WEST AFRICAN CENTRE FOR CELL BIOLOGY OF INFECTIOUS PATHOGENS
DEPARTMENT OF BIOCHEMISTRY CELL AND MOLECULAR BIOLOGY
COLLEGE OF BASIC AND APPLIED SCIENCES
UNIVERSITY OF GHANA

GENETIC ARCHITECTURE OF NON-SYNDROMIC HEARING IMPAIRMENT IN GHANA

This thesis is submitted to the University of Ghana, Legon in partial fulfilment of the requirements for the award of **PhD IN BIOCHEMISTRY DEGREE**

BY

ELVIS TWUMASI ABOAGYE
(10396924)

November 2022

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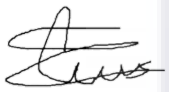
Declaration

Presented in this thesis are studies conducted by me, Elvis Twumasi Aboagye, at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana and the Division of Human Genetics, Faculty of Health Sciences, University of Cape Town. The thesis was supervised by Prof. Ambroise Wonkam (University of Cape Town, and Johns Hopkins University School of Medicine), Prof. Gordon Akanzuwine Awandare (University of Ghana), and Dr. Lucas Amenga-Etego (University of Ghana). I declare that neither part of this work or the whole has been or is being submitted for another degree in this or any other university and that this thesis is my original work (apart from where acknowledgements specify otherwise).

Signed 
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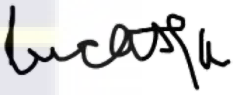
Prof. Ambroise Wonkam

Signed 
Prof. Gordon Akanzuwine Awandare

Signed 
Dr. Lucas N. Amenga-Etego

Acknowledgement

My utmost thanks to the Heavenly Father for His sufficient grace to complete this thesis. Particularly, I am thankful to all the staff of the schools for the deaf, students (participants), siblings, parents, and guardians whose participation means more than they'll ever imagine to achieving the set objectives.

Special thanks and sincere gratitude to Professors Ambroise Wonkam, Gordon Akanzuwine Awandare, and Dr. Lucas Amenga-Etego for their meticulous guidance, informed supervision, and mentorship. Distinct acknowledgements to Dr. S.M Adadey, Dr. Kevin Esoh, Dr. Carmen De Kock, Dr. Vicky Nembaware, Kalinka Propel, Rhiyana Bassier, Dr. Syntia Nchangwi Munung and the entire hearing impairment genetics studies in Africa (HIGenesAfrica) team, their crucial contributions throughout this journey have been very outstanding.

I am very thankful to all faculty members and colleagues at the West African Centre for Cell Biology of Infectious pathogens (WACCBIP), Department of Biochemistry, Cell, and Molecular Biology, College of Basic and Applied Sciences, University of Ghana, and the Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, who dedicated some valuable time to contribute to this study completion.

Additional thanks and appreciation to Professor Neils Ben Quashie, Dr. William Kudzi, and Dr Bartholomew Dzudzor for their fatherly advice, encouragement and providing an invaluable support towards the completion of this degree.

More importantly, this project would not have been completed without the funding support received from the National Institutes of Health (NIH), the Wellcome Trust / African Academy of Sciences, and WACCBIP-World Bank ACE Masters/PhD fellowship (WACCBIP+NCDs).

Dedication

I dedicate this piece to my family and wish to express my love and indebtedness to my loving wife Maame Afua Aboagye and my children (Nana Amafuaa Amell Aboagye, Papa Yaw Okokodurofo Liam Otu Aboagye, and Nana Yaw Obrempong Sewell Twumasi Aboagye) for their patience, understanding and support throughout this study.

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List of abbreviations

ABR	Auditory Brainstem Response
ACMG	American College of Medical Genetics and Genomics
AIFM1	Apoptosis Inducing Factor Mitochondria Associated 1
AMP	Association for Molecular Pathology
ANNOVAR	ANNOtate VARiation
ARHI	Autosomal Recessive Hearing Impairment
ARNSHI	Autosomal Recessive Non-Syndromic Hearing Impairment
AS-PCR	Allele-specific Polymerase Chain Reaction
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
CADD	Combined Annotation Dependent Depletion
CDH23	Cadherin related 23
cDNA	Complementary DNA
CI	Cochlear Implant
ClinVar	Clinical Variant Database
COL4A6	Collagen type IV alpha 6 chain
CT	Computed Tomography
Cx26	Connexin 26
DFNA	Nonsyndromic deafness with Autosomal Dominant Inheritance
DFNB	Nonsyndromic deafness with Autosomal Recessive Inheritance
DFNB1	Nonsyndromic deafness with Autosomal Recessive Inheritance
DFNX	Nonsyndromic deafness with X-Linked Inheritance
DHPLC	Denaturation High-performance Liquid Chromatography

DNA	Deoxyribonucleic Acid
ECBAS	Ethics Committee for Basic and Applied Science
ER	Endoplasmic Reticulum
ExAC	Exome Aggregation Consortium Database
EYA4	EYA Transcriptional Coactivator and Phosphatase 4
GATK	Genome Analysis ToolKits
gDNA	Genomic DNA
GERP	Genomic Evolutionary Rate Profiling
GJB2	Gap Junction Protein Beta 2
GJB4	Gap Junction Protein Beta 4
GJB6	Gap Junction Protein Beta 6
GJC3	Gap Junction Protein Gamma 3
gnomAD	Genome Aggregation Database
GR	Glutathione Reductase
GREB1L	GREB1 like retinoic acid receptor coactivator
GSDME	Gasdermin E
GSH.Px	Glutathione Peroxidase
GSSG	Glutathione Disulfide
GTPBP3	GTP Binding Protein 3
HA	Hearing Aid
HFE	Homeostatic Iron Regulator
HGVS	Human Genome Variation Society
HHL	Hereditary Hearing Loss
HI	Hearing Impairment
HPO	Human Phenotype Ontology

HWE	Hardy–Weinberg Equilibrium
IHCs	Inner Hair Cells
KARS	Lysyl-tRNA synthetase
KCNQ4	Potassium Voltage-gated Channel Subfamily Q member 4
KING	Kinship-based INference for Gwas
LD	Linkage Disequilibrium
LOD	Log of Odds
LRTOMT	Leucine rich transmembrane and O-methyltransferase domain containing
MAF	Minor Allele Frequency
MARVELD2	MARVEL domain containing 2
M-CAP	Mendelian Clinically Applicable Pathogenicity
MCMC	Markov Chain Monte Carlo
MET	Mechano-transduction
MLPA	Multiplex Ligation-dependent Probe Assay
MPS	Massive Parallel Sequencing
MPZL2	Myelin Protein Zero like
MRI Scan	Magnetic Resonance Imaging
MRKH	Mayer–Rokitansky–Küster–Hauser
mRNA	Messenger RNA
MtDNA	Mitochondrial DNA
MTO1	Mitochondrial Transcription Optimization 1
MT-RNR1	Mitochondrial 12S rRNA
MYO15A	Myosin XVA
NADf	North African Deafness
NADPH	Nicotinamide adenine dinucleotide phosphate

NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NIH	National Institutes of Health
NKCC1	Na-K-2Cl cotransporter 1
NOX3	NADPH Oxidase 3
NSHI	Non-Syndromic Hearing Impairment
OAEs	Otoacoustic emissions
OHCs	Outer Hair Cells
OMIM	Online Mendelian Inheritance in Man
OSBPL2	Oxysterol binding protein like 2
OTOF	Otoferlin
PCA	Principal Component Analysis
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
PIP2	Phosphatidylinositol Biphosphate
PLP	Pathogenic or Likely Pathogenic
POU4F3	POU class 4 homeobox 3
PROVEAN	Protein Variation Effect Analyzer
qPCR	Quantitative Polymerase Chain Reaction
RAR	Retinoic Acid Receptor
RHDA3	Renal Hypodysplasia (aplasia 3)
ROS	Reactive Oxygen Species
RT-PCR	Real-time Polymerase Chain Reaction
SCD	Sickle Cell Disease
SHIELD	Shared Harvard Inner-Ear Laboratory database
SIFT	Sorting Intolerant from Tolerant

SLC26A4	Solute Carrier Family 26-member 4
SMPX	Small Muscle Protein X-linked
SNPs	Single Nucleotide Polymorphisms
SNVs	Single Nucleotide Variants
sSA	sub-Saharan Africa
SSCP	Single-strand Conformational Polymorphism
STAT1	Signal Transducer and Activator of Transcription 1
STRs	Short Tandem Repeats
TECTA	Tectorin Alpha
TFB1M	Mitochondrial Transcription Factor 1
TGE	Targeted Genomic Enrichment
TMC1	Transmembrane Channel like 1
TMIE	Transmembrane Inner Ear
TMPRSS3	Transmembrane Serine Protease 3
TRMU	5-Methylaminomethyl-2-thiouridylate methyltransferase
tRNA	Transfer RNA
VQSR	Variant Quality Score Recalibration
WES	Whole-exome sequencing
WFS1	Wolframin ER Transmembrane Glycoprotein
WGS	Whole-genome sequencing
WHO	World Health Organization
ZMYM2	Zinc Finger MYM-type containing 2

Outline of thesis

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

Review of literature

Elvis Twumasi Aboagye, Samuel Mawuli Adadey, Edmond Wonkam-Tingang, Lucas Amenga-Etego, Gordon Akanzuwine Awandare, and Ambroise Wonkam. Global Distribution of Founder Mutations Associated with Non-Syndromic Hearing Impairment. *MDPI, Genes Published.*

Objective 1

Elvis Twumasi Aboagye, Samuel Mawuli Adadey, Kevin Esoh, Mario Jonas, Carmen de Kock, Lucas Amenga-Etego, Gordon Akanzuwine Awandare and Ambroise Wonkam. (2021). Age Estimate of *GJB2*-p.(Arg143Trp) Founder Variant in Hearing Impairment in Ghana, Suggests Multiple Independent Origins across Populations. *MDPI, Biology Published.*

Objective 2

Elvis Twumasi Aboagye, Samuel Mawuli Adadey, Edmond Wonkam-Tingang, Kevin Esoh, Lucas Amenga-Etego, Isabelle Schrauwen, Gordon Akanzuwine Awandare, Suzanne M. Leal and Ambroise Wonkam. Phenotypic Expansion of *MARVELD2* Associated Hearing Impairment: Post-lingual Expression in a Ghanaian Family Segregation a Bi-allelic Novel Variant. *BMC Medical Genomics In peer-review.*

Objective 3

Samuel Mawuli Adadey*, **Elvis Twumasi Aboagye***, Kevin Esoh, Anushree Acharya, Thashi Bharadwaj, Nicole S. Lin, Lucas Amenga-Etego, Gordon Akanzuwine Awandare, Isabelle Schrauwen, Suzanne M. Leal and Ambroise Wonkam. Novel Autosomal Dominant *GREB1L* Variant Associated with Non-Syndromic Hearing Impairment in Ghana. *BMC Medical Genomics, Published.*

Abstract

Background: Despite the genetic heterogeneity in hearing impairment (HI) aetiology, gap junction protein beta 2 (*GJB2*) pathogenic variants have been implicated in more than 50% of autosomal recessive hearing impairment that manifests as an isolated disorder worldwide. The reported pathogenic variants associated with HI are extremely population - specific, demonstrated by variable frequencies and allelic heterogeneity reported in different settings. Deleterious *GJB2* variants associated with HI are described as stemming from either inheriting germline founder mutations or *de novo* (hotspots) mutations. The *GJB2*: c.427C>T-p.(Arg143Trp) founder variant is the predominant cause of non-syndromic hearing impairment (NSHI) in Ghana. However, reported isolated incidence of *GJB2*-p.(Arg143Trp) variant in Asia, Europe, and Middle East though at relatively low frequencies, have raised questions on the variant origin. Therefore, this thesis sought to investigate the provenance and evolutionary history of the *GJB2*: c.427C>T-p.(Arg143Trp) founder variant systematically reported among Ghanaian HI cohorts, and elucidate the array of genetic marker(s) segregating in multiplex and simplex families negative for *GJB2* variants. The hypothesis that the *GJB2*: c.427C>T-p.Arg143Trp founder variant co-evolved as a loss of function or gain of function mutation, positively selected for by malaria endemic in the sub-region was also reviewed.

Method: First-degree kindreds from multiplex and simplex families with NSHI were recruited from Mampong, Bechem, and Sekondi-Takoradi Schools for Deaf in Ghana. Genomic deoxyribonucleic acid (gDNA) was isolated from collected peripheral blood samples of recruited participants (18 multiplex and 23 simplex families). In-house developed polymerase chain reaction-restriction fragment length polymorphism (*NciI* restriction enzyme) was used to screen recruited participants of the most common genetic marker (*GJB2*-p.Arg143Trp). Bi-directional Sanger sequencing and family segregation analysis were performed to validate probands positive for the *GJB2* variant.

Selected unrelated *GJB2*-p.Arg143Trp homozygote participants and hearing controls negative for the founder mutation were whole-exome sequenced to estimate the age and ascertain the origin, and identify the haplotypic backgrounds sustaining the variant segregation in cohorts investigated. Unsolved families negative for the *GJB2* variant were also sent for whole-exome sequencing (WES) (newly recruited and archival families). Illumina DRAGEN Germline developed pipeline and genome analysis toolkit (GATK) were used for alignment and calling variants. Identified variants from *in silico* bioinformatic analysis were validated with bi-directional Sanger sequencing using designed sequence specific primers and family segregation analysis. The implicated mutant protein was modelled by homology modelling, using wild type protein sequence as template on web-based tool (SWISS-MODEL) and visualized in PyMOL.

Results: At least pedigrees for three generational history and audiological assessments of families investigated showed bilateral severe to profound phenotype (sensorineural isolated HI), with one suspected syndromic HI. The estimated age was consistent between the WES-unimputed and imputed computed ancestral informative markers (385 and 380 generations). The *GJB2*-p.(Arg143Trp)-positive individuals carries a unique haplotype background, specific and clearly distinct from the Ghanaian hearing controls, as well as individuals from Japanese and European populations, and shared no common haplotype. Most of the identified markers in linkage disequilibrium (LD) with the founder allele selected for age estimate were relatively absent or rare in Asia and Europe, demonstrating the independent evolutions and multiple ancestries of the variant.

Analysis of WES data identified a novel *MARVELD2*: c.1058dupT-p.(Val354SerfsTer5) duplication on exon 2 as associated with the bilateral post lingual autosomal recessive non-syndromic HI that segregates in a large consanguineous family. *In silico* variant pathogenicity analysis of the identified thymine (T) duplication at position 1058 in exon 2 predicted a protein

with valine substituted by serine at codon 354. This non-synonymous mutation is also projected to change the amino acid reading frame, producing a protein lacking the c-terminal cytoplasmic domain (close to 100 amino acids truncation). The variant was also absent in 46 ethnolinguistic hearing controls and 151 simplex hearing impaired probands screened, as well as hearing loss population database. The observed associated post-lingual severe-profound phenotype in the index case expands the previously reported pre-lingual *MARVELD2* variants associated with HI reported in other populations.

Equally, the WES analysis also defined another novel variant in *GREB1L* [c.3041G>A: p.(Gly1014Glu)] as implicated in DFNA80 (deafness autosomal dominant type 80) in another family. *In silico* variant pathogenicity analysis showed a CADD score of 26.5, and variant absent from human genomic databases such as ClinVar, ClinGen, TopMed and gnomAD. Structural modeling and analysis predicted the variant to likely affect the secondary protein structure that may impact the proteins' function. This was further supported with data showing increased levels of Greb1L expression in mice inner ear during growth and a reduced expression in adulthood, suggesting its importance in early development of the inner ear structures from single cell gene expression resource (gEAR).

Conclusion: This thesis has achieved some important findings on the spectrum of gene(s) variant underlying NSHI segregation and highlighted the specific genetic landscape in Ghana. Carriers of the major genetic contributor to NSHI in Ghana are descendants of an indigenous ancestor who lived about ~9625 years ago, illuminating the selection at the locus in Ghana that predates other reports and demonstrated no shared genetic ancestry between homozygotes cohorts and hearing controls, as well as continental populations investigated. This study also shows the high level of allelic heterogeneity implicated in NSHI. *GJB2* negative families investigated identified novel variants in known HI genes (*GREBIL*, *MARVELD2*, *CDH23*, among others) to segregate with the phenotype. The study findings have contributed to refining

the architecture of NSHI genes in Ghanaian families, and the global knowledge on HI disease – gene pair curation.

CHAPTER ONE

1.0 Introduction

Nearly 60% of congenital hearing impairment (HI) are avoidable and can be prevented by effective implementation of good public healthcare measures that includes, vaccination, improved maternal and childcare practices, and genetic counselling (World Health Organization, 2021). However, the large genetic diversity and allelic heterogeneity in hereditary HI across populations (Camp & Smith, 2019) has challenged the precise diagnosis and implementation of molecular diagnosis with optimal clinical validity in all settings**,** to complement clinical diagnosis especially in sub-Saharan Africa (sSA). In addition, the differential frequencies of reported pathogenic and likely pathogenic (P/LP) variants associated with HI worldwide have been shaped by gene hypermutabilities, population migrations, admixture, structural dynamics, and environmental pressure that may have led to evolutionary selection of some genetic variants (Pereira *et al*., 2021). It is against this background that this thesis aimed to ascertain the provenance and age of the *GJB2*-p.Arg143Trp founder mutation, recurrently associated with HI in Ghana, and elucidate the genetic architecture underlying non-syndromic HI (NSHI) in the Ghanaian population. In this regard, chapter one of this thesis introduces the study, and begins with discussion on the background and the study context, followed by outline of research problems, aims and objectives, through to the relevant questions the study sought to answer, and rationale behind the study, and finally, some anticipated limitations have also been discussed.

Hearing impairment remains the most prevalent human sensory disorder with close to 5% people affected worldwide (Stephenson, 2021). The incidence of HI in sSA has been reported as between 1 to 6 in every 1000 live births (Morton & Nance, 2006) compared to 1.7 per 1000 live births in Europe and America (CDC, 2022). Early detection of HI in infants followed by

the appropriate intervention significantly improve speech, language, cognitive and social development (Ptok, 2004). However, in the sub-region diagnosis is mostly late in children (post-language acquisition age), for example the average age of diagnosis was beyond 3 years in Cameroon (Wonkam *et al.*, 2013), between 6 to 11 years in Ghana (Adadey *et al.*, 2019), and 12 years in Mali (Yalcouyé *et al*., 2021), which are far from the WHO recommended 3 to 6 months (Stephenson, 2021).

Unlike acquired HI, hereditary HI can be non-syndromic (with no other organ or system syndrome accompanying the condition, in 70% of cases) or syndromic (in which the condition is coupled to other organ and/or system defect, in 30% of cases) (Dror & Avraham, 2010; Morton & Nance, 2006). Investigations on the genetic aetiology of HI has provided better understanding of the auditory system normal function as well as the pathophysiological processes that can alter it. Mutations in genes could impact the optimal function of components involved in the hearing process (Dror & Avraham, 2010). The inheritance patterns observed in HI of genetic aetiology is chiefly autosomal recessive (close to 80%), although, autosomal dominant (about 17%), X-linked, and matrilinear pattern of inheritance have also been reported (Smith *et al*., 2005). Reported HI associated pathogenic variants in human populations are diverse, characterized by ethnolinguistic background, genetic landscape, and geographical settings (Mikstiene *et al*., 2016). Of the known HI reported genes that are periodically updated on hereditary hearing loss web page (http://hereditaryhearingloss.org); variants in the *GJB2*, solute carrier family 26 member 4 (*SLC26A4*), and mitochondrial 12S rRNA (*MT-RNR1*) genes are mostly associated with HI in different populations (Hilgert *et al*., 2009; Sheffield & Smith, 2019). Though the frequency of causal gene variants in these genes vary across different populations and ethnicities, *GJB2* variants has been widely reported in most severe to profound HI (Snoeckx *et al*., 2005).

GJB2 gene encodes connexin 26 (Cx26), a transmembrane channel protein. Pathogenic variants in *GJB2* are the preeminent cause of congenital HI across different countries, with high allelic heterogeneity; and more than 400 different variants described to date (Morton & Nance, 2006; Stenson *et al*., 2020). The gamut and allelic frequencies of identified *GJB2* variants are highly population specific (Chan & Chang, 2014; Tsukada *et al*., 2015). *GJB2*: c.35delG-p.(Gly12ValfsTer) is the most dominant variant reported among populations in Europe, North African, Brazil, and America (Denoyelle *et al*., 1997; Frei *et al*., 2002; Gürtler *et al*., 2003), as opposed to *GJB2*: c.235delC-p.(Val37Ile) and *GJB2*: c.109G>A-p.(Leu79CysfsTer) (Abe *et al*., 2000; Hwa *et al*., 2003) in Asia, *GJB2*: c.167delT-p.(Leu56ArgfsTer) (Morell *et al*., 1998) common among Ashkenazi Jews, *GJB2*: c.71G>A-p.(Trp24Ter) (Bouwer *et al*., 2007) in India, and *GJB2*: c.427C>T-p.(Arg143Trp) (Adadey *et al*., 2019; Brobby *et al*., 1998) found in Ghana. In the same vein, the *GJB2*: c.-23+1 G>A accounted for most HI of genetic aetiology among Southwestern (Said *et al*., 2007; Sirmaci *et al*., 2006), and Southern Asia populations (Bajaj *et al*., 2008).

Genetic variations in *SLC26A4* implicated in HI are equally regional and population-specific; *SLC26A4*: c.1246A>C-p.(Thr416Pro) and *SLC26A4:* c.1001G>A-p.(Gly334Glu) are mostly identified in Europe (Campbell *et al*., 2001; Coyle *et al*., 1998), *SLC26A4*: c.2168A>G-p.(His723Arg) in Japan (Tsukamoto *et al*., 2003), and Korea (Park *et al*., 2005), and *SLC26A4*: c.919-2A>G in Taiwan (Wu *et al*., 2005), and Han China (Dai *et al*., 2008) accordingly.

Despite the elucidation of the HI candidate gene markers from extensive and systematic investigations in the past decades, the global equity in regional HI genetic research investment can be improved, as the quality and quantity of studies from numerous regions of the world particularly in Africa are far behind (Hilgert *et al*., 2009). Four major groups have been proposed to represent genetic variation in populations of African descent; North Africa; West, and East Africa; sub-Saharan; and southern regions (Campbell & Tishkoff, 2008; Reed &

Tishkoff, 2006). Though, genetic ancestry investigations of African populations on geographical and ethnolinguistic lines identified more than 13 distinct genetic populations and high degree of admixture, efforts to explore and discover new HI genes are limited (Chakchouk *et al*., 2019; Lebeko *et al*., 2017).

The gap in HI genetic investigation in Africa relative to the globally reported HI causal variants may be a reflection of the lack of resource and capacity driven that may have impacted the utilization for translational benefit in new-born screening (Koffler *et al*., 2015) unlike low-middle-income countries. Strongly, the current HI gene panels have shown cost-effectiveness and good diagnostic rate (about 40%) across all ages, degree of severity and ethnicity (Sloan-Heggen & Smith, 2016), with variations in diagnostic yield linked to multiple factors including phenotype characterization, cultural and ethnolinguistic background, quality metrics of variant classification and interpretation rules applied (Shearer *et al.*, 2019;Seco *et al.*, 2017). In the sub-region, the use of existing panels is limited, since studies that had explored these candidate gene variants contribution to HI in sub-region were inconclusive. Inadequate sample size and lack of effective population description were among reported factors limiting studies in the sub-region for objective generalizations (Kenneson & Boyle, 2004). The reported high heterogeneity of hereditary HI even among affected kindreds with the same variant further compound genetic diagnosis opportunities.

More importantly, the wide HI phenotypic variation reflects the influence of diversity in genetic dynamics and acquired factors to the penetrance and expression of HI population-specific genetic markers. Genetic background differences between populations acts as modifiers of pathogenic or likely pathogenic (P/LP) variants to modulate phenotypic expression, evident in the reported intra-and inter familial variability among some populations (Yousaf *et al*., 2016). The anticipation is that genetic elements modulation of mutant allele may explain the reported incomplete penetrance and expression variability of otherwise highly

monogenic defect clinical manifestation (Ming & Muenke, 2002; Weatherall, 2001). The effect of modifiers could range from worsening to reducing the disorder, or completely suppressing the phenotype near to normal range, onset, and disease progression (Haider *et al*., 2002; Nadeau & Topol, 2006).

Consequently, the discrepancies in the reported associated variants and diversity across populations have lagged the design and implementation of molecular diagnosis, with equal clinical validity, especially in the sSA, coupled to the limited number of HI genetic investigations (Adadey *et al*., 2021). For example, though the North African Deafness (NADf) Chip could simultaneously investigate 58 deafness variants, majority of these variants are specific to the population and not useful for sSA populations (Chakchouk *et al*., 2015).

More importantly, even though the role of inheriting germline founder, and/or *de novo* mutations in HI segregation has been well established, the heterozygote carrier advantage driving the evolutionary selection of the most common variants reported in different populations remains unknown. Considering the varied population background, and heterogeneous genetic aetiology of HI, this research sought to use a comprehensive and representative population approach to investigate the most dominant variant; *GJB2*: c.427C>T-p.(Arg143Trp) among Ghanaians, and further ascertain the contribution of other deleterious alleles to highlight the inherent genetic architecture and structure of NSHI in Ghana, using high resolution data from Next Generation Sequencing (NGS) approaches.

Interestingly, population-specific genetic landscape, migration history, and characteristic dynamics have been linked to HI genetic markers distribution, shaped by bottleneck events in populations history (Petit *et al*., 2001). The common *GJB2* founder variant in Ghana (*GJB2*-p.Arg143Trp) has also been reported at low to moderate frequencies in East Asia, Europe, and Middle East (Tsukada *et al*., 2015). This report generated the multiple independent origins

hypothesis of the founder mutations (Shinagawa *et al*., 2020; Tsukada *et al*., 2015). Investigating the evolutionary age of the variant in Ghana, where the highest frequency of the variant has been reported so far could provide a comprehensive understanding in the demographic dynamics, and epidemiology of the variant distribution across populations. It is therefore imperative to compute ancestry haplotype background analysis in *GJB2*: c.427C>T-p.(Arg143Trp) homozygote chromosomes across populations reporting the variant. In addition, interrogating NGS data of HI cases negative for the *GJB2*: c.427C>T-p.(Arg143Trp) would identify putative new variants in known and novel genes in unresolved familial and simplex cases. Data generated would also aid risk assessment for affected families in making critical reproductive decisions through genetic counselling on management options, and therapy.

It is worth stating that even though the design for participants recruited in this study targeted unbiased ethnic representation to avoid any potential ascertainment bias, families who met the inclusion criteria were not evenly distributed across all ethnic groups represented, and hence may limit the generalizability of the research findings. Moreover, the scope of the genetic investigation sought to systematically use whole-exome and whole-genome sequencing data to resolve genetic variants other than the *GJB2*: c.427C>T-p.(Arg143Trp) that may be responsible for the HI and to generate new hypothesis to further gain insight about the condition. However, the number of families that were resolved by WES limited the available resources to perform WGS for the remaining unsolved families, which would have generated additional data on coding and non-coding variants to elucidate the genetic cause in majority of the affected unresolved cases and investigate other emerging questions.

1.1 Problem Statement

Although systematic HI genetic investigations have identified 125 genes globally (Camp & Smith, 2019), these are restricted to specific populations in Europe, Asia, and America (Vona *et al*., 2015), with very little contribution from studies in the sSA. Thus, the most commonly

reported HI markers had no significant contribution to the disorder in sSA, except for Ghana, where systematic investigation of candidate gene variants had identified the recurrent contribution of *GJB2*: c.427C>T-p.(Arg143Trp) in affected individuals (Adadey *et al*., 2019; Brobby *et al*., 1998; Hamelmann *et al*., 2001). Considering the high healthcare burden, and the fact that populations in Africa have a comparatively high genetic diversity (Gurdasani *et al*., 2015;Wonkam, 2021), there is the need to carry out studies to extensively investigate populations in sSA to elucidate and design gene panels that better represent the spectrum of genetic aetiology in the sub-region (García-García *et al*., 2020) to contribute to the global HI gene curation.

In Ghana, despite the systematic identification of the *GJB2*: c.427C>T-p.(Arg143Trp) variant as the most recurrent NSHI-associated variant, the variant accounted for less than 26% of affected multiplex cases (Adadey *et al*., 2019). The evidence of limited use of NGS (less than 20% in studies across Africa) reported by Adadey *et al* (Adadey *et al*., 2021), suggests that the current largest contributor (*GJB2*-p.Arg143Trp) is only among the array of deleterious alleles, considering HI genetic heterogeneity and ethnic diversity in Africa. The putative genetic cause of the disorder in unresolved families (74%) negative for the *GJB2*: c.427C>T-p.(Arg143Trp) mutation remains unknown. I described in this thesis, the underlying genetic architecture and structure dynamics of the phenotype segregation, and investigations on unresolved NSHI families negative for the most common variants in candidate genes (*GJB2, GJB6, GJB4*, and *GJC3*) in Ghana.

1.2 Aims and Objectives

General Aim

The work described in this thesis investigated the genetic architecture and structure of NSHI segregation in multiplex and simplex cases in Ghana.

Aim 1. To determine the provenance and timing of *GJB2*: c.427C>T-p.(Arg143Trp) founder mutation reported among Ghanaian NSHI.

Hypothesis: *GJB2*: c.427C>T-p.(Arg143Trp) variant evolved in an indigenous Ghanaian ancestor before spreading to other populations.

Rationale: Over 400 different allelic variants in *GJB2* mapped to DFNB1 locus has been associated with NSHI worldwide (Stenson *et al.*, 2020). The distribution *GJB2* deleterious alleles are ethnolinguistic and geographic specific, with observed high prevalence and carrier frequencies linked to founder effect in populations (Abidi *et al*., 2008; Brobby *et al*., 1998; Del Castillo & Del Castillo, 2017; Laer *et al*., 2001; Shinagawa *et al*., 2020; Tsukada *et al*., 2015). The *GJB2*: c.427C>T-p.(Arg143Trp) identified in Ghana is among the top four *GJB2* alleles (variants) reported globally; p.Gly12ValTer (728/3544 variants, 20.5%), p.Arg143Trp (211/1230 variants, 17.2%), p.Glu47Ter (39/1552 variants, 2.5%), and p.Val37Ile (23/1260 variants, 1.8%) (Adadey *et al*., 2021).

Reports of *GJB2*: c.427C>T-p.(Arg143Trp) variant in different populations (Shinagawa *et al*., 2020; Tsukada *et al*., 2015) birthed the concept of multiple independent origins. Haplotype investigations of *GJB2* variants commonly reported among HI Japanese cohorts, estimated the age of *GJB2*: c.427C>T-p.(Arg143Trp) to about 6,500 years in a Japanese ancestor (Shinagawa *et al*., 2020). Since the highest incidence of the variant has been reported in Ghana, relative to the lower frequencies reported in other populations, and considering the significant contribution of the variant to NSHI in Ghana, this study sought to provide clarity on the variant ancestral history, and time of variant evolution in Ghana. Investigating the haplotypic backgrounds on which the variant is carried in unrelated multiplex families co-segregating the variant in Ghana compared to ethnolinguistic Ghanaian hearing controls negative for the

variant, as well as data from publicly available population databases, will illuminate any shared genetic ancestry among populations reporting the variant.

Aim 2. To identify the putative genetic causes of NSHI in unresolved multiplex and simplex cases in Ghana.

Hypothesis: Pathogenic variants in protein coding genes (exome) of the genome can explain NSHI genetic cause in over 50% of unresolved affected individuals.

Rationale: Despite the HI genetic heterogeneity and African population diversity, there is limited use of NGS in HI studies across Africa (Adadey *et al.*, 2021). Of the array of genes implicated in HI globally (Camp & Smith, 2019), about one third (47/124) have been reported in seventeen (17) countries in Africa (Adadey *et al.*, 2021), demonstrating the contribution of African specific population makers, even with the limited studies (Chakchouk *et al*., 2015; Wonkam *et al*., 2020).

To better resolve the causative genetic aetiology of a complex process like mechano-transduction of sound in the ear, the fundamental understanding of the interplay of diverse genes will require a tactful approach. Identification of African specific HI causal genetic markers has tremendous translational application in diagnosis, prognosis, and therapeutic interventions (Vona *et al*., 2015). Though, the introduction of massively parallel sequencing (MPS) technology has revolutionized the face of molecular diagnostics by alleviating gene sequencing bottleneck, the targeted gene method is the most commonly reported approach in studies across the sub-region (Adadey *et al*., 2021). Therefore, the application of WES will identify the putative genetic cause in unresolved cases, with potential translation benefits to reduce delays and uncertainties associated with clinical diagnosis, which will further relieve the associated parental guilt and anxiety, and also generate empirical data to aid better assessment of recurrence risk (Schrauwen *et al*., 2013).

Aim 3. To elucidate the genetic architecture underlying NSHI genes co-segregation among Ghanaian affected families.

Hypothesis: The accumulation of genetic variants, and their segregation within a population is influenced by population-specific genetic structural dynamics.

Rationale: Africa, described as the place where modern humans originated, contains greater population genetic diversity compared to other continents. However, data on HI genetic markers in the continent are sparse. This suggest that the unexplored population genomes have the potential of identifying new HI markers (Chakchouk *et al.*, 2015; Lebeko *et al.*, 2016; Wonkam *et al.*, 2021). More importantly, the contribution of founder variants and other coding region variants linked with gain or loss of gene function (missense variants) have been recognized and refined by reported studies in Africa. However, reports on sporadic, rare, and novel mutations are increasingly missing in African genetic discourse. Equally, *de novo* non-coding variants, mitochondrial DNA mutations, chromosomal abnormalities and HI cases of unknown genetic cause have gained focus in the recent years (Avraham *et al.*, 2022; Brotto *et al.*, 2019; Huang *et al.*, 2023; Tisato *et al.*, 2023). To contextualize population genetic structure and architecture with functional phenotypic evidence, there is the need to characterize the NSHI genetic markers in Ghanaian families for translational utility and clinical care that remains a considerable challenge in the field.

In summary, the beginning chapter introduced the contextual background for carrying out this research, with objectives and research questions outlined, narrowing on the relevance of this study. The anticipated limitations to the study have also been discussed. Chapter two reviewed existing literature to identify the key inherent population-specific modulators of genetic markers associated with NSHI worldwide. The physiology and anatomy of the human ear has been thoroughly discussed in line with the hearing process. Aetiology of HI, with focus on both

acquired and inherited factors linked with the disorder also expatiated in addition to the HI variants characterization. Current literature on the emerging dynamics, architecture, and structure of HI, evident from reported bottle-neck events in the evolutionary history of populations segregating some HI founder mutations also reviewed accordingly. The third chapter contains a systematic review on NSHI founder variants global distribution.

Chapter four follows with a publication on the geographical provenance, and timing of the most common NSHI associated genetic marker (*GJB2*-p.Arg143Trp founder variant) among Ghanaian cohorts. The paper demonstrates sSA unique ancestry proportions with haplotypic analysis in homozygote *GJB2*-p.Arg143Trp affected unrelated individuals compared to ethnolinguistic hearing controls. Chapter five also contains a manuscript on the WES data analysis that elucidated a novel *MARVELD2* duplication of thymine at nucleotide position 1058 associated with post-lingual autosomal recessive NSHI. This novel variant report expands the previously reported pre-lingual *MARVELD2* ARNSHI phenotype to post-lingual defect in a consanguineous Ghanaian family. Chapter six continues with a publication (*BMC Medical Genomics*) of a resolved HI case after WES analysis - *De Novo* Novel Autosomal Dominant *GREB1L* variant as the putative cause of NSHI in a simplex family. In the final chapter, the overall findings were discussed in the context of relevant literature, with insights and inferential conclusions on the general study findings, and current perspective on the identified spectrum of NSHI genes in Ghana.

CHAPTER TWO

2.0 Literature Review

2.1 Background

The genetic and phenotypic heterogeneity of HI like other sensory disorders is linked to a wide spectrum of aetiologies spanning acquired to genetic factors (Dror & Avraham, 2010). Systematic investigations over the years have unravelled the huge contribution of genetics in HI (about 250 genes function in the auditory system). This advancement has provided better understanding of the complex hearing process through candidate genes and linkage studies, mapping microsatellite markers to different chromosomal locations in the genome (Doria *et al*., 2008; Dror & Avraham, 2009). The observed diversity in identified genes with vital role in the auditory system functioning highlights the complexity of hearing organ. In addition, the successful completion and release of human genome by the human genome project further revolutionized medical genetics, presenting promise of personalized medicine, and precision healthcare centred on NGS technologies (Collins & Varmus, 2015).

With the introduction of multiple parallel sequencing (MPS) technologies, the pre-genome era challenge of DNA sequence generation has shifted to demystifying the pathogenic variants, through comprehensive annotations for valid interpretations required for accurate clinical diagnosis (Tucker *et al*., 2009). The advancement of genomics had established high-throughput genetic and physical maps extrapolation, genomic and cDNA libraries for phenotype-genotype correlation studies. Now using linkage analysis, researchers have identified to date enormous list of genes, with constant periodic updates available on hereditary hearing loss website (Camp & Smith, 2019). In this chapter, the relevant literature on events that led to this major paradigm shift to the current understanding of the genetic contribution to NSHI has been reviewed. The

prevailing insights into phenotype-genotype correlation, and implications for genetic diagnosis has also been discussed.

In spite of this significant progress and better understanding of the pathophysiology of HI, the precise genetic causal variant manifesting the phenotype in some simplex cases are unresolved across populations (Wonkam *et al*., 2020;Wonkam *et al*., 2019; Wonkam-Tingang *et al*., 2020). Simultaneously, the characterization of identified HI genetic markers is constraint by the diversity of genes, incomplete penetrance, and variability in expression (Azaiez *et al*., 2018). Historically, not until 1994, only three gene loci were mapped on the human genome to NSHI (Willems, 2000). Nonetheless the high proportion of monogenic cause of NSHI allowed for linkage analysis to establish causality in subsequent studies (Richardson *et al*., 2011). Gene loci for the different NSHI inheritance pattern are denoted DFN (deafness) in the other discovery: DFNA representing autosomal dominant loci; DFNB, referring to autosomal recessive, and DFNX denoting X-linked recessive alleles respectively. Before 1998, 44 loci had been identified (19 DFNA, 21 DFNB, and 4 DFNX) (Willems, 2000). Autosomal dominant (AD) locus was mapped to 5q31 in a large extended Costa Rican family with migration history from Jerez de la Frontera, Spain during the early 1600s (Camp *et al*., 1997). Mitochondrial variant identified in a large Arab-Israeli kindred from pedigree analysis demonstrated maternal inheritance of NSHI (Wei, 2002). The mitochondrial 1555A>G transition mutation implicated in most reports as the common maternally inherited aminoglycoside-induced HI within a highly conserved region on the 12S rRNA gene (Prezant *et al*., 1993).

These strides were achieved through localization studies that involved large multi-generational inbred families, and ethnolinguistic isolated groups across different regions. The high degree of consanguinity in multiplex families investigated, allowed for linkage analysis, and auto-zygosity mapping within pedigrees with significant log of odds (LOD) scores. Following this, a subsequent study by Fukushima *et al* (1995) introduced the concept of mapping autosomal

recessive HI genes in small consanguineous multiplex families with at least three affected individuals (Fukushima *et al*., 1995).

With linkage analysis, single nucleotide variants (SNVs) genotyping, targeted and NGS approaches, researchers have mapped to date over 125 genes as associated with NSHI worldwide (Camp & Smith, 2019). Available NGS technologies coupled to target-enrichment methods have allowed for the systematic identification of novel gene variants association with diverse mode of inheritance (Renkema *et al*., 2014). The conventional Sanger dideoxy sequencing of gene-by-gene approach was thought to be highly expensive and time consuming to explore the plethora of genes associated with HI in families (Sanger *et al*., 1977). Until recently, HI molecular genetic testing was restricted to one or two candidate genes; *GJB2*, *GJB6*, and selected mitochondrial DNA (mtDNA) associated variants (Robin, 2004). Hence the genome capture array method that uses known genes by high-throughput NGS was preferred to overcome the Sanger limitation (Edenberg & Liu, 2009). This classical approach identified a spectrum of dominant, recessive and mitochondrial variants in 20 known HI genes, after excluding probands with variants in *GJB2, SLC26A4* and *MT-RNR1* using targeted gene NGS screening (Bitarafan *et al*., 2020;Chen *et al*., 2015;Yang *et al*., 2013).

Prior to Sanger dideoxy sequencing technique, the classical approach explores multiplex ligation-dependent probe assay (MLPA), an inexpensive, rapid and reliable technique for cost-efficient diagnosis, prime approach for the wide mutation spectrum (Resmerita *et al*., 2020). Yet, this method is also sensitive to impurities, lacking the ability to identify unknown genes and/or gene variants. The target limitation, and sequence length degradation adding to the time and cost constraint associated with Sanger sequencing continues to be a major concern to date (Hömig-Hölzel & Savola, 2012). Using targeted NGS had demonstrated a thorough and cost-effective diagnosis, particularly for known and candidate HI genes (Chen *et al*., 2015; Shearer *et al*., 2011).

Under the current premise, WES has shown much diagnostic value than the phenotype-based Sanger sequencing for HI, blindness, mitochondrial, and movement disorders (Neveling *et al*., 2013). The introduction of NGS since 2006 had displayed great potential in discovering new disease genes on daily basis (Bamshad *et al*., 2011; Wortmann *et al*., 2012). WES technique permits the efficient identification of SNVs, small indels (insertions and/or deletions) and larger indels in the coding regions of genes using a single approach (Bamshad *et al*., 2011). Protein coding genes DNA variants constitute 98.5% of disease - associated variants (Waldern *et al*., 2021), making WES appropriate for HI genetic investigations. Following WES applications, some of the previously reported pathogenic variants based on the information available at the time have been reviewed and re-classified, and annotated as benign due to increased population data on allele frequencies (Lebeko *et al*., 2017; Shearer *et al*., 2014).

Since the introduction of the Sanger sequencing technique in 1997, it has remained the preferred method for variant validation and family segregation analysis in molecular diagnosis (Sanger *et al*., 1977). That notwithstanding, exome sequencing is ideal and cost-effective approach for identifying novel genes; evident by positional cloning experimentations, and the fact that most Mendelian disorders results from protein-coding variants (Chen *et al*., 2015; Teer & Mullikin, 2010). For example, homozygous mapping of exome sequenced data identified DFNB82 locus at 1p13.3 as the causal marker in a Palestinian family with consanguineous history (Walsh *et al*., 2010). Similarly, WES confirmed HI causal variant in a previously implicated DFNA4 family (Chen *et al*., 1995). In a large family with progressive NSHI in Netherlands, MPS and subsequent targeted techniques successfully identified small muscle protein x-linked as the causal gene at the DFNX4 locus (Schraders *et al*., 2011).

Earlier investigations focused on HI recessive inheritance because identifying homozygous or compound heterozygous variants with MPS were ‘easier’ to filter variants that refine prioritization for candidate markers. The impact of advance genetic techniques on investigating

causal HI markers across populations are far reaching. And development in technology in the field has facilitated the current scope of HI genes at an unparalleled rate, with actionable translational benefits in patient care and management, aiding rapid detection, and generating data to improve understanding of the hearing process.

2.2 Anatomy of the ear

The cartilaginous pinna constitutes the outer ear, which is made up of the external section and concha that leads to the canal of auditory system (meatus) (Alberti, 2001). Compared to other animals' ear structure, though the human external ear does not have the same musculature, it has good sound localization and enhancement stimulus especially for high frequency sound (Brownell, 1997; Grothe *et al.,* 2010). The flange and concha collectively increase the acoustic pressure of sound in air over wide frequencies (Maroonroge *et al*., 2000). Aside funnelling sound stimuli to the middle part of the ear, it also performs a protective role on the tympanic membrane from exogenous damage. In addition, the glands in the ear canal produce cerumen to prevent ear debris and bacterial accumulation (McCarter *et al*., 2007). The tympanic membrane and the external section of the ear is separated by an air-filled space consisting of tympanum (lager lower part) and epitympanum (smaller upper part). The tympanic membrane closes the middle ear, and opens through the Eustachian tube by nasopharynx (Park & Roh, 2002). Physiologically, the tympanic membrane link to the Eustachian tube helps to stabilize the pressure between the middle and outer ear sections. Without this optimal balance, the tympanic membrane would vibrate abnormally due to unbalance pressure (Ars & Dirckx, 2016; Seibert & Danner, 2006). The eardrum (cone shaped tympanic membrane), is a partially transparent and oval membrane that terminated the ear canal, pointing inwards toward the inner ear (Volandri *et al*., 2011). Connected to the eardrum are the ossicular chains; the malleus (hammer), incus (anvil), and stapes (stirrup), which are the three smaller bones in the body (Seibert & Danner, 2006) (Figure 2.1).

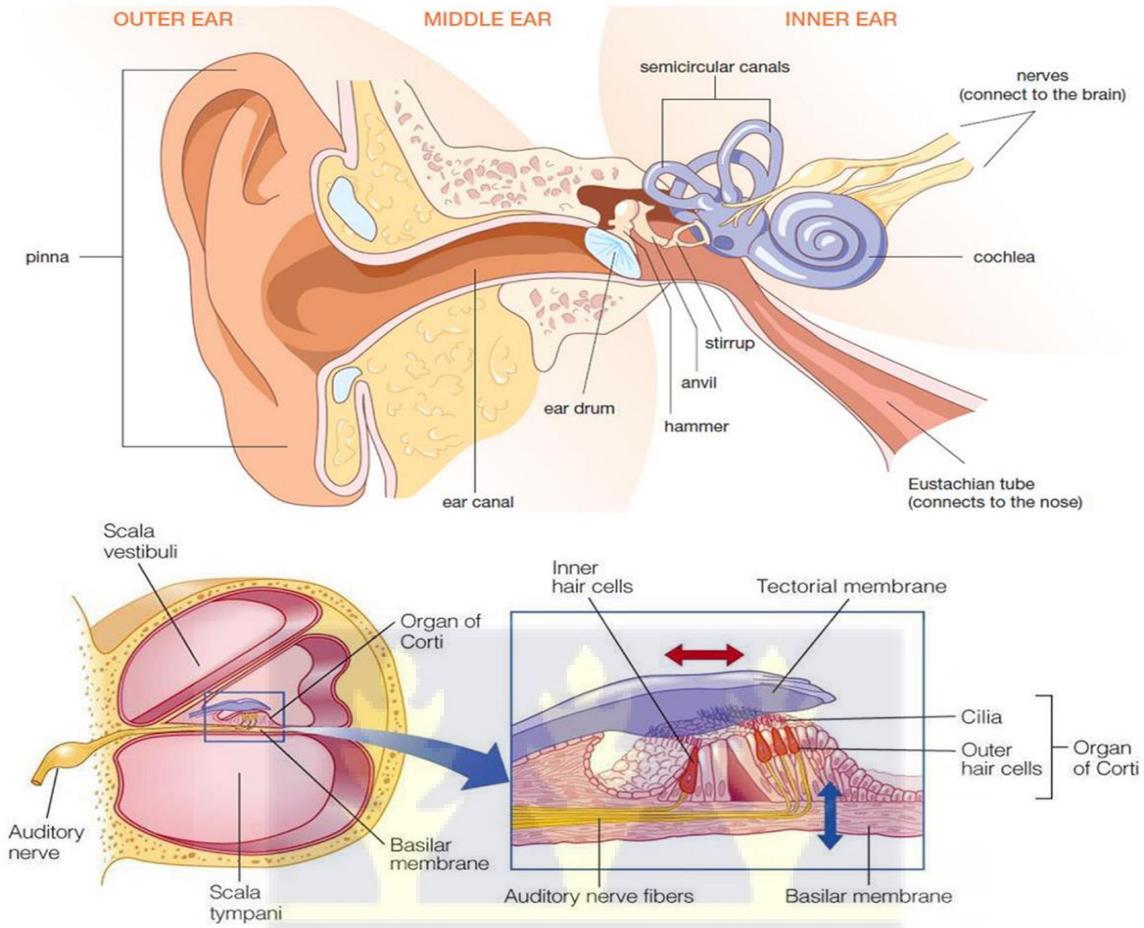

Figure 2.1. Anatomy of the human ear. *Top (outer, middle, and inner); Bottom-left cochlea cross sectional view; and transverse section of the organ of Corti (bottom-right). Source:https://kids.frontiersin.org/articles/10.3389/frym.2015.00008*

The ossicles together with its supporting muscle's primarily relays the sound energy from the eardrum to the inner ear in addition to insulating the inner ear from harmful sound (noise). In response to acoustic pressure, the ossicles under the influence of tympanic membrane move coupled to the reflexes in the middle ear; tensor tympani and stapedius muscles (Alberti, 2001). The ossicles contraction reduces the intensity of sounds transmitted to the inner portion of the ear that shields it from any potential damage. In addition to other ligaments, the tensor tympani and stapedius connected to the malleus and stapes modulates the movement of the ossicular

chain (Frear *et al*., 2018). The malleus manubrium connects to the central part of the tympanic membrane, pulling it inwards and centralizing the middle ear section maintaining the conical shape characteristic of tympanic membrane (Hayes *et al*., 2013). The middle ear lateral wall houses the eardrum, with medial bony wall separating the middle ear portion from the inner wall. The lateral wall is made up of two membranes, oval and round window, which has the anatomical and physiological function of connecting the middle ear and inner ear (Maroonroge *et al*., 2009). The narrow tympanic cavity and irregular rectangular box has thicker edges relative to the middle section. Most of the space in cavity is occupied by the ossicular chain and middle ear muscles, with a small empty space above the ossicles referred to as epitympanum racess (Ars & Dirckx, 2016; Ekdale, 2016).

The tegmen tympani, the thin wall (ceiling of the tympanic cavity) delineates the middle part of the ear from the brain cavity. Located behind middle ear media wall is the bony labyrinth inner ear, described as the most complex and final part of the ear (Maroonroge *et al*., 2009). Anatomically, the semi-circular canals together with the vestibule, and cochlea constitute the inner ear components. Perilymph, an incompressible body fluid fills the space created by the bony labyrinth and the membranous labyrinth partition (Maroonroge *et al*., 2000). Similar in composition with cerebrospinal fluid surrounding the brain is high sodium ions concentration region perilymph, with low potassium ions concentration (Wangemann, 2006). The endolymph contrary to the perilymph, though incompressible, fills the space inside the membranous labyrinth and contains low sodium ion concentration but high potassium ion concentration similar in composition with intracellular fluids inside the cells of the body (Lee & Marcus, 2003). The differences in ionic and chemical compositions of the perilymph and endolymph generates the electro-chemical potential difference optimal for the inner ear sensory cells physiological functioning (Mercier *et al*., 2012).

Structurally, the cochlea together with the vestibular system (utricle, saccule, and semi-circular canals) are the main components of the inner part of the hearing organ. The organ of Corti is the main organ of hearing found in the cochlea (Bruss & Shohet, 2019). The cochlea and the semi-circular canals are situated on the two ends of the inner ear (Hayes *et al*., 2013). The central vestibule is made of the saccule and utricle. Connecting the inner ear to cranial cavity surrounding the brain are two narrow channels; the vestibular and cochlea duct (Ekdale, 2016). It has a structural base consisting of the osseous spiral lamina, with 2½ to 2¾ turns around the bony modiolus (cochlea core) (Maroonroge *et al*., 2000). Situated on the cochlea length are parallel canals, the scala vestibuli, media, and tympani (Wang *et al*., 2016). These are part of the bony labyrinth whiles the scala media forms part of membrane of the labyrinth (Schultz *et al*., 2017). A tiny hole called helicotrema connects the scala vestibuli and scala tympani in the upper part of the cochlear. Acoustic stimulation causes the stapes footplate to mechanically vibrate, which is transmitted to the oval window layer.

This membrane movement pushes to and fro in the perilymph of the scala vestibuli out of the helicotrema, and the scala tympani perilymph (Voss *et al*., 2000). The resultant alternating outward and inward movement of the oval window layer expands outward along with the scala vestibuli containing fluid to scala tympani and vice versa. The scala tympani are separated from the scala vestibuli cochlear duct. Likewise, the scala media and scala vestibuli are partition by the Reissner's layer, whereas the basilar layer creates the barrier between scala media and scala tympani (Kirk & Gosselin-Ildari, 2009; Maroonroge *et al*., 2009). The ductus reuniens connect saccule to the cochlea separating the basilar and Reissner layers by a tiny opening in the scala media (Kirk & Gosselin-Ildari, 2009). The basilar tissue at the scala media base, anchors the spiral lamina at both ends of the spiral ligament. At the top of the cochlea, the spiral lamina is narrow, but wide at the base (Raphael & Altschuler, 2003). However, the basilar layer at the top of the cochlea is wide, more thicker and flaccid compared to the base where it is narrower,

thinner, and more stiffer (Alberti, 2001). The characteristic feature of the membrane gives it the flexibility to respond to the varied frequency vibrations at both ends of the cochlea (apex and base). Basilar layer has the organ of Corti located on it that holds the sensory cells and has a small segment that project onto the spiral lamina (Raphael & Altschuler, 2003). Vibrations of the basilar sheath are converted into neuronal impulses in the organ of Corti that travels via the auditory nerve to the brain (Maroonroge *et al*., 2000; Robles & Ruggero, 2001). The outer and inner hair cells (OHCs and IHCs) are found in the organ of Corti (Alberti, 2001). The afferent and efferent nerve endings connects both hair cells to the nervous system and has variable nerve connections between OHCs and IHCs. The IHCs transmit the sensory impulses through the auditory nerve stimulation, whiles the OHCs performs qualitative and quantitative amplifications (selectivity) (Starlinger *et al*., 2010) (Figure 2.2) .

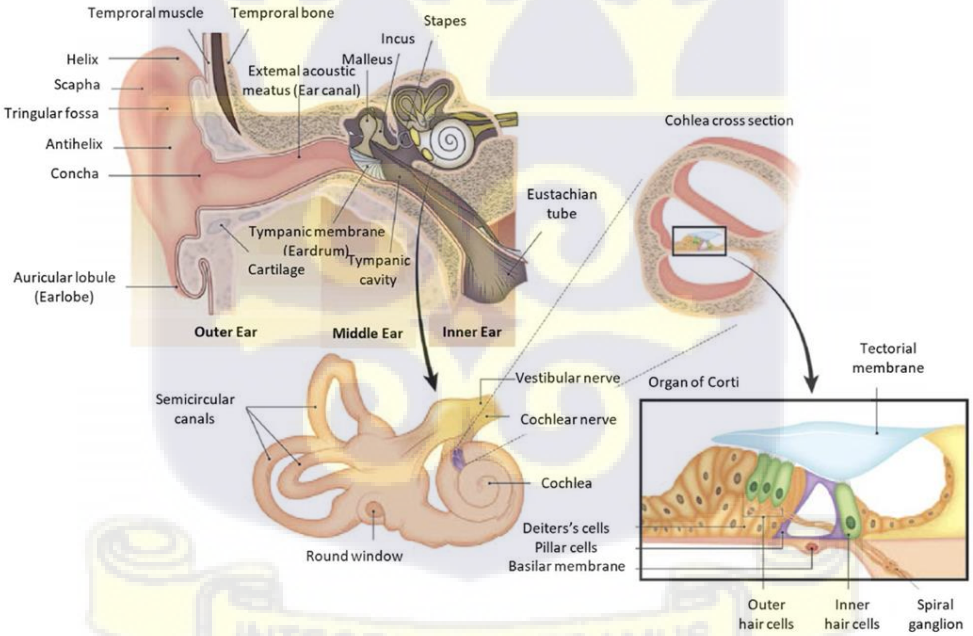

Figure 2.2. Components of the cochlear. *The hearing organ, showing anatomy of the organ of Corti. (Xu et al., 2021).*

2.3 Mechanism of hearing

The human hearing organ have receptors for hearing (in the cochlea), body balance, and spatial orientation (the vestibular system) (Brownell, 1997; Ekdale, 2016). Sound energy travelling as mechanical energy is funnelled through the outer passage of the external ear to the eardrum (tympanic membrane). Usually, this is a complex vibration depending on the frequency and intensity of the stimuli. Acoustic stimuli received by external ear are transferred by the middle components of the ear (incus, malleus, and stapes) to the sensory receptors in the inner ear (Brownell, 1997; Ekdale, 2016). The area of the stapes is small compared to the tympanic membrane, and this physiologically combined with the ossicles lever effect causes acoustic impedance in the middle ear that reduces sound energy reflection (Hayes *et al*., 2013). The stapes articulates tightly with the oval window (essential for the sound pressure transmission), which is continuous with the scala vestibuli of the inner ear. Vibration of the stapes are transferred to the fluid in the oval window. Under optimal conditions, the stapes footplate transmit the compression and rarefaction forces to act on the perilymphatic fluid surrounding the cochlear duct (Kollmeier, 2008) (Figure 2.3).

The perilymph is filled with fluid-like plasma, whereas the endolymph has fluid same as intracellular fluid high in potassium ion (K^+) concentration of up to140 mM. Thus, the optimal electric potential gradient (+85 mV potential) in the endolymph required for triggering hair cells are regulated by action of specialized stria vascularis cells (marginal, intermediate, and basal cells) primarily important in the hearing processes. The marginal cells secrete K^+ in the endolymph, after their uptake, which is mediated by basolateral sodium ion (Na^+)/potassium ion (K^+)/chloride ion ($2Cl^-$) co-transporter. This co-transporter transports $1Na^+$, $2Cl^-$, and $1K^+$ ion directly into the marginal cells, which are release by the apically expressed potassium channels; facilitating the release of the potassium into the endolymph (Zdebik *et al*., 2009) (Figure 2.5).

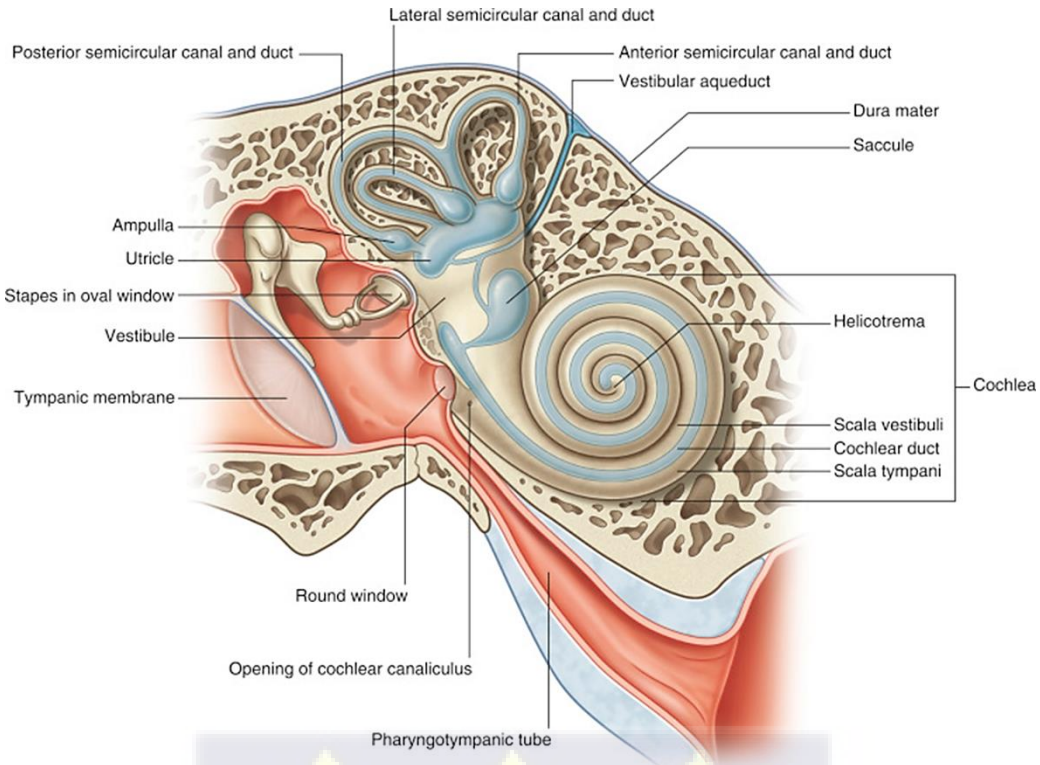

Figure 2.3. Parts of the human ear. *The external ear, particularly the prominent auricle, directs sound into the external auditory canal (meatus). Alternating increases and decreases in air pressure vibrate the tympanum. The vibrations are transmitted across the air-filled three tiny, linked bones: malleus, incus, and stapes. Vibrations of the stapes stimulate the cochlea.* *https://www.sciencedirect.com/topics/veterinary-science-and-veterinary-medicine/middle-ear*

In the organ of Corti, the OHCs and IHCs are highly structured collectively with other supporting and Deiters cells, as well as the basilar and tectorial layers (Maroonroge *et al*., 2009) (Figure 2.4). The basilar layer delimits the cochlear wall at the base from the scala tympani and the Reissner's layer at the top from the scala vestibuli. The stereocilia on OHCs are in close proximity with the tectorial layer, whereas there is a loose contact between stereocilia of the IHCs and the layer (Alberti, 2001; Zdebik *et al*., 2009). The scala media fills the area between the tectorial and Reissner's tissues with endolymph. Ultimately, the stapes vibrations are transmitted via the oval window to instigate a perilymph wave that spreads along the scala vestibuli (Gacek & Gacek, 2003) to the apex of the cochlea. Thus, subsequent movements

cause direct and indirect fluid-mediated shearing forces to act on the stereocilia sensory hair cells in the organ of Corti (Figure 2.4).

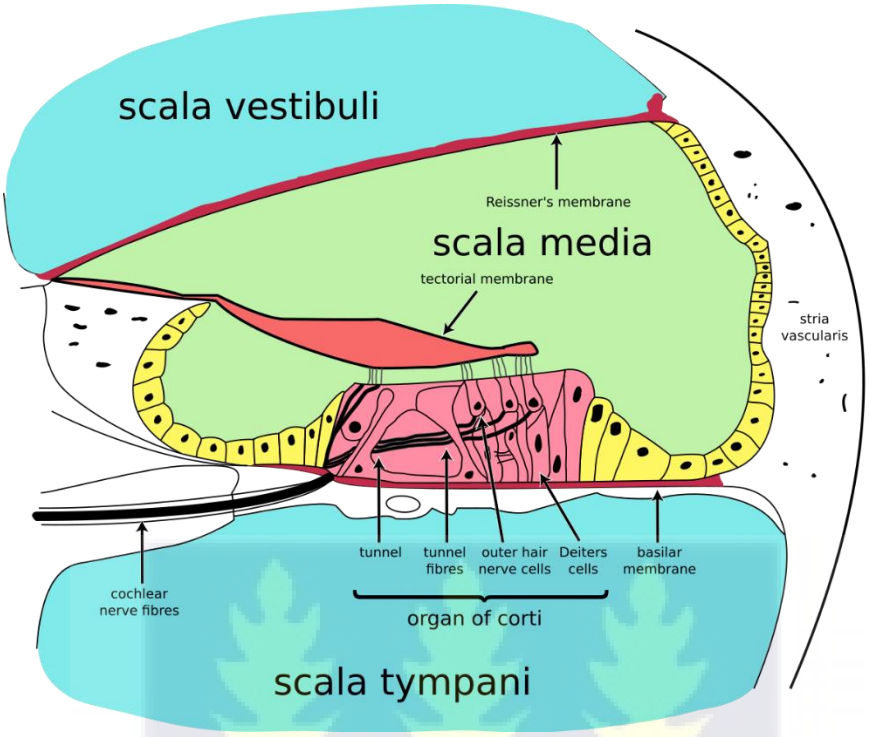

Figure 2.4. Structure of cochlear organ of Corti, OHCs, IHCs, and supporting cells. *Source:* *https://flipper.diff.org/app/items/info/6238*

The waves generated has a characteristic amplitude that reaches its maximum at distinct locations along the cochlea depending on the frequency, which stimulates the sensory cells at these locations. The shearing forces result in the tension of tip links, eventually opening or closing (depending on the direction of movement) mechanosensitive channels at the tip of the shorter stereocilia. Vibrations of the basilar layer bend the OHCs hair bundles which opens and/or closes ion channels, perhaps a transient receptor potential channels, dependent on the direction of the sensory cell bending (Zdebik *et al*., 2009). The membrane at the apex of the stereocilia hair cells are positioned in an organ pipe manner parallel to the longer stereocilia that opens to ion channels and closes when it moves in the opposite direction. Stereocilia and

kinocilia projections on sensory hair cells are in direct communication with the tectorial layer. The vibration of the tectorial membrane displaces the stereocilia that subsequently displaces kinocilia close to the stereocilia. Hair cells depolarization are then triggered by the movement of the kinocilia, leading to an influx and release of neurotransmitters to act on cochlear ganglion (Gacek & Gacek, 2003). Depending on the electrochemical gradient ions, positively charged ions (potassium ions) flow from endolymph to the cytoplasm with ion channels opened. Subsequently, the potassium ions leave the sensory hair cells via localize basolateral potassium voltage-gated channel subfamily Q member 4 (KCNQ4) for homeostasis of ions in the cochlea. Although different explanations on this concept have been postulated for the recovering of potassium ions in the inner ear, the notion that potassium ions that enter hair cells returns to the stria vascularis to be secreted into the scala media via an intracellular pathway seems plausible.

The subsequent depolarization of OHCs induces myosin filament contraction by the action of prestin protein expressed on the lateral layer of the OHCs. This energy dependent process requires mitochondrial abundance to provide the needed energy. The simultaneous shortening and elongation of OHCs amplifies the vibrations strongly at sites characteristic for a defined frequency, thereby depolarizing the IHCs for neurotransmitter release and final activation of afferent nerve fibres for translation and interpretation (Ashmore, 2008). Sound at different frequencies arouse the sensory cells at various locations in the organ of Corti that allows sound travelling at different frequencies to be perceived.

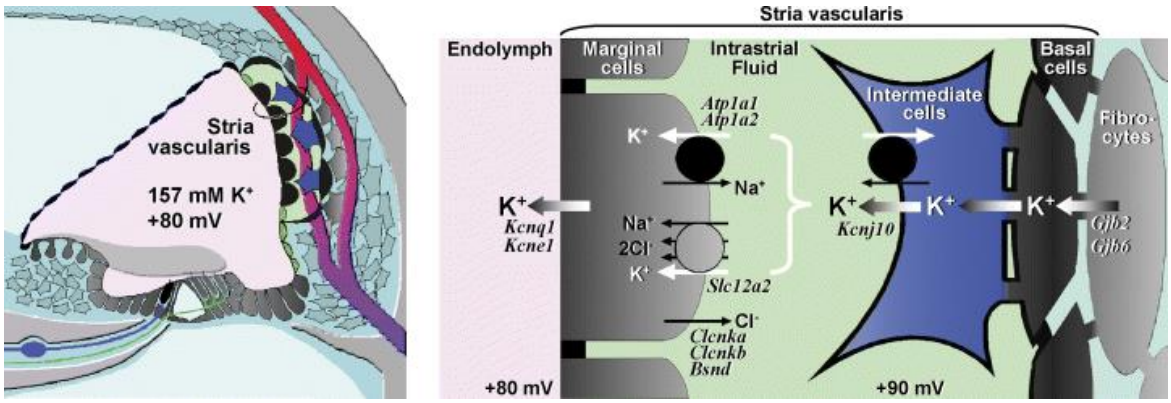

Figure 2.5. Diagrams showing potassium ion secretion. *Right, stria vascularis. Strial marginal cells and basal cells of stria vascularis are connected to each other by tight junctions to form two barriers. The barrier between endolymph and intrastrial fluid consists of strial marginal cells and the barrier between intrastrial fluid and the extracellular fluid spaces in spiral ligament consists of basal cells. The endocochlear potential is generated across the basal cell barrier. Intermediate cells are connected by gap junctions to the inner membrane of basal cells. Source:*https://physoc.onlinelibrary.wiley.com/doi/full/10.1113/jphysiol.2006.112888

2.4.0 Hearing Impairment

Hearing impairment is any loss in the ability to hear sound. In other words, an individual who is unable to hear sound above 25 decibel (dB) in one or both ears is said to be hearing-impaired (Stephenson, 2021). Based on the threshold, HI may be categorized into mild, moderate, moderately severe, severe, or profound (Figure 2.6). Impairment threshold from mild to severe are described as hard of hearing, and persons with profound impairment referred to as deaf (who has very little or cannot hear at all).

Sound is produced by the compression and rarefaction of a medium to generate longitudinal waves, which move from one medium to another. The vibrations produced are at characteristic of sound at different frequencies (measured in hertz (Hz)) that identifies with different sound pitches corresponding to specific amplitudes (measured in pascals). Sound pressure level (SPL) is the apparent sound loudness (Lanvers-Kaminsky *et al*., 2017). Sound energy is the amount

of energy moving sound from its source to the ears or a receiver (for example: a microphone) and is measured in decibels (dB).

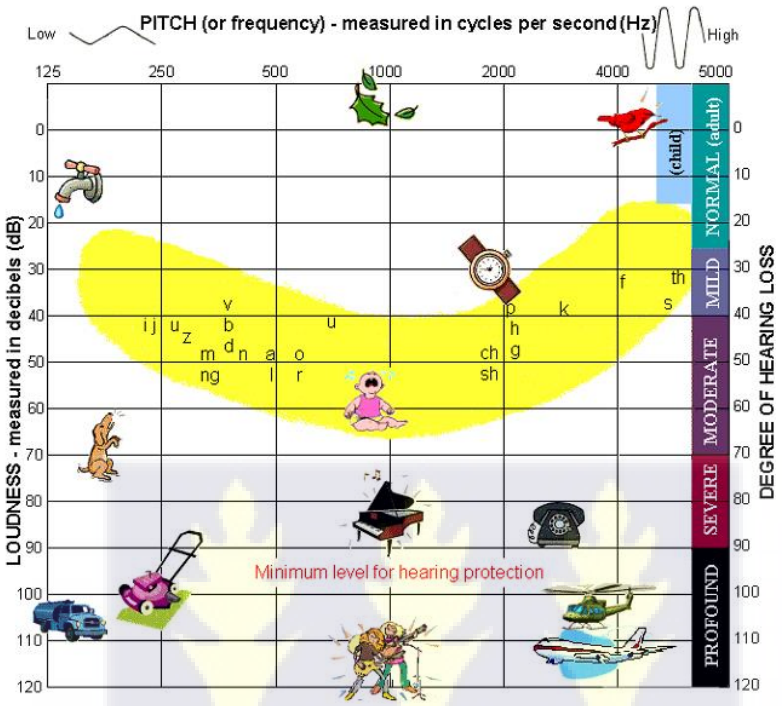

Figure 2.6. The graph shows threshold of hearing impairment degree of severity at different frequencies. *Degree of impairment, relating to every day familiar sounds: -10 dB to 25dB; normal: 25dB to 35dB; mild: 40dB to 55dB; moderate: 55dB to 70dB; moderately severe: 70dB to 90dB; severe: > 90dB; profound. dB = decibels; Hz = hertz, source; https://fixyourears.com/hearing-loss/*

2.4.1 Hearing Impairment Associated Burden

Hearing impairment, if unaddressed may affect many aspects of life including, communication and speech, cognition, education and employment, social and emotional, with economic implications as well (Stephenson, 2021). The constraint and inability to connect and communicate; possibly cause a profound sequelae on language acquisition, development and communication in affected individuals (Patel *et al*., 2021). More importantly, the associated language deprivation could result in delayed cognitive development, which could be avoided with the appropriate intervention at early stage (Ching *et al*., 2017; Moeller & Tomblin, 2015).

In older adults, mental and physical declines with higher risk of age-related dementia are increasingly linked with HI (Rutherford *et al*., 2018).

Particularly, with regards to education and employment, children with hearing disorder in low-middle-income countries have limited access to acquire higher education (Olusanya, 2007). Thus, HI adults face employment challenges. Even for those who are employed, a greater percentage are in lower grades of employment, with lower wages, and face early retirement compared to their hearing peers. Faced with emotional and social challenges, affected persons are vulnerable to social issues like isolation and loneliness at all ages, which is higher in the aged (Mick *et al*., 2014). Inability to comprehend auditory information and maintain balance make affected persons to avoid potential embarrassment in social life, risking cognitive decline and depression. Aside this associated distress experienced by hearing-impaired individuals and the overall financial burden incurred by families. The WHO estimate on annual global cost of unaddressed hearing impairment stands at 980 billion international dollars (excluding cost of care like screening, hearing aids, cochlear implants or rehabilitation); educational support; and cost due to loss of productivity (Stephenson, 2021).

2.4.2 Types of Hearing Impairment

HI can be conductive, sensorineural, mixed or central defect (Figure 2.7) (Cunningham & Tucci, 2017). Conductive disorder is associated with any obstruction of sound-conduction in the external ear and the middle ear. Any impediment of the outer ear canal or immobility of the middle ear bones produce a conductive HI. HI resulting from foreign object, otitis media, perforated eardrum, otosclerosis, and deformities of the external or middle ear constitute conductive cause (Martin & Valentin, 2019). Sensorineural defect on the other hand emanates from any pathological damage to hair cells and stria vascularis structures or the acoustic nerves. Sound waves are normally received from the external and middle parts of the ear before channelling to fluid-filled cochlea in the inner ear. The organ of Corti, at the surface of cochlea

basilar membrane contains sensory cells that produce the nerve signals in responds to sound vibrations (Alberti, 2001).

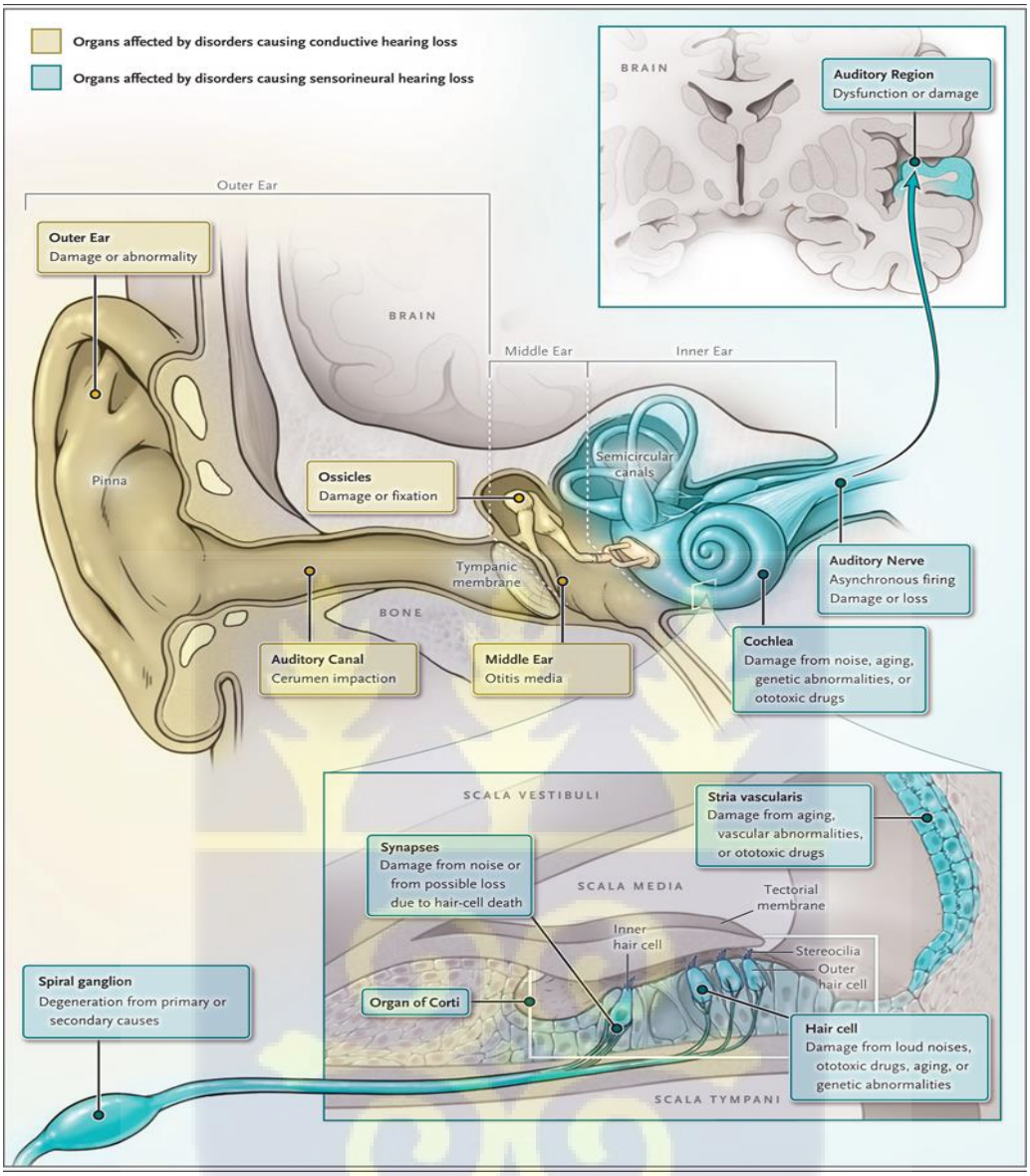

Figure 2.7. Structure of human ear. *Showing the auditory system and damage affecting parts of the system; conductive and sensorineural defect, source: (https://www.nejm.org/doi/full/10.1056/NEJMra1616601)*

Consequently, transduction of mechanical energy generates electrical signals that causes nerve fibres of the acoustic stimulation, which are then transmitted to the brain through the brainstem

for decoding in the cerebral cortex as sound. Sensorineural HI is associated with defect in the perception or interpretation of sound resulting from heritable disease, aging, ear damage from loud noise, prenatal and post-natal infections, skull fractures from accidents, intercranial tumours, and ototoxic medications (Coelho *et al*., 2020). Similarly, pathological condition arising from damage beyond the acoustic nerve and the brainstem are described as the central defect. HI that results from brain tumours, vascular changes that deprives blood supply to the inner ear, stroke syndrome, and erythroblastosis fetalis falls under central HI (Ray *et al*., 2019). Several classifications of HI have been described, which are used to guide diagnosis, prognosis, and treatment.

Table 1.1. Classification of hearing impairment

Criterion	Category / Type
Age of onset	Pre-lingual Post-lingual Age-related (presbycusis)
Aetiology	Acquired Genetic
Clinical phenotype	Syndromic Non-syndromic
Part of ear damage	Conductive Sensorineural Mixed
Disease severity	Mild Moderate Severe Profound
Pattern of inheritance	Autosomal dominant Autosomal recessive X-linked Y-linked Mitochondrial
Symmetry	Unilateral Bilateral
Progression	Progressive /Non progressive

2.4.3 Causes of Hearing Impairment

2.4.3.1 Acquired / Environmental Hearing Impairment

Acquired HI has complex aetiology, however, trauma, infection, ototoxic medications, and auto-immune disorders are the most commonly attributed environmental factors (Cannizzaro

et al., 2014). Bacterial meningitis complication is a well-documented neurological sequela in infants, which can cause mild to profound hearing defect (Richardson *et al*., 1997). Different pathogenic strains of bacteria have been associated with the condition, but the highest incidence result from *Streptococcus pneumoniae* infections (Woolley *et al*., 1999). The *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, among many others accounted for 33.6% frequency of HI in a review (Edmond *et al*., 2010).

Additionally, infections before and after birth from neurotropic viruses (herpes simplex, cytomegalovirus and rubella) have also been implicated chiefly in HI cases (Cannizzaro *et al*., 2014). The virus invading the cochlea and cochlea nerve, latent virus reactivation in the spiral ganglion, and immune-mediated systemic reactions are the proposed pathological mechanisms linked with the disorder (Merchant *et al*., 2008). HI has also been reported in close to 4% of mumps cases, and are frequently associated with unilateral and transient but permanent sensorineural HI in children (Hall & Richards, 1987; Hashimoto *et al*., 2009). Pathological investigations identified lesions and the stria vascularis degeneration, tectorial tissue and organ of Corti in mumps related cases (Everberg, 1957; Lindsay *et al*., 1960).

Ototoxic medicine and compounds have been implicated as impairing cellular function and/or tissue degeneration in the cochlea (Lanvers-Kaminsky *et al*., 2017). Although about 150 drugs have been associated with ototoxic effect, they can be grouped into six (6) different medications; aminoglycosides (Figure 2.8) and platinum-based chemotherapy (Cannizzaro *et al*., 2014; Schacht *et al*., 2012), loop diuretics, quinine, salicylates and vinca alkaloids (Brummett, 1993). While ototoxicity resulting from classes like the diuretics, macrolide antibiotics, and quinine normally disappears when the treatment is discontinued, platinum drugs, such as cisplatin, and aminoglycosides are mostly irreversible after inner ear damage. For most of these compounds, though they are well distinguished compounds and their adverse

contradictions in HI are highly recognized, the precise pathophysiology in auditory defect remains to be completely understood.

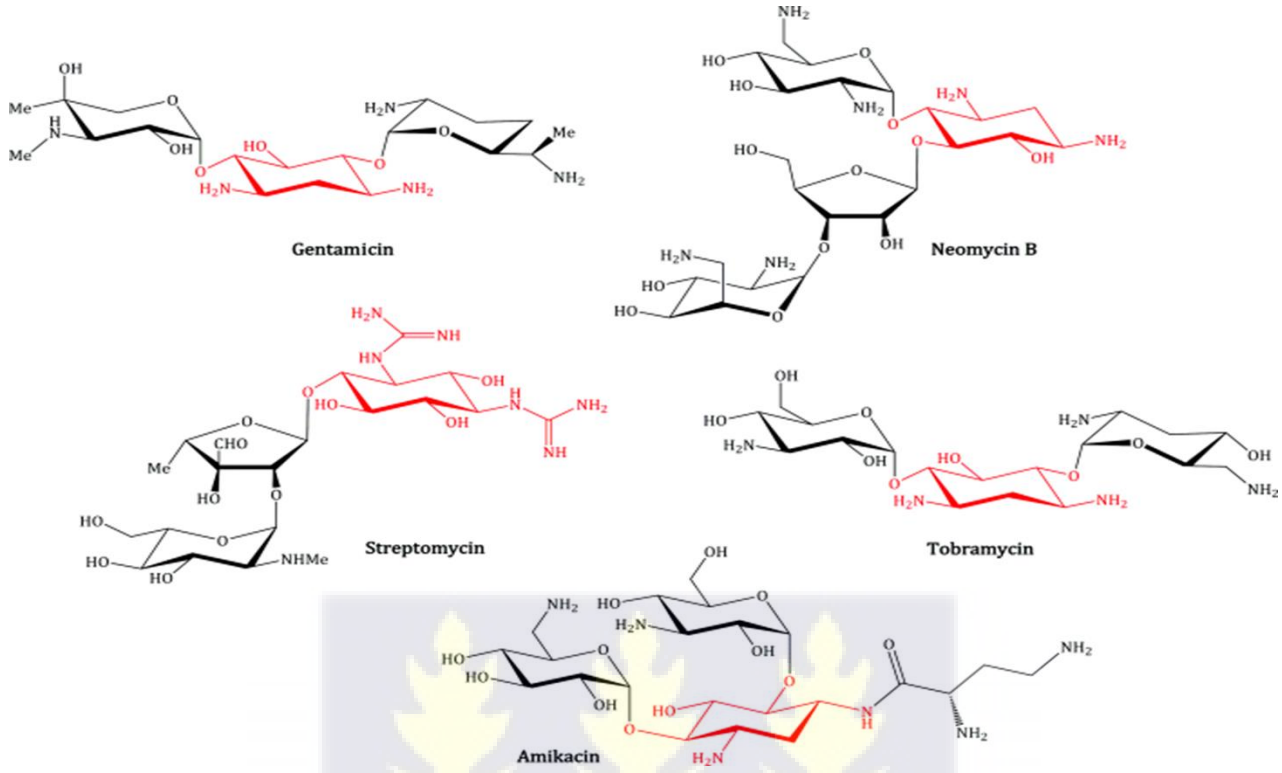

Figure 2.8. Structure of aminoglycoside antibiotics (aminocyclitol ring highlighted in red). *Gentamicin:(2-[4,6-diamino-3-[3-amino-6-[1-(methylamino)ethyl]oxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-5-methyl-4-(methylamino)oxane-3,5- diol);Neomycin((2R,3S,4R,5R,6R)-5-amino-2-(aminomethyl)-6-[(1R,2R,3S,4R,6S)-4,6-diamino-2-[(2S,3R,4S,5R)-4-[(2R,3R,4R,5S,6S)-3-amino-6-(aminomethyl)-4,5-dihydroxyoxan-2-yl]oxy-3-hydroxy-5-(hydroxymethyl)oxolan-2-yl]oxy-3-hydroxycyclohexyl]oxyoxane-3,4-diol);Streptomycin(2-[(1R,2R,3S,4R,5R,6S)-3-(diaminomethylideneamino)-4-[(2R,3R,4R,5S)-3-[(2S,3S,4S,5R,6S)-4,5-dihydroxy-6-(hydroxymethyl)-3-(methylamino)oxan-2-yl]oxy-4-formyl-4-hydroxy-5-methyloxolan-2-yl]oxy-2,5,6-trihydroxycyclohexyl]guanidine);Tobramycin(2S,3R,4S,5S,6R)-4-amino-2-[(1S,2S,3R,4S,6R)-4,6-diamino-3-[(2R,3R,5S,6R)-3-amino-6-(aminomethyl)-5-hydroxyoxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-6-(hydroxymethyl)oxane-3,5-diol):Amikacin((2S)-4-amino-N-[(1R,2S,3S,4R,5S)-5-amino-2-[(2S,3R,4S,5S,6R)-4-amino-3,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4-[(2R,3R,4S,5S,6R)-6-(aminomethyl)-3,4,5-trihydroxyoxan-2-yl]oxy-3-hydroxycyclohexyl]-2-hydroxybutanamide) Source (https://basicmedicalkey.com/aminoglycoside-antibiotics/)*

However, in gram negative bacteria cells in particular, aminoglycosides entry are selectively allowed through membrane transporters porins and mechanosensitive channels (Silver, 2016). Aminoglycosides belongs to a class of polar, polycationic compounds possessing a neamine core containing six-member aminoacyclitol ring that is linked to a glucosaminopyranose ring

through a glycosidic bond. The different aminoglycosides result from position of substituents attachment (position 5 or 6 on ring II), characterized as 4, 5 or 6 aminoglycosides.

Efforts to modify aminoglycosides to inhibit their entry into hair cells postulated the exploitation of existing differences in uptake of the compounds in bacteria and hair cells (O'Sullivan *et al*., 2017). Both mechano-transduction (MET) channel and endocytosis have been proposed as the likely entry mechanism into hair cells (Figure 2.9). During sound transduction, potassium ions move along an electrochemical potential difference into hair cells via mechanical gated channels prevalent in bundles of hair cells (Maoiléidigh & Ricci, 2019). Since the unique gated channels do not restrict cationic carriers as well as other large positive charge molecules like aminoglycosides, it moves into hair cells as well. In addition, endocytosis mechanism as a route of aminoglycosides into hair cells has been described. However, compared to the mechano-transduction channel-mediated, the rate is relatively slow (Figure 2.9). Aminoglycosides filling the cytoplasm rapidly on entry into the hair cells explains the pathophysiology (Alharazneh *et al*., 2011). This has been attributed to the net cationic charge of aminoglycosides, which makes them to attract negatively charged molecules including DNA, RNA, phospholipids, and basic amino acids in proteins. Aside aminoglycosides accumulation in the cytoplasm, build-up in organelles like the endoplasmic reticulum and mitochondrial stress have been implicated (Esterberg *et al*., 2014).

Consistent with the antibacterial activity, the ribosome is another cellular organelle targeted by aminoglycosides in the sensory hair cells (O'Sullivan *et al*., 2017). Ribosomes constitute close to 30% of cellular mass, and are chiefly involved in decoding genetic information during protein synthesis (Kramer *et al*., 2009). Moreover, aminoglycosides also binds to the small subunit of the ribosomal RNA disrupting protein synthesis rate and accuracy in bacteria (Greber *et al*., 2015; Ogle & Ramakrishnan, 2005) (Figure 2.10). Of the two ribosomes (mitochondria and cytoplasmic), strong evidence linking the function of mitochondrial

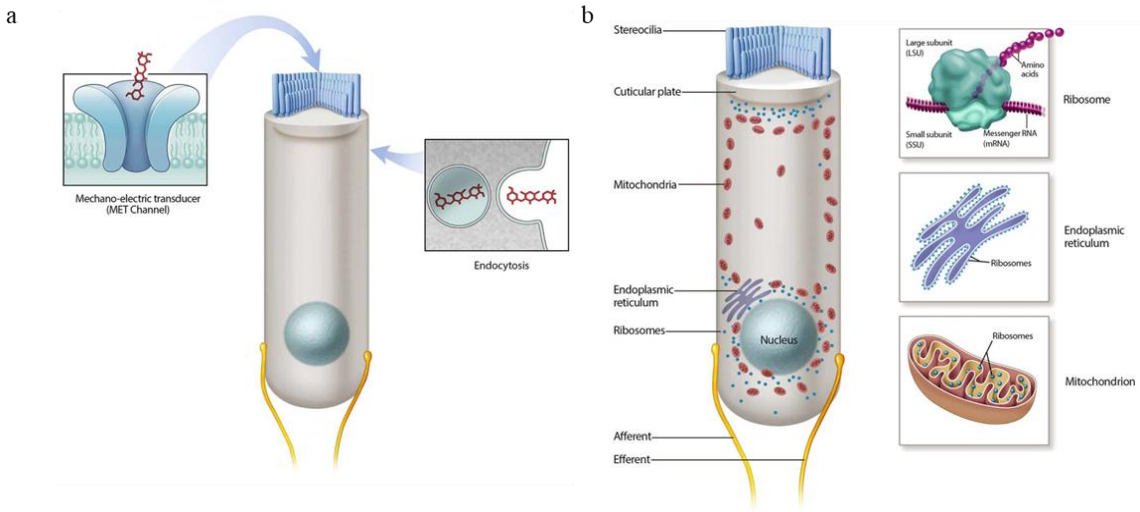

Figure 2.9. Aminoglycoside intracellular mechanisms of actions. *(a) Aminoglycosides mechanisms of entry; the two principal mechanisms by which aminoglycosides flow into hair cells; through mechano-electric transducer (MET) channel and by endocytosis. The MET channel at the tips of hair cell stereocilia facilitates the flow of ions (including aminoglycosides) from the endolymph compartment into hair cells. Endocytosis is mediated by invagination of cell membrane to allow drug entry into hair cells. (b) Shows intracellular mechanisms of aminoglycoside action; other intracellular pathways may be involved in cochlear hair cell death, in addition to mitochondrial and cytosolic ribosome toxicity, mitochondrial and endoplasmic reticulum (ER) calcium signaling, lipid interactions and reactive oxygen species (ROS) production. Source:*https://www.frontiersin.org/articles/10.3389/fncel.2017.00325/full

ribosome in aminoglycoside ototoxicity to allergic reaction in individuals with the mitochondria DNA (mtDNA): m.1555A>G variant (Estivill *et al*., 1998; Hutchin *et al*., 2001). Thus, though the eukaryotic cells mitochondrial ribosomal transcript varies from bacterial ribosome, the presence of mtDNA m.1555A>G allele re-creates an evolutionarily lost allele in the mitochondrial ribosome, making it susceptible to aminoglycosides target (Figure 2.10). Moreover, genetic investigations have added further support for mitochondrial ribosome involvement, with variable penetrance of aminoglycoside-related HI association with nuclear genetic modifiers including mitochondrial transcription optimization 1 (*MTO1*), and GTP binding protein 3 (*GTPBP3*) among others.

The paradigms on ribosomal toxicity favours aminoglycosides antibacterial effect to binding bacterial ribosome that disrupt translation. Recent interests to characterize and develop

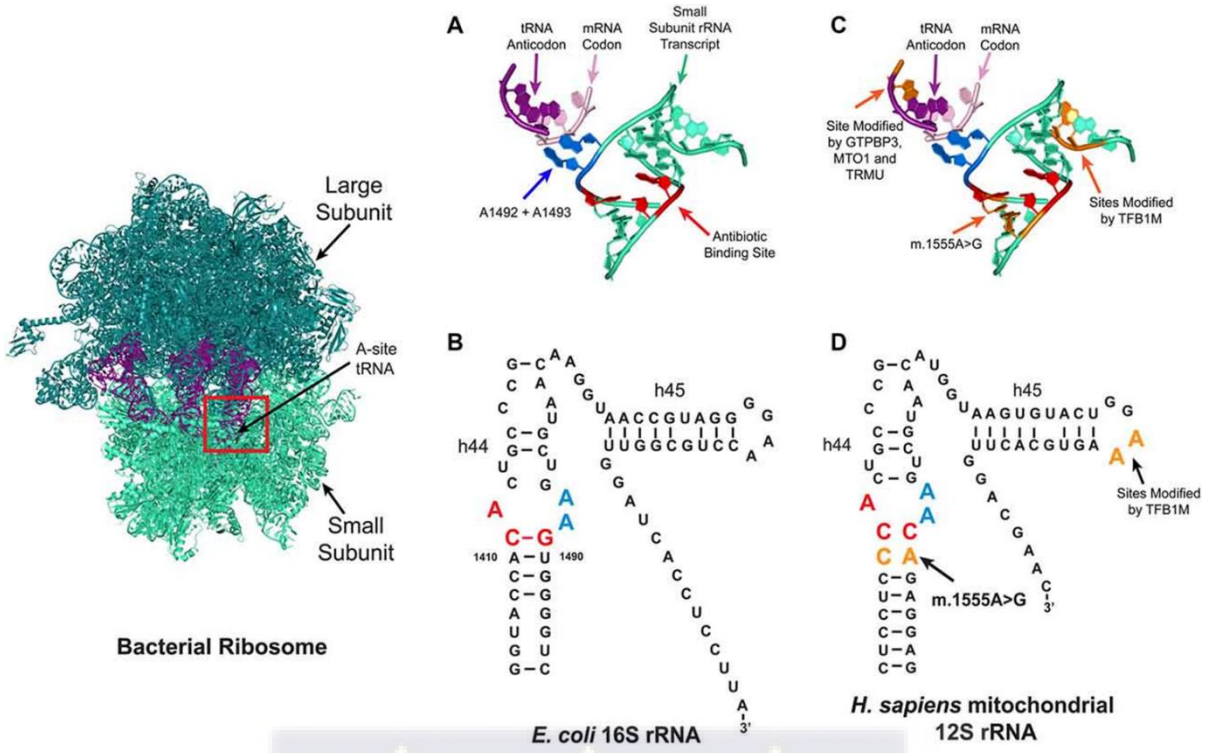

Figure 2.10. Aminoglycosides. *Prokaryotic ribosome binding site by aminoglycosides, primary to the conserved pocket in the A-site (red box) formed by helix h44 of the 16S rRNA in the small ribosomal subunit. The correct tRNA anti-codon matches with the correct mRNA codon at this site. A and B show the tertiary and secondary structures of the bacterial A-site. The nucleotides colored red indicates aminoglycosides conserved binding residues A1408, C1409 and G1491. C and D diagrams depict the tertiary and secondary structures of the mitochondrial A-site. In humans, the mtDNA m.1555A>G allele creates a new base pair in mitochondrial ribosome same as the bacterial ribosome. The level of penetrance are determine by genetic modifiers (enzymes), linked to aminoglycoside-induced HI (TRMU, MTO1, GTPBP3 and TFB1M) (O'Sullivan et al., 2017)*

aminoglycosides drugs with low binding affinity to mitochondrial ribosomes has gained much attention (Matt *et al*., 2012; Perez-Fernandez *et al*., 2014). In addition to mitochondrial damage and cytosolic ribosomes, calcium signalling, degradation of lysosomes, plasma membrane interactions (phosphatidylinositol biphosphate (PIP2) and potassium channels), and production of free radicals have been proposed as other cellular events upon aminoglycosides entry into sensory hair cells (O'Sullivan *et al*., 2017). Moreover, irreversible sensorineural HI (ototoxicity) and kidney damage (nephrotoxicity) are significant side effects of aminoglycoside administration. The incidence of HI is dose-dependent, and parallel to antibacterial efficacy,

with side effects that correlates with aminoglycoside blood concentrations (Chen *et al*., 2013). However, even at standard dose of aminoglycosides at recommended drug concentrations within normal therapeutic range, drug toxicity has been reported (O'Sullivan *et al*., 2017).

This observations led to the notion that probably it is the cumulative effect of the drug, and not the peak and trough drug concentrations, may predict the observed side effects (Cheng *et al*., 2009; Modongo *et al*., 2015). More importantly, for some individuals with mtDNA m.1555A>G mutation, HI side effect manifest following a single dose. For another set of individuals with this allele, mild to moderate HI progresses to severe phenotype after exposure to aminoglycosides (Rahman *et al*., 2012). The prevalence of ototoxicity from existing literature ranged 2 - 25% in patients with hearing disorder and less than 10% for individuals with vestibular disorder (Ariano *et al*., 2008; Huth *et al*., 2015). This prevalence was observed to increase in patients administered on several doses of intravenous aminoglycoside, particularly for tuberculosis and cystic fibrosis treatment, in which case the estimates exceeds more than half (Duggal & Sarkar, 2007; Waters *et al*., 2015).

On cisplatin, its administration is recommended in paediatric and adult solid tumours treatment; immune cell cancer, germ cell cancer, bone cancer, and cancer in the brain among many others (Dilruba & Kalayda, 2016). Ototoxicity, neurotoxicity, and nephrotoxicity are common dose limiting side effects observed in most cisplatin-based chemotherapy (Sheth *et al*., 2017). Though increasing diuresis by hydration is known to reduce nephrotoxicity, no approved treatment for ototoxicity exists currently. Generally, high frequency range, bilateral and permanent HI affecting 22 - 77% children are common in cisplatin-induced ototoxicity (Coradini *et al*., 2007; Knight *et al*., 2005).

Cisplatin causes damage to sensory hair cells, stria vascularis marginal cells, supporting cells, and spiral ligaments (Waissbluth *et al*., 2013). The elucidated molecular mechanism of

cisplatin-induced HI implicated highly reactive oxygen containing molecules and glutathione antioxidant depletion, and its regeneration enzymes, lipid peroxidation, oxidative modification of proteins, and nucleic acids damage among many others (Jamesdaniel *et al*., 2012). Thus, tissues in the cochlea fight metabolic oxidative stress by resulting to its antioxidant defensive systems (glutathione (GSH), glutathione reductase (GR), superoxide dismutase, and catalase) (Gonçalves *et al*., 2013). The antioxidant enzymes expressed in the cochlea; superoxide dismutase catalyses oxygen radical to hydrogen peroxide and molecular oxygen; catalase hydrolyses hydrogen peroxide to molecular oxygen and water; glutathione peroxidase (GSH.Px) catalyse the reduction of hydrogen peroxide and other peroxides. GSH.Px further converts the reduction of glutathione to the oxidized glutathione (GSSG), and in the process detoxifies hydrogen peroxide in the presence of glutathione reductase (Figure 2.11) (Sheth *et al*., 2017)

Cisplatin also induces potassium uptake and secretion into stria vascularis, culminating in sensory hair cells dysfunction that alters the endocolymphatic potential to induce HI (Gentilin *et al*., 2019). Though ototoxicity is generally permanent, a mechanism on reversibility based on animal studies and some cases in humans have demonstrated that initial damage to stria vascularis marginal cells, may recover if reparative processes are allowed to occur. It is the continued accumulation of these compounds that exhausts any chance of recovery, causing permanent destruction of the OHCs and the associated permanent hearing defect (Truong *et al*., 2007). The ototoxicity induced by this antineoplastic agent is also thought to be mediated by ROS increase through NOX3 NADPH oxidation pathway in the cochlear. This mechanism involves ROS generation in inflammatory response and subsequent apoptosis cascade to cause the defect via a signal transducer and activator of transcription 1 (STAT1) activation (Figure 2.11).

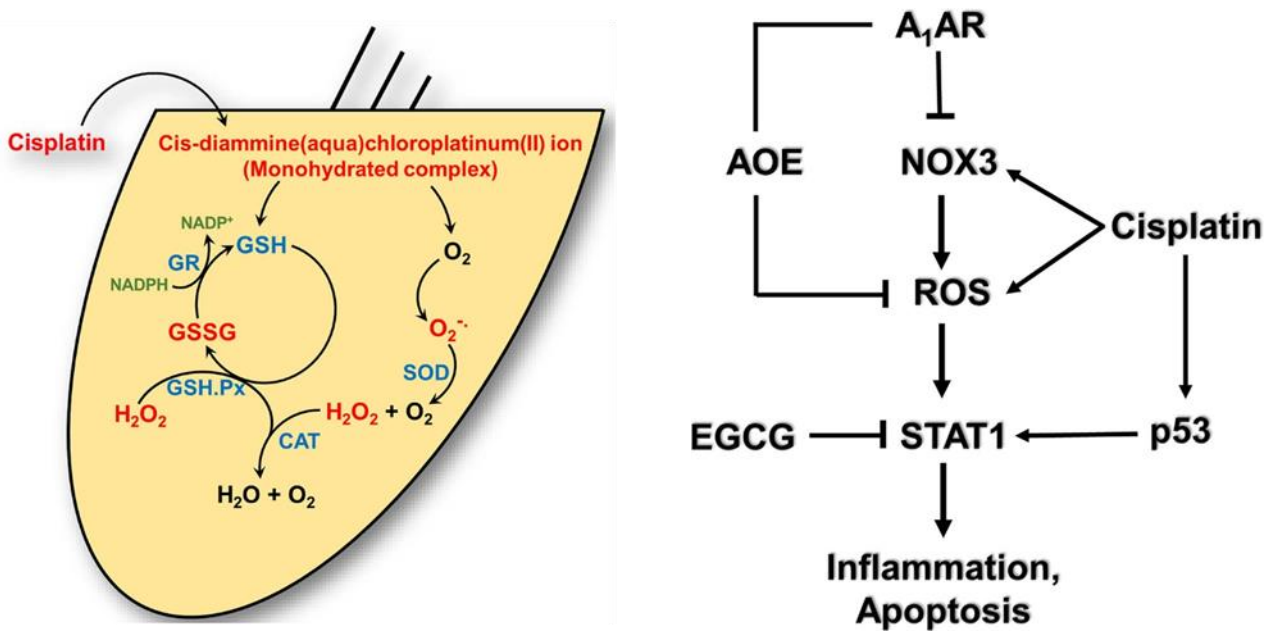

Figure 2.11. Antioxidant defence mechanism of neutralizing cisplatin's interaction and damage to the cochlear. *(A) In the hair cytoplasm, cisplatin is converted to a cis-diammine (aqua) chloroplatinum (II) (a monohydrate cisplatin complex). The reactive platinum species generated react with molecular oxygen (O_2) to produce superoxide, which is detoxified by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) and oxygen. Catalase then detoxifies hydrogen peroxide further to water (H_2O) and oxygen. Simultaneously, cisplatin reactive intermediates readily bind to and oxidize the antioxidant reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione peroxidase (GSH.Px) consumes GSH to produce GSSG in the process of converting H_2O_2 into H2O. Glutathione reductase (GR) reduces GSSG to GSH by using the reduced form of nicotinamide adenine dinucleotide phosphate (NADP+), NAPDH, as cofactor. (B) Cisplatin induced-hearing impairment mechanism and A1 adenosine receptor (A1AR)-dependent otoprotection. NOX3 activation and reactive oxygen species (ROS) generation are mediated by cisplatin. The generated ROS promotes signal transducer and activator of transcription 1 (STAT1) activation, which stimulates the inflammatory process. Activated STAT1 association with active p53 promotes the apoptosis of cochlear cells. The otoprotective effects of A1AR activation is mediated by reducing oxidative stress in the cochlea by activating antioxidant enzymes (AOE) and/or by suppressing the induction of NOX3. EGCG, a known inhibitor of STAT1, has been shown to protect against cisplatin-induced hearing loss. Source: www.frontiersin.org*

2.3.2.2 Genetic / Hereditary Hearing Impairment

Though congenital HI (present at birth) may have diverse aetiology, between 50 - 60% are due to genetic factors (Mason & Herrmann, 1998; Morton & Nance, 2006). The development of the hearing organ and complex physiology relies on the fine balance of complex interactions of different types of cells (del Castillo & del Castillo, 2017). Genetic contribution in HI dates far back in the early seventeenth century when Paulus Zacchias stated that it is necessary to

avoid marriage among the deaf and dumb for the common good, since there is a proof that they produce children like themselves (Cranefield & Federn, 1970). Autosomal recessive HI was identified among affected siblings born to hearing parents in the sixteenth century (Goldstein, 1933). The role of consanguinity in autosomal recessive inheritance pattern suggested by Wilde in 1853 (Wilde, 1853) was later denied by George Darwin in 1875 (Darwin, 1875). However, following the anonymous report that 60% of the population of 'Martha's Vineyard' are descendant of one person, who was deaf in 1877 (Anonymous, 1877), Wilde's proposals became apparent. In line with this, Hartmann presented evidence to demonstrate both autosomal dominant and autosomal recessive pattern of inheritance in HI, and emphasized the importance of parental consanguinity in recessive traits (Hartmann, 1880), which was confirmed later by Politzer (Politzer, 1999). Nonetheless, it was the first comprehensive background investigation of children in deaf schools in Norway compared to their hearing counterparts that reported four times the rate of inbreeding among deaf children's parents.

In addition, they associated high frequency of HI to high degree of consanguinity (Uchermann, 1869). Alexander Graham Bell in a retrospective study involving 2,262 congenital deaf-mute individuals reported 55% to have deaf relatives. His address on the "deaf variety of human race" to the United States of America, National Academy of Sciences, in 1883 embodied the philosophy of neo-Darwinism (Bell, 1884). In the last decade of the 20th century, the advancement in molecular genetic technology and applications identified genes linked to HI accurately mapped to loci in the human genome (Vona *et al*., 2015). The current exponential number of validated HI causal genetic markers, and the increased consciousness of the genetic contribution to congenital, age-related, and noise-induced HI highlights the opportunity for a deeper probe into genetics of HI in contemporary populations.

Connexins (Cx) gene variations encoding the gap junction family proteins are widely associated hereditary HI genes across populations (Wingard & Zhao, 2015). Protein units of

connexins form gap junctions with functional role in providing a direct intracellular conduit of cytoplasm for intercellular communications (Meena & Ayub, 2017). The connexins association in human disorders have been demonstrated by genetic variations in connexin genes affecting many organs and systems, with different conditions linked to the same gene (Rabionet *et al*., 2002). Connexins are made of aggregates of transmembrane hemi-channels (connexons), which join to similar types on adjacent cells to form hydrophilic channels that span lipid bilayer, bridging neighbouring cells for exchange and movement of small molecules (Meena & Ayub, 2017) (Figure 2.12). Gap junctions unlike a typical ion channel, possess large pore size and permit the movement of ions, signalling molecules, and other small molecules with molecular weight less than1.5kDa (Harris, 2001).

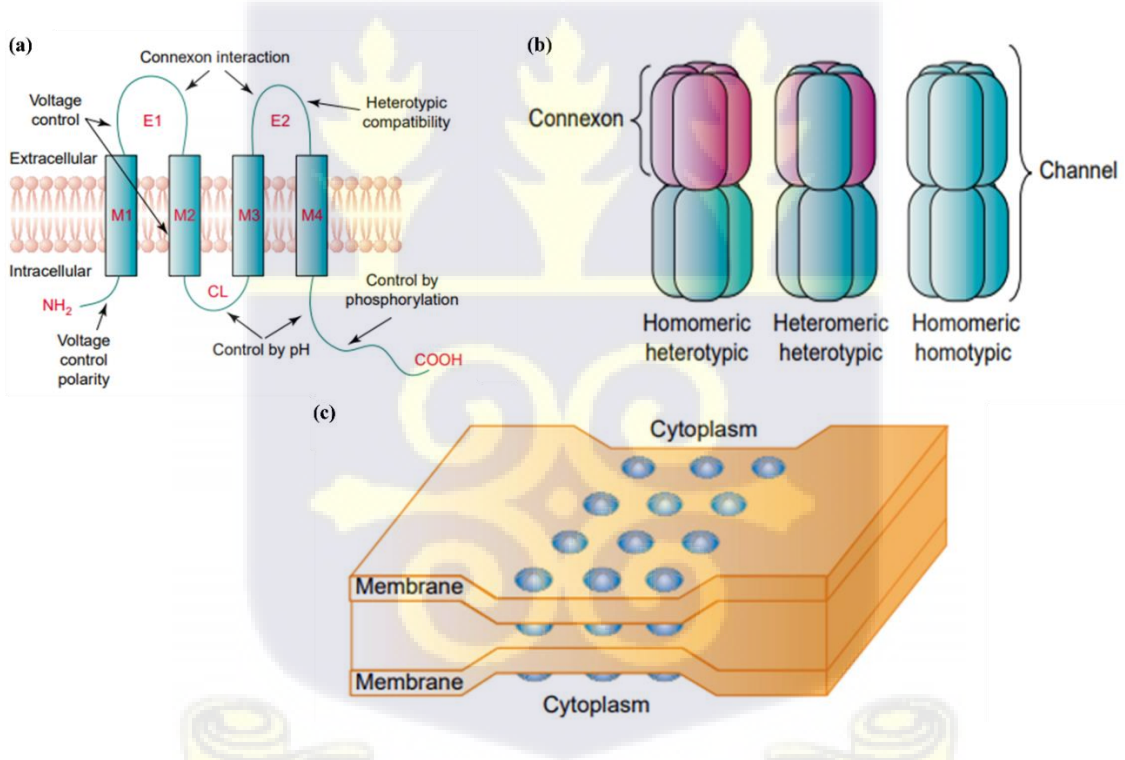

Figure 2.12. The components of gap junction channels. *(a) Topology of a connexin protein in the cytoplasm membrane. (b) Different types of channels depending on the combination of connexin proteins that compose the two hemi-channels or connexons. Colors represent different connexins. (c) Gap junction plaque formed by thousands of connexons from two adjacent cells. CL, intracellular loop; COOH, carboxy terminus; E1 and E2, extracellular domains; M1-4, transmembrane domains; NH_2, N terminus (Meena & Ayub, 2017).*

Over twenty different types of connexins have been identified and classified base on homology and/or molecular weight (α (alpha), β (beta), γ (gamma) and some unspecified). Majority of connexin genes are in groups, such as the *GJB2, GJB6* and *GJA3* mapped to chromosome 13q12 (Rabionet *et al*., 2002). In humans, Cx26 and Cx30 are the commonly reported β-connexins implicated in HI. The six connexin subunits that are formed by polymerization of connexons, can be of the same type (homotypic channel) or different types (heterotypic channel) (Figure 2.12). Connexins allows for the direct intercellular exchange of molecules between adjacent cells, and this is important during embryonic and post-embryonic development, cell proliferation suppression, in addition to varied physiological and pathological function (Wingard & Zhao, 2015). The connexins have been implicated in greater majority of hereditary HI, demonstrating their critical role in the hearing process.

In the cochlea, sound transduction is highly dependent on the optimal homeostasis of fluid in the endolymph and perilymph, which preserves the normal milieu for sensory cell function that sustains the endocochlea potential. The presence of different transport channels (ion and solute), which includes the connexins ensure the homeostasis processes (del Castillo & del Castillo, 2011). Based on studies of cochlea cultures and generated mice models, four mechanisms for maintaining cochlea homeostasis have been described. The Cx26 channels regulate homeostasis by excitotoxic compounds buffer system; distribution of glucose to cochlea cells; calcium ions and adenosine triphosphate signalling; and potassium ions release into the endolymph (see Figure 2.12 and Figure 2.13).

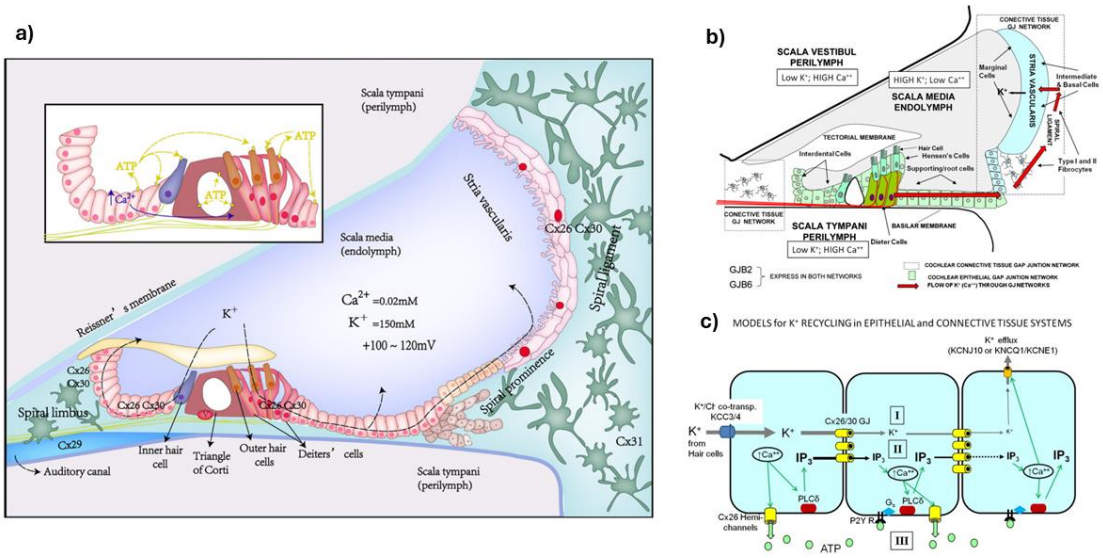

Figure 2.13. Connexins function in cochlea physiology. *(a) Schematic diagrams of Connexons expression, potassium recycling, and ATP-Ca^{2+} signaling in the inner ear. (b). Diagram of the cochlea and its various compartments, showing the proposed return of K^+ (possibly mediated by a propagated Ca^{2+} wave) from the hair cells to the endolymph (red arrows) through the epithelial (green colour) and connective tissue (dotted box) gap junction networks. The former is comprised of Dieters' cells and supporting cells, while the latter is comprised of types I and II fibrocytes in the spiral ligament and basal and intermediate cells of the stria vascularis. Ultimately, released K^+ is taken up by the marginal cells of the stria vascularis and released into the endolymph, thus maintaining the elevated K^+ levels (and high resting potential of ~+100 mV) in this compartment. This is essential for amplifying the responses of hair cells to sound, by increasing the driving potential for K^+ flux across the hair cell membrane when it is activated. (c). Proposed models to explain how K^+ is recirculated includes: (i)Passive K^+ flux through gap junctions. This seems unlikely, as it would decay over a relatively short distance. (ii)A regenerative Ca^{2+} wave that is propagated by IP3 flux between cells. IP3 is regenerated in each cell by IP3-induced Ca^{2+} release from intracellular stores that activates phospholipase C (PLC). Ultimately, in the stria vascularis, K^+ is released into the intrastrial space or the endolymph through KCNJ10 (Kir4.1) or PIP2 regulated (KNCQ1/KCNE1) K+ channels. (iii) A regenerative Ca^{2+} wave that is propagated extracellularly by Ca++ activated release of ATP through connexin hemichannels which then activates P2Y receptors in adjacent cells. These in turn can activate PLCδ, which will generate IP3 and re-initiate the Ca^{2+} response.(Chen et al., 2022; Xu & Nicholson, 2013).*

During sound energy stimulation, the buffering action causes depolarization in the hair cells after the flow of potassium ions. Efflux of potassium ions through basolateral membrane domain potassium channels restores polarization. Active transport facilitates potassium ions transport in supporting cells that prevents the toxic build-up of high potassium ion

concentration in the extracellular milieu. In the epithelium network of gap junctions, *GJB2* deletion caused hair cells death and hearing loss onset, linked to the degeneration of organ of Corti that demonstrate the buffering hypothesis. Also, neurotransmitter glutamate release by receptor actions of hair cells were disperse with the help of Cx26 gap junctions in supporting cells (Cohen-Salmon *et al*., 2002; Sun *et al*., 2009).

The supporting and sensory cells in the organ of Corti requires direct supply of high energy metabolites supplied by blood vessels. However, the avascular epithelium system in the organ of Corti makes it deficient in the high energy demand of cells during sound transduction. Gap junction-mediated diffusion has been shown to assist the movement of glucose from blood to supporting cells (Chang *et al*., 2008). Inositol 1,4,5-triphosphate (IP_3) transport investigation demonstrated the paracrine signalling actions in Cx26 in the cochlea. IP_3 signalling stimulates intracellular calcium ions release, spreading the movements of calcium ions to attached supporting cells via connexins. Subsequent characterization studies on this signalling pathway identified the activity of Cx26 hemichannels in supporting cells. These channels have been implicated in mediating the release of adenosine triphosphate that is controlled by calcium ions, relative to the fluid in the organ of Corti (Beltramello *et al*., 2005). In the stria vascularis, endocochlea potential generated also stimulate the active secretion of potassium ions from the perilymph to the endolymph. Potassium ions are suggested to be transported to the basal and intermediate cells, which is mediated by Cx26 gap junctions aiding these fibrocytes within the network of connective tissues (Lang *et al*., 2007; Marcus *et al*., 2002).

Moreover, *in vitro* functional investigations have shown that gap junction mutations cause different pathological changes that is linked with hearing disorders. Some mutations are known to distort the effective tracking of connexins to cell surface required to create the functional connexin structures (Bicego *et al*., 2006; Wang *et al*., 2003). Other mutations can result in dominant negative effect on wildtype connexon 26 and co-expressed connexon 30 (Bicego *et*

al., 2006; Zhang *et al*., 2011). Some variants affects the efficiency of the resultant gap junction structure formed, though the mutant synthesized protein are able to localizes perfectly to the plasma membrane (Bicego *et al*., 2006). In addition, though some mutations can maintain permeability of ions, small molecules are restricted. There are mutations that can perfectly form homotypic gap junctions but cannot dock to form functional heterotypic channels (Wang *et al*., 2003; Zhang *et al*., 2011). The functional characterization of gap junctions in the cochlear identified potassium ions recycling, transport of food substances and energy, generation of endocochlea potential and optimal potential gradient between the endolymph (intracellular) and perilymph (extracellular), as well as effective cochlea amplification (Chen *et al*., 2014; Gossman & Zhao, 2008; Zhao *et al*., 2006).

2.4.3.3 Genetic Variants associated with NSHI

2.4.3.3.1 Founder Variants associated with NSHI

Inherited disorder of founder origin is a profound example of genetic drift whereby allele frequencies in a larger population is altered in a new population founded by a small subset of individuals in the original population leading to random expansion of new variant. Ordinarily, human conditions associated with disease alleles should be rare in the larger population (larger effective population size) but may become prevalent in the founder population (reduced effective population size due to random genetic drift). This results in individuals carrying along a subset of the genetic information in the original population gene pool prior to their migration. Consequently, the migration as a bottle neck event reduces the degree of genetic diversity in the new population in a phenomenon known as founder effect (Figure 2.14).

The distribution of the most common genetic markers associated with HI are highly population-specific, attributed to the inherent ethnolinguistic and geographical differences in the evolutionary history of global populations (Koohiyan, 2019). The founder phenomenon as described earlier among many other factors have been linked with the segregation of

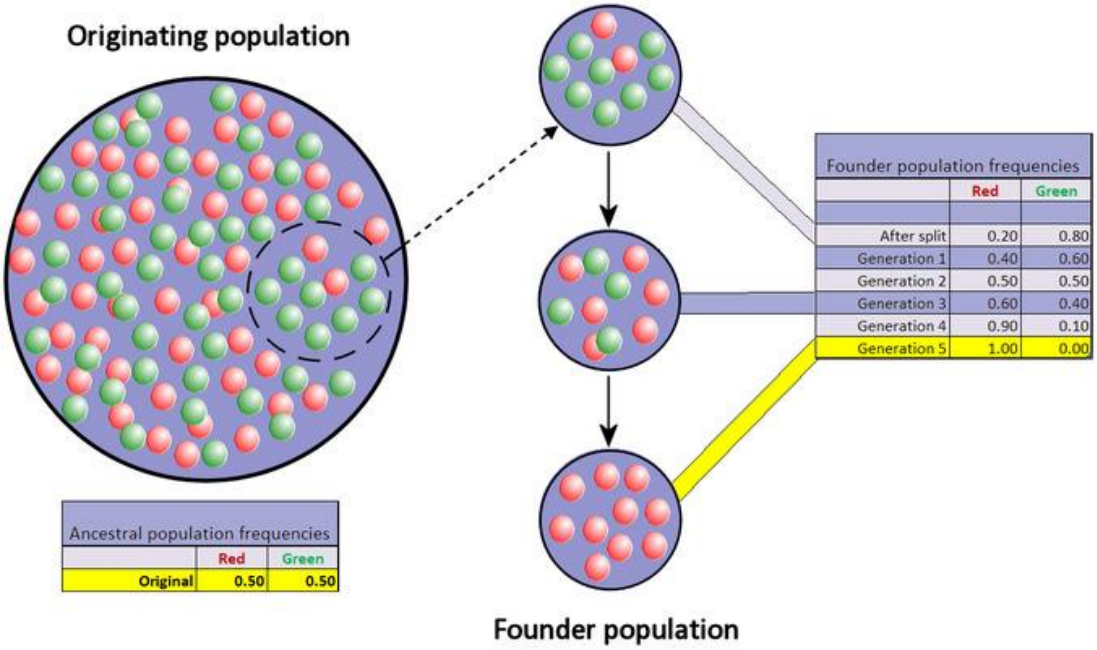

Figure 2.14. Schematic demonstration of founder effect. *The founder effect occurs when part of the population ("founders") separates from the old population to start a new population with different allele frequencies. Source:* https://www.differencebetween.com/difference-between-founder-effect-and-genetic-drift/

evolutionary conserved haplotypes around disease loci. Population bottleneck events and high rates of endogamy in small populations drives the perpetuation of deleterious recessive alleles. Although variations in *GJB2* is well known to account for over 50% of HI genetic aetiology worldwide (Koohiyan, 2019), different deleterious *GJB2* alleles predominate in different populations (Adadey *et al*., 2021; Tsukada *et al*., 2015). The relative variation between the prevalence of specific *GJB2* alleles reflect the regional genetic diversity in the frequencies, even for the most recurrent causal gene variant. The reported causative genes associated with ARNSHI based on reported prevalence are *GJB2, SLC26A4, MYO15A, OTOF, CDH23,* and *TMC1* (Hutchin *et al*., 2005; Ouyang *et al*., 2009). The distribution of these implicated genes demonstrate high diversity across regions, even between ethnic groups (Zytsar *et al.,* 2018) probably linked founder effect (Kabahuma *et al*., 2011). The *GJB2, GJB6,* or gap junction alpha 1 (*GJA1*) genes encode the connexons family proteins that oligomerizes to form

transmembrane channels in vertebrates (Gerido & White, 2004). Globally, *GJB2*: c.35delG-p.(Gly12ValTer2) variant where one guanosine residue is deleted from six repeated guanosine nucleotides at positions 30 and 35 (Figure 2.15) is the most reported cause of HI. This nonsynonymous mutation affects the translation reading frame, and creates a premature stop codon that shortens the full protein synthesis (Pandya *et al*., 2003). The *GJB2*: c.35delG-p.(Gly12ValfsTer2) variant dominates ARNSHI alleles in Australia, Israel, France, Italy, Spain, Lebanon, New Zealand, Morocco, Tunisia, United Kingdom and United States of America at relative frequencies between 28% to 63% (Kelley *et al*., 2002; Lench *et al*., 1998; Scott *et al*., 1998). There are reports of the variant in both multiplex and simplex families in Portugal, north Africa (Algeria), New Zealand, and families in America who comes from northern and southern regions of European (Denoyelle *et al*., 1997; Rabionet *et al*., 2000).

Figure 2.15. Gap junction protein beta 2 (*GJB2*) population-specific founder mutations distribution worldwide *(Chen et al., 2014).*

Despite the wide distribution of the variant among Europeans, North Africans, Middle East, Asian, and American populations, it remains rare in South Asia, Southeast Asia, or East Asia region (Tsukada *et al*., 2015). The comparatively high *GJB2*: c.35delG-p.(Gly12ValfsTer2) carrier frequency reported in some populations favour the founder effect or positive selection for heterozygote advantage, traced to European or Middle East ancestry. Thus accounting for the relative high allelic frequency among Caucasians (Danial-Farran *et al*., 2018; Gasparini *et al*., 2000; Laer *et al*., 2001). The proposed founder effect has been supported by the identification of conserved haplotype background in the peripheral region around the disease locus in homozygote individuals (Laer *et al*., 2001). Y-chromosome haplotype analysis in populations reporting the variant classified the variant to haplogroup I lineage, tracing the variant ancestry in Northern Africa and Europe to Middle East based on the haplogroup cluster analysis (Tsukada *et al*., 2015).

In Monte Santo, 24.1% frequency of *GJB2*: c.35delG-p.(Gly12ValfsTer2) homozygote report is significantly higher than 3.7-7.3% and 0-18.05% reported in Brazil, and other populations (Manzoli *et al*., 2013). However, this was lower than 25.4%, 30.2% and 39.2% reported in Croatia (Medica *et al*., 2005), Italy (Murgia *et al*., 1999), and Bulgaria (Popova *et al*., 2012) respectively. *GJB2*: c.35delG-p.(Gly12ValfsTer2) further accounted for over 85% (82/96) of implicated *GJB2* variants in Tunisia (Riahi *et al*., 2013), similar to 64% in Portugal (Nogueira *et al*., 2011), 68% and 76% in Italy and Algeria (Ammar-Khodja *et al*., 2009; Primignani *et al*., 2009).

Similarly, 43.20% was reported in probands with *GJB2* variants; 38.8% homozygous of c.35delG/c.35delG, 2.7% heterozygous of c.35delG/wild type, 3.7% c.109G>A/wild type, and 2.65% carrier frequency in the hearing control population in Morocco (Abidi *et al*., 2008). Of the 103 non-syndromic hearing-impaired individuals and 100 hearing controls screened in Egypt, six *GJB2*: c.35delG-p.(Gly12ValfsTer2) homozygotes and two heterozygotes mutations

were identified, with high carrier frequency (6.8%) (Gibriel *et al*., 2019; Schrauwen *et al*., 2013). Founder effect was attributed to the higher frequency of *GJB2*: c.35delG-p.(Gly12ValfsTer2) reported among populations in Tunisia, Morocco and Lebanon, traced to Mediterranean origin about 111 generations ago (Belguith *et al*., 2005; Riahi *et al*., 2013). Interestingly, though the most recurrent implicated *GJB2*, *GJB6*, and *GJA1* genes have been explored by some studies in the sub-region, the observed variants are highly divergent, and at low frequencies (Cameroon and South Africa) (Bosch *et al*., 2014), except in North African populations (Riahi *et al*., 2013), and Ghana (Adadey, Manyisa, Mnika, de Kock, *et al*., 2019; Hamelmann *et al*., 2001) (Figure 2.16).

The *GJB2*: c.235delC-p.(Leu79CysfsTer3) mutation, similar to the *GJB2:* c.35delG-p.(Gly12ValfsTer2) is the predominant HI variant in East Asia (Japan, Korea, and China), with low frequencies in Middle East and East Europe, but is absent in Africa (Yan *et al*., 2003). Haplotype analysis of Y-chromosomes of homozygote individuals in different populations clustered with East Asian populations, characteristic of the *GJB2*: c.235delC variant distribution (Tsukada *et al*., 2015). The variant is suggested to have evolved in a common founder approximately 11,500 years ago in Asia (Ohtsuka *et al*., 2003; Yang *et al*., 2013). Contrary to this assertion, some reports suggest that the *GJB2*: c.235delC-p.(Leu79CysfsTer3) mutation might have originated near central region of Eurasia (Baikal Lake or the Altai Sayan) before spreading to East Asia (Dzhemileva *et al*., 2010; Yang *et al*., 2013).

In South Asia (India, Pakistan, and Bangladesh), the *GJB2*: c.71G>A-p.(Trp24Ter) mutation also dominates as the major cause of NSHI, with moderate to low frequencies reported in Middle East and Eastern Europe populations, but relatively rare among African or East Asian populations (Bouwer *et al*., 2007) (Figure 2.16). The proposed founder effect linked to the high prevalence in India trace its origin to roughly 7,880 years ago (Joseph & Rasool, 2009; RamShankar *et al*., 2003). This highlight the earlier reports on *GJB2*: c.71G>A-p.(Trp24Ter)

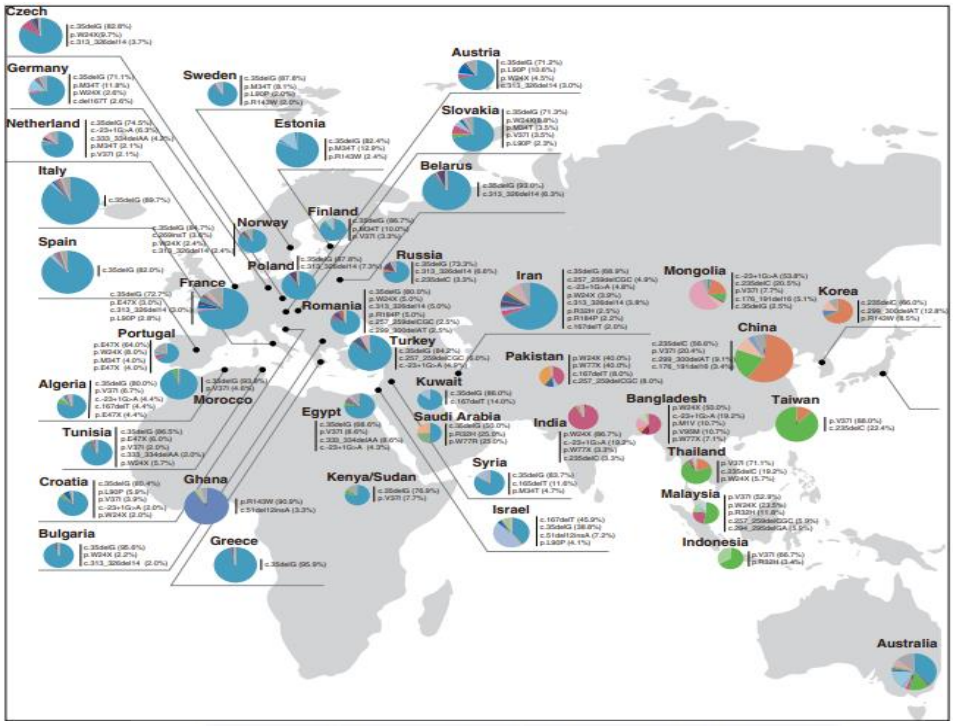

Figure 2.16. The most common hearing impairment-associated variants reported across populations *(Tsukada et al., 2015).*

variant founder effect notion due to its restriction to the Indian sub-continent population (Kelsell *et al*., 1997; Scott *et al*., 1998). Y-chromosome haplotype analysis of population reporting the variant traced the variant lineage to modern-European language speaking countries as carrying the *GJB2*: c.71G>A-p.(Trp24Ter) together with the *GJB2*: c.230G>A-p.(Trp77Ter), which evolved in a sub-group of a lineage traced to Southern Asia population migration. Further analysis of haplotype clusters characterized both variants to India, Pakistan, and Bangladesh origin (Tsukada *et al*., 2015). Similarly, in Thailand, Malaysia, Indonesia, and Taiwan, *GJB2*: c.109G>A-p.(Val37Ile) mutation is the predominant contributor to NSHI (Tsukada *et al*., 2015). Nevertheless, there are reports of the variant at moderate frequencies in Japan, China, Mongolia, Australia, and America NSHI individuals (Shinagawa *et al*., 2020). Studies in North African populations (Egypt, Algeria, Morocco, Kenya/Sudan, and Tunisia) had similar reports, but at relatively low frequencies (Shen *et al*., 2017; Tsukada *et al*., 2015).

There is no report of the variant in sub-Saharan Africa and South Asia (India, Pakistan, and Bangladesh) populations (Tsukada *et al*., 2015).

Likewise, systematic investigation among Ashkenazi Jewish population identified 7.5%, and 4.3% of *GJB2*: c.167delT-p.(Leu56ArgfsTer26) variant as associated with NSHI, with a carrier frequency of 2.8% (Laer *et al*., 2001; Morell *et al*., 1998; Sobe *et al*., 2000). The observed shared unique haplotype background in homozygote individuals favoured an ancient common ancestor in the population demonstrating a founder source. The variant is known to account for close to 60% of all congenital NSHI *GJB2* deleterious alleles in the Jewish population (Laer *et al*., 2001).

The *GJB2*: c.427C>T-p.(Arg143Trp) founder mutation predominantly associated with NSHI in Ghana has been reported in East Asia, Europe, and Middle East (Shinagawa *et al*., 2020; Tsukada *et al*., 2015). And lineage analysis in Y-chromosomes of populations reporting the variant traced the variant to the haplogroup B, which was restricted to sub-Saharan Africa provenance, presumed to have been separated at an early stage of human dispersal (Tsukada *et al*., 2015). Though the distribution of the variant in these populations has been linked to multiple founders, interestingly there are no incidence of the variant in Southeast Asia, South Asia, and North Africa NSHI cohorts.

Alongside *GJB2* variants, large deletion in *GJB6* (del(*GJB6*-D13S1830)) was described in between 5.9% to 9.7% of all NSHI associated alleles reported in Europe (Spain, France, the United Kingdom, Israel), and South America (Brazil) (de Freitas Cordeiro-Silva *et al*., 2011; del Castillo *et al*., 2003; Sirmaci *et al*., 2006). Isolated incidence of the deletion though at lower frequencies (1.3% - 1.4%) were observed in Belgium and Australia (Castillo et al., 2005). Haplotype analysis of markers in linkage disequilibrium (LD) with the deletion revealed a founder source in Western European populations (Marlin *et al*., 2005). In the United States,

two different screening studies reported contrasting findings: a moderate frequency (4.5%) among cohorts in Virginia, relative to 1.6% (low frequency) among individuals in Iowa similar to frequencies reported in Belgium and Australia (del Castillo *et al*., 2003).

In Iran, Schrauwen and colleagues found NSHI affected individuals in three families to share a haplotype of 0.52 Mb, and suggested that the capsular polysaccharide biosynthesis protein 2; *CAPB2*: c.637+1G>T mutation was inherited from a common ancient founder (Schrauwen *et al*., 2012). Genetic variations in transmembrane channel like 1 (*TMC1)* are equally common cause of NSHI in Pakistan, India, and Tunisia, accounting for 3.4 - 5.4% of NSHI familial cases (Kitajiri *et al*., 2007; Kurima *et al*., 2002; Santos *et al*., 2005). Identification of this transversion marker in intronic region in two ethnologically related Iranian HI families who originate from distinct geographic areas suggested founder effect (Davoudi-Dehaghani *et al*., 2013).

Reoccurring founder mutations have also been reported in others genes like the leucine rich transmembrane and O-methyltransferase domain containing (*LRTOMT)* variant c.208C>T and *SLC26A4*:c.1334T>G prevalent in Morocco and Tunisia (Chakchouk *et al*., 2015). For *SLC26A4* mutations, whiles *SLC26A4*: c.2168A>G-p.(His723Arg) is prevalent among Japanese cohorts, in Korea, *SLC26A4*: IVS7-2A>G and p.(His723Arg) are the most reported variants (Park *et al*., 2003; Tsukamoto *et al*., 2003).

2.4.3.3.2 Spontaneous / sporadic gene variants associated with NSHI

Illuminating the molecular mechanisms driving mutational events are important to understanding many biological processes in most genetic conditions (Kondrashov & Kondrashov, 2010). Given that novel NSHI causal genes have repeatedly been discovered, the plethora of identified sporadic genetic markers predisposing to congenital NSHI could be an underestimation. In addition, though the identification of spontaneous mutations are far from

the level of accuracy desired, majority of profound congenital NSHI detected during new-born screening are born to hearing parents (over 90%) (Gu *et al*., 2015; White, 2004).

The most commonly reported sporadic genetic cause of NSHI include variants in *GJB2, OTOG, STRC, SCL26A4, TECTA,* and *SERPIB6* in China, Korea, Japan and United States of America (Francey *et al*., 2012; Jung *et al*., 2017; Khan *et al*., 2019; Kim *et al*., 2013). Two missense mutations in residues conserved in lysyl-tRNA synthetase (*KARS*): c.1129G>A-p.(Asp377Asn) and c.517T>C-p.(Tyr173His) were reported in three families by Santos-Cortez and his colleagues (Santos-Cortez *et al*., 2013). Pathogenicity was demonstrated with evidence of aminoacylation interruption in the inner ear hair cells that prevents binding to tRNA in the inner ear by the *KARS* variant, defining ARNSHI gene causation (Santos-Cortez *et al*., 2013). Like this report, WES investigation associated *de novo* frameshift mutation in oxysterol binding protein like 2 gene (*OSBPL2*) with ADNSHI in a Chinese family. The heterozygote (*OSBPL2*: c.153_154delCT, p.Gln53ArgfsTer100) exon 3 mutation was validated and co-segregated with clinical phenotype in this family, completely absent in the controls (Xing *et al*., 2015). In two Tunisian families, sequencing of *MYO15A* found a nonsense (*MYO15A*:c.4998C>A-p.(Cys1666Ter) and splice site (*MYO15A*:c.9229+1G>A) homozygote mutations as associated with ARNSHI (DFNB3) (Belguith *et al*., 2009).

Likewise, following stringent quality control, and filtration of WES variants and Sanger sequencing validation and family segregation analysis, a novel nonsynonymous variant (*POU4F3*: c.977G>A-p.(Arg326Lys)) in the POU homeodomain of the POU class 4 homeobox 3 gene (*POU4F3*) was identified as the causal variant (Kim *et al*., 2013). *POU4F3* has been described in DFNA15 in five (5) unrelated families, with different autosomal dominant variant (Kim *et al*., 2013). In line with this, genetic investigation of a consanguineous family in China identified a missense variant in the coagulation factor C homolog gene (*COCH*), *COCH:*c.889G>A-p.(Cys162Tyr) as the pathogenic variant that co-segregate with

the phenotype (Gao *et al.*, 2013). Novel *EYA4* missense variant c.1643C>G-p.(Thr548Arg) was also linked with ADNSHI in another Chinese family (Sun *et al*., 2015). Similarly, in a Dutch family, novel tectorin alpha gene (*TECTA*) variant p.(Arg1890Cys) was associated with ADNSHI (Plantinga *et al.*, 2006). Another novel loss-of-function variant in myelin protein zero like (*MPZL2*), c.72delA was reported as the ARNSHI causal variant in three unrelated multiplex cases in Turkey and Iran (Bademci *et al*., 2018). *De novo TMIE*: c.257G>A-p.(Arg86Gln) variant was also implicated in a family in Taiwan (Yang *et al*., 2010). Exome sequencing followed by Sanger sequencing validated a novel homozygous *MYO15A*: c.9316dupC-p.(His3106ProfsTer2) as segregating with ARNSHI family, but completely absent in 200 controls (Xia *et al*., 2015). Two sporadic heterozygote *de novo* mutations in wolframin ER transmembrane glycoprotein, *WFS1*: c.2051C>T-p.(Ala684Val) and c.2590G>A-p.(Glu864Lys) were identified in 3.9% (5/128) NSHI families (Guan *et al*., 2020).

2.4.3.3.3 Mitochondrial DNA variants associated with NSHI

Even though most inherited cases of HI are generally associated with variations in nuclear genes, mtDNA variants contributes to about 5% of post-lingual NSHI as primary cause or predisposing factors (Jacobs *et al*., 2005). Genetic variations in mtDNA could cause structural and functional effects, including alterations in RNA structure, reduced mRNA and tRNA levels, as well as tRNA modifications. Mutations that impact mitochondria energy production for cellular metabolic activities are central to the pathogenicity described in HI, although other mitochondrial functions like reactive oxygen species generation and apoptosis may also contribute to the disease pathogenesis (Zorov *et al*., 1997). The inner ear structures like stria vascularis are energy dependent and requires optimal adenosine triphosphate (ATP) to maintain sodium and potassium ions homeostasis in the endolymph. Thus, deficiencies in molecular production of ATP due to mitochondrial dysfunction in the sensory hair cells and stria vascularis could impact the ions homeostasis (Tekin *et al*., 2001). Therefore, it is

understandable that the cochlea effective functioning is sensitive to mitochondrial dysfunction, the basis of sensorineural HI in mitochondrial diseases (Forli *et al*., 2007).

The mitochondria encoded tRNA leucine 1 (*MT-TL1*) and mitochondrial ribosomal RNA genes (*MT-RNR1*) variants (m.3243A>G and m.1555A>G) are the prevalent HI associated variants reported worldwide (Lehtonen *et al*., 2000; R. Li *et al*., 2004; Young *et al*., 2005). In two independent Spanish studies, high incidence of mtDNA m.1555A>G mutation was identified in pedigrees of 19 (Estivill *et al*., 1998) and 32 families with sensorineural HI (del Castillo *et al*., 2003). The increased prevalence of mtDNA m.1555A>G variant reported amongst Azeri Turkish and Bushehri populations was linked to a likely founder source (Montazer Zohouri & Hashemzadeh-Chaleshtori, 2012). This notion was in contrast to the reported 3% in Japan, 2.6% in Finland (Lehtonen *et al*., 2000), 8.56% in northern China, 0.5 - 2.4% in European or American Caucasians, and 5.3% in Indonesia sensorineural HI cohorts (Guo *et al*., 2008; Malik *et al*., 2003; Xin *et al*., 2013). In addition, the mtDNA variants: m.1494C>T, m.1096T>C, m.7445A>G, m.7510T>C, and m.7511T>C, all in the 12S rRNA gene have been implicated in maternally inherited aminoglycoside induced NSHI (Chu *et al*., 2002; Hutchin *et al*., 2001; Ishikawa *et al*., 2002; Shen *et al*., 2011; Tessa *et al*., 2001; Thyagarajan *et al*., 2000; Xin *et al*., 2013). The mtDNA m.669T>C, and m.827A>G association with NSHI has also been reported among Chinese, Polish, Brazil, Caucasian, Latvian, Iowa and United Arab Emirates populations (Mohamed *et al*., 2020).

2.4.3.3.4 X-Linked (DFNX) variants

X-linked inherited NSHI has been mapped to six (6) loci to date, with five genes identified; leucine-rich repeat extensin-like protein 3 (*LRX3*), POU class 3 homeobox 4 (*POU3F4*), small muscle protein X-linked (*SMPX*), collagen type IV alpha 6 chain (*COL4A6*), and apoptosis inducing factor mitochondria associated 1 (*AIFM1*) (Camp & Smith, 2006). The phosphoribosyl pyrophosphate synthetase 1 (*PRSP1)* gene encodes the enzyme that catalyzes

the phosphoribosylation of ribose 5-phosphate to 5-phosphoribosyl-1-pyrophospahte, a precursor for purine synthesis and nucleotide biosynthesis. Pathogenic variants in *PRSP1* are known to cause Charcot-Marie-Tooth disorder, X-linked recessive type-5, and Arts Syndrome, due to phosphoribosylpyrophosphate synthetase super activity. *PRPS1* loss-of-function variant was associated with X-linked NSHI in a family in Chinese (Liu *et al*., 2010).

POU class 3 homeobox 4 (*POU3F4*) encodes POU-III class of neural transcription factors that functions during the development of inner ear. Defects in the *POU3F4* are also associated with X-linked NSHI. In 1971, Nance *et al* described DFNX2 to X-linked recessive disorder in males characterized by profound mixed HI, with vestibular dysfunction and congenital fixation at the stapes with perilymphatic gusher (Nance *et al*., 1971). HI association with *POU3F4* together with X-linked phenotype was reported in five unrelated affected persons (de Kok *et al*., 1995), with DFNX2 identified in about 50% of families co-segregating X-linked NSHI (Huang *et al*., 2015; Petersen *et al*., 2008).

SMPX variants have been reported in X-linked deafness-four (DFNX4), although with no known functional domain. The encoded protein is proposed to be implicated in the inner ear cells maintenance during mechanical stress. Two different *SMPX* truncating variants were found in two unrelated cases with X-linked pattern of inheritance (Huebner *et al*., 2011). Furthermore, Abdelfatah *et al* had reported possible founder source for a truncating *SMPX* variant associated with DFNX4 in two large multigenerational families in Newfoundland, Canada. Variable phenotype was reported where males developed progressive, severe bilateral HI that affects all frequencies, with female carriers showing variable expression and penetrance; early-onset and post-lingual (Abdelfatah *et al*., 2013).

Findings from a recent study associated apoptosis inducing factor mitochondria associated 1 (*AIFM1)* variants with NSHI in both multiplex and simplex cases that presents with auditory

and late peripheral sensory neuropathies (Zong *et al*., 2015). Hemizygous deletion in *AIFM1* was identified in two Italian male first cousins with DFNX5, born to twin sisters (monozygotic) by fathers with no distant relations (Ghezzi *et al*., 2010). Similarly, Berger *et al*. (2011) also reported a hemizygous mutation in *AIFM1*, which was observed to segregate in another family with the phenotype (Berger *et al*., 2011). Zong *et al* reported the association of different missense mutations in *AIFM1* in 5 unrelated Chinese families with DFNX5 (Zong *et al*., 2015).

Genetic variations in the collagen type IV alpha 6 chain gene (*COL4A6*) have been linked with congenital NSHI in isolated families. *COL4A6* expression in the inner ear basal membrane may have a role in the development of the cochlea. Hemizygous mutation in this gene was identified in four affected male kindreds in a Hungarian family with congenital DFNX6. Female heterozygote carriers are reported to developed mild HI (Rost *et al*., 2014). Malformation in the cochlea was observed in affected individuals who had incomplete cochlea partition that is separates it from the inner ear auditory canal, with some cases, presenting progressive phenotype of varied severity (Rost *et al*., 2014).

2.4.3.3.5 Y-linked (DFNY) variants

Fathers transmit the human Y-chromosome to their sons, generating male-specific pattern of inheritance unique from the rest of the genome. The reported Y-linked pattern of inheritance phenotypes share similar characteristics with autosomal dominant HI, age of onset, degree of severity and audiometric configuration. To date, there are two Chinese deaf families that have described DFNY1 associated NSHI (Wang *et al*., 2004; Wang *et al*., 2009).

2.5 Hearing impairment diagnosis

HI affects all age groups from infants to elderly and can arise from defect in any part of the auditory circuit. For individuals presenting with HI, it is clinically useful to identify disease progression, unilateral or bilateral, and possible of tinnitus and vertigo association (Lasak *et*

al., 2014). Available diagnosis methods for rehabilitation exist for all regardless of the type of HI, described below.

2.5.1 Physical examination

Performing a thorough physical examination with particular detail attention to the ear anatomy is critical to establish HI diagnosis (Isaacson & Vora, 2003). The blockage of the external ear meatus by cerumen or any foreign substance could result in a conductive defect. Abnormalities consistent with clinical syndromes such as, craniofacial, middle or inner ear deformities are important to note (Martin & Hirsch, 2008). Otoscopic examination of the eardrum to check it integrity, translucent property, and the presence or absence of middle ear dysfunction forms a principal part of recommended physical examination for HI diagnosis. Using pneumatic otoscopy, clinicians can ascertain non-mobile tympanic membrane to middle ear effusion, tympanosclerosis, cholesteatoma or any mass in the middle ear. On the other hand, hyper mobile tympanic membrane is indicative of possible ossicular chain disruption. Tympanic membrane that retracts indicate movement with negative pressure, and possible dysfunction of the eustachian tube (Isaacson & Vora, 2003).

The Weber and Rinne test is perform to distinguish conductive from sensorineural defect (Behn *et al*., 2007). To perform the Weber test, the handle of a 512 Hz vibrating tuning fork is pressed to forehead midline, bridge of the nose, and individual report on which ear has loudest sound. Equal sound perception in both ears shows normal hearing or symmetrical HI. If sound is louder in the good ear, the pourer-hearing ear is suspected of sensorineural HI, and the contrast suspects conductive disorder (Lasak *et al*., 2014). With the Rinne test, the 512-Hz tuning fork is positioned on the mastoid bone, and the patient asked to respond when the sound cannot be heard again, with fork on the mastoid bone. Normal or positive test is reported when the sound by air conduction is louder than the one produced by bone conduction, and vice versa occurs if negative Rennie test. For inconclusive, and absence of a simple corrective diagnosis by

physical examination, cases are normally referred for further audiometry investigations and imaging as discussed below.

2.5.2 Imaging

The recommended standard imaging of the brain and inner auditory canal to detect and diagnose HI are the temporal bone computed tomography (CT) and magnetic resonance imaging (MRI) (Martin & Hirsch, 2008). Structural abnormalities like atresia, otitis media, or cholesteatoma are confirm mostly with temporal bone computed tomography. Most importantly, infants with sensorineural disorder, suspected enlargement of vestibular aqueduct syndrome, and any congenital cochlear or vestibular abnormalities requires temporal bone computed tomography imaging (Lasak & Welling, 2000). Conditions like acoustic neuroma (cochlear nerve agenesis) caused by retrocochlea can be diagnosed with gadolinium enhanced magnetic resonance imaging methods. Abnormal temporal bone CT scan and MRI identified 39% of sensorineural HI in children screened (Mafong *et al*., 2002). Using diagnostic imaging yield, Preciado and colleagues demonstrated that persons with sensorineural HI had a lower outcome (21.1%) compared to higher yield (29.9%) observed in persons with severe - profound HI (Preciado *et al*., 2004). In a study that systematically reviewed 50 studies, the reported diagnostic value of temporal bone CT scans ranged 7% to 74% (Chen *et al*., 2014). Abnormalities in temporal bone was reported in 18% of children with profound sensorineural HI, with cochlear dysplasia, and enlarged vestibular aqueduct as the most common anomalies with a pooled diagnostic yield of 30% (Lin *et al*., 2011). The MRI diagnostic imaging was used to evaluation and confirm auditory spectrum neuropathy in 34 to 100 % patients investigated (Kachniarz *et al*., 2015). According to Lin *et al*, 40% of severe to profound sensorineural HI children had abnormal MRI, and 24% MRI imaging results were enough to explain the observed HI (Lin *et al*., 2011).

2.5.3 Audiometric examinations

On suspicion of HI, the degree of impairment needs to be quantified. Common techniques used to ascertain the severity of hearing defect in infants, children and adults includes tympanometry (middle ear evaluation test), otoacoustic emissions (OAEs), which measures sound produce after cochlea stimulation, auditory brainstem response (ABR) testing (evaluates the cochlea and brain networks functioning), behavioural audiometry (measures level of hearing at different tones), speech reception threshold, conventional air and bone audiometry, and speech discrimination score.

Infants and children find it difficult to respond actively in audiometric assessment, and hence passive techniques like tympanometry, otoacoustic emissions, and auditory brainstem response are preferred. Using tympanometry, tympanic membrane compliance and integrity are determined for any likely middle ear effusions and perforations. Effective function of cochlear OHCs by otoacoustic emission test is a standard screening tool in new-borns and young children (Bhatia *et al*., 2013). However, prior to OAEs, external ear canal and middle ear must be normal for reliable acoustic examination. Individuals with auditory neuropathy generally present with normal OAEs but has impairment in the auditory pathway blocking transmission of generated signal, producing abnormal ABR test. Screening by ABR delivers a series of stimuli to the auditory circuit for response, which can be accomplished in sleeping or sedated infants (Nelson *et al*., 2008). In infants and young children, behavioural and/or paly audiometry may be used to provide reliable hearing threshold to aid screening. For adult patients, the conventional air and bone audiometry is preferred in diagnosis.

2.5.4 Genetic testing

Hereditary NSHI extreme genetic heterogeneity makes genetic diagnosis very challenging and time-consuming depending on the technique used (Shearer *et al*., 2010). There is classical consensus of limited clinical utility of genetic testing in unilateral congenital hearing impaired,

unless is syndromic. With technological advances in genetic sequencing techniques, clinical genetic investigations for HI causal factors have evolved from the early single-gene mutation testing to a comprehensive testing of multiple genes, a well-suited strength of NGS. The significance of genetic testing in infants with HI may be inconclusive, due to the array of implicated genes and phenotypic overlap, coupled to variant interpretation bottlenecks. The International Paediatric Otolaryngology Group and ACMG recommends the implementation of genetic testing for congenital HI to be guided by audiological assessment, ethnicity, and clinical examination (Shearer & Smith, 2015).

Though there are consistent efforts to integrate clinical evidence at the gene-level and variant-level, and subsequent interpretation of DNA variants in human genetic conditions, the medical genetics community are limited with both detailed standards for defining gene-disease associations as well as the standard cut-offs of available diagnostic panels (Abou Tayoun *et al*., 2016). To date, 112 laboratories worldwide are registered on the Genetic Testing Registry offering clinical genetic testing of hearing loss (https://www.ncbi.nlm.nih.gov/gtr/all/labs/). The available diagnostic panels among other test includes a list of genes, with a characteristic HI features (degree of severity, age of onset, symmetry, syndromic/non syndromic), pattern of inheritance, and spectrum of mutations (Tayoun *et al*., 2016).

Following a thorough history (family and congenital), physical and audiometric examinations, next steps entail *GJB2* and *GJB6* testing before comprehensive genetic investigation by targeted genome enrichment (TGE) and massive parallel sequencing (MPS) (Alford *et al*., 2014). The TGE+MPS has revolutionized comprehensive genetic diagnosis for NSHI with a single test. Deciding on a specific test option out of the available services could be challenging, however, test-specific difference that could be considered includes, genes considered in analysis, method used for variant detection, inclusion of copy number variation analysis in

methodology, pipeline of analysis and validation, and cost involved and turnaround time (Bean *et al*., 2020).

Based on the patient phenotype, it is important to ascertain if the target gene(s) of interest are captured in the test panel. Whiles the focus of some laboratories is targeted to specific diagnosis, others are expansive with list of genes for both syndromic and non-syndromic HI. The OtoSeq first version included 23 most common genes known to cause NSHI, compared the OtoSCOPE (v7) which targets 133 genes (all HI known genes), including syndromes that initially presents as NSHI. Genetic testing such as the TGE+MPS has a diagnostic rate of about ~40%, making comprehensive genetic investigation the single most appropriate diagnostic test for HI (Shearer & Smith, 2015). Although the comprehensive TGE+MPS is preferred, Sanger dideoxy sequencing approach remains valuable when considering small, selected targets (*GJB2*, *SLC264A*, and *mtDNA*) in a well-defined population or in affected person with a characteristic phenotype. Alternative to the Sanger method is using microarray, however, this offers a narrow detection window, limited by the variants on the chip, even though they are best suited for population enriched variants (Sloan-Heggen & Smith, 2016).

Now, whole-exome sequence technique that captures all coding sequence is a well-established research technique as the new next-generation sequencing for HI genetic testing (Ku *et al*., 2012). WES, with the available commercial exome capture kits have expanded the coverage beyond the exome, to known and unknown to conserved evolutionary non-coding regions, regulatory regions (promoters or enhancers) which are normally not captured (Warr *et al.*, 2015). However, the capture efficiency limitation, coupled to base calling error rates relative to traditional Sanger sequencing remains a challenge. The WES technique provides the ability to analyse simultaneously thousands of genes to detect pathogenic variants in a heterogenous phenotype such as HI.

2.6 Hearing Impairment Treatment

Notwithstanding the enormous scale of HI global burden, available standard management and treatment options primarily use medical devices including hearing aids (HAs) and cochlear implants (CIs). There is no standard pharmacological drug for treatment worldwide (Müller & Barr-Gillespie, 2015). HAs are designed to enhance effects of hearing-impaired sensitivity for better perception of speech, generally recommended for individuals with mild to moderate sensorineural HI (i.e amplifies sound for persons with mild to moderate defect to hear, and listen, communicate effectively). Basically, HAs are very useful and improves the hearing and speech comprehension of hearing-impaired individuals with sensory cell damage aetiology.

There are different types of HAs, and the decision to prescribe one over the other depends on the type and severity of deficit, specific hearing needs, and way of life. Most HAs generally contained basic components including the microphone for picking up the sound; an amplifier that increase the sound received; a receiver that resonates amplified sound through the meatus; and source of power (batteries). HAs are distinguish by the design, amplification technology (analogue or digital), and type specific features. HAs can be grouped into Behind-the-ear (BTE), In-the-ear (ITE), and In-the-canal (ITC). The BTE design is commonly prescribed for children since it can hold different types of earmolds, which requires replacement as children grow. It has most of the components enclosed in a container perfectly placed behind the ear, and connected to an earmold, easy to clean, handled, and are relatively sturdy. In the case of the ITE, all components are enclosed in a shell that fits the outer ear. Their large size fits completely in the canal, easy to handle compared to smaller aids. On the other hand, ITC aids are enclosed in a tiny case that fits partially or fully into the ear canal, with cosmetic and listening advantage relative to the other types. They are however difficult to handle and adjust for some people due to the relatively smaller size.

Cochlear implant is considered the standard clinical care (treatment of choice) (Forli *et al*., 2017; Sladen *et al*., 2017) for patients with severe to profound HI (Boisvert *et al*., 2020). The implant makes use of an electronic device that stimulate the nerves in the auditory system to restore the loss sense of hearing, with the first reported of its use in 1957 (Djourno *et al*., 1957). Contrary to HAs described earlier, CIs bypass damaged part of the ear to stimulate the sensory auditory nerve directly. Generated electrical impulses by the implant stimulation are relayed to the brain for interpretation and perception. Though different from normal hearing and requires time to learn (therapy), it offers users the ability to recognize signals, understand environmental sound and speech in person or on phone. Different findings have demonstrated that children who received CIs at a younger age outperformed their peers with similar degrees of HI with HAs in sound detection and overall auditory perception skills (Kenworthy *et al*., 1990; Nicholas & Geers, 2013).

Successfully, CIs have restored hearing, and improved the quality of life in adults, as well as improving affected children normal development and educational opportunities. Contrary to the conventional HAs sound amplification that may not benefit users as the HI severity progresses to severe or profound, cochlear implantation remains the standard treatment that restores severe to profound HI ("Bilateral Cochlear Implantation," 2018). The implant re-establishes the damaged inner hair cells function by converting the generated acoustical sound into electrical impulses that activates the auditory nerve fibres (Lenarz, 2018). Current advancement has implant systems that are partly implantable and designed with a multitude of added functions parallel to HAs like sound pre-processing, and masking of noise (Lenarz, 2018). This is achieved by perfect intra-cochlear positioning of the implant electrodes that enables for frequency specific stimulation of the sensory nerves for perception of different pitches.

To date implant systems are of two main components; an external component that is situated behind the ear, and a portion that is placed below the skin surgically. The external speech processor functions as a recorder, pre-processor, and transforms the acoustic sound into a rational sequence of electrical signals. Subsequently, the internal implant has the receiver coil that perceive frequency modulation signal; a detector for extraction of the electrical signals; an electrode carrier with varied intra-cochlear connected electrode for transmission of the generated electrical signals to the auditory nerve and telemetric measurement systems (Lenarz, 2018). With technological advancement, CI systems can now do away with a broad range of signal pre-processors like directional mics, noise purging, and acoustic sound analysis, as well as interaural wireless connection of the systems particularly in bilateral and bimodal treatment. Speech processors could be synchronized, and telemetric system that records electrophysiological responses of the implant, electrode resistances, acoustic measurement, and electrical evoked potentials.

Notwithstanding the current technologies in HA and CI, the quality of sound perceived by its users are far from mimicking normal hearing. Impairment in perception of speech, particularly in noisy backgrounds and better appreciation of music remains a great challenge to overcome in the design of these interventions (Bruns *et al*., 2016; Huang *et al*., 2017).

CHAPTER THREE

3.0 Paper 1: Global Distribution of Founder Mutations Associated with Non-Syndromic Hearing Impairment

Elvis Twumasi Aboagye [1,2], Samuel Mawuli Adadey [1,2], Edmond Wonkam-Tingang [2], Lucas Amenga-Etego [1], Gordon Akanzuwine Awandare [1], and Ambroise Wonkam [2,3]

[1] West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, Accra, LG Box 54, Ghana; etaboagye@st.ug.edu.gh; smadadey@st.ug.edu.gh; lucasamenga@gmail.com; and gawandare@ug.edu.gh

[2] Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, Cape Town 7925, South Africa; atelvis45@gmail.com; samuel.adadey@uct.ac.za; wonkamedmond@yahoo.fr; and ambroise.wonkam@uct.ac.za

[3] McKusick-Nathans Institute and Department of Genetic Medicine, John Hopkins University School of Medicine, Baltimore, MD 21205, USA; awonkam1@jhmi.edu

* Correspondence: ambroise.wonkam@uct.ac.za; awonkam1@jhmi.edu (AW), tel.: +27-21-4066-307; +1 (443) 287-0245

3.1 Abstract

The genetic aetiology of non-syndromic hearing impairment (NSHI) is highly heterogeneous with over 124 distinct genes identified. The wide spectrum of implicated genes has challenged the implementation of molecular diagnosis with equal clinical validity in all settings. Differential frequencies of allelic variants in the most common NSHI causal gene, gap junction beta 2 (*GJB2*), has been described as stemming from the segregation of a founder variant and/or spontaneous germline variant hotspots. We aimed to systematically review the global distribution and provenance of founder variants associated with NSHI. The study protocol was registered on PROSPERO, the International Prospective Register of Systematic Reviews, with

the registration number "CRD42020198573". Data from 52 reports, involving 27,959 study participants from 24 countries, reporting 56 founder pathogenic or likely pathogenic (P/LP) variants in 14 genes (*GJB2*, *GJB6*, *GSDME*, *TMC1*, *TMIE*, *TMPRSS3*, *KCNQ4*, *PJVK*, *OTOF*, *EYA4*, *MYO15A*, *PDZD7*, *CLDN14*, and *CDH23*), were reviewed. Varied number short tandem repeats (STRs) and single nucleotide polymorphisms (SNPs) were used for haplotype analysis to identify the shared ancestral informative markers in a linkage disequilibrium and variants' origins, age estimates, and common ancestry computations in the reviewed reports. Asia recorded the highest number of NSHI founder variants (85.7%; 48/56), with variants in all 14 genes, followed by Europe (16.1%; 9/56). *GJB2* had the highest number of ethnic-specific P/LP founder variants. This review reports on the global distribution of NSHI founder variants and relates their evolution to population migration history, bottleneck events, and demographic changes in populations linked with the early evolution of deleterious founder alleles. International migration and regional and cultural intermarriage, coupled to rapid population growth, may have contributed to re-shaping the genetic architecture and structural dynamics of populations segregating these pathogenic founder variants. We have highlighted and showed the paucity of data on hearing impairment (HI) variants in Africa, establishing unexplored opportunities in genetic traits. in populations reporting the variants. International migration, regional and cultural intermarriage, coupled with rapid population growth may have contributed to shaping the genetic architecture and structure of populations reporting deleterious founder alleles. The findings expose a paucity of data on founder mutations in Africa and established unexplored opportunities in NSHI genetic traits.

Keywords: hearing impairment; non-syndromic; genetics; founder variant; gap junction protein beta 2 (*GJB2*); global populations

3.2 Introduction

Despite the multifactorial aetiology of non-syndromic hearing impairment (NSHI), genetic contribution accounts for from 50 to 60% of congenital hearing impairment (HI), with high genetic and allelic heterogeneity (Bliznetz *et al.*, 2017). Of the array of known NSHI-associated genes, pathogenic variants in the gene encoding the gap junction beta 2 protein (*GJB2*) remain the most recurrent cause of NSHI across populations (Mikstiene *et al*., 2016). HI genetic screening and/or testing in at-risk newborns provides evaluative information for accurate diagnosis, for early detection, management, and anticipatory guidance (Borck *et al*., 2012). However, the wide genetic heterogeneity and differential allelic frequencies in NSHI causal markers across populations continue to challenge the implementation of a universal molecular diagnostic tool with equal clinical validity in all settings.

Moreover, in spite of the extensive and systematic investigation of implicated NSHI candidate genes in the past two decades, the global equity in regional HI genetic research investment can be improved, as the quality and quantity of studies from some regions of the world, including Africa, are far behind (Kenneson *et al*., 2002). Among the reported HI-implicated genes, variations in the gap junction beta 2 gene (*GJB2*) (MIM*121011), solute carrier family 26 member 4 (*SLC26A4*) (MIM*605646), and mitochondrial 12S rRNA (*MT-RNR1*) (MIM*561000) are the most associated genes across populations (Hilgert *et al*., 2009). However, whereas *GJB2*: c.35delG-p.Gly12ValfsTer2 is the predominant NSHI causal variant among Europeans, North Africans, Brazilians, and Americans (Denoyelle *et al*., 1997; Frei *et al*., 2002; Gürtler *et al*., 2003), the *GJB2*: c.235delC-p.Leu79CysfsTer3, c.845G>A-p.Met163Val, c.109G>A-p.Val37Ile, c.167delT-p.Lys56Arg, c.71G>A-p.Trp24X (Bouwer *et al*., 2007), and c.427 C>T-p.Arg143Trp (Adadey *et al*., 2019; Brobby *et al*., 1998; Hamelmann *et al*., 2001) are prevalent in Asia, Ashkenazi Jews, India and Ghana, respectively. Similarly, the *GJB2*:c.-23+1G>A is the common NSHI genetic marker reported in Southwest (Said *et al*.,

2007; Sirmaci *et al*., 2006), and South Asians (Bajaj *et al*., 2008). Equally, genetic variations in *SLC26A4* are region and population-specific; while *SLC26A4*:c.1246A>C-p.(Thr416Pro) and c.1001G>A-p.(Gly334Glu) are mostly reported in Europe (Campbell *et al*., 2001; Coyle *et al*., 1998), the c.2168A>G-p.(His723Arg) is common in Japan (Tsukamoto *et al*., 2003), and Korea (Park *et al.*, 2005), with c.919-2A>G in Taiwan (Wu *et al*., 2005), and Han Chinese populations (Dai *et al*., 2008).

The observed differential incidence of deleterious allele segregation with NSHI across populations is largely shaped by genetic architecture and population structural dynamics, including the rate of mutations, immigration/migration, admixture, and unknown environmental pressure that could have led to the likely selection of some pathogenic alleles, described as founder mutations. However, to date, the evolutionary forces driving the persistent segregation of reported deleterious NSHI founder variants across populations re-mains to be fully elucidated. It is imperative to investigate and generate data to better understand the probable evolutionary constraints around the genomic regions of these founder loci. This will further highlight the effective contribution of population size on founder phenomena. We asked the fundamental question as to whether migration, demo-graphic history, and bottleneck events in the history of ethnic groups with higher frequencies of these founder alleles, may have led to the evolution and subsequent segregation of NSHI markers.

In this paper, we have reviewed the distributions and provided a global profile of the reported NSHI founder variants' prevalence, origins, and estimated ages across populations. The study also demonstrated and defined the ancestral evolution and ethnic dynamics of NSHI founder variants while contributing to refining the regional molecular diagnostic tools, as well as setting our future research agenda.

3.3.0 Materials and Methods

We conducted this systematic review of NSHI-associated founder variants following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher *et al*., 2009). In addition, the study protocol was registered on PROSPERO, the International Prospective Register of Systematic Reviews with the registration number; "CRD42020198573".

3.3.1 Search Strategy

The authors performed a literature search on electronic databases (PubMed, Scopus, Google Scholar, Directory of Open Access Journal (DOAJ), African-Wide Information, Global Index Medicus, and the Web of Science) for English language publications on re-ported NSHI causal founder variants. The search covered the prevalence, carrier frequencies, and distribution of ethnic- and population-specific founder variants implicating NSHI segregation across populations. The origin and evolutionary age of the variants traced to their most recent common ancestor were also retrieved. ETA and SMA independently conducted the literature search using selective combinations of the following keywords: "nonsyndromic, non syndromic, non-syndromic, hearing impairment, hearing loss, deafness, founder mutation, founder variant, common haplotype, origin, evolutionary history, estimated age, most common ancestor" on all databases. Additionally, the cited references in the selected articles that met the study inclusion criteria were checked and retrieved until no further study was identified. The consensus and discussions were used to resolve disagreements after the screening.

3.3.2 Selection Criteria and Data Extraction

ETA and SMA conducted the literature search exclusively from 1st July 2020 with periodic updates and only full-text English articles were considered for information retrieval. Other authors (EW and LA) reviewed the selected articles for consistency. Figure 3.1 shows the

standard data extraction flow diagram, detailing the process of article identification, screening, eligibility, and selection. We selected 52 articles reporting 56 founder variants based on the study's inclusion and exclusion criteria. Observational genetic studies (case-control, cohorts, cross-sectional, and case series) reporting ethnic- and population-specific founder variants associated with NSHI were selected for data synthesis. The studies that contained 1) relevant data regarding one or more associated founder variants; 2) evidence of shared haplotypes traced to a common ancestor, origin, and age; 3) estimates of the founder variant, allele and carrier frequencies, and/or prevalence; 4) enough data to compute the prevalence; 5) associated phenotype severity (HI degree and frequency threshold): 6) method used for genetic investigation and/or validation of pathogenicity; 7) heterozygous selective advantage; 8) implications for screening, diagnosis, and prognosis of the condition were critically reviewed. However, after checking for duplicates, observational studies (case reports) reporting rare variants, founder variants with associated syndromic HI phenotypes, qualitative studies, editorials, letters to editors, reviews, communications, studies with overlapping data or unavailable full-text or missing key data, in vitro studies, and animal studies, as well as non-English language publications, were excluded. Information on the evolutionary history and time of variant occurrence and their contemporary regional epidemiology is presented in this review. Geographical and population distributions of variants were further checked by querying available population- and locus-specific databases (VarSome, dbSNP, VarMap, etc). Retrieved variant descriptions, according to the Human Genome Variation Society (HGVS) nomenclature, were checked using the Mutalyzer version 2.0 software and the databases including ClinVar, ClinGen, ExAC, and gnomAD.

3.3.3 Quality Assessment

To rule out bias, two authors (ETA and SMA), using the ascertained search criteria, performed the database search to identify publications included in this systematic review. The consistency

of the selected reports was reviewed by EW and LA and any disagreements were resolved with GA and AW. The data characteristics considered for extraction and analysis include the year of publication, sample size, number of controls, founder mutation, locus, gene, coding impact, prevalence, carrier frequency, estimated age of variant, markers used for haplotype analysis, method used for age estimation, global alternate allele frequency (AAF), pattern of inheritance, ClinVar classification, age of phenotype on-set, and type of defect, among many others. The data synthesis and assessment of the quality of the studies and parameters to include were performed by two independent reviewers (ETA and SMA). The Sohani et al. quality of genetic studies (Q-Genie) tool (Sohani *et al*., 2015), and Hoy *et al*., risk of bias assessment tool (Hoy *et al*., 2012) for prevalence studies were used to assess the study quality. Finally, the relevance of each study outcome informed the quality of the selected publications.

3.3.4 Pathogenicity and Clinical Significance

Although VarSome and InterVar are both bioinformatics web-based tools, built on the American College of Medical Genetics and Genomics (ACMG) / Association of Molecular Pathology (AMP) 2015 guidelines (Richards *et al.*, 2015), each reported founder variant classification based on the ACMG/AMP guidelines was independently reviewed. The ClinVar, dbSNP, and ClinGen classifications of variants were also examined and the allele frequencies from the ExAC and gnomAD databases were extrapolated. In addition, the phenotype-genotype evidence and the relationship between human genetic variants and phenotypes providing strong evidence for clinical significance interpretation, were equally reviewed. Decisions on the putative variants' clinical significance were based on the pathogenic predictions obtained from the databases queried and the clinical reports.

3.4.0 Results

3.4.1 Publication Search Outcome

We extracted a total of 777 publications through the electronic database search. After removing duplicates and a critical screening of titles and abstracts, 121 full-text versions of relevant articles were considered for full-text review. Finally, 52 reports were considered for information retrieval, synthesis, and data analysis, as shown in the flow diagram (Figure 3.1). Data on over 27,959 study participants who were involved in the studies reported in 24 countries were considered for information extraction in this systematic analysis. The details of the retrieved information characteristics extracted from the selected publications in this review are presented in Table 3.1.

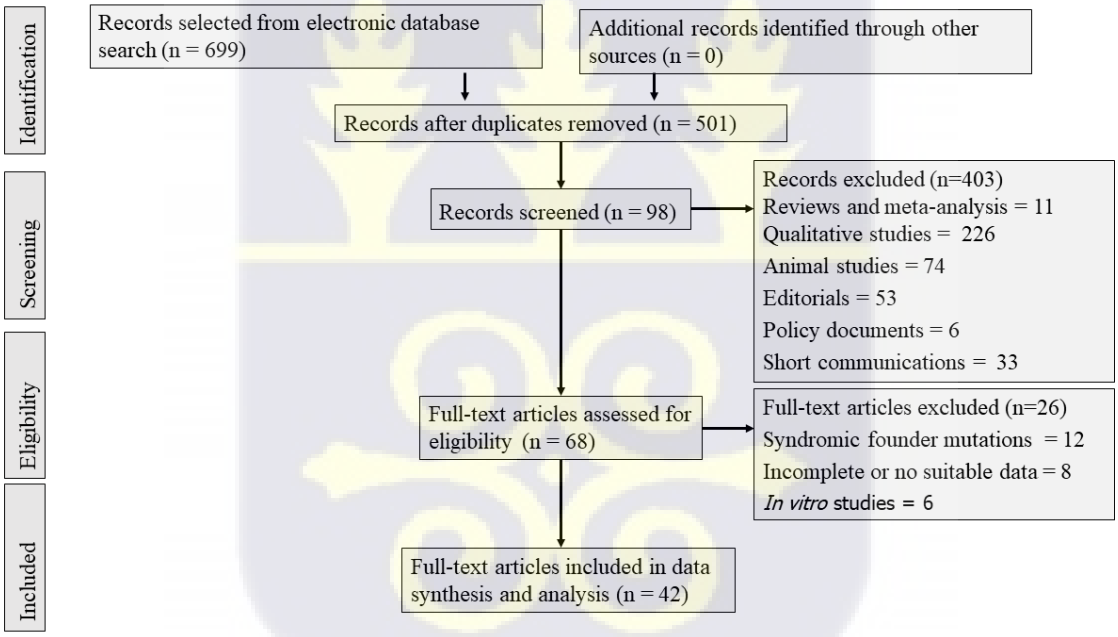

Figure 3.1. Standard information retrieval chat; *schematic flow illustrating extracted articles screening and selection for data extraction, synthesis, and analysis.*

3.4.2 Bias and Quality Assessment

The quality assessment scores for the articles included in the data synthesis are detailed in Table S3.2. The high-risk scores of possible biases for items 3, 4, and 9 (sampling method lacks randomization, non-response bias, and length of shortest prevalence period not applicable to

Table 3.1. NSHI founder mutations allele frequency global distribution, ClinVar, and ACMG/AMP classifications

							ExAC Freq		GnomAD Exomes Freq		
Reference SNP	**Transcript**	**Locus**	**Founder Variant**	**Coding Impact**	**ACMG/AMP**	**ClinVar**	**Ref. Allele**	**Alt. Allele**	**Ref. Allele**	**Alt.Allele**	**Ref**
rs80338948	NM_004004.6	DFNB1	*GJB2*: c.427C>T-p.Arg143Trp	Missense	P (PP5, PM1, PM2, PP3, PM2)	P	0.999835	0.000165	0.999884	0.000116	(Brobby *et al.*, 1998; Shinagawa *et al.*, 2020)
rs80338942	NM_004004.6	DFNB1	*GJB2*: c.167delT-p.Leu56ArgTer26	Frameshift	P (PSV1, PP5)	P	0.999316	0.000684	0.999415	0.000585	(Morell *et al.*, 1998)
rs80338939	NM_004004.6	DFNB1	*GJB2*: c.35delG-p.Gy12Ter	Nonsense	P (PSV1, PM2, PP5)	P	0.993960	0.006040	0.993443	0.006557	(Abidi *et al.*, 2008; Dzhemileva *et al.*, 2010; Janecke *et al.*, 2002; Khandelwal *et al.*, 2009; Kokotas *et al.*, 2008; Laer *et al.*, 2001; Naddafnia *et al.*, 2019; Norouzi *et al.*, 2011; Qi *et al.*, 2007; Tekin *et al.*, 2003; Zytsar *et al.*, 2018)
rs80338943	NM_004004.6	DFNB1	*GJB2*: c.235delC-p.Leu79CysTer3	Frameshift	P (PSVB1 PS3, PP5, PM2)	P	0.999637	0.000363	0.999531	0.000469	(Laer *et al.*, 2001; Ohtsuka *et al.*, 2003; Posukh *et al.*, 2005; Qi *et al.*,

											2007; Yan *et al.*, 2003; Zytsar *et al.*, 2018)
NA	NA	DFNB1	*GJB6*: Δ(GJB6-D13S1830)	Deletion	NR	NR	NR	NR	NR	NR	(Castillo *et al.*, 2005; Castillo *et al.*, 2003)
NA	NA	DFNB1	*GJB2*: delE120	Deletion	NR	NR	NR	NR	NR	NR	(Tekin *et al.*, 2005)
rs104894396	NM_004004.6	DFNB1	*GJB2*: c.71G>A-p.Trp24Ter	Nonsense	P (PSV1, PP55, PM2)	P	0.999423	0.000577	0.999416	0.000584	(Joseph & Rasool, 2009)
rs2274084	NM_004004.6	DFNB1	*GJB2*: c.79G>A-p.Val27Ile	Missense	B (BA1, PP6, BP4, PM1)	B	0.954619	0.045381	0.945994	0.054006	(García Sánchez *et al.*, 2014)
rs104894413	NM_004004.6	DFNB1	*GJB2*: c.131G>A-p.Trp44Ter	Nonsense	P (PS3, PM1, PP3, PM5, PM2)	P	0.999992	0.000008	0.999976	0.000024	(Carranza *et al.*, 2016)
NA	NA	DFNB1	*GJB2*: del(GJB2-D13S175)	Deletion	NR	P	NR	NR	NR	NR	(Bliznetz *et al.*, 2017b)
rs1302739538	NM_004004.6	DFNB1	*GJB2*: c.516G>C-p.Trp172Cys	Missense	P (PP5, PM1, PM2, PP3, PM2)	P/LP	NMR	NR	NR	NR	(Zytsar *et al.*, 2020)
rs1567620939	NM_016239.4	DFNB3	*MYO15A*: c.1171_1177dupGCCATCT - p.Tyr393CysTer41	Frameshift	P (PVS1, PP5, PM2)	P	NR	NR	NR	NR	(Palombo *et al.*, 2017)
rs749136456	NM_016239.4	DFNB3	*MYO15A*: c.4198G>A-p.Val1400Met	Missense	P (PP5, PP3, PM2,BP1)	LP	NR	NR	NR	NR	(Manzoli *et al.*, 2016)
rs28942097	NM_147196.3	DFNB6	*TMIE*: c.250C>T-p.Arg84Trp	Missense	P/LP (PP5, PM3, PP3, PM2)	P/LP	0.999983	0.000017	0.999976	0.000024	(Sirmaci *et al.*, 2006)
rs937270834	NM_138691.3	DFNB7/11	*TMC1*: c.-258A>C	5 Prime UTR Variant	LB (BP4, PM2)	NR	NR	NR	NR	NR	(Davoudi-Dehaghani *et al.*, 2013)
rs121908073	NM_138691.3	DFNB7/11	*TMC1*: c.100C > T-p.Arg34Ter	Nonsense	P (PVS1, PP5, PM2)	P	0.999948	0.000052	0.999944	0.000056	(Bazazzadegan *et al.*, 2011; Ramzan *et al.*, 2020)

rs181949335	NM_032404.2	DFNB8	*TMPRSS3*: c.916G>A-p.Ala306Thr	Missense	P (PP5, PM1, PM5, PP3)	P/LP	0.999827	0.000173	0.999855	0.000145	(Gao *et al.*, 2017)
rs80356605	NM_194248.3	DFNB9	*OTOF*: c.5816G>A-p.Arg1939Gln	Missense	US (PP5, PM2, PP3, BP1)	P	NR	NR	0.999957	0.000043	(Matsunaga *et al.*, 2012)
rs121908354	NM_022124.6	DFNB12	*CDH23*: c.C719T-p.Pro240Leu	Missense	LP (PP5, PM1, PM2, BP4)	P	0.999909	0.000091	0.999960	0.000040	(Kim *et al.*, 2015)
rs74315437	NM_012130.4	DFNB29	*CLDN14*: c.254T>A-p.Val85Asp	Missense	US (PM5, PM2, PP3)	P	0.999992	0.000008	1.000000	0.000000	(Bashir *et al.*, 2013)
rs143797113	NM_012130.4	DFNB29	*CLDN14*: c.488C>T-p.Ala163Val	Missense	US (PM2, PP5)	US, LP	0.999744	0.000256	0.999708	0.000292	(Pater *et al.*, 2017)
rs142846225	NM_012130.4	DFNB29	*CLDN14*: c.414G>A-p.Trp138Ter	Nonsense	P (PVS1, PP5, PM2)	NR	0.999992	0.000008	0.999988	0.000012	*(Mohamed et al.*, 2019)
rs200664140	NM_001195263.2	DFNB57	*PDZD7*: c.490C > T-p.Arg164Trp	Missense	US (PM2, PP5, BP1)	US	NR	NR	NR	NR	(Lee *et al.*, 2019)
rs367688416	NM_001042702.5	DFNB59	*PJVK*: c.406C>T-p.Arg136Ter	Nonsense	P (PSV1, PP5, PM2)	P	0.999982	0.000018	0.999984	0.000016	(Borck *et al.*, 2012)
rs80338940	NM_004004.6	DFNBA1	*GJB2*: c.-23+1G>A	Splice donor	P (PSV1, PP5. PM2)	P	NR	NR	0.999829	0.000171	(Barashkov *et al.*, 2011; Bonyadi *et al.*, 2011; Solovyev *et al.*, 2022; Zytsar *et al.*, 2020)
rs80358272	NM_004700.4	DFNA2	*KCNQ4*: c.211delC-p.Gln71SerTer68	Nonsense	P (PSV1, PM2, PP5)	P	NR	NR	NR	NR	(Naito *et al.*, 2013)
rs1064797088	NM_004004.6	DFNA3A	*GJB2*: c.136G>A-p.Asp46Asn	Missense	P (PM1, PM5, PP3, PP5, PM2)	P	NR	NR	NR	NR	(Bazazzadegan *et al.*, 2011)
rs727505273	NM_001127453.2	DFNA5	*GSDME*: c.991-15_991-13delTTC	Deletion	LP (PP5, PM2, BP4)	P	NR	NR	NR	NR	(Booth *et al.*, 2020; A. Nishio *et al.*, 2014; Park *et al.*, 2010; Yu *et al.*, 2003)

rs757172581	NM_004100.5	DFNA10	*EYA4*: c.1177C>T-p.Gln393Ter	Nonsense	P (PVS1, PM2, PP5)	NR	0.999992	0.000008	0.999996	0.000004	(Kim *et al.*, 2015)
rs138527651	NM_138691.3	DFNA36	*TMC1*: c.1939T>C-p.Ser647Pro	Missense	US (PP5, PM2, PP3)	P	0.999992	0.000008	0.999976	0.000024	(Brownstein *et al.*, 2011)
rs200171616	NM_138691.3	DFNA36	*TMC1*: c.1534C>T-p.Arg512Ter	Nonsense	P (PVS1, PM2, PP3, PP5)	P	0.999802	0.000198	0.999670	0.000330	(Kraatari-Tiri *et al.*, 2022)
NA	NM_138691.3	DFNA36	*TMC1*: c.1627G>A-p.Asp543Asn	Missense	US (PM2, PP3)	US, LP	NR	NR	NR	NR	(Nishio & Usami, 2022)

dbSNP = Single Nucleotide Polymorphism Database, ExAC = Exome Aggregation Consortium, gnomAD = Genome Aggregation Database, ClinVar = Public archive of genomic variation as it relates to human health, ACMG/AMP = American College of Medical Genetics and Genomics and Association of Medical Pathology, Ref. Allele = Reference Allele, Allele Freq. = Allele frequency, NA = Not Available; P = Pathogenic; LP = Likely pathogenic; B = Benign; LB = Likely benign; US = Uncertain significance; and NR = Not reported.

the majority of studies reviewed (the participants are individuals with genetic conditions), respectively) were recorded for most of the publications, which is typical of most genetic studies. Of the remaining points, there was a relatively low risk of bias in the reviewed reports (Table S3.2).

3.4.3 Publications and participants description

Since 1998, there has been an increasing trend in the number of publications that reported NSHI-associated founder variants, with a relatively high number in the past decade. This may be reflective of the current advancement in molecular diagnosis and available and cost-effective massive parallel sequencing Next Generation Sequencing (NGS) technologies. The highest number of reported NSHI-associated founder variants across populations were recorded between 2010 and 2015 (33.9%; n = 19/56), with considerable numbers between 2016 and 2020 as well (26.8%; n = 15/56) (Figure 2A). Of the studies that reported the number of probands (51.8% of studies; n = 29/56), there were 1341 individuals from multiplex families and 1239 simplex cases involved in the genotype and haplotype analysis for the variants investigated (2580 individuals in total). Over 76% of the reports (76.8%; n = 43/56) screened unrelated healthy hearing controls as comparisons for carrier frequencies of putative founder alleles, with 8426 healthy hearing controls screened in total. The age range of the few studies (28.6%; n = 16/56) with data on the age of the participants spanned 0-82 years (pre-lingual and post-lingual) and the mean of means for the reported ages range was 15.6 ± 4.3-42.9 ± 17.1 of the probands of 22.35 years.

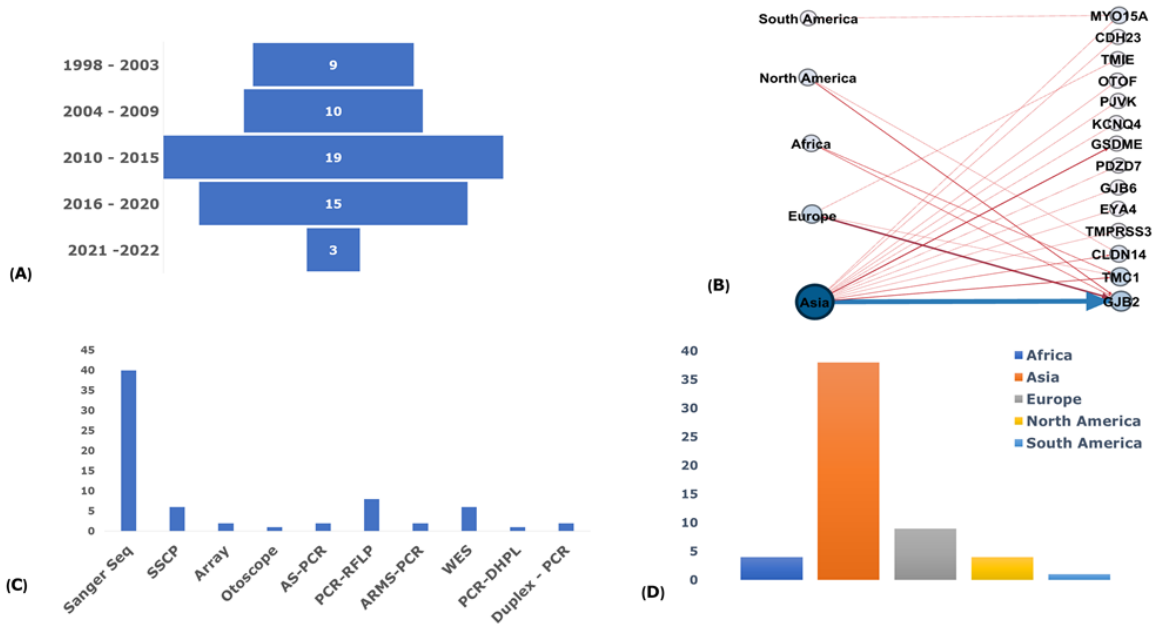

Figure 3.2 Distribution profile of NSHI-associated genes with founder variants; *(A) A bar chart showing the distribution of publications retrieved per year range reporting NSHI-associated P/LP founder variants between 1998 and 2022. (B) A network of reported genes with the mutations plotted against continents from which the mutations were reported. The continents denote the nodes on the left and nodes on the right represent the individual genes harbouring the PLP reported founder mutations. The thick blue line depicts GJB2 as the gene with most reported mutations across the continents. The network was constructed using the Gephi software. (C) Genotyping methods were used to investigate founder mutations for common ancestor or haplotype sharing in individuals segregating the ethnic- and population-specific PLP NSHI founder mutations. At least one of the methods listed was used for the genotype analysis in each of the studies reviewed: Sanger Seq = Sanger sequencing, SSCP = Single-strand conformational polymorphism analysis, Array = Affymetrix GeneChip Array, Otoscope = OtoSCOPE platform, AS-PCR = Allele-specific polymerase chain reaction, PCR-RFLP = Polymerase chain reaction-restriction fragment length polymorphisms, ARMS-PCR = Amplification-refractory mutation system, WES = Whole exome sequencing, PCR-DHPLC = Polymerase chain rection Denaturing High Performance Liquid Chromatography, and Duplex, and PCR = Duplex polymerase chain reaction. (D) A bar chart of the distribution of reviewed articles by continents.*

3.4.4 Non-Syndromic Hearing Impairment Phenotyping

Pure tone audiometry (PTA) provides a snapshot of the level of hearing in both ears and was the most reported method used to assess the studied participants' phenotypes in the studies reviewed. In addition, brainstem electronic response audiometry (BERA), computed tomography (CT), auditory brain stem response (ABR), and magnetic resonance imaging (MRI) were among many other assessment methods used in the phenotype screening of the

participants examined. The degree of HI reported in the analyzed studies showed that the most affected individuals had mild-to-profound HI. Unilateral HI was reported in a few studies that recorded that information. While most of the affected individuals screened in the studies were reported as having non-progressive NSHI, some isolated studies (16.1%; n = 9/56) recorded progressive impairment in the investigated cohorts.

The pre-lingual onset of associated phenotypes was reported in 30 (53.4%; n = 56) studies, compared to 10 (17.9%; n = 56) post-lingual, and as high as 16 (28.6%; n = 56) re-ports not specifying the age of medical diagnosis in the probands investigated. Sensorineural NSHI was the most common type of defect reported (62.5%; n = 35/56) to segregate with pathogenic founder variants among kindreds. Although quite an appreciable number of studies (7.5%; n = 21/56) did not categorize the type of HI defect as either conductive or sensorineural, none of the studies reviewed attributed the NSHI (phenotype)-associated founder variant to a conductive defect in the studied probands. Virtually all the phenotypes described in the examined probands had no evidence of any other associated clinical syndromes (syndromic HI), with only one (1.9%; n = 56) report having no data on the phenotype classification. The autosomal recessive mode of inheritance was the most re-ported pattern of inheritance linked with the segregation of the reported population-specific NSHI-associated founder variants (87.5%; n = 49/56). Little evidence of consanguinity in some of the families investigated for generational history was reported. Autosomal dominant inheritance was also described in five (8.9%; n = 56) studies as associated with NSHI founder mutations and the remaining 3.6% (n = 2/56) of the studies did not re-port the specific pattern of inheritance for the reported founder variant segregation.

3.4.5 Genotyping methods

DNA purified from peripheral blood samples was the most common source of DNA used for all the genetic investigations in the studies reviewed (96.4%; n = 54/56), except in two studies

where buccal mucosa cells were collected in addition to the peripheral blood samples. The targeted gene sequencing approach, specifically the Sanger sequencing using sequence-specific primers, was the most widely used genotyping method in over 90% of the NSHI-associated founder mutations reported (Figure 3.2C). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), single-strand conformational polymorphism (SSCP), whole-exome sequencing (WES), allele-specific PCR (AS-PCR), Array, real-time PCR (RT-PCR), denaturation high-performance liquid chromatography (DHPLC), and targeted panel sequencing (OtoSCOPE) were among other genotyping techniques used for the founder variant genotyping and markers for haplotype investigations in the reviewed studies.

3.4.6 Haplotype and Marker Analysis

To effectively investigate the shared haplotypes for common ancestry and the origin of the putative NSHI-associated founder variants in homozygote individuals, short tandem repeats (STRs) and single nucleotide polymorphism (SNPs) markers were genotyped to identify the shared ancestral informative markers (haplotype markers spanned between 731 bp and 63,759 bp regions around the markers of intertest). Out of the 56 reports reviewed, 20 (35.7%) estimated the evolutionary age of the reported NSHI founder variants (Table S3.1 and S3.3). The single marker method that uses the maximum likelihood-based assumptions (Slatkin & Rannala, 2000), disequilibrium mapping likelihood estimation (DMLE+v.2.3) (Reeve & Rannala, 2002), and genetic clock concept that relies on the decay of markers in linkage disequilibrium and estimates from recombination fractions were among the methods used for the age estimations in the studies reviewed. However, few studies relied on historical events in the history of the affected population to estimate the age of the founder variant. The single marker method uses the basic assumption that two affected individuals are descendants of a local ancestor who introduced the mutation some number of generations ago. Using the size of the haplotype shared by affected individuals, the unknown number of generations is estimated

on each side of the locus (Slatkin & Rannala, 2000). On the other hand, the DMLE+v.2.3 population genetic software uses the Bayesian linkage disequilibrium gene mapping approach, which depends on the linkage disequilibrium between pathogenic mutation and multiple linked markers in affected individuals and unaffected controls (Reeve & Rannala, 2002). The genetic clock principle for mutation age estimation is based on the expected decay of the linkage disequilibrium between the mutation and alleles of neighboring genetic markers due to re-combination.

3.4.7 Regional Distribution of Founder Variants

Most of the NSHI-associated founder variants were reported in Asia (85.7%; n = 48/56), specifically with the majority in Japan, Russia, China, South Korea, Iran, and Pakistan (Figure 3.3 and Figure 3.2B and 2D). For instance, in Japan, seven (7) different founder mutations were reported in five (5) genes (Figure 3.3 and Table S3.1). Europe was the continent with the second most reports (16.1%; n = 9/56); Africa and North America recorded 7.1% (n = 4/56) studies each, while South America had only one (1) publication, respectively (1.8%) (Figure 3.2B and 2D). Two NSHI-associated founder mutations were reported both in Tunisia, India, and the USA. One NSHI-associated founder mutation each was reported to originate from Ghana, Morocco, Mexico, Belgium, Guatemala, Oman, Pakistan, and Greece (Figure 3.3).

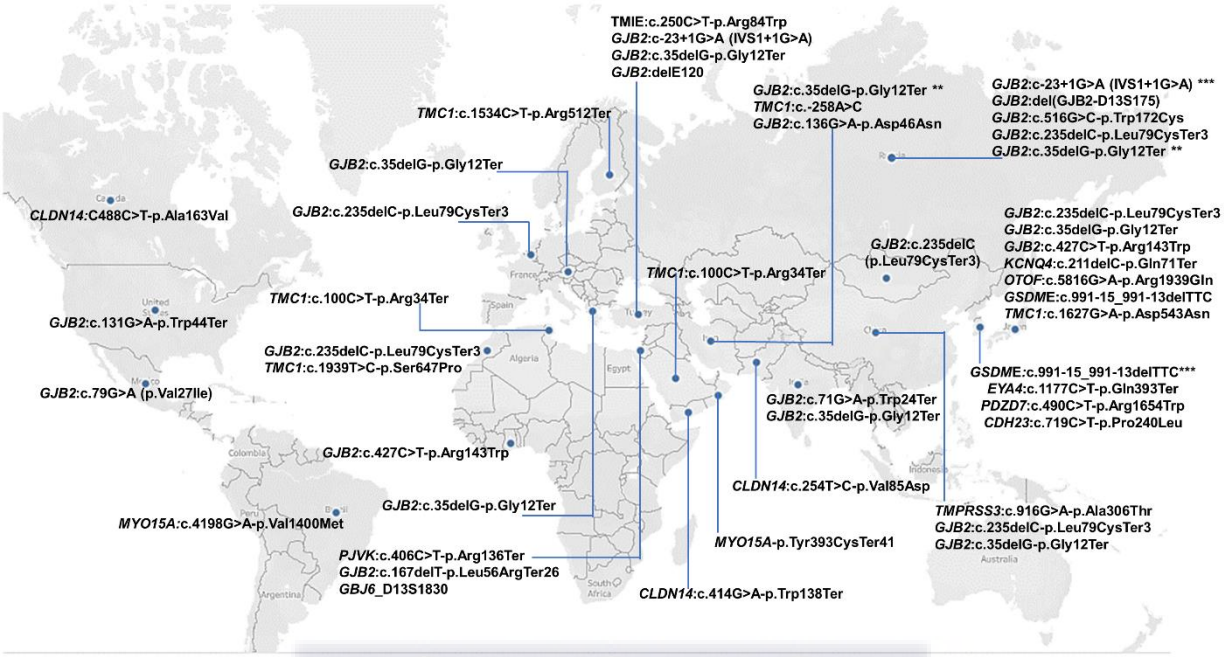

Figure 3.3 A global map showing countries that reported at least one gene with P/LP founder variant in NSHI. *Ethnic- and country-specific reported NSHI founder mutations are depicted with blue dots, with the reported genes and mutations listed in specific countries, respectively. The map was created using Tableau Software* (Tableau, 2016). ** = number of times variant has been reported as founder*

3.4.8 Reported Founder Mutation Genes

The analysis of the implicated genes with reported NSHI founder variants illuminated *GJB2* as the predominant gene with the highest number of reported variants in all regions, further supporting the hypermutability hypothesis (Figure 3.3 and 3.4). The contribution of other gene variants implicated in the segregation of NSHI founder alleles were region, population, and ethnic-specific, also suggesting the occurrence of the local founder effect and/or geographical isolation. *TMC1* and *GSDME* had two different reported variants. Of the studies reviewed, *GJB2* genetic variations remain the most extensively investigated and widely reported causal variants; with 32 different variants reported across populations, representing 57.1% of all NSHI causal variants (32/56) (Figure 3.2B and Table S3.1). In Russia, comparative haplotype analysis favored a common origin of *GJB2*: c.35delG-p.Gly12Ter in the Volga-Ural region and

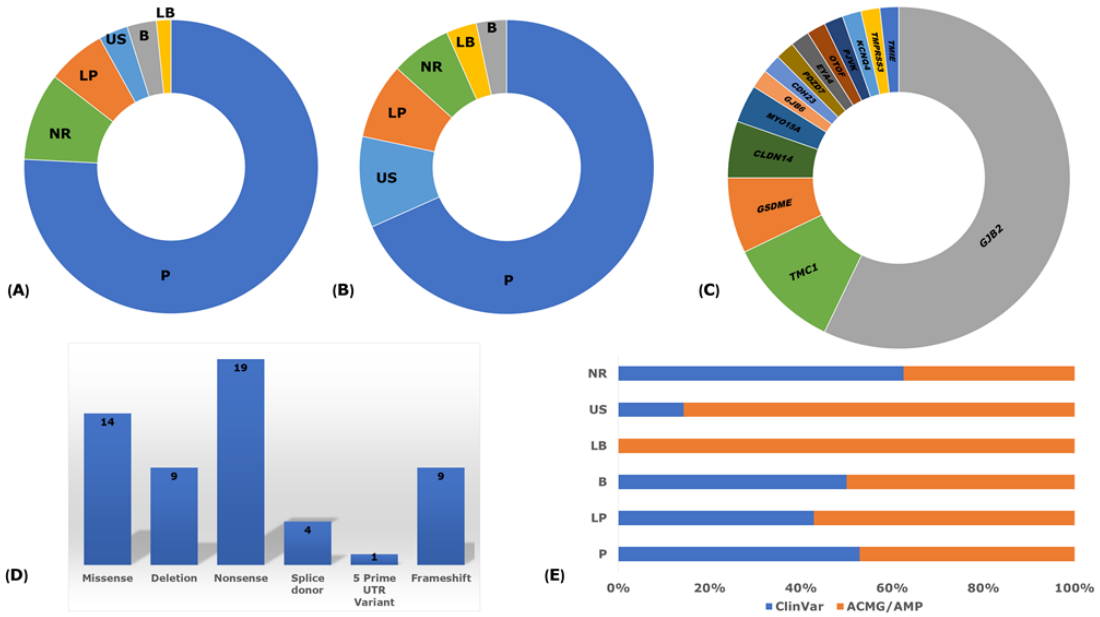

Figure 3.4. Reported variant classification. *(A) ClinVar classification of the reported founder variants. (B) American College of Medical Genetics and Genomics (ACMG) and Association of Medical Pathology (AMP) guideline classification of reported founder variants. (C) A pie chart showing the different genes and proportions reported for NSHI founder mutations. (D) The number, proportions, and types of variants reported. (E) ClinVar and ACMG/AMP proportions compared. P = Pathogenic; LP = Likely pathogenic; B = Benign; LB = Likely benign; US = Uncertain significance; and NR = Not reported. Note: (A) and (B) were normalized by adding "+1" to all values obtained for each category to obtained consistent color coding of sections.*

Siberia and in European ancestry (Zytsar *et al.*, 2018). Subsequent investigation of other GJB2 alleles reported at higher frequencies (c.235delC-p.Leu79CysTer3, c.516G>C-p.Trp172Cys, and c.-23+1G>A) in Southern Siberia identified a shared haplotype to further support the proffered founder role in the variant segregation in that region (Zytsar *et al.*, 2020). The *GJB2* founder variants: c.35delG (19.6%; n = 11/56), c.235delC (12.1%; n = 7/56), IVS1+1G>A (7.1%; n = 4/56), and c.427C>T (3.4%; n = 2/56) were the most common and re-occurring NSHI-associated variants with a putative founder effect across the ethnic groups. All the other reported NSHI-associated founder gene variants relate to specific populations (Table S3.1 and S3.3). Gasparini *et al.*, in 2000, reported a possible common ancestor for the *GJB2*: c.35delG-p. Gly12ValfsTer2 variant and suggested it may have originated in either Europe or in the

Middle East populations; the authors also proposed a probable heterozygote carrier advantage (Gasparini *et al.*, 2000).

In 2001, Van Laer *et al*. showed that the *GJB2*: c.35delG-p.Gly12ValfsTer2 variant may have evolved centuries ago in the white population. They identified the shared haplotype around the locus in homozygote participants from Belgium, the United Kingdom, and the United States that was absent in the population-matched hearing controls negative for the variant (Laer *et al.*, 2001). The West Austria study by Janecke *et al.*, in 2002, report-ed the highest prevalence (72.1%) of the *GJB2*: c.35delG-p.Gly12ValfsTer2 variant (Janecke *et al.*, 2002). Some studies in Turkey identified a higher prevalence of that variant among hearing-impaired individuals compared to controls (Tekin *et al.*, 2001, 2010). The *GJB2*: c.35delG-p.Gly12ValfsTer2 deletion was also reported to originate from China (Qi *et al.*, 2007), Morocco (Abidi *et al.*, 2008), Greece (Kokotas *et al.*, 2008), India (Khandelwal *et al.*, 2009), Iran (Naddafnia *et al.*, 2019; Norouzi *et al.*, 2011), and Russia (Dzhemileva *et al.*, 2010; Zytsar *et al.*, 2018).

The *GJB2*: c.235delC-p.Leu79CysTer3 variant was also predominant among Altaians (South Siberia and Russia). Genetic drift or a founder effect was suggested as the likely underlying molecular basis for the relatively high carrier frequency (4.6%) of the variant observed (Posukh *et al.*, 2005). However, as to whether the Altai region could be one of the multiple founder origins of the widespread *GJB2*: c.235delC-p.Leu79CysTer3 variant in Asia, was uncertain (Posukh *et al.*, 2005). The recent report by Zytsar *et al*. supports the *GJB2*: c.235delC-p.Leu79CysTer3 founder variant ancestry in indigenous Southern Siberia populations, after haplotype reconstruction analysis elucidated the shared haplotype among homozygote-affected individuals (Zytsar *et al*., 2020).

The *GJB2*: c.427C>T-p.Arg143Trp missense variant was first reported as a founder mutation linked to the segregation of NSHI among a population in a village (Adamrobe) in Ghana, West

Africa (Brobby *et al*., 1998). However, Shinagawa et al., after haplotype analysis using markers in linkage disequilibrium with the variant (*GJB2*: c.427C>T-p.Arg143Trp) in a relatively small number homozygous individuals in Japan, suggested phenotype segregation to be a likely founder source in a Japanese ancestor that evolved 6,500 years ago and not a hot spot mutation (Shinagawa *et al*., 2020). Ghanaian families segregating the *GJB2*: c.427C>T-p.Arg143Trp variant are reported to carry a distinct haplotype traced to a common indigenous ancestor some 9,625 years (385 generations) ago (Aboagye *et al.*, 2022), predating the Japanese report. The deleterious allele continuance is suggested to have been sustained at the observed relatively high frequency in Ghana due to the high assortative and complementary marriages among early carriers. The *GJB2*: IVS1+1G>A (c.-23+1G>A) splice donor variant was also reported by publications from Siberia (Zytsar *et al*., 2020) and Turkey (Bonyadi *et al*., 2011) as resulting from a founder effect traced to a common origin in the investigated NSHI families. The details of other gene variants implicated in the reported putative founder variants in different populations reported in the studies reviewed are shown in Table S3.1. In South Korea, Turkey, Russia, Iran, and China, additional founder variants in different genes associated with NSHI were reported (Table S3.1).

3.4.9 Pathological Mechanisms of Implicated Genes

The systematic findings have demonstrated that congenital NSHI primarily results from developmental defects in the cochlear rather than the anticipated hair cell degeneration and endo-cochlear potential reduction (Wingard & Zhao, 2015). The loss of function (LoF) mutation has been suggested as the main pathological mechanism reported in *GJB2* deleterious alleles (the most common cause of NSHI). Similar to this report, the molecular consequences of the reported *GJB2* founder variants, based on their impact on coding (Figure 3.4D) and the type of mutation (nonsense (33.9%; 19/56), missense (25.0%; 14/56), deletion (16.1%; 9/56), frameshift (16.1%; 9/56), and splice donor site (7.1%; 4/56, or a 5 prime UTR variant) sup-

ports the proposed LoF pathological mechanism (Figure 3.4D). Functional studies on *GJB2* variants have revealed varied pathological mechanisms leading to HI. The proposed pathobiological consequence include: i) mutational changes that affect the correct tracking of the gap junction channels to the cell surface (Wang *et al*., 2003), ii) defective intercellular gap junctions (Zhang *et al*., 2011), iii) selective permeability to ions but not small molecules (Bicego *et al*., 2006), and iv) inability to form heterotypic channels (Wang *et al*., 2003), among others. The connexins are expressed chiefly in the organ of Corti's supporting cells, stria vascularis, spiral ligaments, and limbus, as well as in other non-sensory cells and structures in the cochlear (Liu & Zhao, 2008). Two networks of gap junction have been identified, the epithelia gap junctional in the organ of Corti and the connective tissue gap junction network in the cochlear lateral wall. Pathological changes observed in *GJB2* knock-out mice showed cochlear development disorder, hair cell and spiral ganglion degeneration, and inactive cochlear amplification, confirming the loss of function pathology in humans (Cohen-Salmon *et al*., 2002).

The high prevalence of pathogenic *GJB2* alleles in families perpetuating the associated NSHI phenotype across populations regardless of ethnic group led to the suggestion that probable improved fitness (survival benefit) is conferred by these alleles. To investigate this hypothesis, Meyer *et al.* in 2002 reported the relative expression of *GJB2* in the skin of cohorts, with *GJB2* variants having thicker skin, which is plausibly a barrier that improves the ability to prevent pathogen invasion, trauma, and insect bites (Meyer *et al*., 2002). This assertion may be supported by reported *GJB2* variants on the gnomAD population database (Table 3.2). The anticipation is that, under natural selection, the number of 'expected' and 'observed' LoF mutations for a gene should not be significantly different (https://gnomad.broadinstitute.org/ accessed on 10 November 2022). However, this is not the case for this hypermutable *GJB2* gene, where the observed number of LoF SNVs (n = 17) is significantly higher than the

expected number of LoF SNVs (n = 6.5). This observation suggests that unknown genetic modifiers (genetic constraints) and/or environmental pressure may have led to the evolution of variants in the *GJB2* gene across populations. Though haplotype analysis across populations has confirmed the independent evolution of some recurrent *GJB2* alleles and the reported allele frequency is higher than would be expected under neutrality for a pathogenic variant, to date, there is no report correlating any potentially responsible past or present defined endemic condition(s) in the history of the populations reporting deleterious founder alleles (Aboagye *et al*., 2022). To better understand whether these predominant NSHI founder variants are under any kind of selection or coevolved to provide some fitness in early carriers, it may be helpful to further scrutinize the genome wide data of individual's homozygous for these variants across different ethnic groups. In contrast to the distribution of *GJB2* variants on gnomAD, the number of expected LoF SNVs for the other genes were not relatively different from the observed number of LoF SNVs (Table 3.2).

Table 3.2. Founder variant(s) gene constraint metrics extracted from genome aggregation database

Gene	Category	Expected SNVs	Observed SNVs	Constraint Metrics
GJB2	Synonymous	61.1	64	Z = -0.29 o/e = 1.05 (0.86–1.29)
	Missense	137.1	161	Z = -0.72 o/e = 1.17 (1.03–1.34)
	PLoF	6.5	17	pLI = 0 o/e = 2.62 (1.39–1.98)
GJB6	Synonymous	64	69	Z = 0.99, o/e = 1.08 (0.89–1.32)
	Missense	149.6	134	Z = 0.45, o/e = 0.9 (0.78–1.03)
	pLoF	9.3	10	pLI = 0, o/e = 1.07 (0.66–1.74)
GSDME	Synonymous	116.1	128	Z = -0.87, o/e = 1.1 (0.95–1.28)
	Missense	265.4	304	Z = -0.84, o/e = 1.15 (1.04–1.26)
	pLoF	21.6	16	pLI = 0, o/e = 0.74 (0.5–1.13)
CDH23	Synonymous	301.3	290	Z = 0.51, o/e = 0.96 (0.87–1.06)
	Missense	715.2	662	Z = 0.71, o/e = 0.93 (0.87–0.99)
	pLoF	47.1	18	pLI = 0, o/e = 0.38 (0.26–0.57)
CLDN14	Synonymous	77	81	Z = -0.35, o/e = 1.05 (0.88–1.26)
	Missense	154.9	148	Z = 0.2, o/e = 0.96 (0.83–1.09)
	pLoF	6.2	6	pLI = 0, o/e = 0.97 (0.52–1.75)
MYO15A	Synonymous	886.4	826	Z = 1.6, o/e = 0.93 (0.88–0.99)
	Missense	2057.4	1975	Z = 0.65, o/e = 0.96 (0.92–1)
	PLoF	169.1	116	pLI = 0, o/e = 0.69 (0.59–0.8)
PDZD7	Synonymous	138.9	134	Z = 0.32, o/e = 0.96 (0.84–1.11)
	Missense	330	139	Z = -0.37, o/e = 1.06 (0.97–1.16)
	pLoF	22.5	17	pLI = 0, o/e = 0.76 (0.52–1.13)
EYA4	Synonymous	122.5	276	Z = 1.38, o/e = 0.84 (0.72–0.99)
	Missense	333.4	103	Z = 1.12, o/e = 0.83 (0.75–0.92)
	pLoF	7.7	10	pLI = 0.05, o/e = 0.27(0.16–0.45)
PJVK	Synonymous	64.9	57	Z = 0.77, o/e = 0.88 (0.71–1.09)
	Missense	186.1	181	Z = 0.13, o/e = 0.97 (0.86–1.1)
	pLoF	19.1	14	pLI = 0, o/e = 0.73 (0.48–1.15)
TMC1	Synonymous	42.1	146	Z = -, o/e = 1.03 (0.9–1.18)
	Missense	398.2	350	Z = 0.86, o/e = 0.88 (0.81–0.96)
	pLoF	47.6	35	pLI = 0, o/e = 0.74 (0.56–0.97)
KCNQ4	Synonymous	179	156	Z = 1.35, o/e = 0.87 (0.76–0.99)
	Missense	412.2	288	Z = 2.17, o/e = 0.7 (0.63–0.77)
	pLoF	32.1	7	pLI = 0.47, o/e = 0.22(0.12–0.41)
TMPRSS3	Synonymous	101.9	94	Z = 0.62, o/e = 0.92 (0.78–1.09)
	Missense	253.3	212	Z = 0.92, o/e = 0.84 (0.75–0.94)
	pLoF	24.3	17	pLI = 0, o/e = 0.7 (0.48–1.05)
TMIE	Synonymous	31.2	25	Z = 0.87, o/e = 0.8 (0.58–1.12)
	Missense	75.3	74	Z = 0.05, o/e = 0.98 (0.81–1.19)
	pLoF	5.7	6	pLI = 0, o/e = 1.05 (0.57–1.81)
OTOF	Synonymous	508.8	550	Z = -1.44, o/e = 1.08 (1.01–1.16)
	Missense	1223.6	1252	Z = -0.29, o/e = 1.02 (0.98–1.07)
	pLoF	105.2	76	pLI = 0, o/e = 0.72 (0.6–0.88)

pLoF = Probability of loss of function; pLI = Probability of loss of intolerance; o/e = observed/expected; Z = z – score; and SNVs = single nucleotide variants

3.5.0 Discussion

This study systematically reviewed the reported putative P/LP founder variants associated with NSHI worldwide. The review showed a high proportion of recessive founder variants (87.5%; n = 49/56) relative to dominant variants. Consistent with previous reports, Asia and Europe had the most reported NSHI founder variants, with only isolated reports in North America and Africa (Snoeckx *et al*., 2005; Yan *et al*., 2003). This observation may reflect disparities in research priorities and funding distribution and could partly be linked to the epidemiology of HI burden in Asia and the Middle East, as well as the prevalent cultural consanguinity practices in the region (Park *et al*., 2003; Qi *et al*., 2007).

3.5.1 *GJB2* Founder Variants

The finding equally highlights *GJB2* as the most studied and recurrent NSHI gene, implicated with over 57.1% NSHI founder variants, which further demonstrates the gene's variants as the most predominant cause of NSHI worldwide ARNSHI (del Castillo & del Castillo, 2017). This observation intensifies the need to generate a haplotype map on the populations reporting specific NSHI founder variants of multiple origins to clarify the timing and likely evolutionary constraints (modifiers) influencing the variants' segregation across populations.

GJB2 hypermutability (Abe *et al*., 2000; Esoh & Wonkam, 2021; Essamak, 2014; Schrijver, 2004) and the proposed heterozygote selective carrier advantage (Meyer *et al.*, 2002) has been linked to improved fitness for the continuous perpetuation of the phenotype across ethnic groups. Interestingly, the probability of the *GJB2* LoF score, observed / expected (o/e) on gnomAD (https://gnomad.broadinstitute.org/ accessed on 10 November 2022) favors the survival benefit hypothesis proffered on deleterious founder alleles (Meyer *et al.,* 2002). The pLoF score is a continuous measure that describes a gene's tolerance to certain classes of genetic variation. The high *GJB2* (o/e) pLoF score of 2.62 (90% CI: 1.39–1.98) on gnomAD

shows relatively higher observed LoF variants recorded over the years relative to expected LoF variants, suggesting that *GJB2* may be under selection for LoF variants. However, since the pLoF value of 2.63 is outside the 90% credible interval (90% CI: 1.39–1.98), the gene is distinguished as one of the peculiar cases where there are a lot of uncertainties about the constraint metrics due to the number of samples and gene size. On the other hand, a lower o/e score may have suggested a stronger selection against the class of LoF variants, relative to a higher o/e score. However, since the variant count is dependent on the gene size and sample size, relying on the precision of the o/e score may be limited for absolute comparison with other genes. For example, considering the size of *GJB2* (226 aa) against the huge number of variants reported (64 synonymous and 161 non-synonymous variants), further investigations with enough power may be required to elucidate the genetic constraints at the locus of this gene.

Haplotype analysis investigating shared genetic ancestry demonstrated independent multiple origins for some founder variants across ethnic groups. Inheriting the homozygous or compound heterozygous state of the *GJB2*: c.35delG-p.Gly12ValfsTer2 variant accounted for 10-20% of all congenital HI in American Caucasians from North and South Europe (Janecke *et al.*, 2002; Tekin *et al.*, 2001, 2010). Haplotype analysis of the SNPs in linkage disequilibrium with the *GJB2*: c.35delG-p.Gly12ValfsTer2 variant traced the variant's origin to the Middle East, before spreading to Europe and subsequently throughout the region (Laer *et al.*, 2001). Further analysis that computed the haplotypes in homozygote cohorts in Turkey and Iran also suggested a single origin in Anatolia and Iran (Tekin *et al.*, 2003). In China, improved reproductive fitness and ethnic group inter-marriage was cited among the underlying genetic forces for the observed high (11.5%) carrier frequency of the variant (Abe *et al.*, 2000) reported in Chinese cohorts. Though contentious, the variant may have originated in European and North American ancestors or Middle East populations about 100 centuries ago, before spreading to other regions (Laer *et al.*, 2001). Alternatively, perhaps the assertion that it first

arose in Central Asia before it was transferred roughly 11,800 years ago (Barashkov *et al.*, 2011) needs more attention?

Investigating the *GJB2*:c.35delG-p.Gly12ValfsTer2 variant among Moroccan cohorts also presented a shred of strong evidence of three shared markers in linkage disequilibrium with the variant, suggesting a possible evolutionary ancestor in Morocco (Abidi *et al*., 2008). This report favors the idea of multiple independent origins of the variant as opposed to an earlier report that the *GJB2*: c.35delG-p.Gly12ValfsTer2 variant originated from a single Mediterranean ancestor some 10,000 years or nearly 500 generations ago (Laer *et al*., 2001). Ascertaining global haplotypic backgrounds in mutant chromosomes will help identify an integral panel of haplotype diversity and backgrounds sustaining the variant segregation to disambiguate the route of spread of the *GJB2*: c.35delG-p.Gly12ValfsTer2 variant. Generating data on the mutational load across populations reporting the variant using NGS data may illuminate the haplotypic backgrounds carrying the variant and elucidate potential modifiers of phenotype variability. This will, in addition, identify the likely shared genetic ancestry and common backgrounds sustaining the variant distribution across populations.

The *GJB2*: c.235delC-p.Leu79CysfsTer3 variant was the second most common and recurrent *GJB2* reported founder variant. The variant accounted for 65.5% of all *GJB2* mutant alleles and is considered the most prevalent NSHI-associated variant in Asia (Yuan *et al*., 2009). The markers in high-linkage disequilibrium with *GJB2*:c.235delC-p.Leu79CysfsTer3 demonstrated in haplotype screening, traced the variant's origin to a common founder in Altaians, Mongolians, Chinese, and Japanese (Laer *et al*., 2001; Liu *et al*., 2002). In Mongolia, the concurrence of the *GJB2*: c.35delG-p.Gly12ValfsTer2, c.235delC-p.Leu79CysfsTer3, and c.-23+1G>A variants in the study participants (Yan *et al*., 2003) reflected the geographical setting of the population as a crossroad in the migration history. Yan et al. estimated the variant to have evolved in the Baikal area some 11,500 years before it spread to Mongolia, China,

Korea, and Japan by human migration (Yan *et al*., 2003). Although this estimate mirrors the Van Laer et al. report on the c.35delG variant (Laer *et al*., 2001), the Altai region could be the possible origin of the *GJB2*: c.235delC-p.Leu79CysfsTer3 founder variant, before expanding across Asia (Yan *et al*., 2003). The observed discrepancies in the origin and estimated age of the *GJB2*: c.235delC-p.Leu79CysfsTer3 variant in different populations could result from the differences in the age estimation methods, the underlying principles and assumptions, specific ancestral informative markers used for the haplotype analysis, sample size differences, population characteristics, and distinct bottle-neck events in the populations' history and demographic events (Zytsar *et al*., 2020).

The *GJB2*: c.-23+1G>A (IVS1+1G>A) variant accounted for 95% of all deleterious *GJB2* variants examined in the Yakut-affected cohorts (Barashkov *et al*., 2011). A reconstruction of 140 haplotypes with the *GJB2*: c.-23+1G>A (IVS1+1G>A) variant suggested a common ancestor in all the homozygote chromosomes, further supporting the founder source. The estimated age of the variant (~800 years) is consistent with the Turkic-speaking Yakut ancestor's history in Eastern Siberia (Shimkin, 1990). Computing the haplotype in homozygous Turks and Mongolians predicted Central Asia as the origin of the variant before migration spread the carriers of the variant to the Middle East (Tekin *et al*., 2003). Considering the Tuvinians' history of Mongolian influence at different periods in their ethnic formation (Mannai-ool, 2004), the variant may have been introduced into Tuva by ancient Mongolians about 725-4100 years ago, before moving to Siberia. The c.-23+1G>A variant has been reported at varied frequencies (from 0.6% to 58.1%) across populations, from East Asia to Russia and Central Asia, however, there are no reports of the variant in Southeast Asia and sub-Saharan Africa.

The *GJB2*: c.167delT-p.Leu56ArgfsTer26 variant is the most common pathogenic *GJB2* variant in the Ashkenazi Jewish population (Kenna *et al*., 2010; Yan *et al*., 2003). The

hypothesis for a shared origin of this variant within deaf Palestinians and Israelis was supported by the identification of significant markers that were in strong linkage disequilibrium with the *GJB2*: c.167delT-p.Leu56ArgfsTer26 variant in the investigated cohorts (Kenna *et al*., 2010). Morell et al. observed the previously reported highly conserved haplotype markers in the linkage disequilibrium with the variant and proposed a single evolutionary origin among Ashkenazi Jews (Yan *et al*., 2003).

The *GJB2*: c.427C>T-p.Arg143Trp variant remains the most familiar *GJB2* variant associated with NSHI segregation in Ghana, with as high as 37% prevalence (Wonkam *et al.,* 2022). Isolated reports of the variant, though at relatively low frequencies, in East Asia, Europe, and the Middle East traced the origin to Japanese ancestry from about 6,500 years ago (Shinagawa *et al*., 2020; Tsukada *et al*., 2015). This suggests selection at this locus in other populations, together with a predominant founder effect reported in Ghana (Brobby *et al.,* 1998; Hamelmann *et al.,* 2001; Wonkam *et al.,* 2019). The computed haplotype analysis that compared the *GJB2*: c.427C>T-p.Arg143Trp homozygous Ghanaians and SNV data extracted from the 1000 Genomes populations revealed that *GJB2*:c.427C>T-p.Arg143Trp is carried on different haplotype backgrounds in Ghanaian and other populations, particularly in Japan, as well as among populations of European ancestry, providing further support to the multiple independent origins hypothesis (Aboagye *et al.,* 2022). Remarkably, the recent identification of *GJB2*: c.427C>T-p.Arg143Trp in 4.5% (n = 2/44 families) among a Senegalese cohort further strengthens the need to explore more populations for *GJB2* pathogenic variants in West Africa (Dia *et al.,* 2022). This report raises questions on the ancestral and migration histories between Ghana and Senegal. Though the variant has been extensively investigated in Nigerian (Adeyemo *et al.,* 2022), Cameroonian (Wonkam *et al.,* 2020), and Malian (Yalcouyé *et al.,* 2021) HI cohorts, the variant had no contribution to the phenotype in these populations. Considering the distance between Ghana and Senegal relative to the proximity of Nigeria,

Cameroon, and from Mali to Ghana, this report suggests that transatlantic slave transport along the coastal regions of West Africa, drained by forts in Senegal, is likely to have led to the variant's spread to Senegal. However, investigating the haplotypic backgrounds in homozygous Ghanaian and Senegalese cohorts will clarify the hypothesized route of the variant's spread. The observed deficit of reports on P/LP NSHI variants' prevalence in Africa/African Americans relative to other populations in gnomAD, evident in this report, may reflect the limited HI genetic studies and lack of follow-up on clinical cases for genetic testing in West Africa.

3.5.2 Other Genes (Non-*GJB2)* with founder Variants

The haplotype analysis of the SNPs in linkage disequilibrium with the transmembrane inner ear gene (*TMIE*) variant, *TMIE*: c.250C>T-p.Arg84Trp, demonstrated a single origin traced to about 1250 years ago in Turkey. Non-complementary deaf-by-deaf marriage, prevalent in families in Southeastern Anatolia, was linked with this variant segregation in all the affected siblings. The proposed ancestor is thought to have lived after the Arabs came to Southeastern Anatolia and migration possibly introduced the variant into the population (Sırmacı *et al*., 2009). The 3-base polypyrimidine deletion in intron 7 of the gasdermin E gene (*GSDME*), *GSDME*: c.991-15_991-13delTTC, was reported as a founder variant in East Asian families in Korea, China, and Japan (Nishio *et al.*, 2014; Park *et al.*, 2010). Park et al. demonstrated a putative founder effect for the variant after comparing the variant-linked haplotypes between Chinese and Korean families (Park *et al*., 2010). However, this report does not support the European ancestry hypothesis, suggesting a possible hotspot for the mutation due to records of the variant in Toscani, Ashkenazi Jews, and Hungarian families (Booth *et al.*, 2020b). Haplotype investigation for a common ancestor of the transmembrane serine protease 3 gene (*TMPRSS3*) variant, *TMPRSS3*: c.916G>A-p.Ala306Thr, established markers in linkage disequilibrium, suggesting an ancestral founder in China (Gao *et al*., 2017).

The phenotype associated with the *TMPRSS3* founder variant was consistent with a previous report of inter-familial and intra-familial variability, showing a progressive phenotype (Chung *et al*., 2014). An initial report had described the potassium voltage-gated channel subfamily Q member 4 gene (*KCNQ4*) variant, *KCNQ4*: c.211delC, as stemming from a possible hot spot, favorable to mutations rather than the proposed common founder in Japan (Naito *et al*., 2013). Haploinsufficiency and dominant-negative effects were hypothesized as possible mechanisms in pathophysiology and associated phenotype variability. However, the elucidation of this rare mutation in two Lak families, separated by a geographical barrier, led to the founder effect hypothesis (Naito *et al*., 2013).

Carriers of the pejvakin gene (*PJVK*) variant, *PJVK*: c.406C>T-p.Arg136Ter, were reported in two extended families whose ancestors migrated from Sudan to Israel over 150 years ago (Borck *et al*., 2012). The incidence of intellectual disability recorded in the families restricted other families from marrying into these two families (Basel-Vanagaite *et al*., 2007), segregating the founder variant. The high prevalence (56.5%; n = 13/23) of the otoferlin gene (*OTOF*) variant, *OTOF*: c.5816G>A-p.Arg1939Gln, led to the founder source being presumed as having evolved in a single Japanese ancestor (Matsunaga *et al*., 2012). The haplotype analysis found that all the affected chromosomes homozygous for the PDZ domain containing the 7 gene (*PDZD7*) variant, *PDZD7*: c.490C>T-p.Arg164Trp, shared six (6) STRs in linkage disequilibrium. This, together with the observed high carrier frequency, demonstrates a possible founder source relative to the proposed hotspots prone to mutations for the NSHI-associated phenotype segregation (Lee *et al*., 2019). The high level of inbreeding in Oman, coupled with the demographic explosion in the last decade, may have contributed to the myosin XVA gene (*MYO15A*) variant, *MYO15A*: c.1171_1177dupGCCATCT-p.Tyr393CysfsTer41, duplication and subsequent diffusion in the population (Palombo *et al*., 2017). The authors hypothesized East Asia is the likely origin and founder source of the variant *CDH23*: c.C719T-

p.Pro240Leu based on the high frequency of the allele in the Korean pediatric population 85.7% (6/8) (Kim *et al*., 2015). This assertion was confirmed in the haplotype analysis of the *CDH23*: c.C719T-p.Pro240Leu variant that found the variant to be carried on a single haplotype linked to the variant, completely absent in normal-hearing controls (Kim *et al*., 2015). The analysis of the differences between the *CDH23*: c.C719T-p.Pro240Leu variant frequencies of East Asia and other groups showed the variant arose from Central to East Asia about 40,000 years ago (Cavalli-Sforza & Feldman, 2003). The haplotype analysis suggested that the claudin 14 gene (*CLDN14*) variants, *CLDN14*: c.254T>A-p.Val85Asp and c.398T>G-p.Met133ArgfsTer23, as founder variants originated from Pakistan (Bashir *et al.*, 2013). The observed inter- and intra-familial phenotype variability among the affected kindreds with the same *CLDN14* variants were linked to environmental factors and genetic modifiers (Bashir *et al*., 2013). The recent Korean study that analyzed *CDH23*: c.857delC-p.Asn2858GluTer8 and linked microsatellite markers reported a common founder for the variant and suggested evidence of allele decay in the haplotype analysis (Kim *et al*., 2015). The authors argued that, if the suggested founder variant had evolved some time ago, genetic recombination may likely explain the observed shortening in a proportion of the analyzed haplotypes.

3.5.3 General Observations on Founder Variants

Generally, the observed population- and ethnic-specificity of most of the reported NSHI-associated gene variants suggest a possible underlying heterozygous carrier advantage that may be protective against indigenous infection, confer disease protection, and/or provide nutritional benefits (Meyer *et al.*, 2002). Irrespective of the impact of a variant on fitness, most disease-associated founder variants occur at relatively high frequencies, culminating from the effects of possible random genetic drift likely after a historical bottleneck event that is subsequently fueled by consanguineous unions leading to the observed incidence of monogenic recessive disorders in some populations (Searle *et al*., 2012). This phenomenon could be extended to

observations in Asian and Middle Eastern populations with high consanguinity rates. Dynamics such as the size of the founding population, level of isolation due to geographical location, ethnicity, and existing cultural barriers may have contributed to the epidemiology of NSHI-associated founder variant segregation across populations (Nance *et al*., 2000). Moreover, admixture and sporadic mutations may have caused further segregation of some recessive alleles, specifically for *GJB2* founder variants (Arcos-Burgos & Muenke, 2002). However, data that explore the precise mechanism (proposed combination of improved genetic fitness, assortative mating, and unknown genetic modifiers) for the selection or selective sweep of reported HI deleterious alleles among families, and the seemingly heterozygote carrier advantage, remains to be generated (Chong *et al*., 2012; Evans, 2015).

3.6 Conclusion

This report further emphasizes pathogenic variants in *GJB2* as the most prevalent cause of NSHI, widely reported at characteristic high frequencies in Asia, Europe, and North Africa, with sub-Saharan Ghana as the African exception. The study highlights the dearth of HI genetic investigation in Africa, particularly on haplotype conservation in families with suspected founder variants. Considering the observed discrepancies in the origin and estimated age of some reported NSHI-associated founder variants, future studies should focus on the computation of common ancestry haplotypes in the homozygous mutant chromosomes of the predominant founder variants, using Next Generation Sequencing data. We encourage efforts to explore putative founder variants to ascertain allele frequencies and regional distributions and, thus, to facilitate policy recommendations for genetic counselling and genetic testing.

Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes14020399/s1, Table S3.1: Founder variants

reviewed characteristics and population distributions; Table S3.2: Risk of study bias tool assessment results; Table S3.3: Retrieved parameters for systematic analysis.

Author Contributions: Conceptualization and study design, A.W., G.A.A. and L.A.-E.; methodology, E.T.A., S.M.A. and E.W.-T.; validation, E.T.A., S.M.A. and E.W.-T.; formal analysis, E.T.A., S.M.A. and E.W.-T.; data curation, E.T.A. and S.M.A.; writing—original draft preparation, E.T.A., S.M.A. and E.W.-T.; writing—review and editing, E.T.A., S.M.A., E.W.-T., L.A.-E., G.A.A. and A.W.; supervision, A.W., G.A.A. and L.A.-E.; funding acquisition, A.W. and G.A.A. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: Authors declare no conflict of interest.

Funding: This work was supported with funds received from the U.K. government; the National Institutes of Health (NIH), USA, grant number U01-HG-009716 to A.W.; and the African Academy of Science/Wellcome Trust, grant number H3A/18/001 to A.W. Elvis Twumasi Aboagye was also supported by a WACCBIP-World Bank ACE Masters/PhD fellowship (WACCBIP+NCDs: G.A.A). The funders had no influence in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CHAPTER FOUR

4.0 Paper 2: Age Estimate of *GJB2*-p.(Arg143Trp) Founder Variant in Hearing Impairment in Ghana, Suggests Multiple Independent Origins across Populations

Elvis Twumasi Aboagye [1,2], Samuel Mawuli Adadey [1,2], Kevin Esoh [2], Mario Jonas [2], Carmen de Kock [2], Lucas Amenga-Etego [1], Gordon A. Awandare [1] and Ambroise Wonkam [2,3,]

[1] West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, Legon, Accra, Box LG 54, Ghana; [2] Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, Cape Town 7925, South Africa; [3] McKusick-Nathans Institute and Department of Genetic Medicine, John Hopkins University School of Medicine, Baltimore, MD 21205, USA.

4.1 Simple Summary

The incidence of the *GJB2*-p.(Arg143Trp) founder mutation in numerous populations of different ethnolinguistic and geographical backgrounds has generated interest on the provenance and age of the variant, which is predominantly associated with non-syndromic hearing impairment in Ghana. To interrogate the multiple possible independent origins of the *GJB2*-p.(Arg143Trp) variant, we estimated the age of the variant in Ghana by Bayesian inference, using linked single nucleotide variants in whole-exome sequencing data from hearing-impaired unrelated individuals who are homozygous for the *GJB2*-p.(Arg143Trp) variant and nonaffected controls from the same population. We estimated the age of the variant as approximately 9,625 years, from a common indigenous Ghanaian ancestor. Haplotype backgrounds in Ghanaian hearing-impaired individuals were apparently different from those of their Japanese counterparts. We observed low haplotype diversity in the *GJB2*-p.(Arg143Trp) genomic region for homozygous individuals compared to the normal hearing Ghanaian controls, although the recombination rate is relatively high in the region. The *GJB2*-

p.(Arg143Trp)-positive individuals shared no common haplotype with the Ghanaian *GJB2*-p.(Arg143Trp)-negative controls, and as well as no common haplotype with data extracted from populations in 1000 Genomes, further supporting the multiple independent origins of *GJB2*-p.(Arg143Trp) in the global population.

4.2 Abstract

Gap junction protein beta 2 (*GJB2*) (connexin 26) variants are commonly implicated in non-syndromic hearing impairment (NSHI). In Ghana, the *GJB2* variant p.(Arg143Trp) is the largest contributor to NSHI and has a reported prevalence of 25.9% in affected multiplex families. To date, Ghana has the highest *GJB2*-p.(Arg143Trp) frequency reported worldwide. Using whole-exome sequencing data from 32 individuals from 16 families segregating NSHI and 38 unrelated hearing controls with the same ethnolinguistic background, we investigated the date and origin of p.(Arg143Trp) in Ghana using linked markers. With a Bayesian linkage disequilibrium gene mapping method, we estimated *GJB2*-p.(Arg143Trp) to have originated about 9625 years (385 generations) ago in Ghana. A haplotype analysis comparing data extracted from Ghanaians and those from the 1000 Genomes project revealed that *GJB2*-p.(Arg143Trp) is carried on different haplotype backgrounds in Ghanaian and Japanese populations, as well as among populations of European ancestry, lending further support to the multiple independent origins of the variant. In addition, we found substantial haplotype conservation in the genetic background of Ghanaian individuals with biallelic *GJB2*-p.(Arg143Trp) compared to the *GJB2*-p.(Arg143Trp)-negative group with normal hearing from Ghana, suggesting a strong evolutionary constraint in this genomic region in Ghanaian populations that are homozygous for *GJB2*-p.(Arg143Trp). The present study evaluates the age of *GJB2*-p.(Arg143Trp) at 9625 years and supports the multiple independent origins of this variant in the global population.

Keywords: connexin 26 (Cx26); non-syndromic hearing impairment; *GJB2*: c.427C > T-p.(Arg143Trp) founder variant; variant origin; Ghana

4.3 Introduction

Hearing impairment (HI) is the commonest sensory human defect, with an estimated 2.5 billion people predicted to develop some degree of hearing difficulty worldwide by 2050 (Stephenson, 2021). Genetic factors have been implicated in 50 - 60% of congenital HI, of which 80% are non-syndromic (Morton & Nance, 2006; Smith *et al*., 2005). Genetic heterogeneity in HI has been well-demonstrated, with about 124 genes identified to date (Camp & Smith, 2006, 2019). *GJB2* mapped to the DFNB1 locus is the most common gene associated with HI globally (Kenneson *et al*., 2002).

HI associated *GJB2* alleles, are highly population-specific, with *GJB2*-c.35delG-p.(Gly12ValfsTer2) common in populations from Europe and the Middle East (Bliznetz *et al*., 2017b); c.235delC-p.(Leu79CysfsTer3) in East Asia (Yao *et al*., 2012); c.845G>A-p.(Val37Ile) prevalent in South-East Asia (Kim *et al*., 2013); and c.71G>A-p.(Trp24Ter), c.167delT-p.(Leu56ArgfsTer26), and c.427C>T-p.(Arg143Trp) common in India (Bouwer *et al*., 2007), Ashkenazim Jews (Sobe *et al*., 2000), and Ghana (Brobby *et al*., 1998; Hamelmann *et al*., 2001), respectively. Although the molecular basis underlying the increased carrier frequencies of these DFNB1 alleles (0.7 - 2.4%) across populations remains to be elucidated (Adadey *et al*., 2019; Tsukada *et al*., 2015), a possible heterozygote advantage has been proposed. For example, the increased survival of immortalized NEB-1 keratinocyte and NIH 3T3 fibroblast cells for *GJB2*-c.35delG carriers has been reported (Common *et al*., 2004). Moreover, *GJB2*-p.(Arg143Trp) has been suggested to be associated with a thicker skin epidermis and increased sodium and chloride ion concentrations in sweat of *GJB2*-p.(Arg143Trp) homozygous individuals in Ghana, with possibly related increased protection

against insect bites, and provide an unfavourable osmotic milieu for pathogen invasion resistance to malaria (Common *et al*., 2004).

The first observation alluding to multiple independent origins of *GJB2*-p.(Arg143Trp) came from a phylogenetic analysis of Y-chromosomes of populations reporting the variant where no plausible shared ancestry could be established among the populations (Tsukada *et al*., 2015). In 2020, a haplotype analysis of *GJB2* mutations frequently reported in Japanese populations established that the *GJB2*-p.(Arg143Trp) variant evolved as the result of a founder effect. The study suggested that the variant may have occurred as multiple events, and its age was estimated in the Japanese population at 6500 years ago (Shinagawa *et al*., 2020). In Ghana, *GJB2*-p.(Arg143Trp) is the commonest NSHI-associated variant, with a reported prevalence of 25.9% in affected families and a carrier frequency of 1.4% in the general population (Adadey et al., 2019). Reports of a low-to-moderate prevalence of the *GJB2*-p.(Arg143Trp) variant in other populations (Tsukada *et al*., 2015) has raised questions as to whether it originated through multiple events in different populations or whether it originated from a single African (Ghanaian) ancestor and subsequently spread to other populations through migration and/or gene flow. If *GJB2*-p.(Arg143Trp) evolved via multiple independent events, then its higher frequency in Ghana might reflect a much longer presence there. The social, educational, and economic circumstances of the deaf have greatly improved over the past 300 - 400 years, and this is thought to have contributed towards improved fitness among the deaf population (Nance, 2003). Such improved fitness would increase the frequencies of HI gene variants. Nevertheless, such a high frequency could have also been driven by multiple other factors, including natural selection, population expansions, and genetic drift.

In this study, we took advantage of the available whole-exome sequencing (WES) data for Ghanaian families with hearing-impaired relatives to investigate the age of *GJB2*-p.(Arg143Trp) using a linkage disequilibrium-based disease mapping algorithm. We also

performed haplotype analyses in Ghanaian populations, as well as in other continental populations extracted from the 1000 Genomes project database. We show that the age of p.(Arg143Trp) in Ghana predates earlier reports, for example in Japan.

4.4.0 Materials and Methods

4.4.1 The Study Participants

The study was conducted following the Helsinki Declaration for the participants' safety, and the protocol was approved by the Ethics Committee for Basic and Applied Science (ECBAS), University of Ghana (ECBAS 053/19-20).

Genomic DNA (gDNA) was isolated and purified from the peripheral blood samples of 32 NSHI affected individuals homozygous for the *GJB2*-p.(Arg143Trp) variant from 16 Ghanaian families, and 38 unrelated hearing controls negative for the variant and with no family history of deafness were selected from a large study investigating the genetic etiology of congenital hearing impairment in Ghana (Adadey *et al*., 2019). Participants responded to a well-designed structured questionnaire on family history, clinical records, self-reported ethnicity, and photographs of affected individuals taken for clinical confirmation to exclude suspected acquired and syndromic HI. Pedigrees were documented for at least three generations in recruited families. A hearing-impaired individual was defined as someone who is not able to hear well at the normal hearing threshold of 20 dB or better in one or both ears (Camp & Smith, 2019) and with no identified associated clinical condition. Audiological and clinical examinations were performed on all affected kindreds, and they were diagnosed as having profound NSHI (with an average tone over 91dB) using the KUDUwave Audiometer eMoyo, Radioear (Northcliff, Johannesburg, South Africa). Both verbal and signed consent were obtained from all participants or their parents/guardians before recruitment into the study, with guaranteed anonymity for strict scientific use of the samples and data generated.

4.4.2 Whole-Exome Sequencing (WES)

Whole-exome sequencing was performed at Omega Bioservices (Norcross, GA, USA) using a pair-end 150-bp run format on the Illumina HiSeq 2500 platform following the manufacturer's instructions. The Nextera Rapid Capture Exome kit® (Illumina, San Diego, CA, USA) was used to prepare the libraries, and the resulting libraries were hybridized with a 37-Mb probe pool to enrich the exome sequences, as previously described (Usera *et al*., 2020). The generated library sizes ranging from 300 bp to 421 bp were quantified by quantitative PCR (qPCR) before sequencing on an Illumina HiSeq 2500 platform to generate pair-end reads with 100x depth of coverage.

4.4.3 Whole-Exome Sequence Variant Calling

Sequenced data were processed using Omega Bioservices (Norcross, GA, USA) pipelines on the Illumina DRAGEN Germline Pipeline v3.2.8. Briefly, high-quality reads were aligned to the human reference genome GRCh37/hg19 using DRAGEN software version 05.021.408.3.4.12. After sorting and duplicate marking, the base quality scores were recalibrated using the genome analysis toolkit (GATK v4.0.6.0) (Buchan & Jones, 2019). Variants were called using GATKHaplotypeCaller, and individual genomic variant call format (gvcf) files were generated. Joint variant calling was then performed using GATK GenotypeGVCF. The sex of each individual was verified using Plink version 1.9 (Chang *et al*., 2015). The familial relationships for all members were verified using the Kinship-based INference for Gwas (KING) algorithm (Manichaikul *et al*., 2010).

4.4.4 Variant Filtration and Data Quality Control

The GATK variant quality score recalibration (VQSR) was performed on the genotype data, consisting of 291,189 variants. All biallelic single-nucleotide variants (SNVs) that "PASSed" all filters were then extracted into a new VCF file, extracting only those variants with a depth of coverage (DP) > 8 and genotype quality (GQ) > 20. The quality of the data was further

ascertained by computing various quality metrics (i.e., average heterozygosity, singleton by sample, average depth, and the transition/transversion (Ts/Tv) ratio) using bcftools stats and plotted using plot-vcfstats (Danecek *et al*., 2021).

4.4.5 Haplotype Estimation (Phasing) and Genotype Imputation

Haplotype estimation (phasing) was performed using BEAGLE v5.1 (Browning & Browning, 2007). Phasing was performed first without imputation to produce an "unimputed" dataset and then with imputation to produce an "imputed" dataset to mimic the genomic data for age estimation. The phased unimputed data was then merged with 2504 samples from the 1000 Genomes project, phase 3, version 5 (1KGP3v5) dataset to produce a merged superset of 2574 samples using the bcftools --*isec* command, keeping only the variants that were present in both datasets. Further quality control was performed to exclude poor-quality variants by removing variants with minor allele frequency (MAF) < 1%, variants with genotype quality (GQ) < 90%, and variants that failed the Hardy-Weinberg equilibrium (HWE) test at a *p*-value threshold of 1×10^{-6}. Additionally, for the imputed data dataset, all variants with an imputation accuracy below 90% were excluded.

4.4.6 Sample Selection for Age Estimation

A set of 32 samples that were homozygous for the *GJB2*-p.(Arg143Trp) variant was separated into two sets of 16 unrelated individuals (hereafter referred to as R143W-1 and R143W-2, respectively). The KING software was then used to confirm that each set consisted only of unrelated samples. Briefly, identity-by-descent (IBD) reports were computed for each set separately using the --*ibdseq* command. In both datasets, two individuals from two separate families were inferred to be 4th-degree relatives, and one individual of the related pair was therefore excluded, leaving 15 unrelated samples for each set. Thirty-eight (38) unrelated samples that were negative for p.(Arg143Trp) (hereafter referred to as GHA-38) were then selected and merged with R143W-1 and R143W-2 to produce two separate datasets of 53

samples each (hereafter R143W-1N and R143W-2N, respectively). KING software was again used to ascertain that each set consisted only of unrelated samples (Manichaikul *et al*., 2010).

4.4.7 Markers Selection

The Plink1.9 $–r^2$ command was used to compute the linkage disequilibrium (LD) within a 2-Mb region on chromosome 13q12 surrounding the p.R143W variant (1 Mb on each side of the variant) using an LD threshold of 0.1. Four (4) SNVs (rs115802719, rs732021, rs17075877, and rs9578260) in LD with c.427C>T-p.(Arg143Trp) were selected for the age estimation using the phased unimputed dataset. The allele frequencies of the variants were computed for the R143W-1 and R143W-2 and GHA-38 datasets separately. The allele frequencies of the variants for continental populations were extracted from the dbSNP database. In addition, a recombination plot of the 2-Mb region around p.(Arg143Trp) was generated from the HapMap recombination map ("A Haplotype Map of the Human Genome," 2005).

4.4.8 *GJB2*: c.427C>T-p.(Arg143Trp) Variant Age Estimation

The age of *GJB2*: c.427C>T-p.(Arg143Trp) was estimated in the phased unimputed as well as the phased imputed datasets using a nextflow-based (Tommaso *et al*., 2017) workflow (https://github.com/esohkevin/mutationAge/tree/main) (Accessed November 25, 2021) that utilized the Disequilibrium Mapping Likelihood Estimation (DMLE v+ 2.3) algorithm (Reeve & Rannala, 2002). DMLE v+ 2.3 allows a Bayesian inference of the allele age and location based on the LD detected in selected markers, given some population genetic and demographic parameters, including a genetic map (in centimorgans distance), target population average growth rate, and frequency of the mutation in the general population. The program applies the Markov Chain Monte Carlo (MCMC) algorithm to perform massive random simulations to estimate the posterior probability density of the occurrence of each event: mutation location, ancestral state, and allele frequency. In addition, it has a feature that allows multiple chains to be run simultaneously, thereby providing a means of assessing the robustness of the estimation

and pipeline parameters by checking the convergence at the true mutation location if the location is set as unknown and the software is instructed to iterate over it. We therefore set the software to iterate over the mutation location in our data to interrogate the data. Two chains were run for R143W-1N, R143W-2N, and R143W-1N-imputed each, setting 2,000,000 burnin and 1,000,000 main iterations. The allele frequency of the mutation in the general population was estimated at 0.00024 using the world population data (wordometer) (*Ghana Demographics 2020 (Population, Age, Sex, Trends) - Worldometer*, Accessed on 15 June 2021.). The genetic map was computed (using a custom R script) by subtracting the distance of the first (5′-most) marker position from each subsequent position (hence, the first marker position was zero). The distances were then converted to centimorgans (cM) using the approximation 1 cM = 1,000,000 bp. The average population growth rate of 2.15% in Ghana as of 2020 according to The World Bank data was used (*Ghana Population Growth Rate, 1960-2020 - Knoema.Com*, Accessed on 17 August 2021; *Population Growth (Annual %) - Ghana | Data*, Accessed on 17 August 2021).

4.4.9 Principal Component and Ancestry Analyses

The merged set of R143W-1N and 1KGP3v5 data was LD-pruned, such that all variant pairs with LD ≥ 0.2 within any 50-bp region using a window size of 10 SNVs were excluded. Five axes of genetic variations (principal components−PCs) were then computed using the PLINK2 *--pca* command (Chang *et al.*, 2015), and a custom R script was used for visualization. For the global ancestry analysis, 50 samples from each of the 1KGP3v5 populations were randomly selected and merged with R143W-1N and LD-pruned as described above. The ancestral proportions were then computed using Admixture v1.3 (Zhou *et al*., 2011). Admixture utilizes a maximum likelihood approach to estimate the underlying ancestral coefficients and then a moving block bootstrap approach for estimating the standard errors. The analysis was done

with 11 cross-validation runs (K = 1–11) and 300 bootstrap runs. The AncestryPainter perl program was used to visualize the ancestral proportions (Feng *et al.*, 2018).

4.4.10 Haplotype Analysis

Haplotype frequencies were computed for GHA-38, R143W-1, and R143W-2 separately, based on the five markers that were selected for age estimation including p.(Arg143Trp)) using the phased unimputed dataset. Haplotype frequencies were also computed for the R143W-1N + R143W-2N + GHA-38 + 1KGP3v5 merged superset based on the four markers that were in LD with p.R143W, since p.(Arg143Trp) was absent in the 1000 Genomes reference panel.

4.5.0 Results

4.5.1 Characteristics of Study Participants

From the clinical history and examinations, no environmental factors were implicated in the cause of the NSHI in the affected cohorts selected, and no evidence of neurologic (vertigo, dizziness, etc.) or ophthalmologic (distorted vision, photophobia, eye pain, etc.) symptoms were identified. The physical examinations also showed no vestibular or any systemic abnormalities in the affected individuals. At least a history of inter- and intragenerational NSHI phenotype variability was described in many of the unrelated affected families, with most affected kindreds having their first formal audiological assessment performed when it was a requirement for enrollment during school admission. Pedigrees and audiological assessments of representative familial cases showed bilateral profound sensorineural NSHI.

4.5.2 Features of Markers Selected for Age Estimation and Haplotype Analysis

Table 4.1 and Supplementary Table S4.1 show the general features and quality metrics of the four (4) SNVs selected in the WES data in addition to p.(Arg143Trp) for age estimation and haplotype analysis based on LD within the 2-Mb region of c.427C>T-p.(Arg143Trp) respectively. The four markers in the unimputed set occurred in the zinc finger MYM-type

containing 2 gene (*ZMYM2*) and *GJB2* genes (Figure 4.1), spanning a 128.4-Kb region. Eight SNVs that were in LD with p.(Arg143Trp) were selected for the age estimation in the imputed data (Table S4.2). Notably, all the eight markers occurred in *GJB2* (spanning a 193-bp region), the majority of which were intronic variants (Figure 4.1). Apart from p.(Arg143Trp) (rs80338948), only one other marker (rs9578260) was present in both the unimputed and the imputed sets, and these two markers were present in the WES data, while the rest were imputed. In general, LD among the selected markers was low and short-ranged; this was lower for the unimputed data (range: 0.10 - 0.34) as compared to the imputed data (range: 0.36 - 0.38). The low LD around p.(Arg143Trp) was consistent with the relatively high recombination rate around the variant (especially upstream), as revealed by the recombination plot (Figure S4.1).

Table 4.1. Features of linkage disequilibrium-selected variants for the age estimate and haplotype

Marker	Chr:Pos	Ref/Alt	Gene	LD (r^2)	Genetic Map (cM)	Alternate Allele Frequency (AAF)							
						R143W-1N	R143W-2N	GHA-38	AFR	EUR	AMR	Asian	EAS
rs115802719	13:20635310	A/G	*ZMYM2*	0.102	0.00000	0.1875	0.1875	0.0375	0.0253	0.000088	0.0256	0.0000	0.0000
rs7324021	13:20637140	T/G	*ZMYM2*	0.220	0.00183	0.3438	0.3438	0.1125	0.0705	0.05039	0.0700	0.0100	0.0110
rs1705877	13:20641422	A/G	*ZMYM2*	0.105	0.00611	0.2500	0.2500	0.0625	0.1595	0.12264	0.1600	0.1430	0.1600
*rs80338948	13:20763294	G/A	*GJB2*	1.000	0.12798	1.0000*	1.0000 *	0.0000 *	0.0002	0.000024	0.0002	0.0002	0.0002
rs9578260	13:20763754	G/A	*GJB2*	0.341	0.12844	0.9062	0.9375	0.3250	0.2167	0.00082	0.2165	0.0000	0.0000

*AFR = Africa; EUR = Europe; AMR = African Americans; Asian = Asia; EAS = East Asia; RSID = reference SNP ID; Chr:Pos = Chromosome and base pair position; Ref/Alt = Reference and alternate alleles; LD = linkage disequilibrium; cM = Centimorgan; AAF = alternate allele frequency; * = disease allele.*

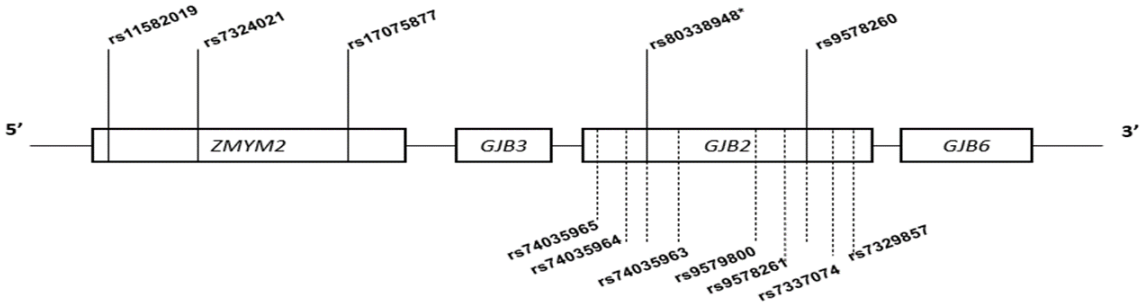

Figure 4.1. Schematic illustration of the five ancestral informative markers (AIMs) on the chromosome 13q12 locus (2-Mb region around the disease mutation) used for the age estimation and haplotype analysis in Ghanaian populations. *Tagged SNVs in linkage disequilibrium were identified within a 128.445-kb region spanning the GJB2: c.427C>T (Arg.R143Trp) locus. One marker in GJB2: c.-22-12C>G and three markers in the zinc finger MYM-type containing 2 gene (ZMYM2): c.3345A>G, c.3037+27T>G, and c.2857A>G, respectively. Unimputed markers (5) are shown above, and imputed markers (7) are shown below, represented by* dotted lines. * = disease allele.

4.5.3 Origin of *GJB2*-p.R143W in Ghana

The age of *GJB2*-p.(Arg143Trp) was estimated using two different marker sets: a five-marker set in the unimputed data and a nine-marker set in the imputed data. There was virtually no difference in the estimates of the R143W-1N and R143W-2N datasets, and therefore, only the results of R143W-1N are herein presented. Using the five-marker unimputed set, the two analysis chains converged at the correct mutation location (0.12798) less than 500,000 iterations into the burnin iterations and remained stable throughout (Figure S4.2a), simultaneously estimating the correct mutation location (Figure 4.2A) while also estimating the mutation age (Figure S4.2b). Figure 4.2b shows the posterior density of the mutation age with a mode of roughly 385 generations (9625 years) and a 95% credible set of ages ranging from 335 to 500 generations (8375–12,500 years), giving a generation time of 25 years. Using the nine-marker imputed set, the two chains converged at the correct location (0.000365; Figure S2b) approximately 1,800,000 iterations into the burnin iterations, while the age of the mutation was estimated at roughly 380 generations (~9500 years) ago, with a credible set of

ages between 315 and 500 generations (7875–12,500 years) (Figure S4.2c). Apparently, the age and location estimates were more stable for the unimputed dataset as compared to the imputed set, likely reflecting the effect of imputation error on the age estimation algorithm. However, the algorithm still equilibrated before the main (good) iterations started, implying that the tool retains the power to estimate the mutation age given that higher values of the burnin iterations are set to allow for enough equilibration time.

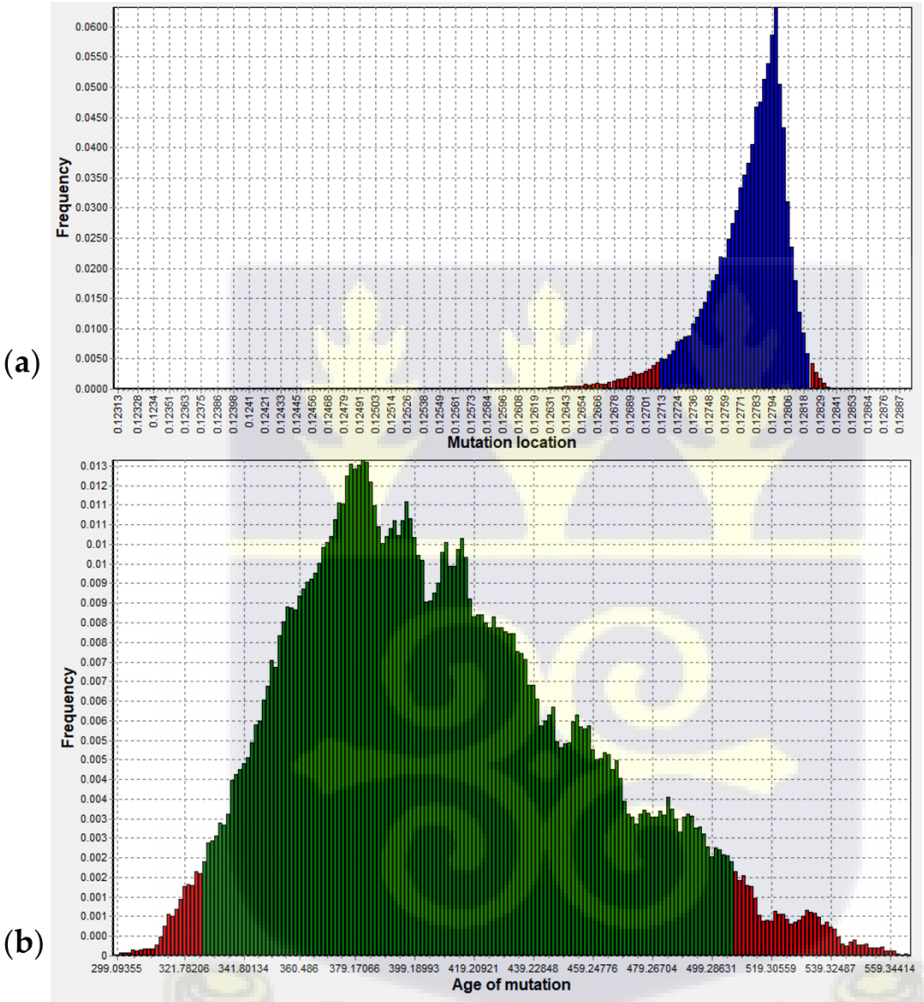

Figure 4.2. DMLE +2.3 estimated age of *GJB2*-p.(Arg143Trp) (c.427C>T) in generations (giving a generational time of 25 years) showing a 95% confidence interval (CI) after two chains: 2,000,000 burnin and 1,000,000 main iterations. *(a) p.(Arg143Trp) mutation location and a 95% CI. (b) p.(Arg143Trp) (c.427C>T) mutation age and a 95% CI for the posterior probability density (the region in green).*

4.5.4 Haplotype and Ancestry Analyses Confirm Independent Origin of p.(Arg143Trp) in Ghana

To further understand how p.(Arg143Trp) may have evolved in Ghana, thirteen (13) unique haplotypes were generated for R143W-1, R143W-2, and GHA-38 separately based on the five-marker set, and nine unique haplotypes were generated for Ghanaian populations and the 1KHP3v5 merged superset, and the haplotype frequencies were compared. Figure 4.3A shows the haplotype distribution among the different Ghanaian sample sets. The R143W-1 and R143W-2 populations exhibited a much lower haplotype diversity in the p.(Arg143Trp) genomic region compared to the GHA-38 (p.(Arg143Trp)-negative) population, possibly reflecting some evolutionary constraints in the region in the genetic background of homozygous p.(Arg143Trp). In addition, the p.(Arg143Trp)-positive samples were clearly distinguished from the GHA-38 samples, as there were no shared haplotypes among the two sample sets (Table S4.3). Interestingly, four (4) of the six (6) haplotypes that carried p.(Arg143Trp) were also homozygous for the rs9578260 intron variant, which has been reported in hearing-impaired cases and classified as benign on ClinVar (https://www.ncbi.nlm.nih.gov/snp/rs9578260#clinicalsignificance) (Accessed December 15, 2021). That is, over 90% of all Ghanaian p.R143W homozygous cases are also homozygous for the *GJB2* intron variant rs9578260. Although rs9578260 is also highly prevalent in p.(Arg143Trp)-negative African populations (MAF = 0.22), it is absent in Asia and extremely rare in Europe. This clearly indicates that p.(Arg143Trp) is carried on different haplotype backgrounds in Ghanaian and Japanese populations, as well as European populations. Moreover, a majority of the markers that were selected for allele age estimation based on LD with p.(Arg143Trp) are either absent or rare in Asia and Europe (Table 4.1), further supporting independent evolutions of p.(Arg143Trp) in the different ancestries. A striking observation was that the haplotypes generated from the four selected markers were strongly ancestry

informative, given that they resolved all the populations in the Ghanaian and 1000 Genomes set at the continental and subcontinental levels (Figure 4.3b). They revealed a higher haplotype diversity in African populations (as expected), as well as an admixture in American populations (PUR, CLM, MXL, and PEL). These observations were consistent with the PCA (Figure 3c) and ancestry analysis (Figure 4.3d). The ancestry analysis at k = 5 resolved all five continental populations in the 1000 Genomes project (AFR, AMR, EUR, SAS, and EAS) (Figure S4.3a), while the Ghanaian populations clustered with African populations near the Mende population from Sierra Leone (MSL) and the Yoruba from Nigeria (YRI). Increasing the k-parameter (number of subpopulations) from k = 8 up to k = 11 in order to resolve the populations further revealed no shared genetic diversity among the Ghanaian and Japanese populations (Figure S4.3b–d), meaning that it is highly unlikely to find shared haplotypes among the populations, further lending support to the notion of an independent origin of p.(Arg143Trp) in Ghana.

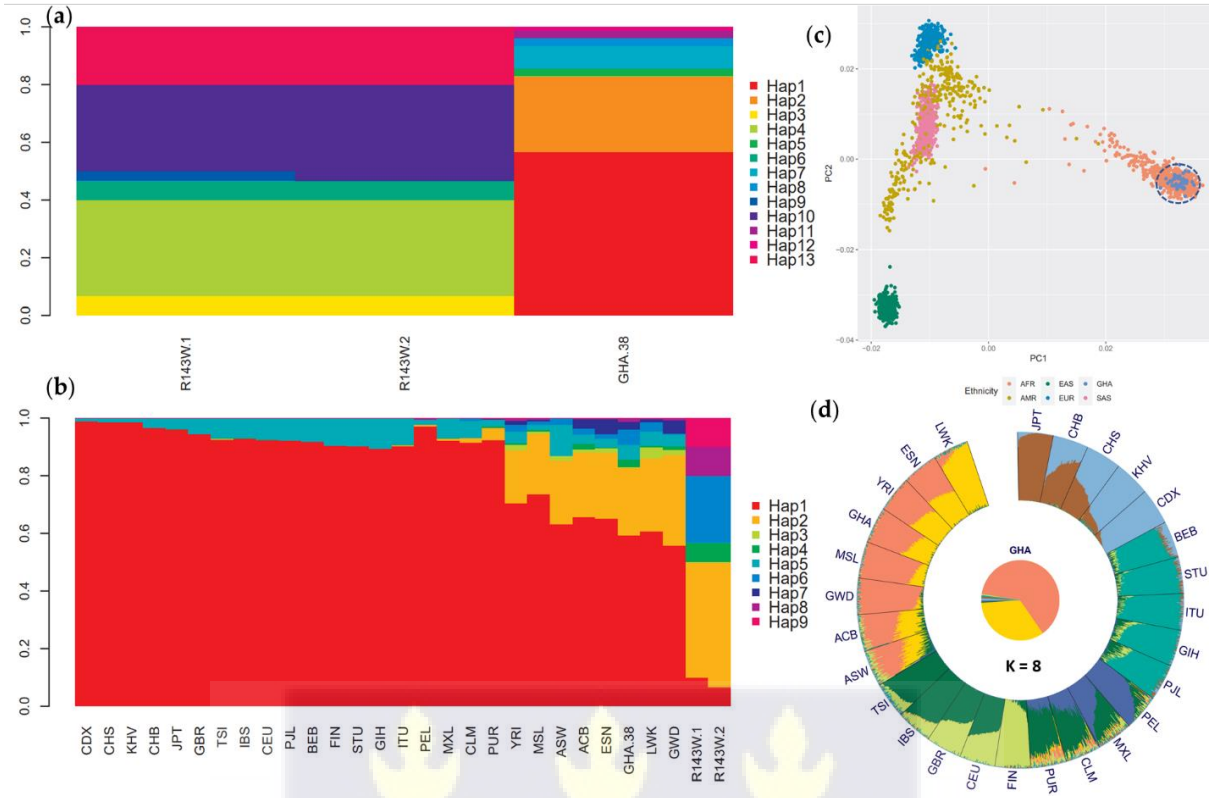

Figure 4.3. Haplotype frequencies in the R143W-1, R143W-2, GHA-38, and 1KGP3v5 populations and a global ancestry analysis. *(a) Haplotype frequencies in Ghanaian samples R143W-1, R143W-2, and GHA-38 based on the 5-marker haplotypes. (b) Haplotype frequencies in Ghanaian (R143W-1, R143W-2, and GHA-38) and 1KGP3v5 populations based on the 9-marker haplotypes. (c) Principal Component Analysis showing the clustering of the continental populations. Ghanaian (GHA) population clustered with the African population as expected. EAS = East Asian Ancestry, AMR = American Ancestry, EUR = European Ancestry, and SAS = South Asian. (d) Bar plot of an unsupervised ADMIXTURE analysis of Ghanaian and the 1000 Genomes populations assuming 8 subpopulations (K). Luhya in Webuye, Kenya (LWK), Esan in Nigeria (ESN), Yoruba in Ibadan, Nigeria (YRI), R143W1-N Ghanaians (GHA), Mende in Sierra Leone (MSL), Gambian in Western Division—Mandinka (GWD), African Caribbean in Barbados (ACB), African Ancestry in SW USA (ASW), Toscani in Italia (TSI), Iberian Populations in Spain (IBS), Utah residents (CEPH) with Northern and Western European ancestry (CEU), British from England and Scotland (GBR), Finnish in Finland (FIN), Puerto Rican in Puerto Rico (PUR), Colombian in Medellín, Colombia (CLM), Mexican ancestry in Los Angeles CA USA (MXL), Peruvian in Lima Peru (PEL), Punjabi in Lahore, Pakistan (PJL), Gujarati Indians in Houston, Texas, USA (GIH), Indian Telugu in the UK (ITU), Sri Lankan Tamil in the UK (STU), Bengali in Bangladesh (BEB), Kinh in Ho Chi Minh City, Vietnam (KHV), Chinese Dai in Xishuangbanna, China (CDX), Han Chinese South (CHS), Han Chinese in Beijing, China (CHB), and Japanese in Tokyo, Japan (JPT).*

4.6 Discussion

The present report shows that the *GJB2*-p.(Arg143Trp) founder variant has been present for approximately ~9625 years in Ghana and most likely evolved independently from its occurrence in Japan (Shinagawa *et al.*, 2020; Tsukada *et al.*, 2015). This suggests that Ghanaian families with the p.R143W variant are likely descendants of an ancestor who lived some ~385 generations ago. The finding elucidates the origin of the founder variant segregating in families with NSHI in Ghana (Dahl *et al.*, 2013; Danilenko *et al.*, 2012; Rabionet *et al.*, 2000).

The much lower haplotype diversity observed in the p.(Arg143Trp) genomic region in p.(Arg143Trp) homozygotes compared to hearing controls (p.(Arg143Trp)-negative) might reflect an evolutionary constraint in the genomic region due to one or more factors, including natural selection and or a higher inbreeding than would be expected under neutrality due to assortative mating among the deaf population. The chromosomal region around *GJB2* is known to contain high recombination rates (Rothrock *et al.*, 2003) and harbor recombinational hotspots, which may explain the high mutability of the *GJB2* gene evident in the reported population-specific *GJB2* variants worldwide (Tsukada *et al.*, 2015). This would further support the notion of multiple independent mutational events of p.(Arg143Trp) and other *GJB2* gene variants in different populations (Adadey *et al.*, 2020; Oliveira *et al.*, 2007).

The factors that may have contributed to maintaining the *GJB2*-p.(Arg143Trp) deleterious allele at an appreciable frequency in Ghana include an unusually higher rate of positive assortative mating in deaf communities and a probable high rate of consanguinity among the early carriers of the variant (Brobby *et al.*, 1998; Hamelmann *et al.*, 2001; Nance *et al.*, 2000). The observed SNVs (in the five-marker haplotype sets) specific to the p.(Arg143Trp) homozygotes and absent in GHA-38 (*GJB2*-p.(Arg143Trp)-negative) individuals could support the consanguineous assertion. The impact of the geographical location, size, and the isolated nature of the early population linked with the variant in Ghana coupled to the high rate

of non-migration among the settlers of the village, fueled by fear of discrimination, stigmatization, and societal neglect, may also explain the high prevalence of the *GJB2*-p.(Arg143Trp) founder variant in this region as compared to elsewhere (Adadey *et al.*, 2020; Bliznetz *et al.*, 2017; Kenneson *et al.*, 2002; Tsukada *et al.*, 2015). Furthermore, the current finding is consistent with reports of the combined impact of improved fitness (Rose, 1975), unknown genetic modifiers, and noncomplementary marriages following sign language introduction that have been implicated in the substantial increase of deafness alleles and could be extended to the perpetuation of the p.(Arg143Trp) alleles in the families investigated (Vona *et al*., 2015).

A heterozygote advantage in genetic conditions like hemochromatosis, sickle cell disease (SCD), and factor V Leiden thrombophilia has been implicated as the driver of the continued segregation of deleterious gene variants in populations. Heterozygote carriers of homeostatic iron regulator (*HFE*): p.(Cys282Tyr) in hemochromatosis are reported to have some protection from iron deficiency (Ganz, 2003; Nicolas *et al*., 2001; Pigeon *et al*., 2001). Sickle cell hemoglobin carriers (HbAS) hardly develop severe malaria episodes (Aidoo *et al*., 2002), and factor V Leiden thrombophilia variant carriers are also reported to be resistant to some bloodstream bacterial infections (Lindqvist & Dahlback, 2008). Similarly, in vivo and in vitro studies have demonstrated skin protection in carriers of the *GJB2*-c.35delG founder variant, thus implicating natural selection as driving the evolution of the variant (Laer *et al*., 2001). Nevertheless, the lack of skin disorders in *GJB2*-associated NSHI cases means that the loss or gain of connexin gene function alone may not impact the epidermis development and function as alluded (Gerido *et al*., 2007). Although our haplotype analysis around the locus suggests it evolved independently, and the allele frequency is higher than would be expected under neutrality for a pathogenic variant, to date, the reported discrepancies in the distribution of the

variant in NSHI cases are yet to be correlated with any past or present defined endemic condition(s) in the history of the populations reported to have the variant.

The current *GJB2*-p.(Arg143Trp) age estimate falls within the period of Holocene climatic changes ~11,650 years ago (around the same time as the emergence of the *GJB2*- c.35delG variant in Anatolia ~10,000 years ago) that coincided with the Neolithic movements and transitioned the natural ecosystem to pastoralism (Jousse, 2004). Archaeological and historical evidence of unfavorable climates in Africa between 7000 and 12,000 years ago show a significant increase in the number of lakes and ponds, breeding pathogenic vectors (Coluzzi, 1999; Soffer & Gamble, 1990). Consequently, human domestication of plants and animals in the subregion and exposure to these conditions may have increased contact with infectious pathogens like the malaria parasite that may have elicited the selection of certain pathogenic variants such as the sickle cell mutation and possibly *GJB2*-p.(Arg143Trp) to protect against the deadly malaria parasite through a heterozygote advantage (Livincstone, 1971; Wiesenfeld, 1967). Therefore, the question of whether *GJB2*-p.(Arg143Trp) has coevolved with malaria deserves further scrutiny with genome-wide data, since connexin mutations causing syndromic sensorineural HI associated with epidermal disorders are coupled with additional variants, and the synergistic effect may be responsible for the observed epidermis differentiation reported in p.Arg143Trp Ghanaians (Man *et al*., 2007; Meyer *et al*., 2002). Preferably, other neighboring African populations with shared geographical and ecological conditions and in whom the mutation has not been explored should also be sampled (Guastalla *et al*., 2009).

The little shared ancestry among the GHA and JPT populations, evidenced by haplotypes in Ghanaian individuals that were absent in the Japanese individuals and vice versa, clearly indicates that the two populations have different LD patterns and haplotype structures in the 2-Mb region around the p.(Arg143Trp) founder locus. This supports the independent evolution of *GJB2*-p.(Arg143Trp) in the two populations. Moreover, the absence of the p.(Arg143Trp)

tag variant rs9578260 in Japanese populations, whereas it is highly prevalent in African populations, further suggests that *GJB2*-p.(Arg143Trp) is carried on different haplotype backgrounds in both populations and therefore may have highly likely evolved independently in these populations. The four-marker haplotypes were strongly ancestry informative and resolved populations in Ghana and the 1000 Genomes set at the continental and subcontinental levels. The unique haplotype proportions in *GJB2*-p.(Arg143Trp) homozygotes compared to the hearing controls may allow the use of the four tagged SNVs as surrogate secondary markers for the *GJB2*-p.(Arg143Trp) pathogenic variant in Ghana. Thus, this study shed light on the origin of the most common *GJB2*-p.(Arg143Trp) variant associated with the most NSHI in Ghana. Yet, how the *GJB2*-p.(Arg143Trp) variant has been maintained at appreciable frequencies in the Ghanaian population remains a puzzle that would most likely be delineated using whole-genome data from Ghanaian HI, as well as hearing populations.

A global haplotype analysis with the disease allele would have been ideal to provide haplotype blocks of the evolved allele with other markers in LD with it across populations. However, the absence of the disease mutation in the 1KGP3v5 database restricted the haplotype analysis to the use of four tagged markers that together form strong ancestry informative haplotypes. In addition to the absence of accurate population records, the study used raw observed population growth rates without considering the past population demographic history and the possibility of natural selection, which might influence the growth rates for the age estimation.

4.7 Conclusions

We demonstrated in this study the unique ancestral haplotype background in *GJB2*-p.(Arg143Trp) homozygotes individuals, confirming no significant shared genetic clusters between Ghana (sSA) ancestry and the highly homogenous Asian populations. The carriers perpetuating the deleterious allele (*GJB2*-p.(Arg143Trp)) in Ghana are descendants of an indigenous ancestor about ~385 generations ago. The high recombinational events around the

disease locus may explain the shortening of the haplotype and possible balanced selection evident in the relatively low LD observed in the mutated chromosomes. This study strongly supports that the *GJB2*-p.(Arg143Trp) variant evolved independently and segregated in the affected families, preserved by a selective mating pattern and consanguinity within the populations.

4.8 Supplementary Materials

The following supporting information can be downloaded at www.mdpi.com/xxx/s1: Figure S4.1: Pattern of linkage disequilibrium and recombination in the 2-Mb region around p.R143W. Figure S4.2: Estimation of p.(Arg143Trp) location and age for the unimputed dataset (A,B) and the imputed data set (C,D). Figure S3: Ancestry analysis for the Ghana and 1000 Genomes populations. Table S4.1: Quality metrics of the markers selected for the age estimation and haplotype analysis. Table S4.2: Quality metrics of imputed SNVs markers and the accuracy scale. Table S4.3: Haplotypes and haplotype frequencies in Ghanaian p.(Arg143Trp)-positive and p.(Arg143Trp)-negative populations based on five markers that were in linkage disequilibrium with p.(Arg143Trp). Table S4.4: Excel 1KGP3v5 WES haplotype frequencies and diversity.

4.8.1 Appendix A

Table S4.1 Quality metrics of markers selected for age estimation and haplotype analysis

Marker	Chr: Pos	MQ	DP	AvGQ	VQSLOD	FILTER	BIOTYPE
rs115802719	13:20635310	249.92	9622	89.78	7.35	PASS	Exonic
rs7324021	13:20637140	250	7530	78.15	4.98	PASS	Intronic
rs17075877	13:20641422	248.84	9149	85.73	5.72	PASS	Exonic
***rs80338948**	13:20763294	249.37	13226	88.56	5.1	PASS	Exonic
rs9578260	13:20763754	249.44	17623	79.17	6.77	PASS	Intronic

*Chr:Pos=Chromosome and base pair position; MQ=Mapping quality; DP=Depth of coverage; AvGQ=Average genotype quality; VQSLOD=Variant quality score log of the odds; * = Disease allele*

4.8.2 Appendix B

Table S4.2. Quality metrics of imputed SNVs markers and accuracy scale

Marker	Chr:Pos	Ref/Alt	Gene	Biotype	LD (r^2)	Genetic Map (cM)	MAF (1KGP3 AFR populations)	Imputation accuracy (scale: 0 - 1)
rs7329857	13:20762929	G/A	*GJB2*	3'UTR	0.38	0.000000	A= 0.2196	0.85
rs7337074	13:20762936	T/A	*GJB2*	3'ÚTR	0.38	0.000007	A = 0.0002	0.85
***rs80338948**	13:20763294	G/A	*GJB2*	Exonic	1.000	0.000365	A= 0.0002	1
rs9578260	13:20763754	G/A	*GJB2*	Intronic	0.38	0.000825	A = 0.0002	1
rs9578261	13:20764147	C/A	*GJB2*	Intronic	0.36	0.001218	T = 0.0002	0.99
rs9579800	13:20764312	T/A	*GJB2*	Intronic	0.36	0.001383	A = 0.0002	0.99
rs74035963	13:20764722	A/G	*GJB2*	Intronic	0.36	0.001793	G = 0.0002	0.99
rs74035964	13:20764815	T/C	*GJB2*	Intronic	0.36	0.001886	C = 0.0002	0.99
rs74035965	13:20764850	C/T	*GJB2*	Intronic	0.36	0.001921	T = 0.0002	0.99

*Computed genetic map distance in centimorgans (cM), and minor allele frequencies (MAF) in the reference 1000 Genome reference panel, phase 3, version 5 database (1KGP3v5); Computed linkage disequilibrium (r2) for each marker respectively. RSID=reference SNP ID; Chr:Pos=Chromosome and base pair position; Ref/Alt=Reference and alternate alleles; LD=linkage disequilibrium; cM=Centimorgan; AAF=alternate allele frequency; * = the disease mutation*

4.8.3 Appendix C

Table S4.3. Haplotypes and haplotype frequencies in Ghanaian p.Arg143Trp-positive and p.Arg143Trp-negative populations based on five markers that were in linkage disequilibrium with p.Arg143Trp

Marker and Ref/Alt allele					Hap	Hap Name	Frequency		
rs115802719	**rs7324021**	**rs1705877**	**rs80338948**	**rs9578260**	**-**	**-**	**GHA-38**	**R143W-1**	**R143W-2**
A/G	**T/G**	**A/G**	**G/A**	**G/A**	**-**	-			
0	0	0	0	0	00000	Hap1	0.57	0	0
0	0	0	0	1	00001	Hap2	0.26	0	0
0	0	0	1	0	00010	Hap3	0	0.067	0.067
0	0	0	1	1	00011	Hap4	0	0.333	0.33
0	0	1	0	1	00101	Hap5	0.026		0
0	0	1	1	1	00111	Hap6	0	0.067	0.067
0	1	0	0	0	01000	Hap7	0.079	0	0
0	1	0	0	1	01001	Hap8	0.026	0	0
0	1	0	1	0	01010	Hap9	0	0.033	0
0	1	0	1	1	01011	Hap10	0	0.3	0.33
1	0	1	0	0	10100	Hap11	0.026	0	0
1	0	1	0	1	10101	Hap12	0.013	0	0
1	0	1	1	1	10111	Hap13	0	0.2	0.2

The "0s" and "1s" against each marker indicate whether the marker is homozygous for the reference allele (0) or homozygous for the alternate allele (1). Ref/Alt=Reference and alternate alleles; Hap = Haplotype.

4.8.4 Appendix E

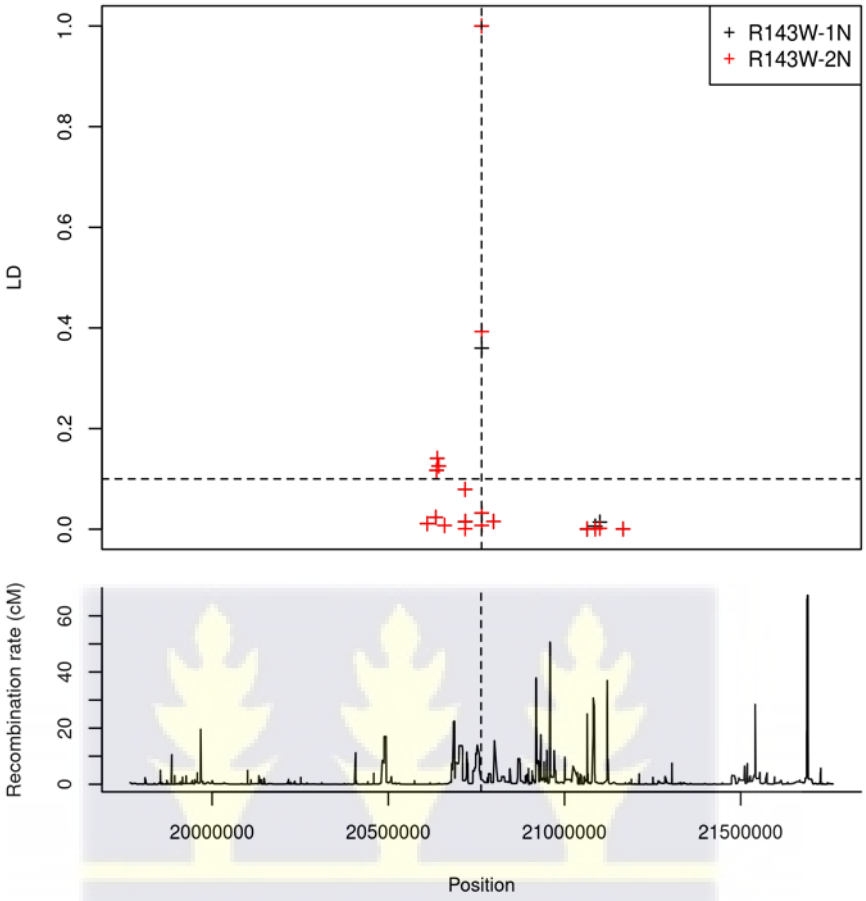

Figure S4.1. Pattern of linkage disequilibrium and recombination in the 2 Mb region around *GJB2*-p.(Arg143Trp). *Distribution of markers in linkage disequilibrium with the GJB2:c.427C>T-p.Arg143Trp founder variant (single nucleotide variants upstream and downstream), and the rate of recombination within the 2 Mb genomic region.*

4.8.5 Appendix F

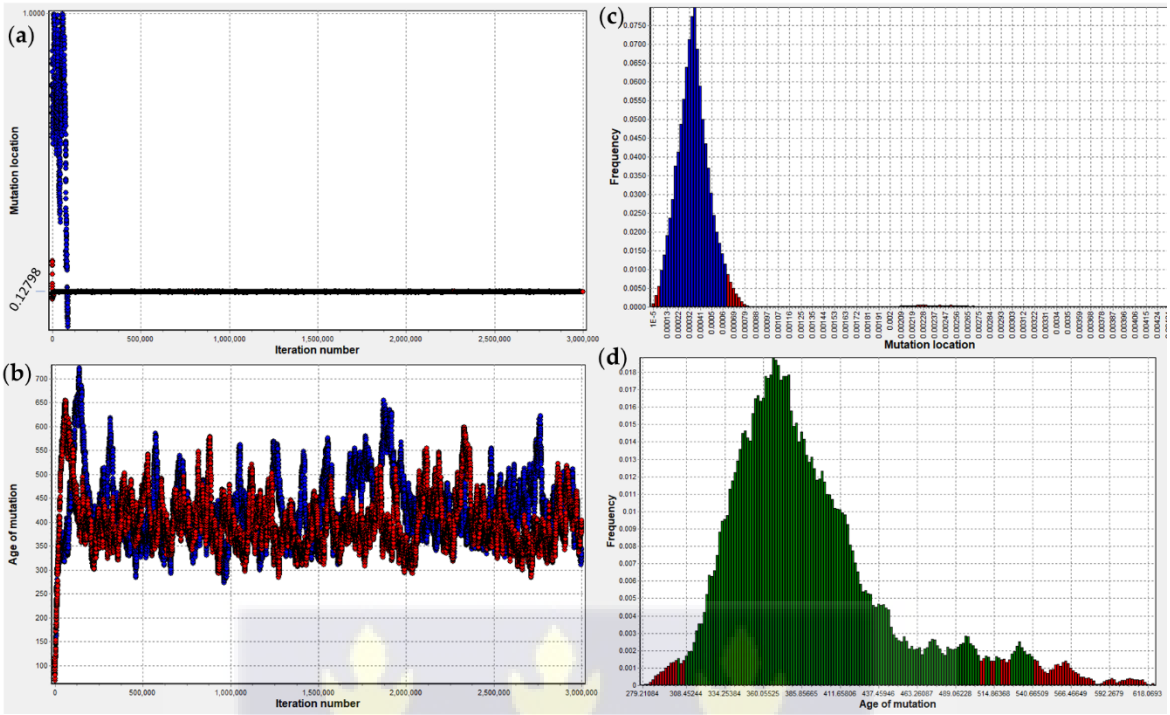

Figure S4.2. Estimation of p.(Arg143Trp) location and age for the unimputed data set; *((a) and (b)) and the imputed data set ((c) and (d)). The two chains converged at the correct mutation location ((a) and (c)) while estimating the allele age ((b) and (d). In the unimputed set (b), Chain 2 stably estimated the allele age, and this was apparent by its more precise estimation of the allele location from the very beginning of the burnin iterations (a).*

4.8.6 Appendix G

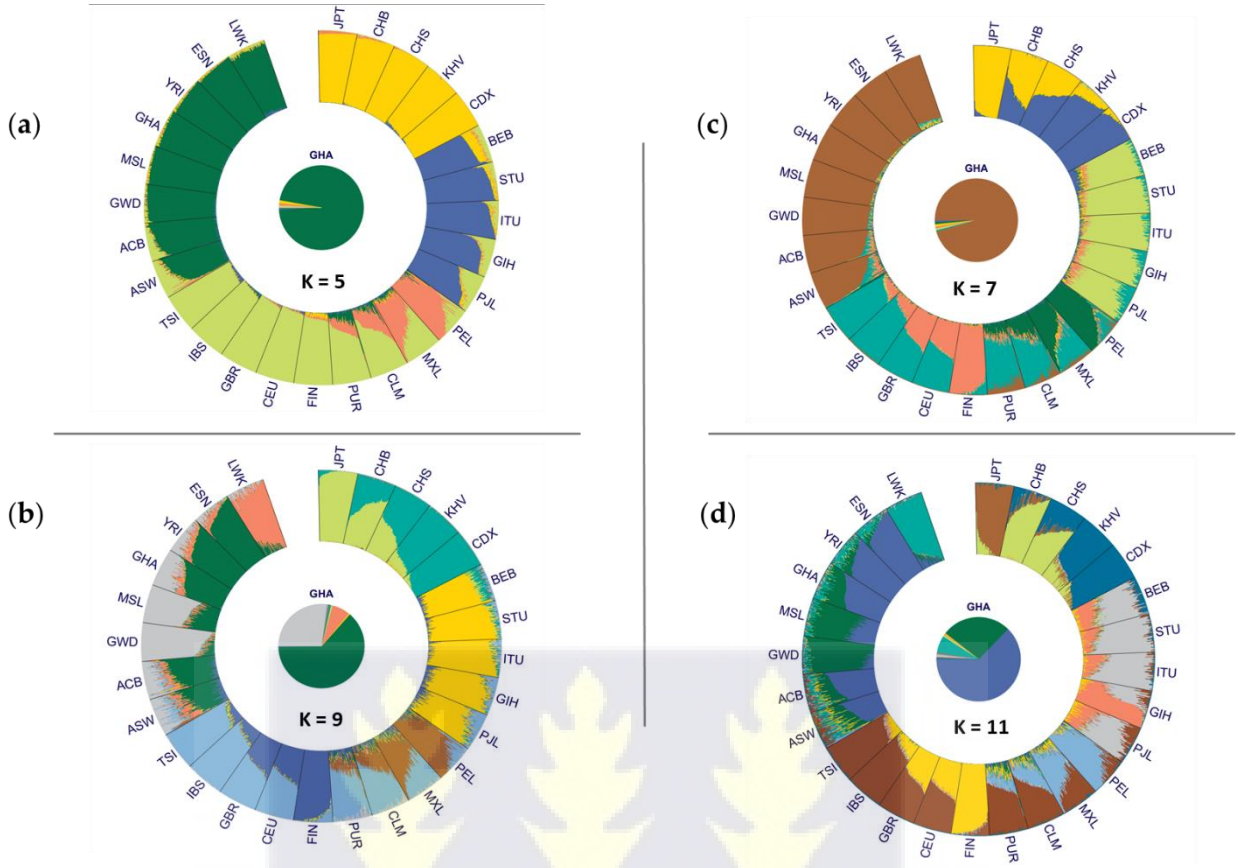

Figure S4.3. Ancestry analysis for Ghana and 1000 Genomes populations. *At k = 5, all continental populations are resolved. European, American, as well as East Asian populations are further resolved at k = 7. From K = 8 to k = 11, all the continental ancestries are further resolved into sub-populations reflecting geographic separation. For instance, the LWK population in East Africa is clearly distinguished from all other African populations which are all from West Africa.*

4.9 Author Contributions

Conceptualization and study design, A.W., G.A.A., and L.A..; participant recruitment; E.T.A. and S.M.A.; methodology, E.T.A., K.E., and S.M.A.; validation, E.T.A., S.M.A., K.E., and M.J.; formal analysis, K.E., E.T.A., S.M.A., and M.J.; software, K.E.; investigation, E.T.A., S.M.A., and C.dK.; resources, A.W. and G.A.A.; data curation, E.T.A., K.E., S.M.A., and M.J.; writing—original draft preparation, E.T.A., K.E., and S.MA.,; writing - review and editing, E.T.A., S.M.A., K.E., M.J., C.dK., L.A.., G.A.A., and A.W.; visualization, E.T.A., C.dK., and M.J.; supervision, A.W., G.A.A., and L.A..; project administration, C.dK., G.A.A., and A.W.; and funding acquisition, A.W. and G.A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported with funds received from the UK government, the National Institutes of Health (NIH), USA, grant number U01-HG-009716, to A.W, and the African Academy of Science/Wellcome Trust, grant number H3A/18/001, to A.W.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of College for Basic and Applied Sciences (ECBAS), protocol code ECBAS 053/19-20; approved on 28 October 2021).

Informed Consent Statement: Informed consent was obtained from all subjects (parents and guardians) involved in the study. Signed written informed consent was obtained from the participant(s) to publish this manuscript.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to acknowledge the study participant, heads, and staff of schools for the deaf in Ghana, the Ghana Health Service, and the Ghana Education Service for their support.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

CHAPTER FIVE

5.0 Manuscript 1. Phenotypic Expansion of *MARVELD2* Associated Hearing Impairment: Post-lingual Expression in a Ghanaian Family Segregation a Bi-allelic Novel Variant

Elvis Twumasi Aboagye [1,3], Samuel Mawuli Adadey [1,3], Edmond Wonkam-Tingang [3], Kevin Esoh [3], Lucas Amenga-Etego [1], Isabelle Schrauwen [2], Gordon Akanzuwine Awandare[1], Suzanne M. Leal [2] and Ambroise Wonkam[3,4]

[1.] West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, Legon-Accra Box LG 54, Ghana [2.] Centre for Statistical Genetics, Sergievsky Center, Taub Institute for Alzheimer's disease and the Aging Brain, and the Department of Neurology, Columbia University Medical Centre, New York, NY 10032, USA [3.] Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, Cape Town 7925, South Africa [4.] McKusick-Nathans Institute and Department of Genetic Medicine, John Hopkins University School of Medicine, Baltimore, MD 21205, USA

5.1 Abstract

Systematic investigation of implicated candidate genes has identified gap junction protein beta 2 gene (*GJB2*): c.427C>T-p.(Arg143Trp) founder marker as the major contributor (25.9%) to non-syndromic hearing impairment (NSHI) in Ghana. Using Next Generation Sequencing in hearing-impaired studies is limited in Africa, despite the high population genetic diversity, and genetic heterogeneity of HI. This means that we are missing on the full spectrum of gene(s) variants/allele(s) co-segregating with HI among affected families in the sub-region. Whole-exome sequencing was carried out to identify the causal genetic marker segregating post-lingual NSHI in a multiplex family in Ghana with consanguineous history. The putative marker identified was further screened in ethnologically matched hearing controls (n = 46), and a group of unrelated individuals with simplex NSHI (n = 151) by direct Sanger sequencing. The mutant

protein was modelled with a resolved wildtype protein (PDB ID: 5N7K) as template, using homology modeling on SWISS-MODEL. A literature review of the implicated *MARVELD2* variants associated with HI was performed. Homozygous MARVEL domain containing 2 (*MARVELD2)*: c.1058dupT-p.(Val354SerfsTer5) frameshift variant on exon 2 segregates with the phenotype in this family. This bi-allelic novel *MARVELD2* (*MIM 610572*) variant was not identified in the 46 matched controls, and 151 unrelated sporadic cases screened. Equally, the variant was absent on publicly available databases including ClinVar, hereditary hearing loss homepage, ClinGen, online Mendelian inheritance in man, and human phenotype ontology searched. The identified *MARVELD2*: c.1058dupT-p.(Val354SerfsTer5) variant predicted to truncate the encoded protein, is the putative pathogenic variant associated with the intra-family bilateral ARNSHI. A review on the gene association with ARNSHI identified isolated reports of twelve different *MARVELD2* variants from seven countries. The bi-allelic *MARVELD2* nonsense mutation [c.1058dupT-p.(Val354SerfsTer5)] identified in this multiplex consanguineous Ghanaian family segregating bilateral, sensorineural, post-lingual ARNSHI, is novel, and adds to the global knowledge in refining HI disease-gene pair curation.

Keywords: Non-syndromic Hearing Impairment; *MARVELD2*, Tricellulin (TRIC); Ghana.

5.2 Introduction

Hearing impairment (HI) is a widespread sensory human disorder that affects 34 million children, globally (WHO, 2020). The incidence of the disorder range 1 - 6 in every 1000 live births in sub-Saharan Africa (sSA) (Morton & Nance, 2006). Early diagnosis of HI is highly recommended as critical to ensuring timely access to required early interventions related to speech and language development, and to addressing the general psycho-social well-being of the affected individuals, and families. However, diagnosis is mostly late in children (post language acquisition age) in developing countries. The mean age for medical diagnosis in Cameroon for example was 3.3 years (Wonkam *et al.*, 2013), and between 6 to 11 years in

Ghana (Adadey et al., 2019). Genetic variation generally explains about 50% of congenital HI (Angeli *et al*., 2012). Pathogenic gene variants associated with HI in human populations are diverse and associated with ethnic background, geographical location, and type of HI (syndromic or non-syndromic) (Smith *et al*., 2005). Autosomal recessive pattern of inheritance is the main mode of inheriting HI of genetic etiology (observed in 80% of reported cases). However, autosomal dominant, X-linked, and mitochondrial pattern of inheritance have also been reported (Mikstiene *et al*., 2016). Currently, over 121 genes and 151 loci are associated with HI; 58 autosomal dominant genes loci, 87 autosomal recessive genes loci, and 6 loci for X-linked genes (Camp & Smith, 2019). HI can be characterized as conductive, sensorineural, or mixed; syndromic or non-syndromic; pre-lingual or post-lingual, progressive, or non-progressive (Brobby *et al.*, 1998a; Liu *et al.*, 2002; Morton & Nance, 2006).

In Ghana, the first genetic investigation reported *GJB2*-p.Arg143Trp founder mutation in 11 familial cases from a deaf village with a characteristic high prevalence of deafness (Adadey *et al*., 2019; Brobby *et al*., 1998; Hamelmann *et al*., 2001). We recently used WES analysis to explore other genetic variants in *GJB2* negative families in Ghana (Wonkam *et al*., 2022), that revealed, for the first time a post-lingual expression of bi-allelic *MARVELD2* not previously described nonsense mutation [c.1058dupT-p.(V354SfsTer5)] in a Ghanaian family segregating NSHI. In the present paper we refined the phenotypic description of that family compared with other previously reported cases and reviewed the literature on all *MARVELD2*-assocaited HI cases globally and investigated the variants in ethnologically Ghanaian matched healthy normal hearing controls (n = 46), and unrelated simplex NSHI individuals (n = 151).

5.3.0 Material and Methods

5.3.1 Ethical Approvals

The study was conducted following the ethical principles of the declaration of Helsinki guiding medical research. The study received approval from the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB CPN 006/16-17), the Faculty of Health Sciences' Human Research Ethics Committee, University of Cape Town (HREC 104/2018), the Columbia University's Institutional review board (IRB-AAAS2343), and the University of Ghana's Ethics Committee for Basic and Applied Sciences (ECBAS 053/19-20). The study protocol was explained in preferred native language (and signed language interpretation) for full comprehension and signed and/or verbal consent obtained before participant recruitment. The study was performed with strict adherence to the guiding principles of ensuring participants' safety.

5.3.2 Patients' recruitment and sample collection

The participants enrolled in this study are members of a large Ghanaian family segregating post lingual NSHI (Figure 5.1A). This multiplex consanguineous family was recruited through our community engagement program and excluded to have no *GJB2* coding region pathogenic variants ,as previously reported (Adadey *et al*., 2019). Medical records and clinical history of participants thoroughly examined by a clinical geneticist, and a specialist; ear, nose, and throat (ENT). Participants' medical history and responses to a structured questionnaire were used to assess probands of suspected known environmental factors that may contribute to the phenotype. All three affected and available unaffected kindred hearing threshold assessed by conducting pure tone audiometry and the recommendation number 02/1 of the Bureau International d'Audiophonologie (BIAP), guidelines used for severity classification (*BIAP - International Bureau of Audiophonology*).

5.3.4 Whole exome sequencing

Three affected and one unaffected kindred gDNA were whole-exome sequenced at Omega Bioservices (Norcross, GA, USA) using pair-end 150bp run format on an Illumina HiSeq 2500 platform Illumina. Sequence libraries were prepared using Nextera Rapid Capture Exome kit® (Illumina, San Diego, CA, USA), after which they were hybridized with a 37Mb probe pool to enrich exome sequences.

5.3.5 Whole exome sequence analysis

Reads obtained were analyzed following the Illumina DRAGEN Germline Pipeline v3.2.8. After in-house quality control check, the resultant high-quality reads were aligned to the genome human reference consortium built 37 (GRCh37/hg19) using the Illumina DRAGEN 05.021.408.3.4.12 software. Genomic variant call format (gVCF) files were generated after sorting and marking duplicates. Joint calling of variants computed using the genome analysis toolkit (GATK) software v4.0.6.0. GATK CombineGVCFs and GenotypeGVCFs commands were used for the joint calling to generate a VCF file that was recalibrated using GATK VariantRecalibrator command. Generated variants were filtered by variant quality score recalibration using ApplyVQSR command that flagged low-quality variants. To solve the possible genetic marker co-segregating among the observed late – onset ARNSHI in this multiplex consanguineous family, the VCF file variants were annotated and filtered with ANNOVAR as described by Wang et al (Wang *et al*., 2010). The autosomal mode of inheritance was first considered for the variant filtering since it conformed to the pedigree analysis of the family (Figure 5.1A). To elucidate the causal variant, rare and novel variants were filtered using alternate allele frequency (AAF) threshold < 0.005 for recessive single nucleotide variants and < 0.0005 dominant single nucleotide variants (SNVs) on available public databases, including genome aggregation database (gnomAD), and exome aggregation consortium database (ExAC). Clinical significance and deleterious prediction were performed

for the variants identified using bioinformatic tools on the dbNSFP v3.0 database. Sorting Intolerant from Tolerant (SIFT), Protein Variation Effect Analyzer (PROVEAN), MutationTaster, MutationAssessor, Mendelian Clinically Applicable Pathogenicity (M-CAP) score, Polymorphism Phenotyping v2 (PolyPhen-2) x 2, and Deleterious Annotation of genetic variants using Neural Networks (DANN) score, among many others were used to validate the pathogenicity of putative variants.

To further confirm the MARVELD2 conservation in different species, evolutional conservation of substituted amino acid in the affected domain was assessed using phyloP, SiPhy, and Genomic Evolutionary Rate Profiling (GERP), and phastCons scores as depicted in Figure 5.2D. The association between identified HI candidate genes was reviewed on the hereditary hearing loss homepage (HHL) (Camp & Smith, 2019), human phenotype ontology (HPO), Online Mendelian Inheritance in Man (OMIM), and ClinVar databases. Putative variants in genes chiefly expressed in the ear with high predicted deleterious effects, altering the protein structure and function were considered pathogenic or likely pathogenic (P/LP).

5.3.6 Sanger sequencing validation

Sequence specific primers for the *MARVELD2* were designed using the National Center for Biotechnology Information (NCBI) primer Basic Local Alignment Search Tool (BLAST), covering the region with our variant of interest. Designed primers were optimized using the Oligo-analyzer tool designed by Integrated DNA Technologies, Inc. (Iowa, USA). *MARVELD2* exon 2 was PCR amplified using designed primer pair, forward: (5'-GAGAATACTGGGTGTGGTGGAG-3') and reverse: (5'-ACAGACATAATAATGCAGCCAACAA-3'), with cycling conditions; denaturation, annealing, and extension (95 °C, 60 °C, and 72 °C). Bi-directional Sanger sequencing with the BigDye™ Terminator v3.1 Cycle Sequencing Kit was performed on PCR amplicons and

sequence analyzed using ABI 3130XL Genetic Analyzer® (Applied Biosystems, Foster City, CA, USA). FinchTV v1.4.0 and UGENE v34.0 software were used to check sequenced files quality and analysis of AB1 files obtained as described by Okonechinikov and colleagues (Okonechnikov *et al.*, 2012). The putative *MARVELD2*: c.1058dupT-p.(Val354SerfsTer5) implicated variant was investigated in 151 unrelated simplex Ghanaian cases with NSHI, and additional 46 apparently healthy ethno-linguistic matched controls with normal hearing, who had no known history (personal or family) of HI, randomly recruited from the general population.

5.3.7 Multiple sequence analysis of MARVELD2 protein

The protein sequence of the *MARVELD2* gene of different organisms were retrieved from the NCBI database. Using the UGENE v34.0 software (Okonechnikov *et al.*, 2012), ClustalW multiple sequence alignment was conducted with the downloaded protein sequences and *MARVELD2*: c.1058dupT-p.(Val354Serfser5) variant protein sequence. The aligned MARVELD2 proteins sequence was analyzed for evolutional conservation of amino acids at the variant site (Figure 5.2E).

5.3.8 MARVELD2 protein model analysis

The wildtype protein model solved by Schuetz *et al* (Schuetz *et al.*, 2017) was retrieved from the protein database (PDB) and visualized in PyMOL. Homology protein modeling approach was employed to model the mutant protein using a web-based tool, SWISS-MODEL (Biasini *et al.*, 2014). The wildtype protein (PDB ID: 5N7K) was used as a template for the mutant protein modeling. Solved mutant predicted structure was downloaded from the SWISS-MODEL and visualized in PyMOL, and the protein alignment function of PyMOL used to align the wildtype protein to mutant protein (Figure 5.2C).

5.4.0 Results

5.4.1 Phenotypic description of affected family

The affected kindreds (IV:3 and IV:4) were found to have post-lingual profound HI at the time of enrollment and sample collection. These two patients were documented to have developed hearing disorder at age 12 years. The last affected male (IV:6) at the time of interview was found to be "hard of hearing", with onset of the condition at age 8 years. All three affected siblings were good at lip reading perfectly since the hearing disorder onset after post-language acquisition age (at 8 and 12 years) and were communicating verbally before the onset of the condition. The unaffected family members had normal hearing. The 151 unrelated individuals from simplex families age ranged; 5 to 26 years (mean age of 17.7 years), and 51.6% (78) females, all had sporadic congenital NSHI.

5.4.2 Results of WES

The WES achieved an average target region of about 181x and about 96% of the target regions having more than 10x coverage. WES data analysis using filtering methods detailed in chapter six, section 6.3.2 discovered the novel variant in *MARVELD2*: c.1058dupT-p.(V354SfsTer5) on exon 2 as a putative cause of the observed phenotype in homozygous state in all the three affected kindred. The variant has not been reported on the 1000 genome, gnomAD, and UK10K database. Following the ACMG guidelines for the variants interpretation and annotation (Green *et al.*, 2013), the variant was suggested as pathogenic, and segregates with the phenotype within the family further supporting it as the putative causal variant. In addition, the thymine (T) duplication at position 1058 in exon 2 causes a frameshift, which produced a protein with valine substituted by serine at codon 354 (amino acid position 354). The non-synonymous mutation changes the reading frame, causing a truncation of the synthesized to lose a cytoplasmic domain, confirming the associated pathology.

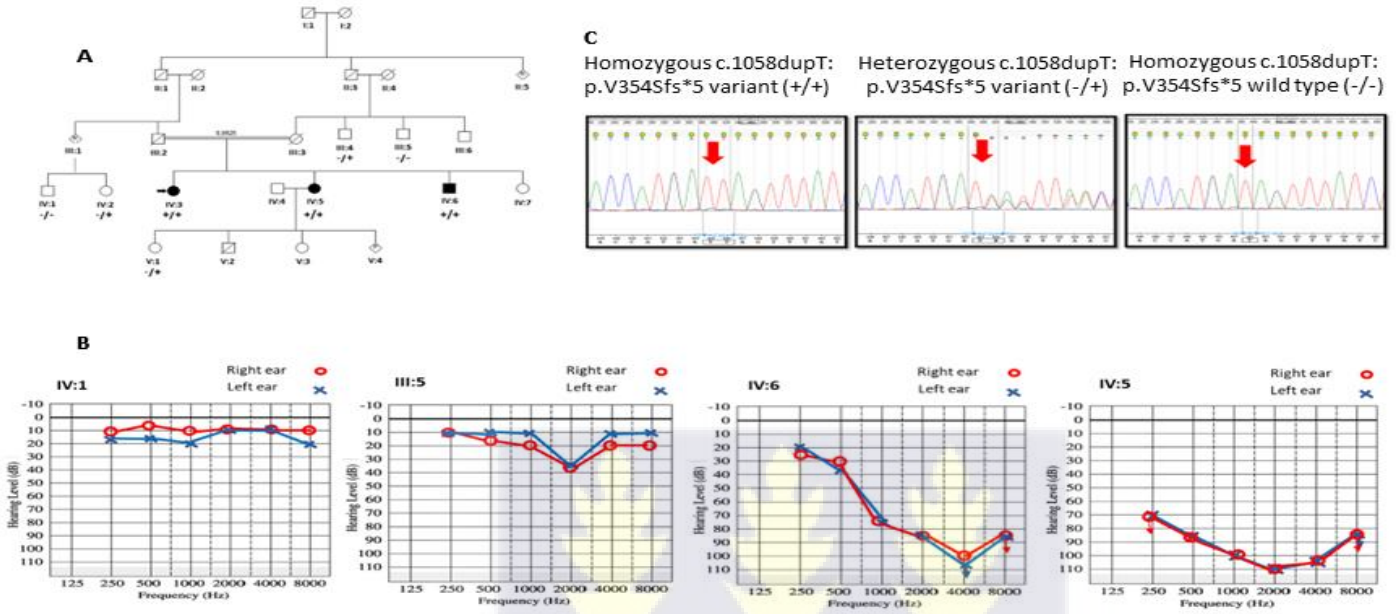

Figure 5.1. Family pedigree of large consanguineous multiplex case. *A novel bi-allelic MARVELD2: c.1058dupT-p.(V354SfsTer5) variant, phenotype-genotype correlation, and direct Sanger sequencing validation: Panel A; The genotypes of the family members are represented with +/+; -/+; and -/- which denotes homozygote for the pathogenic variant, heterozygote, and homozygote wild type, respectively. Individuals homozygous for this mutation (IV:3, 34years, IV:5, 31years and IV:6, 27 years) showed post-lingual hearing impairment, homozygote wildtype and heterozygous with normal hearing. Symbols filled denotes affected persons, squares and circles represents males and females; Panel B; Audiograms of the family members as indicated with the pedigree number on top of each audiogram. Carriers or family member(s) heterozygote of the mutation as illustrated by (IV;1 and III;5) showed normal hearing and affected kindred homozygous of the mutation having severe (IV:6) to profound (IV:5), bilateral sensorineural ARNSHI; Panel C; Representative Sanger sequence chromatogram of the various genotypes. c.1058dupT-p.(Val354SerfsTer5) pathogenic variants. Post-lingual ARNSHI in the family co-segregate with the identified bi-allelic MARVELD2: c.1058dupT-p.(Val354SerfsTer5) variant.*

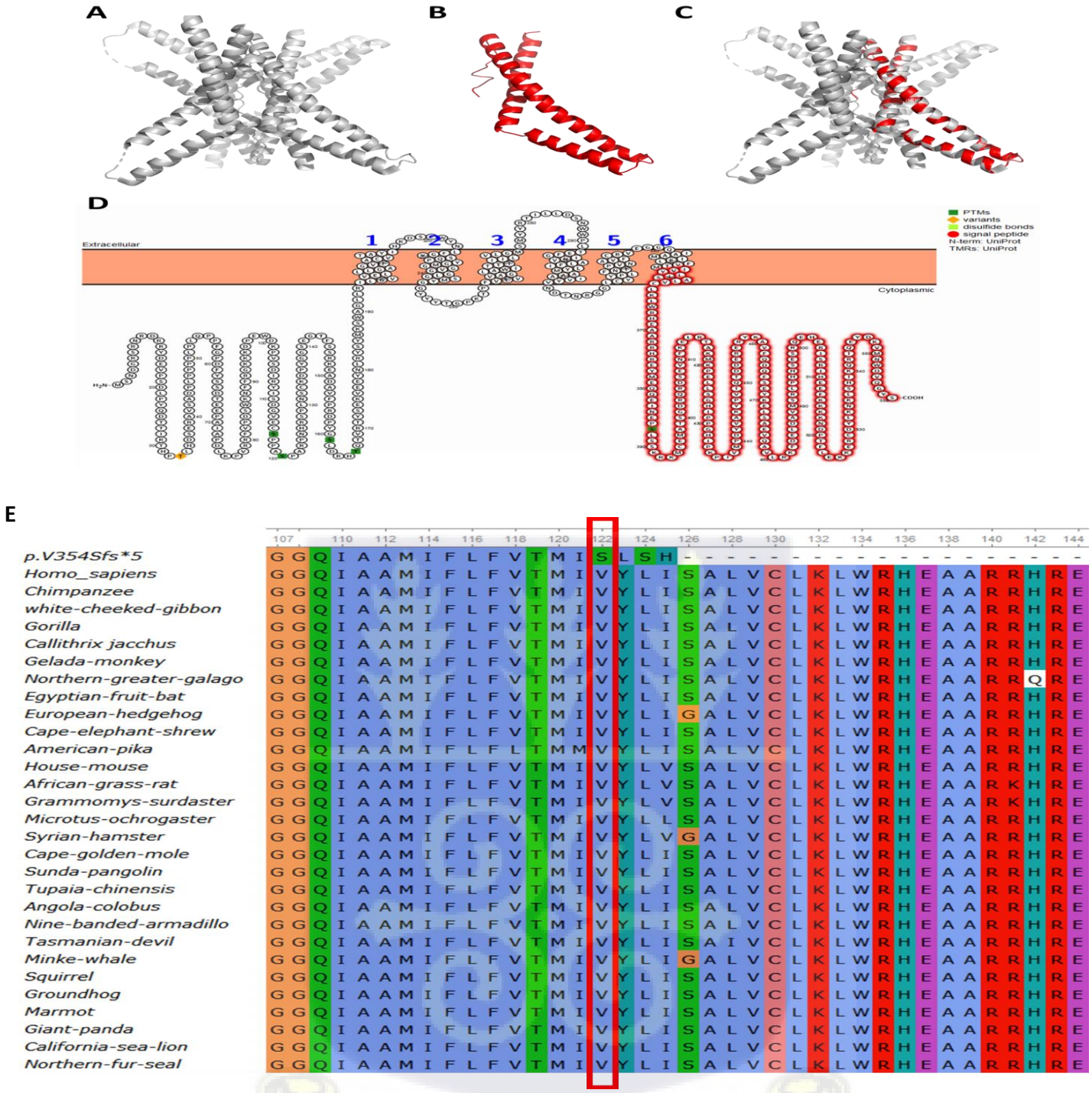

Figure 5.2. MARVELD2: p.V354SfsTer5 protein model and MARVELD2 protein *(A) Structure of wildtype MARVELD2 protein. (B) Structure of MARVELD2: p.V354SfsTer5 mutant protein. (C) Alignment of wildtype and mutant MARVELD2: p.V354SfsTer5 proteins. (D) Schematic diagram of the primary structure of the MARVELD2 protein showing the truncated cytoplasmic region highlighted in red. (E) Multiple sequence alignment of MARVELD2 protein of different species. A red rectangle is used to highlight the position of the MARVELD2: c.1058dupT: p.V354SfsTer5 variant*

5.4.3 Phenotype-genotype correlation

All the hearing-impaired family members (Figure 5.1: IV.3, IV.5 and IV.6) were found to have the homozygous (+/+) form of the pathogenic variant *MARVELD2*: c.1058dupT-p.(Val354SerfsTer5). The first two affected individuals (IV:3 and IV:5 on Figure 5.1A) were found to have profound HI with pure tone average of 100 and 98 dB respectively, in the better ear depicted in Figure 5.1B. Unlike the first two siblings, the third affected family member (IV:6) was diagnosed of severe HI with a pure tone average of 63 dB in the better ear. There was no clinically significant difference in phenotype between the hearing of individuals with the heterozygous genotype (p.(Val354Val)/p.(Val354SerfsTer5)) and the homozygous wildtype (p.(Val354Val)/p.(Val354Val)). Inference from the genotype-phenotype correlation follows recessive pattern of inheritance, requiring two mutant alleles for the expression of the HI phenotype.

Table 5.1. Review of pathogenic *MARVELD2* mutations identified to date

Variant	Amino acid change	County of Origin	Age of onset: (Pre-lingual/post - lingual)	Variable Expression (Yes or No)	Mode of Inheritance (Syndromic/Non-Syndromic)	References
c.1058dupT	p.(V354SfsTer5) exon 2	Ghanaian	Post-lingual	Severe to Profound (Yes)	Autosomal Recessive (Non-syndromic)	Present study
c.1183-1G>A	Splice-acceptor site exon 4	Pakistani	N/A	Profound (Yes)	(Non-syndromic)	Riazuddin *et al* 2006
c.1331+1G>A	Splice donor site on intron 4	Pakistan, Iranian	Pre-lingual	Profound (Yes)	Autosomal Recessive (Non-syndromic)	Chishti *et al* 2008, Sadeghi *et al* 2020.
c.1331+2T > C	Splice-donor site on intron 4	Pakistan, Czech, Roma, Slovak Roma, Hungarian Roma	Pre-lingual	Severe to Profound (No)	Autosomal Recessive (Non-syndromic)	Riazuddin *et al* 2006, Nayak et al 2015, Safka Broˇzkov et al, 2012, Chishti *et al* 2008, Mašindová *et al*, 2015, Atik *et al* 2015,
c.1331+2deITGAG	Splice–donor site on exon 4	Pakistani	N/A	Profound (No)	Autosomal Recessive (Non-syndromic)	Riazuddin *et al* 2006
c.1498C > T	Nonsense codon on exon 5 p.(Arg500Ter)	Pakistani	N/A	Severe to Profound (Yes)	Autosomal Recessive (Non-syndromic)	Riazuddin *et al* 2006
c.1543delA	p.(Lys517ArgfsTer16) exon 6	Iranian	Pre-lingual/Congenital	Profound (No)	Autosomal Recessive (Non-syndromic)	Babanejad *et al* 2012
Exon 4-5 deletion	p.C395-Q501del In-frame del	Pakistani	Pre-lingual	Severe to Profound (Yes)	Autosomal Recessive (Non-syndromic)	Nayark *et al* 2015
c.1555delinsAA	p.(Asp519LysfsTer12) exon 7 (frameshift)	Iranian	N/A	Moderate to Profound (Yes)	Autosomal Recessive (Non-syndromic)	Taghipour-Sheshdeh *et al* 2018

c.949C>G	p.(Arg317Gly) exon 2	Chinese	-	Mild to profound (-)	Non-syndromic	Zheng *et al* 2019
c.772G>A	p.(Val258Met) exon 2	Chinese	-	Mild to profound (-)	Non-syndromic	Zheng *et al* 2019
c,730G>A	p.(Gly244Arg) exon 2	Chinese	-	Mild to profound (-)	Non-syndromic	Zheng *et al* 2019
c.1006C>T	p.(Arg335Trp) exon 2	Chinese	-	Mild to profound (-)	Non-syndromic	Zheng *et al* 2019

5.4.4 Evolutional conservation of the MARVELD2 protein

To evaluate the pathological significant effect of the nonsense mutation (substituted amino acid to stop codon) at this position, and the general conservation of the MARVELD2 amino acid sequence in different species. Downloaded protein sequence of 20 different species together with the mutant MARVELD2 sequence was aligned by ClustalW multiple sequence alignment, and the domain containing the mutation of interest analyzed. The multiple sequence analysis showed MARVELD2 as a highly conserved protein across different species, supporting its functional importance in the organisms studied. Valine substituted was highly conserved in all species sequence at amino acid sequence position 354 of the protein sequences compared (Figure 5.2E).

5.4.5 Validation of WES

Bidirectional Sanger sequencing analysis of the three affected and unaffected kindred confirmed the presence of the *MARVELD2*: c.1058dupT-p.(Val354SerfsTer5) in a homozygous state (affected) and heterozygote carrier (unaffected) individuals. Three unaffected members in the family were heterozygous and two homozygous for the wildtype (Figure 5.1C). The variant was not identified in all 151 unrelated participants with HI from simplex families in the larger study including healthy controls (n = 46) screened for the mutation.

5.4.6 MARVELD2 protein model analysis

We obtained a high-quality model for the wild type MARVELD2 protein (PDB ID: 5N7K) from the PDB database (Figure 5.2A). Modeled novel mutant p.(Val354SfsTer5) protein based on the 5N7K template, produced a Ramachandran plot favored and outlier scores of 99.10% and 0.00% respectively (Figure S5.1). The quality estimated scores of the mutant model were within the acceptable range (Figures S5.1B, S5.1C, and S5.1D). Aligning the wildtype (shown in gray) to the mutant protein (shown in red) demonstrates the quality of mutant protein model

obtained (Figure 5.2C). The mutation resulted in a truncated and non-functional MARVELD2 protein. As expected, the mutation caused a truncation in the protein length apparent in the superimposed structures (Figure 5.2C). A domain search on the InterProScan server revealed that the truncation results in the loss of the cytoplasmic domain which contains the occludin domain, necessary for localization to tight junctions (Furuse *et al*., 1993). This was also demonstrated by the folding model of the protein produced using Protter shown in Figure 5.2D (https://wlab.ethz.ch/protter/).

5.5 Discussion

DFNB1 *(GJB2)* remains the most common locus linked with HI, accounting for close to 50% of ARNSHI in Europe (Mehra *et al*., 2009; Tabatabaiefar *et al*., 2011). We have previously reported close to 26% prevalence of *GJB2*-p.Arg143Trp founder variant among ARNSHI Ghanaian cohorts, as the most common HI associated variant from systematic investigation of the connexins (Adadey *et al*., 2019). Other candidate gene variants contribution to the segregation of HI in about 70% of unresolved multiplex Ghanaian families negative for *GJB2* variant has been reported recently (Adadey *et al*., 2020; Wonkam *et al*., 2022). This report expands the previously reported *MARVELD2* HI-associated phenotype with particular focus on a consanguineous Ghanaian family expressing sensorineural, post-lingual bilateral ARNSHI, with intra-family phenotype variability (severe to profound). This is the first pathogenic *MARVELD2* associated variant in Africa, which contributes to advancing the disease-gene pair curation worldwide.

To date, this novel thymine duplication report, brings to thirteen *MARVELD2* known pathogenic variants elucidated to be associated with ARNSHI in families globally (Brozkova *et al*., 2012; Chishti et *al*., 2008; Ikenouchi *et al*., 2005; Mašindová *et al*., 2015; Nayak *et al*., 2015; Riazuddin *et al*., 2006; Sadeghi *et al*., 2020; Tabatabaiefar *et al*., 2011; Taghipour-

Sheshdeh *et al*., 2019; Zheng *et al*., 2019) (Table 5.1). Affected individuals homozygote for this bi-allelic mutation (p.Val354SerfsTer5/Val354SerfsTer5) showed bilateral, sensorineural intra-family variable ARNSHI (severe to profound) phenotype (Figure 5.1B) consistent with previously reported *MARVELD2* variants associated ARNSHI described in other populations (Nayak *et al*., 2015; Riazuddin *et al*., 2006). However, conflicting reports that observed no variation in phenotype severity among affected kindreds have been reported (Chishti *et al*., 2008; Riazuddin *et al*., 2006; Zheng *et al*., 2019), relative to the present observation. The threshold of phenotype severity in affected kindred identified here were non-progressive (Taghipour-Sheshdeh *et al*., 2019), and had no correlation with age (Chishti *et al*., 2008; Riazuddin *et al*., 2006), similar to other reports. While some authors have attributed the observed phenotype variability (even for individuals with the same variant) to environmental factors (Nayak *et al*., 2015), suggestions of possible contribution of genetic modifiers as modulating this varied phenotype thresholds among affected individuals, like the index case, remains to be explained (Riazuddin *et al*., 2006). Autosomal recessive inheritance identified from the pedigree analysis in the present study, linked with post-lingual onset of ARNSHI, replicate findings in most affected families in Pakistan (Ramzan *et al*., 2005; Riazuddin *et al*., 2006). Contrary to this phenotype, different reports on *MARVELD2* variants associated HI cases, segregating autosomal recessive non-syndromic pre-lingual HI have also been reported previously (Babanejad *et al*., 2012; Chishti *et al*., 2009; Nayak *et al*., 2015). The differences in reported age of onset of *MARVELD2* variants associated ARNSHI phenotype may reflect the diversity and population specific mutations, and the predicted pathogenicity in the same protein corresponding to the associated phenotype variations. Aside the most common and reoccurring *MARVELD2* mutation (c.1331+2T>C) that has been reported in nine (9) different families in different regions (Brozkova *et al*., 2012; Chishti *et al*., 2009; Riazuddin *et al*., 2006). All the

reported pathogenic *MARVELD2* mutations reported to date are population specific, a possible basis of the current inconsistencies in reported *MARVELD2* associated ARNSHI phenotypes.

The c.1058dupT variant reported here is the second report of *MARVELD2* pathogenic exon 2 mutation to be associated with ARNSHI, in addition to the Chinese study (Zheng *et al*., 2019), relative to the exon 4, 5, 6, and 7 (Chishti *et al*., 2009; Riazuddin *et al*., 2006; Taghipour-Sheshdeh *et al*., 2019) variants identified earlier. *MARVELD2* exon 2 encodes all the 4 transmembrane domains in tricellulin protein (Kamitani *et al*., 2015) (Figure 2D). Exon 2 mutation that truncate the normal complete translation of full-length amino acid sequence (588 amino acid), resulting from a frameshift produce non-functional tricellulin, like the present report.

Full-length MARVELD2 has functional role vital for high rigidity of the tight junctions (TJs) in the organ of Corti (Riazuddin *et al*., 2006). Riazuddin *et al* demonstrated HI association with *MARVELD2* mutations in humans and mice, and generated *Tric-/-* mice that showed an early-onset progressive HI (Riazuddin *et al*., 2006). Another *MARVELD2* phenotype expression study that used a *Marveld2* knock-in mice observed different pathological disorders in varied organs other than the inner ear (Nayak *et al*., 2015). The observed phenotype was linked to cochlea hair cells destruction caused by epithelial barrier function disturbance in the inner ear (Kamitani *et al*., 2015). Comparing the wide hearing disorder observed in modified mice, affected individuals had no other organ involvement other than the ARNSHI (Brozkova *et al*., 2012; Chishti *et al*., 2009; Riazuddin *et al*., 2006). Complex expression of superfluous tight junction (TJ) in humans is understood to evade the wide phenotype characteristic of *Marveld2* mutant mice, and this has been postulated to explain the inconsistencies observed in tricellulin deficiency mice and human phenotypes (Nayak *et al*., 2015).

Four exon 4 splice site (Riazuddin *et al*., 2006), two nonsense (Babanejad *et al*., 2012; Chishti *et al*., 2009) *MARVELD2* variants from systematic reports reported, and the thymine duplication mutation in the index family are predicted to produce a truncated protein lacking the cytosolic domain for effective interaction with the three scaffolding proteins; Zonula Occludens (ZOs); ZO1, ZO-2 and ZO-3 (Kamitani *et al*., 2015; Li *et al*., 2005). This predicted pathogenicity is in tandem with most reported causal variants predicted to have pathogenic effect in other populations; ranging from nonsense protein truncating mutations (Babanejad *et al*., 2012; Chishti *et al*., 2009) to in-frame deletions (Nayak *et al*., 2015) and splice site (Ramzan *et al*., 2005) variations that affects mRNA processing and translation. MARVELD2/TRIC is concerted in tricellular tight junctions (tTJs) in the cochlea epithelium; the endolymph and perilymph fluid-filled compartment of varied ionic compositions in the organ of Corti, prime for optimal sound transduction (Higashi *et al*., 2013). Pathogenic variants in *MARVELD2* affecting the transmembrane tricellulin protein are grouped mostly in the cytoplasmic tail of tricellulin (Higashi *et a*l., 2013; Ramzan *et al*., 2005; Taghipour-Sheshdeh *et al*., 2019). Akin to the predicted truncated protein caused by the c.1058dupT mutation, the MARVELD2 produced lacks the tricellulin transmembrane domains and cytoplasmic tail, in-line with other pathogenic variants described in Pakistan (Nayak *et al*., 2015; Ramzan *et al*., 2005; Riazuddin *et al*., 2006), Iran (Babanejad *et al*., 2012; Tabatabaiefar *et al*., 2011; Taghipour-Sheshdeh *et al*., 2019), Czech Roma (Brozkova *et al*., 2012), and Slovak Roma (Mašindová *et al*., 2015).

Furthermore, the non-synonymous p.Val354SerfsTer5 mutation is predicted to produce a protein lacking transmembrane and C-terminal occluding-ELL domains (Figure 5.2D) that binds the scaffolding proteins, as the predicted pathogenic effect associated with phenotype reported earlier (Babanejad *et al*., 2012; Chishti *et al*., 2009). Variations in tricellulin affects the occludin domain interactions, interfering with its localization to TJs (Li *et al*., 2005). The

inability of these mutant tricellulin proteins to incorporate into cytosolic scaffolds with ZO proteins (Riazuddin *et al*., 2006) forms the cellular basis of the observed dysfunction in the inner ear. TRIC together with other TJ proteins, provide the seal control of lateral ion diffusion between apical and basolateral plasma membrane domains required for normal hearing (Kitajiri *et al*., 2007). Pathogenic variants in *MARVELD2* that alter the structural integrity of TJ's expressed in the inner ear sensory epithelium, may cause selective inhibition of the paracellular permeability to ions and small substances, which affects the optimum intracellular milieu required for normal functioning of cochlear hair cells (Riazuddin *et al*., 2006). MARVELD2/TRIC provides the required connection between epithelial cells, with physiological function in creating the epithelial barrier that controls paracellular flux of ions and solutes, important in maintaining the inner ear fluid ion gradient, ideal for sound mechano-transduction and normal hearing (Ikenouchi *et al*., 2005).

5.6 Conclusion

This report provides the first case of *MARVELD2* variant associated ARNSHI in Ghana (sub-region) and Africa at large. The elucidated *MARVELD2*: c.1058dupT-p.(Val354SerfsTer5) in this multiplex consanguineous Ghanaian family segregating post-lingual bilateral ARNSHI, is novel, expanding the associated phenotype and adds to the global knowledge, refining HI disease-gene pairs curation. The identified novel *MARVELD2* mutation associated with ARNSHI, needs to be studied in other unresolved multiplex familial cases for better understanding of its role and contribution to HI.

5.7 Author Contributions

Conception of the project: A.W, S.M.L, G.A.A, and L.E; recruitment and molecular experiments: E.T.A and S.M.A; exclusion of *GJB2* and *GJB6* variants: E.T.A; bioinformatics analysis: K.K.E., L.A. and I.S.; *in silico* analysis of the pathogenicity of variants: E.T.A.,

S.M.A., K..E., L.A. and I.S; protein modelling: E.T.A., K.E., and S.M.A; writing of the first draft of the manuscript: E.T.A; review and editing: all authors; supervision of the whole project: L.A., S.M.L., G.A.A. and A.W. All authors have agreed to the final version of the manuscript.

Funding: This study was possible thanks to funding from the Wellcome Trust, grant number 107755Z/15/Z to GAA and AW (co-applicants); NIH, USA, grant number U01-HG-009716 to AW, the African Academy of Science/Wellcome Trust, grant number H3A/18/001 to AW, and the National Institute of Deafness and other Communication Disorders grants R01 DC01165, DC003594 and DC016593 to S.M.L. The funders were not involved in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments: Authors are grateful to family members for their participation in this research.

Conflicts of Interest: The authors declare no competing interest.

Supplementary Material

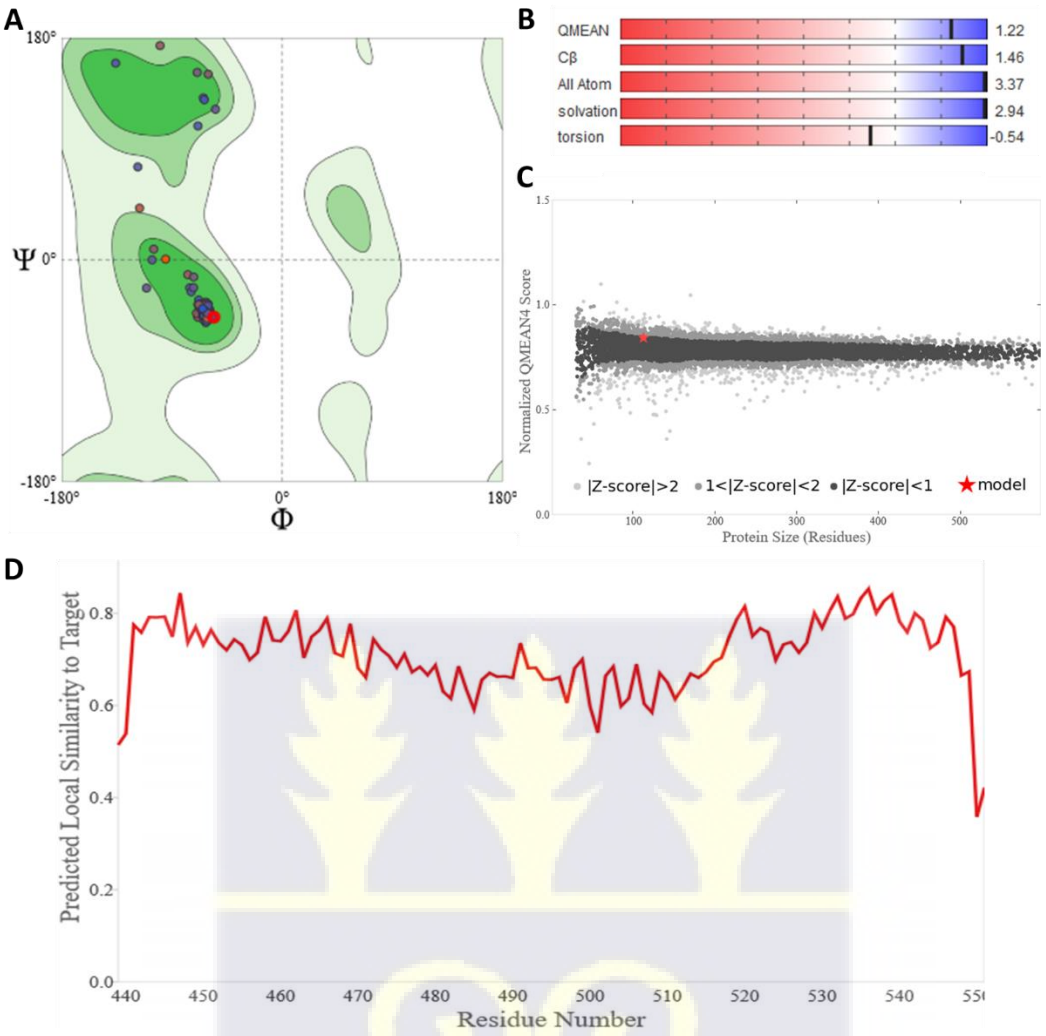

Figure S5.1. Quality assessment of MARVELD2: p.V354SfsTer5 mutant; *(A) Ramachandran plot of p.Val354SerfsTer5 mutant protein model. (B) Estimated protein model quality. (C) Comparison of the solved mutant model with non-redundant set of PDB protein structures. (D) Local quality estimate of the p.Val354SerfsTer5 mutant protein.*

CHAPTER SIX

6.0 Paper 3: Novel Autosomal Dominant *GREB1L* Variant Associated with Non-Syndromic Hearing Impairment in Ghana

Samuel Mawuli Adadey [1,2,#], **Elvis Twumasi Aboagye** [1,2,#], Kevin Esoh [2], Anushree Acharya[3], Thashi Bharadwaj[3], Nicole S. Lin[3], Lucas Amenga-Etego [1], Gordon Akanzuwine Awandare[1], Isabelle Schrauwen[3], Suzanne M. Leal [3,4] and Ambroise Wonkam [2,5]

[1.] West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, Accra LG 54, Ghana [2], Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, Cape Town 7925, South Africa [3], Center for Statistical Genetics, Sergievsky Center, the Department of Neurology, Columbia University Medical Centre, New York, NY 10032, USA [4], Taub Institute for Alzheimer's disease and the Aging Brain, Columbia University Medical Centre, New York, NY 10032, USA [5], McKusick-Nathans Institute and Department of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

[#] These authors contributed equally to the manuscript

6.1 Abstract

Background: Childhood hearing impairment (HI) is genetically heterogeneous with many implicated genes, however, only a few of these genes are reported in African populations. **Methods:** This study used exome and Sanger sequencing to resolve the possible genetic cause of non-syndromic HI in a Ghanaian family. **Results:** We identified a novel variant c.3041G > A: p.(Gly1014Glu) in *GREB1L* (DFNA80) in the index case. The *GREB1L*: p.(Gly1014Glu) variant had a CADD score of 26.5 and was absent from human genomic databases such as TopMed and gnomAD. In silico homology protein modeling approaches displayed major structural differences between the wildtype and mutant proteins. Additionally, the variant was predicted to probably affect the secondary protein structure that may impact its function. Publicly available expression data shows a higher expression of *Greb1L* in the inner ear of mice during development and a reduced expression in adulthood, underscoring its

importance in the development of the inner ear structures. **Conclusion:** This report on an African individual supports the association of *GREB1L* variant with non-syndromic HI and extended the evidence of the implication of *GREB1L* variants in HI in diverse populations.

Keywords: Hearing impairment, *GREB1L*; Ghana.

6.2 Background

Globally, over 124 genes have been implicated in hearing impairment (HI) (Camp & Smith, 2020) with connexins, especially *GJB2*, accounting for over 50% of autosomal recessive cases (Adadey *et al.*, 2020; Chan & Chang, 2014). A recent meta-analysis of HI genes from Africa identified 46 HI genes from 17 African countries confirming the heterogeneity of HI (Adadey *et al*., 2021). The most frequently associated genes with HI from Africa were *GJB2*, MYO15A, and ATP6V1B1. However, the contribution of connexins to the HI in sub-Saharan Africa is negligible except for a few cases from some countries including Ghana (Adadey *et al.*, 2019; Wonkam, 2015; Wonkam *et al.*, 2015). In Ghana, *GJB2*:p.(Arg143Trp) was reported as a founder variant (Brobby *et al.*, 1998b) and recent studies have shown that the variant accounts for about 25-42% of familial cases (Adadey *et al.*, 2019; Wonkam *et al.*, 2022).

Growth Regulation by Estrogen in Breast cancer 1 Like gene (*GREB1L*), a pre-migratory neural crest regulator, plays a vital role in early metanephros and genital development (Plouhinec *et al.*, 2014; Schrauwen *et al.*, 2018). Wild-type *GREB1L* functions in retinoic acid receptor (RAR) gene co-activation and the development of vestibulocochlear, renal system, genital tract, and ventricular tract (Brophy *et al.*, 2017). Variations in *GREB1L* have been implicated in renal hypodysplasia (aplasia 3) (RHDA3), Autosomal Dominant hearing impairment (DFNA80), and Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome (Barffour & Kwarkoh, 2021; Brophy *et al.*, 2017; Schrauwen *et al.*, 2018). Variations in *GREB1L* implicated in HI have been shown to be either autosomal dominantly inherited (with or without

reduced penetrance) or *de novo* (Kim *et al.*, 2022; Schrauwen *et al.*, 2018, 2020). Patients with HI due to variant in *GREB1L* typically display bilateral profound non-syndromic (NS) HI. Additionally, when imaging data are available cochleovestibular abnormalities are observed (Schrauwen *et al.*, 2018). When performed, a normal renal ultrasound and urine analysis were observed in investigated NSHI probands with *GREB1L* variants (Schrauwen *et al.*, 2020). In this study, we present a novel case with a heterozygous *GREB1L* p.(Gly1014Glu) variant associated with congenital profound NSHI in a Ghanaian family. Thus, describing the ninth family with a novel variant contributing to the expansion of the genetic and demographic spectrum of *GREB1L* pathogenic variants.

6.3.0 Materials and Methods

6.3.1 Study Participants

The family with an affected proband was part of a large study aimed at investigating the association of genetic markers to congenital hearing impairment among patients at the schools for the Deaf in Ghana. The affected family member was screened and negative for GJB2 pathogenic variants (Adadey *et al.*, 2019). In combination with a structured questionnaire, the medical records of the proband were evaluated to rule out any potential environmental or acquired cause of HI. The proband was examined by a medical geneticist and an ear, nose, and throat specialist. Urine analysis was conducted for the proband using Urine Test Paper Strips. To assess the hearing thresholds, pure-tone audiometric examination was performed using KUDUwave™ Audiometer eMoyo Technologies (Johannesburg, Gauteng, South Africa) (Figure 6.1). Genomic DNA (gDNA) was extracted from venous blood obtained from 3 family members [II:4, III:7, and III:9) (Figure 6.1)] QIAamp DNA Blood Maxi Kit® (Qiagen, USA).

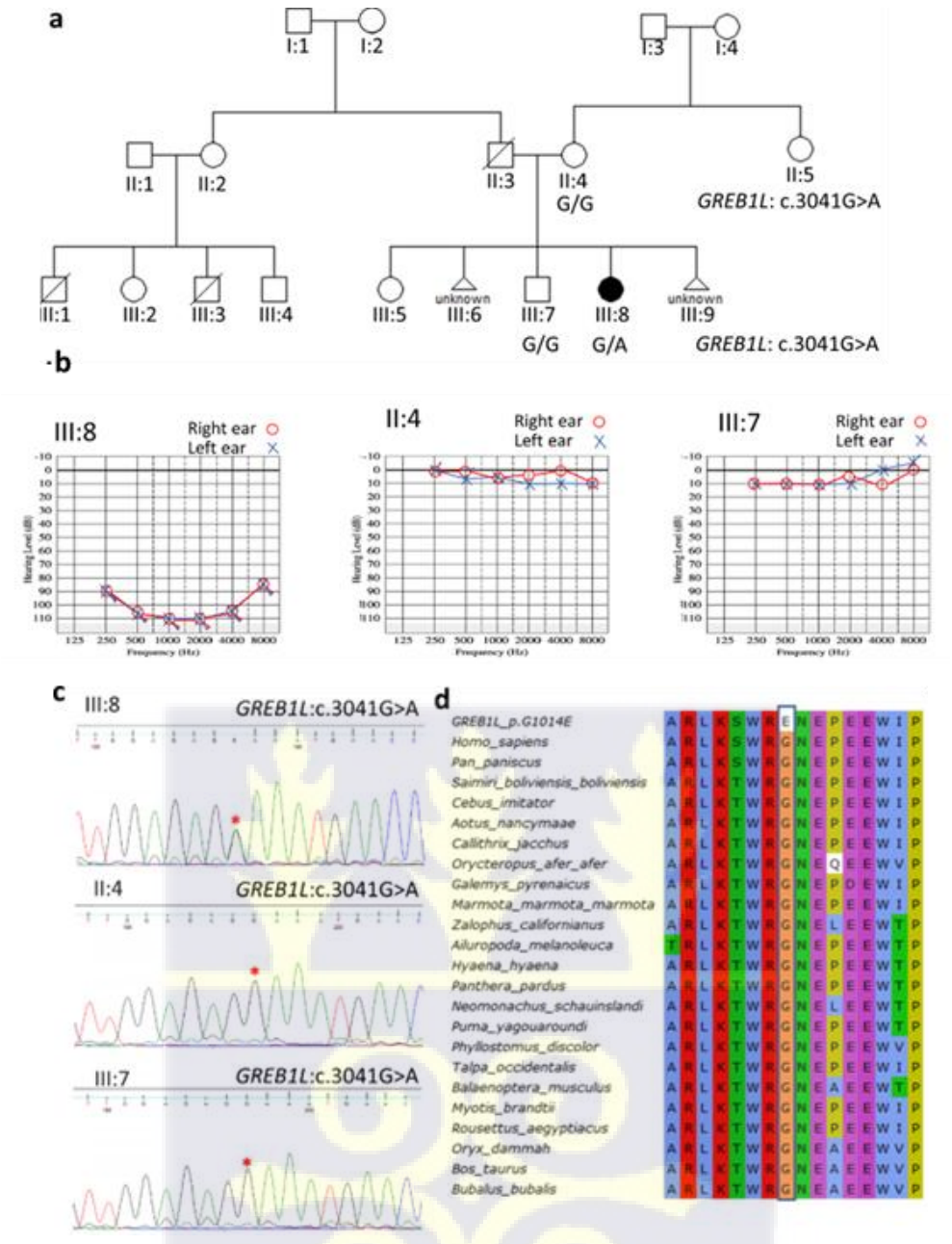

Figure 6.1. Pedigree, hearing impairment phenotype, and Sanger sequence validation of GREB1L: c.3041G > A variant. *A Pedigree and genotype of the affected child (III:8), her mother (II) and brother. Squares represent males and circles females. The individual with the filed symbol is affected and clear symbols represent individuals without hearing impairment. B Pure tone audiograms of affected and unaffected family members. C Chromatograms showing GREB1L: c.3041G > A genotypes. D GREB1L protein sequence alignment has shown conservation of the amino acid at position 1014*

6.3.2 Exome sequencing

Whole exome sequencing (WES) was performed on the gDNA obtained from the affected proband. Briefly, Quantus Fluorometer (Promega, Madison, WI) was used to assess the quality

of the gDNA prior to the exome sequencing. Exome libraries were prepared using SureSelect V4 + UTR 71 Mb All Exon Capture Kit (Agilent Technologies, Inc., Santa Clara, CA, USA), ~ 3-5 µg of the DNA was fragmented with ultrasound using a Covaris® instrument (Covaris, Inc., Woburn, MA, USA). Sequencing of the libraries was performed on Illumina HiSeq 2000 (Illumina, San Diego, CA) to produce paired end reads of 100 bp. The Illumina BaseSpace app suite was used for exome sequencing mapping and variant calling. The sequence reads were aligned to the human reference genome (hg19/GRCh37) using Illumina DRAGEN Germline Pipeline version 05.021.408.3.4.12. Reads were sorted and marking of duplicates was performed using Picard. The Genome Analysis Toolkit (GATKv4.1.7) software package (McKenna et al., 2010) was used to conduct joint variant calling for single nucleotide variations (SNV) and Insertion/Deletions (Indels). The sex of the family member that underwent WES was verified using plink (version 1.9) (Manichaikul *et al.*, 2010; Purcell *et al.*, 2007). Last, copy number variants (CNV) were called using the copy number inference from exome reads algorithm (CoNIFER) (Krumm *et al.*, 2012).

6.3.3 Annotation and Filtering

We performed annotation and filtering using an in-house pipeline built on ANNOVAR as described previously (Wang *et al.*, 2010). After checking known pathogenic variants for NSHI regardless of their frequency, filtering of SNVs and indels was performed using Genome Aggregation Database (gnomAD) (Karczewski & Francioli, 2017) with a population-specific minor allele frequency of < 0.005 [for homozygous and potentially compound heterozygous variants and variants on the X chromosome AR] and < 0.0005 for heterozygous variants. Synonymous and intronic variants that were not close to a splice site region were removed. Variants that met the above criteria were further prioritized based on in silico prediction scores from Sorting Intolerant From Tolerant (SIFT); MutationTaster; combined annotation dependent depletion (CADD); Genomic Evolutionary Rate Profiling (GERP++);

polymorphism phenotyping v2 (PolyPhen-2); and deleterious annotation of genetic variants using neural networks (DANN). The variants were further assessed with information from Hereditary Hearing Loss Homepage (HHL), Online Mendelian Inheritance in Man (OMIM), Human Phenotype Ontology (HPO), and ClinVar databases. Allele frequencies were also assessed using the TOPMed Bravo database. The American College of Medical Genetics and Genomics and Association (ACMG-AMP) guidelines for HI (Oza *et al.*, 2018) were followed to evaluate clinical significance. CNVs were annotated and filtered with AnnotSV (Geoffroy *et al.*, 2018) and an in-house pipeline that interrogates BioMart (Smedley *et al.*, 2009) and the Database of Genomic Variants (MacDonald *et al.*, 2014).

6.3.4 Sanger Sequence Validation of the Candidate Variant from WES

To verify segregation of the *GREB1L*: c.3041G > A: p.(Gly1014Glu) candidate variant, Sanger sequencing was performed for all the family members from whom a DNA sample was obtained. Allele specific primers (Forward: AAACTACAGCCCTCGTTCCT, Reverse: CCTTGAGGGGTGCAGGAATAG) were used to PCR amplify the region of the GREB1L gene containing the variant. The PCR amplicons were cleaned using exonuclease 1 and alkaline phosphatase after which they were sequenced using BigDye™ Terminator v3.1 Cycle Sequencing Kit. The sequencing products were resolved and analyzed by ABI 3130XL Genetic Analyzer® (Applied Biosystems, Foster City, CA, USA). The Sanger sequence data files were analyzed using FinchTV v1.4.0, and UGENE v34.0.

6.3.5 GREB1L Protein Modelling

The 3D structure of GREB1L [1923 amino acids (aa) long] predicted by the highly accurate AlphaFold method was retrieved as a PDB file from the AlphaFold protein structure database and used as a template for the modeling of the mutant [*GREB1L*: c.3041G > A: p.(Gly1014Glu)] structure using SwissModel. Since the downstream structure refinement by GalaxyWeb (Seok *et al.*, 2021) required the structure to be ≤ 1000aa, the protein was truncated

by removing 600aa from the N-terminal and 369aa from the C-terminal to retain a 954aa sequence that contained the mutant site. The truncation was further deemed to be desirable since there were no apparent interactions between the variant site and distant residues in the wildtype AlphaFold-predicted structure. In addition, the wildtype glycine residue (G1014) resides within a region in the protein that was predicted with high confidence by AlphaFold. The refined mutant truncated structure and the full-length wildtype structure were then analyzed using PyMol (DeLano, 2002). The truncated wildtype and mutant proteins were further analyzed on PROTTER (Omasits *et al.*, 2014) and PSIPRED v4.0 programs (Buchan & Jones, 2019) to determine the location and predictive effect of the variant on secondary structure formation.

6.3.6 Expression of Greb1l in Mouse Inner Ear

To examine the expression of GREB1 in the mouse inner ear, we obtained and analyzed single cell RNA-seq data at different developmental stages from a publicly available database, gene Expression Analysis Resource (gEAR) suite (Orvis *et al.*, 2021). The gEAR suite is a data deposition, display, analysis, and interrogation database which consist of expression data of different organisms such as mouse, human, rat, and zebrafish (Orvis *et al.*, 2021).

6.4.0 Results

6.4.1 Clinical Evaluation

No known syndrome was identified in the proband when examined by a medical geneticist. At the time of sample collection, the affected participant did not have any clinical signs of renal/kidney dysfunction, and no abnormal urine analysis parameter was observed. The affected individual (III:8, Figure 6.1) presented with profound bilateral, symmetrical sensorineural NSHI while the unaffected family members had normal hearing (Figure 6.1b).

We were unable to perform any imaging to assess inner ear malformations which may lay at the basis of the NSHI in the proband.

6.4.2 *GREB1L*: c.3041G>A-p.(Gly1014Glu) identified through exome sequencing

Whole exome sequence data was generated from gDNA samples from one affected (III:8) family member. The analysis of the exome data identified a mono-allelic *GREB1L* variant (NM_ 001142966.2: c.3041G > A) in the affected individual (Table 6.1). The variant was predicted as variant of uncertain significance with some pathogenic evidence based on the ACMG guidelines (Green *et al.,* 2013) and Varsome (Kopanos *et al.,* 2019), and it was predicted to be pathogenic by different bioinformatic predictive tools including SIFT, PolyPhen, and FATHMM (Additional file 6.1: Table S6.1). In addition, the variant is absent from the Deafness Variation Database (DVD), gnomAD and TopMed databases and its position is conserved amongst species (phyloP100way = 5.3). Family members with normal hearing were homozygote wildtype (G/G) (Figure 6.1a). The identified missense c.3041G > A variant was confirmed by Sanger sequencing (Figure 6.1c). No relevant CNVs were identified.

Table 6.1. *GREB1L* variants associated HI

Family type	cDNA change*	amino acid change	Mode of inheritance	CADD score (v1.6)	TopMed	gnomAD v3.1.2	Hearing loss	Inner ear Imaging Phenotype	Country	Reference
Isolated	c.347C>T (rs1333304296)	p.(Thr116Ile)	AD**	27.2	Absent	Absent	Profound bilateral SNHI	Bilateral cochlear aplasia, bilateral dysplastic vestibule, and semicircular canals; bilateral cochlear nerve aplasia	Egypt	(Schrauwen *et al.*, 2020)
Familial	c.848A>G	p.(Asn283Ser)	AD	16.2	Absent	Absent	Profound bilateral SNHI	NA	Pakistan	(Schrauwen *et al.*, 2020)
Isolated	c.982C>T (rs1555648043)	p.(Arg328*)	de novo/ AD**	36.0	Absent	Absent	Profound bilateral SNHI	Case 1: Bilateral cochlear aplasia, bilateral dysplastic vestibule, and semicircular canals; bilateral cochlear nerve aplasia. Case 2: Cochlear hypoplasia type 1 (right): Cochlear aplasia with dilated vestibule (left)	US, Korea	(Kim *et al.*, 2022; Schrauwen *et al.*, 2018)
Isolated	c.1079T>A	p.(Leu360*)	AD**	36.0	Absent	Absent	Profound bilateral HI	Cochlear aplasia with dilated vestibule (right): between common cavity and Cochlear aplasia with dilated vestibule (left)	Korea	(Kim *et al.*, 2022)
Isolated	c.3041G>A	p.(Gly1014Glu)	AD or de novo	26.5	Absent	Absent	Profound bilateral SNHI	NA	Ghana	This study
Isolated	c.4368G>T	p.(Glu1410fs)	de novo	36.0	Absent	Absent	Profound bilateral SNHI	Unilateral cochlear aplasia, unilateral incomplete partition type I, bilateral dysplastic vestibule, and semicircular	US	(Schrauwen *et al.*, 2018)

								canals; bilateral cochlear nerve aplasia		
Isolated	c.5618T>C	p.(Leu1873Pro)	AD**	28.9	Absent	Absent	Profound bilateral HI	Bilateral common cavity	Korea	(Kim *et al.*, 2022)

AD = Autosomal Dominantly inherited; NA: Not Available, CADV = cochlear aplasia with dilated vestibule, R = right, L = left

6.4.3 *In silico* GREB1L protein analysis

Protein sequence alignment was conducted to study the evolutionary conservation of amino acid position 1014 of the GREB1L protein (Figure 6.1d). The position 1014 was found in the intracellular domain of the protein (Figure 6.2a), and glycine (G) was conserved at this position for all the species studied except for *Xenopus tropicallis* and *Takifugu rubripes* (Figure 6.1d) which are evolutionarily distant from mammals. It is however worth mentioning that the amino acid at this position was conserved in the mammalian species studied, suggesting its importance to the structure of *GREB1L* protein. The change from a neutral amino acid, glycine, to a negatively charged glutamate (E) at this position likely affected the protein structure and function. Analysis of the protein models showed that there was an excellent superimposition between the full-length wildtype structure and the mutant structure (Figure 6.2b). Comparison of the superimposed structures revealed several secondary structural changes among the wildtype and mutant structures including: the gain of a short helix in the mutant structure in a region that formed a loop in the wildtype structure between residues serine-1469 and glycine-1474 (i.e., ^{1469}SSMLG^{1474}) (Fig. 2c), a shortening of a helix around residues leucine-1559 and tyrosine-1560 (i.e., ^{1559}KY^{1560}) and a shortening of a beta strand between residues leucine-1544 and valine-1549 (i.e., ^{1544}LHLLVV^{1549}) in the mutant (Figure 6.2d), the extension of a helix between residues aspartate-1093 and glycine-1096 (i.e., ^{1093}DLSG^{1096}) and between threonine-923 and threonine-924 (i.e., ^{923}TT^{924}) in the mutant (Figure 6.2e and f). PSIPRED, a bioinformatic tool, also predicted that the E1014 residue in the mutant forms a helix which is absent in the wildtype (blue rectangles in Additional file 6.1: Fig. S6.1). Other major secondary structural changes were found when the wildtype was compared to the mutant (red rectangles in Additional file 6.1: Figure S6.1).

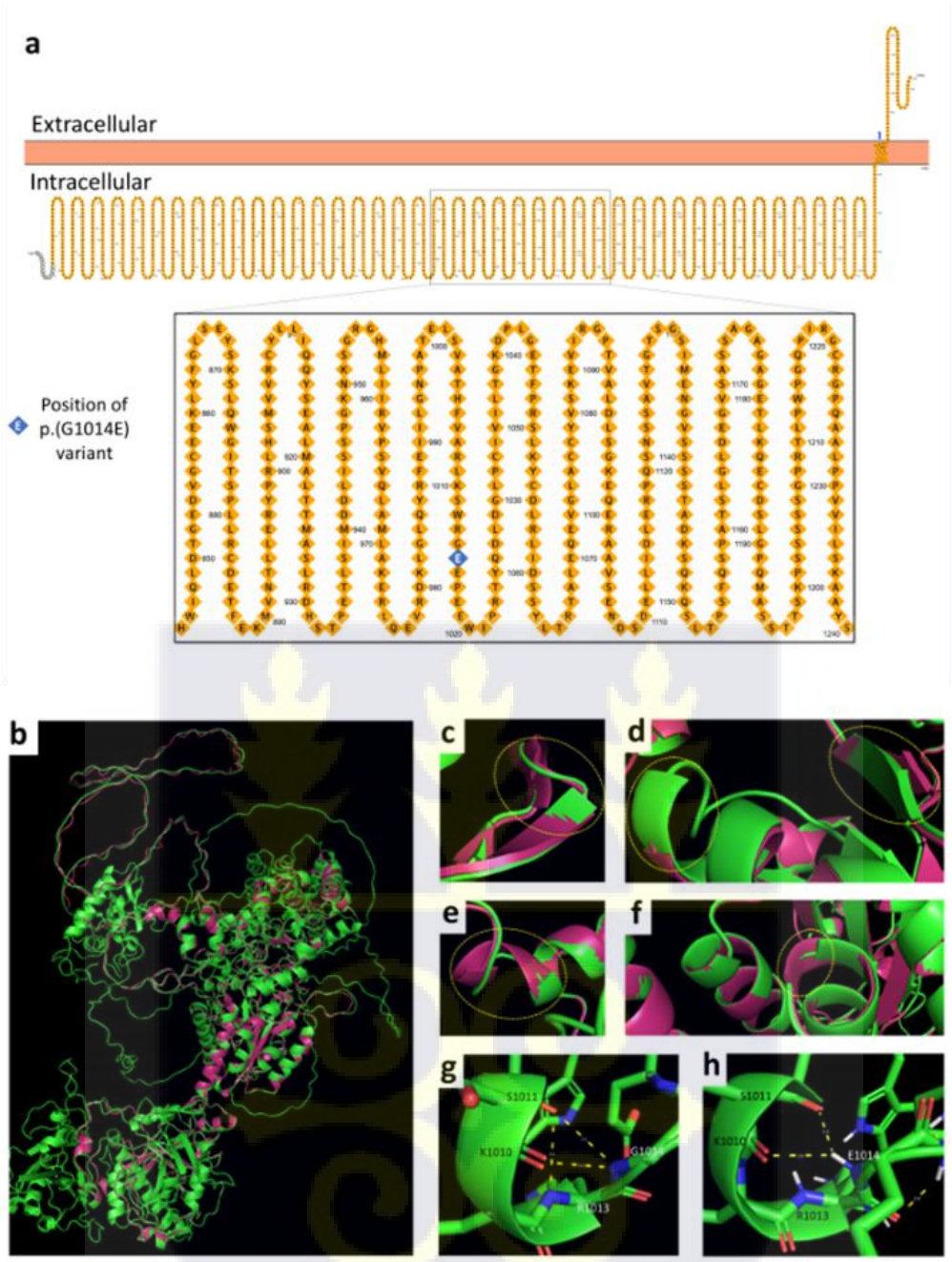

Figure 6.2. In silico GREB1L protein analysis showing structural variations between the modelled wildtype and mutant proteins. *A. Cellular organization of GREB1L as predicted using PROTTER [28], an online bioinformatic tool. Homology modelling represented in B Superimposition of full-length wildtype and truncated mutant GREB1L structures. C Formation of helix at 1469SSMLG1473 in the mutant. D shortening of helix at 1559KY1560 and beta strand at 1544LHLLVV1549 in the mutant. E and F Extension of helices at 1093DLSG1096 and 923TT924 in the mutant. Yellow circles denote sites of different secondary structural changes of the mutant compared to the wildtype structures. Hydrogen-bond formation of G wildtype and H mutant residues with nearby residues. H-bond between K1010 and R1013 residues is lost in the mutant, and the H-bond lengths are shortened in the mutant compared to the wildtype.*

The wildtype G^{1014} residue is predicted by AlphaFold to form two hydrogen (H-) bonds with lysine-1010 (K^{1010}) and serine-1011 (S^{1011}), while K^{1010} forms another H-bond with arginine-1013 (R^{1013}) (Figure 6.2g). Interestingly, although the H-bonds formed with the K^{1010} and S^{1011} residues are retained in the mutant structure, the H-bond bond between the K^{1010} and R^{1013} residues is lost (Figure 6.2h). In addition, the H-bonds formed by the mutant E^{1014} residue appear to be shorter (hence stronger) than those formed by the wildtype residue. Therefore, it is probable that the mutant residue imposes a change in the geometry of nearby residues, particularly the arginine residue. Indeed, the mutant E^{1014} residue is larger than the wildtype G^{1014} residue in addition to being polar charged.

6.4.4 Expression of Greb1l in Mouse Inner Ear

Our study further explored single cell RNA-seq (scRNA-seq) data from gEAR to study Greb1l expression in the developing inner ear (Orvis et al., 2021), as scRNA-seq data had not been interrogated in previous studies. The scRNA-seq data covered expression of Greb1l in spiral, glia, and hair cells obtained at six developmental stages (E15.5, P1, P8, P12, P14, and P30). Greb1l expression was the most prominent in the developing spiral ganglion, where it was upregulated at the E15.5 developmental stage decreased over the developmental stages (Additional file 6.1: Figure S6.2). In the glia and hair cells, it was up regulated at P8 and P12 developmental stages respectively (Additional file 6.1: Figure S6.2). The Greb1l expression data supports its critical role in the development and functioning of the inner ear and cochlear nerve.

6.5.0 Discussion

Using WES, we identified a previously unreported variant in *GREB1L* [c.3041G > A: p.(Gly1014Glu)], associated with the NSHI phenotype in an individual of African ancestry from Ghana, and a wider investigation in other African countries and diaspora is needed. This finding

presents the first report of a *GREB1L* variant association with HI from sub-Saharan Africa. The identified heterozygous missense p.(Gly1014Glu) variant was predicted as a possible loss of function mutation that suggests haploinsufficiency as the pathological basis for the associated phenotype, and this is consistent with earlier *GREB1L* reports (Sanna-Cherchi *et al.,* 2017; Schrauwen *et al.*, 2018).

Variants in *GREB1L* were associated with HI in individuals from the US, Egyptian, Korean, and Pakistani populations (Kim et al., 2022; Schrauwen *et al.*, 2018, 2020). Two *de novo GREB1L* pathogenic variants; p.(Glu1410fs) and p.(Arg328*) were associated with profound HI, cochlear aplasia, incomplete partition type I (IP-I) and cochleovestibular nerve malformations (Schrauwen *et al.*, 2018). An inherited missense variant; p.(Asn283Ser) was found to segregate with HI in a Pakistani family with 3 members having profound bilateral NSHI (Schrauwen *et al.*, 2020). Similarly, a missense variant [p.(Thr116Ile)] in the gene was reported in NSHI with bilateral cochlear aplasia and cochlear nerve aplasia in Egyptian family (Schrauwen *et al.*, 2020). Recently, three additional Korean cases with severe inner ear malformations were reported with rare heterozygous *GREB1L* variants [p.(Arg328*), p.(Leu360*), p.(Leu1873Pro)] (Kim *et al.*, 2022) (Table 6.1). Most of the GREB1L variants associated with HI (6/7) were in the intracellular domain of the protein and only one variant was found in the extracellular domain. The extracellular domain variant [p.(Leu1873Pro)] was associated with a less severe inner ear malformation (Table 6.1; Additional file 6.1: Figure S6.3). The increased severity of inner ear malformations observed in patients with intracellular domain variants may be due to the reduction or disruption of the protein's gene regulatory activity which is associated with this domain (Rae *et al.*, 2005).

GREB1L (OMIM: 617,782) is located on 18q11.1-q11.2 of the human genome and has been implicated in autosomal dominant deafness and renal hypodysplasia/aplasia. Although the precise

function of GREB1L remains uncertain, its involvement in the neural crest suggests its associated disorders are neurocristopathies (Vega-Lopez *et al.,* 2018). *GREB1L* was predicted to be involved in retinoic acid signaling based on its similarity with *GREB1* (Brophy *et al.,* 2017). The molecular mechanism of HI pathogenesis of *GREB1L* remains unclear, however, *Greb1L* knockout mice were reported to develop severe craniofacial abnormalities and RNA-Seq data from laser capture micro-dissected (LCM) mouse tissues during craniofacial development shows that Greb1l was preferentially expressed at the early stages of mouse development (Brunskill *et al.,* 2014; Schrauwen *et al.,* 2018). In zebrafish, greb1l has been implicated in Hoxb1 and Shha signaling, with critical role in pathways in the inner ear and cranial nerve development (Sanna-Cherchi *et al.,* 2017; Vogel *et al.,* 2016; Webb *et al.,* 2012). The inner ear imaging examination for the proband would have been relevant for the comprehensive description of the associated phenotype however, it was not conducted due to major challenges faced at the time of participant recruitment and sample collection. Yet, we believe it is important to report these cases in the African population, even though we are typically more limited in clinical phenotyping in these populations.

Similar to the gene's involvement in multiple tissues development as seen in mice studies (Tommaso *et al.,* 2017), pleiotropic effects are seen in humans. Several studies associated variants in *GREB1L* to congenital kidney malformations/agenesis (Boissel *et al.,* 2018; Tomasi *et al.,* 2017; Herlin *et al.,* 2019), urogenital adysplasia, and Mayer-Rokitansky-Kuster-Hauser syndrome (Barffour & Kwarkoh, 2021; Jacquinet *et al.,* 2020) suggesting its role in the functioning of the urogenital systems. However, at the time of sample collection, the proband did not show any sign/symptoms of urogenital disorders. Furthermore, urine analysis showed no signs of kidney/urinary tract disorders in the proband.

Previous studies have shown that *GREB1L* pathogenic variants exhibits a maternal bias inheritance which may be explained by imprinting or low male fertility due to *GREB1L* variants (Tomasi *et al.*, 2017; Schrauwen *et al.*, 2020). The mother of the index case was unaffected, which does not favor the maternal bias observation from the previous reports. The variant p.(Gly1014Glu) identified in this study may be a de novo variant since it was absent in the unaffected mother and brother. Biological samples were not obtained from the deceased father of the affected child and hence his genotype is unknown. Nonetheless, the low rate of paternal inheritance of *GREB1L* variants (Tomasi *et al.*, 2017; Herlin *et al.*, 2019) and absence from gnomAD/TopMed supports our claim of the *GREB1L*: p.(Gly1014Glu) as a likely *de novo* variant.

6.6.0 Conclusion

Using exome sequencing, we identified a variant in *GREB1L* [p.(Gly1014Glu)] as the possibly associated genetic cause of HI in a Ghanaian individual with profound HI. In silico techniques predicted the novel missense substitution as the likely cause of pathogenicity which led to the observed HI phenotype. This was evident in major structural difference observed between the wildtype and mutant *GREB1L* modelled protein, which is likely to affect the protein function. *GREB1L* variants should be investigated in other African populations and its inclusion in hearing panels should be considered.

Availability of data and materials

GREB1L: p.(Gly1014Glu) Sanger sequence generated from the proband was submitted to GenBank with the accession code ON390796. Data on *GREB1L*: p.(Gly1014Glu) variant has been added to dbSNP and will be publicly available when the next dbSNP Build (B156) is released (https://www.ncbi.nlm.nih.gov/snp/). All other relevant data supporting the key findings of this study are available within the article and its Supplementary Material. Due to lack of ethical

approval, individual-level whole-exome sequence data cannot be made publicly available; however, it can be obtained from the corresponding author [A.W.] upon reasonable request.

Abbreviations

HI	Hearing impairment
GREB1L	GREB1-like retinoic acid receptor coactivator
PLP	pathogenic or likely pathogenic
CADD	Combined Annotation Dependent Depletion
NSHI	non-syndromic hearing impairment
MRKH	Mayer–Rokitansky–Küster–Hauser
WES	Whole exome sequencing
SNV	Single-nucleotide variant
AD	Autosomal dominant
GERP++	Genomic Evolutionary Rate Profiling
DANN	deleterious annotation of genetic variants using neural networks
HHL	Hereditary Hearing Loss Homepage
OMIM	Online Mendelian Inheritance in Man
HPO	Human Phenotype Ontology
ACMG-AMP	American College of Medical Genetics and Genomics and Association for Molecular Pathology
GEO	Gene Expression Omnibus
SHIELD	Shared Harvard Inner-Ear Laboratory database

Acknowledgements: We are extremely grateful to all participants who gave their consent and samples for the accomplishment of the objectives of this project.

Ethical consideration: The study was conducted in accordance with the Helsinki Declaration for participant's well-being and safety. We obtained ethical clearance from the Noguchi Memorial Institute for Medical Research Institutional Review Board (IRB), University of Ghana (NMIMR-

IRB CPN 006/16-17), College of Basic and Applied Sciences, Ethics Committee for Basic and Applied Sciences (ECBAS 053/19-20), University of Ghana, and Faculty of Health Sciences Human Research Ethics Committee, University of Cape Town (HREC 104/2018). The study was explained to all participants in their preferred language and a written informed consent was obtained from each participant prior to their enrolment on the project.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

Funding: This work was supported with funds received from the U.K. government; the National Institutes of Health (NIH), USA, grant number U01-HG-009716 to A.W; and the African Academy of Science/Wellcome Trust, grant number H3A/18/001 to A.W. The funders had no influence in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Contributions

Conception of the project: AW, SML, IS, GAA; participant recruitment and molecular experiments: ETA and SMA; bioinformatics analysis: AA, TB, NSL, IS, KE, LA; in silico analysis of the pathogenicity of variants: ETA, SMA, IS, KE; protein modeling: KE, ETA, SMA; writing of the first draft of the manuscript: ETA, SMA; review and editing: all authors; supervision of the whole project: SML, LA, GAA, IS and AW. All authors have agreed to the final version of the manuscript.

Supplementary Material

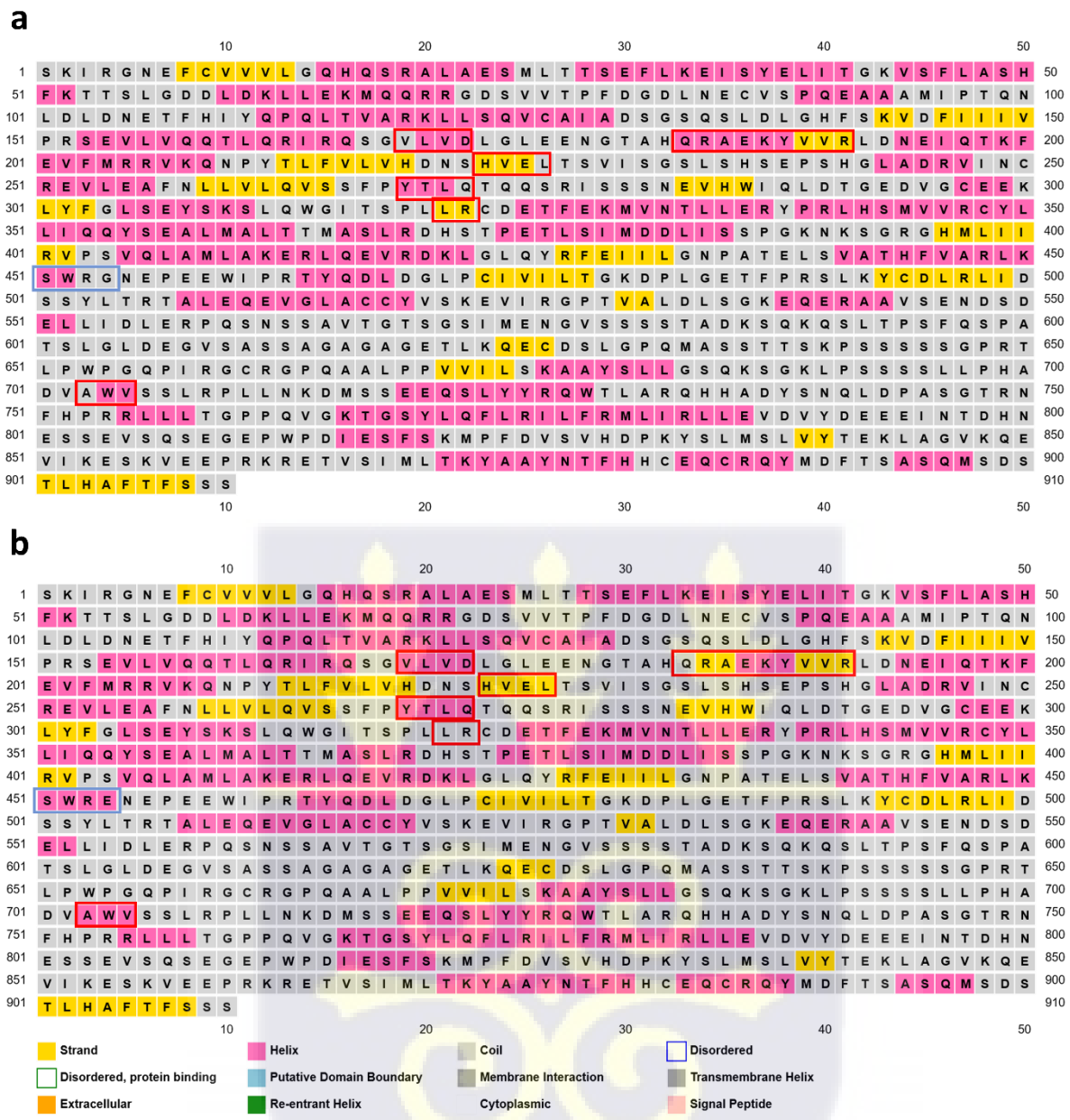

Figure S6.1 Secondary structure prediction of GREB1L protein. *The effect of the variant on secondary structure formation was examined using PSIPRED (Buchan & Jones, 2019), a bioinformatic tool. Predicted secondary structures for the (A) wildtype and (B) mutant proteins. Blue rectangles were used to indicate the absence and presence of a helix at the mutation site of the wildtype and mutant proteins respectively. Red rectangles were used to highlight the sites where differences were observed in the structures of the wildtype compared to the mutant.*

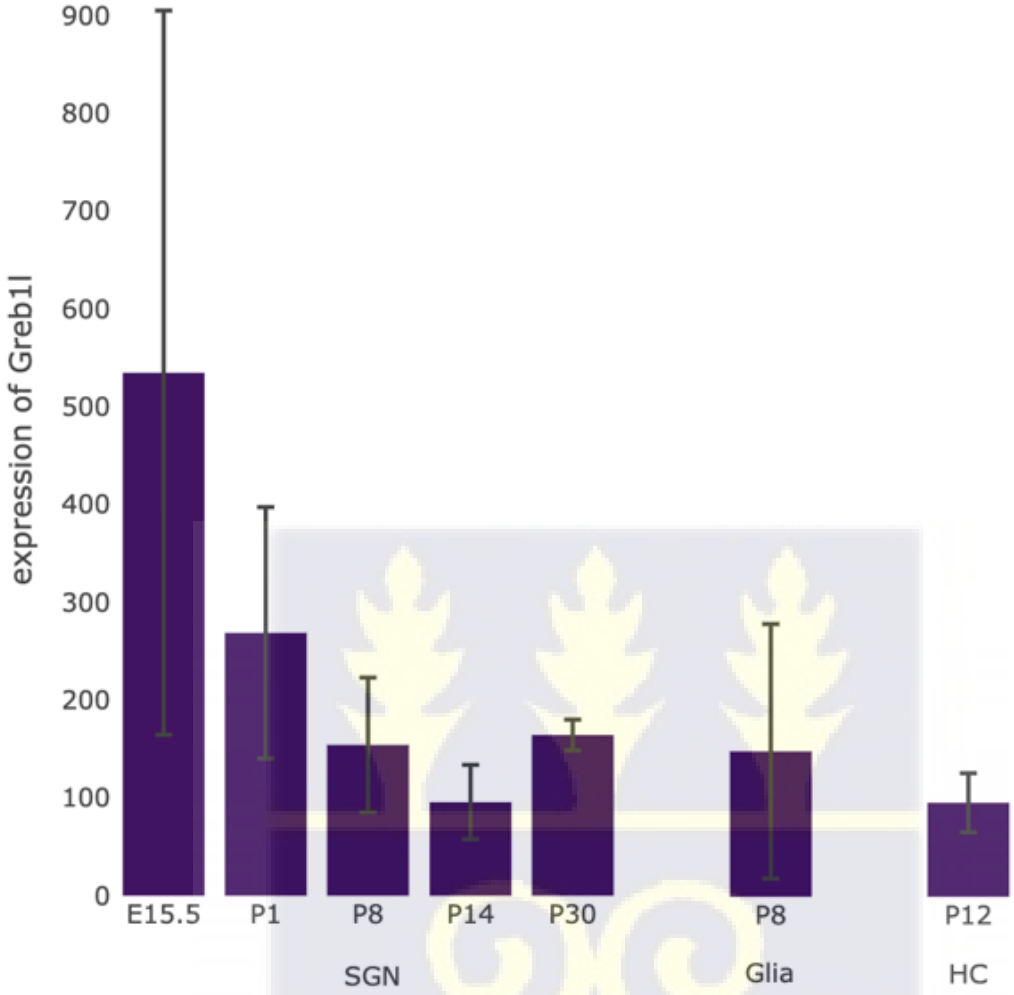

Figure S6.2. Single cell RNA expression of Greb1l at different developmental stages in the mouse inner ear. *The spiral ganglion (SGD), glia, and hair cell (HC) RNA-seq data sets were retrieved from gEAR (Orvis et al., 2021).*

Supplementary Table 6.1. In silico prediction of clinical significance/pathogenicity

Variant database/browser or prediction tool	Evidence/Score	Prediction
ACMG	PM2_supporting, PP3_supporting, PS2_moderate	Uncertain significance
SIFT	0.024	Damaging
PolyPhen	0.82	Damaging
PolyPhen-2	0.99	Damaging
FATHMM-MKL	0.9779	Damaging
FATHMM-XF	0.089	Neutral
MutationTaster	0.81	Disease causing
EIGEN	0.857	Pathogenic
CADD score	26.5	Deleterious
MetaLR	0.54	Damaging
Revel	0.36	Benign
PrimateAI	0.54	Tolerated
PhyloP100way_vertebrate	5.305	Evolutionally conserved
GERP_RS	5.42	Conserved across multiple species
EIGEN	0.74	Pathogenic

CHAPTER SEVEN

7.0 General Discussion

To better understand the genetic contribution to congenital and/or early onset NSHI in Ghana, whole-exome sequencing was performed followed by bi-directional Sanger sequencing in multiplex and simplex cases. Following variant quality filtration, annotations and prioritization, and standard ACMG guidelines, *GJB2* cumulatively accounted for between 37-42% in families investigated combining previous studies and further re-evaluation from current data. These finding replicates earlier reports (Adadey *et al*., 2019; Brobby *et al*., 1998; Hamelmann *et al*., 2001) of *GJB2* mutation association with NSHI in Ghana, as an African exception similar to the gene's contribution to HI among Caucasians (Laer *et al*., 2001; Tsukada *et al*., 2015; Vona *et al*., 2015). Despite a recent report of *GJB2* variants identification among Senegalese HI cohorts, where the founder mutation (*GJB2*: c.427 C>T-p.Arg143Trp) reported in Ghana accounted for 4.5% (2/44) of families investigated (Dia *et al*., 2022). Consistent with *GJB2* deleterious alleles that are ethnic and population specific, *GJB2*: c.94C>T-p.(Arg32Cys) was observed in 25% (11/44 families) of individuals investigated, of which 80% (12/15) had consanguineous family history (Dia *et al*., 2022).

This Senegalese report has generated interest to further explore the contribution of *GJB2* variants in the sub-region, and compute haplotype diversity to investigate the ancestral background of homozygote individuals in Senegal and elucidate the migration route of the variant spread. Apart from the North African countries, the contribution of *GJB2* mutations to HI has been limited to the p.Arg143Trp founder mutation in Ghana (Brobby *et al*., 1998; Hamelmann *et al*., 2001), and *GJB2*: c. del35G and Arg32Cys in Mauritania (Moctar *et al*., 2016) until the recent report of the *GJB2* variants in Senegal (Dia *et al*., 2022).

Aside *GJB2*, variants in *CDH23* (10/105; 9.5%) and *MYO15A* (8/105; 7.6%) were the most recurrent gene mutations associated with HI out of 23 known HI genes implicated in families investigated. This observation further supports prioritization of these two genes in addition to *GJB2* for clinical considerations in Ghana (Wonkam *et al*., 2022). Variants in *CDH23* has been reported as important HI contributor in different populations, with relatively high frequencies in Asia (Japan and Korea) (Astuto *et al.*, 2002; Xu *et al.*, 2019). ARNSHI and Usher syndrome type 1D association with variants in *CDH23* has been described (Siemens *et al*., 2002). Two variants identified by this report has been associated with HI previously in Spain (Bujakowska *et al.*, 2014) and Ireland (Zhao *et al*., 2015) c.5237G>A-p.(Arg1746Gln), and in French Canadians from Quebec c.2206C>T-p.(Arg73 6Ter) (Ebermann *et al*., 2007). Notably, the non-syndromic phenotype focus of this study may indeed have recruited non-syndromic participants, however it is possible the associated syndromic disorder may have been overlooked considering the age of some affected participants during the time of recruitment and general assessment. A specific case is the onset of retinitis or vision loss in Usher syndrome (*CDH23* variants) which may have been present but at subclinical and missed at the time of examination (Wonkam *et al*., 2022).

Globally, *MYO15A* variants contribution to severe-to-profound ARNSHI is ranked third to fourth (Rehman *et al.*, 2016; Zhang *et al.*, 2019). Comparing this finding to previous reports of *MYO15A* HI associated variants in the continent, the current frequency is higher than the 2.0% reported in Nigeria, 2.0% in South Africa, 2.3% in Tunisia, and 3.0% in Morocco (Yan *et al.*, 2016). However, a study in Egypt had reported 15.3% frequency (Farjami *et al*., 2020) higher than what we observed. This further underscores the significance of *MYO15A* gene in the hearing process (Budde *et al*., 2020). WES investigation identified *MYO15A* variants in 22.2% Cameroonian HI individuals with putative genetic aetiology suggesting the gene as the most common cause of HI (Wonkam *et al*.,

2020). The elucidated four variants implicated by this study among our cohorts [c.4778A>G-p.(Glu1593Gly), c.6551G>C-p.(Cys2184Ser), c.9947A>G-p.(Gln3316Arg), and c.6518C>T-p.(Ser2173Phe)] are consistent with novel variants reported by most studies (Farjami *et al*., 2020).

The calcium and integrin binding family member 2 (*CIB2*) (OMIM:605564) mapped to chromosome 15q25.1 encodes calcium and integrin binding proteins containing 3 or 4 EF-hand domains that changes conformation on binding to calcium ions. By this affinity for calcium ions, it facilitates intracellular calcium signalling, with conserved roles in calcium homeostasis (Riazuddin *et al.*, 2012). *CIB2* also referred to as DNA-dependent protein kinases (DNA-PK) functions in double-strand DNA breaks repair and V(D)J recombination during lymphoid development. Cloning and expression investigation by RT-PCR analysis had demonstrated the ubiquitous expression in varied human tissues and can cause DFNB48 or Usher syndrome 1J (USH1J). Wide expression of *CIB2* in human and mouse (adult and embryonic) tissues, including cells in the inner ear and retina has been described (Riazuddin *et al.*, 2012).

In 54 Pakistani families with DFNB48, Riazuddin *et al.* (2012) identified homozygous *CIB2*: c.272T>C-p.(Phe91Ser) as implicated in the phenotype segregation. Subsequent report found two additional homozygous *CIB2* mutations in other families with DFNB48: c.297C>G-p.(Cys99Trp) and c.368T>C-p.(Ile123Thr). *CIB2* mutations is thought to account for close to 7.25% of Pakistani families with ARSHI (Riazuddin *et al*., 2012). Consistent with the Pakistani family, we identified homozygous *CIB2*: c.89T>C-p.(Lys30Pro) as the causal variant for ARSHI in a multiplex family. WES of families in Nigeria identified CIB2 variant in suspected compound heterozygote with another variant (Adeyemo *et al*., 2022).

In addition, *Cib2* knockdown in zebrafish showed mutant phenotypes, including marked reduction in the quantity and function of mechanosensory hair cells that failed to respond to acoustic stimuli,

and unable to remain upright during swimming. In Drosophilia, *Cib2*-related gene decreased photo response amplitude and impaired responses to flicker stimuli at high frequencies. The observed phenotypes suggested loss of *Cib2* function to cause defects in calcium regulation and may likely contribute to mechano-transduction deficiencies and defects in photoreceptor maintenance (Riazuddin *et al*., 2012). Similarly, in adult mouse, *Cib2* was observed to localized in the cytoplasm of supporting cells and found to be concentrated in sensory hair cells stereocilia tips as well as vestibular hair cells. This localization may reflect its functioning in calcium signalling that regulates mechano-electrical transduction. *Cib2* was also found in portions of photoreceptor cells and in pigmented epithelium of the retina.

Radixin (*RDX*: 179410), a cytoskeletal protein with structural role in linking actin to the plasma membrane has been associated with DFNB24. *RDX* pathogenic variants had been reported in three Pakistani families as implicated in DFNB24 phenotype. *In silico* prediction of the variants suggested disruption or truncation of the actin-binding domain of the protein. In consanguineous Pakistani families, homozygous *RDX*: c.1732G>A-p.(Asp578Asn), c.463C>T-p.(Gln155Ter), and one-base insertion in *RDX* (1404insG) were associated with DFNB24 (Khan *et al*., 2007). The mutation disrupts the actin binding domain that alters the reading frame, causing a frameshift to truncate protein synthesis premature, which results in a protein that lacks the actin-binding domain (Khan *et al*., 2007). In an Iranian consanguineous family with DFN24 (congenital severe -to-profound), a homozygous intron transition *RDX*: (689 +1G-A) was implicated in 4 siblings. The variant was predicted to culminate in premature or nonsense-mediated decay of the mRNA. Subsequent screening identified the variant in 53 Iranians and 133 European hearing controls (Shearer *et al*., 2009) in contrast to the earlier pathogenicity. Consistent with these reports, we report *RDX*: (NM_001260495.1): c.427G>A-p.(Asp143Asn) in a Ghanaian family in compound

heterozygote state with a known pathogenic *CDH23* variant. Though ACMG classify the variant as variant of uncertain significance (VUS), with conflicting interpretation or pathogenicity on ClinVar, this report from Ghana adds to the two previous submissions and contributes to the global gene-pair curation. On structure analysis, a substitution of aspartate to asparagine side chain is not a large change and is likely to or not to affect the protein function. However, since aspartate residue at amino acid 490 position has high conservation score across 200 species (conservation = 0.9), its functional significance in the protein cannot be ignored, despite the low disease propensity (0.99) and natural variant allele frequency of 0.0001988 (https://bio.tools/VarMap). The ARSHI (DFNB24) associated mutations may cause changes in the inner ear nerve receptors, affecting the nerve pathways and interaction with the brainstem that relays sound signals received.

Using immunocytochemistry assays of inner hair cells, Pataky *et al*. (2004) showed *RDX* expression in chicken, frog, mouse, and zebrafish hair bundles. After further microscopic analysis, the authors suggested that *RDX* likely function as anchor of the actin filaments pointed ends to the stereocilia membrane. In addition, mouse homozygous for targeted *RDX* mutation exhibited mild degenerative changes in the liver, with hyperbilirubinemia (Kikuchi *et al.*, 2002). Profound deafness associated with progressive stereocilia hair cells degeneration cochlea was observed in adult homozygotes mouse (http://www.informatics.jax.org/marker/MGI:97887).

This study illuminates the proof of concept that additional gene and variant discovery investigations are needed, particularly for the unexplored populations in sub-Saharan Africa known to harbour higher genetic diversity. NSHI genetic heterogeneity burden remains enriched when characterized by ancestry, type of variant, level of expression, penetrance, mode of inheritance, and with subtle phenotypic variability (inter- and intra-familial), emphasizing the significance of genetic factors and modifiers contribution to the underlying genetic structure. The array of genes

elucidated and enriched by the discovery of candidate novel variants in known HI genes set demonstrate the multigenic function in the hearing process and widens the phenotype presentations.

Given the allelic heterogeneous status of HI (Camp & Smith, 2019), using NGS platforms, particularly WES have recently gained attention with enhanced variant discovery rate (Bamshad *et al.*, 2011; Yan et *al*., 2013). Cumulatively, the overall positive rate of *GJB2* from systematic reports by the group demonstrated *GJB2* contribution of close to 50% in families investigated (Adadey *et al*., 2019; Adadey, Wonkam-Tingang, *et al*., 2020; Wonkam *et al*., 2022).

The estimated age of the most predominant founder variant in Ghana shows selection in earlier than the report in Japan, further illuminating the independent origin of the variant in Ghana (Aboagye *et al*., 2022). Understanding how this deleterious allele is distributed across different settings and the underlying haplotypic background carrying the variant segregation has clarified the multiple independent origin hypothesis on the variant ancestry and epidemiology (Shinagawa *et al*., 2020; Tsukada *et al*., 2015). Though a recessive trait, and the mutation load is expected to increase by consanguineous history, and likely to be fuelled by high selective mating, the observed high prevalence may have been counteracted by a strong genetic force (gene flow) due to population expansion.

Interestingly, all the haplotypes of *GJB2*-p.Arg143Trp homozygous samples appear to have derived from two haplotypes, Hap2 (in which only rs9578260 is mutated) and Hap3 (in which only p.Arg143Trp is present). Hap2 and Hap3, in turn clearly evolved independently from Hap1 (the root haplotype in which none of the position is mutated), Supplementary Table S4.5, and S4.6. Hap2 being the first to have evolved given its higher frequency (Supplementary Table S4.4). This opens a new set of questions, such as: did p.Arg143Trp evolved at least two independent times in Ghana? Or did it occur once in the Hap3 background and then subsequently transferred to the Hap2

background by recombination? Given the higher frequency of the rs9578260 as compared to p.Arg143Trp, it seems plausible that it emerged earlier on the evolutionary timescale (Supplementary Table S4.4, and S4.5). Nevertheless, considering that rs9578260 is a benign intron variant on which there is conceivably no appreciable evolutionary pressure, it implies that p.Arg143Trp could still have emerged first, however, it remained low in the population due to its associated reduced relative fitness (deleterious effect).

Historically, the human race has undergone sweeping changes in population size dating over 100,000 years owing to range expansions, bottleneck events and stages of rapid growth (Henn *et al*., 2012, 2012; Nielsen *et al*., 2017). The impact of such demographic fluctuations on the efficacy of natural selection, relative to genetic drift towards the frequency and distribution of deleterious alleles across populations has been proffered (Charlesworth, 2009). Reported improved fitness coupled to non-complementary unions among early carriers, and unknown genetic forces has been implicated as sustaining the high frequency of deleterious *GJB2* variants across populations (Aboagye *et al*., 2022). Theoretical optimal fitness, which is dependent on the rate of mutation, model of dominance, and selection balance remains to be fully understood in *GJB2* deleterious variants. Furthermore, the high rate of mutation in the *GJB2* under a recessive dominance model may have undergone severe fluctuations towards new equilibrium prior to the demographic changes during human domestication and pasteurization.

An important novelty of the work is the elucidation of novel gene variants (*MARVELD2* and *GREB1L*) that adds to the global HI variant curation. The implication of the novel variants in families investigated were supported by clinical and segregation data extensively discussed earlier. Using WES to investigate HI families in Ghana resolve higher (over 60%) number of *GJB2*-negative families segregating NSHI. The identification of novel variant in known HI genes that

segregates with the phenotype in *GJB2*-negative families further suggests that future clinical diagnostic attempts should consider NGS, and preferably WES for clinical diagnosis in families negative for *GJB2* pathogenic variant in Ghana (Wonkam *et al*., 2022).

Moving on, is imperative to conduct functional investigation on the *MARVELD2* duplication novel and *GREBIL* variants, and if feasible, to design and produce animal models to investigate these variants further to complement the data presented in this study. For instance, cochlear hair cell degeneration; frequent hair cells lost in the cochlea with no morphological changes at P21, deafness; early-onset rapidly progressive HI development, with no balance or gait abnormalities observed in mice. With the current phenotype expansion of post-lingual NSHI report, it will be useful to characterize the phenotypes for the available model mutant deaf mouse, i.e., Marveld2$^{tm1.1Sria}$, Marveld2$^{tm1a(EUCOMM)Wtsi}$, Marveld2$^{tm1b(EUCOMM)Wtsi}$, and Marveld2 ^{tm1Sats}, in line with the present clinical evidence described in this work that clearly strengthens its significance to the genetics of human HI (http://www.informatics.jax.org/marker/MGI:2446166).

In addition, this study had identified HI-associated variants not described in previous investigations. More importantly, the sheer number and family size investigated and the capacity to recruit these sizable individuals across Ghana is another strength of the study. Generated exome sequence for multiple first-degree siblings and parents facilitated the segregation analysis and validation. The data generated will also contribute to available exomes resource on individuals form the sub-region, which may accelerate future investigation of deleterious variants in actionable genes in African populations.

7.1 Limitations of studies in thesis

1. Whole exome sequencing target close to 97% of all exons in the genome, of which about ~10% are likely not to be covered fully to reliably call some variants, particularly

heterozygous variants (Parla *et al*., 2011). Despite improved variant analysis pipeline which likely achieved high sensitivity and specificity from exome sequence analysis (https://www.asperbio.com/ngs-service/limitations-of-the-exome-sequencing/) that minimized the chance of false positive and false negative relative to SNP array technology, rare variants at the probe target site are likely to affect analytical sensitivity of this approach in solving the genetic cause of NSHI in families investigated.

2. The limited ability associated with WES to detect variants in poorly sequenced GC rich regions, large deletions/duplications, insertions, rearrangements, translocations, and copy numbers (structural variants), failure to detect non-coding or splice site variants, epigenetic factors, mosaic mutations, and unannotated exons missing in commercial exome sequence reagents used (due to incomplete annotation of human genome), among other factors may have impacted the ability to detect additional variants in families investigated.
3. For some families that required re-contacting and re-phenotyping for further validation of putative markers, it was very difficult locating some first-degree relatives (members) to complete the family cohort for phenotyping and sampling, which affected subsequent segregation analysis.
4. There were instances where a rare heterozygous PLP variants in known recessive HI associated genes where identified. Though the identification of such variants may be a false positive or coincidental, it is also likely the sequencing might have missed the second pathogenic variant to confirm the suspected compound heterozygote state.

7.2 Conclusions

This thesis investigated the genetic spectrum of genetic factors associated with NSHI in Ghana. Analysis of computed shared haplotypes in unrelated affected individuals homozygote for the most

common genetic marker; *GJB2*: c.427C>T-p.(Arg143Trp) founder marker traced its evolution and origin to an indigenous common ancestor about 385 generations ago. This finding shows the selection at this locus that segregates with the phenotype and is consistent with previous reports of a founder effect. And this also agrees with the assumptions that the elevated frequency of the founder variant in Ghana may demonstrate existing degree of genetic subdivisions, even among carriers in Ghana, resulting from genetic drift, possibly due to geographical isolation of early carriers. This work has generated data to support the hypothesis that the frequent *GJB2* mutations are derived from founder effects and/or mutational hots spot. Considering the high ethnic and population specific frequencies of observed *GJB2* deleterious alleles across different populations, this study has illuminated the independent origin of the *GJB2*: c.427C>T-p.(Arg143Trp) variant in Ghana, further supporting both founder effect and/or hot spot mutation as implicated in the variant spread in populations reporting the variant.

To solve the genetic etiology of NSHI in families negative for connexins deleterious variants, WES was performed in affected and unaffected kindred recruited. WES investigation of recruited families identified and solved the causal markers among connexin genes-negative families segregating NSHI. The study uncovers and underlines the high level of locus heterogeneity for HI genes. Most affected cases negative for connexin pathogenic variants had deleterious variants in known HI genes (*GREB1L*, *MARVELD2*, *CDH23*, among others), with novel variant that segregates with HI. This suggests that future genetic diagnostic efforts should consider NGS, and if possible, WES for *GJB2*-negative families. The findings of this work have contributed to defining the genetic landscape of mutations among Ghanaian NSHI families, and the global understanding on the genetics of HI in Africa.

7.3 Recommendations

1. Despite the stated limitations, WES ought to be considered for routine genetic investigation and clinical diagnosis based on the practical advantage of its robustness in screening larger populations, with a higher potential of discovering novel candidate gene(s) variant(s) in heterogenous conditions like HI. The technique could be recommended for genetic testing in infants who fail new-born hearing test to complement clinical examinations for early detection and intervention. Continuous efforts targeted at exploring populations with dearth of data on the genetic contribution to NSHI will help expand the current array of actionable gene(s) and/or variant(s) particularly for underrepresented ethnic groups in Ghana.
2. The high proportion of single parents observed in families investigated (broken marriages/homes), of which the majority were attributed to giving birth to HI children, calls for an extensive nationwide sensitization on the genetic contribution to the phenotype to help minimize this worrying and growing trend.
3. On translation of the study findings for clinical benefits, there is an urgent need to unify health care delivered by multiple agencies in the country, particularly with a policy to develop national registry for genetic conditions database. The absence of this vital resource restricts efforts to perform any significant nationwide statistical estimates such as the incidence, prevalence, and distribution of genetic conditions, since the localized data are not representative enough. Going forward, a national registry is long overdue to help compute important epidemiological statistics and inferences on genetic conditions (disease associated mutations) in Ghana.
4. The prevention, diagnosis, management, and treatment of genetic diseases should be recognised as a silent epidemic that requires a national response in addressing it, instead of the existing localized hospital-based interventions.

5. Considering the current report on the contribution of *GJB2*: c.427C>T-p.(Arg143Trp) and carrier frequency (0.7%) in Ghana, it imperative to reflect and ask why the suggested screening for *GJB2*-p.Arg143Trp published health policy considerations has not been tabled for discussion for adoption in the Ghana, to aid the expansion of the existing premarital counselling to include *GJB2* founder variant carrier testing similar to the sickle cell disease.

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APPENDIX A

Candidate contributed to the published articles listed below.

(1). Adadey, S. M., Wonkam-Tingang, E., Alves de Souza Rios, L., **Aboagye, E. T**., Esoh, K., Manyisa, N., De Kock, C., Awandare, G. A., Mowla, S., & Wonkam, A. (**2022**). Cell-based analysis of *CLIC5A* and *SLC12A2* variants associated with hearing impairment in two African families. *Frontiers in Genetics*, *13*. https://www.frontiersin.org/articles/10.3389/fgene.2022.924904

(2). Wonkam, A., Adadey, S. M., Schrauwen, I., **Aboagye, E. T**., Wonkam-Tingang, E., Esoh, K., Popel, K., Manyisa, N., Jonas, M., deKock, C., Nembaware, V., Cornejo Sanchez, D. M., Bharadwaj, T., Nasir, A., Everard, J. L., Kadlubowska, M. K., Nouel-Saied, L. M., Acharya, A., Quaye, O., … Leal, S. M. (**2022**). Exome sequencing of families from Ghana reveals known and candidate hearing impairment genes. *Communications Biology*, *5*(1), 1–16. https://doi.org/10.1038/s42003-022-03326-8

(3). Adadey, S. M., Schrauwen, I., **Aboagye, E. T**., Bharadwaj, T., Esoh, K. K., Basit, S., Acharya, A., Nouel-Saied, L. M., Liaqat, K., & Wonkam-Tingang, E. (**2021**). Further confirmation of the association of *SLC12A2* with non-syndromic autosomal-dominant hearing impairment. *Journal of Human Genetics*, *66*(12), 1169–1175. https://www.nature.com/articles/s10038-021-00954-6

(4). Adadey, S. M., Wonkam-Tingang, E., **Aboagye, E. T.,** Quaye, O., Awandare, G. A., & Wonkam, A. (**2021**). Hearing loss in Africa: Current genetic profile. *Human Genetics*. https://doi.org/10.1007/s00439-021-02376-yA

APPENDIX B (Data Collection Sheet)

QUESTIONNAIRE

Date: ……../……../……. Study participant's **Code number**……

1 **Socio-Demographics**

1.1 Name: ……

1.2 Date of birth: ……/……/……

1.3 Sex: *(Please encircle code) 1*. Male 2. Female

1.4 Ethnic origin

…….

1.4.1 Cultural origin if known:

……...

1.5 Level of education *(Please encircle code)*

1. Primary	2. Secondary	3. Tertiary	4. Special (not main stream school)	5. None/pre-school	6. Other (no formal education)

1.6 Phone number (Preferably three telephone numbers):

…….

2. Exclusion of an Acquired/Environmental Etiology

2.1 Congenital Infection Yes (1) No (2) Unknown (3) *Circle the relevant one if known subsection*

Toxoplasmosis Rubella CMV Syphilis

2.2 Usage of ototoxic drugs during pregnancy (streptomycin, gentamicin):

Yes (1) No (2) Unknown (3)

2.3 Radiation therapy during the 1st trimester of pregnancy Yes (1) No (2) Unknown (3)

2.4 Prematurity	Yes (1)	No (2)	Unknown (3)
2.5 Neonatal asphyxia	Yes (1)	No (2)	Unknown (3)
2.6 Meningitis	Yes (1)	No (2)	Unknown (3)
2.7 Measles	Yes (1)	No (2)	Unknown (3)
2.8 Ototoxity	Yes (1)	No (2)	Unknown (3)
2.9 Noise pollution	Yes (1)	No (2)	Unknown (3)
2.10 Small for age	Yes (1)	No (2)	Unknown (3)
2.11 Nuclear jaundice	Yes (1)	No (2)	Unknown (3)
2.12 Mumps	Yes (1)	No (2)	Unknown (3)

2.13 Chronic otitis	Yes (1)	No (2)	Unknown (3)
2.14 Trauma	Yes (1)	No (2)	Unknown (3)

Comments:

Researcher: The Condition is likely to be

1. Environmental 2. Genetic 3. To be confirmed by Doctor *(Please encircle code)*

Note: IF ENVIRONMENTAL, EXCLUDE THE PATIENT FROM THE STUDY Comments:

3. **Phenotype of the Hearing Impairment**

3.1 Onset of hearing problem *(Please encircle)*

1. From birth
2. Suddenly (over three days)
3. Quickly (weeks and months)
4. Gradually (years)

3.2 Age – Hearing impairment noticed ……

3.3 Age - Medical diagnosis:

…….

3.4 Technique used for diagnosis: *(Pleas encircle)*

1. Audiometry (hearing test/over ears)

2. ABR (auditory brainstem response, put to sleep/brain images)

3.5 Degree of hearing loss: (Only complete if audiogram is available)

Grade of Impairment	Corresponding audiometric ISO Value	Performance	Right ear	Left ear
0- No impairment	25 dB or lower (better ear)	No or very slight hearing problems. Able to hear whispers.		
1- Slight impairment	26 –40 dB (better ear)	Able to hear and repeat words spoken in normal voice at 1m		
2- Moderate Impairment	41-60 dB (better ear)	Able to hear and repeat words spoken in raised voice at 1m.		

3- Severe impairment	61-80 dB (better ear)	Able to hear some words when shouted into better ear.		
4- Profound impairment, including deafness	81 dB or greater (better ear)	Unable to hear and understand even a shouted voice.		

3.6 Localization of the hearing loss *(Please refer to the table above and encircle code/s)*

1. One side

   If on one side: 1. Right 2. Left

2. Both sides

   If on both sides: 1. Symmetrical *(the same)* 2. Asymmetrical *(not the same)*

3.7 Mechanism of hearing problem *(Please encircle code)*

1. Sensorineural 2. Conductive 3. Mixed

3.8 Management of the hearing loss

3.8.1 Hearing aid Yes (1) No (2) Unknown (3)

3.8.2 Cochlear implant Yes (1) No (2) Unknown (3)

3.8.3 Surgery Yes (1) No (2) Unknown (3)

3.8.4 Speech therapy aid Yes (1) No (2) Unknown (3)

3.8.5 Alternative communication (sign language, lip reading) Yes (1) No (2) Unknown (3)

Comments

4. **Clinical History**

4.1.1 Intellectual Disability/Learning difficulties Yes (1) No (2) Unknown (3)

4.1.2 Seizures Yes (1) No (2) Unknown (3)

4.1.3 Goitre Yes (1) No (2) Unknown (3)

4.1.4 Diabetes mellitus Yes (1) No (2) Unknown (3)

4.1.5 Diabetes insipidus Yes (1) No (2) Unknown (3)

4.1.6 Syncope Yes (1) No (2) Unknown (3)

4.1.7 Hirschprung's disease Yes (1) No (2) Unknown (3)

4.1.8 Kidney disease Yes (1) No (2) Unknown (3)

Specify …….

4.1.9 Haematuria Yes (1) No (2) Unknown (3)

4.1.10 Visual impairment Yes (1) No (2) Unknown (3)

Specify……

4.1.11 Other Yes (1) No (2) Unknown (3)

Specify…….

5. Family History/ Pedigree (At Least 3 Generations)

5.1 Consanguinity Yes (1) No (2) Unknown (3)

Comments

5.2 Likely mode of Inheritance *(Please encircle code)*

1. AR 2. AD 3. X-linked 4. Mitochondrial 5. Unknown

Associated Anomalies

6.0 Anthropometrics

6.1.1 Weight: ……kg 6.1.2 Height: …….cm

6.1.3BP……. /……. mmHg 6.1.4 COH: …….cm/……. Centile

Doctor: The Condition is likely to be

1. Environmental 2. Genetic *(Please encircle code)*

Note: IF ENVIRONMENTAL, EXCLUDE THE PATIENT FROM THE STUDY
Comments:

6.1.2 Craniofacial dysmorphism:	Yes (1)	No (2)	Unknown (3)
6.1.2.1 Auricle malformation	Yes (1)	No (2)	Unknown (3)
6.1.2.2 Atresia of the EAM	Yes (1)	No (2)	Unknown (3)
6.1.2.3 Preauricular sinus	Yes (1)	No (2)	Unknown (3)
6.1.2.4 Microtia	Yes (1)	No (2)	Unknown (3)
6.1.2.5 Branchial fistula	Yes (1)	No (2)	Unknown (3)
6.1.2.6 Cleft palate	Yes (1)	No (2)	Unknown (3)
6.1.2.7 Branchial cyst	Yes (1)	No (2)	Unknown (3)
6.1.2.8 Microphtalmia	Yes (1)	No (2)	Unknown (3)
6.1.2.8 Microphtalmia	Yes (1)	No (2)	Unknown (3)

6.1.2.10 Macrocephaly	Yes (1)	No (2)	Unknown (3)
6.1.2.11 Goitre	Yes (1)	No (2)	Unknown (3)
6.1.2.12DystopiaCanthorum	Yes (1)	No (2)	Unknown (3)
6.1.2.13Heterochromia	Yes (1)	No (2)	Unknown (3)
6.1.2.14Whitehair/forelock	Yes (1)	No (2)	Unknown (3)

6.1.3 Skin defect	Yes (1)	No (2)	Unknown (3)
6.1.3.1 Ichthyosis	Yes (1)	No (2)	Unknown (3)
6.1.3.2 Neurofibromas	Yes (1)	No (2)	Unknown (3)
6.1.3.3 Atrichosis/Hypotrichosis	Yes (1)	No (2)	Unknown (3)
6.1.3.4 Skin depigmentation	Yes (1)	No (2)	Unknown (3)
6.1.3.5 Keratoderma	Yes (1)	No (2)	Unknown (3)
6.1.4 Limb defect	Yes (1)	No (2)	Unknown (3)

6.1.5 Other abnormality Specify ……

Otoscopy …….

6.1.7 Photographs taken Yes (1) No (2)

Investigations (if available)

Investigation	Results
Kidney Ultrasound	
Electrocardiogram	
Eye test	
Fasting blood sugar	
Urine Dipstick	

Type of Hearing Impairment

8.1 Syndromic Yes (1)

No (2) Unknown (3)

8.2 Non-syndromic Yes (1) No (2) Unknown (3)

8.3 Other condition ……

9 Genetic Investigations

9.2 Previous DNA Extraction in the index case Yes (1) No (2) Unknown (3)

9.2.1If yes, please indicate:……

GJB2 mutation excluded

GJB6 mutation excluded

Whole exome sequencing

Whole Genome sequencing

9.3 Previous DNA extraction in family member(s) Yes (1) No (2) Unknown (3)

9.3.1 If yes, please indicate:

GJB2 mutation excluded

GJB6 mutation excluded

Whole exome sequencing

Whole Genome sequencing

9.3. Any positive genetic testing on the index case Yes (1) No (2) Unknown (3)

9.3.1 If yes, please indicate: …….

APPENDIX C (Consent and Assent Forms)

UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

Ethics Committee for Basic and Applied Sciences (ECBAS)

Official Use only Protocol number

PROTOCOL ADULT CONSENT FORM

Section A- BACKGROUND INFORMATION

Title of Study:	**Genetic Markers of Autosomal Recessive Non-Syndromic Hearing Impairment in Ghana**
Principal Investigator:	**Elvis Twumasi Aboagye**
Certified Protocol Number	**ECBAS 053/19-20**

Section B– CONSENT TO PARTICIPATE IN RESEARCH

General Information about Research

Deafness refers to the total or partial loss of the ability to hear sound. This difficulty in hearing can be acquired through illness or other environmental causes. In other words, a person may be born hearing but will develop deafness after an illness due to infections, or damage to the hearing organ through accidents, exposure to loud sound, drug actions, birth complications, among others. However, hearing problems can also be passed on from parents to children in a family, which we term inherited (genetic cause). In most cases, hearing parents give birth to a child who cannot hear, clearly showing how a child may inherit "bad" genes from both parents to cause deafness. Between these two causes of hearing problems, the genetic cause is of more concern now. This is due to the reduction in environmental causes, resulting from improve health care delivery practices and good workplace regulations. Many current hearing problems are caused by inheriting "bad" genes, which are hidden in a family until a child inherits it from his/her parents. This study seeks to investigate the specific genetic cause of deafness that runs in families and isolated children's cases in Ghana. We are therefore requesting your participation, and to allow us to take about 2 to 4 teaspoons of whole blood samples for genetic investigation of deafness in families.

Study procedure

Deoxyribonucleic acid (DNA) would be extracted from participants blood samples collected. After quality control and purity checked, familial and isolated cohorts in addition to ethnic grouped matched controls will be screen for the most common gap junction beta 2 gene variant reported to be linked to deafness in Ghana. Unrelated individual positive and negative for the variant will be used to investigate any other gene variants linked to the deafness in both familial and isolated cases, and as well investigate the ancestral history of this common variant in Ghana.

Benefits of the study

There is no direct benefit for participation in this study, but indirectly your participation will help determine and confirm heritable genetic cause of deafness in Ghana. Data generated in the study population will help the entire Ghanaian community by helping to identify genetic markers to be screen for early childhood diagnosis, genetic screening, management, and where possible treatment of this life transforming genetic condition.

Risk of the study

Your participation in this study does not increase your risk beyond the usual risk of infection that you may experience during venipuncture for blood sample collection, or the minimal discomfort associated with it. However, this risk will be minimized because the blood collection will be performed by a qualified and trained phlebotomist following standard laboratory procedures.

Confidentiality

All data collected will be securely protected in participant folders with no link to the individual or families' members recruited in the study. All samples and participant records will be coded with study identification numbers and stored in locked cabinets with limited access by only the study team.

Compensation

There is no financial compensation for participating in this study. However, affected families will gain a deeper understanding of the condition by participating in this study and findings that have direct clinical benefit/risk will be communicated to affected families in feedback engagement.

Withdrawal from Study

Your participation in this study is completely voluntary, and you have the right to say no to this study and can withdraw from the study at any time without penalty or need to explain yourself to the study team. Your participation in this study could be terminated by the study

investigators if your condition is determined to be due to environmental factors and not genetics, which is the focus of this study.

Contact for Additional Information

If you have any enquiries on this research study or need additional information, you may contact any of the following.

Prof. Gordon A. Awandare, West African Centre for Cell Biology of Infectious Pathogens, Department of Cell and Molecular Biology, University of Ghana, Post Office Box LG 54, Legon-Accra. E-mail: gawandare@ug.edu.gh.

Dr. Lucas Amenga-Etego, West African Centre for Cell Biology of Infectious Pathogens, Department of Cell and Molecular Biology, University of Ghana, Post Office Box LG 54, Legon-Accra. E-mail: lamenga-etego@ug.edu.gh

Prof. Ambroise Wonkam, University of Cape Town, Anzio Road-Observatory-7925, Cape Town South Africa, E- mail: ambroise.wonkam@uct.ac.za.

If you have any issues on your rights as a participant, you can contact the address below:

Administrator, Ethics Committee for Basic and Applied Sciences
College of Basic and Applied Sciences
University of Ghana
P. O. Box LG 68
Legon – Accra
IP No.: 3014, Email: ethicscbas@ug.edu.gh.

Section C- VOLUNTEER AGREEMENT

"I have read or have had someone read all of the above to me, asked questions, and received answers regarding participation in this study, and I am willing to give consent for me, my child/ward to participate in this study. I have not waived any of my rights by signing this consent form. Upon signing this consent form, I will receive a copy for my personal records."

_____

Name of Volunteer _____

Date (dd/mm/yyyy)

_____

Signature or mark of volunteer

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered, and the volunteer has agreed to take part in the research.

_____

Name of witness _____

Date (dd/mm/yyyy)

_____

Signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual

_____

Name of Person who obtained Consent

_____ _____

Signature of Person who obtained Consent Date (dd/mm/yyyy)

UNIVERSITY OF GHANA

Official Use only Protocol number

COLLEGE OF BASIC AND APPLIED SCIENCES

Ethics Committee for Basic and Applied Sciences (ECBAS)

PROTOCOL PARENTAL CONSENT FORM

Section A- BACKGROUND INFORMATION

Title of Study:	**Genetic Markers of Autosomal Recessive Non-Syndromic Hearing Impairment in Ghana**
Principal Investigator:	**Elvis Twumasi Aboagye**
Certified Protocol Number	**ECBAS 053/19-20**

Section B– CONSENT TO PARTICIPATE IN RESEARCH

General Information about Research

Deafness refers to the total or partial loss of the ability to hear sound. This difficulty in hearing can be acquired through illness or other environmental causes. That is a person may be born hearing but will develop deafness after an illness or results from damage to the hearing organ by infections, accidents, exposure to loud sound, drug actions, birth complications, among others. However, hearing problems can also be passed on from parents to children in a family, which we termed inherited (genetic cause). In most cases, hearing parent gives birth to a child who cannot hear, clearly showing how a child may inherit each copy of "bad" genes from both parents to cause deafness. Between these two causes of hearing problems, genetics are of more concern now. This is due to the reduction in environmental factors contribution with improve health care delivery practices and good workplace regulations. Many current hearing problems are caused by genetics which is hidden in a family until a child is inherited from parents. By this study, we seek to investigate the specific genetic causes of deafness in familial and isolated children's cases in Ghana. We will request you allow and make yourself available for about 2 to 4 teaspoons of blood samples to be taken for genetic investigation.

Benefits of the study

There is no direct benefit for participation in this study, but indirectly his/her participation will help determine and confirm inheritable genetic cause of the deafness. Data generated in the

study population will also help in early childhood diagnosis, genetic screening, management, and treatment in Ghana**.**

Risk of the study

Your wards participation in this study is at no risk, except the minimal risk of infection because of venipuncture for blood sampling and associated minimal discomfort, but this procedure will be done by a qualified and trained personnel (phlebotomist) to ensure that this does not happen.

Confidentiality

All data collected will be securely protected with no link to the individual participants' identity in the study. Samples will be coded with study identification numbers and treated as highly confidential and data analysis open to only the research team.

Compensation

There is no financial compensation for participating in this study. However, affected families will gain a deeper understanding of the condition by participating in this study and findings that have direct clinical benefit/risk will be communicated to affected families in feedback engagement.

Withdrawal from Study

Your wards participation in this study in completely voluntary, and he/she has the free will and right to withdraw from the study with no penalty or compensation at any point in the study to research team.

Termination of Participation by the Researcher

Your wards participation in this study could be terminated if the condition is determined to be caused by environmental factors instead of the genetic cause the focus of this study.

Contact for Additional Information

Prof. Gordon A. Awandare, West African Centre for Cell Biology of Infectious Pathogens, Department of Cell and Molecular Biology, University of Ghana, Post Office Box LG 54, Legon-Accra. E-mail: gawandare@ug.edu.gh. Phone contact: *+233 (0)30 393 3223 or +233 (0)543717697*

Dr. Lucas Amenga-Etego, West African Centre for Cell Biology of Infectious Pathogens, Department of Cell and Molecular Biology, University of Ghana, Post Office Box LG 54, Legon-Accra. E-mail: lamenga-etego@ug.edu.gh

Prof. Ambroise Wonkam, University of Cape Town, Anzio Road-Observatory-7925, Cape Town South Africa, E- mail: ambroise.wonkam@uct.ac.za.

If you have any issues on your rights as a participant, you can contact the address below:

Administrator, Ethics Committee for Basic and Applied Sciences
College of Basic and Applied Sciences
University of Ghana
P. O. Box LG 68
Legon – Accra
IP No.: 3014
Email: ethicscbas@ug.edu.gh

Section C- VOLUNTEER AGREEMENT

"I have read or have had someone read all of the above, asked questions, and received answers regarding participation in this study, and I am willing to give my consent for me, my child/ward to participate in this study. I have not waived any of my rights by signing this consent form. Upon signing this consent form, I will receive a copy for my personal records."

_____

Name of Volunteer

_____ _____

Signature or mark of volunteer Date

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered, and the volunteer has agreed to take part in the research.

_____

Name of witness

_____ _____

Signature of witness Date

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____

Name of Person who obtained Consent

_____ _____

Signature of Person who obtained Consent Date

UNIVERSITY OF GHANA

Official Use only Protocol number

COLLEGE OF BASIC AND APPLIED SCIENCES

Ethics Committee for Basic and Applied Sciences (ECBAS)

PROTOCOL CHILD (12 to 17 years) ASSENT FORM

Section A- BACKGROUND INFORMATION

Title of Study:	**Genetic Markers of Autosomal Recessive Non-Syndromic Hearing Impairment in Ghana**
Principal Investigator:	**Elvis Twumasi Aboagye**
Certified Protocol Number	**ECBAS 053/19-20**

Section B– ASSENT TO PARTICIPATE IN RESEARCH

Introduction

My name is **Elvis Twumasi Aboagye,** and I am from the **Department of Biochemistry, Cell and Molecular Biology, University of Ghana-Legon**. I am conducting a research study entitled "**Investigating Genetic Markers of Autosomal Recessive Non-Syndromic Hearing Impairment in Ghana**". In addition to your parents agreeing for you to participate in this study, I am asking you if you are willing to take part in this study to help me learn and understand more about deafness. This will take 30 minutes maximum of your time.

General Information

Deafness refers to the total or partial loss of the ability to hear sound in one or both ears. This difficulty in hearing can be acquired (accidental, illness, infections) or inherit from parents (genetic). In majority of the cases, hearing parent gives birth to a child who cannot hear, clearly showing how a child may inherit some genes from both parents to cause deafness. Between these two causes of hearing problems, the genetic is of more concern now. Most of the current hearing problems are caused by genetic which is hidden in a family until a child inherit from parents. By this study, we seek to investigate the specific genetic causes of deafness in familial and isolated children's cases in Ghana.

If you agree to be in this study, you will be asked to provide responses to some questions and donate 2 to 4 teaspoons of blood sample for the genetic investigation.

Possible Benefits

There is not direct financial benefit for participating in this study, but your participation will help provide more information on the causal genetic factors contributing to deafness in families and individuals in Ghana, which will help in deafness diagnosis and management.

Possible Risks and Discomforts

There are minimal risks of infection because of venipuncture for blood sampling and the usual associated minimal discomfort, but this procedure will be done by a qualified and trained personnel to ensure that this is minimize.

Confidentiality

Your information will be kept confidential. No one will be able to know how you responded to the questions and your information will be anonymous.

Compensation

There is no financial compensation for participating in this study. However, affected families will gain a deeper understanding of the condition by participating in this study and findings that have direct clinical benefit/risk will be communicated to affected families in feedback engagement.

Voluntary Participation and Right to Leave the Research

Your participation in this study is completely voluntary. You may say no to this study or withdraw your participation at any time if you feel uncomfortable. No one will be angry with you if you do not want to participate.

Contacts for Additional Information

You may ask me any questions about this study. You can call me at any time on the phone 0546793468 or talk to me the next time you see me.

Please talk about this study with your parents before you decide whether to participate. I will also ask permission from your parents before you are enrolled into the study. Even if your parents say “yes” you can still decide not to participate.

Your rights as a Participant

This research has been reviewed and approved by the Institutional Review Board of the Ethical Committee of Basic and Applied Science (ECBAS-IRB). If you have any questions about your rights as a research participant, you can contact the IRB Office between the hours of 8am-5pm through the landline *+233-(0)-302-213820* email addresses: ethicscbas@ug.edu.gh.

VOLUNTARY AGREEMENT

By making a mark or thumb printing below, it means that you understand and know what activities will be done and what is expected of you in this study. If you do not want to participate in this study, please do not sign this assent form. You (or your parents) will be given a copy of this form after you have signed it.

This assent form which describes the benefits, risks and procedures for the research titled "Investigating Genetic Markers of Autosomal Recessive Non-Syndromic Hearing Impairment in Ghana" has been read and or explained to me. I have been given an opportunity to ask any questions about the research, which were answered to my satisfaction. I agree to participate.

Child's Name…… Researcher's Name……

Child's Mark/Thumbprint……. Researcher's Signature……

Date: …… Date……..

APPENDIX D (Ethical Approval)

UNIVERSITY OF GHANA
ETHICS COMMITTEE FOR BASIC AND APPLIED SCIENCES (ECBAS)
P. O. Box LG 1195, Legon, Accra, Ghana

Ref. No: ECBAS 053/19-20

14th October, 2021.

Mr. Elvis Twumasi Aboagye
Department of Biochemistry,
Cell & Molecular Biology
University of Ghana
Legon, Accra

Dear Mr. Twumasi Aboagye,

ECBAS 053/19-20: GENETIC MARKERS OF AUTOSOMAL RECESSIVE NONSYNDROMIC HEARING IMPAIRMENT IN GHANA.

This is to inform you that the above referenced study has been presented to the Ethics Committee for Basic and Applied Sciences for a full board review and the following actions taken subject to the conditions and explanation provided below:

Expiry Date:	04/09/2022
On Agenda for:	Continuous Review
Date of Submission:	05/08/2021
ECBAS Action:	Approved
Reporting:	Annually

Please accept my congratulations.

Yours sincerely,

Professor Daniel Bruce Sarpong
ECBAS Chairperson

ETHICS COMMITTEE FOR BASIC AND APPLIED SCIENCES
APPROVED
28 OCT 2021
UNIVERSITY OF GHANA