Nevertheless, the test will be very useful in Ghana, since other *GJB2* variants that the test cannot discriminate against are absent from the Ghanaian population.

We sequenced and analyzed *GJB6*, *GJB4*, and *GJC3* genes to search for gene variants associated with HI. From the study population, we identified no *GJB6* variant. One *GJC3* mutation was found in both control and hearing-impaired samples hence this variant may not associate with HI. Out of the seven *GJB4* variants identified, one (p.Asn119Thr/ rs190460237) was found to be “likely pathogenic” while the rest six were either benign or of uncertain significance. No variant was found in the familial cases segregated with the HI phenotype. Protein modelling revealed some changes in the protein structure of the mutant (p.Asn119Thr) compared to the wild type, specifically the protein-ligand

binding prediction showed that the mutant (p.Asn119Thr/c.356A>C) protein and had the extra binding affinity to N-Ethyl-5'-Carboxamido Adenosine (DB03719). This ligand (DB03719) is known to be a cAMP/cGMP phosphodiesterase (PDE) inhibitor as well as adenosine A (2A) receptor agonist.

7.3. Recommendations

1. Consenting hearing-impaired students from 11/15 schools for the deaf and deaf participants in Adamorobe were enrolled onto the project. The study could not identify hearing-impaired patients who were not enrolled in these schools; hence, these children were missed during recruitment. It is therefore recommended that future studies should involve the four non-participating schools for the deaf and hearing-impaired patients who are not in the regular schools for the deaf.
2. The *GJB2*-Arg143Trp-NciI-RFLP test should be used to investigate the carrier frequency in a larger population of hearing controls in Ghana. We recommend the adoption of the *GJB2*-Arg143Trp-NciI-RFLP test by the newborn hearing screening (NHS) program in Ghana to serve as a first of level screening for genetic HI.
3. It is recommended that modern genomic approaches such as next-generation sequencing (NGS) should be used to interrogate other HI genes in patients that tested negative for the investigated connexin genes. The NGS techniques would facilitate the discovery of novel HI genes as well as the identification of the major HI gene variants in Ghana towards to design and development of Ghanaian-specific HI microarray chips. Targeted NGS panels can also be developed to screen the HI genes found in the African population. It is also important to mention that there is a need to study the molecular mechanisms of pathogenesis of the identified gene variants using cell and animal models.

4. Community/public engagement has been initiated to educate and obtain information from society on the genetics of HI. It is recommended that policymakers and health professionals are committed to incorporate genetic screening into the NHS program. We have written a policy document (Appendix C) to aid policymaker's engagement.

Bibliography

Abe, S., Nishio, S. Y., Yokota, Y., Moteki, H., Kumakawa, K., & Usami, S. I. (2018). Diagnostic pitfalls for GJB2-related hearing loss: A novel deletion detected by Array-CGH analysis in a Japanese patient with congenital profound hearing loss. *Clin Case Rep, 6*(11), 2111-2116. doi:10.1002/ccr3.1800

Adadey, S. M., Awandare, G., Amedofu, G. K., & Wonkam, A. (2017). Public health burden of hearing impairment and the promise of genomics and environmental research: A case study in Ghana, Africa. *Omics: a journal of integrative biology, 21*(11), 638-646. doi:10.1089/omi.2017.0145

Adadey, S. M., Manyisa, N., Mnika, K., de Kock, C., Nembaware, V., Quaye, O., . . . Wonkam, A. (2019). GJB2 and GJB6 Mutations in Non-Syndromic Childhood Hearing Impairment in Ghana. *Front Genet, 10*, 1-10. doi:10.3389/fgene.2019.00841

Adadey, S. M., Tingang Wonkam, E., Twumasi Aboagye, E., Quansah, D., Asante-Poku, A., Quaye, O., . . . Wonkam, A. (2020). Enhancing Genetic Medicine: Rapid and Cost-Effective Molecular Diagnosis for a GJB2 Founder Mutation for Hearing Impairment in Ghana. *Genes, 11*(2), 132.

Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P., . . . Sunyaev, S. R. (2010). A method and server for predicting damaging missense mutations. *Nature Methods, 7*(4), 248-249.

Ahmad, S., Tang, W., Chang, Q., Qu, Y., Hibshman, J., Li, Y., . . . Lin, X. (2007). Restoration of connexin26 protein level in the cochlea completely rescues hearing in a mouse model of human connexin30-linked deafness. *Proceedings of the National Academy of Sciences, 104*(4), 1337-1341.

Ahn, J. H., Lee, H.-S., Kim, Y.-J., Yoon, T. H., & Chung, J. W. (2007). Comparing pure-tone audiometry and auditory steady state response for the measurement of hearing loss. *Otolaryngology—Head and Neck Surgery, 136*(6), 966-971.

Akil, O., Seal, R. P., Burke, K., Wang, C., Alemi, A., During, M., . . . Lustig, L. R. (2012). Restoration of hearing in the VGLUT3 knockout mouse using virally mediated gene therapy. *Neuron, 75*(2), 283-293.

Al-Abri, R., Al-Balushi, M., Kolethekkat, A., Bhargava, D., Al-Alwi, A., Al-Bahlani, H., & Al-Garadi, M. (2016). The accuracy of IOS device-based uHear as a screening tool for hearing loss: a preliminary study from the Middle East. *Oman medical journal, 31*(2), 142.

Alexandrino, F., de Oliveira, C. A., Magalhaes, R. F., Florence, M. E., de Souza, E. M., & Sartorato, E. L. (2009). Connexin mutations in Brazilian patients with skin disorders with or without hearing loss. *Am J Med Genet A, 149a*(4), 681-684. doi:10.1002/ajmg.a.32765

Alkowari, M. K., Vozzi, D., Bhagat, S., Krishnamoorthy, N., Morgan, A., Hayder, Y., . . . Abdulhadi, K. (2017). Targeted sequencing identifies novel variants involved in autosomal recessive hereditary hearing loss in Qatari families. *Mutat Res, 800-802*, 29-36. doi:10.1016/j.mrfmmm.2017.05.001

Allen, S. B., & Goldman, J. (2018). Hearing, Inner Ear, Syndromic Sensorineural Loss *StatPearls [Internet]*: StatPearls Publishing.

Alshuaib, W. B., Al-Kandari, J. M., & Hasan, S. M. (2015). Classification of Hearing Loss. *Update On Hearing Loss*, 29.

Amedofu, G. K. (2002). Hearing-impairment among workers in a surface gold mining company in Ghana. *Afr J Health Sci, 9*(1-2), 91-97.

Amedofu, G. K., Ocansey, G., & Antwi, B. B. (2006). Characteristics of hearing-impairment among patients in Ghana*. *Afr J Health Sci, 13*(1), 110-116.

Amedofu, K., Brobby, G. W., & Ocansey, G. (1999). Congenital non-syndromal deafness at Adamarobe, an isolated Ghanaian village: Prevalence, incidence and audiometric characteristics of deafness in the village (Part I). *J Gh Sci Assoc, 1*(2). doi:10.4314/jgsa.v1i2.17806

Angeli, S. I., Suarez, H., Lopez, A., Balkany, T. J., & Liu, X. Z. (2011). Influence of DFNB1 status on expressive language in deaf children with cochlear implants. *Otology & neurotology: official publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology*, *32*(9), 1437.

Antoniadi, T., Pampanos, A., & Petersen, M. B. (2001). Prenatal diagnosis of prelingual deafness: carrier testing and prenatal diagnosis of the common GJB2 35delG mutation. *Prenat Diagn*, *21*(1), 10-13. doi:10.1002/1097-0223(200101)21:1<10::aid-pd968>3.0.co;2-h

Asma, A., Ashwaq, A., Norzana, A. G., Atmadini, A. M., Ruszymah, B. H., Saim, L., & Wahida, I. F. (2011). The association between GJB2 mutation and GJB6 gene in non syndromic hearing loss school children. *Med J Malaysia*, *66*(2), 124-128.

Awuah, P. (2012). Tinnitus among patients in Ghana. *Journal of Biology, Agriculture and Healthcare*, *2*(6), 120-125.

Awuah, P., Duah, I. M., & Amedofu, G. K. (2012). Prevalence of Hearing Loss in Patients with Acute Otitis Media. *Journal of Science and Technology (Ghana)*, *32*(1). doi:10.4314/just.v32i1.2

Bademci, G., Cengiz, F., Foster Ii, J., Duman, D., Sennaroglu, L., Diaz-Horta, O., . . . Alper, H. (2016). Variations in multiple syndromic deafness genes mimic non-syndromic hearing loss. *Scientific Reports*, *6*, 1-7. doi:https://doi.org/10.1038/srep31622

Bainbridge, J. W., Mehat, M. S., Sundaram, V., Robbie, S. J., Barker, S. E., Ripamonti, C., . . . Gardner, P. J. (2015). Long-term effect of gene therapy on Leber's congenital amaurosis. *New England Journal of Medicine*, *372*(20), 1887-1897.

Baratloo, A., Hosseini, M., Negida, A., & El Ashal, G. (2015). Part 1: Simple Definition and Calculation of Accuracy, Sensitivity and Specificity. *Emerg (Tehran)*, *3*(2), 48-49.

Barnard, J. M., Fisher, L. M., Johnson, K. C., Eisenberg, L. S., Wang, N.-Y., Quittner, A. L., . . . Team, C. I. (2015). A Prospective, Longitudinal Study of US Children Unable to Achieve Open-Set Speech Recognition Five Years after Cochlear Implantation.

Otology & neurotology: official publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, *36*(6), 985.

Beck, C., Perez-Alvarez, J. C., Sigruener, A., Haubner, F., Seidler, T., Aslanidis, C., . . . Schmitz, G. (2015). Identification and genotype/phenotype correlation of mutations in a large German cohort with hearing loss. *Eur Arch Otorhinolaryngol*, *272*(10), 2765-2776. doi:10.1007/s00405-014-3157-5

Behlouli, A., Bonnet, C., Abdi, S., Hasbellaoui, M., Boudjenah, F., Hardelin, J.-P., . . . Zenati, A. (2016). A novel biallelic splice site mutation of TECTA causes moderate to severe hearing impairment in an Algerian family. *International Journal of Pediatric Otorhinolaryngology*, *87*, 28-33. doi:10.1016/j.ijporl.2016.04.040

Beyer, E. C., & Berthoud, V. M. (2018). Gap junction gene and protein families: Connexins, innexins, and pannexins. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1860*(1), 5-8.

Beyer, E. C., Paul, D. L., & Goodenough, D. A. (1990). Connexin family of gap junction proteins. *J Membr Biol*, *116*(3), 187-194.

BIAP. (1997). Classification audiométrique des déficiences auditives. *Recommandation biap 02/1 bis.* Retrieved from http://www.biap.org/index.php?option=com_content&view=article&id=5%3Arecommandation-biap-021-bis&catid=65%3Act-2-classification-des-surdites&Itemid=19&lang=en

Birkenhäger, R., Aschendorff, A., Schipper, J., & Laszig, R. (2007). [Non-syndromic hereditary hearing impairment]. *Laryngo-rhino-otologie*, *86*(4), 299-309; quiz 310-293.

Boateng, C. A., & Amedofu, G. K. (2004). Industrial noise pollution and its effects on the hearing capabilities of workers: A study from saw mills, printing presses and corn mills. *Afr J Health Sci*, *11*(1-2), 55-60.

Bosch, J., Lebeko, K., Nziale, J. J., Dandara, C., Makubalo, N., & Wonkam, A. (2014a). In search of genetic markers for nonsyndromic deafness in Africa: a study in Cameroonians and Black South Africans with the GJB6 and GJA1 candidate genes. *OMICS, 18*(7), 481-485. doi:10.1089/omi.2013.0166

Bosch, J., Noubiap, J. J., Dandara, C., Makubalo, N., Wright, G., Entfellner, J. B., . . . Wonkam, A. (2014b). Sequencing of GJB2 in Cameroonians and Black South Africans and comparison to 1000 Genomes Project Data Support Need to Revise Strategy for Discovery of Nonsyndromic Deafness Genes in Africans. *OMICS, 18*(11), 705-710. doi:10.1089/omi.2014.0063

Brobby, G. (1988). Causes of congenital and acquired total sensorineural hearing loss in Ghanaian children. *Tropical doctor, 18*(1), 30-32. doi:10.1177/004947558801800112

Brobby, G. W., Muller-Myhsok, B., & Horstmann, R. D. (1998). Connexin 26 R143W mutation associated with recessive nonsyndromic sensorineural deafness in Africa. *N Engl J Med, 338*(8), 548-550. doi:10.1056/NEJM199802193380813

Brown, K. K., & Rehm, H. L. (2012). Molecular diagnosis of hearing loss. *Curr Protoc Hum Genet, Chapter 9*(1), Unit 9 16. doi:10.1002/0471142905.hg0916s72

Bult, C. J., Blake, J. A., Smith, C. L., Kadin, J. A., & Richardson, J. E. (2019). Mouse genome database (MGD) 2019. *Nucleic Acids Research, 47*(D1), D801-D806.

Burke, W. F., Warnecke, A., Schoner-Heinisch, A., Lesinski-Schiedat, A., Maier, H., & Lenarz, T. (2016). Prevalence and audiological profiles of GJB2 mutations in a large collective of hearing impaired patients. *Hear Res, 333*, 77-86. doi:10.1016/j.heares.2016.01.006

Calistri, A., & Palù, G. (2015). Editorial commentary: Unbiased next-generation sequencing and new pathogen discovery: undeniable advantages and still-existing drawbacks: Oxford University Press.

Chakchouk, I., Zhang, D., Zhang, Z., Francioli, L. C., Santos-Cortez, R. L. P., Schrauwen, I., & Leal, S. M. (2019). Disparities in discovery of pathogenic variants for autosomal

recessive non-syndromic hearing impairment by ancestry. *European journal of human genetics, 27*(9), 1456-1465.

Chakrabarti, S., & Ghosh, N. (2019). Prevalence of Severe and Profound Sensorineural Hearing Loss in School Children in West Bengal, India. *Indian Journal of Otolaryngology and Head & Neck Surgery*, 1-8.

Chan, D. K., & Chang, K. W. (2014). GJB2-associated hearing loss: systematic review of worldwide prevalence, genotype, and auditory phenotype. *Laryngoscope, 124*(2), E34-53. doi:10.1002/lary.24332

Chang, Q., Wang, J., Li, Q., Kim, Y., Zhou, B., Wang, Y., . . . Lin, X. (2015). Virally mediated Kcnq1 gene replacement therapy in the immature scala media restores hearing in a mouse model of human Jervell and Lange-Nielsen deafness syndrome. *EMBO molecular medicine, 7*(8), 1077-1086.

Charaziak, K., & Shera, C. (2019). Methods for Correcting Otoacoustic Emission Measurements: Google Patents.

Chen, K., Wu, X., Zong, L., & Jiang, H. (2018). GJB3/GJB6 screening in GJB2 carriers with idiopathic hearing loss: Is it necessary? *Journal of Clinical Laboratory Analysis, 32*(9), e22592. doi:10.1002/jcla.22592

Chen, P., Chen, H., Fu, S., Chen, G., & Dong, J. (2012). Prevalence of GJB6 mutations in Chinese patients with non-syndromic hearing loss. *International Journal of Pediatric Otorhinolaryngology, 76*(2), 265-267. doi:10.1016/j.ijporl.2011.11.018

Chibisova, S., Markova, T., Alekseeva, N., Yasinskaya, A., Tsygankova, E., Bliznetz, E., . . . Tavartkiladze, G. (2018). Epidemiology of hearing loss in children of the first year of life. *Vestnik Otorinolaringologii, 83*(4), 37-42. doi:10.17116/otorino201883437

Ching, T. Y., Dillon, H., Button, L., Seeto, M., Van Buynder, P., Marnane, V., . . . Leigh, G. (2017). Age at intervention for permanent hearing loss and 5-year language outcomes. *Pediatrics, 140*(3), e20164274. doi:e20164274

Choi, Y. (2012). *A fast computation of pairwise sequence alignment scores between a protein and a set of single-locus variants of another protein.* Paper presented at the

Proceedings of the ACM Conference on Bioinformatics, Computational Biology and Biomedicine.

Choi, Y., & Chan, A. P. (2015). PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics, 31*(16), 2745-2747.

Choi, Y., Sims, G. E., Murphy, S., Miller, J. R., & Chan, A. P. (2012). Predicting the functional effect of amino acid substitutions and indels. *PLoS One, 7*(10), 1-13.

Common, J. E., O'Toole, E. A., Leigh, I. M., Thomas, A., Griffiths, W. A., Venning, V., . . . Kelsell, D. P. (2005). Clinical and genetic heterogeneity of erythrokeratoderma variabilis. *Journal of investigative dermatology, 125*(5), 920-927.

Cone-Wesson, B., Dowell, R. C., Tomlin, D., Rance, G., & Ming, W. J. (2002). The auditory steady-state response: comparisons with the auditory brainstem response. *Journal of the American Academy of Audiology, 13*(4), 173-187.

Copley, G., & Friderichs, N. (2010). An approach to hearing loss in children. *South African family practice, 52*(1), 34-39. doi:https://doi.org/10.1080/20786204.2010.10873928

Cullen, R. D., Buchman, C. A., Brown, C. J., Copeland, B. J., Zdanski, C., Pillsbury III, H. C., & Shores, C. G. (2004). Cochlear implantation for children with GJB2-related deafness. *Laryngoscope, 114*(8), 1415-1419.

Cunningham, F., Achuthan, P., Akanni, W., Allen, J., Amode, M. R., Armean, I. M., . . . Boddu, S. (2018). Ensembl 2019. *Nucleic Acids Research, 47*(D1), D745-D751.

Dalamon, V., Florencia Wernert, M., Lotersztein, V., Craig, P. O., Diamante, R. R., Barteik, M. E., . . . Elgoyhen, A. B. (2013). Identification of four novel connexin 26 mutations in non-syndromic deaf patients: genotype-phenotype analysis in moderate cases. *Mol Biol Rep, 40*(12), 6945-6955. doi:10.1007/s11033-013-2814-x

De Andrés, F., Terán, S., Hernández, F., Terán, E., & LLerena, A. (2016). To genotype or phenotype for personalized medicine? CYP450 drug metabolizing enzyme genotype–phenotype concordance and discordance in the Ecuadorian population. *Omics: a journal of integrative biology, 20*(12), 699-710.

de Freitas Cordeiro-Silva, M., Barbosa, A., Santiago, M., Provetti, M., Dettogni, R. S., Tovar, T. T., . . . Louro, I. D. (2011). Mutation analysis of GJB2 and GJB6 genes in Southeastern Brazilians with hereditary nonsyndromic deafness. *Molecular biology reports, 38*(2), 1309-1313.

del Castillo, F. J., & del Castillo, I. (2011). The DFNB1 subtype of autosomal recessive non-syndromic hearing impairment. *Front Biosci, 16*, 3252-3274.

del Castillo, I., Moreno-Pelayo, M. A., Del Castillo, F. J., Brownstein, Z., Marlin, S., Adina, Q., . . . Chamberlin, G. P. (2003). Prevalence and evolutionary origins of the del (GJB6-D13S1830) mutation in the DFNB1 locus in hearing-impaired subjects: a multicenter study. *The American Journal of Human Genetics, 73*(6), 1452-1458. doi:10.1086/380205

del Castillo, I., Villamar, M., Moreno-Pelayo, M. A., del Castillo, F. J., Alvarez, A., Telleria, D., . . . Moreno, F. (2002). A deletion involving the connexin 30 gene in nonsyndromic hearing impairment. *New England Journal of Medicine, 346*(4), 243-249. doi:10.1056/NEJMoa012052

DeLano, W. L. (2002). Pymol: An open-source molecular graphics tool. *CCP4 Newsletter on protein crystallography, 40*(1), 82-92.

Denoyelle, F., Marlin, S., Weil, D., Moatti, L., Chauvin, P., Garabedian, E. N., & Petit, C. (1999). Clinical features of the prevalent form of childhood deafness, DFNB1, due to a connexin-26 gene defect: implications for genetic counselling. *Lancet, 353*(9161), 1298-1303. doi:10.1016/s0140-6736(98)11071-1

Diaz-Horta, O., Duman, D., Foster II, J., Sırmacı, A., Gonzalez, M., Mahdieh, N., . . . Menendez, I. (2012). Whole-exome sequencing efficiently detects rare mutations in autosomal recessive nonsyndromic hearing loss. *PLoS One, 7*(11), e50628. doi:10.1371/journal.pone.0050628

DiStefano, M. T., Hemphill, S. E., Oza, A. M., Siegert, R. K., Grant, A. R., Hughes, M. Y., . . . Chapin, A. (2019a). ClinGen expert clinical validity curation of 164 hearing loss gene–disease pairs. *Genetics in Medicine, 21*(10), 2239-2247.

DiStefano, M. T., Hemphill, S. E., Oza, A. M., Siegert, R. K., Grant, A. R., Hughes, M. Y., . . . Amr, S. S. (2019b). ClinGen expert clinical validity curation of 164 hearing loss gene-disease pairs. *Genet Med, 21*(10), 2239-2247. doi:10.1038/s41436-019-0487-0

Du, Y., Wu, X., & Li, L. (2006). Mechanisms of bacterial meningitis-related deafness. *Drug Discovery Today: Disease Mechanisms, 3*(1), 115-118.

Eiberger, J., Kibschull, M., Strenzke, N., Schober, A., Büssow, H., Wessig, C., . . . Lautermann, J. (2006). Expression pattern and functional characterization of connexin29 in transgenic mice. *Glia, 53*(6), 601-611.

Evans, W. H., & Martin, P. E. (2002). Gap junctions: structure and function. *Molecular membrane biology, 19*(2), 121-136.

Fang, Y.-K., Chien, K.-Y., Huang, K.-Y., Cheng, W.-H., Ku, F.-M., Lin, R., . . . Tang, P. (2016). Responding to a Zoonotic Emergency with Multi-omics Research: Pentatrichomonas hominis Hydrogenosomal Protein Characterization with Use of RNA Sequencing and Proteomics. *Omics: a journal of integrative biology, 20*(11), 662-669.

Figueroa-Ildefonso, E., Bademci, G., Rajabli, F., Cornejo-Olivas, M., Villanueva, R. D. C., Badillo-Carrillo, R., . . . Tekin, M. (2019). Identification of Main Genetic Causes Responsible for Non-Syndromic Hearing Loss in a Peruvian Population. *Genes (Basel), 10*(8), 581. doi:10.3390/genes10080581

Foerst, A., Beutner, D., Lang-Roth, R., Huttenbrink, K.-B., von Wedel, H., & Walger, M. (2006). Prevalence of auditory neuropathy/synaptopathy in a population of children with profound hearing loss. *International Journal of Pediatric Otorhinolaryngology, 70*(8), 1415-1422. doi:10.1016/j.ijporl.2006.02.010

Frei, K., Ramsebner, R., Hamader, G., Lucas, T., Schoefer, C., Baumgartner, W. D., . . . Kirschhofer, K. (2004). Lack of association between Connexin 31 (GJB3) alterations and sensorineural deafness in Austria. *Hear Res, 194*(1-2), 81-86. doi:10.1016/j.heares.2004.03.007

Fuse, Y., Doi, K., Hasegawa, T., Sugii, A., Hibino, H., & Kubo, T. (1999). Three novel connexin26 gene mutations in autosomal recessive nonsyndromic deafness. *Neuroreport, 10*(9), 1853-1857. doi:10.1097/00001756-199906230-00010

Fykerud, T. A., Kjenseth, A., Schink, K. O., Sirnes, S., Bruun, J., Omori, Y., . . . Leithe, E. (2012). Smad ubiquitination regulatory factor-2 controls gap junction intercellular communication by modulating endocytosis and degradation of connexin43. *Journal of Cell Science, 125*(17), 3966-3976.

Gao, X., & Dai, P. (2014). Impact of next-generation sequencing on molecular diagnosis of inherited non-syndromic hearing loss. *Journal of Otology, 9*(3), 122-125.

Gao, Z., Lu, Y., Ke, J., Li, T., Hu, P., Song, Y., . . . Ma, F. (2016). Application of SNPscan in Genetic Screening for Common Hearing Loss Genes. *PLoS One, 11*(10), e0165650. doi:10.1371/journal.pone.0165650

García-García, G., Berzal-Serrano, A., García-Díaz, P., Villanova-Aparisi, R., Juárez-Rodríguez, S., de Paula-Vernetta, C., . . . Millán, J. M. (2020). Improving the Management of Patients with Hearing Loss by the Implementation of an NGS Panel in Clinical Practice. *Genes, 11*(12), 1467.

Gasmelseed, N. M., Schmidt, M., Magzoub, M. M., Macharia, M., Elmustafa, O. M., Ototo, B., . . . Meyer, C. G. (2004). Low frequency of deafness-associated GJB2 variants in Kenya and Sudan and novel GJB2 variants. *Human Mutation, 23*(2), 206-207. doi:10.1002/humu.9216

Gasparini, P., Rabionet, R., Barbujani, G., Melchionda, S., Petersen, M., Brøndum-Nielsen, K., . . . Fortina, P. (2000). High carrier frequency of the 35delG deafness mutation in European populations. *European journal of human genetics: EJHG, 8*(1), 19.

Gazzaz, B., Weil, D., Rais, L., Akhyat, O., Azeddoug, H., & Nadifi, S. (2005). Autosomal recessive and sporadic deafness in Morocco: high frequency of the 35delG GJB2 mutation and absence of the 342-kb GJB6 variant. *Hear Res, 210*(1-2), 80-84. doi:10.1016/j.heares.2005.08.001

Ge, J., Elferich, J., Goehring, A., Zhao, H., Schuck, P., & Gouaux, E. (2018). Structure of mouse protocadherin 15 of the stereocilia tip link in complex with LHFPL5. *Elife, 7*, e38770.

Gettelfinger, J. D., & Dahl, J. P. (2018). Syndromic hearing loss: a brief review of common presentations and genetics. *Journal of pediatric genetics, 7*(01), 001-008.

Goodenough, D. A., & Paul, D. L. (2009). Gap junctions. *Cold Spring Harbor perspectives in biology, 1*(1), a002576.

Gorlin, R. J., Toriello, H. V., & Cohen, M. M. (1995). *Hereditary hearing loss and its syndromes*: Oxford University Press, USA.

Groce, N. E. (1997). Women with disabilities in the developing world: Arenas for policy revision and programmatic change. *Journal of Disability Policy Studies, 8*(1-2), 177-193.

GSL_Biotech. (2018). SnapGene Viewer | Free software for plasmid mapping, primer design, and restriction site analysis. . Retrieved from https://www.snapgene.com/

Gu, X., Guo, L., Ji, H., Sun, S., Chai, R., Wang, L., & Li, H. (2015). Genetic testing for sporadic hearing loss using targeted massively parallel sequencing identifies 10 novel mutations. *Clinical Genetics, 87*(6), 588-593. doi:10.1111/cge.12431

Gyamfi, C. K., Amankwaa, I., Owusu Sekyere, F., & Boateng, D. (2016). Noise Exposure and Hearing Capabilities of Quarry Workers in Ghana: A Cross-Sectional Study. *J Environ Public Health, 2016*, 7054276. doi:10.1155/2016/7054276

Hamelmann, C., Amedofu, G. K., Albrecht, K., Muntau, B., Gelhaus, A., Brobby, G. W., & Horstmann, R. D. (2001). Pattern of connexin 26 (GJB2) mutations causing sensorineural hearing impairment in Ghana. *Hum Mutat, 18*(1), 84-85. doi:10.1002/humu.1156

Helzner, E. P., Cauley, J. A., Pratt, S. R., Wisniewski, S. R., Zmuda, J. M., Talbott, E. O., . . . Simonsick, E. M. (2005). Race and sex differences in age-related hearing loss: The Health, Aging and Body Composition Study. *Journal of the American Geriatrics Society, 53*(12), 2119-2127.

Henkel, C. K. (2018). The Auditory System *Fundamental Neuroscience for Basic and Clinical Applications* (5th Edition ed., pp. 306-319): Elsevier Inc.

Hertzano, R., & Elkon, R. (2012). High throughput gene expression analysis of the inner ear. *Hearing Research, 288*(1), 77-88.

Hodgson, A., Smith, T., Gagneux, S., Akumah, I., Adjuik, M., Pluschke, G., . . . Genton, B. (2001). Survival and sequelae of meningococcal meningitis in Ghana. *International Journal of Epidemiology, 30*(6), 1440-1446.

House, W. F. (1976). Cochlear implants. *Annals of Otology, Rhinology & Laryngology, 85*(3_suppl), 3-3.

Huang, S., Huang, B., Wang, G., Yuan, Y., & Dai, P. (2015). The relationship between the p.V37I mutation in GJB2 and hearing phenotypes in Chinese individuals. *PLoS One, 10*(6). doi:10.1371/journal.pone.0129662

Huang, S. A., & Lie, J. D. (2013). Phosphodiesterase-5 (PDE5) inhibitors in the management of erectile dysfunction. *Pharmacy and therapeutics, 38*(7), 414-419.

Ijaduola, G. (1982). The problems of the profoundly deaf Nigerian child. *Post graduate document. Africa, 4*, 180-184.

Ingham, N. J., Pearson, S. A., Vancollie, V. E., Rook, V., Lewis, M. A., Chen, J., . . . Lam, C. C. (2019). Mouse screen reveals multiple new genes underlying mouse and human hearing loss. *PLoS biology, 17*(4).

Ionita-Laza, I., McCallum, K., Xu, B., & Buxbaum, J. D. (2016). A spectral approach integrating functional genomic annotations for coding and noncoding variants. *Nature Genetics, 48*(2), 214-220.

Jacquemont, S., Coe, B. P., Hersch, M., Duyzend, M. H., Krumm, N., Bergmann, S., . . . Eichler, E. E. (2014). A higher mutational burden in females supports a "female protective model" in neurodevelopmental disorders. *The American Journal of Human Genetics, 94*(3), 415-425.

Jaiganesh, A., De-la-Torre, P., Patel, A. A., Termine, D. J., Velez-Cortes, F., Chen, C., & Sotomayor, M. (2018). Zooming in on cadherin-23: Structural diversity and potential mechanisms of inherited deafness. *Structure, 26*(9), 1210-1225. e1214.

James, M., Kumar, P., & Ninan, P. J. (2018). A study on prevalence and risk factors of hearing Impairment among newborns. *International Journal of Contemporary Pediatrics, 5*(2), 304-309. doi:http://dx.doi.org/10.18203/2349-3291.ijcp20180018

Javidnia, H., Carson, N., Awubwa, M., Byaruhanga, R., Mack, D., & Vaccani, J. P. (2014). Connexin gene mutations among Ugandan patients with nonsyndromic sensorineural hearing loss. *Laryngoscope, 124*(9), E373-E376.

Jiang, H., Chen, J., Shan, X. J., Li, Y., He, J. G., & Yang, B. B. (2014). Prevalence and range of GJB2 and SLC26A4 mutations in patients with autosomal recessive non-syndromic hearing loss. *Molecular Medicine Reports, 10*(1), 379-386. doi:10.3892/mmr.2014.2148

Jonas Brännström, K., & Olsen, S. Ø. (2017). The acceptable noise level and the pure-tone audiogram. *American Journal of Audiology, 26*(1), 80-87.

Jung, J., Lee, J. S., Cho, K. J., Yu, S., Yoon, J. H., Yung Gee, H., & Choi, J. Y. (2017). Genetic Predisposition to Sporadic Congenital Hearing Loss in a Pediatric Population. *Sci Rep, 7*, 45973. doi:10.1038/srep45973

Kabahuma, R. I., Ouyang, X., Du, L. L., Yan, D., Hutchin, T., Ramsay, M., . . . Liu, X.-Z. (2011). Absence of GJB2 gene mutations, the GJB6 deletion (GJB6-D13S1830) and four common mitochondrial mutations in nonsyndromic genetic hearing loss in a South African population. *International Journal of Pediatric Otorhinolaryngology, 75*(5), 611-617.

Karami-Eshkaftaki, R., Ahmadinejad, F., Aghaei, S., Moghim, H., Hashemzadeh-Chaleshtori, M., & Jami, M.-S. (2017). Hearing loss: A review on molecular genetics and epidemiologic aspects. *International Journal of Epidemiologic Research, 4*(2), 166-172.

Kasakura-Kimura, N., Masuda, M., Mutai, H., Masuda, S., Morimoto, N., Ogahara, N., . . . Matsunaga, T. (2017). WFS1 and GJB2 mutations in patients with bilateral low-frequency sensorineural hearing loss. *Laryngoscope, 127*(9), E324-E329. doi:10.1002/lary.26528

Kathryn, H. (2015). The Human Auditory System *Handbook of Clinical Neurology* (Vol. 129, pp. 479-494): Elsevier.

Kegele, J., Hurth, H., Lackner, P., Enimil, A., Sylverkin, J., Ansong, D., . . . Schmutzhard, J. (2015). Otoacoustic emission testing in Ghanaian children with sickle-cell disease. *Trop Med Int Health, 20*(9), 1209-1212. doi:10.1111/tmi.12533

Kelley, P., Harris, D., Comer, B., Askew, J., Fowler, T., Smith, S. D., & Kimberling, W. (1998). Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss. *The American Journal of Human Genetics, 62*(4), 792-799.

Kelsell, D. P., Di, W.-L., & Houseman, M. J. (2001). Connexin mutations in skin disease and hearing loss. *The American Journal of Human Genetics, 68*(3), 559-568.

Kelsell, D. P., Dunlop, J., Stevens, H. P., Lench, N. J., Liang, J. N., Parry, G., . . . Leigh, I. M. (1997). Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. *Nature, 387*(6628), 80-83. doi:10.1038/387080a0

Kim, M.-A., Cho, H.-J., Bae, S.-H., Lee, B., Oh, S.-K., Kwon, T.-J., . . . Kim, U.-K. (2016a). Methionine sulfoxide reductase B3-targeted in utero gene therapy rescues hearing function in a mouse model of congenital sensorineural hearing loss. *Antioxidants & redox signaling, 24*(11), 590-602.

Kim, S. Y., Kim, A. R., Kim, N. K., Lee, C., Kim, M. Y., Jeon, E.-H., . . . Choi, B. Y. (2016b). Unraveling of Enigmatic Hearing-Impaired GJB2 Single Heterozygotes by Massive Parallel Sequencing: DFNB1 or Not? *Medicine, 95*(14). doi:10.1097/MD.0000000000003029

Kitcher, E. D., Ocansey, G., Abaidoo, B., & Atule, A. (2014). Occupational hearing loss of market mill workers in the city of Accra, Ghana. *Noise Health, 16*(40), 183-188.

Knox, C., Law, V., Jewison, T., Liu, P., Ly, S., Frolkis, A., . . . Neveu, V. (2010). DrugBank 3.0: a comprehensive resource for 'omics' research on drugs. *Nucleic Acids Research, 39*(suppl_1), D1035-D1041.

Ko, J., Park, H., Heo, L., & Seok, C. (2012). GalaxyWEB server for protein structure prediction and refinement. *Nucleic Acids Research, 40*(W1), W294-W297.

Kooshavar, D., Tabatabaiefar, M. A., Farrokhi, E., Abolhasani, M., Noori-Daloii, M. R., & Hashemzadeh-Chaleshtori, M. (2013). Digenic inheritance in autosomal recessive non-syndromic hearing loss cases carrying GJB2 heterozygote mutations: assessment of GJB4, GJA1, and GJC3. *Int J Pediatr Otorhinolaryngol, 77*(2), 189-193. doi:10.1016/j.ijporl.2012.10.015

Kopanos, C., Tsiolkas, V., Kouris, A., Chapple, C. E., Albarca Aguilera, M., Meyer, R., & Massouras, A. (2019). VarSome: the human genomic variant search engine. *Bioinformatics, 35*(11), 1978-1980. doi:10.1093/bioinformatics/bty897

Kumar, P., Henikoff, S., & Ng, P. C. (2009). Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc, 4*(7), 1073-1081. doi:10.1038/nprot.2009.86

Kusters, A. (2012). Being a deaf white anthropologist in Adamorobe: Some ethical and methodological issues. *Sign languages in village communities: Anthropological and linguistic insights*, 27-52.

Laird, D. W., Naus, C. C., & Lampe, P. D. (2017). SnapShot: Connexins and disease. *Cell, 170*(6), 1260-1260. e1261.

Laleh, M. A., Naseri, M., Zonouzi, A. A. P., Zonouzi, A. P., Masoudi, M., Ahangari, N., . . . Nejatizadeh, A. (2017). Diverse pattern of gap junction beta-2 and gap junction beta-4 genes mutations and lack of contribution of DFNB21, DFNB24, DFNB29, and DFNB42 loci in autosomal recessive nonsyndromic hearing loss patients in Hormozgan, Iran. *J Res Med Sci, 22*, 99. doi:10.4103/jrms.JRMS_976_16

Lalwani, A. K., Jackler, R. K., Sweetow, R. W., Lynch, E. D., Raventos, H., Morrow, J., . . . Leon, P. E. (1998). Further characterization of the DFNA1 audiovestibular phenotype.

Archives of Otolaryngology-Head & Neck Surgery, *124*(6), 699-702. doi:DOI 10.1001/archotol.124.6.699

Landrum, M. J., Lee, J. M., Benson, M., Brown, G. R., Chao, C., Chitipiralla, S., . . . Jang, W. (2018). ClinVar: improving access to variant interpretations and supporting evidence. *Nucleic Acids Research*, *46*(D1), D1062-D1067.

Lasisi, A. O., Bademci, G., Foster, J., 2nd, Blanton, S., & Tekin, M. (2014). Common genes for non-syndromic deafness are uncommon in sub-Saharan Africa: a report from Nigeria. *Int J Pediatr Otorhinolaryngol*, *78*(11), 1870-1873. doi:10.1016/j.ijporl.2014.08.014

Le Roux, T., Louw, A., Vinck, B., & Tshifularo, M. (2015). Profound childhood hearing loss in a South Africa cohort: Risk profile, diagnosis and age of intervention. *International Journal of Pediatric Otorhinolaryngology*, *79*(1), 8-14. doi:https://doi.org/10.1016/j.ijporl.2014.09.033

Lebeko, K., Bosch, J., Noubiap, J. J., Dandara, C., & Wonkam, A. (2015). Genetics of hearing loss in Africans: use of next generation sequencing is the best way forward. *Pan Afr Med J*, *20*, 383. doi:10.11604/pamj.2015.20.383.5230

Lebeko, K., Sloan-Heggen, C., Noubiap, J., Dandara, C., Kolbe, D., Ephraim, S., . . . Leal, S. (2016). Targeted genomic enrichment and massively parallel sequencing identifies novel nonsyndromic hearing impairment pathogenic variants in Cameroonian families. *Clinical Genetics*, *90*(3), 288-290.

Lebon, G., Warne, T., Edwards, P. C., Bennett, K., Langmead, C. J., Leslie, A. G., & Tate, C. G. (2011). Agonist-bound adenosine A 2A receptor structures reveal common features of GPCR activation. *Nature*, *474*(7352), 521-525.

Leithe, E., Kjenseth, A., Sirnes, S., Stenmark, H., Brech, A., & Rivedal, E. (2009). Ubiquitylation of the gap junction protein connexin-43 signals its trafficking from early endosomes to lysosomes in a process mediated by Hrs and Tsg101. *Journal of Cell Science*, *122*(21), 3883-3893.

Leithe, E., Mesnil, M., & Aasen, T. (2018). The connexin 43 C-terminus: A tail of many tales. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1860*(1), 48-64.

LeMasurier, M., & Gillespie, P. G. (2005). Hair-cell mechanotransduction and cochlear amplification. *Neuron*, *48*(3), 403-415.

Leong, I. U., Stuckey, A., Lai, D., Skinner, J. R., & Love, D. R. (2015). Assessment of the predictive accuracy of five in silico prediction tools, alone or in combination, and two metaservers to classify long QT syndrome gene mutations. *BMC medical genetics*, *16*(1), 34.

Li, Q., & Wang, K. (2017). InterVar: clinical interpretation of genetic variants by the 2015 ACMG-AMP guidelines. *The American Journal of Human Genetics*, *100*(2), 267-280.

Lidén, G., & Kankkunen, A. (1969). Visual reinforcement audiometry. *Acta Oto-Laryngologica*, *67*(2-6), 281-292.

Liu, X. Z., Xia, X. J., Adams, J., Chen, Z. Y., Welch, K. O., Tekin, M., . . . Balkany, T. (2001). Mutations in GJA1 (connexin 43) are associated with non-syndromic autosomal recessive deafness. *Human molecular genetics*, *10*(25), 2945-2951.

Lopez-Bigas, N., Melchionda, S., Gasparini, P., Borragan, A., Arbones, M. L., & Estivill, X. (2002). A common frameshift mutation and other variants in GJB4 (connexin 30.3): Analysis of hearing impairment families. *Hum Mutat*, *19*(4), 458. doi:10.1002/humu.9023

López-Bigas, N., Olivé, M., Rabionet, R., Ben-David, O., Martínez-Matos, J. A., Bravo, O., . . . Avraham, K. B. (2001). Connexin 31 (GJB3) is expressed in the peripheral and auditory nerves and causes neuropathy and hearing impairment. *Human molecular genetics*, *10*(9), 947-952.

López-Bigas, N., Melchionda, S., Gasparini, P., Borragán, A., Arbonés, M. L., & Estivill, X. (2002). A common frameshift mutation and other variants in GJB4 (connexin 30.3): Analysis of hearing impairment families. *Human Mutation*, *19*(4), 458-458.

Lotthammer, J., Walujkar, S., Wimalasena, L. N., & Sotomayor, M. M. (2020). In-Silico Electrophysiology of Inner-Ear Mechanotransduction Channel Models. *Biophysical Journal*, *118*(3), 276a.

Lucotte, G., Bathelier, C., & Champenois, T. (2001). PCR test for diagnosis of the common GJB2 (connexin 26) 35delG mutation on dried blood spots and determination of the carrier frequency in France. *Mol Cell Probes, 15*(1), 57-59. doi:10.1006/mcpr.2000.0335

Luo, J., Bai, X., Zhang, F., Xiao, Y., Gu, L., Han, Y., . . . Wang, H. (2017). Prevalence of Mutations in Deafness-Causing Genes in Cochlear Implanted Patients with Profound Nonsyndromic Sensorineural Hearing Loss in Shandong Province, China. *Annals Of Human Genetics, 81*(6), 258-266. doi: 10.1111/ahg.12207

Ma, Y., Zheng, Y., Li, G., Lu, T., & Nie, M. (2018). Auditory development in 1-5 years prelingual hearing loss children after cochlear implantation. *Lin chuang er bi yan hou tou jing wai ke za zhi= Journal of clinical otorhinolaryngology, head, and neck surgery, 32*(12), 905. doi:10.13201/j.issn.1001-1781.2018.12.006

Madell, J. R. (2008). Using behavioral observation audiometry to evaluate hearing in infants from birth to 6 months. *Pediatric audiology: Diagnosis, technology, management*, 54-64.

Mahmood, K., Jung, C.-h., Philip, G., Georgeson, P., Chung, J., Pope, B. J., & Park, D. J. (2017). Variant effect prediction tools assessed using independent, functional assay-based datasets: implications for discovery and diagnostics. *Human genomics, 11*(1), 10.

Mani, R. S., Ganapathy, A., Jalvi, R., Srisailapathy, C. R. S., Malhotra, V., Chadha, S., . . . Anand, A. (2009). Functional consequences of novel connexin 26 mutations associated with hereditary hearing loss. *European journal of human genetics, 17*(4), 502-509. doi:10.1038/ejhg.2008.179

Marfoh, A. (2011). *Prevalence of hearing impairment in the Offinso municipality.* SCHOOL OF GRADUATE STUDIES, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY.

Margolis, R. H., Wilson, R. H., Popelka, G. R., Eikelboom, R. H., Swanepoel, D. W., & Saly, G. L. (2016). Distribution Characteristics of Air-Bone Gaps–Evidence of Bias in Manual Audiometry. *Ear and Hearing, 37*(2), 177.

Martin, P. E., Blundell, G., Ahmad, S., Errington, R. J., & Evans, W. H. (2001). Multiple pathways in the trafficking and assembly of connexin 26, 32 and 43 into gap junction intercellular communication channels. *Journal of Cell Science, 114*(21), 3845-3855.

Martínez-Saucedo, M., Rivera-Vega, M. R., Gonzalez-Huerta, L., Urueta-Cuellar, H., Toral-López, J., Berruecos-Villalobos, P., & Cuevas-Covarrubias, S. (2015). Two novel compound heterozygous families with a trimutation in the GJB2 gene causing sensorineural hearing loss. *International Journal of Pediatric Otorhinolaryngology, 79*(12), 2295-2299. doi:10.1016/j.ijporl.2015.10.030

Mathe, E., Olivier, M., Kato, S., Ishioka, C., Hainaut, P., & Tavtigian, S. V. (2006). Computational approaches for predicting the biological effect of p53 missense mutations: a comparison of three sequence analysis based methods. *Nucleic Acids Research, 34*(5), 1317-1325.

Matsunaga, T. (2009). Value of genetic testing in the otological approach for sensorineural hearing loss. *The Keio journal of medicine, 58*(4), 216-222.

McPherson, B., & Holborow, C. (1985). A study of deafness in West Africa: the Gambian hearing health project. *International Journal of Pediatric Otorhinolaryngology, 10*(2), 115-135. doi:https://doi.org/10.1016/S0165-5876(85)80024-0

Moore, D. R. (1991). Anatomy and physiology of binaural hearing. *Audiology, 30*(3), 125-134.

Morell, R. J., Kim, H. J., Hood, L. J., Goforth, L., Friderici, K., Fisher, R., . . . Ostrer, H. (1998). Mutations in the connexin 26 gene (GJB2) among Ashkenazi Jews with nonsyndromic recessive deafness. *New England Journal of Medicine, 339*(21), 1500-1505.

Mulwafu, W., Kuper, H., & Ensink, R. (2016). Prevalence and causes of hearing impairment in Africa. *Tropical Medicine & International Health, 21*(2), 158-165.

Murray, E., McCabe, P., Heard, R., & Ballard, K. J. (2015). Differential diagnosis of children with suspected childhood apraxia of speech. *Journal of Speech, Language, and Hearing Research, 58*(1), 43-60. doi:DOI: 10.1044/2014_JSLHR-S-12-0358

Nagata, K. K. (2003). Gender and disability in the Arab region: The challenges in the new millennium. *Asia Pacific Disability Rehabilitation Journal, 14*(1), 10-17.

Nance, W. E., Liu, X.-Z., & Pandya, A. (2000). Relation between choice of partner and high frequency of connexin-26 deafness. *The Lancet, 356*(9228), 500-501.

Neumann, K., Chadha, S., Tavartkiladze, G., Bu, X., & White, K. R. (2019). Newborn and Infant Hearing Screening Facing Globally Growing Numbers of People Suffering from Disabling Hearing Loss. *International Journal of Neonatal Screening, 5*(1), 7.

Ng, P. C., & Henikoff, S. (2001). Predicting deleterious amino acid substitutions. *Genome research, 11*(5), 863-874.

Ng, P. C., & Henikoff, S. (2002). Accounting for human polymorphisms predicted to affect protein function. *Genome Res, 12*(3), 436-446. doi:10.1101/gr.212802

Ng, P. C., & Henikoff, S. (2003). SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Research, 31*(13), 3812-3814. doi:10.1093/nar/gkg509

NIH. (2015, May 2015). How Do We Hear? *National Institutes on Deafness and Other Communication Disorders (NIDCD)*. Retrieved from https://www.nidcd.nih.gov/health/how-do-we-hear

Norouzi, V., Azizi, H., Fattahi, Z., Esteghamat, F., Bazazzadegan, N., Nishimura, C., . . . Smith, R. (2011). Did the GJB2 35delG mutation originate in Iran? *American Journal of Medical Genetics Part A, 155*(10), 2453-2458.

Noubiap, J.-J., Djomou, F., Njock, R., Toure, G. B., & Wonkam, A. (2014). Waardenburg syndrome in childhood deafness in Cameroon. *South African Journal of Child Health, 8*(1), 3-5.

Nyako, E. A. (2013). Prevalence of Hearing Impairment at the Korle-Bu Teaching Hospital. *A DISSERTATION SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON*.

Nyarko, E. A. (2013). *Prevalence of Hearing Impairment at the Korle-Bu Teaching Hospital.* University of Ghana.

Nyst, V. A. S. (2007). *A descriptive analysis of Adamorobe sign language (Ghana)*: LOT.

Oh, S. K., Choi, S. Y., Yu, S. H., Lee, K. Y., Hong, J. H., Hur, S. W., . . . Kim, U. K. (2013). Evaluation of the pathogenicity of GJB3 and GJB6 variants associated with nonsyndromic hearing loss. *Biochim Biophys Acta, 1832*(1), 285-291. doi:10.1016/j.bbadis.2012.05.009

Ohtsuka, A., Yuge, I., Kimura, S., Namba, A., Abe, S., Van Laer, L., . . . Usami, S. (2003). GJB2 deafness gene shows a specific spectrum of mutations in Japan, including a frequent founder mutation. *Hum Genet, 112*(4), 329-333. doi:10.1007/s00439-002-0889-x

Okonechnikov K, G. O., Fursov M, the_UGENE_team. (2012). Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics, 28*(8), 1166-1167. doi:doi:10.1093/bioinformatics/bts091

Olusanya, B. O., Neumann, K. J., & Saunders, J. E. (2014). The global burden of disabling hearing impairment: a call to action. *Bulletin of the World Health Organization, 92*, 367-373. doi: http://dx.doi.org/10.2471/BLT.13.128728

OMIM. (2017). Online Mendelian Inheritance in Man®. Retrieved from http://omim.org/

Oxenham, A. J. (2018). How we hear: The perception and neural coding of sound. *Annual review of psychology, 69*.

Padma, G., Ramchander, P., Nandur, U., & Padma, T. (2009). GJB2 and GJB6 gene mutations found in Indian probands with congenital hearing impairment. *Journal of genetics, 88*(3), 267-272. doi:https://doi.org/10.1007/s12041-009-0039-5

Pandya, A., Arnos, K. S., Xia, X. J., Welch, K. O., Blanton, S. H., Friedman, T. B., . . . Nance, W. E. (2003). Frequency and distribution of GJB2 (connexin 26) and GJB6 (connexin 30) mutations in a large North American repository of deaf probands. *Genet Med, 5*(4), 295-303. doi:10.1097/01.GIM.0000078026.01140.68

Paznekas, W. A., Boyadjiev, S. A., Shapiro, R. E., Daniels, O., Wollnik, B., Keegan, C. E., . . . Hannibal, M. C. (2003). Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. *The American Journal of Human Genetics, 72*(2), 408-418.

Pejaver, V., Urresti, J., Lugo-Martinez, J., Pagel, K. A., Lin, G. N., Nam, H.-J., . . . Iakoucheva, L. M. (2017). MutPred2: inferring the molecular and phenotypic impact of amino acid variants. *BioRxiv, 10*(1101), 1-28. doi:doi: https://doi.org/10.1101/134981

Pérez-Palma, E., May, P., Iqbal, S., Niestroj, L.-M., Du, J., Heyne, H. O., . . . Palotie, A. (2020). Identification of pathogenic variant enriched regions across genes and gene families. *Genome research, 30*(1), 62-71.

Pinto, Y. M., Elliott, P. M., Arbustini, E., Adler, Y., Anastasakis, A., Bohm, M., . . . Charron, P. (2016). Proposal for a revised definition of dilated cardiomyopathy, hypokinetic non-dilated cardiomyopathy, and its implications for clinical practice: a position statement of the ESC working group on myocardial and pericardial diseases. *European Heart Journal, 37*(23), 1850-1858. doi:10.1093/eurheartj/ehv727

Qi, H., Chen, C., Zhang, H., Long, J. J., Chung, W. K., Guan, Y., & Shen, Y. (2018). MVP: predicting pathogenicity of missense variants by deep learning. *BioRxiv*, 259390.

Qin, H., Shao, Q., Igdoura, S. A., Alaoui-Jamali, M. A., & Laird, D. W. (2003). Lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43 in gap junctional intercellular communication-deficient and-competent breast tumor cells. *Journal of Biological Chemistry, 278*(32), 30005-30014.

Ramchander, P. V., Panda, K. C., & Panda, A. K. (2010). Mutations in the connexin 29 gene are not a major cause of nonsyndromic hearing impairment in India. *Genetic testing and molecular biomarkers, 14*(4), 539-541.

Ratbi, I., Hajji, S., Ouldim, K., Aboussair, N., Feldmann, D., & Sefiani, A. (2007). The mutation 35delG of the gene of the connexin 26 is a frequent cause of autosomal-recessive non-syndromic hearing loss in Morocco. *Archives De Pediatrie, 14*(5), 450-453. doi:10.1016/j.arcped.2006.12.004

Reva, B., Antipin, Y., & Sander, C. (2007). Determinants of protein function revealed by combinatorial entropy optimization. *Genome biology*, *8*(11), R232.231-R232.215.

Reva, B., Antipin, Y., & Sander, C. (2011). Predicting the functional impact of protein mutations: application to cancer genomics. *Nucleic Acids Research*, *39*(17), e118-e118.

Robson, C. D. (2006). Congenital hearing impairment. *Pediatric radiology*, *36*(4), 309-324.

Roche, J. P., & Hansen, M. R. (2015). On the horizon: cochlear implant technology. *Otolaryngologic clinics of North America*, *48*(6), 1097-1116.

Rodriguez-Paris, J., & Schrijver, I. (2009). The digenic hypothesis unraveled: the GJB6 del (GJB6-D13S1830) mutation causes allele-specific loss of GJB2 expression in cis. *Biochemical and Biophysical Research Communications*, *389*(2), 354-359.

Rousso, H. (2015). Education for All: a gender and disability perspective.

Rudman, J. R., Kabahuma, R. I., Bressler, S. E., Feng, Y., Blanton, S. H., Yan, D., & Liu, X. Z. (2017). The genetic basis of deafness in populations of African descent. *J Genet Genomics*, *44*(6), 285-294. doi:10.1016/j.jgg.2017.03.008

Sabag, A. D., Dagan, O., & Avraham, K. B. (2005). Connexins in hearing loss: a comprehensive overview. *Journal of Basic and Clinical Physiology and Pharmacology*, *16*(2-3), 101-116.

Sacheli, R., Delacroix, L., Vandenackerveken, P., Nguyen, L., & Malgrange, B. (2013). Gene transfer in inner ear cells: a challenging race. *Gene therapy*, *20*(3), 237-247.

Sáez, J. C., Berthoud, V. M., Branes, M. C., Martinez, A. D., & Beyer, E. C. (2003). Plasma membrane channels formed by connexins: their regulation and functions. *Physiological reviews*, *83*(4), 1359-1400.

Salih, M. A., Dirar, A. I., Ibrahim, N. O., Osman, M. H., Sabeel, S. M., Yusif, N. A., . . . Elagib, A. A. (2014). Mutation in (GJB3 and GJB4) Genes involved in Deafness in two Sudanese Families Using Next Generation Sequencing. *International Journal of Computational Bioinformatics and In Silico Modeling Int J Comput Bioinfo In Silico Model*, *3*(3), 388-392.

Samanich, J., Lowes, C., Burk, R., Shanske, S., Lu, J., Shanske, A., & Morrow, B. E. (2007). Mutations in GJB2, GJB6, and mitochondrial DNA are rare in African American and Caribbean Hispanic individuals with hearing impairment. *American Journal of Medical Genetics Part A, 143*(8), 830-838. doi:10.1002/ajmg.a.31668

Sarant, J. Z., Holt, C. M., Dowell, R. C., Rickards, F. W., & Blamey, P. J. (2008). Spoken language development in oral preschool children with permanent childhood deafness. *J Deaf Stud Deaf Educ, 14*(2), 205-217. doi:10.1093/deafed/enn034

Schade, G., Kothe, C., Ruge, G., Hess, M., & Meyer, C. G. (2003). [Non-invasive screening for GJB2 mutations in buccal smears for the diagnosis of inherited hearing impairment]. *Laryngorhinootologie, 82*(6), 397-401. doi:10.1055/s-2003-40538

Schmutzhard, J., Lackner, P., Helbok, R., Hurth, H. V., Aregger, F. C., Muigg, V., . . . Kremsner, P. (2015). Severe malaria in children leads to a significant impairment of transitory otoacoustic emissions--a prospective multicenter cohort study. *BMC Med, 13*, 125. doi:10.1186/s12916-015-0366-8

Schrauwen, I., Sommen, M., Corneveaux, J. J., Reiman, R. A., Hackett, N. J., Claes, C., . . . Van Camp, G. (2013). A sensitive and specific diagnostic test for hearing loss using a microdroplet PCR-based approach and next generation sequencing. *American Journal of Medical Genetics Part A, 161*(1), 145-152.

Schrijver, I. (2004). Hereditary non-syndromic sensorineural hearing loss: transforming silence to sound. *The Journal of molecular diagnostics, 6*(4), 275-284.

Schwarz, J. M., Cooper, D. N., Schuelke, M., & Seelow, D. (2014). MutationTaster2: mutation prediction for the deep-sequencing age. *Nature Methods, 11*(4), 361-362.

Shan, J., Chobot-Rodd, J., Castellanos, R., Babcock, M., Shanske, A., Parikh, S. R., . . . Samanich, J. (2010). GJB2 mutation spectrum in 209 hearing impaired individuals of predominantly Caribbean Hispanic and African descent. *International Journal of Pediatric Otorhinolaryngology, 74*(6), 611-618.

Shearer, A. E., DeLuca, A. P., Hildebrand, M. S., Taylor, K. R., Gurrola, J., Scherer, S., . . . Smith, R. J. H. (2010). Comprehensive genetic testing for hereditary hearing loss using

massively parallel sequencing. *Proceedings of the National Academy of Sciences of the United States of America, 107*(49), 21104-21109. doi:10.1073/pnas.1012989107

Shearer, A. E., Hildebrand, M. S., Sloan, C. M., & Smith, R. J. (2011). Deafness in the genomics era. *Hearing Research, 282*(1-2), 1-9.

Shearer, A. E., Hildebrand, M. S., & Smith, R. J. H. (1993). Hereditary Hearing Loss and Deafness Overview. In M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. H. Bean, K. Stephens, & A. Amemiya (Eds.), *GeneReviews((R))*. Seattle (WA): University of Washington, Seattle.

Shearer, A. E., & Smith, R. J. (2012). Genetics: advances in genetic testing for deafness. *Current opinion in pediatrics, 24*(6), 679.

Sheffield, A. M., & Smith, R. J. (2019). The Epidemiology of Deafness. *Cold Spring Harbor perspectives in medicine*, 1-16. doi:10.1101/cshperspect.a033258

Shen, M. y., & Sali, A. (2006). Statistical potential for assessment and prediction of protein structures. *Protein science, 15*(11), 2507-2524.

Shihab, H. A., Gough, J., Cooper, D. N., Day, I. N., & Gaunt, T. R. (2013a). Predicting the functional consequences of cancer-associated amino acid substitutions. *Bioinformatics, 29*(12), 1504-1510.

Shihab, H. A., Gough, J., Cooper, D. N., Stenson, P. D., Barker, G. L., Edwards, K. J., . . . Gaunt, T. R. (2013b). Predicting the functional, molecular, and phenotypic consequences of amino acid substitutions using hidden Markov models. *Human Mutation, 34*(1), 57-65.

Shihab, H. A., Gough, J., Mort, M., Cooper, D. N., Day, I. N., & Gaunt, T. R. (2014). Ranking non-synonymous single nucleotide polymorphisms based on disease concepts. *Human genomics, 8*(11), 1-6. doi:http://www.humgenomics.com/content/8/1/11

Shin, W.-H., Lee, G. R., Heo, L., Lee, H., & Seok, C. (2014). Prediction of protein structure and interaction by GALAXY protein modeling programs. *Bio Design, 2*(1), 1-11.

Shinagawa, J., Moteki, H., Nishio, S.-y., Noguchi, Y., & Usami, S.-i. (2020). Haplotype analysis of GJB2 mutations: founder effect or mutational hot spot? *Genes, 11*(3), 250.

Šimundić, A.-M. (2009). Measures of diagnostic accuracy: basic definitions. *Ejifcc, 19*(4), 203. doi:PMC4975285

Sinnathuray, A., Raut, V., Awa, A., Magee, A., & Toner, J. (2003). A review of cochlear implantation in mitochondrial sensorineural hearing loss. *Otology & Neurotology, 24*(3), 418-426.

Skoe, E., & Kraus, N. (2010). Auditory brainstem response to complex sounds: a tutorial. *Ear and Hearing, 31*(3), 302.

Sloan-Heggen, C. M., Bierer, A. O., Shearer, A. E., Kolbe, D. L., Nishimura, C. J., Frees, K. L., . . . Smith, R. J. H. (2016). Comprehensive genetic testing in the clinical evaluation of 1119 patients with hearing loss. *Hum Genet, 135*(4), 441-450. doi:10.1007/s00439-016-1648-8

Smith, J. R., Hayman, G. T., Wang, S.-J., Laulederkind, S. J., Hoffman, M. J., Kaldunski, M. L., . . . Ellanki, S. L. (2020). The Year of the Rat: The Rat Genome Database at 20: a multi-species knowledgebase and analysis platform. *Nucleic Acids Research, 48*(D1), D731-D742.

Smith, R. J., Bale, J. F., & White, K. R. (2005). Sensorineural hearing loss in children. *The Lancet, 365*(9462), 879-890.

Snoeckx, R. L., Huygen, P. L., Feldmann, D., Marlin, S., Denoyelle, F., Waligora, J., . . . Van Camp, G. (2005). GJB2 mutations and degree of hearing loss: a multicenter study. *Am J Hum Genet, 77*(6), 945-957. doi:10.1086/497996

Srinivas, M., Jannace, T. F., Cocozzelli, A. G., Li, L., Slavi, N., Sellitto, C., & White, T. W. (2019). Connexin43 mutations linked to skin disease have augmented hemichannel activity. *Scientific Reports, 9*(1), 1-11.

Srinivas, M., Verselis, V. K., & White, T. W. (2018). Human diseases associated with connexin mutations. *Biochimica et Biophysica Acta Biomembranes, 1860*(1), 192-201. doi:10.1016/j.bbamem.2017.04.024

Stevens, G., Flaxman, S., Brunskill, E., Mascarenhas, M., Mathers, C. D., Finucane, M., & Global Burden of Disease Hearing Loss Expert, G. (2013). Global and regional hearing

impairment prevalence: an analysis of 42 studies in 29 countries. *Eur J Public Health, 23*(1), 146-152. doi:10.1093/eurpub/ckr176

Sundaram, L., Gao, H., Padigepati, S. R., McRae, J. F., Li, Y., Kosmicki, J. A., . . . Shon, J. (2018). Predicting the clinical impact of human mutation with deep neural networks. *Nature Genetics, 50*(8), 1161-1170.

Tang, W., Zhang, Y., Chang, Q., Ahmad, S., Dahlke, I., Yi, H., . . . Lin, X. (2006). Connexin29 is highly expressed in cochlear Schwann cells, and it is required for the normal development and function of the auditory nerve of mice. *Journal of Neuroscience, 26*(7), 1991-1999.

Tang, Y.-Q., Lee, S. A., Rahman, M., Vanapalli, S. A., Lu, H., & Schafer, W. R. (2020). Ankyrin Is An Intracellular Tether for TMC Mechanotransduction Channels. *Neuron*.

Tavtigian, S. V., Deffenbaugh, A. M., Yin, L., Judkins, T., Scholl, T., Samollow, P. B., . . . Thomas, A. (2006). Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. *Journal of Medical Genetics, 43*(4), 295-305.

Tayoun, A. N. A., Mason-Suares, H., Frisella, A. L., Bowser, M., Duffy, E., Mahanta, L., . . . Amr, S. S. (2016). Targeted droplet-digital PCR as a tool for novel deletion discovery at the DFNB1 locus. *Human Mutation, 37*(1), 119-126.

Tekin, M., Bogoclu, G., Arican, S. T., Orman, M. N., Tastan, H., Elsobky, E., . . . Akar, N. (2005). Evidence for single origins of 35delG and delE120 mutations in the GJB2 gene in Anatolia. *Clin Genet, 67*(1), 31-37. doi:10.1111/j.1399-0004.2004.00334.x

Tekin, M., Duman, T., Boğoçlu, G., İncesulu, A., Çomak, E., Ilhan, I., & Akar, N. (2003). Spectrum of GJB2 mutations in Turkey comprises both Caucasian and Oriental variants: roles of parental consanguinity and assortative mating. *Human Mutation, 21*(5), 552-553. doi:10.1002/humu.9137

Tingang Wonkam, E., Chimusa, E., Noubiap, J. J., Adadey, S. M., JV, F. F., & Wonkam, A. (2019). GJB2 and GJB6 Mutations in Hereditary Recessive Non-Syndromic Hearing Impairment in Cameroon. *Genes (Basel), 10*(11). doi:10.3390/genes10110844

Van Camp G, & Smith, R. (2020, 22 Aug 2019). Hereditary Hearing Loss Homepage. Retrieved from https://hereditaryhearingloss.org/

VanSlyke, J. K., & Musil, L. S. (2002). Dislocation and degradation from the ER are regulated by cytosolic stress. *The Journal of cell biology, 157*(3), 381-394.

Venkatesh, M., Moorchung, N., & Puri, B. (2015). Genetics of non syndromic hearing loss. *Medical Journal Armed Forces India, 71*(4), 363-368.

Vinken, M. (2015). Introduction: connexins, pannexins and their channels as gatekeepers of organ physiology. *Cellular and Molecular Life Sciences, 72*(15), 2775-2778.

Vona, B., Nanda, I., Hofrichter, M. A., Shehata-Dieler, W., & Haaf, T. (2015). Non-syndromic hearing loss gene identification: A brief history and glimpse into the future. *Mol Cell Probes, 29*(5), 260-270. doi:10.1016/j.mcp.2015.03.008

Vos, T., Allen, C., Arora, M., Barber, R. M., Bhutta, Z. A., Brown, A., . . . Chen, A. Z. (2016). Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *The Lancet, 388*(10053), 1545-1602.

Vos, T., Barber, R. M., Bell, B., Bertozzi-Villa, A., Biryukov, S., Bolliger, I., . . . Dicker, D. (2015). Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet, 386*(9995), 743-800. doi: DOI: 10.1016/S0140-6736(15)60692-4

Waldmuller, S., Muller, M., Rackebrandt, K., Binner, P., Poths, S., Bonin, M., & Scheffold, T. (2008). Array-based resequencing assay for mutations causing hypertrophic cardiomyopathy. *Clinical chemistry, 54*(4), 682-687.

Waller, B., Larsen-Reindorf, R., Duah, M., Opoku-Buabeng, J., Edwards, B., Brown, D., . . . Basura, G. (2017). Otolaryngology outreach to Komfo Anokye Teaching Hospital: a medical and educational partnership. *The Journal of Laryngology & Otology, 131*(7), 608-613. doi:10.1017/S0022215117000330

Wang, H., Wu, K., Yu, L., Xie, L., Xiong, W., Wang, D., . . . Wang, Q. (2017). A novel dominant GJB2 (DFNA3) mutation in a Chinese family. *Scientific Reports, 7*. doi:10.1038/srep34425

Wang, W.-H., Yang, J.-J., Lin, Y.-C., Yang, J.-T., & Li, S.-Y. (2010a). Novel expression patterns of connexin 30.3 in adult rat cochlea. *Hearing Research, 265*(1-2), 77-82.

Wang, W. H., Yang, J. J., Lin, Y. C., Yang, J. T., Chan, C. H., & Li, S. Y. (2010b). Identification of Novel Variants in the Cx29 Gene of Nonsyndromic Hearing Loss Patients Using Buccal Cells and Restriction Fragment Length Polymorphism Method. *Audiology and Neuro-Otology, 15*(2), 81-87. doi:10.1159/000231633

Webb, B., & Sali, A. (2017). Protein structure modeling with MODELLER *Functional Genomics* (pp. 39-54): Springer.

Werling, D. M., & Geschwind, D. H. (2015). Recurrence rates provide evidence for sex-differential, familial genetic liability for autism spectrum disorders in multiplex families and twins. *Molecular autism, 6*(1), 27.

WHO. (2014). *Deafness and hearing loss*. Retrieved from World Health Organization Media Center: http://www.who.int/mediacentre/factsheets/fs300/en/

WHO. (2019, 20 March 2019). Deafness and hearing loss. Retrieved from https://www.who.int/news-room/fact-sheets/detail/deafness-and-hearing-loss

WHO. (2020). Prevention of blindness and deafness *Prevention of blindness and deafness (PBD)*. Retrieved from https://www.who.int/pbd/deafness/hearing_impairment_grades/en/

Willems, P. J. (2000). Genetic causes of hearing loss. *New England Journal of Medicine, 342*(15), 1101-1109.

Wilson, B. S., Tucci, D. L., Merson, M. H., & O'Donoghue, G. M. (2017a). Global hearing health care: new findings and perspectives. *The Lancet, 390*(10111), 2503-2515.

Wilson, B. S., Tucci, D. L., Merson, M. H., & O'Donoghue, G. M. (2017b). Global hearing health care: new findings and perspectives. *Lancet*. doi:10.1016/S0140-6736(17)31073-5

Wingard, J. C., & Zhao, H.-B. (2015). Cellular and deafness mechanisms underlying connexin mutation-induced hearing loss–a common hereditary deafness. *Frontiers in cellular neuroscience, 9*, 202.

Wittlieb-Weber, C. A., Haude, K. M., Fong, C.-T., & Vinocur, J. M. (2016). A novel GJA1 mutation causing familial oculodentodigital dysplasia with dilated cardiomyopathy and arrhythmia. *HeartRhythm case reports, 2*(1), 32-35.

Wong, S. H., Wang, W. H., Chen, P. H., Li, S. Y., & Yang, J. J. (2017). Functional analysis of a nonsyndromic hearing loss-associated mutation in the transmembrane II domain of the GJC3 gene. *International Journal of Medical Sciences, 14*(3), 246-256. doi:10.7150/ijms.17785

Wonkam, A. (2015). Letter to the editor regarding "GJB2, GJB6 or GJA1 genes should not be investigated in routine in non syndromic deafness in people of sub-Saharan African descent". *International Journal of Pediatric Otorhinolaryngology, 79*(4), 632-633. doi:10.1016/j.ijporl.2015.01.012

Wonkam, A., Bosch, J., Noubiap, J. J., Lebeko, K., Makubalo, N., & Dandara, C. (2015). No evidence for clinical utility in investigating the connexin genes GJB2, GJB6 and GJA1 in non-syndromic hearing loss in black Africans. *S Afr Med J, 105*(1), 23-26. doi:10.7196/samj.8814

Wonkam, A., Noubiap, J. J. N., Djomou, F., Fieggen, K., Njock, R., & Toure, G. B. (2013). Aetiology of childhood hearing loss in Cameroon (sub-Saharan Africa). *European Journal of Medical Genetics, 56*(1), 20-25. doi:10.1016/j.ejmg.2012.09.010

Wright, A. (1991). The aetiology of childhood deafness in Sierra Leone. *The Sierra Leone Medical and Dental Association Journal, 6*, 31-45.

Wu, B. L., Lindeman, N., Lip, V., Adams, A., Amato, R. S., Cox, G., . . . Platt, O. (2002). Effectiveness of sequencing connexin 26 (GJB2) in cases of familial or sporadic childhood deafness referred for molecular diagnostic testing. *Genet Med, 4*(4), 279-288. doi:10.1097/00125817-200207000-00006

Wu, C.-C., Lee, Y.-C., Chen, P.-J., & Hsu, C.-J. (2008). Predominance of genetic diagnosis and imaging results as predictors in determining the speech perception performance outcome after cochlear implantation in children. *Archives of pediatrics & adolescent medicine*, *162*(3), 269-276.

Wu, C.-M., Ko, H.-C., Tsou, Y.-T., Lin, Y.-H., Lin, J.-L., Chen, C.-K., . . . Wu, C.-C. (2015). Long-term cochlear implant outcomes in children with GJB2 and SLC26A4 mutations. *PLoS One*, *10*(9).

Wu, C. C., Liu, T. C., Wang, S. H., Hsu, C. J., & Wu, C. M. (2011). Genetic characteristics in children with cochlear implants and the corresponding auditory performance. *Laryngoscope*, *121*(6), 1287-1293.

Wu, X., Gao, X., Han, P., & Zhou, Y. (2018). Identification of causative variants in patients with non-syndromic hearing loss in the Minnan region, China by targeted next-generation sequencing. *Acta Oto-Laryngologica*, 1-8.

Wu, Z., Grillet, N., Zhao, B., Cunningham, C., Harkins-Perry, S., Coste, B., . . . Fettiplace, R. (2017). Mechanosensory hair cells express two molecularly distinct mechanotransduction channels. *Nature neuroscience*, *20*(1), 24.

Yan, D., Xiang, G. X., Chai, X. P., Qing, J., Shang, H. Q., Zou, B., . . . Liu, X. Z. (2017). Screening of deafness-causing DNA variants that are common in patients of European ancestry using a microarray-based approach. *PLoS One*, *12*(3), e0169219. doi:ARTN e0169219

10.1371/journal.pone.0169219

Yan, Y.-j., Li, Y., Yang, T., Huang, Q., & Wu, H. (2013). The effect of GJB2 and SLC26A4 gene mutations on rehabilitative outcomes in pediatric cochlear implant patients. *European Archives of Oto-Rhino-Laryngology*, *270*(11), 2865-2870.

Yang, J.-J., Liao, P.-J., Su, C.-C., & Li, S.-Y. (2005). Expression patterns of connexin 29 (GJE1) in mouse and rat cochlea. *Biochemical and Biophysical Research Communications*, *338*(2), 723-728.

Yang, J. J., Wang, W. H., Lin, Y. C., Weng, H. H., Yang, J. T., Hwang, C. F., . . . Li, S. Y. (2010). Prospective variants screening of connexin genes in children with hearing impairment: genotype/phenotype correlation. *Hum Genet, 128*(3), 303-313. doi:10.1007/s00439-010-0856-x

Yang, T., Guo, L., Wang, L., & Yu, X. (2019). Diagnosis, intervention, and prevention of genetic hearing loss *Hearing Loss: Mechanisms, Prevention and Cure* (pp. 73-92): Springer.

Yin, Y., Butler, C., & Zhang, Q. (2021). Challenges in the application of NGS in the clinical laboratory. *Human Immunology*.

Yu, Q., Wang, Y., Chang, Q., Wang, J., Gong, S., Li, H., & Lin, X. (2014). Virally expressed connexin26 restores gap junction function in the cochlea of conditional Gjb2 knockout mice. *Gene therapy, 21*(1), 71-80.

Zakpala, R. N., Armah, F. A., Sackey, B. M., & Pabi, O. (2014). Night-time decibel hell: mapping noise exposure zones and individual annoyance ratings in an urban environment in ghana. *Scientifica (Cairo), 2014*, 892105. doi:10.1155/2014/892105

Zelante, L., Gasparini, P., Estivill, X., Melchionda, S., D'Agruma, L., Govea, N., . . . Fortina, P. (1997). Connexin26 mutations associated with the most common form of non-syndromic neurosensory autosomal recessive deafness (DFNB1) in Mediterraneans. *Hum Mol Genet, 6*(9), 1605-1609. doi:10.1093/hmg/6.9.1605

Zhang, W., Kim, S. M., Wang, W., Cai, C., Feng, Y., Kong, W., & Lin, X. (2018). Cochlear gene therapy for sensorineural hearing loss: current status and major remaining hurdles for translational success. *Frontiers in Molecular Neuroscience, 11*, 221.

Zheng-Fischhöfer, Q., Schnichels, M., Dere, E., Strotmann, J., Loscher, N., McCulloch, F., . . . Nagy, J. I. (2007). Characterization of connexin30. 3-deficient mice suggests a possible role of connexin30. 3 in olfaction. *European journal of cell biology, 86*(11-12), 683-700.

Zheng, J., Ying, Z., Cai, Z., Sun, D., He, Z., Gao, Y., . . . Guan, M.-X. (2015). GJB2 mutation spectrum and genotype-phenotype correlation in 1067 Han Chinese subjects with non-

syndromic hearing loss. *PLoS One, 10*(6), e0128691. doi: 10.1371/journal.pone.0128691

Zhou, Y., Li, C., Li, M., Zhao, Z., Tian, S., Xia, H., . . . Chen, J. (2019). Mutation analysis of common deafness genes among 1,201 patients with non-syndromic hearing loss in Shanxi Province. *Molecular genetics & genomic medicine, 7*(3), 1-8. doi:DOI:10.1002/mgg3.537

Appendix A

Table A1: *GJB2* variations in humans

GJB2 variant	rs number	Clinical significance	Type of variant
p.Arg75 = (c.225G>T)	rs149137695	Benign	Missense variant
5`UTR -15C>T	rs72561725	Benign	5-prime UTR variant
c.-22-12C>T	rs9578260	Benign	Intronic variant
c.-57G>T	rs191461105	Benign	Intronic variant
c.IVS1-15C>T	rs72561725	Benign	5-prime UTR variant
E114G (341A>G)	rs2274083	Benign	Missense variant
p.A49A (c.147C>T)	rs1431278544	Benign	Missense variant
p.F83L (c.249C>G)	rs111033218	Benign	Missense variant
p.I111T (c.332T>C)	rs1316789942	Benign	Missense variant
p.L89L	rs727503067	Benign	Missense variant
p.T123N (c.368C>A)	rs111033188	Benign	Missense variant
pH94H (c.282C>T)	rs766998544	Benign	Missense variant
R127H (c.380G>A)	rs111033196	Benign	Missense variant
V153I (c.457G>A)	rs111033186	Benign	Missense variant
V27I (79G>C)	rs2274084	Benign	Missense variant
p.Arg216fs (c.645delT)	rs1555341794	Likely pathogenic	Frame shift variant
E110K (c.328G>A)	-	Likely pathogenic	Missense variant
F69L (c.205T>C)	-	Likely pathogenic	Missense variant
I82N (c.245T>A)	-	Likely pathogenic	Missense variant
L10P (c.29T>C)	-	Likely pathogenic	Missense variant
M34V (c.100A>G)	-	Likely pathogenic	Missense variant
N206H (c.616A>C)	-	Likely pathogenic	Missense variant
N206T (c.617A>C)	-	Likely pathogenic	Missense variant
p. Leu205Pro (c. 614T>C)	-	Likely pathogenic	Missense variant
p.A148P (c.442G>C)	-	likely pathogenic	Missense variant

Continuation of Table A1: *GJB2* variations in humans

GJB2 variant	rs number	Clinical significance	Type of variant
p.A78I (c.232_233delGCinsAT)	-	likely pathogenic	Missense variant
p.A78S (c.232G>T)	-	Likely pathogenic	Missense variant
p.A78T (c.232G>A)	-	likely pathogenic	Missense variant
p.A88P (c.262G>C)	-	Likely pathogenic	Missense variant
p.A88S (c.262G>T)	-	Likely pathogenic	Missense variant
p.D117H (c.349G>C)	-	likely pathogenic	Missense variant
p.D159Y (c.475G>T)	rs373684994	Likely pathogenic	Missense variant
p.E42K (c.124G>A)	-	Likely pathogenic	Missense variant
p.F31I (c.91T>A)	-	Likely pathogenic	Missense variant
p.F31L (c.93T>G)	-	Likely pathogenic	Missense variant
p.G109V (c.326G>T)	-	Likely pathogenic	Missense variant
p.G160R (c.468G>C)	-	Likely pathogenic	Missense variant
p.G21R (c.61G>C)	-	Likely pathogenic	Missense variant
p.I121n (c.362T>A)	-	likely pathogenic	Missense variant
p.I30F (c.88A>T)	-	Likely pathogenic	Missense variant
p.I30L (c.88A>C)	-	Likely pathogenic	Missense variant
p.I33N (c.98T>A)	-	Likely pathogenic	Missense variant
p.K105R (c.314A>G)	-	Likely pathogenic	Missense variant
p.K112M (c.335A>T)	-	Likely pathogenic	Missense variant
p.K41R (c.122A>G)	rs866127155	likely pathogenic	Missense variant
p.K61Q (c.181A>C)	-	Likely pathogenic	Missense variant
p.L90V (c.268C>G)	-	Likely pathogenic	Missense variant
p.M151R (c.452T>G	-	likely pathogenic	Missense variant
p.N14D (c.40A>G)	rs1476034902	Likely pathogenic	Missense variant

Continuation of Table A1: *GJB2* variations in humans

GJB2 variant	rs number	Clinical significance	Type of variant
p.N54S (c.161A>G)	-	likely pathogenic	Missense variant
p.P175H (c.524C>A)	-	Likely pathogenic	Missense variant
p.P175T (c.523C>A)	-	Likely pathogenic	Missense variant
p.P70S (c.208C>T)	-	likely pathogenic	Missense variant
p.P87del (c.257_259 del CGC)	-	Likely pathogenic	Frame shift variant
p.Q80H (c.240G>C)	-	Likely pathogenic	Missense variant
p.Q80L (c.239A>T)	-	Likely pathogenic	Missense variant
p.Q80R (c.239A>G)	-	Likely pathogenic	Missense variant
p.S72C (c.215C>G)	-	Likely pathogenic	Missense variant
p.S85P (c.253T>C)	-	likely pathogenic	Missense variant
p.S85Y (c.254C>A)	-	Likely pathogenic	Missense variant
p.T186A (c.556A>G)	rs899636227	Likely pathogenic	Missense variant
p.T18A (c.52A>G)	-	likely pathogenic	Missense variant
p.T8M c.23C>T	rs529500747	Likely pathogenic	Missense variant
p.V178A (c.533T>C)	rs568612627	Likely pathogenic	Missense variant
p.V193E (c.578T>A)	-	Likely pathogenic	Missense variant
p.V198M (c.592G>A)	-	Likely pathogenic	Missense variant
p.V63G (c.188T>G)	-	Likely pathogenic	Missense variant
p.V84A (c.251T>C)	-	Likely pathogenic	Missense variant
p.V84W (c.250_251delGTinsTG)	-	Likely pathogenic	Missense variant
p.W133G (c.397T>G)	-	Likely pathogenic	Missense variant
p.Y142del (c.424_426delTTC)	-	Likely pathogenic	Frame shift variant
pD159N (c.475G>A)	rs373684994	Likely pathogenic	Missense variant
R32G (c.94C>G)	-	Likely pathogenic	Missense variant

Continuation of Table A1: *GJB2* variations in humans

GJB2 variant	rs number	Clinical significance	Type of variant
S19R	-	Likely pathogenic	Missense variant
V43L (c.127G>T)	-	Likely pathogenic	Missense variant
V43M (c.127G>A)	-	Likely pathogenic	Missense variant
Y155T (c.463_464delTAinsAC)	-	Likely pathogenic	Missense variant
p.L213F	-	Likely pathogenic	Missense variant
(p.Arg127Cysfs*85 (c.377_378insATGCGGA)	-	Pathogenic	Frame shift variant
313_326del14 (p.Lys105GlyfsTer5)	rs111033253	Pathogenic	Frame shift variant
508_509insAACG	rs773528125	Pathogenic	Frame shift variant
c.-23+1G>A	rs80338940	Pathogenic	5-prime UTR variant
c.-23G>T	rs786204734	Pathogenic	5-prime UTR variant
c.291_292insA (p.R98Tfs*4)	-	Pathogenic	Frame shift variant
c.30delG	-	Pathogenic	Frame shift variant
c.35delG	rs80338939	Pathogenic	Frame shift variant
c.575_576del (c.575delCA) (p.Thr192SerfsTer17)	rs1057517521	Pathogenic	Frame shift variant
delE119 (c.355-357delGAG)	rs80338947	Pathogenic	Frame shift variant
E114K (c.340G>A)	rs201855069	Pathogenic	Missense variant
E147* (c.439G>T)	-	Pathogenic	Stop gain
F115C (c.344T>G)	rs1265940668	Pathogenic	Missense variant
G160D (c.479G>A)	rs1003116265	Pathogenic	Missense variant
G45E (134G>A)	rs72561723	Pathogenic	Missense variant
I203T (608T>C)	rs76838169	Pathogenic	Missense variant
I20T (c.59T>C)	rs1057517519	Pathogenic	Missense variant
I82M (c.246C>G)	rs781534323	Pathogenic	Missense variant
K15T (c.44A>C)	rs111033217	Pathogenic	Missense variant

Continuation of Table A1: *GJB2* variations in humans

GJB2 variant	rs number	Clinical significance	Type of variant
K221N (c.663G>C)	rs375599392	Pathogenic	Missense variant
L89P (c.265C>T)	rs765921870	Pathogenic	Missense variant
M151L (c.451A>T)	rs1356838160	Pathogenic	Missense variant
M163L (c.487A>C)	rs80338949	Pathogenic	Missense variant
M163V (c.487A>G)	rs80338949	Pathogenic	Missense variant
N206S (c.617A>G)	rs111033294	Pathogenic	Missense variant
p. Trp44GlyfsX38* (c. 129delG)	-	Pathogenic	Frame shift variant
p.A171T (c.511G>A)	rs201004645	Pathogenic	Missense variant
p.A197S (c.589G>T)	rs777236559	Pathogenic	Missense variant
p.A40E (c.119C>A)	rs111033296	Pathogenic	Missense variant
p.A49V (c.146C>T)	rs1057517976	Pathogenic	Missense variant
p.A78V (c.233C>T)	rs1481417187	Pathogenic	Missense variant
p.C169Nfs*42 (504insAACG)	-	Pathogenic	Frame shift variant
p.C169R (c.505T>C)	rs760489970	Pathogenic	Missense variant
p.C169Y (c.506G>A)	rs774518779	Pathogenic	Missense variant
p.C202F (c.605G>T)	rs104894406	Pathogenic	Missense variant
p.C211* (c.633T>A)	-	Pathogenic	Stop gain
p.C53R	rs1555341986	Pathogenic	Missense variant
p.D159V (c.476A>T)	rs28931592	Pathogenic	Missense variant
p.D179H (c.535G>C)	rs28931595	Pathogenic	Missense variant
p.D179N (c.535G>A)	rs28931595	Pathogenic	Missense variant
p.D46N (c.136G>A)	rs1064797088	Pathogenic	Missense variant
p.D50N (c.148G>A)	rs28931594	Pathogenic	Missense variant
p.D66H (c.196G>C)	rs104894403	Pathogenic	Missense variant

Continuation of Table A1: *GJB2* variations in humans

GJB2 variant	rs number	Clinical significance	Type of variant
p.del120E (c.358_360delGAG)	rs80338947	Pathogenic	Frame shift variant
p.E110DfsX4 (c.327_328delGG)	-	Pathogenic	Frame shift variant
p.E110GfsX2 (c.329delA)	-	Pathogenic	Frame shift variant
p.E119K (c.355G>A)	rs150529554	Pathogenic	Missense variant
p.E129* c.385G>T	rs397516875	Pathogenic	Stop gain
p.E129K (c.385G>A)	rs397516875	Pathogenic	Missense variant
p.E147K (c.439G>A)	rs767178508	Pathogenic	Missense variant
p.E47* (139G>T)	rs104894398	Pathogenic	Stop gain
p.E47K (c.139G>A)	rs104894398	Pathogenic	Missense variant
p.F106L (c.318C>A)	rs779358271	Pathogenic	Missense variant
p.F191L (c.571T>C)	rs397516878	Pathogenic	Missense variant
p.G109E (c.326G>A)	rs374572413	Pathogenic	Missense variant
p.G12C (c.34G>T)	rs104894408	Pathogenic	Missense variant
p.G12D (c.35G>A)	rs1801002	Pathogenic	Missense variant
p.G12R (c.34G>C)	rs104894408	Pathogenic	Missense variant
p.G12V (c.35G>T)	rs1801002	Pathogenic	Missense variant
p.G130A (c.389G>C)	rs779018464	Pathogenic	Missense variant
p.G130D (c.389G>A)	rs779018464	Pathogenic	Missense variant
p.G160S (c.468G>A)	rs34988750	Pathogenic	Missense variant
p.G160S (c.478G>A)	rs34988750	Pathogenic	Missense variant
p.G200A (c.599G>C)	-	Pathogenic	Missense variant
p.G200R (c.598G>A)	rs786204597	Pathogenic	Missense variant
p.G4D (c.11G>A)	rs111033222	Pathogenic	Missense variant
p.G59A (c.176G>C)	rs104894404	Pathogenic	Missense variant
p.G59S (c.175G>A)	rs104894410	Pathogenic	Missense variant

Continuation of Table A1: *GJB2* variations in humans

GJB2 variant	rs number	Clinical significance	Type of variant
p.Gly59AlafsTer18 (c.176-191del16)	rs750188782	Pathogenic	Frame shift variant
p.H100RfsTer14 (c.299_300delAT)	rs111033204	Pathogenic	Frame shift variant
p.H100Y (c.298C>T)	rs143343083	Pathogenic	Missense variant
p.I203K (c.608TC>AA)	-	Pathogenic	Missense variant
p.I20M (c.60T>G)	rs749693224	Pathogenic	Missense variant
p.I30V (c.88A>G)	rs374625633	Pathogenic	Missense variant
p.I33T (c.98T>C)	rs575453513	Pathogenic	Missense variant
p.I35S (c.104T>G)	rs756467247	Pathogenic	Missense variant
p.I9Dfs*39 (c.23_24insA)	-	Pathogenic	Frame shift variant
p.K112EfsX2 (c.334_335delAA)	rs756484720	Pathogenic	Frame shift variant
p.K122I (c.365A>T)	rs111033295	Pathogenic	Missense variant
p.K168* (c.502A>T)	-	Pathogenic	Stop gain
p.K168R (c.503A>G)	rs200104362	Pathogenic	Missense variant
p.K168R (c.503A>G)	rs200104362	Pathogenic	Missense variant
p.K224Q (c.670A>C)	rs111033194	Pathogenic	Missense variant
p.L213*	-	Pathogenic	Stop gain
p.L214P (c.641T>C)	NM_004004.6:c.641T>C	Pathogenic	Missense variant
p.L36P (c.107T>C)	rs587783644	Pathogenic	Missense variant
p.L56Rfs (c.167delT)	rs80338942	Pathogenic	Frame shift variant
p.L76P (c.227C>T)	rs111033361	Pathogenic	Missense variant
p.L79Cfs (235delC)	rs80338943	Pathogenic	Frame shift variant
p.L79Cfs*3 (c.233delC/G)	rs80338943	Pathogenic	Frame shift variant
p.L79P (236T>C)	rs1555341957	Pathogenic	Missense variant
p.L81V (c.241C>G)	rs145216882	Pathogenic	Missense variant
p.L90Lfs*101 (c.269dupT)	rs730880338	Pathogenic	Frame shift variant

Continuation of Table A1: *GJB2* variations in humans

GJB2 variant	rs number	Clinical significance	Type of variant
p.L90P (c.269T>C)	rs80338945	Pathogenic	Missense variant
p.M195I (c.585G>C)	rs570552952	Pathogenic	Missense variant
p.M195V (c.583A>G)	rs532203068	Pathogenic	Missense variant
p.M1I (3G>T)	-	Pathogenic	Start loss
p.M1V (c.1A>G)	rs111033293	Pathogenic	Missense variant
p.M34* (c.99delT)	rs1566528961	Pathogenic	Stop gain
p.M34L (c.100A>T)	rs564084861	Pathogenic	Missense variant
p.M34T (c.101T>C)	rs35887622	Pathogenic	Missense variant
p.M34T (c.101T>C)	rs35887622	Pathogenic	Missense variant
p.M34T (c.101T>C)	rs35887622	Pathogenic	Missense variant
p.M93I (c.279G>A)	rs397516871	Pathogenic	Missense variant
p.N170Kfs*40 (c.509insA)	rs749675121	Pathogenic	Frame shift variant
p.N62 = (c.186C>T)	rs397516869	Pathogenic	Missense variant
p.N62Ifs*40 (c.184_185insT)	-	Pathogenic	Frame shift variant
p.N7* (c. 19C>T)	rs111033451	Pathogenic	Stop gain
p.P70A (c.208C>G)	rs200023879	Pathogenic	Missense variant
p.P70A (c.208C>G)	rs200023879	Pathogenic	Missense variant
p.Q124* (c.370C>T)	rs397516874	Pathogenic	Stop gain
p.Q57* (c.169C>T)	rs111033297	Pathogenic	Stop gain
p.Q7* (c.19C>T)	rs111033451	Pathogenic	Stop gain
p.Q80* (c.238C>T)	rs199883710	Pathogenic	Stop gain
p.Q80P (c.239A>C)	rs727504302	Pathogenic	Missense variant
p.R104fsX6 (c.310del14)	-	Pathogenic	Frame shift variant
p.R127C (c.379C>T)	rs727503066	Pathogenic	Missense variant
p.R127L (c.380G>T)	rs111033196	Pathogenic	Missense variant
p.R143N (c.428G>A)	rs104894401	Pathogenic	Missense variant
p.R143Q (c.428G>A)	rs104894401	Pathogenic	Missense variant

Continuation of Table A1: *GJB2* variations in humans

GJB2 variant	rs number	Clinical significance	Type of variant
p.R143W (427C>T)	rs80338948	Pathogenic	Missense variant
p.R143W (c.427C>T)	rs80338948	Pathogenic	Missense variant
p.R165W (c.493C>T)	rs376898963	Pathogenic	Missense variant
p.R184P (c.551G>C)	rs80338950	Pathogenic	Missense variant
p.R184Q (c.551G>A)	rs80338950	Pathogenic	Missense variant
p.R184W (c.550C>T)	rs998045226	Pathogenic	Missense variant
p.R216Ifs*17 (c.645-648delTAGA)	rs587783647	Pathogenic	Frame shift variant
p.R32Afs*3 (c.93delT)	-	Pathogenic	Frame shift variant
p.R32C (c.235delC)	rs371024165	Pathogenic	Missense variant
p.R32C (c.94C>T)	rs371024165	Pathogenic	Missense variant
p.R32H (c.95G>A)	rs111033190	Pathogenic	Missense variant
p.R32L (c.95G>T)	rs111033190	Pathogenic	Missense variant
p.R32S (c.94C>A)	rs371024165	Pathogenic	Missense variant
p.R75Q (c.224G>A)	rs28931593	Pathogenic	Missense variant
p.R98W (c.292C>T)	rs104894402	Pathogenic	Missense variant
p.S139N (c.416G>A)	rs76434661	Pathogenic	Missense variant
p.T123PfsX45 (c.363delC)	rs1330280423	Pathogenic	Frame shift variant
p.T137Nfs*73 (c.408InsA)	-	Pathogenic	Frame shift variant
p.T18_G21del+I23DfsX23 (c.51_62del+65dup)	rs886037624	Pathogenic	Frame shift variant
p.T86M (c.257C>T)	rs1291519904	Pathogenic	Missense variant
p.T86R (c.257C>G)	rs1291519904	Pathogenic	Missense variant
p.V13Cfs*35 (c.34_35insG)	rs80338939	Pathogenic	Frame shift variant
p.V13CfsX35 (c.35dup)	rs80338939	Pathogenic	Frame shift variant
p.V144Dfs*59 (c.431-450del19)	-	Pathogenic	Frame shift variant

Continuation of Table A1: *GJB2* variations in humans

GJB2 variant	rs number	Clinical significance	Type of variant
p.V167M (c.499G>A)	rs111033360	Pathogenic	Missense variant
p.V226D (c.677T>A)	rs773846324	Pathogenic	Missense variant
p.V43Cfs*39 (c.127delG)	-	Pathogenic	Frame shift variant
p.V52Afs*29 (c.155_158delTCTG)	-	Pathogenic	Frame shift variant
p.V63A (c.188T>C)	rs727504309	Pathogenic	Missense variant
p.V63L (c.187G>A)	rs370696868	Pathogenic	Missense variant
p.V84L (c.250G>C)	rs104894409	Pathogenic	Missense variant
p.V84L (c.250G>T)	rs104894409	Pathogenic	Missense variant
p.V84M (c.250G>A)	rs104894409	Pathogenic	Missense variant
p.V95M (c.283G>A)	rs111033299	Pathogenic	Missense variant
p.Val13CysfsTer35 (c.35insG/35dupG)	rs80338939	Pathogenic	Frame shift variant
p.Val198GlnfsTer11 (c.592_600delins17)	rs111033335	Pathogenic	Stop gain
p.Val91SerfsTer11 (c.269InsT)	rs730880338	Pathogenic	Frame shift variant
p.W133* (c.399G>A)	rs777225786	Pathogenic	Stop gain
p.W134* (c.402G>A/c.402delG)	rs1195692356	Pathogenic	Stop gain
p.W24* (c.71G>A)	rs104894396	Pathogenic	Stop gain
p.W3* (c.9G>A)	rs111033401	Pathogenic	Stop gain
p.W3LfsX45 (c.7dup)	-	Pathogenic	Frame shift variant
p.W44C (c.132G>A)	rs104894407	Pathogenic	Missense variant
p.W77* (c.230G>A)	rs104894395	Pathogenic	Stop gain
p.W77* (c.231G>A)	rs80338944	Pathogenic	Stop gain
p.W77R (c.229T>C)	rs104894397	Pathogenic	Missense variant
p.W77R (c.229T>C)	rs104894397	Pathogenic	Missense variant
p.Y136Tfs*32 (c.405delC)	-	Pathogenic	Frame shift variant
p.Y152* (c.456C>A)	rs111033420	Pathogenic	Stop gain
p.Y155* (c.465T>A)	rs772264564	Pathogenic	Stop gain
p.Y68C (c.203A>G)	rs397516870	Pathogenic	Missense variant
p.Y97* (c.290dupA)	rs786204491	Pathogenic	Stop gain

Continuation of Table A1: *GJB2* variations in humans

GJB2 variant	rs number	Clinical significance	Type of variant
p.Y97* (Ins A 290-291)	rs786204491	Pathogenic	Stop gain
pI71T (c.212T>C)	rs1373154561	Pathogenic	Missense variant
pQ57* (c.169C>T)	rs111033297	Pathogenic	Stop gain
R75W (c.223C>T)	rs104894402	Pathogenic	Missense variant
S199F	rs771748289	Pathogenic	Missense variant
S19T (c.56G>C)	rs80338941	Pathogenic	Missense variant
T55N (c.164C>A)	rs1064797089	Pathogenic	Missense variant
T8M (c.23C>T)	rs529500747	Pathogenic	Missense variant
V37I (109G>A)	rs72474224	Pathogenic	Missense variant
Val91SerfsX11 (c.269insT)	rs730880338	Pathogenic	Frame shift variant
W172*	rs1302739538	Pathogenic	Stop gain
W44* (131G>A)	rs104894407	Pathogenic	Stop gain
W44L (131G>T)	-	Pathogenic	Missense variant
Y136* (c.408C>T)	rs786204690	Pathogenic	Stop gain
c.-28T>C	rs141962118	Uncertain significance	Intronic variant
c.-40T>A	rs778602324	Uncertain significance	Intronic variant
c.-41G>A	-	Uncertain significance	5-prime UTR variant
c.-60C>T	-	Uncertain significance	5-prime UTR variant
c.IVS1 -1G>A	rs768338285	Uncertain significance	5-prime UTR variant
p.D159= (c.477C>T)	rs935617755	Uncertain significance	Missense variant
p.F106F (c.318C>T)	-	Uncertain significance	Missense variant
p.F146F (c.438C>T)	rs750795475	Uncertain significance	Missense variant
p.G4D (c.11G>A)	rs111033222	Uncertain significance	Missense variant
p.T186T (c.558G>A)	rs547195492	Uncertain significance	Missense variant
p.T5A (c.12A>C)	-	Uncertain significance	Missense variant
p.V198V (c.594G>A)	rs773768026	Uncertain significance	Missense variant

Continuation of Table A1: *GJB2* variations in humans

***GJB2* variant**	**rs number**	**Clinical significance**	**Type of variant**
p.V63V (c.189G>T)	-	Uncertain significance	Missense variant
p.W172R (c.514T>A)	rs770330002	Uncertain significance	Missense variant
pI118= (c.354C>T)	rs1260220204	Uncertain significance	Missense variant
pV52L (c.154G>C)	rs1555341987	Uncertain significance	Missense variant
T5= (c.15G>A)	rs757226502	Uncertain significance	Missense variant
p.L213S	-	Undetermined	Missense variant
W172C	rs1302739538	Undetermined	Missense variant

Appendix B

Molecular methods used to investigate HI gene variants

Polymerase chain reaction (PCR)

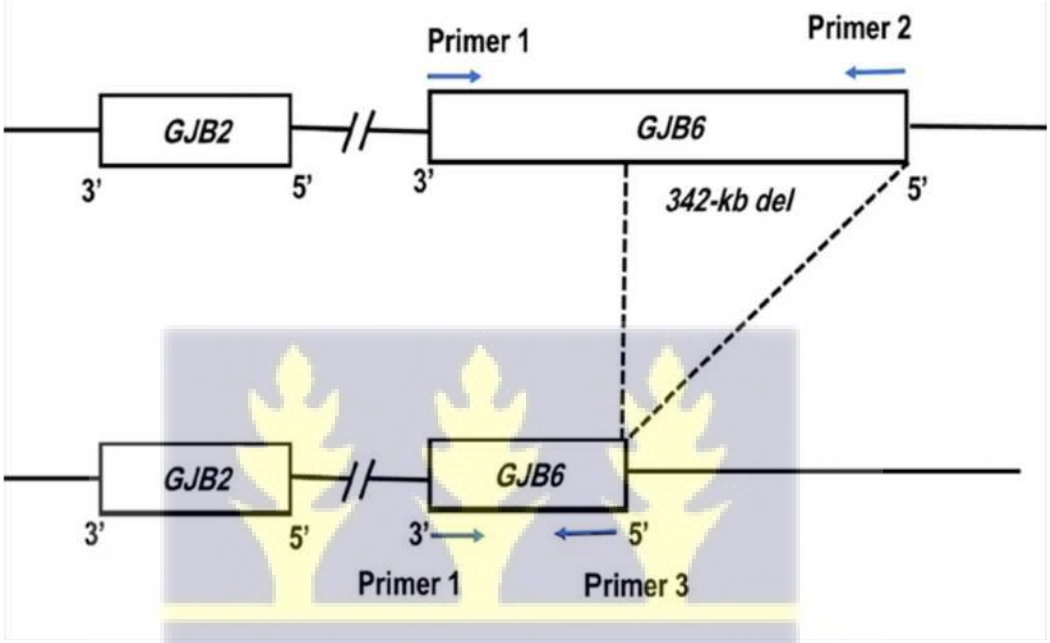
Allele-specific primers (Table S5.1 and section 6.3.3.) were used to amplify the coding regions of *GJB2*, *GJB4*, and *GJC3*. A 25µl total reaction volume was prepared from 5µl of 1x buffer, 1µl (200µM) deoxynucleotide triphosphates (dNTPs), 0.5µl of 0.4µM of each primer, 0.02U/µl of GoTaq polymerase, and 2µl of 50ng/µl of gDNA. The cycling conditions for 36 cycles were as follows: denaturation temperature of 95 for 3 minutes, the annealing temperature of 60 °C for 30 seconds, extension temperatures of 70 °C for 1 minute. The PCR products were resolved on 2% agarose gel prior to Sanger sequencing.

Screening for del(*GJB6*-D13S1830)

A multiplex PCR was conducted with allele-specific primers listed in Figure B1 to assess the del(*GJB6*-D13S1830) variant within the Ghanaian population. A total reaction volume of 25µl was prepared with the following constituents: 5µl of 1x buffer, 1µl (200µM) deoxynucleotide triphosphates (dNTPs), 0.5µl of 0.4µM of each primer, 0.02U/µl of GoTaq polymerase, and 2µl of 50ng/µl of gDNA. The reaction was carried out in a thermal cycler with a denaturation temperature of 95 °C for 3 minutes, the annealing temperature of 58 °C for 30 seconds, extension temperatures of 70 °C for 1 minute. A total of 36 cycles was completed for each sample. The PCR products were resolved on a 2% agarose gel.

Primer 1 and 2 targets the wild-type gene without the deletion while primer 1 and primer 3 targets the deletion (Figure B1). This implies that the wild type will give a larger band

size of about 681bp compared to the deletion which will give a smaller band size of about 486bp.



Primer name	sequence
Δ(GJB6-D13S1830) Primer 1	TTTAGGGCATGATTGGGGTGATTT
Δ(GJB6-D13S1830) Primer 2	CACCATGCGTAGCCTTAACCATTTT
Δ(GJB6-D13S1830) Primer 3	TCATCGGGGGTGTCAACAAACA

Figure B1: Schematic representation of primers for Δ(GJB6-D13S1830) genotyping

Sanger sequencing

The forward and reverse primers used for the PCR were used to separately Sanger sequence the various genes. Exactly 5µl of the PCR products were cleaned using 0.2µl exonuclease 1 (Exo1) and 0.5µl alkaline phosphatase (FAST-AP) in each reaction mixture. The reaction mixture was incubated at 37°C for 1 hour and 75°C for 15 minutes to stop the reaction.

The cleaned PCR products were used in a chain termination PCR with reagents and conditions similar to the PCR described earlier. Instead of dNTPs,

dideoxyribonucleotides (ddNTPs) were used in the PCR-sequencing reaction. The sequencing products were resolved using ABI 3130XL Genetic Analyzer® (Applied Biosystems, Foster City, CA), in the Division of Human Genetics, University of Cape Town, South Africa.

Appendix C

Screening for *GJB2*-R143W associated hearing impairment: implications for health policy and practice in Ghana

Running head: Policy statement on *GJB2*-R143W newborn testing in Ghana

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C1.0. Abstract

Genetic factors significantly contribute to the burden of hearing impairment (HI) in Ghana, as there is a high carrier frequency (1.5%) of the connexin 26 gene founder variant *GJB2*-R143W in the healthy Ghanaian population. *GJB2*-R143W mutation account for nearly 26% of causes in families segregating congenital non syndromic HI. With HI associated with high genetic fitness, this indicates that Ghana will likely sustains an increase in the number of individuals living with inheritable HI. There is a universal newborn hearing screening (UNHS) program in Ghana. However, this program does not include genetic testing. Adding genetic testing of *GJB2*-R143W mutation at the population, prenatal and neonatal stages may lead to guiding genetic counselling for individuals and couples, early detection of HI for at-risk infants, and improvement of medical management, including speech therapy and audiologic intervention, as well as provision of the needed social service to enhance parenting, and education for children with HI. Based on published research on the genetics of HI in Ghana, we *recommend that the UNHS program should include genetic screening for the GJB2-R143W gene variant for newborns who did not pass the initial UNHS tests*. This will require an upgrade and resourcing of public health infrastructures to implement the rapid and cost-effective *GJB2*-R143W testing, followed by appropriate genetic and anticipatory guidance for medical care.

Keywords: Health policy, *GJB2*-R143W founder mutation, hearing impairment, newborn screening; Ghana.

C1.1. Key messages

1. *GJB2*-R143W founder mutation is the major cause associated with HI in Ghana, with relatively high population carrier frequency of 1.45% amongst healthy Ghanaians.

2. *GJB2*-R143W account for nearly 26% of causes in families segregating congenital non-syndromic HI

3. There is a rapid test designed for screening for the *GJB2*-R143W variant. We recommend that this test be adopted as part of the universal newborn hearing screening (UNHS) program in Ghana.

4. Early testing for the *GJB2*-R143W variant could lead to early detection of HI and the provision of medical and social services that will help improve the quality of life of affected individuals.

5. Cochlear implants should be developed in Ghana, as the method of choice for correcting HI in children diagnosed before the age of language development.

C2.0. Background

Hearing impairment (HI) is the partial or complete loss of hearing in an individual. Globally, about 1-2 of every 1000 persons, present with some form of HI (James *et al.*, 2018). In Africa, approximately 6 out of every 1000 live births will have HI (Olusanya *et al.*, 2014). The global prevalence is expected to increase to about 900 million by 2050 (WHO, 2019). Persons with HI cannot hear sound within the "normal" audible range of hearing (Oxenham, 2018), and this impacts on their ability to effectively communicate with people around them. It could also affect their quality of life, including access to

education, healthcare, and other basic social services (Copley & Friderichs, 2010; Sarant *et al.*, 2015).

A common form of HI is Non-syndromic hearing impairment (NSHI). NSHI is not associated with any known clinical symptoms (Birkenhäger *et al.*, 2007) and presents in different forms and degrees of severity ranging from hearing loss in one or both ears to difficulties in understanding soft speech, and inability to hear very loud noises. In some cases, the degree of hearing loss may become worse with age. NSHI could be caused by a variety of factors, some of which may be genetic or environmental (Wonkam *et al.*, 2013). The genetic causes are mainly associated to the connexin 26 gene (*GJB2*) mutation which is mostly inherited in an autosomal recessive pattern (Lebeko *et al.*, 2015), whilst environmental causes include, but not limited to exposure to loud sound, infectious diseases, and certain health conditions. In Ghana, meningitis is the main environmental cause of childhood HI, while the main genetic cause is the inheritance of the *GJB2*-R143W variant (Adadey *et al.*, 2019; Adadey *et al.*, 2020; Brobby *et al.*, 1998; Hamelmann *et al.*, 2001).

Ghana is an English-speaking West African country with an estimated population of about 30,280,482 people (Ghana_Statistical_Service, 2009). About 50% and 15% of rural and urban dwellers in Ghana are living in poverty (Cooke *et al.*, 2016). To reduce poverty, the government of Ghana has made commitments towards expanding and ensuring free access to formal education (Adu Boahen & Yamauchi, 2018; Ekundayo, 2018; Salifu *et al.*, 2018). In spite of these efforts, an educational performance gap exists between students in urban schools compared to rural schools, which is possibly as a result of an uneven distribution of educational facilities and resources (Takyi *et al.*, 2019). Similarly, schools for the deaf are mostly underprivileged and have inadequate resources

for effective teaching and learning. Hence, hearing-impaired students are part of the marginalized individuals who receive minimum attention from the government (Nortey, 2009). The structure of the Ghanaian society does not effectively support disabilities (Nortey, 2009) especially the negative cultural perception of hearing-impaired people (Boadi, 2017), and this affects the participation of hearing-impaired individuals within the society. The low participation of these individuals negatively affects their psychosocial health and makes them feel inferior in many situations (Nortey, 2009).

Access to quality health care is a fundamental human right in Ghana, but it is often inaccessible to the hearing-impaired (Senayah *et al.*, 2019). Hearing-impaired patients face major barriers such as finance, the proximity of the facilities, and lack of sign language interpreters when accessing health care in Ghana (Ganle *et al.*, 2016; Senayah *et al.*, 2019). A recent study among young hearing-impaired adults underscores the need for health care professionals to be trained to communicate using sign language (Senayah *et al.*, 2019). There is an uneven distribution of health facilities in the country, underdeveloped communities travel longs kilometers on foot to access health services in the city centers (Kwasi Ofosu, 2012). In addition to the above challenges, the majority of Ghanaian health facilities cannot effectively diagnose HI at an early age where interventions are most needed (Jatto *et al.*, 2018; Kankam *et al.*, 2017). To date, there is no routine clinical investigation of HI genes in Ghana as well as cochlear implants for affected Ghanaians. There is a need therefore to have informed policy on genetic screening for HI in Ghanaian infants who fail universal newborn hearing tests and the provision of early interventions.

C2.1. Universal Newborn Hearing Screening

The universal newborn hearing screening (UNHS) has been implemented in several countries (Baroch, 2003; Bezuidenhout *et al.*, 2018; Hyde, 2005) with the aim of diagnosing HI in newborn babies to give appropriate interventions, follow-up tests, or treatments to children with permanent HI (Bezuidenhout *et al.*, 2018). The UNHS program is also referred to as early hearing detection and intervention (EHDI) program (Hyde, 2005). The methods used in the UNHS are non-invasive quick tests to assess the physiological status of the infant's ear and are often conducted soon after birth. The procedure for the UNHS consists of presenting soft sounds (clicks) to through the ears of the baby using automated auditory brainstem response (AABR) or automated otoacoustic emissions (AOAEs). The child's response to the sound presented is measured by a sensor through the scalp. Special devices with inbuilt algorithms are used to evaluate the auditory brainstem response (ABR) of the child. In most cases, the children who did not pass the first UNHS test are scheduled for a second test and subsequently referred to a specialist when no response is obtained from the initial tests (Bezuidenhout *et al.*, 2018; Hyde, 2005).

C3.0. Hearing Impairment: A condition of public health significance in Ghana

Hearing impairment adversely affects the cognitive development of children (Sarant *et al.*, 2015), making it challenging for them to learn vocabulary, grammar, and other aspects of verbal communication (Copley & Friderichs, 2010; Sarant *et al.*, 2015). This significantly impacts their education and in some instances, persons with HI are considered a social and economic burden to their families and community (Emmett & Francis, 2015). For example, over 80% of the deaf children in Ghana are born to hearing parents, and their parents, siblings, and friends struggle to communicate with them (Boadi, 2017). Equally, one-on-one interviews by us with some deaf children in Ghana

revealed that they feel neglected and unloved by their parents. Other studies in Ghana have reported difficulties by persons with HI to access social services such as healthcare and education, or to socially adapt to their communities (Agyire-Tettey *et al.*, 2017; Boadi, 2017; Senayah *et al.*, 2019). This could be frustrating for both the deaf children and other people in the community.

Early diagnosis of HI could lead to the early introduction of intervention that could support speech, language, and cognitive development for deaf children (Sarant *et al.*, 2015). Empirical Studies in South Africa and the United Kingdom, have demonstrated that hearing-impaired children when diagnosed early and given the appropriate intervention, especially within the first six months after birth, are likely to have similar cognitive and language development as hearing children of the same age group (Copley & Friderichs, 2010; Lovett *et al.*, 2010; Swanepoel *et al.*, 2007). However, in Ghana, the majority of hearing-impaired children are only able to have comprehensive hearing tests after the age of 6 years (Adadey *et al.*, 2019), when they would have passed the age of language development. This impacts negatively on the effectiveness of any interventions that may be introduced to improve on their quality of life.

To facilitate early detection and management of HI in children, several countries have introduced Universal newborn hearing screening (UNHS) into their clinical programs. However, UNHS is still not available in many African countries and many newborn screening programs tend to rely on ontological (ENT) examination to detect hearing loss in infants (Copley & Friderichs, 2010). Unfortunately, these diagnostic procedures are not able to provide conclusive results in infants (Kanji & Khoza-Shangase, 2018). Although UNHS was introduced in Ghana in the early 1970s, this service is still largely unavailable in most health centers across the country (Jatto *et al.*, 2018; Kankam *et al.*,

2017). Failure to effectively roll out the UNHS in Ghana could be due to a variety of reasons including high cost of testing, limited infrastructural capacity, and human resources to man the service.

Genetic testing may at this time be costly for populations in sub-Saharan Africa. However, we are of the opinion that we could leverage existing knowledge and genetic programs on HI in Ghana to introduce a cost-effective genetic test for HI as part of the national UNHS package. For example, it is already established that the *GJB2 gene* accounts for over a quarter (26%) of familial HI cases in Ghana (Adadey *et al.*, 2019), suggesting that 1 out of every 4 hearing-impaired families in Ghana are likely to have the *GJB2* gene variants. The reported carrier frequency of nearly 1.5% suggests that among every 145 Ghanaians (without HI), two are likely to pass on a defective *GJB2* gene to their children. Therefore, genetic screening for hearing loss may identify, at an early stage, children who are likely to develop HI.

In Ghana, there is no clinical investigation for HI genes especially in the UNHS program. A number of genetic sequencing platforms for HI are now commercially available (Shearer & Smith, 2012). However, their use in resource limited countries may be practically challenging because of limited human and infrastructural capacity to support genetic sequencing as part of routine clinical processes (Schade *et al.*, 2003; Schrauwen *et al.*, 2013; Tayoun *et al.*, 2016). Given this practical challenge, we propose that UNHS Ghana adopts and uses a rapid and effective diagnostic tool for screening for the *GJB2*-R143W variant (Adadey *et al.*, 2020). This diagnostic tool was recently developed following genetic studies on HI in Ghana. Unlike most commercially available tools, this diagnostic test is based on the restriction fragment length polymorphism technique and therefore does not require the use of sequencing technology. The tool has the potential

to identify the common genetic cause (*GJB2*-R143W) of HI among Ghanaians and can effectively be used as a first-line genetic testing tool. Adding genetic testing of *GJB2*-R143W mutation at the population, prenatal and neonatal level may lead to guiding genetic counseling for individuals and couples, early detection of HI for at-risk infants, and improvement of medical management, including speech therapy and audiologic intervention, as well as provision of the needed social service to enhance parenting, and education for children with HI. As the UNHS will identified more children with HI, this will further the rationale to develop a cochlear implant service in Ghana, as the method of choice for hearing restoration in children diagnosed before the age of language development.

C4.0. Policy Recommendations

UNHS is an important strategy for reducing the burden of HI. Although it has been introduced in Ghana, it does not incorporate yet the option for genetic HI testing (Kennedy *et al.*, 2005; Thompson *et al.*, 2001). Therefore, despite the strong evidence for the major contribution of *GJB2*-R143W mutations to HI, Ghanaian children are unable to receive early HI genetic diagnosis (Adadey *et al.*, 2019). Therefore, we recommend the following:

1. Early screening of Ghanaian children for hearing impairment should be introduced in pediatric programs across the country. For this to be possible, hearing assessment centers in Ghana, as well as the existing Community-Based Health Planning and Services *(CHPS) compounds,* should be equipped with the necessary logistics and human resources to complement the UNHS program.

2. Children who are found to have HI from the UNHS should be tested for mutations in *GJB2* and more especially R143W mutation since it accounts for the majority

of HI in Ghana. Figure 1 shows an outline of recommended for HI screening among newborn babies.

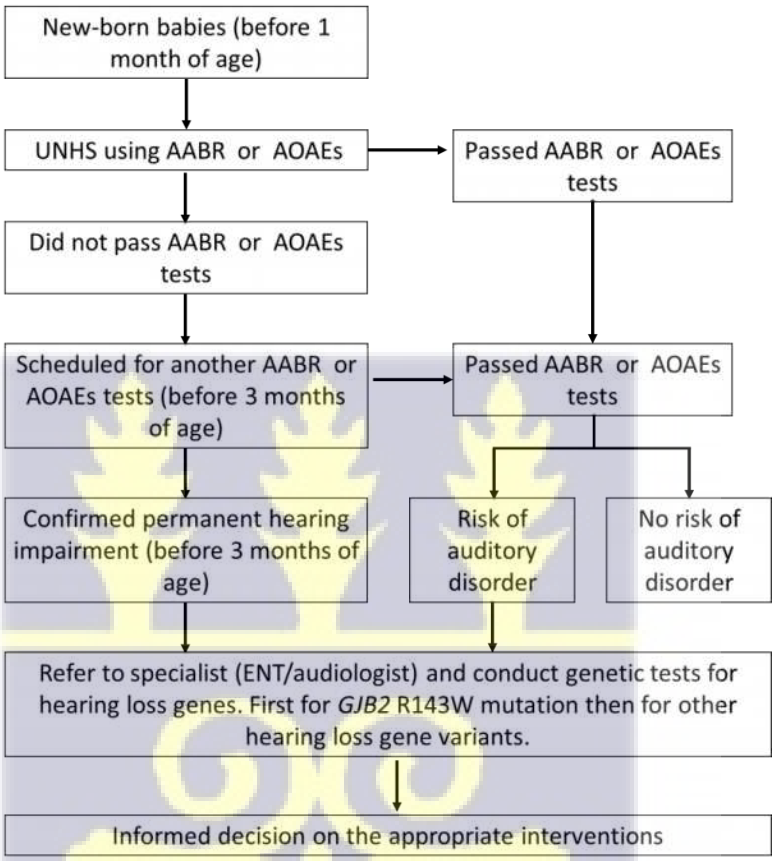

Figure B1: Flow diagram of recommended screening for early detection of HI. *(UNHS = universal newborn hearing screening; AABR = auto-mated auditory brainstem response; AOAEs = auto-mated otoacoustic emissions)*

3. Laboratory diagnosis services should implement the recently developed *GJB2*-R143W cost-effective for HI in Ghana (Adadey *et al.*, 2020). This will relatively decrease the cost of HI genetic screening in Ghana compared to existing costs in other African countries. This is because our cost-effective screening tool was developed based on a simple and inexpensive RFLP technic to screen for the common R143W mutation which accounts for over 26% of familial HI cases in Ghana.

4. Health services should develop genetic services including genetics counselling for HI, to accompany the UNHS program.

5. Appropriate intervention programs should be planned accordingly. This will include a cochlear implant services in Ghana, is this a standard treatment for genetic HI in high resources countries such as the United Kingdom (Lovett *et al.*, 2010). This is however not the case in Ghana. This may be due to the inability of Ghanaian health centers to properly diagnose genetic HI. With the implementation of the suggestions above, the HI children under the UNHS scheme can be well characterized and given the appropriate interventions such as provision of hearing aids, cochlear implants, speech therapy, or early language aids. Intervention programs should extend to develop social resources to enhance parenting including Sign language courses for families, equip school for the deaf in Ghana to improve educational attainment for affected children, and wide speech-language interventions for children and families.

C5.0 Conclusion

Hearing impairment is a noncommunicable sensory disorder of major public health concern in Ghana. The majority of congenital hearing impairment in Ghana is caused by genetic factors, of which *GJB2*-R143W is a major contributor. Genetic screening for *GJB2*-R143W in newborns in Ghana would offer families with options for proper intervention which will improve the living standards and quality of life of deaf children.

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Statement of Ethics

This manuscript did not contain the involvement human or animal participants.

Disclosure Statement

The authors declare no conflicts of interest.

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Author Contributions

Author Contributions: Conceptualization, A.W., G.A.A., and S.M.A.; writing—original draft preparation, S.M.A., G.A.A., A.W.; writing-review and editing, S.M.A., O.Q., G.K.A., G.A.A., and A.W.; supervision, A.W., G.A.A., G.K.A., and O.Q.; funding acquisition, A.W. and G.A.A. All authors contributed important intellectual content presented and have read and agreed to the published version of the manuscript.

B6.0. References

Adadey, S. M., Manyisa, N., Mnika, K., De Kock, C., Nembaware, V., Quaye, O. Q., . . . Wonkam, A. (2019). GJB2 and GJB6 mutations in non-syndromic childhood hearing impairment in Ghana. *Frontiers in Genetics, 10*, 1-10.

Adadey, S. M., Tingang Wonkam, E., Twumasi Aboagye, E., Quansah, D., Asante-Poku, A., Quaye, O., Wonkam, A. (2020). Enhancing Genetic Medicine: Rapid and Cost-Effective Molecular Diagnosis for a GJB2 Founder Mutation for Hearing Impairment in Ghana. *Genes (Basel), 11*(2). doi:10.3390/genes11020132

Adu Boahen, E., & Yamauchi, C. (2018). The effect of female education on adolescent fertility and early marriage: Evidence from free compulsory universal basic education in Ghana. *Journal of African Economies, 27*(2), 227-248.

Agyire-Tettey, E. E., Cobbina, M., & Hamenoo, E. S. (2017). Academic Challenges of Students with Hearing Impairment (SHIs) in Ghana. *Disability, CBR & Inclusive Development, 28*(3), 127-150.

Baroch, K. A. (2003). Universal newborn hearing screening: fine-tuning the process. *Curr Opin Otolaryngol Head Neck Surg, 11*(6), 424-427.

Bezuidenhout, J., Khoza-Shangase, K., De Maayer, T., & Strehlau, R. (2018). Universal newborn hearing screening in public healthcare in South Africa: Challenges to implementation. *South African Journal of Child Health, 12*(4), 159-163.

Birkenhäger, R., Aschendorff, A., Schipper, J., & Laszig, R. (2007). [Non-syndromic hereditary hearing impairment]. *Laryngo-rhino-otologie, 86*(4), 299-309; quiz 310-293.

Boadi, F. (2017). *Parents' perception on the education of the deaf–the perspective of parents of children with deafness at Ashanti school for the deaf, Jamasi, Ghana.*

Brobby, G. W., Muller-Myhsok, B., & Horstmann, R. D. (1998). Connexin 26 R143W mutation associated with recessive nonsyndromic sensorineural deafness in Africa. *N Engl J Med, 338*(8), 548-550. doi:10.1056/NEJM199802193380813

Cooke, E., Hague, S., & McKay, A. (2016). The Ghana poverty and inequality report: Using the 6th Ghana living standards survey. *University of Sussex*.

Copley, G., & Friderichs, N. (2010). An approach to hearing loss in children. *South African family practice, 52*(1), 34-39. doi:https://doi.org/10.1080/20786204.2010.10873928

Ekundayo, O. S. (2018). The right to free and compulsory primary education in Ghana: Lessons for other African countries. *JL Pol'y & Globalization, 69*, 105.

Emmett, S. D., & Francis, H. W. (2015). The socioeconomic impact of hearing loss in US adults. *Otology & neurotology: official publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 36*(3), 545.

Ganle, J. K., Otupiri, E., Obeng, B., Edusie, A. K., Ankomah, A., & Adanu, R. (2016). Challenges women with disability face in accessing and using maternal healthcare services in Ghana: a qualitative study. *PLoS One, 11*(6).

Ghana_Statistical_Service. (2009). Demography: Population Projection. *Social and Demography Demography*. Retrieved from https://www.statsghana.gov.gh/nationalaccount_macros.php?Stats=MTA1NTY1NjgxLjUwNg==/webstats/s679n2sn87

Hamelmann, C., Amedofu, G. K., Albrecht, K., Muntau, B., Gelhaus, A., Brobby, G. W., & Horstmann, R. D. (2001). Pattern of connexin 26 (GJB2) mutations causing sensorineural hearing impairment in Ghana. *Hum Mutat, 18*(1), 84-85. doi:10.1002/humu.1156

Hyde, M. L. (2005). Newborn hearing screening programs: Overview. *Journal of otolaryngology, 34*(2), S70.

James, M., Kumar, P., & Ninan, P. J. (2018). A study on prevalence and risk factors of hearing Impairment among newborns. *International Journal of Contemporary Pediatrics, 5*(2), 304-309. doi:http://dx.doi.org/10.18203/2349-3291.ijcp20180018

Jatto, M. E., Ogunkeyede, S. A., Adeyemo, A. A., Adeagbo, K., & Saiki, O. (2018). Mothers' perspectives of newborn hearing screening programme. *Ghana Med J, 52*(3), 158-162.

Kanji, A., & Khoza-Shangase, K. (2018). Objective Hearing Screening Measures: An Exploration of a Suitable Combination for Risk-Based Newborn Hearing Screening. *J Am Acad Audiol, 29*(6), 495-502. doi:https://doi.org/10.3766/jaaa.16155

Kankam, K. S., Owusu, I., Dogbe, J. A., & Opoku, V. K. (2017). *Challenges of Screening and Early Detection of Hearing Impairment among Children in some selected hospitals in Kumasi Metropolis.* (MSc), University of Science and Technology

Kennedy, C., McCann, D., Campbell, M. J., Kimm, L., & Thornton, R. (2005). Universal newborn screening for permanent childhood hearing impairment: an 8-year follow-up of a controlled trial. *The Lancet, 366*(9486), 660-662.

Kwasi Ofosu, D. (2012). *Assessing the spatial distribution of health facilities in the Eastern Region of Ghana.*

Lebeko, K., Bosch, J., Noubiap, J. J., Dandara, C., & Wonkam, A. (2015). Genetics of hearing loss in Africans: use of next generation sequencing is the best way forward. *Pan Afr Med J, 20*, 383. doi:10.11604/pamj.2015.20.383.5230

Lovett, R. E., Kitterick, P. T., Hewitt, C. E., & Summerfield, A. Q. (2010). Bilateral or unilateral cochlear implantation for deaf children: an observational study. *Archives of Disease in Childhood, 95*(2), 107-112.

Nortey, D. A. (2009). *Barriers to social participation for the deaf and hard of hearing in Ghana.* The University of Bergen.

Olusanya, B. O., Neumann, K. J., & Saunders, J. E. (2014). The global burden of disabling hearing impairment: a call to action. *Bulletin of the World Health Organization, 92*, 367-373. doi: http://dx.doi.org/10.2471/BLT.13.128728

Oxenham, A. J. (2018). How we hear: The perception and neural coding of sound. *Annual review of psychology, 69*.

Salifu, I., Boateng, J. K., & Kunduzore, S. S. (2018). Achieving free compulsory universal basic education through school feeding programme: Evidence from a deprived rural community in northern Ghana. *Cogent Education, 5*(1), 1509429.

Sarant, J. Z., Harris, D. C., & Bennet, L. A. (2015). Academic outcomes for school-aged children with severe–profound hearing loss and early unilateral and bilateral cochlear implants. *Journal of Speech, Language, and Hearing Research, 58*(3), 1017-1032.

Schade, G., Kothe, C., Ruge, G., Hess, M., & Meyer, C. G. (2003). [Non-invasive screening for GJB2 mutations in buccal smears for the diagnosis of inherited

hearing impairment]. *Laryngorhinootologie, 82*(6), 397-401. doi:10.1055/s-2003-40538

Schrauwen, I., Sommen, M., Corneveaux, J. J., Reiman, R. A., Hackett, N. J., Claes, C., . . . Van Camp, G. (2013). A sensitive and specific diagnostic test for hearing loss using a microdroplet PCR-based approach and next generation sequencing. *American Journal of Medical Genetics Part A, 161*(1), 145-152.

Senayah, E. A., Mprah, W. K., Opoku, M. P., Edusei, A. K., & Torgbenu, E. L. (2019). The accessibility of health services to young deaf adolescents in Ghana. *The International journal of health planning and management, 34*(1), e634-e645.

Shearer, A. E., & Smith, R. J. (2012). Genetics: advances in genetic testing for deafness. *Current opinion in pediatrics, 24*(6), 679.

Swanepoel, D. W., Louw, B., & Hugo, R. (2007). A novel service delivery model for infant hearing screening in developing countries: Un modelo novedoso para la prestación de servicios de tamiz auditivo infantil en países en desarrollo. *Int J Audiol, 46*(6), 321-327. doi:https://doi.org/10.1080/14992020601188583

Takyi, S. A., Amponsah, O., Asibey, M. O., & Ayambire, R. A. (2019). An overview of Ghana's educational system and its implication for educational equity. *International Journal of Leadership in Education*, 1-26.

Tayoun, A. N. A., Mason-Suares, H., Frisella, A. L., Bowser, M., Duffy, E., Mahanta, L., . . . Amr, S. S. (2016). Targeted droplet-digital PCR as a tool for novel deletion discovery at the DFNB1 locus. *Hum Mutat, 37*(1), 119-126.

Thompson, D. C., McPhillips, H., Davis, R. L., Lieu, T. A., Homer, C. J., & Helfand, M. (2001). Universal newborn hearing screening: summary of evidence. *JAMA, 286*(16), 2000-2010.

WHO. (2019, 20 March 2019). Deafness and hearing loss. Retrieved from https://www.who.int/news-room/fact-sheets/detail/deafness-and-hearing-loss

Wonkam, A., Noubiap, J. J. N., Djomou, F., Fieggen, K., Njock, R., & Toure, G. B. (2013). Aetiology of childhood hearing loss in Cameroon (sub-Saharan Africa). *European journal of medical genetics*, *56*(1), 20-25. doi:10.1016/j.ejmg.2012.09.010

Appendix D (Ethical and admirative clearance)

GHANA EDUCATION SERVICE

(SPECIAL EDUCATION DIVISION)

P.O. Box NT.451
Accra-Newtown
specialedgh@gmail.com

In case of reply the number and date of this letter should be quoted.
My Ref. No .SE./239/7
Your Ref. No

3RD August, 2016

THE DIRECTOR
WEST AFRICAN CENTRE FOR CELL BIOLOGY
OF INFECTIOUS PATHOGENS
COLLEGE OF BASIC AND APPLIED SCIENCES
UNIVERSITY OF GHANA
P.O.BOX LG.54
LEGON, ACCRA

Dear Sir/Madam,

RE: REQUEST FOR RESEARCH VISIT TO SCHOOLS FOR THE DEAF

Your letter dated 27th July, 2016 on the above subject matter refers: has been received.

Approval is given to Mr. Samuel Mawuli Adadey to visit Schools for the Deaf as per attached list for his research project.

We will inform the schools involved to facilitate his research activity.

Thank you.

ANTHONY BOATENG
DIRECTOR

INTEGRI PROCEDAMUS

GHANA EDUCATION SERV

(SPECIAL EDUCATION DIVISION)

P.O. Box NT.451
Accra-Newtown
specialedgh@gmail.com

In case of reply the number and date of this letter should be quoted.

My Ref. No .SE./239/7

Your Ref. No

3RD August, 2016

HEADS OF SELECTED SCHOOLS FOR THE DEAF

RESEARCH AUTHORIZATION

I write to introduce Mr. Samuel Mawuli Adadey who is a first year PhD student of the West African Centre for Cell Biology of Infectious Pathogens(WACCBIP), University of Ghana.

Mr. Mawuli Adadey is to conduct research on "**The genetics of congenital pre-lingual non-syndronic hearing impairment in Ghana".**

Heads of Schools for the Deaf (list attached) are kindly requested to give Mr. Adadey the needed support and assistance to facilitate his research work.

Thank you

ANTHONY BOATENG
DIRECTOR

Cc: MR. SAMUEL MAWULI ADADEY
WACCBIP
University of Ghana, Legon.

THE DIRECTOR
WACCBIP
University of Ghana, Legon.

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University of Ghana

INSTITUTIONAL REVIEW BOARD

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My Ref. No: DF.22
Your Ref. No:

7th September, 2016

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 006/16-17 **IORG 0000908**

On 7th September 2016, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting reviewed and approved your protocol titled:

TITLE OF PROTOCOL : **The Genetics of congenital pre-lingual non-syndromic hearing impairment in Ghana**

PRINCIPAL INVESTIGATOR : **Samuel Mawuli Adadey, PhD Cand.**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 6th September, 2017. You are to submit annual reports for continuing review.

Signature of Chair: .....

Mrs. Chris Dadzie
(NMIMR – IRB, Chair)

Appendix D

Approved Questionnaire

QUESTIONNAIRE
Department of Biochemistry Cell and Molecular Biology
School of Basic and Applied Science
University of Ghana, Legon

Title: THE GENETICS OF CONGENITAL PRE-LINGUAL NON-SYNDROMIC HEARING IMPAIRMENT IN GHANA

Study participant's code number: ..... *Date: .....*

Sex: ..... Age: .....
Date of Birth: ..... Unit: .....

Please tick the option that best describes your situation

1. Do you have hearing problem? Yes No
2. When did you first begin to experience your hearing problem?
   a. ____ from birth
   b. ____ suddenly (over three days)
   c. ____ quickly (weeks and months)
   d. ____ gradually (months or years)?
3. On which ear is your hearing problem?
   a. ____ on the right,
   b. ____ on the left,
   c. ____ on both sides?
4. Have you ever worn a hearing aid? Yes No
5. Have you ever had ear surgery? Yes No
   a. If yes, please describe:
6. Have you been exposed to noise during school, work or recreation? Yes No
7. Do you have any family members with hearing problems? Yes No
   a. If yes, how many .....
8. Do you have any family members with hearing aids? Yes No
9. Do you ever experience ringing or hear other noises in your ears? Yes No
10. Have you ever been treated for cancer? Yes No
11. Have you ever been treated for diabetes? Yes No
12. Have you ever suffered ear trauma or head injury? Yes No
13. Have you ever had to be on IV antibiotics for serious ear infections? Yes No
14. Are you currently on any hearing loss medications? Yes No

15. Please fill the table below as it describes your situation

Description	Both ears	Left ear	Right ear	None
Fullness or stuffiness in your ears?				
Pain in your ears?				
Drainage from your ears?				

1

Distortion of sound?				
Sensitivity to sound?				

16. Do you have any medical history of prenatal and postnatal exposure to:
    a. Rubella? Yes No
    b. Cytomegalovirus? Yes No
    c. Viral or bacterial infection? Yes No
    d. Meningitis? Yes No
17. Were you born premature (before 9 months)? Yes No
18. Do you have previous treatment using potentially ototoxic drugs or antibiotics such as streptomycin or gentamicin? Yes No