Identifying Genes and Novel Variants Involved in Non-syndromic Hearing Impairment, and Assessment of the Psychosocial Burden of Hearing Impairment in Cameroon

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Declaration

I, Edmond Wonkam Tingang, hereby declare that this dissertation/thesis is based on my original work (except where acknowledgments indicate otherwise) and that neither the whole work nor any part of it has been or is being submitted for another degree in this or any other university.

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List of publications included in this thesis

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1. **Wonkam Tingang, E.**, Noubiap, J. J., F. Fokouo, J. V., Oluwole, O. G., Nguefack, S., Chimusa, E. R., and Wonkam, A. (2020). Hearing Impairment Overview in Africa: The Case of Cameroon. *Genes*, 11(2), 233. https://doi.org/10.3390/genes11020233. (Status: *published*)
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3. Hotchkiss, J., Manyisa, N., Adadey, S. M., Oluwole, O. G., **Wonkam, E.**, Mnika, K., Yalcouye, A., Nembaware, V., Haendel, M., Vasilevsky, N., Mulder, N. J., Jupp, S., Wonkam, A., & Mazandu, G. K. (2019). The Hearing Impairment Ontology: A Tool for Unifying Hearing Impairment Knowledge to Enhance Collaborative Research. *Genes*, *10*(12). https://doi.org/10.3390/genes10120960. (Status: *published*)
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Preface

The World Health Organisation estimated in 2018 that approximately 466 million people globally live with disabling hearing impairment (HI) (6.1% of the world population), of whom 34 million are children and 49 million live in sub-Saharan Africa (SSA). Although the incidence of HI in SSA is six times higher (6 per 1000 live births) than that of high-income countries, little is known about its genetics in this region. Indeed, variants in *GJB2* that are known to be the major contributors to HI in Europeans, Asians, and Arabs were shown to be infrequent in populations of African descent. Additionally, the possible involvement of *DMD* gene in HI in humans has not been extensively studied despite previous studies, suggesting its implication in the hearing process in the mouse; also, the genetics and clinical profile of patients with Duchenne muscular dystrophy (DMD) in Africa have been understudied. Lastly, HI in Africa is sometimes considered as originating from a curse, and persons with HI are faced with issues (including discrimination and limited access to proper management) that need to be addressed and remedied.

Research concept

The study concept and all the experiments were conceived by the candidate in conjunction with the supervisor A/Professor Emile Chimusa and the co-supervisor Professor Ambroise Wonkam. The research proposal including methods for patients' recruitment, samples, and data collection, experiments, and analysis was designed by the candidate with the advice and guidance of the supervisors. Besides, drafting the full manuscripts and incorporating revisions from co-authors and journals' reviewers were performed by the candidate.

Data collection

All necessary activities (such as community engagements, schools and hospitals visitations) prior to the target population identification were solely conducted by the candidate. Contacting and recruiting participants, obtaining informed consent, and performing clinical interviews and physical examination of patients were done by the candidate, who is a board-certified medical doctor. Also, the sampling of biological materials, and in-depth interviews (for the qualitative part of our study) were conducted in full by the candidate. Lastly, retrospective reviewing of patients' medical records when necessary was performed by the candidate.

Experimentation and data analysis

DNA isolation, polymerase chain reactions, and sequencing experiments were performed in full by the candidate. The primary analysis of data was performed by the candidate, and in silico prediction of the pathogenicity of variants was performed by the candidate. Bioinformatics analysis and identification of the putative variants were performed by our

collaborators from the University of Columbia, New York, USA, with the close implication of the candidate. The work and contribution of these collaborators to the study are indicated in each publication where applicable. Transcription and translation of in-depth interviews, as well as coding and qualitative data analysis were done in full by the candidate.

Publications

Synthesis of all the results and drafting of manuscripts of the publications included in this thesis were executed by the candidate, after which revisions from all co-authors were similarly incorporated before submission to the journal by the principal investigator. After review, all reviewer comments were addressed by the candidate in conjunction with the supervisors. The detailed role of the candidate in the publications included is stated in each chapter.

To improve knowledge on the genetics of HI and its psychosocial burden in Cameroon, the research work proposed for this thesis was written and published during my registration as a PhD candidate. We have thus chosen this thesis format i.e. "thesis by publication" which would scholarly contribute to the field of hearing impairment genetics. The sixth chapter of this thesis, however, is presented in the traditional format and includes an introduction, materials and methods, results, discussion, and conclusions sub-sections.

The inclusion of published work also aimed to: 1) highlight the candidate's proficiency of new knowledge in the relevant field, which will also strengthen his research and academic career; 2) enhance his candidacy for continued financial support from bodies that support this research work i.e. Hearing Impairment Genetics Studies in Africa (HI-GENES Africa), Genetic Medicine of African Populations (GeneMAP), and the University of Cape Town; 3) enable participation in and attendance at academic conferences and training workshops. The candidate successfully attended several specialised national and international conferences in South Africa, Rwanda, and the United States of America, including the prestigious American Society of Human Genetics annual congress, that awarded him, on a competitive basis, a travel fellowship to attend the meeting; Last, 4) these publications constitute essential parts of a consistent body of research that fulfil the Faculty of Health Sciences policies. These policies promote the publication of thesis research work as much as possible to disseminate knowledge generated and further improve the profile of the institution and the candidate.

This thesis is presented in four cohesive parts: 1) the current status of the knowledge on various aspect of HI in Africa with a focus on Cameroon, as well as the contribution of connexin genes to non-syndromic HI (NSHI) in humans worldwide; 2) application of targeted gene screening to investigate the contribution of common NSHI-genes (*GJB2* and *GJB6*) and *DMD* to HI in Cameroon; 3) Identification of two new genes involved in NSHI in Africans through the use of whole exome sequencing technique; 4) a qualitative part that addresses the challenges

faced by hearing impaired people in Cameroon, their understanding of the causes of HI, and how challenges can be overcome. The candidate has met all requirements and approval of UCT's Doctoral Degrees Board, under Rules GP6.7 as follows:

1. The candidate's proposal to include publications in the current thesis was approved by the UCT Faculty of Health Sciences Doctoral Degree Board.
2. The thesis contains an adequate summary, introduction, a chapter on the aims and objectives, a comprehensive academic discussion of the results as a whole, forming the basis of the conclusions and perspectives drawn from this research.
3. Each results chapter with publications included is preceded by a synopsis of how the publications directly tie to the aims and objectives of the project, as well as to the thesis as a whole.
4. All included publications were written and published during the candidate's tenure as a PhD student since 2018.

The candidate,

Edmond Wonkam Tingang

Abstract

Background

Hearing impairment (HI) is the most common sensory disability and occurs in about 1 per 1000 live births in high-income countries, with a much higher incidence of up to 6 per 1000 live births in sub-Saharan Africa (SSA). HI can be due to environmental or genetic causes, and in many cases, it is not possible to establish a definite aetiology. Hereditary HI contributes to 30% to 50% of HI cases in SSA. Hereditary HI can be syndromic or non-syndromic, depending on whether it is associated with additional abnormalities in other organs or not. Non-syndromic HI (NSHI) accounts for 70% of hereditary hearing loss, and is genetically highly heterogeneous, with approximately 170 loci and 121 genes identified to date. Studies in European and Asian populations have identified pathogenic variants in *GJB2* (MIM: 121011), and *GJB6* (MIM: 604418) genes as the major contributors to autosomal recessive NSHI (ARNSHI). The genetic aetiology of HI in Cameroon is unclear, as previous studies have found no contribution of *GJB2* and *GJB6* genes to NSHI in Cameroon. However, patients included in those studies consisted of both familial and isolated cases, therefore, underlying environmental/multifactorial causes in some cases cannot be excluded (especially for the isolated cases).

Six loci for X-linked HI have been described to date, including DFNX3 (Xp21.2), where *DMD* is located. Variants in *DMD* in humans are known to be responsible for Duchenne muscular dystrophy (DMD; MIM: 310200), and Becker muscular dystrophy (BMD; MIM: 300376), an X-linked recessive disorder. Previous studies have demonstrated that mdx mice, (an animal knockout model for DMD), have an increased threshold for hearing when compared to wildtype mice. However, the contribution of *DMD* to HI in humans has not been extensively studied. Besides, most of the previous studies on DMD were conducted in Caucasians, Asians, and Arabs; therefore, little is known about the features of this condition in Africans.

Parents of children with HI tend to face challenges of parenting especially in terms of communication and social interaction. In Africa, parent’s perceived causes of deafness vary from environmental factors to mysterious (“evil forces”) or superstitious beliefs. Also, the attitude of the society towards people with HI does not encourage their participation and involvement in the community, as they face overt discrimination.

Aim and methods

The aim of this project was to examine the genetic aetiologies of HI in the Cameroonian population, and undercover the challenges faced by persons with HI in Cameroon and their understanding of the causes of HI. This was addressed by 1) Establishing the current status

of knowledge on HI in Africa (in terms of prevalence, aetiologies, and genetics aspects) with a particular focus on Cameroon, and assessing the contribution of connexin genes to HI in humans at a global level, through systematic literature reviews; 2) Revisiting the contribution of *GJB2* and *GJB6* genes to NSHI in 29 multiplex Cameroonian families with NSHI and with strong evidence of non-environmental causes, through targeted gene sequencing and specific multiplex polymerase chain reaction (PCR); 3) Using multiplex ligand-dependent probe amplification (MLPA) technique to investigate the most common variants associated with DMD in Cameroon and assess their possible implication in HI in humans; 4) Performing whole exome sequencing (WES) on 2 Cameroonian multiplex families with NSHI and who tested negative for pathogenic variants in *GJB2* and *GJB6*, to identify the underlying causative genes; 5) Performing in-depth interviews to gain an understanding of the challenges faced by people with HI in Cameroon, their understanding of the causes of hearing impairment (HI), and how challenges could be remedied to improve the quality of life of persons with HI.

Results

Literature reviews

Our first systematic review showed that HI is a public health issue in Cameroon, especially in the elder population where the prevalence of HI is 14.8% in people aged 50 years and more. Environmental factors, including meningitis, impacted wax, and age-related disorders are the leading aetiologies of HI in Cameroon as in many other SSA countries, contributing 52.6% to 62.2% of HI cases. Hereditary HI comprises 0.8% to 14.8% of all cases in Cameroon, and in 32.6% to 37% of HI cases, the origin remains unknown. This contrasts with findings from high-income countries where hereditary HI constitutes the main aetiology of HI, contributing to approximately 50% of cases. NSHI is the most frequent clinical entity and accounts for 86.1% to 92.5% of cases of hereditary HI in the Cameroonian population. No pathogenic variant was described in *GJB6* gene, and the prevalence of pathogenic variants in *GJB2* ranged from 0% to 0.5%. The prevalence of pathogenic variants in other known NSHI genes was <10% in Cameroonian probands. The second systematic review which assessed the contribution of connexin genes to HI worldwide confirms their lower implication in NSHI in African populations, and suggests that only *GJB2* and *GJB3* are recognised and validated HI genes.

Targeted gene screening

We recruited a total of 93 patients with HI from 41 multiplex families. HI was sensorineural in 51 out of 54 (94.4%) patients. Pedigree analysis suggested autosomal recessive inheritance in 85.4% (35/41) of families. The disease was likely inherited in an autosomal dominant and mitochondrial mode in 12.2% (5/41) and 2.4% (1/41) of families, respectively. Most HI participants were non-syndromic (92.5%; 86/93). Four patients from two families presented

with type 2 Waardenburg syndrome, and three cases of type 2 Usher syndrome were identified in one family. By direct gene sequencing of the coding region of *GJB2*, no variants were found in any of the 29 families with NSHI. Additionally, through a specific multiplex PCR, the *GJB6*-D3S1830 deletion which contributes to 9.7% of NSHI cases in Europeans was not identified in any of the patients with HI.

Subsequently, a total of 17 males with DMD from 14 families were recruited, aged 14 ± 5.1 (8–23) years. The mean age at onset of symptoms was 4.6 ± 1.5 years, and the mean age at diagnosis was 12.1 ± 5.2 years. Proximal muscle weakness was noted in all patients and calf hypertrophy in the large majority of them (88.2%; 15/17). Flexion contractures were particularly frequent on the ankle (85.7%; 12/14). Wasting of the shoulder girdle and thigh muscles was present in 50% (6/12) and 46.2% (6/13) of patients, respectively. No patient presented with HI. The MLPA found that deletions of at least one exon in *DMD* occurred in 45.5% of patients (5/11), while duplications were observed in 27.3% (3/11). Both variant types were clustered between exons 45 and 50, and the proportion of de novo variant was estimated at 18.2% (2/11).

Whole exome sequencing

We submitted DNA samples from five members of a multiplex non-consanguineous Cameroonian family segregating prelingual and progressive ARNSHI for WES. We identified novel bi-allelic compound heterozygous pathogenic variants in *CLIC5* (MIM: 607293). The variants identified, i.e. the missense [NM_016929.5:c.224T>C; p.(Leu75Pro)] and the splicing (NM_016929.5:c.63+1G>A), were validated using Sanger sequencing in all seven available family members and co-segregated with HI in the three family members with HI. The three affected individuals were compound heterozygous for both variants, and all unaffected individuals were heterozygous for one of the two variants. Both variants classify as pathogenic by the American College of Medical Genetics (ACMG) guidelines for classification of variants and are absent from the genome aggregation database (gnomAD), UK10K, Greater Middle East (GME) database, and the Single Nucleotide Polymorphism Database (dbSNP), as well in 122 healthy controls from Cameroon. We also did not identify these pathogenic variants in 118 unrelated sporadic cases of NSHI from Cameroon.

A second multiplex family was also screened through the use of WES, followed by direct Sanger sequencing in additional patients and control participants. We identified a heterozygous novel missense variant [NM_001174116.2:c.918G>T; p.(Gln306His)] in *DMXL2* (MIM:612186) which was transmitted in an autosomal dominant manner, and co-segregates with congenital/prelingual profound to total non-syndromic sensorineural HI in a family from Cameroon. The described family showed a variable expressivity of the HI phenotype. The

p.(Gln306His) variant which substitutes a highly conserved glutamine residue is predicted deleterious by various bioinformatics tools and is absent from several genome databases including genome aggregation database (gnomAD), and trans-omics for precision medicine (TOPMed) database. This variant was neither found in 121 healthy controls without personal or family history of HI, nor 112 sporadic cases of NSHI from Cameroon.

Our study identified novel variants in *CLIC5* and *DMXL2* in two Cameroonian families, and provided only the second report of variants in these genes worldwide; thus, strengthening the case for these two genes as candidate genes for NSHI in humans.

The psychosocial burden of HI

We performed in-depth interviews with 10 HI professionals (healthcare workers, and educationists), and 10 persons affected by HI (persons with HI, and caregivers). The results show that in this study population, the cause of HI is attributed to a variety of causes, including genetics, environmental factors, and a spiritual curse. There were reported cases of stigma and discrimination with persons with HI in the Cameroonian population sometimes seen as having a "mental disorder". Our participants also highlighted the difficulty that persons with HI have in accessing the necessary education and healthcare services, and suggested the need for policymakers and researchers to develop strategies to improve the social integration of persons with HI and their access to basic social services. This includes 1) Increased awareness amongst the general population, 2) the establishment of more special schools, and 3) building and equipping facilities for proper management of HI.

Conclusions

Our project confirms that variants in *GJB2* and *GJB6* genes do not contribute significantly to NSHI in the Cameroonian population. Also, variants in *DMD* that were shown to be associated with an increased hearing threshold in mice, do not seem to be implicated in HI in Cameroon, neither in previous human studies (although they did not objectively assess hearing using standardized testing methods). Despite the first symptoms of DMD occurring in infancy, the diagnosis is frequently made later in adolescence, indicating an underestimation of the number of cases of DMD in Cameroon. Future screening of deletions and duplications in patients from Cameroon should focus on the distal part of the *DMD* gene. Subsequently, this study successfully identified the candidate genes in two Cameroonian multiplex families with NSHI through the use of WES, and thus highlights the efficacy of next-generation sequencing techniques in resolving HI cases in Cameroonians and in cases where no pathogenic variants are found in common HI-genes. Additionally, our project which confirms that *CLIC5* and *DMXL2* genes are associated with HI in humans advocate for the inclusion of these two genes in diagnostic gene panels for NSHI in clinical settings. Last, this study shows the difficult social

interaction and access to proper management faced by persons with HI in Cameroon, and highlights the need to educate populations on the causes of HI for a better acceptance of persons with HI in the Cameroonian society.

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3.2. Adadey, S. M., **Wonkam-Tingang, E.**, Twumasi Aboagye, E., Nayo-Gyan, D. W., Boatemaa Ansong, M., Quaye, O., Awandare, G. A., & Wonkam, A. (2020). Connexin Genes Variants Associated with Non-Syndromic Hearing Impairment: A Systematic Review of the Global Burden. *Life*, 10(11), 258. https://doi.org/ 10.3390/life10110258. 27

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Abbreviations

aa	amino acid
AAS	African Academy of Science
ABR	Auditory Brainstem Responses
ACMG	American College of Medical Genetics and Genomics
AD	Autosomal Dominant
ADA	Adaptive boosting
ALT	Alanine Transaminase
AMP	Association for Molecular Pathology
ANNOVAR	Annotate Variation
AR	Autosomal Recessive
ARISE	Africa Regional International Staff/student Exchange
ARMS	Amplification-Refractory Mutation System
ARNSHI	Autosomal Recessive Non-syndromic Hearing Impairment
AST	Aspartate Transaminase
BIAP	Bureau International d'AudioPhonologie
BLAST	Basic Local Alignment Search Tool
BLASTp	protein-protein Basic Local Alignment Search Tool
BMD	Becker Muscular Dystrophy
BWA	Burrows-Wheeler Aligner
CA	California
CADD	Combined Annotation Dependent Depletion
Chrom	Chromosome
CK	Creatine Kinase
CLIC	Chloride Intracellular Channel
CLIC5	Chloride Intracellular Channel-5
DANN	Deleterious Annotation of genetic variants using Neural Networks
dbNSFP	database of human Nonsynonymous Single nucleotide polymorphisms and their Functional Predictions
dbSNP	Single Nucleotide Polymorphism database
DELTAS	Developing Excellence in Leadership, Training and Science
DHPLC	Denaturating High-Performance Liquid Chromatography

DMD	Duchenne Muscular Dystrophy
DMXL2	DmX-like protein 2
EIEE81	Early Infantile Epileptic Encephalopathy-81
ENT	Ear Nose and Throat
ER	Endoplasmic Reticulum
FATHMM-MKL	Functional Analysis Through Hidden Markov Models
FSL	French Sign Language
GA	Georgia
GATK	Genome Analysis ToolKit
GeneMAP	Genetic Medicine of African Populations
GERP	Genomic Evolutionary Rate Profiling
GME	Greater Middle East
gnomAD	genome Aggregation Database
GST	Glutathione S-Transferase
HAART	Highly Active Antiretroviral Therapy
Het	Heterozygous
HGVS	Human Genome Variation Society
HHH	Hereditary Hearing loss Homepage
HI	Hearing Impairment
HI-GENES	Hearing Impairment Genetics Studies
HIV	Human Immunodeficiency Virus
HPO	Human Phenotype Ontology
HSF	Human Splice Finder
HUGO	Human Genome Organization
IBM	International Business Machines Corporation
Indel	Insertion/deletion
ISCN	International System for human Cytogenetic Nomenclature
KID	Keratitis–Ichthyosis–Deafness
KING	Kinship-based INference for Gnome wide association studies
LRT	Likelihood Ratio Test
MAF	Minor Allele Frequency
M-CAP	Mendelian Clinically Applicable Pathogenicity

MD	Meniere Disease
MDR-TB	MultiDrug-Resistant Tuberculosis
MIM	Mendelian Inheritance in Man
MLPA	Multiplex Ligand-dependent Probe Amplification
MOY6	Myosin VI
mRNA	messenger RNA
MSA	Multiple Sequence Alignment
NCBI	National Centre for Biotechnology Information
NGS	Next-Generation Sequencing
NIH	National Institutes of Health
NSHI	Non-Syndromic Hearing Impairment
OAE	Otoacoustic Emission Test
OAV	Oculo-Auriculo-Vertebral
OMIM	Online Mendelian Inheritance in Man
PACE	Polymerase chain reaction Allele Competitive Extension
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PEPNS	PolyEndocrine-PolyNeuropathy Syndrome
phyloP	phylogenetic *P*-values
PLP	Pathogenic or Likely Pathogenic
PolyPhen	Polymorphism Phenotyping
PRISMA	Preferred Reporting Items for Systematic Review and Meta-Analysis
PROSPERO	International Prospective Register of Systematic Reviews
PROVEAN	Protein Variation Effect Analyzer
PSI-BLAST	Position-Specific Iterative Basic Local Alignment Search Tool
PTA	Pure Tone Audiometry
PTPRQ	Protein Tyrosine Phosphatase Receptor Q
RC3	RabConnectin-3α
RDX	Radixin
RefSeq	Reference Sequence
REVEL	Rare Exome Variant Ensemble Learner
RF	Random Forest

RFLP	Restriction Fragment Length Polymorphism
SD	Standard Deviation
SIFT	Sorting Tolerant from Intolerant
Siphy	Site-specific phylogenetic analysis
SNV	Single Nucleotide Variant
SPSS	Statistical Package for the Social Sciences
SSA	Sub-Saharan Africa
SSCP	Single-Strand Conformational Polymorphism
TOPMed	Trans-Omics for Precision Medicine
TPRN	Taperin
UK	United Kingdom
USA	Unite States of America
WACCBIP	West African Centre for Cell Biology of Infectious Pathogens
WES	Whole-Exome Sequencing
WHO	World Health Organisation
WS	Waardenburg Syndrome
Wt	Wild type
yo	years old

Chapter 1: Introduction

Hearing impairment (HI) is considered disabling when the loss of hearing is greater than 40dB in the better hearing ear in adults (15 years or older) and greater than 30 dB in the better hearing ear in children (0 to 14 years) [1]. Approximately 466 million people worldwide live with disabling HI as of 2018 (6.1% of the world population); whereby 34 million of these are children, and 49 million live in sub-Saharan Africa (SSA) [1]. WHO estimates that the number of people with disabling HI will grow over the years to up to 630 million by 2030 and over 900 million in 2050 [1]. The incidence of HI in high-income countries is approximately 1.1 in 1000 live births [2], which is six times lower than that of low- and middle-income countries where the incidence is estimated at 6 in 1000 live births [3]. HI constitute the most common sensory disability, and when occurring in childhood is associated with impaired language acquisition, learning, and speech development [4].

The aetiologies HI can be environmental (acquired) or genetic, and in many cases, it is not possible to establish a definite aetiology. In developing countries, the environment contributes significantly more to the aetiology of HI than in the developed world [5]. This is attributed to limited access to healthcare services that are often not adequately equipped [5]. Meningitis is the leading cause of environmental HI in Cameroon, contributing to up to 34.4% of HI cases within a cohort of 584 patients. Other environmental aetiologies of HI observed in Cameroon include ototoxicity, mumps, measles, and rubella infection [6]. Hereditary HI contributes from 30 to 50% of HI cases in SSA [5]. Hereditary HI may be syndromic whereby there are other clinical features associated with the loss of hearing. Conversely, HI may be non-syndromic whereby the loss of hearing is the only observed clinical feature [5]. Syndromic HI accounts for up to 30% of hereditary HI cases. Over 400 HI-associated syndromes have been described; including Waardenburg syndrome, Branchiootorenal syndrome, Usher syndrome, Pendred syndrome, Keratitis-Ichthyosis-Deafness syndrome, and Alport syndrome [5,7].

Non-syndromic HI (NSHI) accounts for approximately 70% of hereditary HI cases [7], and is mainly inherited in an autosomal recessive matter (~80%) [8]. Autosomal dominant inheritance is less frequent but not negligible, as it represents about 18% of all NSHI cases, while X-linked and mitochondrial inheritances constitute 1-3% and <1% of cases, respectively [8,9]. NSHI is a highly genetic heterogeneous condition, with about 170 NSHI loci and 121 genes identified to date [10]. Variants that are involved in monogenic HI mainly occur in the genes that control the components of the human auditory system [9]. The most prevalent variants associated with autosomal recessive non-syndromic HI (ARNSHI) have been found within the connexin genes, and they have been implicated amongst European, Asian, and Arab populations [5].

GJB2 (on chromosome 13q12) is the most common gene associated with ARNSHI in Europeans and Asians, and accounts for almost 50% of cases [7]. The most common *GJB2* variant is c.35delG which is seen in up to 70% of ARNSHI cases [5] and is prevalent throughout Europe, North Africa, and the Middle East, as well as areas populated largely by immigrants from these regions. Other variants are prevalent in specific populations, including c.167delC among the Ashkenazim in Israel and p.R143W in Ghana [11]. Outside of the Caucasian European population, c.35delG is rarely implicated in hearing loss. This has resulted in the hypothesis that c.35delG is a founder variant amongst populations of Caucasian descent, rather than a mutational hot spot in *GJB2* [5,12]. Apart from connexin genes, other common genes implicated in HI in European and Asian populations include *SLC26A4* (implicated in Cochlear Homeostasis), *MYO15A* (involved in *Cellular Organization), OTOF (involved in* Neural transmission), *TMC1, CDH23* (implicated in Cellular Organization), and *TMPRSS3* [13].

Ideally, the team evaluating and treating the deaf individual should consist of an otolaryngologist, an audiologist, a clinical geneticist, and a paediatrician. The expertise of an educator of the Deaf, a neurologist, and a paediatric ophthalmologist may also be required. Regardless of its aetiology, uncorrected hearing loss has consistent sequelae [8]. Auditory deprivation through the age of two years is associated with poor reading performance, poor communication skills, and poor speech production. Moreover, educational intervention is insufficient to completely remediate these deficiencies. In contrast, early auditory intervention, whether through amplification, otologic surgery, or cochlear implantation, is effective [8].

Rationale for this study

Variants in *GJB2* which are known to be the major contributors to NSHI in Europeans and Asians do not seem to play a significant role in HI in African populations [14–17]. Indeed, 205 Cameroonians and Black South Africans with congenital non-syndromic deafness were screened for *GJB2* variants in 2014. A pathogenic variant [*GJB2*-c.424_426delTTC, p.(F142del)] and a variant of uncertain significance [*GJB2*-c.499G>A, p.(V167M)] were detected in two unrelated Cameroonian participants. Both variants were detected in a single individual in the heterozygous state [14]. Also, no pathogenic or likely pathogenic (PLP) variant in *GJB2* was found in a cohort of 44 probands from Nigeria with non-syndromic deafness [18]. These results suggest that some other genes and variants are associated with NSHI within sub-Saharan African populations, and given the high genetic heterogeneity of NSHI, next-generation sequencing (NGS) techniques appear as the most effective way to identify them [5]. Additionally, patients included in the previous studies that assessed the contribution of *GJB2* variants to NSHI in Cameroon consisted of both familial and isolated cases, with

therefore a high probability of environmental aetiologies in many cases. There is thus a need to revisit the contribution of *GJB2* variants to familial cases of NSHI from Cameroon.

Targeted sequencing panels that include > 100 HI genes have detected a consistently lower rate of pathogenic and likely pathogenic (PLP) variants in sporadic HI cases of African ancestry e.g. African Americans (26%), and Nigerians and Black South Africans (4%), compared to >70% for Europeans and Asians [19,20]. However, the detection rate was 70% for 10 mutiplex Cameroonian families [21]. Moreover, the prevalence of ARNSHI PLP variants, using data from the Genome Aggregation Database (gnomAD) database [22] were estimated to account for ARNSHI in 5.2 per 100,000 individuals for Africans/African Americans, compared to 96.9 per 100,000 individuals for Ashkenazi Jews based on sequence data [23]. Therefore, there is an urgent need to investigate HI in populations of African ancestry, particularly multiplex families, using NGS, to improve knowledge on variants and genes which underlie NSHI in African populations.

Six loci for X-linked HI have been described to date, including the DFNX3 (Xp21.2) locus which contains *DMD* [24]. Variants in the latter are known to be associated with an X-linked recessive disorder called Duchenne muscular dystrophy (DMD) [25]. Studies from mice found that the knockout mouse model for *DMD* (the *mdx* mouse) presents an increased threshold for hearing as compared to the wildtype mouse [26]. Also, dystrophin (the protein encoded by *DMD*) was found to be expressed in the cochlear of pig and normal mice, but absent from *mdx* mice [27]. These results suggested that *DMD* might play a role in the hearing process. The possible implication however of *DMD* in HI in humans has not been extensively studied nor proven yet. Last, most studies on DMD in Africa were conducted in the Northern and Southern parts of the continent. Therefore, the clinical presentation and genetics of this condition in some Africa populations are not well known.

In Africa, parents of children with HI attribute the HI to a variety of causes, from environmental factors to mysterious ("evil forces") or superstitious beliefs [28,29]. Also, people with HI in some African communities are faced with issues of discrimination and decreased social interaction [30]. Indeed, a previous study from Cameroon reported discrimination against people with HI as they are considered incapable of engaging in activities such as family meetings and decision-making [30]. Besides, family exclusion and social isolation of persons with HI were reported as the main challenges faced by people with HI in a study from Burundi [31]. It is therefore critical to understand the challenges faced by persons with HI in African communities, their perception of the causes of HI, and how these challenges can be remedied.

Chapter 2: Aims and Objectives

This study was aimed at identifying the genes that contribute to NSHI in Cameroon, through the subsequent use of targeted gene screening and a wider approach i.e. whole exome sequencing (WES). Also, we sought to identify the challenges faced by persons with HI in Cameroon, and determine ways to overcome these challenges.

Objectives

1. Systematic literature search:
    1.1. Establish the current status of knowledge on HI in Africa with a particular focus on Cameroon,
    1.2. Determine at the global level the contribution of connexin genes to HI in humans.
2. Recruit familial and isolated cases with NSHI of putative genetic origin from Cameroon.
3. Revisit the contribution of connexin genes to hereditary HI cases in Cameroon.
4. Determine the clinical presentation of DMD in Cameroon and the most common variants associated with this condition, and explore the possible implication of *DMD* in HI in humans.
5. Use NGS techniques to solve NSHI cases in Cameroon:
    5.1. Generate WES data on families with NSHI families who tested negative for pathogenic variants in *GJB2*.
    5.2. Analyse these data through the use of bioinformatics tools, to identify the causative genes and variants
6. Determine the social perception of HI in Cameroon, and understand the challenges faced by persons with HI in Cameroon, as well as their expectations.

Chapter 3: Literature Reviews

Synopsis: This chapter presents the recent scientific knowledge on various aspects (including the prevalence, aetiology, clinical presentation and genetics) of HI in Africa with a focus on Cameroon, and the contribution of connexin genes to HI in humans at a global level. This chapter includes two peer reviewed systematic review articles.

3.1. **Wonkam Tingang, E.**, Noubiap, J. J., F. Fokouo, J. V., Oluwole, O. G., Nguefack, S., Chimusa, E. R., and Wonkam, A. (2020). Hearing Impairment Overview in Africa: The Case of Cameroon. *Genes*, 11(2), 233. https://doi.org/10.3390/genes11020233.

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OGO: Critically revised the successive versions of the manuscript

ERC: Supervised the review and contributed to compile the revisions

AW: Conceived and supervised the review, and compiled the revisions

Hearing Impairment Overview in Africa: the Case of Cameroon

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Abstract: The incidence of hearing impairment (HI) is higher in low- and middle-income countries when compared to high-income countries. There is therefore a necessity to estimate the burden of this condition in developing world. The aim of our study was to use a systematic approach to provide summarized data on the prevalence, etiologies, clinical patterns and genetics of HI in Cameroon. We searched PubMed, Scopus, African Journals Online, AFROLIB and African Index Medicus to identify relevant studies on HI in Cameroon, published from inception to 31 October, 2019, with no language restrictions. Reference lists of included studies were also scrutinized, and data were summarized narratively. This study is registered with PROSPERO, number CRD42019142788. We screened 333 records, of which 17 studies were finally included in the review. The prevalence of HI in Cameroon ranges from 0.9% to 3.6% in population-based studies and increases with age. Environmental factors contribute to 52.6% to 62.2% of HI cases, with meningitis, impacted wax and age-related disorder being the most common ones. Hereditary HI comprises 0.8% to 14.8% of all cases. In 32.6% to 37% of HI cases, the origin remains unknown. Non-syndromic hearing impairment (NSHI) is the most frequent clinical entity and accounts for 86.1% to 92.5% of cases of HI of genetic origin. Waardenburg and Usher syndromes account for 50% to 57.14% and 8.9% to 42.9% of genetic syndromic cases, respectively. No pathogenic mutation was described in *GJB6* gene, and the

prevalence of pathogenic mutations in *GJB2* ranged from 0% to 0.5%. The prevalence of pathogenic mutations in other known NSHI genes was <10% in Cameroonian probands. Environmental factors are the leading etiology of HI in Cameroon, and mutations in most important HI genes are infrequent in Cameroon. Whole genome sequencing therefore appears as the most effective way to identify variants associated with HI in Cameroon and sub-Saharan Africa in general.

Keywords: hearing impairment; prevalence; etiologies; genetics; Cameroon; Africa

3.1.1. Introduction

Hearing impairment (HI) is considered disabling when the loss of hearing is greater than 40 dB in the better-hearing ear in adults (15 years or older) or greater than 30 dB in the better-hearing ear in children (0 to 14 years) [32]. The World Health Organization (WHO) estimated in 2018 that approximately 466 million people globally live with disabling HI (6.1% of the world population), of whom 34 million are children and 49 million live in sub-Saharan Africa [32]. HI affects up to 6 per 1000 live births in sub-Saharan Africa, with a lower incidence of about 1 per 1000 live births in developed countries [2,3].

According to WHO, HI can be classified as mild, moderate, severe or profound when the pure tone average ranges from 26 to 40 dB, 41 to 60 dB, 61 to 80 dB or is over 81 dB, respectively [32]. HI can be due to environmental or genetic causes, and in many cases it is not possible to establish a definite etiology [33,34]. Environmental factors such as meningitis, measles or ototoxicity are the leading causes of HI in low- and middle-income countries, while their burden is lower in high income countries [5,34–36]. This is attributed to poor healthcare systems that are not always adequately equipped to prevent, screen and manage causes of HI [5]. Genetic factors contribute to 30% to 50% of HI cases in sub-Saharan Africa [5]. HI of genetic origin can be syndromic or non-syndromic, depending on whether it is associated with additional abnormalities in other organs or not [5,37,38]. Non-syndromic hearing impairment (NSHI) accounts for 70% of hereditary hearing loss [7]. Over 400 syndromes with HI have been described, including Waardenburg syndrome, branchiootorenal syndrome, Usher syndrome, Pendred syndrome, keratitis–ichthyosis–deafness syndrome and Alport syndrome [5,7,39].

GJB2 (on chromosome 13q12) is the most commonly associated gene with NSHI in European and Asian populations and accounts for almost 50% of cases [7,40,41]. The most common *GJB2* mutation is c.35delG, which represents about 70% of *GJB2* mutated alleles in those populations [5,42]. Other mutations are prevalent in specific populations, including 167delC among the Ashkenazim in Israel and p.R143W in Ghana [11,43,44].

Regardless of its etiology, uncorrected HI has sequelae [8]. Undetected and untreated hearing loss can result in poor reading performance, poor communication skills and poor speech production [8,45]. Educational intervention is insufficient to completely remediate these deficiencies. In contrast, early auditory intervention is effective, whether through amplification, otologic surgery or cochlear implantation [8].

Some systematic reviews have estimated the global and the regional burden of HI [46,47]; however, there is a lack of data on national estimates of the prevalence of this condition in many countries, especially in Africa. Therefore, there is a call to action for each country to estimate its national burden and to develop specific programs for the prevention and the management of HI. The aim of the present review was to use a systematic approach to provide summarized data on the prevalence, etiologies, clinical patterns and genetics of HI in Cameroon, a sub-Saharan African country.

3.1.2. Materials and Methods

This review is reported in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) statement [48] and is registered in the International Prospective Register of Systematic Reviews (PROSPERO, registration number: CRD42019142788).

3.1.2.1. Selection Criteria

We included observational studies published from inception to 31 October, 2019 that report data on the prevalence, etiologies, clinical characteristics or genetics of HI in Cameroon. For duplicate studies, the most comprehensive and/or recent article with the largest sample size was considered. Qualitative studies, letters to the editor, reviews and commentaries were excluded. Studies with either unavailable full text or missing key data that could not be accessed after a reasonable request from corresponding authors, or before the end of the data extraction process, were also excluded.

3.1.2.2. Search Strategy

We searched PubMed, Scopus, and African-specific databases (African Journals Online, AFROLIB and African Index Medicus) for relevant articles. Keywords used for the search included: “hearing loss”, “hearing impairment”, “deafness”, “deaf” and “Cameroon”. All the search strategies are represented in the Supplementary Materials (Tables 3.3–3.7).

The reference lists of all eligible studies and of relevant literature reviews were screened, and specific researchers active in the field of hearing loss in Cameroon were contacted to identify additional sources of information.

3.1.2.3. Selection of Studies

Titles and abstracts obtained from searches were imported into the software Zotero, version 5.0.64, for the removal of any duplicates. Regarding our inclusion and exclusion criteria, one author (EWT) screened unduplicated titles and abstracts before reviewing the full text of all selected studies for final inclusion. A second author (JJN) verified that the study screening and selection process was performed correctly. Any disagreement between the two authors was solved through discussion and consensus.

3.1.2.4. Data Extraction Process

Using a predesigned data extraction sheet, one researcher (EWT) extracted data from relevant studies. A second researcher (JJN) checked the accuracy of the data extraction process, with any discrepancy resolved through discussion and consensus. Extracted data included: the last name of first author, the year of publication, the region(s) where the study was conducted, setting (hospital, school for the deaf, community), study design, data collection (prospective versus retrospective), study population, proportion of males, mean or median age, age range, sample size, tool used to diagnose HI, number of cases of HI (for prevalence studies), proportions of different types of hearing loss (sensorineural, conductive, mixed), proportions of different levels of hearing loss (mild, moderate, severe, profound), distribution of etiologies, inheritance patterns for genetic cases, clinical patterns with details (syndromic versus non-syndromic), data on molecular testing including genotyping method, targeted genes or mutations and pathogenic variants found. For some studies, relevant proportions were calculated from their raw data. Data were summarized narratively.

3.1.2.5. Assessment of Methodological Quality

Two investigators (EWT and ERC) assessed the risk of bias and the quality of included studies using the quality of genetic studies (Q-Genie) tool developed by Sohani et al. [49] for genetic studies and the risk of bias assessment tool for prevalence studies developed by Hoy et al. [25] for the other studies. Discrepancies were solved by discussion and consensus.

3.1.3. Results

3.1.3.1. The Review Process

Initially, 333 records were identified through database searches. After the removal of duplicates, titles and abstracts of 289 studies were screened, of which 255 records were excluded. Full texts of the remaining 34 records and of papers identified through other sources were scrutinized for final inclusion. A total of 17 articles were judged to be eligible and were included in the review (Figure 3.1).

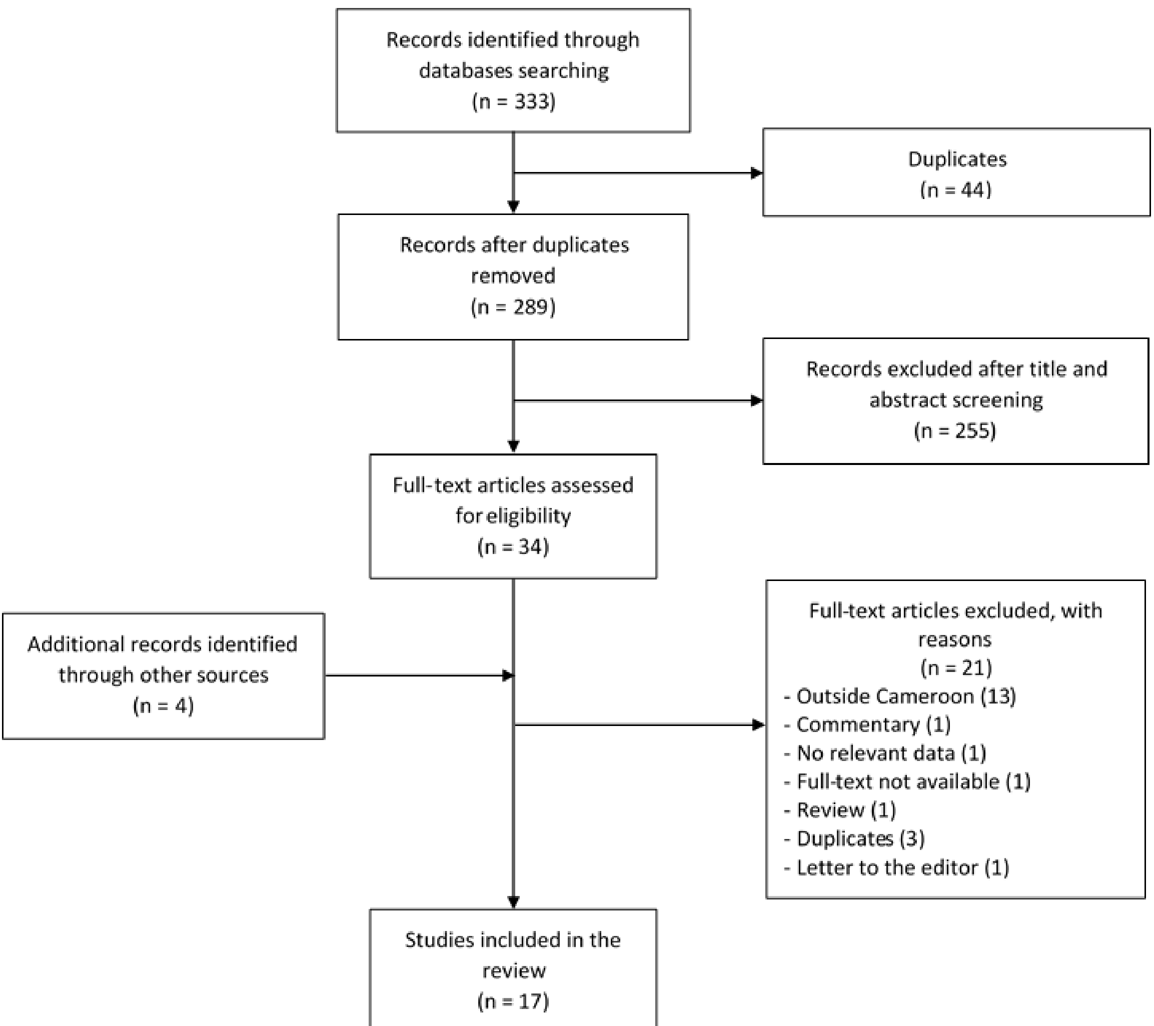

Figure 3.1: Flow chart of studies selection

3.1.3.2. Characteristics of Included Studies

The characteristics of the included studies are summarized in Table 3.1. The majority of the studies were school- and/or hospital-based. Patients included in these studies were globally recruited from 9 of the 10 administrative regions of Cameroon and were recruited from both urban and rural areas. The male proportion ranged from 0% to 74.4%, and the mean age ranged from 3.5 to 43.2 years. Pure tone audiometry (PTA) was the most frequent tool used to diagnose HI. The study quality was high, moderate and low in ten [14,51–59], three [60–62] and four [63–66] studies, respectively.

Table 3.1: General characteristics of the included studies

First Author's Name, Publication Year	Area	Regions	Study Setting	Study Design	Data Collection	Study Population	Male (%)	Mean Age (Years)	Age Range (Years)	Sample Size	Diagnosis Tool	Quality
Fokouo, 2015 **[51]**	urban	center region	hospital	case-control	prospective	patients followed up for HIV infection.	28.3	33.4 ± 7.7	15–49	180	PTA	high
Wonkam, 2013 **[52]**	urban and rural	7 regions	school and hospital	cross sectional	prospective	patients with childhood deafness	54.1	11 *	1–32	582	PTA and ABR	high
Lebeko, 2017 **[53]**	urban and rural	7 regions	schools and hospital	cross sectional	prospective	patients with non-syndromic hearing impairment of either putative genetic origin or unknown origin	NR	NR	NR	57	PTA	high
Djomou, 2016 **[63]**	urban	center region	hospital	cross sectional	retrospective	patients admitted at the ENT unit for sensorineural emergencies	NR	43.2 ± 17	16–66	22	PTA	low
Tingang Wonkam, 2019 **[54]**	urban and rural	8 regions	school and community	cross sectional	prospective	familial hearing impairment cases	45.2	18 ± 10.4	1–50	93	PTA and ABR	high
Trotta, 2011 **[60]**	rural	Far-North region	school	cross sectional	prospective	patients with prelingual hearing loss	74.4	NR	>5	70	PTA	moderate
Wonkam, 2013 **[55]**	NR	NR	school	Case report	prospective	patients suffering from KID syndrome	0	3.5 ± 2.12	2–5	2	PTA and ABR	high

Kuaban, 2015 **[61]**	urban	2 regions	hospital	longitudinal	prospective	MDR-TB (multidrug-resistant tuberculosis) patients, treated with a standardized 12-months regiment, including kanamycin.	51.3	33.7	17–68	150	PTA	moderate
Jivraj, 2014 **[64]**	rural	Northwest region	school	cross sectional	prospective	students at two schools, including a school for the deaf	58.2	11.8 ± 2.8	NR	320	NR	low
Bosch, 2014 **[56]**	urban and rural	7 regions	school and hospital	cross sectional	prospective	patients with deafness of either putative genetic origin or unknown origin and that were shown not to have mutation in *GJB2* gene	52	12.11	NR	75	PTA	high
Ferrite, 2017 **[57]**	rural	Northwest region	community	cross sectional	prospective	general population of the Fundong Health District, Northwest Cameroon	40.8	24.4	0–80+	3567	PTA and OAE	high
Bosch, 2014 **[14]**	urban and rural	7 regions	school and hospital	cross sectional	prospective	patients with deafness of either putative genetic origin or unknown origin	NR	NR	NR	180	PTA	high

Lebeko, 2016 **[58]**	urban and rural	NR	school and hospital	cross sectional	prospective	families with at least two individuals with ARNSHI who were negative for pathogenic variants in *GJB2* and *GJB6*	53.8	NR	NR	26	PTA	high
Chiabi, 2004 **[65]**	rural	East region	hospital	cross sectional	retrospective	patients admitted and treated for severe malaria	55.3	2.7	0–15	387	self-reported	low
Trébucq, 2018 **[62]**	urban	NR	hospital	longitudi nal	prospective	MDR-TB (multidrug-resistant tuberculosis) patients, treated with a standardized 9-months regiment, including kanamycin	NR	NR	≥18	176	PTA	moderat e
Cockburn, 2014 **[66]**	rural	Northwest region	community	cross sectional	prospective	people living in the Northwest region of Cameroon	43.3	NR	0–70+	18 878	self-reported	low
Noubiap, 2014 **[59]**	urban and rural	7 regions	schools and hospital	cross sectional	prospective	patients suffering from Waardenburg syndrome	50	12.2 ± 7	6–25	6	PTA	high

ABR, auditory brainstem response test; ARNSHI, autosomal recessive non-syndromic hearing impairment; ENT, ear nose and throat; KID, keratitis–ichthyosis–deafness; NR, not reported; OAE, otoacoustic emission test; PTA, pure tone audiometry; * The number shown here is the median.

3.1.3.3. Prevalence of Hearing Impairment in Cameroon

None of the studies included in this review reported on the national prevalence of HI in Cameroon. However, the regional estimates reported by these studies give an insight into the public health burden of this condition in Cameroon. In 2013, a community-based study was carried out in a health district located in the Northwest region of Cameroon and included the general population of that area. A total of 3567 individuals were recruited and screened through PTA and otoacoustic emission test. They were aged 0 to over 80 years old, with a male proportion of 40.8%. The overall prevalence of HI in this population was estimated at 3.6% (95% CI: 2.8–4.6) [57]. The prevalence was relatively low at 1.1% among children (<18 years), was high as 6.5% among adults (≥18 years old), and rose rapidly to a level of 14.8% in participants aged 50 years and more [57]. The prevalence of HI in this health district seems to be greater than the overall prevalence in the Northwest region of Cameroon, which was estimated at 0.9% in a study including 18,878 participants from that region and where HI was diagnosed on a self-reported base [66].

In a retrospective cross-sectional study conducted at the Yaoundé teaching hospital, the authors included patients who visited the ear nose and throat (ENT) unit from January 2010 to July 2014 for sensorineural emergencies, which includes sudden sensorineural hearing loss, Bell's palsy and acute vertigo [63]. A total of 22 patients were included in the study, with a mean age of 43.2 ± 17 (range 16–66) years. The prevalence of sudden sensorineural hearing loss, which was defined as an NSHI occurring within 72 h, was estimated at 9.1% (2 out of 22) in this group of patients [63].

3.1.3.4. Audiometric Characteristics of Hearing Impairment in Cameroon

In Cameroon, sensorineural HI is the most frequent pathophysiological type, and is accounted for in 61.7% to 94.4% of all HI cases, while mixed and conductive HI are found in 5.6% to 20% and 0% to 18.3% of cases, respectively [51,52,54]. HI tend to be more severe in school settings, where profound HI (≥81 dB) accounts for 93.5% to 98.2% of cases [52,54,60], than in community settings, where profound HI is observed in only 9% of cases, the majority (76%) being moderate HI (41–60 dB) [57]. Bilateral HI represent 36% to 100% of cases [51,52,54,60]; and in the case-control study performed in a referral hospital of Yaoundé, HI was left-sided and right-sided in 43% and 21% of participants, respectively [51].

3.1.3.5. Etiologies of Hearing Impairment in Cameroon

Environmental factors are the leading causes of HI in Cameroon, and account for 52.6% to 62.2% of HI cases [52,57]. Hereditary HI is responsible for 0.8% to 14.8% of cases, and in 32.6% to 37% of HI cases the origin remains unknown (Table 3.2) [52,57].

Table 3.2: Etiologies of hearing impairment in Cameroon and comparison to other African countries.

Country	Cameroon	Cameroon	Sierra Leone	Gambia	Ghana	Nigeria
Year of publication	2013	2017	1991	1985	2019	1982
Reference	[52]	[57]	[35]	[67]	[44]	[68]
Number of patients	582	127	354	259	1104	298
Hereditary	14.8%	0.8%	–	8.1%	21.3%	13.1%
Meningitis	34.4%	–	23.9%	31.7%	3.9%	11%
Impacted wax	–	31.5%	–	–	–	–
Age-related HI	–	22.8%	–	–	–	–
Noise-induced HI	–	1.5%	–	–	–	–
Measles	4.3%	–	4.1%	1.9%	0.9%	13%
Rubella	0.5%	–	–	1.5%	0.2%	2%
Mumps	2.1%	–	16.7%	–	0.5%	3%
Ototoxicity	6%	–	20.8%	–	–	9%
Other	5.3%	6.4%	–	2.3	13.1%	7.7%
Unknown	32.6%	37%	34.8%	54.4%	60.1%	41.2%

HI, hearing impairment.

In a cohort including 582 hearing-impaired children from 7 of the 10 administrative regions of Cameroon, meningitis was the leading environmental etiology and was implicated in 34.4% of HI cases (Table 3.2). Infectious diseases that are preventable by vaccination (meningitis, measles, rubella and mumps) represented 41.3% of cases [52]. A cross-sectional study performed in 2014 emphasized the link between rubella infection and deafness [64]. The authors recruited 320 children from two different schools in the Northwest region of Cameroon, including one school for the deaf. They found that hearing-impaired children were seven times more likely to have positive rubella IgG serology (48.7%) than children with normal hearing (7.4%; $p < 0.0001$) [64].

In a community-based study undertaken in 2013 in Fundong health district, impacted wax and age-related HI were the most frequent environmental etiologies and accounted for 31.5% and 22.8% of HI cases, respectively (Table 3.2). Age-related HI was particularly prevalent in elderly patients and was implicated in 31% of HI cases in patients aged 50 years or more [57]. Severe malaria has been identified as a cause of deafness in a retrospective cross-sectional study performed in 2004 [65]. The authors assessed the outcome of severe malaria in 387 patients admitted and treated in a regional hospital of the East region of Cameroon. Among the 317 patients who recovered, neurological sequelae were observed in six patients, of which three had deafness [65].

Ototoxicity due to anti-tuberculosis drugs also plays an important role in the etiology of HI in Cameroon. A longitudinal study performed in two hospitals, located in two different regions of Cameroon, assessed the outcome of anti-tuberculosis treatment in multidrug-resistant tuberculosis (MDR-TB) patients. Patients were treated with a standardized 12-month drug regimen, including gatifloxacin, clofazimine, prothionamide, ethambutol and pyrazinamide

throughout, supplemented by kanamycin and isoniazid during an intensive phase of a minimum of 4 months. Participants underwent audiometry testing at baseline and after 4 months treatment. A total of 150 participants were recruited, and their mean age was 33.7 years (range 17–68). Among the 106 patients with audiometry data available, 46 (43.4%) presented HI after a 4 month follow-up [61]. A similar study performed in nine African countries (Cameroon, Burkina Faso, Burundi, Benin, Democratic Republic of Congo, Central Africa Republic, Ivory Coast, Niger, and Rwanda), assessed the adverse effects of anti-tuberculosis drugs in MDR-TB patients, treated with a standardized 9-month regimen, including moxifloxacin, clofazimine, ethambutol and pyrazinamide throughout, supplemented by kanamycin, prothionamide and high-dose isoniazid during an intensive phase of a minimum of 4 to a maximum of 6 months. Of the 491 patients with audiometry results available at both month 0 and month 4, 56 (11.4%) had severe hearing deterioration at month 4 [62].

With reference to a case-control study undertaken in a referral hospital in Yaoundé, HIV infection seems to be one of the etiological factors of HI in the Cameroonian population. Included in the aforementioned study were 90 HIV-infected individuals divided in three subgroups: 30 highly active antiretroviral therapy (HAART)-naive patients, 30 patients receiving first-line HAART and 30 patients receiving second-line HAART, as well as 90 apparently healthy participants as controls. The prevalence of HI in the HIV-positive group was higher than the HIV negative group (27.2% vs. 5.6%; $p = 0.04$) [51]. Additionally, HIV-positive patients had a significant increase in pure tone averages when compared with the HIV-negative patients. No significant difference was found in terms of pure tone averages and susceptibility to hearing loss between HAART-naive patients and those receiving HAART [51].

3.1.3.6. Hearing Impairment of Genetic Origin

Clinical Patterns

Concerning hereditary HI, NSHI is the most prevalent clinical entity in Cameroon, and accounts for 86.1% to 92.5% of cases, while syndromic HI represents 7.5% to 13.9% of cases [52,54].

Waardenburg syndrome (WS) is the most frequent syndromic HI in the Cameroonian population; it represents 1% of all HI cases, 4.3% to 7% of HI of genetic origin, and 50% to 57.14% of genetic syndromic cases [52,54,59]. All cases of WS described in the Cameroonian population are type 2 (without dystopia canthorum). Clinical signs included: congenital severe to profound sensorineural HI, hypopigmentation of the skin (usually on the trunk), premature canitis, isochromic sapphire-blue eyes and in some cases heterochromia iridis that can be segmental or complete (Figure 3.2) [52,54,59].

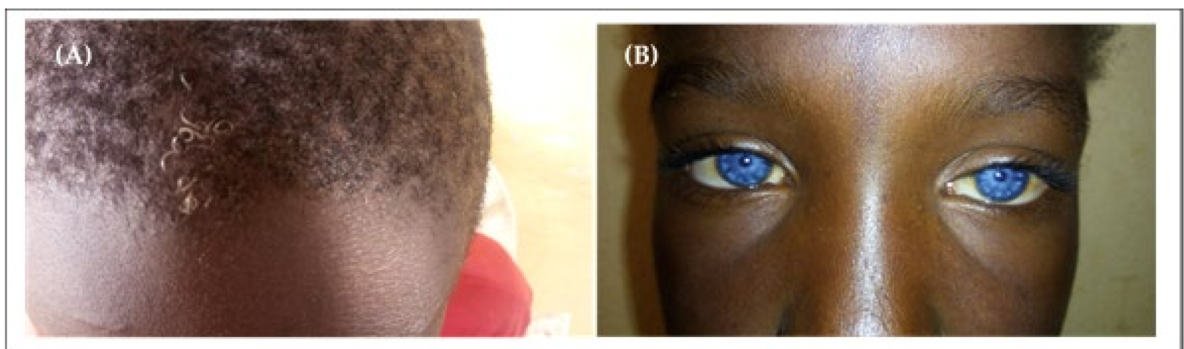

Figure 3.2: Illustration of some clinical signs found in Cameroonian patients with Waardenburg syndrome. (**A**) Premature white hair; (**B**) Sapphire-blue eyes (extracted from the study by Tingang Wonkam et al. [54]).

Usher syndrome is the second most frequent syndromic HI in the Cameroonian population. It accounts for 8.9% to 42.9% of all syndromic HI cases [52,54]. Three cases of type 2 Usher syndrome have been described with clinical signs of retinitis pigmentosa, including night vision impairment and constricted visual field present in affected patients, in addition to HI [54]. A case of type 1 Usher syndrome was reported, and clinical signs included those of type 2, associated with vestibular dysfunction [52].

Two cases of keratitis–ichthyosis–deafness (KID) syndrome were reported. PTA in these patients showed bilateral profound sensorineural HI. Physical examination revealed generalized ichtyosis and erythrokeratodermia, palmoplantar keratoderma, rippled hyperkeratotic plaques on the knees, elbows and ankles (reducing the mobility of the affected joints), hypotrichosis, alopecia, and hyperkeratosis lesions in the external auditory canal. Ophthalmologic examination revealed a mild vascularizing keratitis which explained photophobia and reduced visual acuity. Oral examination showed dental dysplasia, and histopathological examination of the skin revealed an acanthotic dyskeratosis (Figure 3.3) [52,55].

One case of oculo-auriculo-vertebral (OAV) spectrum, presenting with unilateral facial hypoplasia, anti-mongoloid slant of the palpebral fissures, microtia and preauricular tags, a slight degree of mental retardation, vertebral anomalies and deafness, and a case of Pendred syndrome that presented with a postlingual progressive sensorineural deafness and hypothyroid goitre have also been described [52].

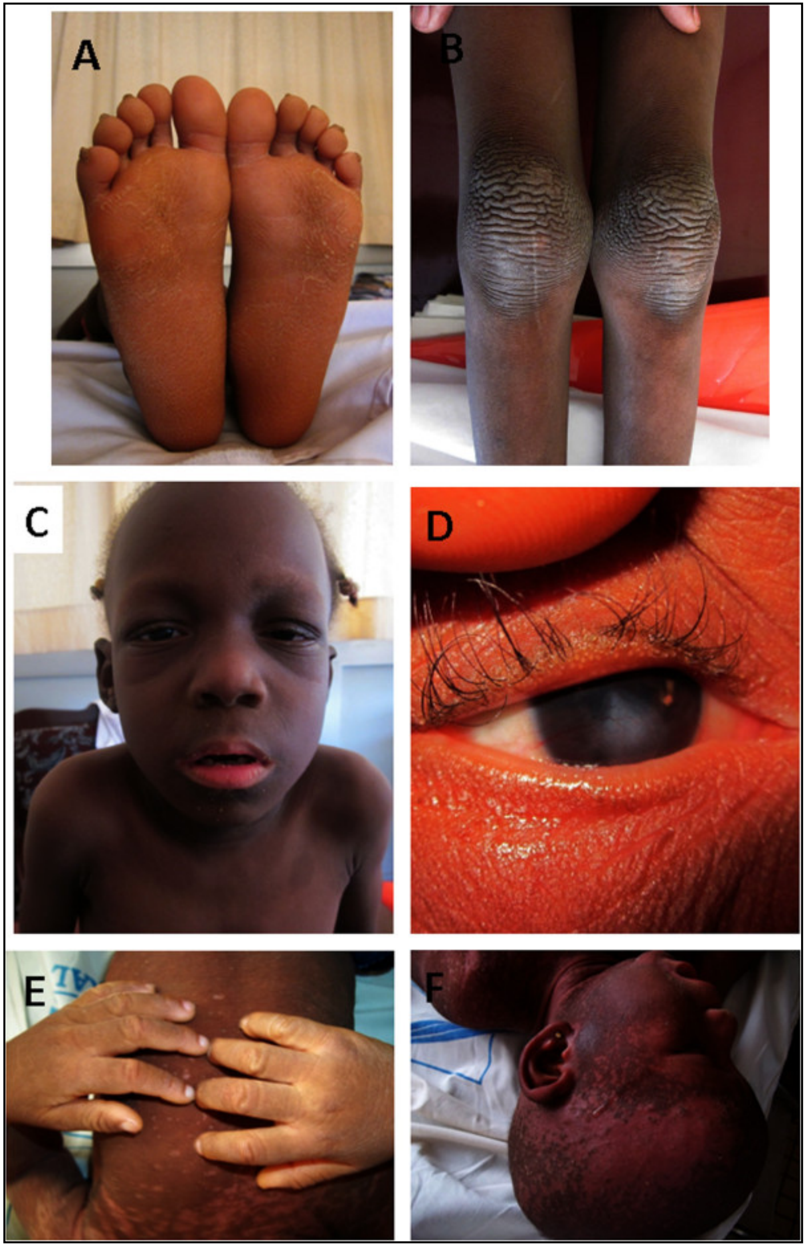

Figure 3.3: Illustrations of some clinical features of the two Cameroonian KID cases (Case 1; panels A–D; Case 2 panels E and F). (**A**) Keratoderma of the soles; (**B**) Rippled hyperkeratotic plaques on the knees; (**C**) Hypotrichosis of the eyelashes and eyebrows; (**D**) Mild vascularizing keratitis; (**E**) Hyperkeratosis of the hands; (**F**) Alopecia, hypotrichosis, ichthyosiform erythrokeratoderma (extracted from the paper by Wonkam et al. [55]).

Inheritance Pattern

Based on pedigree analysis, studies reported autosomal recessive inheritance to be the most frequently observed pattern of inheritance, accounting for 82.8% to 87.2% of cases of HI of genetic origin. Autosomal dominant and mitochondrial inheritance were less frequent and were observed in 9.3% to 10.7% and 0% to 6.5% of cases, respectively. Pedigree-based consanguinity was estimated at 6.5% to 13.1% of cases of hereditary HI in the Cameroonian population (Figure 3.4) [52,54].

Gene Variants and Hearing Impairment

Non-syndromic Hearing Impairment

Several studies assessed the contribution of known HI genes to HI in the Cameroonian population. Mutations in *GJB2* and *GJB6* genes have a prevalence close to zero in the Cameroonian population. In 2011, a cohort of 70 deaf children from the Far-North region of Cameroon were screened for mutations in *GJB2* and *MTRNR1* (mitochondrial DNA) genes, by direct sequencing. No mutations were found in *GJB2*, and a *MTRNR1* variant of unknown pathogenicity (m.G1462T) was present in one patient [60]. Additionally, a cohort of 180 Cameroonians with non-syndromic deafness of either putative genetic origin or unknown origin were screened for *GJB2* mutations in 2014. A pathogenic mutation, c.424_426delTTC (p.F142del), and a mutation of uncertain significance, c.499G>A (p.V167M), were both detected in one patient, in the heterozygous form [14]. Subsequently, 75 Cameroonian patients with non-syndromic deafness were analyzed through direct Sanger sequencing of the entirety of the coding regions of *GJB6* gene and *GJA1* pseudo-gene; the large-scale *GJB6*-D3S1830 deletion was also investigated. No pathogenic mutations were detected in either *GJB6* or *GJA1*, nor was the *GJB6*-D3S1830 deletion detected [56].

Recently, 29 non-syndromic hearing-impaired families, showing a clear segregation of the disease within the family, with at least two affected family members and with strong evidence of non-environmental etiology, were recruited. They were screened for mutations in the coding exon (exon2) of *GJB2*, and for the 342-kb deletion (*GJB6*-D3S1830) in *GJB6*. None of these 29 families exhibited the del(*GJB6*-D13S1830) mutation or any of the reported disease-causing mutations in *GJB2*. However, the *GJB2* variant of uncertain significance, c.499G>A (p.V167M), was present in one family in the heterozygous form [54].

In 2016, a panel of 116 HI genes (OtoSCOPE® platform) was interrogated through targeted genomic enrichment and massively parallel sequencing. In 7 out of 10 families investigated (70%), 12 putatively pathogenic variants were identified in six NSHI genes (*CHD23, LOXHD1, MYO7A, SLC26A4, OTOF,* and *STRC*) [58]. Five of the 12 identified variants (41.6%) were novel, whereas the remaining seven variants have been shown to be involved in NSHI in populations outside of sub-Saharan Africa. All identified variants segregated with HI phenotype [58]. The prevalence of these newly identified mutations was assessed in a cohort of 57 non-syndromic hearing-impaired individuals from Cameroon. Only variants *OTOF* NM_194248.2:c.766-2A>G and *MYO7A* NM_000260.3:c.1996C>T (p.Arg666Stop) were found in 3 (5.3%) and 5 (8.8%) patients, respectively [53]. All these variants were in a heterozygous state in all cases and are thus unlikely to explain, alone, the cause of HI in these patients [53].

Keratitis–Ichtyosis–Deafness Syndrome

The coding exons (exon2) of *GJB2* of the two unrelated Cameroonian patients with KID syndrome were screened through direct sequencing. A pathogenic missense mutation, c.148G>A, resulting in a putative amino acid change from aspartic acid (GAC) to asparagine (AAC) in codon 50, p.Asp50Asn, was identified in the heterozygous form in both patients [55]. The "NM_004004.6:c.148G>A" mutation was not present in more than 180 unrelated individuals who were screened for recessive deafness mutations, nor in 60 healthy control persons of Cameroonian origin [55].

3.1.4. Discussion

To our knowledge, this systematic review is the first report summarizing data on the prevalence, etiologies and genetics of HI in Cameroon. Our study confirms the lack of nationwide studies evaluating the prevalence or the incidence of HI in Cameroon.

When using a threshold of 40 dB in adults and 35 dB in children, the population-based prevalence of HI in Cameroon is about 3.6% and increases with age. It is low at 1.1% in children, is up to 6.5% in adults and rises to a level of 14.8% in participants aged of 50 years or more. This is consistent with report from the WHO which estimated that the prevalence of disabling HI in sub-Saharan Africa is about 4.5% in the general population, 1.9% in children and 6.4% in adults [32]. HI prevalence varies greatly across studies in sub-Saharan Africa. Population-based prevalence of HI ranges from 5% in Mozambique to 9.1% in Sierra Leone and 18% in Uganda [69–71].

Several factors contribute to this prevalence variation across studies, including different study settings (community-, school- or hospital-based) and the use of different hearing test techniques [72]. When using PTA as the hearing test technique, different cut-offs used to diagnose HI would also contribute to prevalence variation across studies. Using a low cut-off such as 25 dB would identify milder HI and would produce a higher prevalence, whilst a pure tone cut-off at 40 dB would result in a lower prevalence as only moderate and severe HI would be identified [72]. The prevalence of HI in a population-based study from South Africa was estimated at 12.3% when using a cut off of 25 dB, while it was low at 4.6% when the cut off was 30 dB in children and 40 dB in adults [73]. Additionally, different mean ages of the study populations across studies will also lead to different prevalence across these studies, since HI prevalence was shown to increase with age [32]; therefore, the older the population, the higher the prevalence. The prevalence of HI in a tertiary health institution in Southwestern Nigeria was estimated at 7.5% in children (≤ 15 years old), whilst it was up to 34.5% in adults (>15 years old) [74].

Our study confirms that environmental and preventable factors play a major role in the etiology of HI in Cameroon, as is the case in other sub-Saharan African countries (Table 1). Environmental factors contribute to 65.5%, 45.7% and 37.5% of HI cases in Sierra Leone, Nigeria and Gambia, respectively [35,67,68]. Infectious diseases that are preventable by vaccination (meningitis, measles, rubella and mumps) were the leading environmental etiologies and represented 41.3% of cases, which is in line with findings from Sierra Leone and Gambia, where preventable infectious diseases were the leading environmental cause of HI and contributed to 44.7% and 35.1% of HI cases, respectively [35,67]. The substantial implication of preventable diseases in the etiology of HI in sub-Saharan Africa highlights the weakness of health care systems, and the need for sub-Saharan African countries to develop strategies to reduce the prevalence of HI that is due to preventable diseases. Strategies to fight HI in sub-Saharan Africa should include reinforcement of immunization programs, adequate equipment in health care facilities, and early diagnosis and proper management of HI-causing diseases.

In this review, impacted wax and age-related HI were identified as the second and the third most frequent causes of HI, respectively, after meningitis. They were also found to be important causes of HI in other sub-Saharan African populations. Impacted wax was highly prevalent in Nigerian children and was responsible for 53% of HI cases, whilst it was less prevalent but not negligible in Uganda and Zimbabwe, where it was implicated in 10% and 13% of HI cases, respectively [47]. This accentuates the important role of primary healthcare facilities in the prevention of HI, as impacted wax can be easily treated through primary healthcare services [57]. Presbycusis, also called age-related HI, can be defined as a progressive, bilateral and symmetrical sensorineural HI, due to age-related degeneration of inner ear structures [75]. It is a multifactorial disorder, with the implication of both environmental and genetic factors [75,76]. Presbycusis was implicated in 22.7% of HI cases in an elderly population from Nigeria [77].

As previously reported by other studies from sub-Saharan African countries, the etiology of HI remains unknown in approximately one-third of HI cases in the Cameroonian population (Table 1). This can be explained by the poor quality of medical records and limited clinical, biological, genetic and morphological (e.g., CT scan) investigational capacities in most settings [52]. The low contribution of genetic factors to HI was also reported in other sub-Saharan African countries (Table 1). This can be attributed to limited access to molecular screening in some sub-Saharan African countries, as many cases of HI classified as being of unknown origin might be of genetic origin [78]. There is therefore a need to intensify research on the genetics of HI in sub-Saharan Africa to establish a comprehensive list of the most frequent mutations associated with HI in these populations, as many cases of genetic HI cases presenting as

sporadic cases will be classified as of unknown origin in the absence of molecular diagnosis [52].

Our study identified WS as the most prevalent syndromic HI in Cameroon, in line with another study performed on African subjects, in which 62% of black children with syndromic deafness from Southern Africa presented with WS [79]. WS is defined as an auditory–pigmentary disorder which affects the iris, hair and skin's pigmentary deposits [5]. WS has four subgroups categorized according to the presence or absence of coexisting abnormalities [7]. In WS type 1, dystrophia canthorum is observed in every patient, while there is no dystrophia canthorum in WS type 2 [7]. In WS type 3, upper extremity abnormalities and the findings of type 1 are found [7]. In WS type 4, there are pigmentation abnormalities, Hirschsprung disease and the findings of type 2 [7]. All the cases reported in the Cameroonian population were type 2 WS. Type 1 WS was described in some African populations, including 18 cases from Kenya [80] and 31 cases from South Africa [81]. No genetic exploration was performed on cases of WS described in the Cameroonian population, however, the most common genes incriminated in the etiology of WS in previous studies include *PAX, EDRNB, SOX10, MITF* and *EDN3* [7,82–85].

This review confirms that *GJB2* and *GJB6* genes, the major contributors to NSHI in Caucasian and Asian populations [86–91], are not significant in NSHI in the Cameroonian population. Apart from the Ghanaian population, where the *GJB2* founder mutation p.R143W (c.427C>T) was shown to be highly prevalent [43] and implicated in approximately 25% of familial cases and 8% of isolated cases of HI [44], our study is consistent with previous reports in other populations of African descent [17,92]. Mutations in *GJB6*, including the 342-kb deletion, *GJB6*-D13S1830, were not reported in cohorts of 182, 44 and 401 probands from South Africa, Nigeria and Ghana, respectively [17,44,93]. As in the Cameroonian population, disease-causing mutations in *GJB2* are also less prevalent or absent in populations from Kenya, Sudan, Uganda, Nigeria, Mauritania and South Africa [14,15,17,34,93,94].

Targeted gene sequencing identified putatively pathogenic variants in 7 out of 10 Cameroonian families (70%), and the prevalence of these variants was <10% in 82 probands from Cameroon and South Africa. This result highlights the low contribution of other known HI genes to NSHI in sub-Saharan African populations, and the promise of next generation sequencing (NGS) in finding variants associated with HI in these populations. Next generation sequencing techniques have demonstrated their efficacy in resolving HI cases across many populations. Targeted gene sequencing was able to identify pathogenic variants that co-segregate with HI in 46.7%, 52.9% and 60% of Japanese, Turkish and Chinese families, respectively, which had presented NSHI and which did not have pathogenic mutations in common HI genes [95–97].

Because of the high genetic heterogeneity of NSHI, NGS appears to be the most effective method to identify variants associated with non-syndromic deafness in African populations.

3.1.5. Strengths and Limitations

Of the 17 studies included in this review, only two prevalence studies were population-based, and they were both conducted in just one of the ten administrative regions of Cameroon. Therefore, we do not have a national estimate of the prevalence of HI in Cameroon. Additionally, in one of these two population-based studies, HI cases were diagnosed on a self-reported base, allowing a potential bias in the estimate of the prevalence of HI in that region.

However, the present review is, to the best of our knowledge, the first report assessing the burden of HI in Cameroon. This study has public health implications, since it will raise awareness among policy-makers and will help them develop strategies to reduce the burden of HI (including implementation of a newborn screening program, reinforcement of the immunization program and the improvement of equipment available at health facilities). Additionally, this review should encourage the funding of research projects that aim to identify gene variants associated with HI in the Cameroonian population, as our study showed that known HI genes do not have a significant role in HI in this population.

3.1.6. Conclusions

The prevalence of HI in Cameroon is similar to the WHO estimate of HI prevalence in sub-Saharan Africa. Environmental factors are the leading causes of HI in Cameroon, with meningitis, impacted wax and age-related disorder being the most common causes. Concerning HI of genetic origin, autosomal recessive inheritance is the most frequently observed inheritance pattern. NSHI is the most common clinical entity, and Waardenburg and Usher syndromes have been identified as the most frequent syndromic HI in the Cameroonian population. The high implication of diseases that are preventable by vaccination (meningitis, measles, rubella and mumps) in the etiology of HI highlights the need to reinforce the current national immunization program in Cameroon, and the very low implication of known HI genes in the Cameroonian population shown by our study highlights the need for next-generation sequencing techniques to identify novel variants that are associated with HI in populations of African descent.

3.1.7. Research Perspectives

There is an obvious need for population-based study at a national level to estimate the prevalence and incidence of HI in Cameroon. Additionally, whole genome sequencing data should be generated from non-syndromic hearing-impaired patients and analyzed in order to identify genes that contribute to NSHI in Cameroon. Functional studies should also be

performed to understand the molecular mechanisms underlying the pathogenicity of HI in the Cameroonian population.

Author Contributions: A.W. conceived the study. E.W.T. and J.J.N. developed the protocol. E.W.T. did the literature search, selected studies and extracted relevant data. E.W.T. and J.J.N. analyzed and interpreted the data. E.W.T. issued the first draft of the paper, J.J.N., J.V.F.F., O.G.O., E.R.C. and A.W. critically revised successive drafts of the manuscript. E.R.C. and A.W. supervised the project and compiled the revisions. All the authors approved the final version of the manuscript. E.W.T. is the guarantor of this review.

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Conflicts of Interest: Authors declare that no competing interests exist.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1:

Table 3.3: Search strategy in Pubmed

Search step	Search terms	Hits
1	"Hearing impairment" OR "hearing loss" OR deaf OR deafness	99 075
2	Cameroon	
3	#1 and #2	31
4	#3 and **Search limits:** from inception to October 31th 2019	31

Table 3.4: Search strategy in Scopus

Search step	Search terms	Hits
1	"Hearing impairment" OR "hearing loss" OR deaf OR deafness	146 211
2	Cameroon	
3	#1 and #2	38
4	#3 and **Search limits:** publication year < 2020	38

Table 3.5: Search strategy in AFROLIB

Search step	Search terms	Hits
1	"Hearing impairment" "hearing loss" "deaf" "deafness" "Cameroon"	15
2	#1 and **Search limits:** publication date ≤ October 31th 2019	8

Table 3.6: Search strategy in African Index Medicus

Search step	Search terms	Hits
1	“Hearing impairment” “hearing loss” “deaf” “deafness” “Cameroon”	83
2	#1 and **Search limits:** publication date ≤ October 31th 2019	81

Table 3.7: Search strategy in African Journals Online

Search step	Search terms	Hits
1	“Hearing impairment” OR “hearing loss” OR deaf OR deafness	268
2	#1 and **Search limits:** from inception to October 31th 2019	175

Table 3.8: Age at diagnosis of hearing impairment in Cameroon (extracted from the study by Wonkam et al. *[52]*)

Age of onset		Number of cases, n (%)
Prelingual	(Before 2 years old)	437 (75.1)
Perilingual	(Between 2 and 4 years)	116 (20)
Postlingual	(After 4 years)	29 (4.9)

Table 3.9: Categories of hearing loss in Cameroon with pure tone audiometry, according to the BIAP classification (extracted from the study by Wonkam et al. [52]).

Category of hearing loss	Sensorineural hearing loss	Mixed hearing loss	Total, n (%)
Severe I (71–80 dB)	14	11	25 (4.8)
Severe II (81–90 dB)	39	14	53 (10.1)
Profound I (91–100 dB)	140	27	167 (31.9)
Profound II (101–110 dB)	156	16	172 (32.8)
Profound III (111–120 dB)	71	11	82 (15.6)
Total (>120 dB)	25	0	25 (4.8)
Total, n (%)	445 (84.9)	79 (15.1)	524 (100)

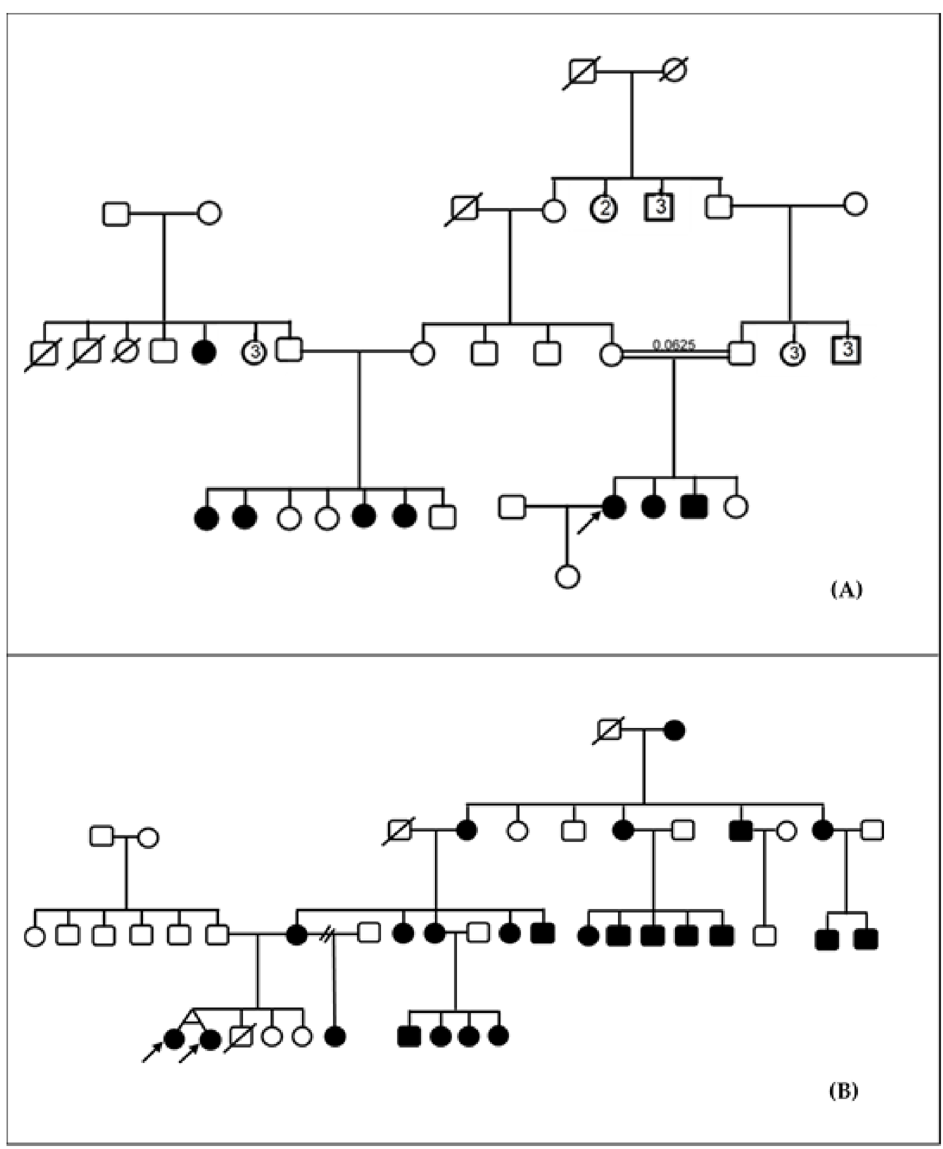

Figure 3.4: Inheritance of familial hearing impairment in Cameroon. (A) Pedigree of a consanguineous family with autosomal recessive non-syndromic hearing impairment. (B) Pedigree of a family with non-syndromic hearing impairment suggestive of mitochondrial inheritance. Arrows here indicate the probands (Extracted from the study by Tingang Wonkam et al. [54]).

3.2. Adadey, S. M., **Wonkam-Tingang, E.**, Twumasi Aboagye, E., Nayo-Gyan, D. W., Boatemaa Ansong, M., Quaye, O., Awandare, G. A., & Wonkam, A. (2020). Connexin Genes Variants Associated with Non-Syndromic Hearing Impairment: A Systematic Review of the Global Burden. *Life*, 10(11), 258. https://doi.org/ 10.3390/life10110258.

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Co-Authors Contribution:

AW and **SMA**: Conceptualization.

SMA, EWT, ETA, DWNG, and **MBA**: literature search.

SMA, EWT, and **AW**: Data extraction and original draft preparation.

SMA, EWT, ETA, DWNG, MBA, OQ, GAA, and **AW**: writing-review and editing, and agreement to the published version of the manuscript.

AW, GAA, and **OQ:** Supervision.

AW and **GAA:** Funding acquisition.

Connexin Genes Variants Associated with Non-Syndromic Hearing Impairment: A Systematic Review of the Global Burden

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Abstract: Mutations in connexins are the most common causes of hearing impairment (HI) in many populations. Our aim was to review the global burden of pathogenic and likely pathogenic (PLP) variants in connexin genes associated with HI. We conducted a systematic review of the literature based on targeted inclusion/exclusion criteria of publications from 1997 to 2020. The databases used were PubMed, Scopus, Africa-Wide Information, and Web of Science. The protocol was registered on PROSPERO, the International Prospective Register of Systematic Reviews, with the registration number "CRD42020169697". The data extracted were analyzed using Microsoft Excel and SPSS version 25 (IBM, Armonk, New York, United States). A total of 571 independent studies were retrieved and considered for data extraction with the majority of studies (47.8% (n = 289)) done in Asia. Targeted sequencing was found to be the most common technique used in investigating connexin gene mutations. We identified seven connexin genes that were associated with HI, and *GJB2* (520/571 publications) was the most studied among the seven. Excluding PLP in GJB2, GJB6, and *GJA1* the other connexin gene variants (thus *GJB3*, *GJB4*, *GJC3*, and *GJC1* variants) had conflicting association with HI. Biallelic *GJB2* PLP variants were the most common and widespread variants associated with non-syndromic hearing impairment (NSHI) in different global populations but absent in most African populations. The most common GJB2 alleles

found to be predominant in specific populations include; p.Gly12ValfsTer2 in Europeans, North Africans, Brazilians, and Americans; p.V37I and p.L79Cfs in Asians; p.W24X in Indians; p.L56Rfs in Americans; and the founder mutation p.R143W in Africans from Ghana, or with putative Ghanaian ancestry. The present review suggests that only *GJB2* and *GJB3* are recognized and validated HI genes. The findings call for an extensive investigation of the other connexin genes in many populations to elucidate their contributions to HI, in order to improve gene-disease pair curations, globally.

Keywords: connexin; gap junction protein; gene variant; *GJB2*; systematic review

3.2.1. Introduction

Hearing impairment (HI) is the most common sensorineural disability worldwide, with a global prevalence of 1.3 per 1000 population [33,98]. It occurs in about 1 per 1000 live births in high-income countries, with a much higher incidence of up to 6 per 1000 in the lower-income countries [3]. According to the World Health Organization, 466 million people are living with HI and about 900 people will be affected by the year 2050 [32]. Depending on the degree of severity, HI can be classified as mild, moderate, severe, or profound when the pure tone average ranges from 26 to 40 dB, 41 to 60 dB, 61 to 80 dB or is over 81 dB, respectively [99]. It is estimated that approximately 50% of congenital profound HI cases are of genetic origin [7]. If there are no other distinguishing clinical findings, HI is classified as non-syndromic [100]. About 80% of non-syndromic HI (NSHI) cases are inherited in an autosomal recessive mode, while an autosomal dominant pattern of inheritance is observed in 18% of cases [8]. In the remaining 2% of cases, the mode of inheritance is either X-linked or mitochondrial [8].

Non-syndromic HI is extremely heterogeneous, with approximately 170 loci and 121 genes identified so far [10]. Studies in European and Asian populations have identified mutations in connexin genes as the major contributors to NSHI [11,101]. Connexins (Cx) are a homogeneous family of proteins expressed in a large variety of tissues in the human body and known for their assembly into intercellular channels, called gap junctions [102]. Twenty-one different human connexin genes have been reported so far, each coding for a transmembrane protein with the same protein topology [102]. Connexins have four transmembrane domains (TM), TM1–TM4, connected by two extracellular loops (E), E1, and E2, which mediate docking [103]. The N- and C-termini, and a loop connecting TM2 and TM3 are on the cytoplasmic side of the plasma membrane [103].

Connexins are synthesized in the endoplasmic reticulum (ER) and oligomerize in the ER/Golgi or trans-Golgi network to form hexameric hemichannels or connexons [102]. Connexons are transported to the plasma membrane, where they can act as functional channels by

themselves, or move to regions of cell contact and find a partner hemichannel from an adjacent cell to form a complete gap junction channel [103]. Gap junctions play an important role in cell-cell communication and homeostasis in various tissues, by mediating a direct exchange of ions and other small molecules up to 1 kDa (including a variety of second messengers, metabolites, but also small linear peptides) between the cytoplasms of adjacent cells [102].

To date, mutations in four connexin genes including *GJB2* (Cx26), *GJB3* (Cx31), *GJB4* (Cx30.3), and *GJB6* (Cx30) have been associated with sensorineural HI [104–106]. These four connexins were shown to be expressed in the inner ear, and some studies supported their role in potassium removal and recycling in the ear, as well as a possible role for nutrient passage in the cochlea [107]. *GJB2*-related sensorineural HI can occur alone or in association with hyperproliferative skin disorders, as in the case in Keratitis-ichthyosis-deafness syndrome and Bart-Pumphrey syndrome [52,108,109]. It is has been shown that digenic inheritance of recessive deafness by mutations in *GJB2* and *GJB6*, or *GJB2* and *GJB3* can occur [11]. In other words, deafness can be caused by the addition of a mutation in one allele of GJB2 and one allele of *GJB6* or *GJB3*, indicating an interaction of these connexins in the cochlea [102]. *GJB6* coding region variants have been proven not to cause HI using mouse models, however, the large deletions of the *GJB6* gene especially *GJB6*-D13S1830 were implicated as causal factors of HI. The cis-acting element upstream of *GJB2* and *GJB6* gene is disrupted by the large genomic deletions abolishing the expression of *GJB2* gene which is responsible for the development of HI [110].

GJB2 and *GJB6* genes have been well studied in Europeans and Asians, with c.35delG identified as the most prevalent *GJB2* mutations associated with NSHI [101]. However, the other NSHI-causing connexin genes (i.e., *GJB3* and *GJB4*) have not been extensively studied [102,111]. Using a systematic review approach, we provided summary data on connexin gene variants associated with HI, and specifically the global contribution of connexin genes to NSHI.

3.2.2. Results

Of the 2592 studies that were screened, 571 articles were downloaded and analyzed. The 571 articles comprised publications that dated as far back as 1997 to recent publications in 2020 (Figure 3.5A). The analysis suggested that there were few studies on connexin gene variants association with HI in first the three years of the study timeframe (1997, 1998, and 1999), followed by a drastic increase in the last two decades. The year 2015 recorded the highest number of publications on connexin gene variants (Figure 3.5A).

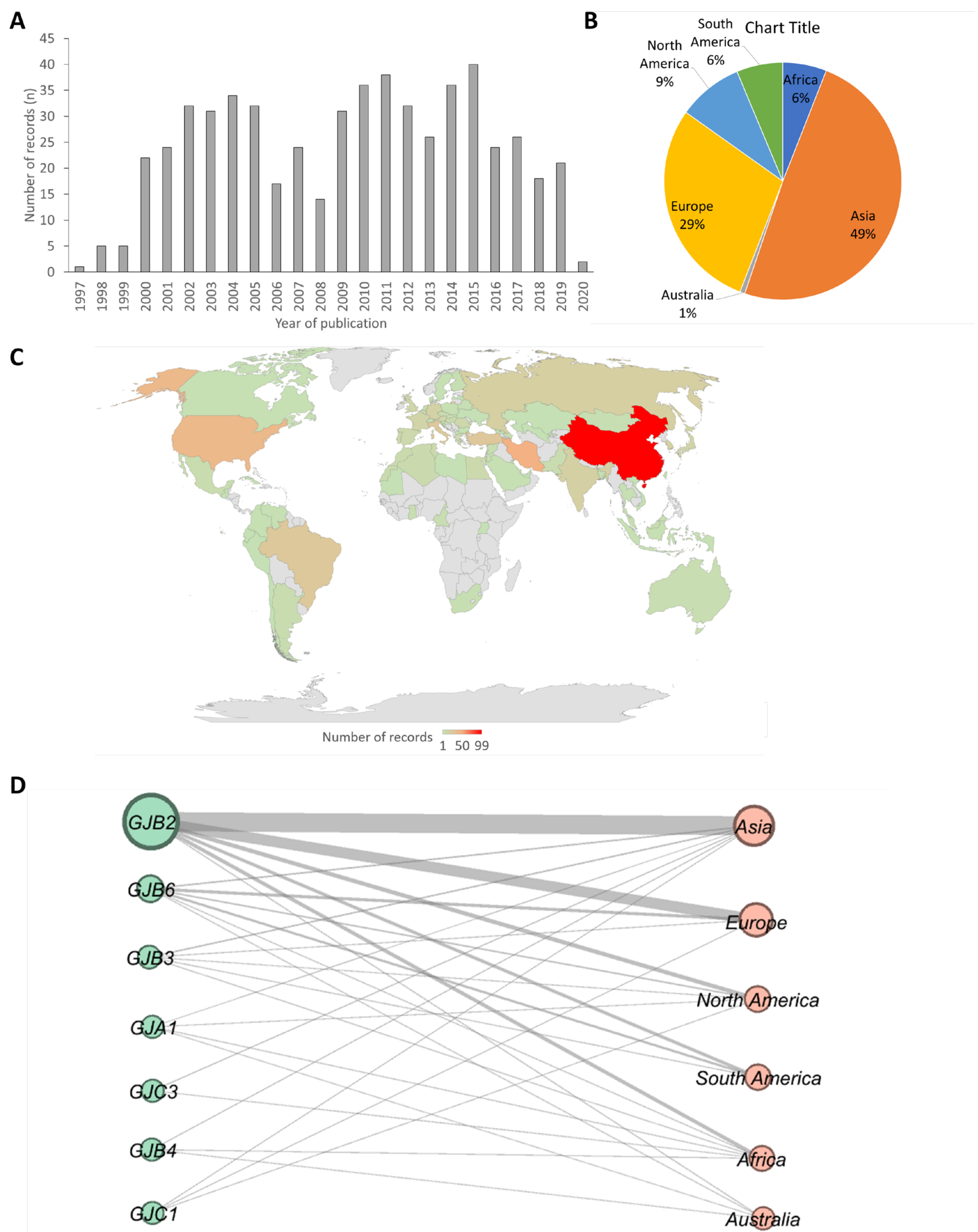

Figure 3.5: Geographical distributions of the studies included in this review. (**A**) A bar chart showing frequency of articles by the year of publication. (**B**) A pie chart of distribution of articles from which data were extracted by continent. (**C**) A map of countries showing the number studies that reported at least one connexin gene variant. The gray regions have no record included in this study. Different shades of blue were used to represent the number of studies retrieved and reviewed per country with the darkest shade of blue as the highest number and the lightest as the smallest number. The number written on the map denotes the number of studies. The map was created in Microsoft Excel (Office 365 education license under the University of Cape Town, South Africa) (**D**) Network of connexin gene plotted against continents from which they were reported. The nodes on the left (green) and the right (pink) correspond to connexin genes and continents respectively. The size of the nodes and

the thickness of the lines between nodes are proportional to the number of publications. The network was built using the open-source software Gephi [112].

Most of the articles retrieved were from Asia (47.8% (n = 289)) with China (99/289) recording the highest number of articles. Australia had the least number (0.7% (n = 4)) of retrieved articles (Figure 3.5B,C). There were relatively few studies from Africa (6% (n = 35), compared to other continents (Figure 3.5). Asia had reports on all connexins (7) found in this review, while Europe and Australia reported on 4 connexins. Africa, North America, and South America had reports of 6, 5, and 3 connexins respectively (Figure 3.5D). We identified *GJB2* as the most widely studied connexin in all the continents (Figure 3.5D).

We identified a cocktail of methods used by the researchers to investigate connexin gene variants in hearing-impaired patient samples. Several studies employed two or more approaches while others depended on a single approach. Targeted sequencing was the most common method followed by polymerase chain reaction (PCR) techniques. The targeted sequencing studies were mostly by Sanger sequencing where one or more primer sets were used to amplify and sequence the coding regions of the connexins under investigation. The analysis of the primer sets used for sequencing revealed their uniqueness for each study (Table S1). Among the PCR techniques were amplification-refractory mutation system (ARMS), PACE™ (PCR Allele Competitive Extension), and multiplex PCR. A few studies used next-generation sequencing techniques (NGS) such as whole-exome sequencing (NGS) and NGS panel sequencing (Figure 3.6).

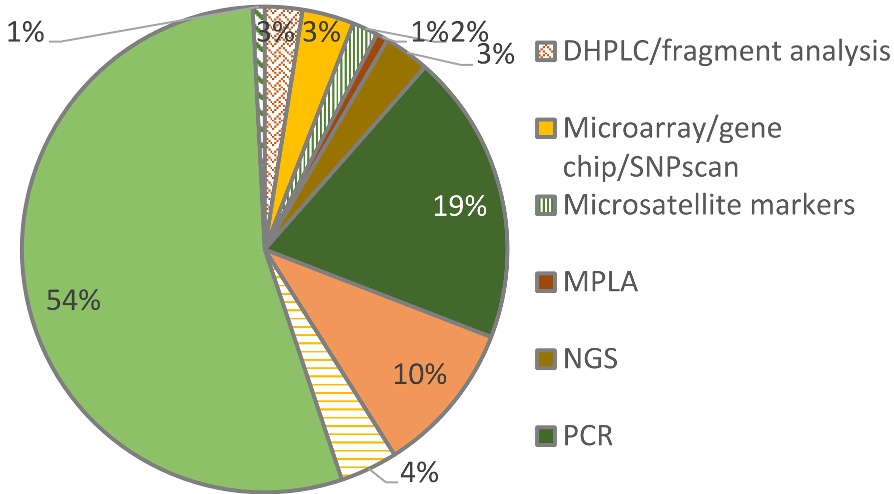

Figure 3.6: Methods used to investigate connexin gene variants. Among the methods are denaturing high-performance liquid chromatography (DHPLC), multiplex ligation-dependent probe amplification (MLPA), polymerase chain reaction (PCR), next-generation sequencing (NGS), restriction fragment length polymorphism (RFLP), and single-strand conformational polymorphism (SSCP).

3.2.2.1. Connexin 26 (*GJB2*)

We identified 337 variants in *GJB2* from the review of the publications included in this study. Analysis of the clinical significance of these variants gave 124 (37.2%), 53 (15.9%), 46 (13.8%), and 25 (7.5%) pathogenic, likely pathogenic, uncertain significance, and benign variants, respectively (Figure 3.7). Asia (*n* = 288; 48%) was the highest contributor of pathogenic (PLP) variants followed by Europe (*n* = 166; 28%), North America (*n* = 52; 9%), Africa (*n* = 48, 8%); South America (*n* = 37; 6%) and Australia (*n* = 4; 1%). The three databases used in this study did not have data on the clinical significance of 13 out of the 333 variants, implying that some of these variants may be novel (Table S2).

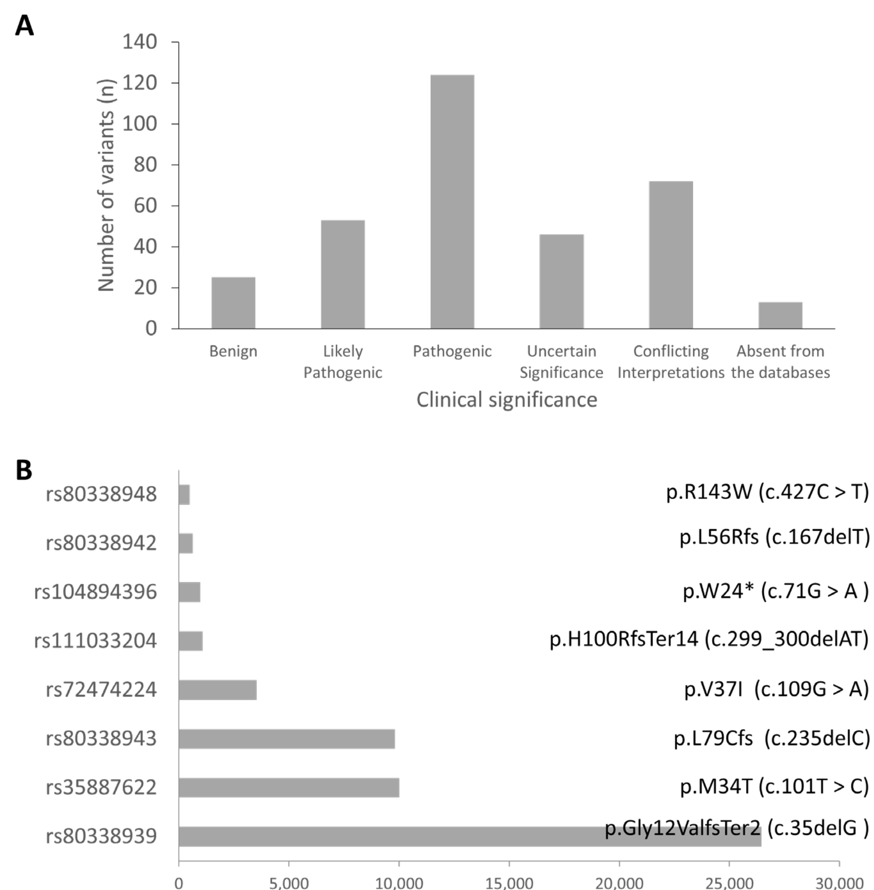

Figure 3.7: Common *GJB2* variants. (**A**) Clinical significance of identified variants. (**B**) The top eight *GJB2* variants ranked based on the total number of alleles.

The pathogenic variants were sorted based on the number of reported alleles to identify the commonly reported variants. The variants with more than 450 reported alleles were considered as the commonly associated *GJB2* variants. Based on the number of reported alleles, p.Gly12ValfsTer2 (c.35delG) was ranked as the most commonly reported *GJB2* variant, found in 26,429 (15.1%) out of 175,491 investigated alleles. The frequencies of the other common *GJB2* mutations were 10,009/82,805 (12.1%), 9813/277,116 (3.5%),

3520/127,802 (2.8%), 972/54,394 (1.8%), 641/54,279 (1.2%), 1080/10,7855 (1.0%), and 497/90,305 (0.6%) for p.M34T (c.101T>C), p.L79Cfs (c.235delC), p.V37I (c.109G>A), p.W24X (c.71G>A), p.L56Rfs (c.167delT), p.H100RfsTer14 (c.299_300delAT), and p.R143W (c.427C>T), respectively (Table S3). Further analysis of the p.Gly12ValfsTer2 (c.35delG) showed its widespread and high prevalence in European countries, and North African countries and some parts of Brazil and America. In Asia, p.V37I (c.109G>A and p.L79Cfs (c.235delC)) variants were the most frequently reported *GJB2* variant. On a country-wise analysis, we observed that the highest number of p.Gly12ValfsTer2 (c.35delG) and p.M34T (c.101T>C) mutated alleles were from the United Kingdom (Figure 4). The highest number of mutated alleles for p.L79Cfs (c.235delC), p.V37I (c.109G>A), and p.H100RfsTer14 (c.299_300delAT) were recorded in China. India, the United States of America, and Ghana recorded the highest number of p.W24X (c.71G>A), p.L56Rfs (c.167delT), and p.R143W (c.427C > T) mutated alleles, respectively (Figure 3.8).

We extracted data on known PLP variants in *GJB2* (p.W44*: c.131G>A (North America), c.IVS1+1G>A (Russia), p.W172*: c.516G>A (Siberia), p.W172C: c.516G>C (Siberia) p.W172R, and c.514T>A (Siberia) that are prevalent in isolated ethnic groups. The majority of mutated c.IVS1+1G>A variant was recorded in Asia with high frequencies from Yakutia, Siberia, and Russia (Table S3). The other three rare *GJB2* variants (p.W172*: c.516G>A, p.W172C: c.516G>C, p.W172R, and c.514T>A) which are at the same amino acid position were common in the Asian countries with Russia having the highest frequency (Table S4).

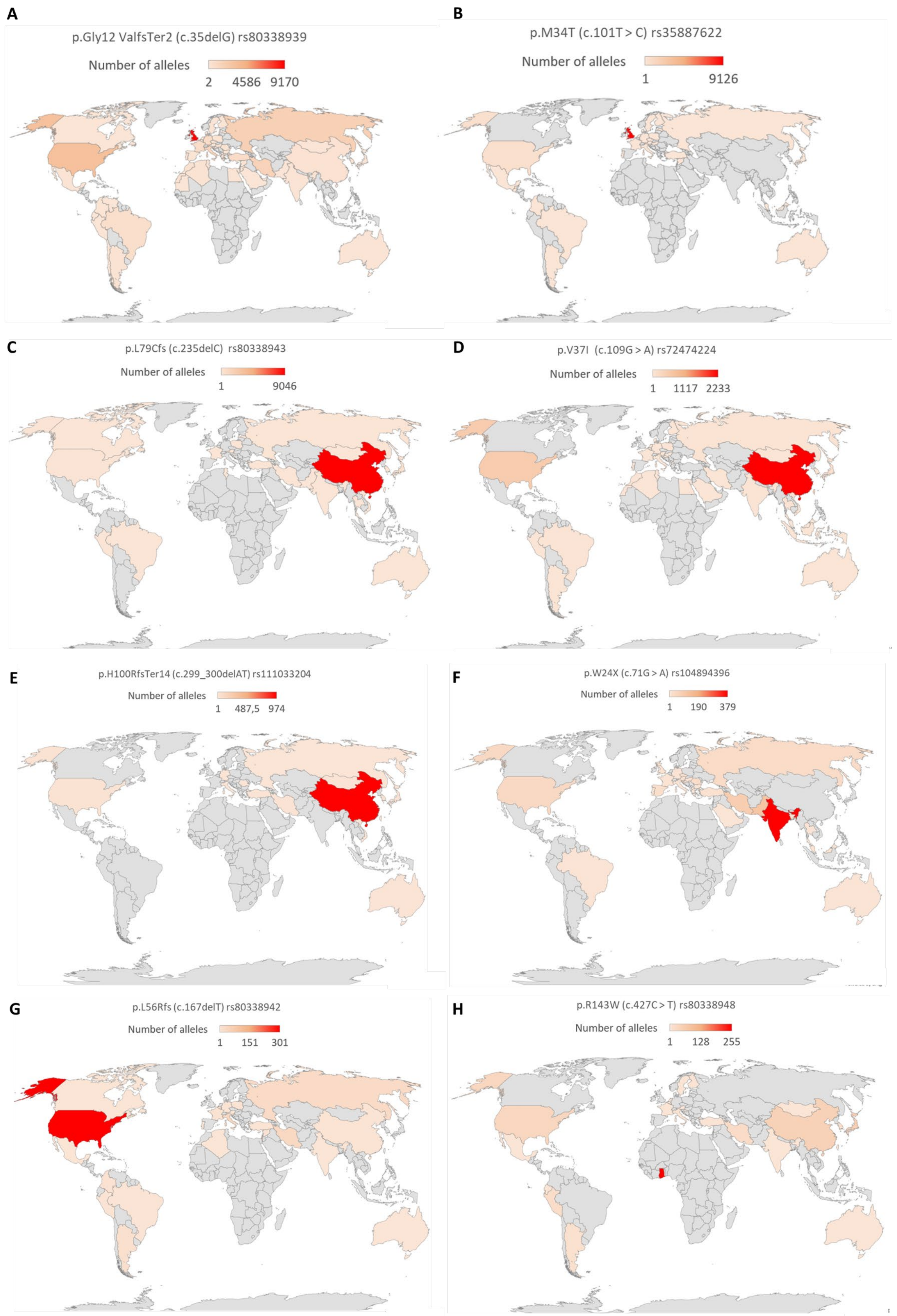

Figure 3.8: Global distribution of common *GJB2* variants. A graph showing the total number of reported alleles of (**A**) p.Gly12ValfsTer2 (c.35delG), (**B**) p.M34T (c.101T>C), (**C**) p.L79Cfs/c.235delC, (**D**) p.V37I/c.109G>A, (**E**) p.H100RfsTer14/

c.299_300delAT, (**F**) p.W24X/c.71G>A, (**G**) p.L56Rfs/ c.167delT, and (**H**) p.R143W/ c.427C>T. The countries were colored with a gradient from red (highest number of alleles) to brown (lowest number of alleles). Countries shaded grey either had no reports or no alleles. The map was created by the authors in Microsoft Excel (Office 365 education license of the University of Cape Town, South Africa).

3.2.2.2. Connexin 30 Gene (*GJB6*)

We selected reports of 18 variants in *GJB6*, including two large genomic deletions. Most coding region variants in *GJB6* were predicted as benign or uncertain significance (n = 12 (85.7%)). Two variants were predicted as pathogenic; *GJB6*: p.A40V/c.119C>T/rs780320724 from Taiwan [113] and *GJB6*: p.T5M/c.14C>T/rs104894414 from Germany [114], and Iran [115], respectively (Table 3.10). The del(*GJB6*-D13S1830) was the most common *GJB6* variant reported from 31 countries from all continents, with virtually no case from Africa. High allele frequencies were particularly reported in France and Spain (Figure 3.9). We found few studies that reported del(*GJB6*-D13S1854) variation among the hearing-impaired; these studies were from Argentina, Colombia, Portugal, and Brazil.

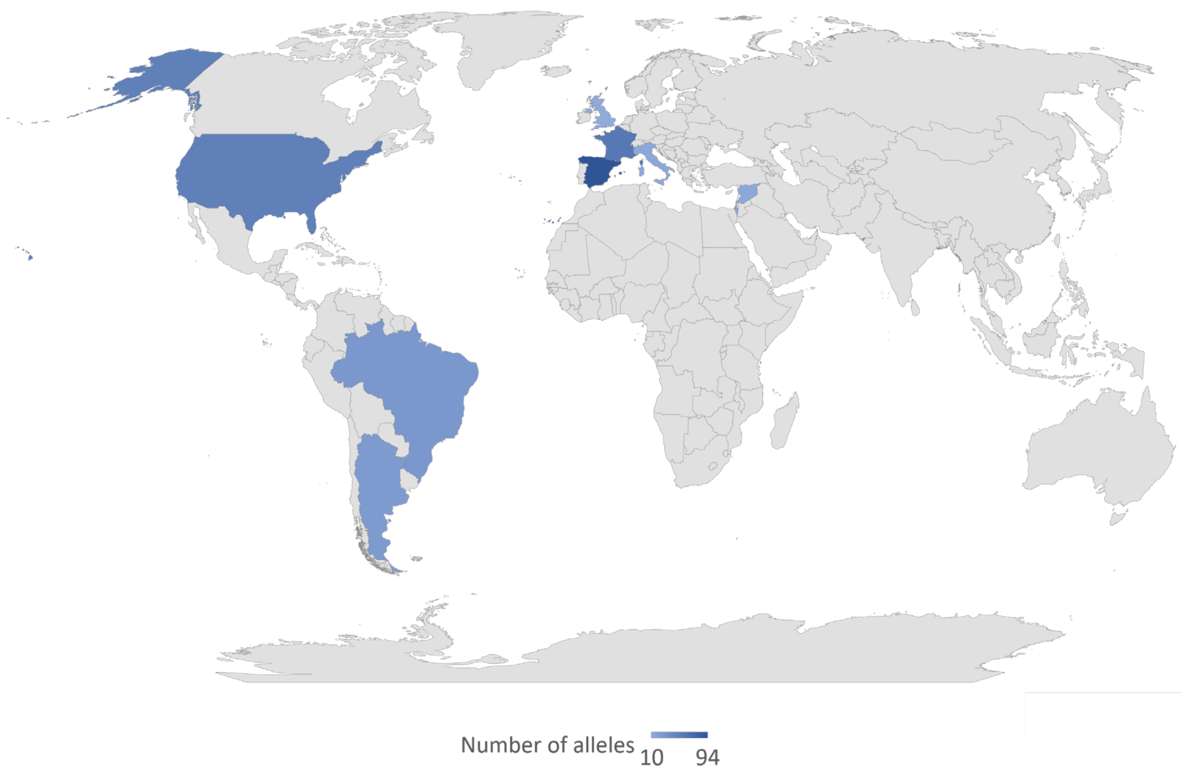

Figure 3.9: Global distribution of del(*GJB6*-D13S1830). The variant del(*GJB6*-D13S1830) was reported in all the countries highlighted in blue color. The intensity of the blue color denotes the frequency of reported alleles. The map was created by the authors in Microsoft Excel (Office 365 education license of the University of Cape Town, South Africa).

3.2.2.3. Connexin 31 Gene (*GJB3*)

We identified reports of variants of *GJB3* from nine countries with Korea having the highest number of reported alleles. The variant with the highest number of reported alleles was found to be synonymous and was predicted to be benign. None of the variants was predicted pathogenic although they were identified in hearing-impaired populations. Based on the databases used, we predicted the variants as benign, with uncertain significance or conflicting interpretations. Two variants (c.547G>A/rs74315318 and c.497A>G/rs121908851) were predicted as pathogenic by only one database with conflicting interpretations from the other databases; it was, therefore, difficult to conclude on their pathogenicity (Table 3.11).

3.2.2.4. Connexin 30.3 Gene (*GJB4*)

In this review, we identified studies from 5 countries that reported *GJB4* variants. The majority of identified *GJB4* variants had conflicting clinical significance since they had different interpretations in the databases used. VarSome predicted seven variants as pathogenic or likely pathogenic and InterVar predicted an additional variant as pathogenic. The pathogenic variants of VarSome were not predicted as pathogenic by InterVar and vice versa (Table 3.12).

3.2.2.5. Connexin 29 Gene (*GJC3*)

We identified seven *GJC3* variants from 4 countries (Table 3.13) which were predicted as benign or of uncertain significance with no PLP variant found.

3.2.2.6. Connexin 43 Gene (*GJA1*)

Twelve (12) variants in *GJA1* were reported by different researchers of which three were predicted to be pathogenic or likely pathogenic (PLP). The pathogenic variants were reported in hearing-impaired patients from America and Asia, the variants reported from Africa (South Africa) were predicted as benign or uncertain significance. *GJA1* c.932delC variant had the highest number of alleles reported from Australia. Four (4) out of the 11 *GJA1* variants were reported in a study from South Africa (Table 3.14).

Table 3.10: Global distribution of connexin 30 (*GJB6*) gene variants.

Country/ Territory	Number of Alleles *	Protein Change	Nucleotide Change	Reference Number	Clinical Significance				Reference
					Intervar	Varsome	ClinVar	Verdict	
Taiwan	1/520	p.A40V	c.119C > T	rs780320724	Likely Pathogenic	Likely Pathogenic	Pathogenic	Pathogenic	[113]
Malaysia	3/NA	-	366delT	-	-	-	-	-	[116]
Germany	1/376	-	682insA	-	-	-	-	-	[117]
Uganda	2/230	p.N113K	c.339T > A	rs143766955	Benign	Likely Benign	Benign	Benign	[94]
Uganda	1/230	c.476A>G	p.N159S	rs35277762	Benign	Likely Benign	Benign	Benign	[94]
Malaysia	2	p.E101K	c.301G > A	rs571454176	Likely Benign	Uncertain Significance	Uncertain Significance	Uncertain Significance	[116]
Malaysia	1/NA	p.A148D	c.443_444 delC AinsAC	-	-	Uncertain Significance	-	Uncertain Significance	[116]
Malaysia	1/NA	p.Q124H	-	-	Uncertain Significance	Uncertain Significance	-	Uncertain Significance	[116]
Slovenia	1/144	p.M203V	c.607A > G	rs200674715	Uncertain Significance	Likely Benign	Benign	Benign	[118]
Germany	1/376								[117]
Korea	1/394	p.I248V	c.742A > G	rs747371119	Uncertain Significance	Uncertain Significance	Uncertain Significance	Uncertain Significance	[119]
Qatar	1/NA	p.P70L	c.209C > T	rs727505123	Uncertain Significance	Uncertain Significance	Uncertain Significance	Uncertain Significance	[120]
Korea	1/394	p.P87P	c.261A > T	rs777309137	Likely Benign	Likely Benign	-	Benign	[119]
Germany	6/396	p.T5M	c.14C > T	rs104894414	Likely Pathogenic	Uncertain Significance	Pathogenic	Pathogenic	[114]
Malaysia	1/NA	p.R32Q	c.95G > A	rs766604251	Uncertain Significance	Uncertain Significance	-	Uncertain Significance	[116]
Germany	1/376	p.V190A	c.569 T > C	rs780513857	Uncertain Significance	Uncertain Significance	Uncertain Significance	Uncertain Significance	[116,117]
Malaysia	1/NA	p.I145H	c.433_434 delA TinsCA	-		Uncertain Significance	-	Uncertain Significance	[116]

*, The numerators in this column represent the number of mutated alleles, and the denominators the total number of screened alleles. NA, not applicable (the authors were not clear on the total number of alleles they have screened), InterVar, VarSome, and ClinVar are databases to assess the clinical significance of the variants.

Table 3.11: Global distribution of connexin 31 (*GJB3*) gene variants.

Country/ Territory	Number of Alleles	Protein	Nucleotide Change	rs Number	Clinical Significance				Reference
					Intervar	Varsome	ClinVar	Verdit	
Germany	2/376	p.K56Q	c.166 A > C	rs746219527	Uncertain Significance	Likely Benign	Uncertain Significance	Uncertain Significance	[117]
Germany	1/376	p.R101Q	c.302G > A	rs765605645	Uncertain Significance	Likely Benign	-	Conflicting Interpretations	[117]
Germany	1/376	p.R106H	c.317 G > A	rs369979083	Uncertain Significance	Uncertain Significance	Likely Benign	Uncertain Significance	[117]
Tunisia	1/NA	p.R32W	c.94C > T	rs1805063	Benign	Benign	Likely Benign	Benign	[121]
Austria	2/90								[122]
USA	2/126								[123]
Brazil	2/NA								[124]
Tunisia	4/NA	p.N119N	c.357C > T	rs41310442	Benign	Benign	Benign	Benign	[121]
Austria	4/90								[122]
China	7/186								[125]
USA	1/126								[123]
Morocco	1/390								[126]
Korea	36/424								[119]
China	1/216	p.N166S	c.497A > G	rs121908851	Uncertain Significance	Likely Benign	Pathogenic	Conflicting Interpretations	[127]
Korea	1/20	p.A194T	c.580G > A	rs117385606	Benign	Benign	Benign	Benign	[128]
China	2/216								[127]
Korea	7/430								[119]
Taiwan	4/506								[129]
China	2/NA								[130]
China	3/206	p.V84I	c.250G > A	rs145751680	Benign	Benign	Benign	Benign	[131]
Korea	7/424								[119]
Taiwan	1/506								[129]
Korea	1/40								[132]
China	1/NA								[130]
Austria	11/90	p.N266N	c.798C > T	rs35983826	Benign	Benign	Benign	Benign	[122]
USA	10/126								[123]
China	4/186								[125]
China	12/170								[130]
China	2/186	p.S11S	c.33C > T	rs112499125	Likely Benign	Benign	Likely Benign	Benign	[125]
USA	2/126	p.N67N	c.201C > T	-	Likely Benign	Uncertain Significance	-	Conflicting Interpretations	[123]

Korea	1/424	pV27M	c.79G > A	rs775072109	Uncertain Significance	Benign	-	Conflicting Interpretations	[119]
Korea	1/424	p.V43M	c.127G > A	rs761320902	Uncertain Significance	Likely Benign	-	Conflicting Interpretations	[119]
Korea	415/430		c.813+43C > A	rs41266429	-	Benign	Benign	Benign	[119]
Korea	351/430		c.813+53G > A	rs476220	-	Benign	Benign	Benign	[119]
China	2/4	p.E183K	c.547G > A	rs74315318	Likely Pathogenic	Benign	Conflicting Interpretations	Conflicting Interpretations	[133]
Taiwan	1/506								[129]
China	2/4	p.R180 *	c.538C > T	rs74315319	Uncertain Significance	Benign	Uncertain Significance	Uncertain Significance	[133]
Taiwan	2/506	p.L10R	c.29T > G	-	Uncertain Significance	Uncertain Significance	-	Uncertain Significance	[129]
Taiwan	1/506	p.T18I	c.53C > T	rs755025684	Uncertain Significance	Benign	-	Conflicting Interpretations	[129]
Brazil	1/NA	p.49delK	c.1227C > T	-	-	-	-	-	[124]
Australia	3/520	p.V174M	c.520G > A	rs749431664	Uncertain Significance	Uncertain Significance	-	Uncertain Significance	[129]
Brazil	2/414	p.Y177D	c.529T > G	rs80297119	Benign	Benign	Benign	Benign	[134]
Brazil	2/4								[124]
China	2/186	p.G256S	c.766G > A	-	Likely benign	Uncertain significance		Conflicting Interpretations	[125]

*, The numerators in this column represent the number of mutated alleles, and the denominators the total number of screened alleles. NA, not applicable (the authors were not clear on the total number of alleles they have screened), InterVar, VarSome, and ClinVar are databases to assess the clinical significance of the variants.

Table 3.12: Connexin 30.3 (*GJB4*) gene variants.

. Protein Change	Nucleotide Change	rs Number	Clinical Significance				Ghana	Australia	Iran	China
			Intervar	Varsome	InterVar	Verdit	[111]	[113]	[135]	[106]
p.C169 *	c.507C > A	rs79193415	Uncertain Significance	Pathogenic	-	Conflicting Interpretations	-	2/NA	-	1/506
p.C169C	c.507C > T	rs79193416	Likely Benign	Uncertain Significance	-	Conflicting Interpretations	-	-	2/144	
p.E67L	c.199G > A	rs368331423	Uncertain Significance	Benign	-	Conflicting Interpretations	-	-	-	1/506
p.G126T	c.376G > A	rs146979528	Likely Pathogenic	Benign	-	Conflicting Interpretations	-	-	-	2/506
p.H221Y	c.661C > T	rs1223189096	Uncertain Significance	Likely Benign	-	Conflicting Interpretations	-	-	-	1/506

p.R101H	c.302G > A	rs375702737	Likely Pathogenic	Likely Benign	-	Conflicting Interpretations	-	1/520	-	-
p.R103C	c.307C > T	rs9426009	Benign	Benign	-	Benign	-	-	1/144	-
p.R124W	c.370C > T	rs373126632	Likely Pathogenic	Benign	-	Conflicting Interpretations	-	1/520	-	1/506
p.R227W	c.679C > T	rs185327282	Uncertain Significance	Likely Benign	-	Conflicting Interpretations	-	-	1/144	-
p.R22C	c.64C > T	rs776245625	Likely Pathogenic	Likely Benign	-	Conflicting Interpretations	-	1/520	-	1/506
p.R98C	c.292C > T	rs200602523	Likely Pathogenic	Benign	-	Conflicting Interpretations	-	2/520	-	1/506
p.T233L	c.698C > A	-	Uncertain Significance	Likely Benign		Conflicting Interpretations	-	-	-	1/506
p.V37M	c.109G > A	rs146378222	Benign	Benign	Uncertain Significance	Benign	-	2/520	-	2/506
p.V74M	c.220G > A	rs771048190	Likely Pathogenic	Likely Benign	-	Conflicting Interpretations	-	1/520	-	-
p.N119T	c.356A > C	rs190460237	Likely Pathogenic	Uncertain Significance	-	Conflicting Interpretations	2/400	-	-	-
p.E204A	611A > C	rs3738346	Benign	Benign	Benign	Benign	70/400	-	-	-
p.R151S	c.451C > A	rs78499418	Benign	Benign	-	Benign	58/400	-	-	-
p.T172T	c.516T > C	rs111693060	Benign	Benign	Benign	Benign	13/400	-	-	-
p.K123K	c.369G > A	rs142843509	Likely Benign	Benign	Likely Benign	Benign	2/400	-	-	-
p.R101R	c.303C > G	rs138184343	Likely Benign	Benign	Benign	Benign	15/400	-	-	-
p.Q80*	c.238C > T	rs114429815	Benign	Benign	Benign	Benign	3/400	-	-	-

*, The numerators in this column represent the number of mutated alleles, and the denominators the total number of screened alleles. NA, not applicable (the authors were not clear on the total number of alleles they have screened), InterVar, VarSome, and ClinVar are databases to assess the clinical significance of the variants.

Table 3.13: Connexin 29 (*GJC3*) gene variants

							*Number of Alleles			
							Ghana	Taiwan	India	China
Protein Change	Nucleotide Change	rs Number	Intervar	Varsome	ClinVar	Verdict	[111,136]	[129]	[136]	[137]
p.I190N	c.569T > A	rs121908693	Uncertain Significance	Uncertain Significance	-	Uncertain Significance			1/246	
p.R15G	c.43C > G	-	Uncertain Significance	Uncertain Significance	-	Uncertain Significance				1/506
p.W77S	c.230G > C	-	Uncertain Significance	Uncertain Significance	-	Uncertain Significance				1/506
p.L17S	c.525T > G	rs752804324	Likely benign	Likely benign		Likely benign				2/506
-	c.781 + 62G > A	rs116853822	-	Likely Benign	-	Likely Benign		10/520		8/506
p.M1R	c.2T > G	-	Uncertain Significance	Uncertain Significance	-	Uncertain Significance		3/520		3/506
p.E269D	c.807A > T	rs763649019	Uncertain Significance	Likely Benign	Uncertain Significance	Uncertain Significance		1/520		
p.P164S	c.490C > T	rs73405465	Benign	Benign	-	Benign	53/400			

*, The numerators in this column represent the number of mutated alleles, and the denominators the total number of screened alleles, InterVar, VarSome, and ClinVar are databases to assess the clinical significance of the variants.

Table 3.14: *Connexin 43* (GJA1*) gene variants.*

Country/ Territory	* Number of Alleles	Protein	Nucleotide Change	rs Number	Clinical Significance				Reference
					Intervar	Varsome	ClinVar	Verdict	
Taiwan	1/520	p.S69P	c.205T > C	-	Likely pathogenic	Likely pathogenic	-	pathogenic	[113]
Taiwan	16/520	-	c.932delC	-	-	-	-	-	[113]
Taiwan	2/520	-	c.976C > T	-	-	-	-	-	[113]
Taiwan	1/506	p.L181F	c.543G > C	-	Likely pathogenic	Likely pathogenic	-	pathogenic	[129]
Cameroon	2/134	-	c.-16-51A > G	rs189167598	-	Benign	-	Benign	[56]
South Africa	1/46	p.N63N	c.189T > C	rs139688042	Likely benign	Uncertain Significance	-	Conflicting Interpretations	[56]
South Africa	1/46	p.N122N	c.366T > C	-	Likely benign	Uncertain Significance	-	Conflicting Interpretations	[56]

Cameroon	11/134	p.R239R	c.717G > A	rs57946868	Benign	Uncertain Significance	-	Conflicting Interpretations	[56]
South Africa	2/46								
South Africa	1/46	p.A253V	c.758C > T	rs17653265	Benign	Benign	Benign	Benign	[56]
USA	6/52	p.L11Y	c.31–32 delCTinsTA	-	-	Likely pathogenic	-	Likely pathogenic	[138]
USA	2/20	p.V24A	c.71T > C	-	Likely pathogenic	Likely pathogenic	-	pathogenic	[138]

*, The numerators in this column represent the number of mutated alleles, and the denominators the total number of screened alleles, InterVar, VarSome, and ClinVar are databases to assess the clinical significance of the variants.

3.2.2.7. Connexin 45 (*GJC1*)

Connexin 45 was previously given the gene symbol *GJA7* but the Human Genome Organization (HUGO) Gene Nomenclature Committee [139] has given it the symbol *GJC1.* We identified a multi-site study that reported *GJC1* variants from three different populations: the USA, Turkey, and the UK [140]. None of the reported variants were predicted pathogenic although they were identified in hearing-impaired cohorts. We observed conflicting interpretations of the variants' clinical significance based on the predictions from the databases used (Table 3.15).

3.2.2.8. Summary of the Global Allele Frequencies of the Common Connexin Genes Pathogenic (PLP) Variants Associated to Hearing Impairment (HI)

We calculated the allele frequency of the reported PLP variants in connexin genes in patients and controls for each continent, in order to assess the global contribution of these variants associated with HI. Asia had the highest allele frequencies of the common *GJB2* variants in the hearing controls compared to the other continents. *GJB2:*p.V37I:c.109G>A had the highest allele frequency (200/3478 (5.8%)) among the control group from Asia (200/3478 (5.8%)) and North America (11/588 (1.9%)). In Africa, *GJB2*:p.Gly12ValfsTer2:c.35delG and p.R143W:c.427C>T recorded the highest frequencies in Ghana. In Europe, among the eight common *GJB2* variants, p.Gly12ValfsTer2:c.35delG (Table 3.16). We did not find reports of carriers of the *GJB6* large deletions and *GJA1* PLP variants.

Table 3.15: Connexin 45 (*GJC1*) gene variants.

Country	USA		Turkey	UK
Reference	[140]		[140]	[140]
Protein change	p.L71L	p.T302T	p.D297N	L304L
Nucleotide change	c.213C>T	c.906C > T	c.889G > A	c.912G > T
rs number	rs61749924	rs2229395	-	-
*Number of alleles	2/168	13/120	4/194	1/80
Intervar	Likely benign	Likely benign	Uncertain significance	Likely benign
Varsome	Uncertain significance	Uncertain significance	Uncertain significance	Uncertain significance
ClinVar	-	-	-	-
Verdict	Conflicting Interpretations	Conflicting Interpretations	Conflicting Interpretations	Conflicting Interpretations

*, The numerators in this column represent the number of mutated alleles, and the denominators the total number of screened alleles, InterVar, VarSome, and ClinVar are databases to assess the clinical significance of the variants.

Table 3.16: Summary of the global allele frequencies of the common pathogenic (PLP) variants in connexin genes associated to hearing impairment (HI).

		*Patients (#Chrom/Total #Chrom (Allele Frequency))						*Controls (#Chrom/Total #Chrom (Allele Frequency))					
Gene	**Variant**	**Africa**	**Asia**	**Australia**	**Europe**	**North America**	**South America**	**Africa**	**Asia**	**Australia**	**Europe**	**North America**	**South America**
GJB2	p.Gly12 ValfsTer2 (c.35delG)	770/3848 (20.0%)	5917/71,209 (8.3%)	50/104 (48.1%)	15,616/65,019 (24.0%)	3237/26,976 (12.0%)	822/8191 (10.0%)	12/1604 (0.7%)	35/4684 (0.7%)		197/21,978 (0.9%)	6/988 (0.6%)	
	p.M34T (c.101T>C)	2/272 (0.7%)	89/4902 (1.8%)	5/104 (4.8%)	9418/47,909 (19.7%)	451/25,118 (1.8%)	44/4500 (1.0%)				13/1886 (0.7%)	5/588 (0.9%)	
	p.L79Cfs (c.235delC)	2/262 (0.8%)	9666/250,680 (3.9%)	31/520 (6.0%)	32/4382 (0.7%)	80/20,406 (0.4%)	2/866 (0.2%)		35/4908 (0.7%)		1/1886 (0.1%)	1/588 (0.1%)	
	p.V37I (c.109G>A)	24/1192 (2.0%)	2833/81,139 (3.5%)	8/104 (7.7%)	95/13,227 (0.7%)	530/27,288 (1.9%)	30/4852 (0.6%)	3/640 (0.5%)	200/3478 (5.8%)			11/588 (1.9%)	
	p.H100Rfs Ter14 (c.299_300delAT)	0/0	1046/85,332 (1.2%)	7/520 (1.3%)	7/2936 (0.2%)	20/19,067 (0.1%)	0/0		3/1264 (0.2%)				
	p.W24X (c.71G>A)	0/0	666/22,464 (3.0%)	3/104 (2.9%)	249/12,523 (2.0%)	47/17,055 (0.3%)	7/2248 (0.3%)		11/320 (3.4%)				
	p.L56Rfs (c.167delT)	2/50 (4.0%)	240/17,350 (1.4%)	1/104 (1.0%)	93/12,141 (0.8%)	275/21,540 (1.3%)	30/3094 (1.0%)		7/2690 (0.3%)		1/1886 (0.1%)		
	p.R143W (c.427C>T)	255/1298 (19.6%)	154/62,605 (0.2%)	1/104 (1.0%)	17/2977 (0.6%)	35/21,189 (0.2%)	35/2132 (1.6%)	2/290 (0.7%)					
GJB6	Del (*GJB6*-D13S1830)	1/204 (0.5%)	31/3096 (0.1%)	2/68 (2.9%)	186/7778 (2.4%)	36/1498 (2.4%)	44/4516 (1.0%)	0/198	0/782		0/1502	0/230	0/1508
	Del (*GJB6*-D13S1854)				1/782 (0.1%)		10/2524 (0.4%)						
GJA1	p.L11Y (c.31–32 delCTinsTA)				6/52 (11.5%)						0/200		
	p.V24A (c.71T>C)				2/20 (10.0%)						0/200		
	p.L181F (c.543G>C)		1/506 (0.2%)						0/240				
	p.S69P (c.205T>C)		1/520 (0.2%)						0/240				

*, The numerators in this column represent the number of mutated alleles, and the denominators the total number of screened alleles. #chrom = number of chromosomes, InterVar, VarSome, and ClinVar are databases to assess the clinical significance of the variants.

3.2.3. Discussion

To the best of our knowledge, this paper is, to date, the most comprehensive review on the contribution of connexin gene variants in HI, globally. Connexin channels regulate the transport of small signaling molecules between cells to aid the proper functioning of the tissue/organ systems in the body [141]. Our review found that more than 570 studies were conducted globally on connexin-related HI investigations with most studies performed in Asia, while relatively few have been done in Africa.

Most studies used targeted sequencing, but the decline in next-generation sequencing cost has accelerated the discovery of novel disease gene variants through available high-throughput targeted panels or whole-exome sequencing technologies investigating several gene targets in a single test [142–144]. Indeed, there was a clear migration from non-sequencing approaches such as denaturing high-performance liquid chromatography (DHPLC), multiplex ligation-dependent probe amplification (MLPA), PCR, restriction fragment length polymorphism (RFLP), and single-strand conformational polymorphism (SSCP) to sequencing techniques or a combination of sequencing and non-sequencing techniques.

Connexin 26 gene (*GJB2*, OMIM:121011) located on chromosome 13q12.11 is known to be expressed in different tissues including the cochlear of humans [145], mouse, and rat [146]. *GJB2* gene variants were the most common genetic factors associated with NSHI among several populations [44,147], however, they are rare in African, and African American populations [148]. Similarly, it was clear from our review that *GJB2* is the most investigated gene and had the highest number of pathogenic variants identified among hearing-impaired patients. The most common pathogenic variants (*GJB2*: p.Gly12ValfsTer2, p.M34T, p.L79Cfs, p.V37I, p.H100RfsTer14, p.W24X, p.L56Rfs, and p.R143W) appeared to be localized to specific populations, due to a founder effect [11,149].

In a previous review by Chan and Chang in 2014, 216 original *GJB2* research articles reporting not less than 10 probands were retrieved and analyzed [11]. In our current review, 571 original research publications on connexins associated with HI were considered of which 566 articles reported on *GJB2* associated HI. The previous report was from 63 countries [11], while in this study, we retrieved *GJB2* publications from 106 countries. The differences in the number of publications and countries involved can be explained by the time difference between the previous report and the present study. Also, we did not exclude case reports, contrary to the previous report. In contrast to the report from Chan and Chang, Australia, and not Africa, had the lowest contribution of *GJB2* variants. Moreover, Asia was identified as the highest contributor while the previous report had Europe as the highest contributor of *GJB2* PLP to HI [11]; this can be attributed to the increasing interest and number of genetic researches in all

parts of the world. Despite the above differences, the commonly reported PLP *GJB2* variants were similar in both studies. Furthermore, our study and the studies from Chan and Chang and Tsukada reported a similar ethnic-specific spectrum of the common PLP variants in *GJB2* [11,149].

The most common *GJB2* variant is p.Gly12ValfsTer2 (c.35delG) which is frequently reported among populations in Europe, the Middle East, Australia, North, and South America [11]. We observed widespread of this variant across the globe but it was almost absent in sub-Saharan Africa although there were studies from Ghana [44,150], Cameroon [14,54] and South Africa [14] that investigated this variant in African populations. Morocco is an exception, where five independent studies identified biallelic c.35delG mutation in hearing-impaired patients [34,151–154]. The spread of the variant from Europe and North Africa to North and South America seems to follow migration patterns [11].

Second to *GJB2*: c.35delG is *GJB2*- p.M34T (c.101T>C) which was found to be most prevalent in the United Kingdom (UK). The carrier rate of *GJB2*: p.M34T was calculated at 2.69% in the UK, which was almost twice the carrier rate of *GJB2*-c.35delG (1.36%). In the United States of America, the carrier rate for the *GJB2*: p.M34T variant was found to be 2.3% [155]. The high carrier rates of variants suggested the possibility of heterozygous advantage. However, the audiometric characterization of *GJB2*-p.M34T carriers was not different from homozygous hearing individuals. Hence there is no effect on the hearing ability of the carriers [156].

In the present review, we identified three variants (*GJB2*: p.L79Cfs/c.235delC, p.V37I/c.109G>A, and p.H100RfsTer14/c.299_300delAT) with very high allele frequencies from Asia compared to other continents. These variants were absent in sub-Saharan African countries but were found in a few cases in some North African countries. The Chinese population was found to have a high prevalence of *GJB2*: c.235delC [157] with frequencies of about 14.7% homozygous among a hearing-impaired sub-population, and 16.1% heterozygous in the hearing population [158]. The carrier frequency of *GJB2*: c.235delC is similar to that of the entire Asian population [157,158], and a high prevalence of that variant was reported in Japan [159,160], Korea [161], and Taiwan [162].

The *GJB2*: p.V37I variant was described as a polymorphism by some researchers while others consider it a potential disease-causing missense mutation [163]. The high carrier frequency of the variant among hearing controls informs the polymorphism argument, however individual homozygous of the variant had HI [164]. Compound heterozygosity of the *GJB2*: p.V37I variant and other known *GJB2* pathogenic variants produced mild to severe HI. It was proposed that the milder phenotype was due to the *GJB2*-p.V37I allele [165]. We have identified several independent studies that reported the variant in hearing-impaired individuals, implying that the

variant is likely disease-causing. In addition, *GJB2*: p.V37I was predicted as pathogenic by CinVar, Varsome [166], and InterVar [167]. The majority of *GJB2*: p.V37I mutated alleles were identified among Asians and mostly Chinese [163,164]. The third most common *GJB2* variant associated with HI in Asia was c.299_300delAT with an estimated allele frequency of 3.89% [160,168]. Although this variant is very prevalent in China, it appears that this variant is not common in other populations [169].

The truncating *GJB2* mutation p.W24X is the predominant mutation among the Indian and European Gypsy populations [170–172]. The *GJB2*: p.W24X was the most commonly observed mutation and accounted for about 95% of all *GJB2* mutations found in the Indian population with a carrier frequency of 2.4% [172]. The mutation was proposed to be a founder effect and confirmed through haplotype analysis of the flanking markers of the *GJB2* gene [170,172].

GJB2: c.167delT was reported to be common in the Eurasian populations and postulated to have a single origin of allele due to the observed conserved haplotypes around the mutation [173]. Although the mutation was prevalent in the territories of the Middle East [173–175], we found a high number of alleles with PLP in the United States of America. The fourth most common *GJB2* mutation in the American population is *GJB2*: c.167delT and was found to account for about 3.6% of cases. The variant was more prevalent in the White-American population compared to other populations [176].

As, an exception in populations of African ancestry, the *GJB2*: p.R143W is a founder mutation reported first in a Ghanaian village known for its extremely high number of deaf people. To date, *GJB2*: p.R143W mutation is still the most common HI gene in Ghana [44,150,177]. In 1998, there was a study that reported the homozygous form of the mutation in all 11 families investigated [43]. An update from the Ghanaian village in 2020 found 7 out of 8 families with the homozygous mutation, the 8th family had the heterozygous form [177]. The phenotype-genotype correlation from both studies showed that patients with biallelic *GJB2:* p.R143W had profound HI while no difference was observed between the carriers and normal hearing participants [43,177]. In our review, we identified studies from the United States of America and Asia that had reports of patients with the mutated *GJB2:* p.R143W. Considering that the p.R143W mutation has a high frequency in Ghana, it is likely that the mutation emerged in this population and was introduced in the other populations with African ancestry in the diaspora via ancient and/or recent migration events, specially through the Black African transatlantic slave trade.

In our current review, we identified some rare *GJB2* variants that were absent from the databases used. Although these variants were identified in hearing-impaired participants, they

may not be considered as HI variants since there are no functional and/or population studies on these variants. A recent report showed that rare variants within the coding region of *GJB2* gene and other HI genes are associated with Meniere disease (MD) [178]. The signs and symptoms of MD are sensorineural hearing loss, tinnitus, and episodic vertigo which are sometimes common to patients with NSHI [179]. Meniere disease was reported to be extremely rare in individuals of African descent which supported the negligible contribution of *GJB2* variants to HI in sub-Saharan Africa [180].

Rare known PLP variants in *GJB2* were found to displace some degree of ethnic specificity in some populations. Similar to previous reports [181,182], we identified extremely high frequencies of *GJB2*: c.IVS1+1G > A from Yakutia and Russia; the high prevalence of the variant within the Yakutia population can be seen in the high carrier rate of 10% [182]. This is also suggestive of a potential accumulation of *GJB2* pathogenic variants in this population and calls for public health attention [181]. The Siberian population which comprises Russia, Kazakhstan, boarders of Mongolia and China was found to have the rare *GJB2*: p.W172C (c.516G>C). We analyzed other variants (p.W172* (c.516G>A), p.W172R (c.514T>A)) at the same site of mutation, and similar to our observation, a previous study reported an extremely high frequency of 62.9% from these populations [183]. Other population-specific *GJB2* rare variants found were p.W44X, p.Q7X, and p.S199F which are common to North America [157,176,184], Ecuador [185], and Colombia [186] respectively.

Although not in high numbers, we identified pathogenic *GJB2* intronic variants that were reported in hearing-impaired patients. These variants were c.IVS1-1G>A [187], c.IVS1-15C>T [157], c.IVS1+12G>A [188], c.IVS1+27G>C [189], and c.-23+1G>A. The –23+1G>A variant was reported in studies from Russia [190], Poland [191], China [192], India [172], United States of America [155], and with a high prevalence in Turkey [193].

GJB6 (OMIM:604418) encodes connexin 30, which is part of the family of proteins that form gap junction channels. The location of *GJB6* in the human genome is chromosome 13q12.11 (the same as *GJB2*). The *GJB6* gene is expressed mainly in the brain and skin [194,195] with about 76% protein identity when compared to human *GJB2* [196]. The *GJB6* gene has been associated with Clouston syndrome and hearing impairment. The association of *GJB6* coding region variations to HI has recently been refuted [110]; however, previous reports from Taiwan [113] Germany [114], and Iran [115] identified pathogenic variants within the coding regions of the gene. Six *GJB6* large genomic deletions have been found and previously reported, they are: >920kb deletion [197], 179 kb deletion [198], 131 kb deletion [199], del(*GJB2*-D13S175), del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) [200]. In addition to the six, there was a report of a seventh *GJB6* deletion, del(*GJB6*-D13S1834), in February 2020 [201]. We identified the

large genomic deletions, *GJB6*-D13S1830, and *GJB6*-D18S1854 as the frequently reported *GJB6* variants. In many cases, the large deletions were in trans with pathogenic *GJB2* variants and similar to previous observations [110]. Unlike the *GJB6* coding region variations, the large deletions disrupt a 5′ cis-acting element upstream of both genes. The disruption of the cis-acting element abolishes the expression of *GJB2* and hence is responsible for the phenotype [202,203]. The previously reported *GJB6* knockout mice had significant reduction in the *GJB2* expression which was the cause of HI [204]. Mice models with only the coding region of *GJB6* deleted were found to have normal hearing which provided evidence that the coding region plays no role in the development of HI [198,199].

Our analysis of the global distribution of *GJB6*-D13S1830 confirmed a previous observation by del Castillo et al. [17,205] with a high prevalence of the variant in North America, South America, and Europe with no record from Asia, Australia, and Africa. This variant is known to be frequent in Spain, France, the United Kingdom, Israel, and Brazil [206], and similarly, we observed that the highest number of alleles were from Spain and France. The absence of *GJB6*-D13S1830 from the Asian, Australian, and African populations is indicative of a population-specific spread of the variant and it would inform future studies as well as inform public health policies.

Connexin 43 (*GJA1*, OMIM:121014) is located on chromosome 6 (6q21-q23.2) [138] of the human genome and has been implicated in a number of diseases but mainly in oculodentodigital dysplasia [206–208] with pleiotropic phenotypes [209]. It should be noted that numerous pathogenic variants that have been linked to HI were found in the *GJA1* pseudogene, which could explain their lack of representation in HI-associated genes. Indeed, *GJA1* pseudogene has the features of an expressed gene [210]. The messenger RNA of *GJA1* gene was identified in tumor cells which was contrary to the characteristics of pseudogenes (inability to produce functional mRNA and proteins) [211]. Also, *GJA1* is ubiquitously expressed in many human tissues and cells [212]. Although *GJA1* has been associated with HI, only a few pathogenic variants (*GJA1*: p.L11Y, p.V24A, p.L181F, and p.S69P) were reported in deaf patients from the USA [138] and Taiwan [129]. This gene's contribution to HI is not conclusive considering the low number of patients with the pathogenic variants, and more data from diverse populations are needed to refine the gene-disease pair curation. It is possible that the voltage-gating mechanism of connexin 43 may be affected by the pathogenic variants, culminating in defective gap junction channels. The expression pattern from the mouse genome database has shown that *GJA1* is expressed in the auditory system and may play key roles in hearing and the functioning of the ear [138,213].

The connexin 45 gene, *GJC1* (OMIM:608655), is located on chromosome 17q21.31. Connexin 45 is a candidate HI gene expressed in the auditory system as part of the connexin proteins; however, the mouse genome database has no report of its association with the HI phenotype [213]. Cardiovascular disorders are the most common phenotypes associated with *GJC1* [214]. We found a research effort to identify gene variants in *GJC1* among hearing-impaired participants [140]. The authors studied participants from 3 different populations but did not find any pathogenic variants from the HI cohorts studied. It is imperative to screen a larger population for *GJC1* mutations to investigate the gene's contribution to HI.

In humans, *GJB3* (connexin 31 gene, OMIM:603324) found on chromosome 1p34.3, encodes gap junction beta 3 protein [215]. The gene has been associated with two major conditions erythrokeratodermia variabilis et progressive (MIM:133200) and non-syndromic hearing loss. In our review, we found only a few hearing-impaired participants with mutations in *GJB3*, at low allele frequencies. However, only two likely pathogenic variants (*GJB3*-p.E183K/c.547G>A/rs74315318 and *GJB3*-p.N166S/c.497A>G/rs121908851) were reported from publications considered [216]. We could not conclude on the clinical significance of these variants since the 3 databases gave conflicting clinical significance [166,167,217]. Functional analysis suggested an expression overlap and possible interactions between connexin 26 and connexin 31 in the cells at the tip of the spiral limbus. It was further demonstrated that the two connexins formed heterotypic channels in the [127]. The data from the above functional studies confirmed a digenic form of NSHI. In addition, a biallelic mutation in any of the two connexins can result in NSHI. Confirming the digenic claim, *GJB2* p.V37I/p.L213S/*GJB3* p.V84I was found to co-segregate with NSHI in a family [131]. To fully understand the role of *GJB3* in HI, there is a need to study many populations as well as conduct further functional assays to assess the ironic and biochemical functions of the gene and its mutant forms.

Like other connexins, *GJB4* (OMIM:605425) encodes connexin 30.3 which oligomerizes to form gap junction channels. Unlike the common connexins, the contribution of *GJB4* to HI remains unknown. There were considerable efforts by five groups of researchers identified in this review to investigate *GJB4* mutations in deaf populations. The clinical significance of the identified variants of *GJB4* had conflicting clinical significance; six variants were predicted as pathogenic or likely pathogenic on not more than one of the three databases used [166,167,217]. Functional genomics using mouse models showed that the auditory system of *GJB4* null mice is unaffected, the mutant mice had normal hearing when assessed by brain stem-evoked potentials [218]. However, the gene was found to be expressed in the mouse auditory system [213] and rat cochlear [219] suggesting its role in hearing.

The association of *GJC3* (a gene that encodes connexin 29, OMIM:611925) to NSHI remains unclear although a few studies have associated variations in connexin 29 to NSHI. These variants as presented in our results were predicted to have uncertain clinical significance. However, data from the Mouse Genome Informatics has shown that *GJC3* is expressed in the auditory system and has hearing loss as one of its phenotypes [213]. Functional studies using *GJC3* null mice showed reduced maturation of the hearing threshold. Noise-induced HI has been reported in *GJC3* null mice [213], while another study conducted with adult mice did not find any difference in the hearing threshold of *GJC3* null and wildtype mice [220]. To conclude on the contribution of *GJC3* to HI, we recommend screening more deaf populations across the globe.

There was a limited number of publications retrieved on *GJB3*, *GJB4*, *GJC3, GJA1*, and *GJC1* which was a major challenge encountered in this study. Therefore, it was difficult to determine whether these genes should be considered or catalogued as HI genes. Apart from *GJB2,* which is globally known to be associated with HI, there is a need for more studies from different populations as well as functional studies for a concluding decision to be made on *GJB4*, *GJA1, GJC3*, and *GJC1.* The Hearing Loss Home Page [10] has categorized *GJB3* as an autosomal dominant HI gene. Although *GJB6* was previously considered as a HI gene, recent studies have disproved its classification as a HI gene. The present review suggests that only *GJB2* and *GJB3* are recognized and validated HI genes.

3.2.4. Materials and Methods

3.2.4.1. Search Terms

We reviewed publications on connexin genes variants implicated in human HI and the distributions of the common variants across the globe. Also, we evaluated the methods used to investigate NSHI-implicated gene variants. The protocol was registered on PROSPERO, International Prospective Register of Systematic Reviews with the registration number "CRD42020169697". Two independent reviewers conducted the literature search on PubMed, Scopus, Africa-Wide Information, and Web of Science databases. The search term used in the study comprised of three major components: the first component was (connexins OR "gap junction alpha protein" OR "Gap junction alpha proteins" OR "Gap junction beta-protein" OR "Gap junction proteins" OR connexin OR GJB OR GJA), the second component was ("hearing loss" OR "non-syndromic sensorineural hearing loss" OR "non-syndromic deafness" OR "non-syndromic hearing impairment" OR "hearing impairment" OR deafness) and the last component was (genetic OR gene OR genes) OR ("genetic loci" OR genes OR "genetic diseases" OR "genetic markers). Each component of the search term was joined with "AND"

to obtain the resultant search term which was used to retrieve publications from the databases used (Figure 3.10).

3.2.4.2. Data Extraction

Two independent reviewers conducted the literature search between 1 April to 31 May 2020 and 2592 full-length articles were selected based on the inclusion and exclusion criteria outlined below.

Inclusion criteria:

- Publications on human hearing impairment;
- Publications on the genetics of non-syndromic hearing impairment;
- Publications reporting on connexins association with NSHI.

Exclusion criteria:

- Studies that are not on human hearing impairment;
- Review or meta-analysis publications;
- Policy papers;
- Publications that are not on connexin hearing impairment;
- Publications on environmental and/or syndromic hearing impairment;
- Publications focusing on in silico analysis.

The search with the keywords gave 874, 992, 31, and 695 results from PubMed, Scopus, Africa-Wide Information, and Web of Science databases, respectively. A blinded screening was undertaken by the two reviewers (SMA, and EWT, first and second authors of this manuscript) using the titles as the first-level screening followed by the abstracts. The search results were downloaded into EndNote referencing software and duplicates removed (Figure 6). The data extraction was conducted independently by the two reviewers and compared to remove any form of bias. The following data elements were extracted: (1) location and date; (2) connexin genes investigated; (3) the number of mutant alleles; (4) methods for genetic screening. The data extracted were manually captured onto Microsoft Excel sheets and analyzed using Microsoft Excel (Office 365 education license under the University of Cape Town, South Africa) and SPSS version 25 (IBM, Armonk, New York, United States). A third person (AW) who is an expert in the field was consulted in times of disagreement between the individual judgments during the screening and data extraction process.

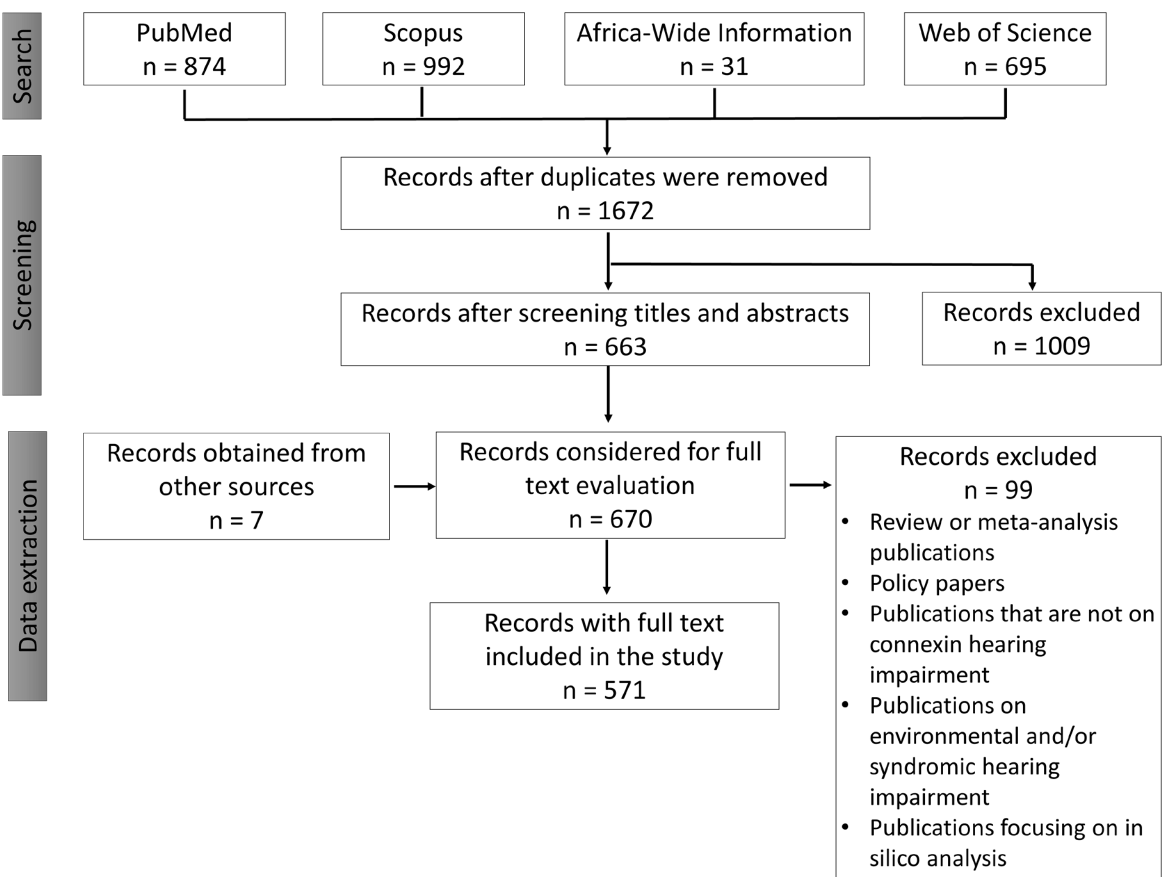

Figure 3.10: Flow diagram illustrating the screening of articles obtained after the literature search.

3.2.4.3. Quality Assessment

To avoid any form of bias, two independent reviewers (SMA and EWT) synthesized the data and assessed the quality of the documents included, using the quality of genetic studies (Q-Genie) tool developed by Sohani et al. [49] for genetic studies and the risk of bias assessment tool for prevalence studies developed by Hoy et al. [50] for the other studies. Discrepancies were solved by discussion and consensus. An expert (AW) was consulted to resolve disagreements between the judgment of the reviewers, by discussion and consensus. The quality assessment was conducted at the outcome level of each study. The studies were assessed for selective outcome reporting and whenever there was evidence of this, the effect of the selective outcome reporting on the study results was further analyzed.

3.2.4.4. Clinical Significance

The clinical significance of the identified variants was assessed on three databases InterVar [167], VarSome [166], and ClinVar [217]. Both VarSome and InterVar are bioinformatic web-based tools build on the American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology (AMP) 2015 guidelines and are useful for clinical interpretation of human genetic variants [166,167]. ClinVar is also a web-based database that

provides evidence and relationships between human variants (found in biological sample) and phenotypes which serve as strong evidence for clinical significance interpretation [217]. Our judgement on the clinical significance of each variant was based on the prediction from the 3 databases mentioned above.

3.2.5. Conclusions

The present comprehensive review on the contribution of connexins genes in HI, globally, found most investigations performed in populations from China, with relatively few studies from African populations. In most populations, except Africans, common *GJB2* pathogenic variants that were found were p.Gly12ValfsTer2 and p.M34T (commonly among Europeans), p.L79Cfs, p.V37I, and p.H100RfsTer14 (mostly in Asians), p.W24X among Indians, p.L56Rfs among Americans, and p.R143W particularly in Ghanaians, the African exception for *GJB2* variants. These *GJB2* variants exhibited population-specific prevalence due to founder effects. The second most common HI-associated connexin was *GJB6.* We identified two main deletions in *GJB6* (*GJB6*-D13S1830 and *GJB6*-D13S1854), that were predicted to be pathogenic, however, the coding region variants of *GJB6* are no longer considered as causes of HI. From the review, we identified 11 *GJA1* variants of which 3 were predicted to be pathogenic but their pathogenicity needs to be confirmed with more data from multiple populations. The *GJB4* variants found from the reports that were used for this review mostly had conflicting clinical significance, but the majority were predicted pathogenic by one of the 3 databases used. None of the *GJC1*, *GJB3,* and *GJC3* variants were predicted pathogenic. Most researchers used targeted sequencing approaches to investigate connexin genes associated with HI. The present review suggests that only *GJB2* and *GJB3* are recognized and validated HI genes. It is likely that the wide use of whole-exome sequencing, particularly in understudied African populations, will rapidly increase the identification of novel HI-associated gene variants and improve disease-gene pairs curation, globally.

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Abbreviations

GJB2	Gap junction beta-2
GJB6	Gap junction beta-6
GJB3	Gap junction beta-3
GJB3	Gap junction beta-4
GJC3	Gap junction gamma-3
GJA1	Gap Junction Alpha 1
GJC1	Gap junction gamma 1

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1 (given its large content, the supplementary materials for this specific manuscript has been placed at the end of the thesis, in the appendices).

Chapter 4: Results – Targeted gene screening

Synopsis: This chapter explores the use of targeted gene screening to solve HI cases, and includes two original articles. The first one assessed the implication of *GJB2* and *GJB6* genes to hereditary recessive NSHI in Cameroon, while the second presents the clinical and genetic aspects of Duchenne muscular dystrophy in Cameroon and assesses the possible implication of *DMD* gene in HI in humans.

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

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Candidate's Contribution: Performed patients recruitment, molecular experiments, audiometry testing, audiometry data analysis, and issued the first draft of the manuscript.

Co-Authors Contributions:

AW: Conceived the project;

ETW: Performed the recruitment and molecular experiments.

JJN: Supervised the recruitment;

ETW and **SMA:** Performed molecular analysis of the *GJB2* and GJB6 genes.

ETW: and **JVFF:** Interpreted audiometric data.

AW and **EC:** Supervised the project.

ETW, EC, JJN, SMA, JVFF, and **AW:** revised successive draft of the manuscript, and agreed to the published version of the manuscript.

GJB2 and *GJB6* mutations in Autosomal Recessive Non-syndromic Hearing Impairment (DFNB) in Cameroon

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Abstract: This study aimed to investigate *GJB2* (connexin 26) and *GJB6* (connexin 30) mutations associated with familial non-syndromic childhood hearing impairment (HI) in Cameroon. We selected only families segregating HI, with at least two affected individuals and with strong evidence of non-environmental causes. DNA was extracted from peripheral blood, and the entire coding region of *GJB2* was interrogated using Sanger sequencing. Multiplex PCR and Sanger sequencing were used to analyze the prevalence of the *GJB6*-D3S1830 deletion. A total of 93 patients, belonging to 41 families, were included in the analysis. Hearing impairment was sensorineural in 51 out of 54 (94.4%) patients. Pedigree analysis suggested autosomal recessive inheritance in 85.4% (35/41) of families. Hearing impairment was inherited in an autosomal dominant and mitochondrial mode in 12.2% (5/41) and 2.4% (1/41) of families, respectively. Most HI participants were non-syndromic (92.5%; 86/93). Four patients from two families presented with type 2 Waardenburg syndrome, and three cases of type 2 Usher syndrome were identified in one family. No *GJB2* mutations were found in any of the 29 families with non-syndromic HI. Additionally, the *GJB6*-D3S1830 deletion was not identified in any of the HI patients. This study confirms that mutations in the *GJB2* gene and the del(*GJB6*-D13S1830) mutation do not contribute to familial HI in Cameroon.

Keywords: hearing impairment; genetics; *GJB2* and *GJB6;* Cameroon; Africa

4.1.1. Introduction

Hearing impairment (HI) is a disabling congenital disease with the highest rate for age-standardized disability of life in the world [221]. Globally, congenital HI has a prevalence of 1.3 per 1,000 population [33], and is accounted for in about 1 per 1,000 live births in developed countries, with a much higher incidence of up to 6 per 1,000 live births in sub-Saharan Africa [3]. Genetic factors contribute from 30 to 50% of hearing impairment cases in sub-Saharan Africa [5]. In 70% of neonates who fail newborn hearing screens (NBHS) and are presumed to have inherited HI, there are no other distinguishing physical findings and the HI is classified as non-syndromic [39]. Among non-syndromic (NS) HI, nearly 80% of cases are inherited in an autosomal recessive (AR) mode [222,223].

Non-syndromic hearing impairment (NSHI) is an extremely heterogeneous trait, with approximately 170 NSHI loci and 112 genes identified to date [10]. Nevertheless, studies in European and Asian populations have identified pathogenic variants in *GJB2* (encoding connexin 26) and *GJB6* (encoding connexin 30) as the major contributors to autosomal recessive NSHI (ARNSHI) [11], with *GJB2*-c.35delG being the most prevalent variant (20–57%) found in cases of autosomal recessive non-syndromic hearing impairment (ARNSHI) [223,224]. The *GJB6*-D13S1830 deletion was identified in up to 9.7% of cases, and thus is the second largest contributor to the genetic aetiology of NSHI in European populations, either with homozygous presentation, or when present in addition to a *GJB2* mutation on the opposite allele [89,225].

Cameroon is a sub-Saharan African country, covering an area of 475,442 km^2, with a 2017 census reporting a population of 24,053,727 [226]. Cameroon is frequently referred to as “Africa in miniature”, because of its many geographical and cultural attributes, its population and linguistic diversity (there are more than 200 distinct local languages in the country) [6], and its vast genetic diversity that mimics that of Africa [227].

Previous studies have found no contribution of the *GJB2* and *GJB6* genes to HI in Cameroon [14,228,229]. However, the patients included in those studies were chosen indiscriminately, and consisted of both familial and isolated cases, with a high likelihood of environmental causes in many cases. In this study we revisit the contribution of the *GJB2* and *GJB6* genes to HI in Cameroon by only focusing investigations into cases that showed a clear pattern of inheritance within families.

4.1.2. Materials and Methods

4.1.2.1. Ethical Approval

The study was performed in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Institutional Research Ethics Committee for Human Health of the Gynaeco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon (No723/CIERSH/DM/2018), and the University of Cape Town's Faculty of Health Sciences' Human Research Ethics Committee (HREC 104/2018). Written and signed informed consent was obtained from all participants who were 21 years of age or older, and from parents or guardians in cases of minors, with verbal assent from participants, including permission to publish photographs.

4.1.2.2. Participants' selection

Hearing impaired patients were recruited from eight of the ten administrative regions of Cameroon, from schools for the deaf, and in the community, following procedures previously reported in Cameroon [6]. Briefly, all participants' detailed personal and family histories were obtained, medical records were reviewed by a general practitioner, a medical geneticist, and an ENT specialist when possible, and relevant data were extracted, including three-generation pedigrees and perinatal histories. A general systemic and otological examination and audiological evaluation were performed, including pure tone audiometry. We followed the recommendation number 02/1 of the Bureau International d'Audiophonologie (BIAP), Belgium, to classify hearing levels [6,230]. Only HI individuals belonging to families segregating hearing impairment, with at least two affected individuals and with strong evidence of non-environmental causes were recruited.

4.4.2.3. Molecular analysis

Genomic DNA samples were extracted from peripheral blood, following the manufacturer's instructions for the available commercial kit (Puregene Blood Kit®(Qiagen, Alameda, CA, USA)), at the Biochemical Laboratory of the Centre Pasteur du Cameroun, Yaoundé, Cameroon, or using the Chemagic extraction protocol, in the division of Human Genetics, University of Cape Town, South Africa.

Previously reported primers for the *GJB2* gene were evaluated using BLAST® (NIH, USA) and other software, as recommended [14]. The entire coding region of the *GJB2* gene (exon 2) was amplified, followed by sequencing using an ABI 3130XL Genetic Analyzer® (Applied Biosystems, Foster City, CA), in the Division of Human Genetics, University of Cape Town, South Africa.

Detection of del(*GJB6*-D13S1830) was performed using the method and primers described by del Castillo et al. [89,225]. The entire coding region of *GJB6* was amplified using the method described by Chen et al. [231]. The PCR results were validated by Sanger sequencing of 10% of the samples.

4.1.2.4. Data Analysis

Data analysis was performed through the use of descriptive statistics.

4.1.3. Results

4.1.3.1. Participant demographics

We recruited a total of 93 patients, belonging to 41 families. Their mean age was 18 ± 10.4 (1–50) years. The male/female ratio was 0.82 (42/51). Hearing impairment was congenital in 62 patients (66.7%), and the mean age at medical diagnosis was 3.2 ± 3.3 (1–22) years.

4.1.3.2. Audiometric characteristics

A pure tone audiometry was performed in 54 of our 93 patients. Hearing impairment was sensorineural in 51 out of 54 (94.4%) patients and mixed in 3 patients; no patients exhibited a conductive hearing impairment. All of our patients had bilateral hearing impairment, and the majority had profound to total hearing impairment (n=51; 94.4 %) (Table 4.1).

Table 4.1: Audiometric classification of hearing impairment, according to the Bureau International d'Audiophonologie (BIAP) recommendation

Level of hearing loss	n*	Percentage (%)
Severe I (71–80 dB)	01	1.8
Severe II (81–90 dB)	02	3.7
Profound I (91–100 dB)	04	7.4
Profound II (101–110 dB)	13	24.1
Profound III (111–119 dB)	23	42.6
Total (≥120 dB)	11	20.4
Total	54	100

*Number of patients.

4.1.3.3. Inheritance pattern

Pedigree analysis suggested that autosomal recessive inheritance was the most frequently observed pattern of inheritance and accounted for 85.4% (35/41) of families (Figure 4.1A). In 12.2% (5/41) and 2.4% (1/41) of families, HI were likely inherited in an autosomal dominant and mitochondrial mode, respectively (Figure 4.1B). Consanguinity was present in three families (3/41; 7.3%). The inbreeding coefficient was 0.0625 in one of these families (Figure 4.1A), and 0.0156 in the other two families. A total of six participants (6/93; 6.5%) were thus born from consanguineous union.

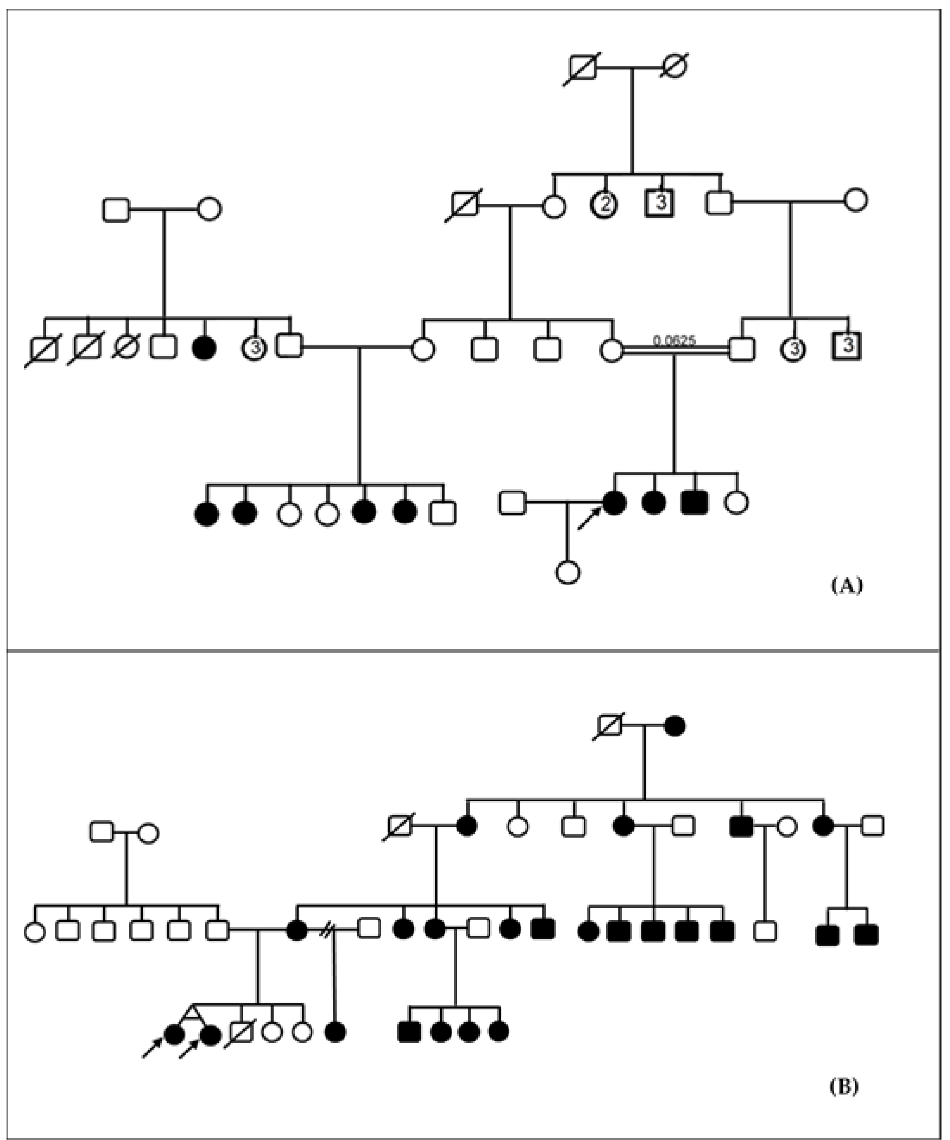

Figure 4.1: Inheritance of familial hearing impairment in Cameroon. (**A**) Pedigree of a consanguineous family with autosomal recessive non-syndromic hearing impairment. (**B**) Pedigree of a family with non-syndromic hearing impairment suggestive of mitochondrial inheritance. Arrows here indicate the probands.

4.1.3.4. Non-syndromic and syndromic hearing Impairment

The majority of our participants exhibited non-syndromic hearing impairment, which accounted for 92.5% (86/93) of cases, for a total of 38 families (Figure 4.2).

Four patients from two families in our cohort presented type 2 Waardenburg syndrome (without dystopia canthorum). The main clinical signs included: hearing impairment, patches of hypopigmented skin, sapphire-blue eyes, and premature white hair (Figure 4.3).

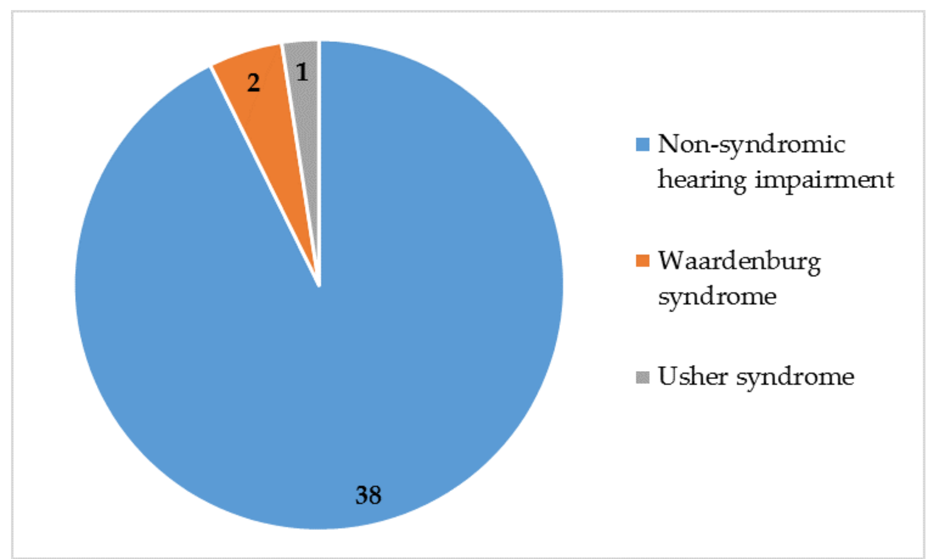

Figure 4.2: Non-syndromic and syndromic hearing impairment. N = 41 families

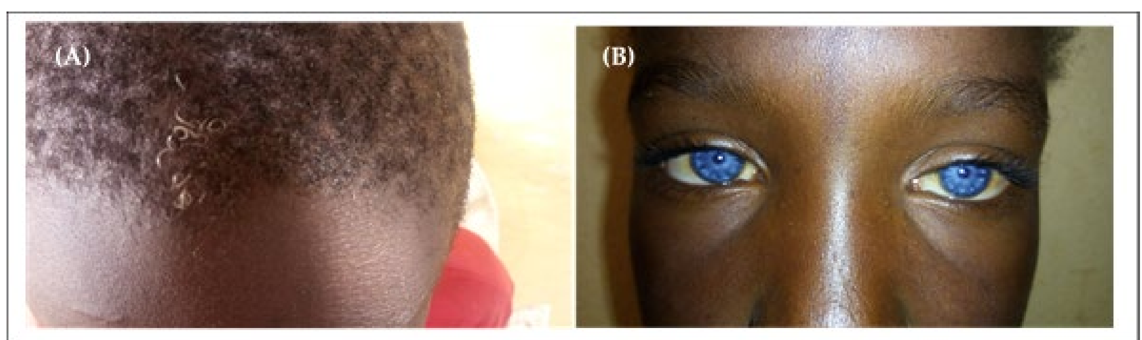

Figure 4.3: Waardenburg syndrome in our cohort. (**A**) Premature white hair; (**B**) Sapphire-blue eyes

Three cases of type 2 Usher syndrome (without vestibular areflexia), from one family, were identified; in addition to hearing impairment, clinical signs of retinitis pigmentosa were present, including night vision impairment and constricted visual field.

4.1.3.5. Molecular Analysis Results of *GJB2* and *GJB6*

A total of 29 families with segregating recessive non-syndromic hearing impairment were tested for mutations in *GJB2* and for the del(*GJB6*-D13S1830) mutation. None of the families exhibited the del(*GJB6*-D13S1830) mutation, or any of the reported disease-causing mutations in *GJB2.* However, a *GJB2* variant of uncertain significance, NM_004004.6: c.499G>A (p.V167M), was present in one family in the heterozygous form (Figure 4.4).

4.1.4. Discussion

This report is the most compressive study of the role of *GJB2* and *GJB6* in familial HI in Cameroon, and confirmed the non-implication of these genes in non-syndromic HI in that

country; this is consistent with previous reports in selected isolated HI cases of putative genetic origin [14,228]. By carefully and stringently selecting only multiplex families in the current studies, our results consolidate previous findings. In addition, we recruited in nearly all the schools, as well as in the community, for the deaf in 8/10 provinces representing about 90% of the population; therefore, we are confident that the sample is representative of the population in Cameroon.

The low implication of the *GJB2* gene in non-syndromic hearing impairment has also been demonstrated in other populations of African descent. The 35delG mutation, which constitutes almost 50% of all *GJB2* mutations in Caucasians [86–88], was not reported in 406, 356, 182, and 126 probands from Kenya, Ghana, South Africa, and Uganda, respectively [15,17,94,150]. Moreover, no pathogenic mutations in *GJB2* were found in a cohort of 44 probands from Nigeria with non-syndromic deafness [93], and only 7% of a cohort of 127 American probands of Hispanic or African descent with bilateral non-syndromic hearing impairment presented a disease causing mutation in *GJB2* [232]. However, an African exception is the Ghanaian population, where the *GJB2* founder mutation p.R143W (c.427C>T) was shown to be highly prevalent in that population [43], and accounted for nearly 25% of familial cases and 8% of isolated cases of HI [44]. The *GJB2* variant, c.499G>A (p.V167M), of uncertain significance according to the ClinVar database, was present in a family in our cohort, and has previously been described by our group in the Cameroonian population [14]. It has also been described in the USA [233] and in China [234]. The *GJB2* variant c.499G>A (p.V167M) could thus be considered to require further investigation.

The most common mutation in *GJB6* is a 342-kb deletion, *GJB6*-D13S1830, which causes non-syndromic HI when homozygous, or when present on the opposite allele to a *GJB2* mutation [17,92]. The del(*GJB6*-D13S1830) mutation is the second most frequent genetic cause of non-syndromic prelingual hearing impairment in the Spanish population (after the 35delG mutation in *GJB2*) [89]. This deletion is also prevalent in France, Brazil, and Israel [225,235,236], but is rare or absent in Italy (including Sicily), Romania, Iran, and India [42,223,237–240]. This deletion was also absent in Nigerians [93] and in Ghanaians [44]. In order to identify any other mutations different from del(*GJB6*-D13S1830), the coding region of *GJB6* was sequenced in African probands from Cameroon, South Africa and Uganda, however, this revealed no additional pathogenic mutations [94,229].

Our findings support previous reports that *GJB2* and *GJB6* do not play a significant role in non-syndromic hearing impairment in most populations of African descent. Interestingly, genetic testing through targeted genomic enrichment and massively parallel sequencing of 116 genes were used to screen 10 multiplex families with non-syndromic hearing impairment. In seven of

the 10 families (70%), 12 pathogenic variants were identified in six genes, and nearly half of these variants were novel [21]. Therefore, due to the highly heterogeneous genetic nature of NSHI, next generation sequencing would be the most effective way to identify variants associated with non-syndromic deafness in the African populations [5], and all the families investigated in the present study should be subjected to whole exome sequencing in order to potentially identify variants in known genes as well as novel genes. Indeed, based on the identification of specific inner ear transcripts, it is estimated that more than 1,000 NSHI genes are still to be identified [241].

The study also confirms Waardenburg syndrome as the most common cause of syndromic hearing impairment in Cameroon, in line with previous reporting in other African populations [59]; these families, as well as the singular family displaying mitochondrial inheritance, should also warrant further molecular investigation.

4.1.5. Conclusions

The present study showed that hereditary hearing impairment in Cameroon is mostly non-syndromic, congenital, sensorineural, and inherited in an autosomal recessive mode. Additionally, this study identified Waardenburg and Usher syndromes as the most common syndromic hearing impairments in Cameroon. This study confirmed that mutations in the *GJB2* gene, and the del(*GJB6*- D13S1830) deletion are not implicated in familial non-syndromic hearing impairment in Cameroon. Future studies should employ whole genome sequencing approaches and functional genomics studies to identify other genes which may be implicated in the hearing impairment observed in these families.

Author Contributions: AW conceived the project; ETW performed the recruitment and molecular experiments. JJN supervised the recruitment; ETW and SMA performed molecular analysis of the *GJB2* and *GJB6* genes. ETW and JVFF interpreted audiometric data. AW and EC supervised the project. All the authors agreed to the final manuscript.

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Acknowledgements: We thank patients and their families for their participation in this project. We also thank schools and associations for the deaf for their help during the process of identification and recruitment of families.

Conflicts of Interest: We declare that no competing interests exist.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1:

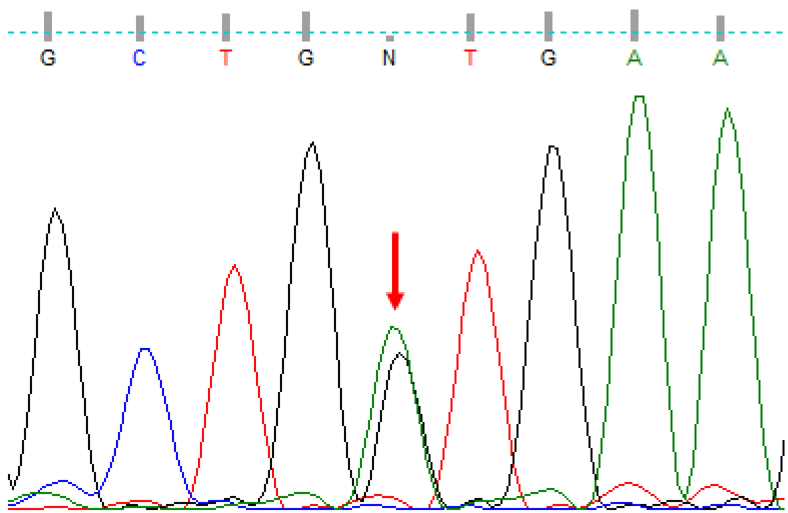

Figure 4.4: GJB2 variant of uncertain significance, c.499G>A (p.V167M), present in a family in the heterozygous form

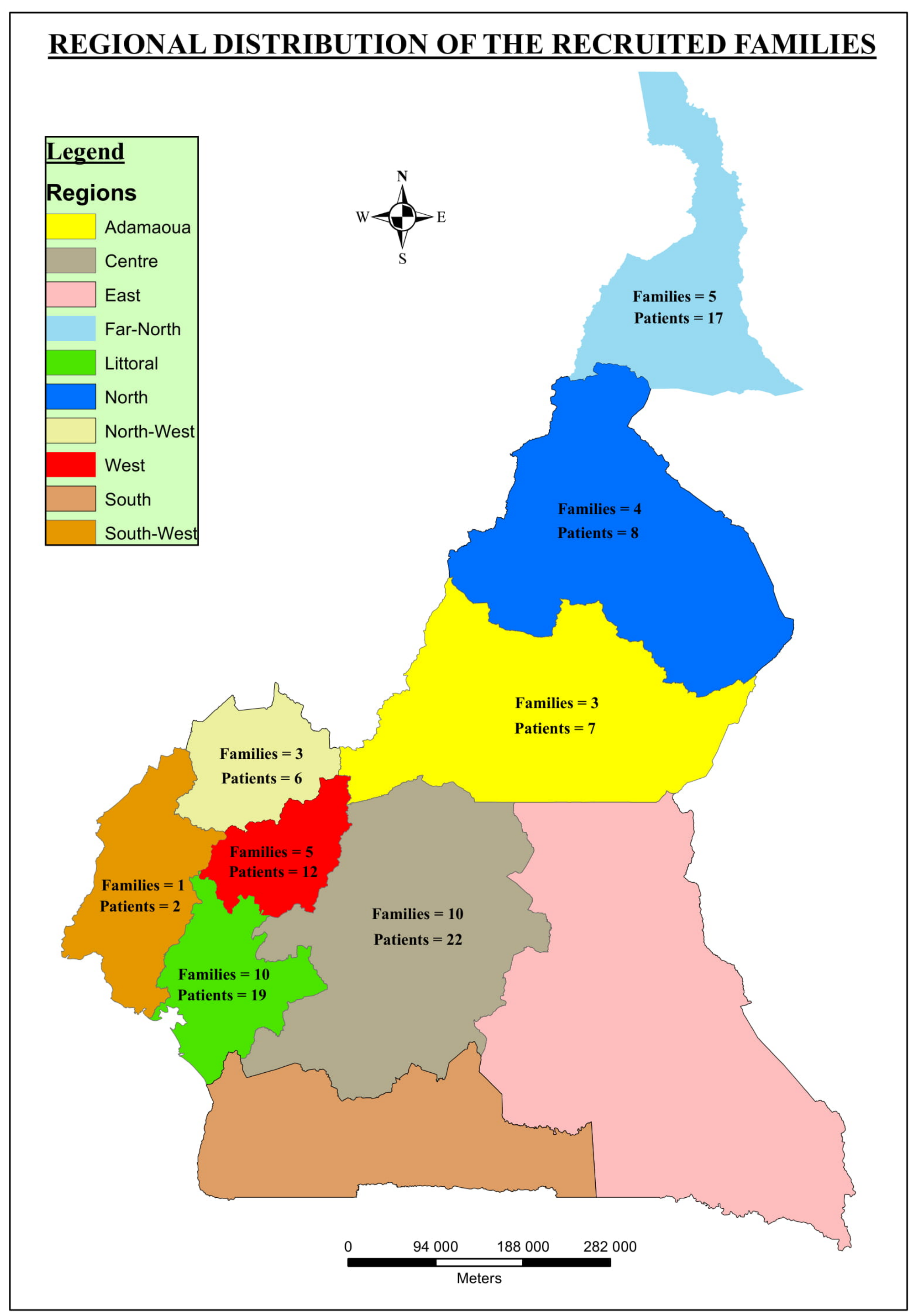

Figure 4.5: Regional distribution of families

4.2. **Wonkam-Tingang, E.**, Nguefack, S., Esterhuizen, A. I., Chelo, D., and Wonkam, A. (2020). *DMD*-related muscular dystrophy in Cameroon: Clinical and genetic profiles. *Molecular Genetics & Genomic Medicine*, e1362. https://doi.org/10.1002/mgg3.1362.

Nature of Publication: Original Full Journal Article.

Journal/Publisher: Molecular Genetics and Genomic Medicine; © Wiley Periodicals, LLC; ISSN: 2324-9269; Peer-reviewed.

Candidate's Contribution: Designed the study protocol, performed clinical interview and physical examination of patients, compiled all the results and issued the first draft of the manuscript.

Co-Authors Contributions:

AW and **SN:** Conceived the project

EWT: Developed the protocol.

EWT, SN, DC, and **AW:** Took medical histories and performed clinical examinations of patients.

AIE: Performed molecular analyses.

EWT: Compiled all the results and issued the first draft of the manuscript.

SN, AIE, DC, and **AW:** Critically revised successive drafts of the manuscript.

SN and **AW:** Supervised the project and compiled the revisions.

Rationale for including this manuscript in the thesis: Previous studies have demonstrated a significant sensorineural hearing loss associated with muscular dystrophy in the mdx mouse (a DMD mouse model) [26]. Additionally, the dystrophin protein was detected in the cochlea of guinea pigs and normal mice, but was absent in *mdx* mice [27]. Besides, Aberrant glycosylation of α-dystroglycan, a protein that interacts with dystrophin (the protein encoded by *DMD*) at the basal lamina leads to a group of disorders called "muscular dystrophy dystroglycanopathy" (MD-DG) [242]. Two mouse models for MD-DG were shown to exhibited congenital, non-progressive, and mild-to-moderate sensorineural HI in auditory brainstem response [242]. Also, HI was found to be associated with some muscular dystrophies in humans, including facioscapulohumeral muscular dystrophy, and limb-girdle muscular dystrophy [243–245]. Last, six loci for X-linked HI have been described to date, including the DFNX3 (Xp21.2) locus, which contains *DMD* [24]. No previous studies explored the possible implication of *DMD* variants in HI in humans.

DMD-related Muscular Dystrophy in Cameroon: Clinical and Genetic Profiles

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Abstract

Background

Most of the previous studies on Duchenne Muscular Dystrophy (DMD) were conducted in Caucasian, Asian and Arab populations. Therefore, little is known about the features of this disease in Africans. In this study, we aimed to determine the clinical characteristics of DMD, and the common mutations associated with this condition in a group of Cameroonian patients.

Methods

We recruited DMD patients and performed a general physical examination on each of them. Multiplex ligand-dependant probe amplification was carried out to investigate exon deletions and duplications in the *DMD gene* (OMIM: 300377) of patients and their mothers.

Results

A total of 17 male patients from 14 families were recruited, aged 14 ± 5.1 (8 – 23) years. The mean age at onset of symptoms was 4.6 ± 1.5 years, and the mean age at diagnosis was 12.1 ± 5.2 years. Proximal muscle weakness was noted in all patients and calf hypertrophy in the large majority of them (88.2%; 15/17). Flexion contractures were particularly frequent on the ankle (85.7%; 12/14). Wasting of shoulder girdle and thigh muscles was present in 50% (6/12) and 46.2% (6/13) of patients, respectively. No patient presented with hearing impairment. Deletions in *DMD* (OMIM: 300377) occurred in 45.5% of patients (5/11), while duplications were observed in 27.3% (3/11). Both mutation types were clustered between exons 45 and 50, and the proportion of *de novo* mutation was estimated at 18.2% (2/11).

Conclusion

Despite the first symptoms of DMD occurring in infancy, the diagnosis is frequently made later in adolescence, indicating an underestimation of the number of cases of DMD in Cameroon. Future screening of deletions and duplications in patients from Cameroon should focus on the distal part of the gene.

Keywords: Duchenne muscular dystrophy; clinical patterns; genetics; Cameroon; Africa

4.2.1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder, caused by mutations in the *DMD gene* (OMIM: 300377), leading to the synthesis of a defective dystrophin protein [25]. Dystrophin, in association with other muscle membrane proteins, makes a dystrophin-associated-protein-complex that stabilizes the sarcolemmal membrane [25,246,247]. In the absence or reduction of dystrophin, the plasma membrane is fragile and predisposed to tearing and fragmentation during muscle fibre contraction [25,247]. DMD has a world incidence of 1 in 3500 live male births [248], and a global prevalence of 4.8 per 100,000 males [249]. DMD prevalence in sub-Saharan Africa has been estimated at 1/100,000 males [250]. DMD is the most common recessive X-linked disease [251].

Most boys with DMD present symptoms between 3 and 5 years of age [252,253]. Delayed motor milestones, repeated falls, gait abnormalities, and difficulty climbing stairs are the most common symptoms at onset [252,253]. Subsequently, proximal and distal muscle weakness,

calf hypertrophy, muscle atrophy, and contractures with orthopaedic deformities occur [254]. In the early stages of DMD, the weakness of proximal muscles manifests by affected patients 'climbing up their own bodies with their hands' (Gower's sign) when rising from the floor to the standing position [255]. Serum creatine kinase (CK) levels and hepatic transaminases are usually elevated, reflecting active muscle degeneration [252,256]. The severity of the disease is due to the eventual development of cardiac and respiratory abnormalities [257,258].

DMD (OMIM: 300377) is the largest known human gene, spanning 2.2 mbp and containing 79 exons [251,259]. Deletions account for approximately 65% of *DMD* mutations that have been observed, while duplications have been measured in approximately 6%–10% of DMD patients. The remaining 30%–35% consist of small deletions, insertions, point mutations, or splicing mutations [251,252]. These mutations either disrupt the reading frame or generate a premature stop codon [259]. Deletions and duplications can occur anywhere in the gene, but they concentrate between exons 45–55 and exons 2–10, respectively [259,260]. Mutations maintaining the reading frame of *DMD* are often associated with a milder form of the disease called Becker muscular dystrophy (BMD) [252]. Approximately one-third of DMD cases are due to *de novo* mutations [252,261].

Six loci for X-linked hearing impairment (HI) have been described to date, including DFNX1 (Xq22.3), DFNX2 (Xq21.1), DFNX3 (Xp21.2), DFNX4 (Xp22.12), DFNX5 (Xq26.1), and DNFX6 (Xq22.3) [24]. In 1994, Lalwani et al. [262] reported a family with X-linked non-syndromic hearing impairment (NSHI) that mapped to region Xp21.2, which contains the *DMD* locus. A few years later, Pfister et al. [263] reported a second family, from Turkey, with HI that mapped to the same locus. Interestingly, Raynor and Mulroy demonstrated that *mdx* mice, an animal model for DMD, have an increased threshold for hearing when compared to normal mice [26]. Additionally, the dystrophin protein was detected in the cochlea of guinea-pigs and normal mice, but was absent in *mdx* mice [27]. Based on these results, these authors suggested that dystrophin might play a role in the hearing process, and recommended a systematic hearing screening of all DMD/BMD patients [262,263].

Most of the previous studies on DMD were conducted in Caucasian, Asian and Arab populations [252,264–266]. Therefore, little is known about the features and the burden of this condition in Africans. This study aimed to determine the clinical features of DMD and the most common mutations associated with this condition in Africans, and to assess a possible association with HI in a group of patients from Cameroon.

4.2.2. Materials and Methods

4.2.2.1. Ethical Compliance

This study was conducted in accordance with the Declaration of Helsinki. Ethical approval was granted by the Institutional Research Ethics Committee for Human Health of the Gynaeco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon (No. 723/CIERSH/DM/2015, and No. 137/CIERSH/DM/2018), and the University of Cape Town's Faculty of Health Sciences' Human Research Ethics Committee (HREC 484/2019). Written and signed informed consent was obtained from all participants who were 21 years of age or older, and from parents or guardians in cases of minors, with verbal assent from participants, including permission to publish data.

4.2.2.2. Participants selection

We included patients admitted and treated for DMD, in the paediatric neurology unit of the Gynaeco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon, from January 2008 to May 2015. This cross-sectional study had a retrospective component, from January 2008 to February 2015, mostly focused on participant recruitment and clinical data collection. It also included a prospective phase from February 2015 to May 2015, mainly dedicated to molecular analysis and clinical examination of patients who were still alive.

For all participants, detailed personal and family histories were collected. Medical records were reviewed by a medical team comprising of a general practitioner, a medical geneticist, a neuro-paediatrician and a cardio-paediatrician when possible. Relevant data were collected, including three-generation pedigrees, psychomotor development, age at onset, symptoms at onset, and age at diagnosis. A general physical examination was also performed.

4.2.2.4. Biochemical analyses

Biochemical measurements were obtained within 8 hours of drawing a peripheral blood sample in the biochemistry laboratory of the Centre Pasteur du Cameroon (CPC), Yaoundé, Cameroon. Participants were asked to refrain from excessive physical exercise for 48 hours prior to the blood sample collection. Analyses for CK, aspartate transaminase (AST), and alanine transaminase (ALT) were performed on the Vitros® autoanalyzer (Ortho-clinical diagnostics, Johnson and Johnson, Rochester, NY, USA) following the manufacturer's instructions and standard operating procedures. Enzymes activity levels were interpreted based on published standard age- and gender-matched normal values [267–270].

4.2.2.5. Molecular analyses

Molecular analyses were performed in the National Health Laboratory Service, Groote Schuur Hospital and University of Cape Town, South Africa. DNA was isolated from peripheral blood using the salting out method as reported by Miller et al. [271]. Multiplex ligand-dependant probe amplification (MLPA) was carried out using P034 and P035 probes from MRC-Holland (Amsterdam, Netherlands) in both patients and parents. The 79 exons of *DMD* were analysed for the presence of exon deletions and/or duplications. The procedures and analyses were carried out as previously published [272,273]. The consequences of the identified mutations on the reading frame of *DMD* were obtained through online search from Leiden Muscular Dystrophy pages [274]. The GenBank reference sequence used for the annotation of variants is NM_004006.2.

4.2.2.6. Definitions

The diagnosis of DMD was based on the presence of characteristic clinical signs (calf hypertrophy, muscle weakness, contractures, toe walking, frequent falls, difficulties climbing stairs, Gowers' sign), elevated serum muscle enzymes' activities, and molecular analysis in some cases.

The hearing of our patients was assessed during clinical examination and was based on the ability of the patient to perceive speech with a normal voice. According to the recommendation number 02/1 bis of the Bureau International d'Audiophonologie (BIAP), Belgium [275], an individual with a pure tone average of less than 41 dB can perceive speech with a normal voice. However, when the average tone is equal to or more than 41 dB, the person can only perceive speech if the voice is loud. The latter situation also corresponds to adult disabling HI, as stated by the World Health Organisation [99]. Based on this recommendation, only patients that could not perceive speech with a normal conversational voice were candidates for a formal audiology assessment.

A mother was considered as an obligate carrier in any of the following situations: she has one affected son as well as another affected male from her maternal lineage [276]; she has two affected sons, or an affected son and a carrier daughter [276]; on molecular analysis, both the mother and an affected son have a mutation in *DMD*.

4.2.2.7. Data analysis

Descriptive statistic and non-parametric tests were used for comparisons, and p-values < 0.05 were considered statistically significant.

4.2.3. Results

4.2.3.1. Participants' Demographics

A total of 17 patients from 14 families were investigated. All participants were male, aged 14 ± 5.1 years (range: 8 – 23 years). The majority of our patients (82.4%; 14/17) were aged < 20 years (Figure 4.6). Nine patients were re-examined by the research team, while the other patients' clinical data were extracted from their pre-existing medical records.

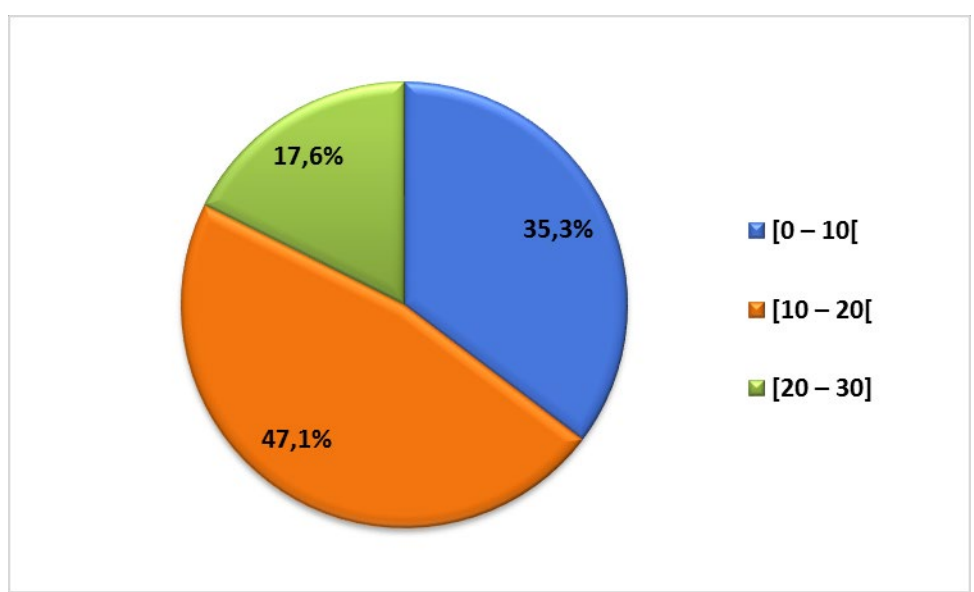

Figure 4.6: Distribution of our study population according to age range (years). N = 17 patients.

4.2.3.2. Medical History

Psychomotor Development

The mean age at onset of autonomous walking was 15.8 ± 4.1 months (range: 10 – 24 months). Six of our patients (35.3%; 6/17) had a history of delayed motor milestone development and 2 (11.8%; 2/17) had a history of delayed language acquisition.

Onset and Diagnosis

The mean age at onset of symptoms was 4.6 ±1.5 years (range: 1 – 6 years; Table 4.6), and the mean age at diagnosis was 12.1 ± 5.2 years (range: 4 – 23 years; Table 4.7). Symptoms present at the onset of the disease are reported in Figure 4.7. The most common ones were toe walking, difficulties climbing stairs, and frequent falls, which were present in 64.7% (11/17), 47.1% (8/17) and 41.2% (7/17) of patients, respectively.

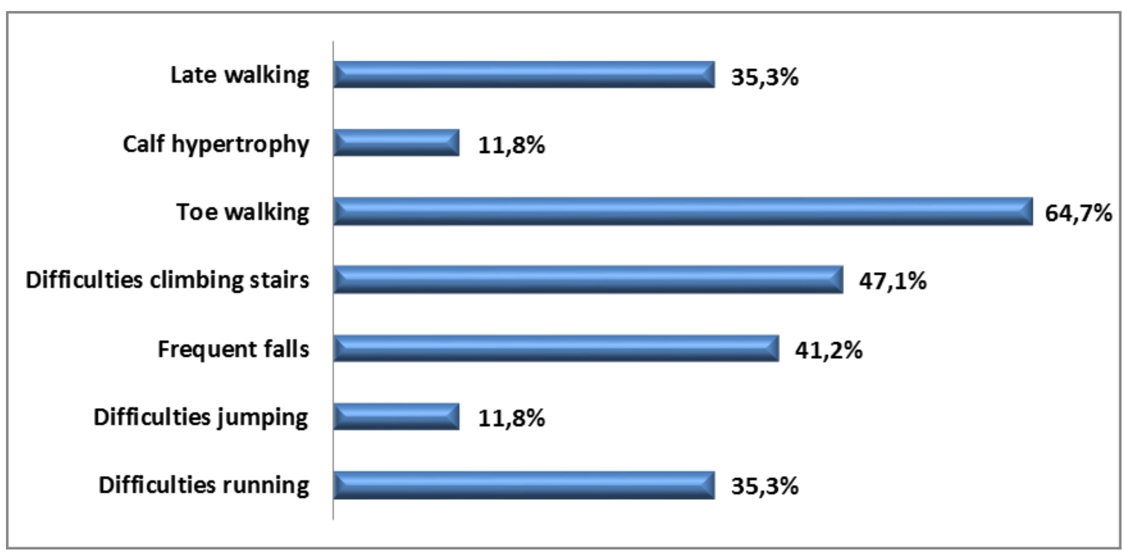

Figure 4.7: Clinical signs at onset of the disease. N = 17 patients.

Loss of Ambulation

A proportion of patients (52.9%; 9/17) had expectedly lost ambulation at the time of the study. The mean age at loss of ambulation was 10.9 ± 2.1 years (range: 8 – 14 years).

4.2.3.3. Motor and Orthopaedic Abnormalities

A wide range of motor abnormalities was observed in our patients and are summarized in Table 4.2. All patients who were still able to walk presented difficulties climbing stairs or running, frequent falls, toe walking, and inability to rise from the floor without using their arms (Gowers' sign). All participants presented with proximal muscle weakness, particularly in the lower limbs. The large majority of participants had calf hypertrophy (88.2%; 15/17). Contracture in ankle plantar flexion (equine foot) was also observed in the majority of patients (85.7% 12/14). Muscle wasting of the shoulder girdle and thigh were noted in 50% (6/12) and 46.2% (6/13) of patients, respectively. Scoliosis was noted in 41.7% (5/12) of patients.

4.2.3.4. Other Clinical Abnormalities

None of our patients presented with HI. They were all able to perceive speech with normal voice, meaning that their pure tone averages were less than 41 dB. Two patients (20 years old, and 21 years old) had clinical signs of heart failure (Table 4.3), and their echocardiograms further highlighted a dilated cardiomyopathy. Symptoms suggestive of obstructive sleep apnoea and nocturnal hypoventilation, (fatigue, morning headaches, daytime sleepiness, snoring, and abrupt awakening accompanied by choking) were present in 2 patients (Table 4.3), including one (the 23 years old patient) who presented with respiratory insufficiency. Three patients had at least two gastrointestinal symptoms, and two others had urological symptoms (Table 4.3).

Table 4.2: Motor and orthopaedic abnormalities

Clinical signs	n/N	Frequency (%)
Myalgia	5/11	45.5
Cramps	4/11	36.4
Difficulties climbing stairs	6/6	100
Difficulties running	7/7	100
Frequent falls	7/7	100
Toe walking	8/8	100
Gowers' sign	8/8	100
Scoliosis	5/12	41.7
Contractures		
Elbow	5/12	41.7
Wrist	4/10	40
Fingers	2/9	22.2
Hips	6/11	54.5
Knees	7/13	53.8
Ankles (Equine foot)	12/14	85.7
Hypertrophy		
Tongue	2/11	18.2
Deltoid	4/12	33.3
Triceps	2/11	18.2
Quadriceps	2/10	20
Calf	15/17	88.2
wasting		
Neck muscles	3/10	30
Shoulder girdle muscles	5/12	41.7
Arm muscles	4/10	40
Forearm muscles	3/9	33.3
Pelvic girdle muscles	6/12	50
Tight muscles	6/13	46.2
Leg muscles	4/10	40
Neck weakness		
Neck flexors	7/9	77.8
Neck extensors	6/9	66.7
Sternocleidomastoid	6/9	66.7
Upper limbs weakness		
Deltoid	9/9	100
Pectorals	9/9	100
Biceps	10/12	83.3
Triceps	8/10	80
Wrist flexors	5/9	55.6
Wrist extensors	5/9	55.6
Lower limbs weakness		
Iliopsoas	14/14	100
Gluteus maximus	14/14	100

Hip adductors	14/14	100
Hip abductors	14/14	100
Quadriceps	14/14	100
Hamstrings	14/14	100
Tibialis anterior	7/10	70
Gastrocnemius	7/10	70

N, number of patients in whom the clinical sign was checked; n, number of patients in whom the clinical sign was found

Table 4.3: Other clinical findings

Clinical signs	n/N	Frequency (%)
Signs of heart failure		
Dyspnoea	3/10	30
Orthopnoea	2/10	20
Displaced apex beat	2/10	20
Tachycardia	3/10	30
Third heart sound	2/9	22.2
Gallop rhythm	2/9	22.2
Heart murmur	2/9	22.2
Abnormal pulse	1/9	11.1
Symptoms of sleep-disordered breathing		
Morning headaches	2/9	22.2
Daytime sleepiness	2/9	22.2
Fatigue	2/9	22.2
Snoring	2/9	22.2
Abrupt awakenings and choking	2/9	22.2
Trouble concentrating	1/9	11.1
Night-time sweating	1/9	11.1
Gastrointestinal symptoms		
Abdominal distention	3/9	33.3
Constipation	3/9	33.3
Dysphagia	1/9	11.1
Regurgitation	1/9	11.1
Urological symptoms		
Urinary frequency	2/9	22.2
Urinary urgency	1/9	11.1
Nocturia	2/9	22.2
Enuresis	3/9	33.3

N, number of patients in whom the clinical sign was checked; n, number of patients in whom the clinical sign was found

4.2.3.5. Enzymes

Mean serum enzymes' activities are reported in Table 4.4. CK levels were above normal range values for age in all patients (N = 17) and screened mothers (N = 4). All patients (N = 17) had

CK levels 10 times higher than the maximal limits for age, including four patients (23.5%; 3/17) with CK activities 100 times higher than the upper normal limit. Serum ALT activities were raised in all patients (N = 15), and AST activities were raised in almost all patients (93.3%; 14/15).

Table 4.4: Serum muscle enzymes levels

Enzymes	N	Mean values	Min.	Max.	Normal ranges
CK (U/L)	17	8171.2 ± 7545.3	837	31872	30 – 150
AST (U/L)	15	183.4 ± 133.0	45	558	8 – 50
ALT (U/L)	15	243.1 ± 163.7	51	606	4 – 49
Maternal CK (U/L)	4	434.5 ± 450.6	155	1104	20 – 140

ALT, alanine transaminase; AST, aspartate transaminase; CK, creatine kinase; Min., minimal value; Max., maximal value; N, number of participants in whom serum enzymes activities have been measured.

4.2.3.6. Genetic Profile

Inheritance Patterns

In five families (5/14; 35.7%; Figure 4.8, Figure 4.10-4.21), the pedigrees were compatible with an X-linked recessive inheritance pattern, with at least two affected boys. While in the remaining nine families (9/14; 64.3%), the patient was the only affected person, suggesting a *de novo* mutational event (Figure 4.8; Figure 4.10-4.21). None of our patients were the offspring of a consanguineous relationship.

Mutations Associated with DMD in our Cohort

MLPA was performed in 11 patients, and four mothers. Exon deletions were found in five patients (45.5%; 5/11), and duplications found in three (27.3%; 3/11). In the remaining three patients, the MLPA did not reveal any mutation. Of the five patients in whom deletions occurred, three had deletions of six exons (exons 45 to 50), one patient displayed deletions of three exons (exons 48 to 50), and the remaining patient had deletions of 36 consecutive exons (exons 8 to 43). With regards to duplications, two patients had duplications of six exons (exons 45 to 50) while the last patient had duplications of seven exons (exons 3 to 9). The impact of the identified mutations on the reading frame of *DMD* is reported in Table 4.5. Most commonly affected exons for both deletions and duplications were exons 45 to 50 (Figure 4.9). No correlation was found between mutation location and motor impairments.

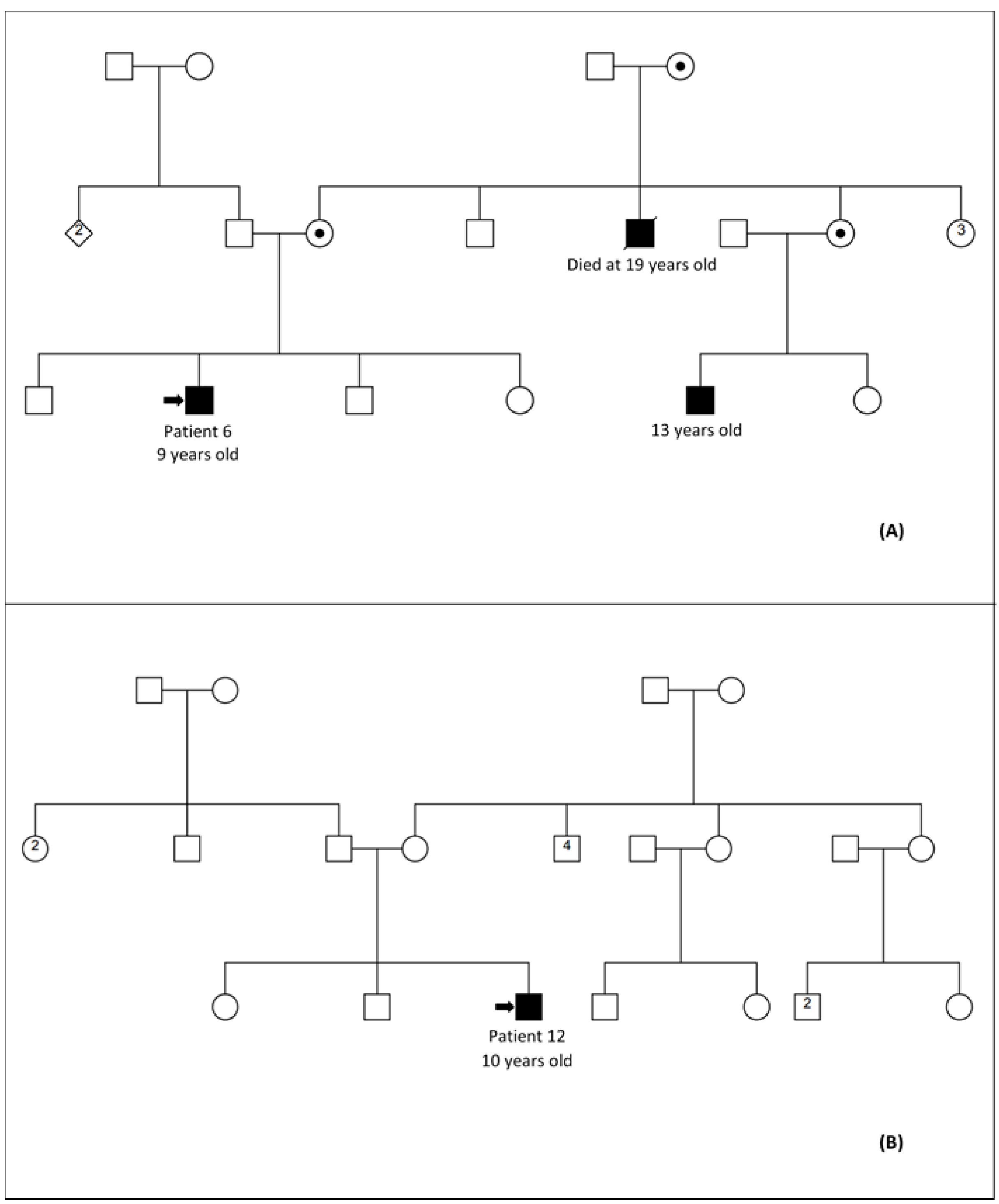

Figure 4.8: Inheritance pattern of DMD in our cohort. (**A**) Pedigree of a multiplex family (family 5), suggestive of X-linked inheritance. The proband here has a cousin who exhibits clinical signs of DMD, and a deceased uncle who has presented the same clinical sign. (**B**) Pedigree of a family (family 9) in which the disease seems to be of sporadic occurrence. Arrows indicate the proband.

Table 4.5: List of mutations identified in our cohort

Mutation	HGVS nomenclature*	Translational effect*	ISCN (2013) nomenclature¥	n
Deletion exons 45-50	c.6439-?_7309+?del	Frame-shift	rsa Xp21.2(31,819,975-31,968,514)x0	03
Deletion exons 48-50	c.6913-?_7309+?del	Frame-shift	rsa Xp21.2(31,819,975-31,875,376)x0	01
Deletion exons 8-43	c.650-?_6290+?del	Frame-shift	rsa Xp21.2(32,287,529-32,699,293)x0	01
Duplication exons 45-50	c.6439-?_7309+?dup	Frame-shift	rsa Xp21.2(31,819,975-31,968,514)x2	02
Duplication exons 3-9	c.94-?_960+?dup	In-frame	rsa Xp21.2(32,697,870-32,849,820)x2	01

HGVS, Human Genome Variation Society; ISCN, International System for Human Cytogenetic Nomenclature; n, number of patients carrying the mutation; rsa, region-specific assay; *Data obtained through online search from Leiden Muscular Dystrophy pages [274], RefSeq: NM_004006.2; ?The actual breakpoint is unknown, ¥Corresponding genomic coordinates were retrieved from Leiden Muscular Dystrophy pages [274], build GRCh38/hg38.

Carrier Status

Five mothers were from multiplex families and were thus designated obligate carriers. Molecular testing was performed for four mothers. Duplications were found in two mothers (including 1 from a multiplex family) while, in the remaining two, deletions identified in their respective sons were not found in their own *DMD* genes, suggesting that these were *de novo* events.

Combining pedigree analysis and molecular testing, we established that of the 14 mothers included in our cohort, six (related to nine of the patients) were carriers, two (two patients) were non-carriers, and a definite status could not be defined for the remaining six (six patients). The proportion of *de novo* mutations in this study population was therefore estimated at 25% (2/8) in families with positive molecular analysis, corresponding to 18.2% (2/11) among all patients.

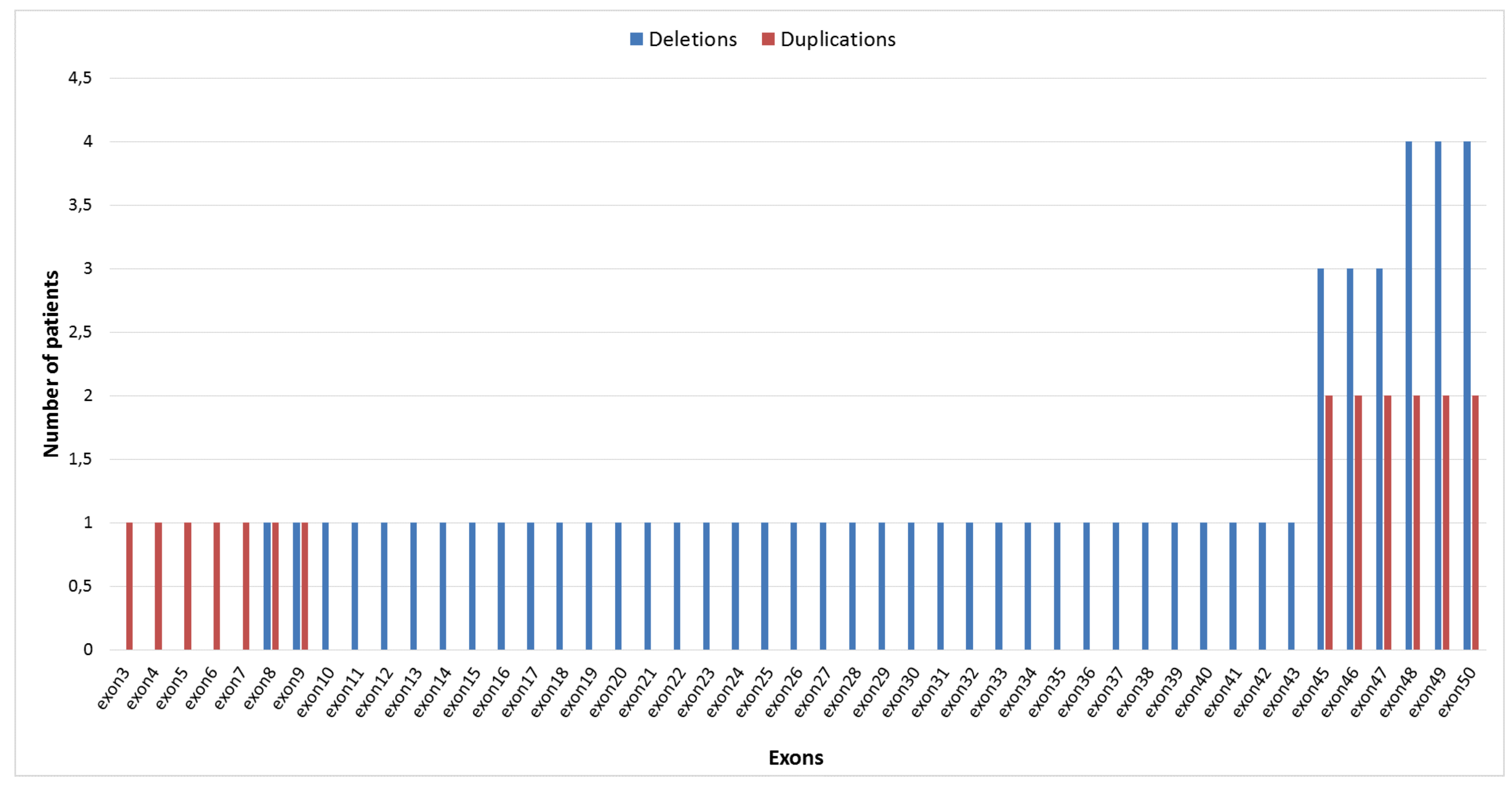

Figure 4.9: Distribution of the nature of mutations in patients according to affected exons

4.2.4. Discussion

This study is one of the rare reports from Central Africa that describes clinical and genetic profile of *DMD*-related Muscular Dystrophy. Despites the molecular testing been performed in relatively limited number of patients (11/17), the data provide the much-needed insight into the features of this condition among Black Africans.

The mean age at onset of the first symptoms at 4.6 years (Table 4.6) was similar to that previously described in India, where the mean at onset of the disease was found to be 4.1 years [253]. This is, however, higher than the 2.4 years reported by Alvarez Leal et al. [277] in Mexico. Similar symptoms at the onset of the disease, including delayed motor milestone, toe walking, frequent falls, late walking, and difficulties running and climbing stairs were reported previously in the United States of America (USA), Italy and Kuwait [278–280]. In this study, despite the occurrence of symptoms in infancy among patients, the clinical diagnosis of DMD was only established at a later age (12.1 ± 5.2 years), aided by the establishment of a medical genetic service in Cameroon [281]. This late diagnosis (Table 4.7) suggests a possible underestimation of the actual number of cases of DMD in Cameroon. It also further highlights the need to raise awareness amongst clinicians, in order to improve early referral, diagnosis, and management of the condition, and the need to build capacity for the molecular diagnosis of DMD and other genetic conditions in general in Cameroon.

The distribution of muscle weakness, muscle wasting, hypertrophy, and contractures noted in the study participants is similar to that described in multiple populations from India, Korea, and Tunisia [254,282–284]. CK levels are comparable to those reported in the literature [252]. The variable expression in terms of age of onset and clinical severity was observed, even between patients from the same family, has also been reported before [254,282,285]. Spinal deformity is common in muscular dystrophies, usually occurring after the loss of walking ability [286]. The mean age at loss of ambulation in our study population was 10.9 years, which is in line with findings from studies conducted in other populations, including France [287]. Similar to the findings described in this group of Cameroonian patients with DMD, heart failure and respiratory insufficiency were shown in previous reports to occur at an advanced stage of the disease [288,289].

Like the present study, DMD and BMD have been described in a few African countries. A group of 11 patients from Nigeria (a West African country) were reported some decades ago [290]. Clinical signs described in this group of patients were similar to those reported in our study, including frequent falls, Gowers' sign, calf hypertrophy, muscle weakness, and contracture. However, the mean age at onset of symptoms in that Nigerian cohort was 7.7 years, which is higher than the 4.6 years reported in the present study. In 2014, a cohort of

six patients from Rwanda (an East African country) presenting with the DMD phenotype was described [291]. The clinical and biological findings (CK levels) were similar to those described in our cohort. Like in the present study, they used MPLA to screen their patients for mutations in *DMD*, with a detection rate of 66.7% (4/6), compared to 72.7% (8/11) in our study. These findings highlighted the efficacy of MLPA in detection copy number variations in *DMD*. A nine years old boy presenting typical clinical signs of DMD was described in Tanzania, as well as a familial case of BMD [292,293]. A large cohort of DMD and BMD patients (*n* = 128) from Southern Africa was reported, with a prevalence of deletion of 46%, which is similar to the 45.5% reported in the present study [250].

Previous studies have demonstrated a significant sensorineural hearing loss associated with muscular dystrophy in the mdx mouse model [26]. Mutations in families with DFNX3 deafness might occur in a novel gene located within the Xp21.2 locus [262], however, among families previously reported as having segregating HI that mapped to the *DMD* gene locus (Xp21.2), none presented with any of the typical clinical signs of DMD [262,263]. Additionally, although HI was found to be associated with some muscular dystrophies, including facioscapulohumeral muscular dystrophy, and limb-girdle muscular dystrophy [243–245], it was not clinically proven in humans affected with DMD [294]. This study concurs with these findings.

This study confirms that deletions in *DMD* are the most frequent mutations associated with the disease in Cameroon. The prevalence of deletions in *DMD* gene varies greatly across populations i.e. 37%, 64% and 86% among Israeli, Caucasians, and Arabs, respectively [295–297]. Previous studies have demonstrated that 50% to 82% of deletions occur within the distal region of *DMD* gene, including exons 44 to 55 [295,297–299]; this is in line with the findings of the present study, as 80% (4/5) of our patients had deletions between exons 45 and 50. The frequency of duplications in our cohort (27.3%; 3/11) was higher than that of populations from the Netherlands, China, and India, in which duplications were found in 7%, 8.4% and 9% of DMD patients, respectively [299–301].

The exonic distribution of duplications in our patients also differed from previous reports. In two of our three patients (66.7%) with duplications, mutations occurred between exons 45 and 50, while previously published papers reported that duplications are most commonly clustered toward the 5’ end of the gene, between exons 2 and 25 [299,300,302,303]. In three of our patients (27.3%; 3/11) in which MLPA was performed, no mutation was identified, suggesting the occurrence of small mutations that still need to be investigated (including insertions, small deletions, single nucleotide variants, and splice site mutations). These small mutations, which are ideally screened through sequencing techniques, were found in 19%, 22%, and 34.2% of

DMD patients from China, Japan, and Spain, respectively [302–304]. Point mutations were described in two Western African families from Mali [305].

Seven patients presented an out-of-frame mutation, and their clinical profiles were compatible with a severe form of DMD. One patient presented an in-frame duplication of exons 3-9. Although this 10-year-old patient has not yet lost ambulation, he could not walk without support or crutches, and his phenotype was too severe to be associated to BMD. Exceptions to the reading-frame rule have also been described in the Rwandan cohort, where two patients with the DMD phenotype presented an in-frame deletion [291], and in Canada were in-frame duplications were reported in two DMD patients [306]. Three of the eight patients who had not yet lost ambulation did not benefit from a molecular screening. Two of them were 8 years old, and the last one was 12 years old. Although their phenotypes were compatible with the severe form of the DMD, the possibility of BMD could not be excluded. BMD is less frequent than DMD, with a prevalence in sub-Saharan Africa estimated at 1/755,000 males [250]. BMD patients have a milder phenotype, with a lower progression as compared to DMD patients. The first symptoms appear around 12 years of age, and the loss of ambulation never occurs before 16 years old [291]. BMD is most commonly due to in-frame mutations in *DMD* [307]. An Eastern African family from Tanzania presenting BMD was reported, with an in-frame deletion of exons 45-48 [292].

The proportion of *de novo* mutations in the present study was estimated at 18.2%, which is lower than the theoretical estimate of one-third predicted by Haldane's rule [308], probably due to the relatively modest sample size. The frequency of new mutants amongst patients with DMD ranges from 16.4% to 39.5% in Caucasians and Asians [309–311], and is up to 62.2% in Latin Americans [312]. In four of our carrier mothers, CK levels were above the normal range values, which means that the assay of CK activity may be a reliable and cost-effective criterion to determine carrier status [276,313], especially in sub-Saharan African settings where molecular diagnosis facilities are not always available. The determination of carrier status is critical for the genetic counselling [314]. Women at risk of being carriers in families included in our study were offered a retrospective molecular screening and were informed of the existence of antenatal molecular diagnosis, which is now possible in Cameroon [281].

Limitations of this study include the clinical assessment of hearing of our patients as it was solely based on conversational voice. As a result, patients with mild/moderate HI could have been missed. Additionally, retrospective clinical data extraction from medical records was incomplete due to poor medical archiving. In addition, intellectual disability was not formally assessed; intellectual disability has been estimated to occur in approximately 32% of DMD patients [315]. Lastly, molecular analysis was not performed for all patients.

Targeted sequencing of the whole *DMD* gene should be performed in future to identify small mutations associated with DMD in the Cameroonian population, which were not detected by MLPA. Nevertheless, the study had some benefits for these families with a rare disease that deserves to be the object of more attention in Africa. Affected families were encouraged to form support groups to raise awareness amongst the general population and policymakers, to share their experience of the disease, and to help each other face their challenges. Further studies are needed to estimate the national prevalence and incidence of DMD in Cameroon.

4.2.5. Conclusion

The study described a relatively late diagnosis of DMD in Cameroon, despite clinical signs and symptoms similar to those described in various other populations around the world. HI was not associated with DMD in our study population. Exon deletions are the most frequent mutations associated with DMD in Cameroon and occur in almost half of patients. The frequency of duplications in the Cameroonian population seems to be higher than that reported in other populations. We suggest that future screening of deletions and duplications in patients from Cameroon, when analysis of all the 79 exons of the *DMD* gene is not feasible, should be focused on the distal part of the gene between exons 45 and 50. More awareness of this rare genetic disease is needed among the general population, policy-makers, and clinicians in order to build capacity for the molecular diagnosis of genetic conditions in both Cameroon and the rest of Africa.

Authors' contributions: A.W. and S.N. conceived the project and E.W.T. developed the protocol. E.W.T., S.N., D.C., and A.W. took medical histories and performed clinical examinations of patients. A.I.E. performed molecular analyses. E.W.T. compiled all the results and issued the first draft of the manuscript. S.N., A.I.E., D.C., and A.W. critically revised successive drafts of the manuscript. S.N. and A.W. supervised the project and compiled the revisions. All authors agree to the final version of the manuscript.

Conflict of interest: We declare that no competing interests exist.

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Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supporting Information: Additional Supporting Information may be found online in the supporting information tab for this article (see Appendix 1).

Chapter 5: Results – Whole exome sequencing identified novel variants in NSHI-candidate genes in Cameroonians

Synopsis: This chapter includes two original articles, and describes the use of WES to identify candidate NSHI-genes in Cameroonians. The first publication reports on *CLIC5* gene as a novel NSHI-gene in the Cameroonian population, and the second reports on *DXML2* gene, another novel NSHI-gene in Cameroonians.

5.1. **Wonkam-Tingang, E**., Schrauwen, I., Esoh, K. K., Bharadwaj, T., Nouel-Saied, L. M., Acharya, A., Nasir, A., Adadey, S. M., Mowla, S., Leal, S. M., & Wonkam, A. (2020). Bi-Allelic Novel Variants in *CLIC5* Identified in a Cameroonian Multiplex Family with Non-Syndromic Hearing Impairment. *Genes*, 11(11), 1249. https://doi.org/ 10.3390/genes11111249.

Nature of Publication: Original Full Article

Journal/Publisher: GENES

Candidate's Contribution: Performed patients recruitment, molecular analyses, in silico analysis of the pathogenicity of candidate variants, and issued the first version of the manuscript.

Co-Authors Contributions:

AW and **SML:** Conception of the project.

EWT: Recruitment and molecular experiments.

EWT and **SMA:** Exclusion of *GJB2* and *GJB6* variants.

IS, TB, LMNS, and **AA:** Bioinformatics analysis.

EWT, IS, and **KKE:** In silico analysis of the pathogenicity of variants

KKE, AN, and **SM:** Protein modelling.

EWT: Issued of the first draft of the manuscript

SML and **AW:** Supervision of the whole project.

EWT, IS, KKE, TB, LMNS, AA, AN, SMA, SM, SML, and **AW:** Review and editing, agreed to the published version of the article.

Bi-allelic Novel Variants in *CLIC5* Identified in a Cameroonian Multiplex Family with Non-syndromic Hearing Impairment.

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Abstract: DNA samples from five members of a multiplex non-consanguineous Cameroonian family segregating prelingual and progressive autosomal recessive non-syndromic sensorineural hearing impairment underwent whole exome sequencing. We identified novel bi-allelic compound heterozygous pathogenic variants in *CLIC5*. The variants identified, i.e. the missense [NM_016929.5:c.224T>C; p.(L75P)] and the splicing (NM_016929.5:c.63+1G>A), were validated using Sanger sequencing in all seven available family members and co-segregated with HI in the three hearing impaired family members. The three affected individuals were compound heterozygous for both variants, and all unaffected individuals were heterozygous for one of the two variants. Both variants were absent from the gnomAD, UK10K, GME, and dbSNP databases as well in 122 apparently healthy controls from Cameroon. We also did not identify these pathogenic variants in 118 unrelated sporadic cases of NSHI from Cameroon. In silico analysis showed that the missense variant *CLIC5* -p.(L75P) substitutes a highly conserved amino acid residue (leucine), and is expected to alter the stability, the structure, and the function of the CLIC5 protein, while the splicing variant

CLIC5 - (c.63+1G>A) is predicted to disrupt a consensus donor splice site and alter the splicing of the pre-mRNA. This study is the second report, worldwide, to describe *CLIC5* involvement in human hearing impairment, and thus confirms *CLIC5* as a novel non-syndromic hearing impairment gene that should be included in targeted diagnostic genes panels.

Keywords: non-syndromic hearing impairment; *CLIC5*; Africa.

5.1.1. Introduction

Hearing impairment (HI) is the most common sensory disability and is accounted for in about 1 per 1000 live births in high-income countries, with a much higher incidence of up to 6 per 1000 live births in sub-Saharan Africa [3]. When occurring in childhood, HI is associated with impaired language acquisition, learning, and speech development, and affects ~34 million children worldwide (World Health Organisation) [4]. Approximately 30 to 50% of HI cases in Africa have a genetic origin [5,316]. Non-syndromic hearing impairment (NSHI) accounts for about 70% of HI cases of genetic origin and is inherited on an autosomal recessive (AR) mode in approximately 80% of cases [8].

Variants in *GJB2* and *GJB6* genes, which are the major contributors to NSHI in Europeans, Asians, and Arabs, are infrequent in most populations of African descent, with a prevalence close to zero [14,229,317]. NSHI is highly genetically heterogeneous [5,316]. To date, about 170 loci and 121 genes have been identified as being associated with NSHI (hereditary hearing loss homepage). Targeted sequencing panels that include > 100 HI genes have detected a consistently lower rate of pathogenic and likely pathogenic (PLP) variants in sporadic HI cases of African ancestry e.g. African Americans (26%), and Nigerians and Black South Africans (4%), compared to >70% for Europeans and Asians [19,20]. However, the detection rate was 70% for 10 mutiplex Cameroonian families [21]. Moreover, the prevalence of ARNSHI pathogenic and likely pathogenic (PLP) variants, using data from the Genome Aggregation Database (gnomAD) database [22] were estimated to account for ARNSHI in 5.2 per 100,000 individuals for Africans/African Americans, compared to 96.9 per 100,000 individuals for Ashkenazi Jews based on sequence data [23]. Therefore, there is an urgent need to investigate HI in populations of African ancestry, particularly multiplex families, using next generation sequencing, to improve knowledge on variants and genes which underlie NSHI in African populations.

In this study we generated whole exome sequence (WES) data for samples obtained from a multiplex non-consanguineous Cameroonian family, segregating progressive ARNSHI, and identified novel bi-allelic PLP variants in *CLIC5* in the locus DFNB103. This gene was previously reported to be associated with HI in a single Turkish family [318]. This gene

encodes a member of the chloride intracellular channel (CLIC) family of chloride ion channels. The encoded protein associates with actin-based cytoskeletal structures and may play a role in multiple processes including hair cell stereocilia formation, myoblast proliferation, and glomerular podocyte and endothelial cell maintenance. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene (provided by RefSeq). The corresponding mutant mouse model (jbg mouse) which has an intragenic deletion in *CLIC5* resulting in a truncated protein presents with progressive hearing impairment and vestibular dysfunction [319].

5.1.2. Materials and Methods

5.1.2.1. Ethics Approval

This study was performed in respect of the Declaration of Helsinki. Ethical approval was granted by the University of Cape Town's Faculty of Health Sciences' Human Research Ethics Committee (HREC 484/2019), the Institutional Research Ethics Committee for Human Health of the Gynaeco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon (No. 723/CIERSH/DM/2018) and the Institutional review board of Columbia University (IRB-AAAS2343). Written and signed informed consent was obtained from all participants who were 21 years of age or older, and from parents in cases of minors, with verbal assent from participants.

5.1.2.2. Participants' Recruitment

The participants' selection process has been previously reported [52]. The hearing-impaired members of the Cameroonian family (Family 24, Figure 5.1A) were identified through a community engagement program for the deaf. For all hearing-impaired participants, detailed personal history, and medical records were reviewed by a general practitioner, a medical geneticist, and an ear, nose and throat (ENT) specialist. A general systemic and otological examination was performed, including pure tone audiometry. We followed the recommendation number 02/1 of the Bureau International d'Audiophonologie (BIAP), Belgium.

Genomic DNA samples were extracted from peripheral blood, using the chemagic extraction protocol, in the division of Human Genetics, University of Cape Town, South Africa. Additionally, a group of 118 unrelated Cameroonian individual living with sporadic NSHI of putative genetic origin (Table 5.1) were recruited, to investigate the frequencies of pathogenic variants that could be found. All hearing impaired family members were previously investigated for variants in *GJB2* (through direct sequencing of the entire coding region of *GJB2*), and the *GJB6*-D13S1830 deletion (using a multiplex polymerase chain reaction), and were negative [317].

A total of 122 ethno-linguistically matched Cameroonian controls without personal of familial history of HI were randomly recruited among blood donor at Central Hospital Yaounde, Cameroon.

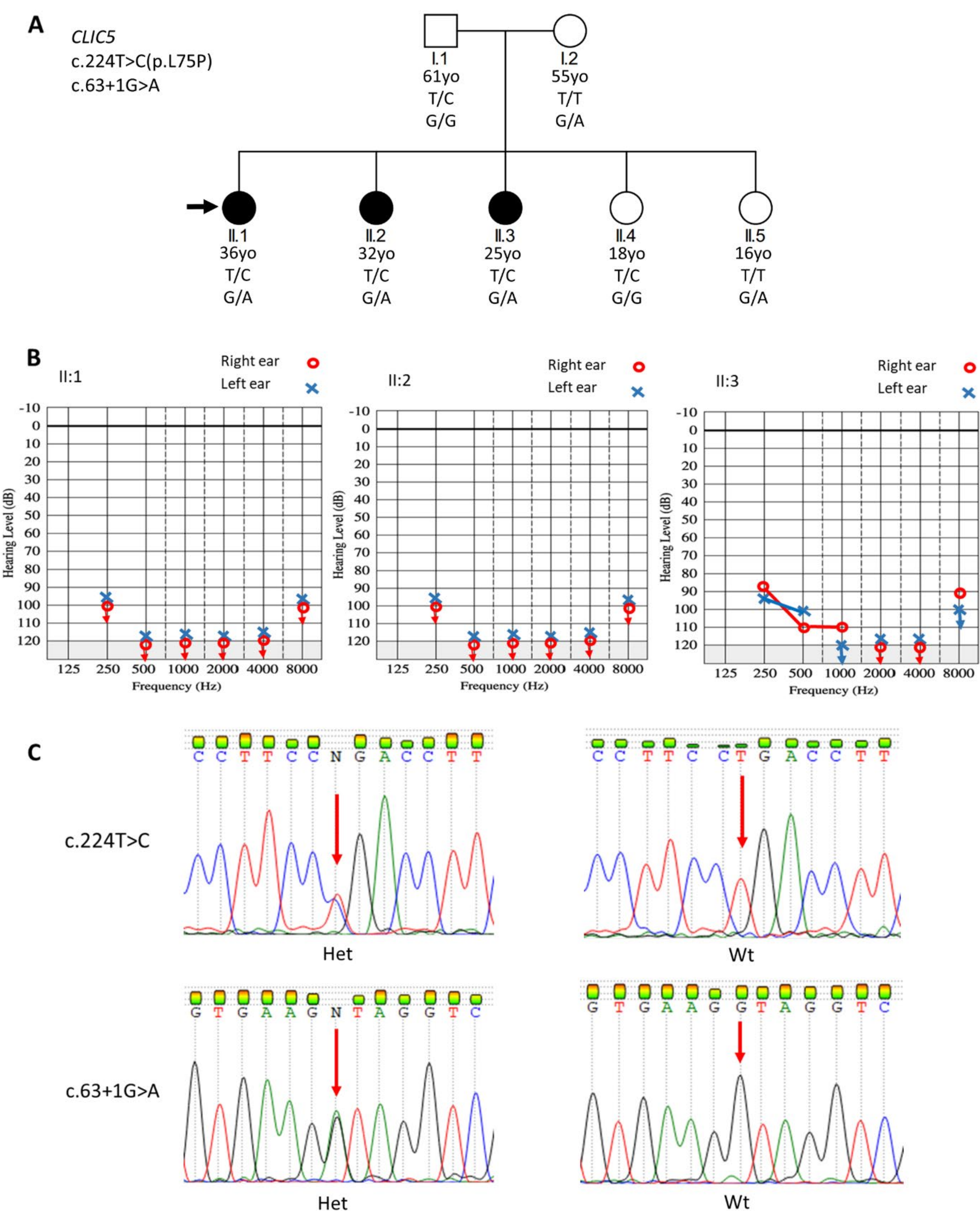

Figure 5.1: Pedigree of the non-consanguineous family, audiological phenotypes, and electropherogram data of the pathogenic variants in CLIC5. (A) The pedigree is suggestive of an autosomal recessive mode of inheritance. The missense CLIC5 variant (NM_016929.5:c.224T>C) and the splicing CLIC5 variant

(NM_016929.5:c.63+1G>A) variants co-segregated with HI, as compound heterozygous. The black arrow indicates the proband. (B) Air conduction of the pure tone audiometry performed for hearing impaired family members. Participants II.1, II.2, and II.3 presented with a bilateral profound HI. (C) Sanger sequencing chromatograms, showing the reference and the alternate alleles of both the missense and the splicing variants. The red arrows indicate the nucleotides affected by the variants. Het, heterozygous for the variant allele; Wt, wild type (homozygous for the reference allele); yo, years old.

5.1.2.3. Whole Exome Sequencing and Data Analysis

DNA samples from five family members were exome sequenced at Omega Bioservices (Norcross, GA, USA); these samples were obtained from two affected individuals (Figure 5.1A, II.1, and II.3), their parents (I.1, and I.2), and one unaffected sibling (II.4). Library preparation was performed with an Illumina Nextera Rapid Capture Exome kit® (Illumina, San Diego, CA, USA) following the manufacturer's instructions, and the resulting libraries were hybridized with a 37Mb probe pool to enrich exome sequences. Sequencing was performed on an Illumina HiSeq 2500 sequencer using the pair-end 150bp run format. Sequencing data were processed using the Illumina DRAGEN Germline Pipeline v3.2.8. Briefly, high-quality reads were aligned to the human reference genome GRCh37/hg19 using the DRAGEN software version 05.021.408.3.4.12, and after sorting and duplicate marking, variants were called, and individual gvcf files were generated. Joint single nucleotide variant (SNV) and Insertion/Deletion (Indel) variant calling was performed using the genome analysis toolkit (GATK) software v4.0.6.0 [320]. Sex of each individual was verified using plinkv1.9 [321]. Familial relationships for all members were verified via Identity-by-Descent sharing (plinkv1.9) and the Kinship-based INference for Gwas (KING) algorithm [321,322].

5.1.2.4. Annotation and Filtering Strategy

Variants were annotated and filtered using ANNOVAR [323] and custom scripts. Variants were first prioritized based on the inheritance model, considering both AR and autosomal dominant (AD) modes of inheritance. Subsequently, rare variants with a minor allele frequency (MAF) < 0.005 (for AR) and < 0.0005 (for AD) in all populations of the genome aggregation database (gnomAD) were retained. Known pathogenic HI variants listed in ClinVar were also retained regardless of their frequencies. dbNSFP v3.0 was used to annotate with 17 bioinformatic tools to predict the deleterious effects of the identified variants [324]. Coding variants were evaluated using SIFT, polymorphism phenotyping v2 (PolyPhen-2) x 2, MutationAssessor, the likelihood ratio test (LRT), Mendelian clinically applicable pathogenicity (M-CAP) score, REVEL, MutPred, protein variation effect analyzer (PROVEAN), MetaSVM, and MetaLR, while MutationTaster, Eigen, Eigen-PC, functional analysis through Hidden Markov models (FATHMM-MKL), combined annotation dependent depletion (CADD) score, and deleterious

annotation of genetic variants using neural networks (DANN) score were used to annotate both coding and non-coding variants [324].

Adaptive boosting (ADA) and random forest (RF) scores derived from dbscSNV v1.1 were used to predict the deleterious effect of variants within splicing consensus regions (−3 to +8 at the 5' splice site and −12 to +2 at the 3' splice site) [324,325]. We used phyloP, Genomic Evolutionary Rate Profiling (GERP), SiPhy, and phastCons scores to estimate the evolutionary conservation of the nucleotides and amino acid (aa) residues at which the variants occurred [324,326,327]. The hereditary hearing loss homepage (HHL), online Mendelian inheritance in man (OMIM), human phenotype ontology (HPO), and ClinVar databases were used to determine if there were any existing association between the identified variants and genes and HI. Candidate variants were considered when: 1) they occurred in known HI genes (and genes expressed in the inner ear); 2) have a predicted effect on protein function or pre-mRNA splicing (nonsense, missense, start-loss, frameshift, splicing, start-loss, etc.); and 3) they co-segregated with the HI phenotype within the family.

5.1.2.5. Sanger Sequencing

Sanger sequencing was performed for all the available family members (I.1, I.2, II.1, II.2, II.3, II.4, and II.5; Figure 5.1A), and from Cameroon 118 unrelated sporadic NSHI cases (Table 5.1) and 122 apparently healthy controls that were previously recruited as blood donors at Yaoundé Central Hospital. Primers to target our variants of interest in exon3 (forward 5'-GAAGGAACATACTGGGGCGA-3'; reverse 5'-AGCGCATTTTTGTTAGGCAGA-3') and at the exon1-intron1 boundary (forward 5'-CTCTGAGCGAAAGAGAGAAAGAG-3'; reverse 5'-ACTTGTTGCTCCCACGACC-3') of *CLIC5* gene were validated using NCBI BLAST. The optimal annealing and extension temperatures for the PCR were 60 °C and 70 °C for 30 s and 1 min, respectively. PCR-amplified DNA products were Sanger sequenced using a BigDye™ Terminator v3.1 Cycle Sequencing Kit and an ABI 3130XL Genetic Analyzer® (Applied Biosystems, Foster City, CA, USA) in the Division of Human Genetics, University of Cape Town, South Africa. Sequencing chromatograms were manually checked using FinchTV v1.4.0, and aligned in UGENE v34.0 to the *CLIC5* reference sequence (ENSG00000112782; retrieved from Ensembl browser).

5.1.2.6. Evolutionary Conservation of Amino Acids and Secondary Structure Analysis

We performed a multiple sequence alignment (MSA) of human CLIC5 with non-human similar proteins to provide more evidence on the evolutionary conservation of the amino acid residue at which our candidate missense variant occurred. A PSI-BLAST search against the non-redundant protein database of CLIC5 was performed. Non-redundant, non-synthetic, CLIC5 proteins from all the different species in the 500 BLAST hits were manually retrieved as FASTA

files. The MSA was performed using CLUSTAL Omega v1.2.4 [328] and the MSA file was visualized using Jalview v2.10.5 [329]. Furthermore, PSIPRED v4.0 [330] and Swiss-Model [331] were used to assess the secondary structural features of both protein forms. Also, the InterPro [332] database was queried via the InterProScan web service [333] to identify domains and potential domain changes for both protein forms separately.

5.1.2.7. Protein Modelling

A 3D modeling was performed on the longest isoform of the *CLIC5* gene as following: a homology model of the longest isoform (410 amino acids) of wild-type and mutant *CLIC5* [NM_001114086.1: c.701T>C:p.(L234P)] were constructed using program MODELLER based on the available crystal structure of human chloride intracellular channel protein 5 (PDB ID: 6Y2H) as a template [334]. PYMOL viewer was used for structural visualization and image processing.

5.1.3. Results

5.1.3.1. Participants Phenotypes

A total of seven individuals from "Family 24" were recruited, including three affected individuals (II.1: 36 years old, II.2: 32 years old, and II.3: 25 years old), their parents (I.1: 61 years old, and I.2: 55 years old), and two unaffected siblings (II.4: 18 years old, and II.5: 16 years old) (Figure 5.1A). The most likely mode of inheritance for the NSHI is AR. From the medical history, no environmental factors were identified as a possible cause of HI, and no HI participant has a history of ophthalmological (blurred or distorted vision, photophobia, eye pain, etc.) or neurological (vertigo, dizziness, etc.) symptoms. Additionally, no vestibular, neurologic, or any other systemic abnormalities were detected by physical examination. A history of prelingual and progressive HI was described for all three affected pedigree members; however, before this study, no formal audiological assessment was performed for any of the family members. Audiological assessment of the three affected individuals revealed bilateral profound sensorineural HI (Figure 5.1B).

5.1.3.2. WES Identification of Candidate Gene and Variants

The average target region coverage was about 225X, with 96.30% of the target region being covered to a depth of 10X or more. After applying our various filtering criteria described in the methods section, two candidate variants were found to occur in a known HI gene (*CLIC5;* MIM:607293) and to co-segregate with HI phenotype. These two variants which occurred in a compound heterozygous state are missense variant NM_016929.5:c.224T>C, and splice-site variant NM_016929.5:c.63+1G>A. The NM_016929.5:c.224T>C variant leads to the substitution of a leucine by a proline amino acid residue at position 75

[NM_016929.5:p.(L75P)] and is predicted to be damaging by 16 of the 17 bioinformatics tools used (Table 5.2). The NM_016929.5:c.63+1G>A variant, which occurs in a canonical donor splice site was predicted damaging by most of the tools that can be used to evaluate non-coding variants, including MutationTaster, FATHMM-MKL, Eigen-PC, CADD, and DANN (Table 5.2). Both variants were predicted as occurring in conserved positions of the genome and were both absent from gnomAD, UK10K, Greater Middle East (GME) variome project, and dbSNP databases (Table 5.2). Based on human splice finder server (HSF v3.1) and NNSPLICE 0.9, the variant NM_016929.5:c.63+1G>A is predicted to break the consensus 5' donor site "AAGGTAGGT" (which is altered due to the variation "AAGATAGGT") and probably alter the splicing of the pre-mRNA. The NM_016929.5:c.63+1G>A variant might thus alter normal protein synthesis and function through various mechanisms. Based on the American college of medical genetics (ACMG) guidelines for the interpretation of sequence variants, both variants were classified as pathogenic (NM_016929.5:c.63+1G>A: PSV1, PP1-S, PM2, and PP3 and NM_016929.5:c.224T>C: PM2, PP3, PM3, PP1, and PP1-S) [335,336]. In addition to *CLIC5,* only the *CEP250* gene shows compound heterozygous synonymous variants that co-segregate with hearing impairment (Table 5.3), which was unlikely the cause of the disease.

5.1.3.3. Sanger Sequencing Confirmation of Variants

Sanger sequencing confirms these candidate variants and their co-segregation with HI phenotype (Figure 1A & C). The three affected individuals (II.1, II.2, and II.3) were compound heterozygous for both variants, the father (I.1) and an unaffected daughter (II.4) were heterozygous for the missense variant, and the mother (I.2) and the other unaffected daughter (II.5) were both heterozygous for the splice-site variant (Figure 5.1A). Neither of these variants was detected in 122 controls or 118 sporadic NSHI cases (Table 5.1) from Cameroon.

5.1.3.4. Analysis of the CLIC5 – NM_016929.5(CLIC5):p.(L75P) Variant on the Protein

Evolutionary Conservation of Amino Acids

The NCBI PSI-BLAST search of CLIC5 (NP_058625.2) against the non-redundant protein database found the variant position p.(L75P) to be highly conserved across all non-human species retrieved in the top 500 BLAST hits (Figure 5.2). As expected, there was substantial conservation across an extensive aa block (on which the variant resides) which forms the thioredoxin/GST-N-terminal binding domain. This was consistent with the GERP and PhyloP scores for conservation indicating a strong evolutionary and functional constraint on the region.

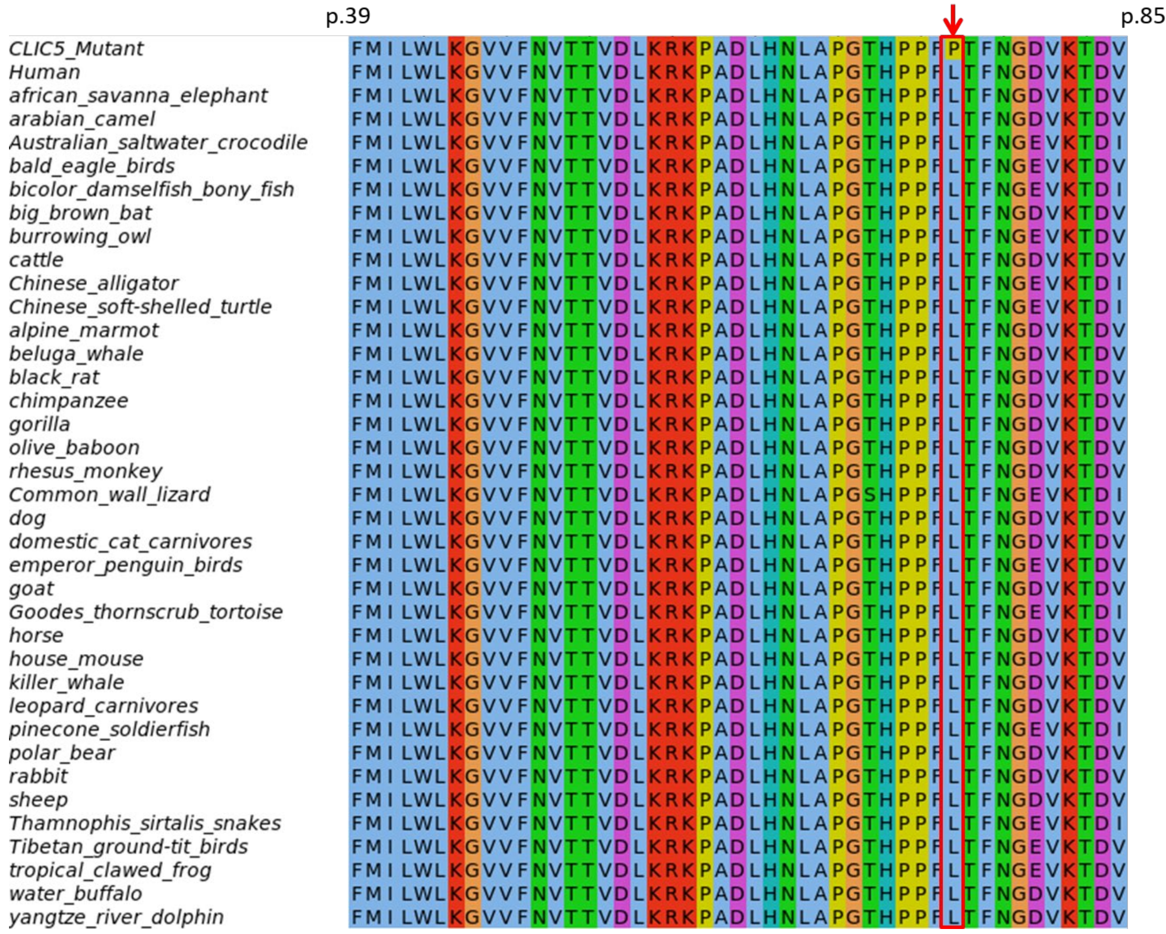

Figure 5.2: Evolutionary conservation of the CLIC5:p.(L75P) variant position (indicated by the red arrow).

Protein Modelling: Secondary Structure Analysis and Domain Search

A significant attenuation of the protein secondary structural features was predicted for the NM_016929.5(*CLIC5*):p.(L75P) variant using the PSIPRED v4.0 server whereby; there was abolishment of the β4 strand (Figure 5.3, and Figure 5.4 red box) and multiple changes affecting the lengths of β strands and several helices were inflicted (Figure 5.4 black boxes). Using Swiss-Model, a similar distortion in the secondary structure of the mutant protein was observed; shortening of the β4 strand although no β-strand loss was apparent. A domain search by InterProScan (InterPro v80.0) predicted the loss of the N-terminal GST domain due to the variant (Figure 5.5). This domain loss was also predicted to lead to abrogation of CLIC5's protein binding function (GO:0005515). Model parameters were refined and showed improvement in model qualities (Table 5.4).

Last, we performed 3D modelling of the wild-type and mutant long isoform of CLIC5 (Figure 5.3). The NM_016929.5:c.224T>C missense variant is located in a β-sheet in the extracellular domain of the long isoform of CLIC5 [NM_001114086.1:c.701T>C:p.(L234P)] (Figure 5.3c). We found that there was a local perturbation in the hydrophobic interaction of nearby residues

at position 234 of the CLIC5 protein (Figure 5.3d, f). Pro234 affects the shortness of the nearby β-sheet conformation in the mutant protein as shown in figure 3f. There was also a difference observed on the surface charges distribution between wild-type and mutant (Figure 5.3e, g).

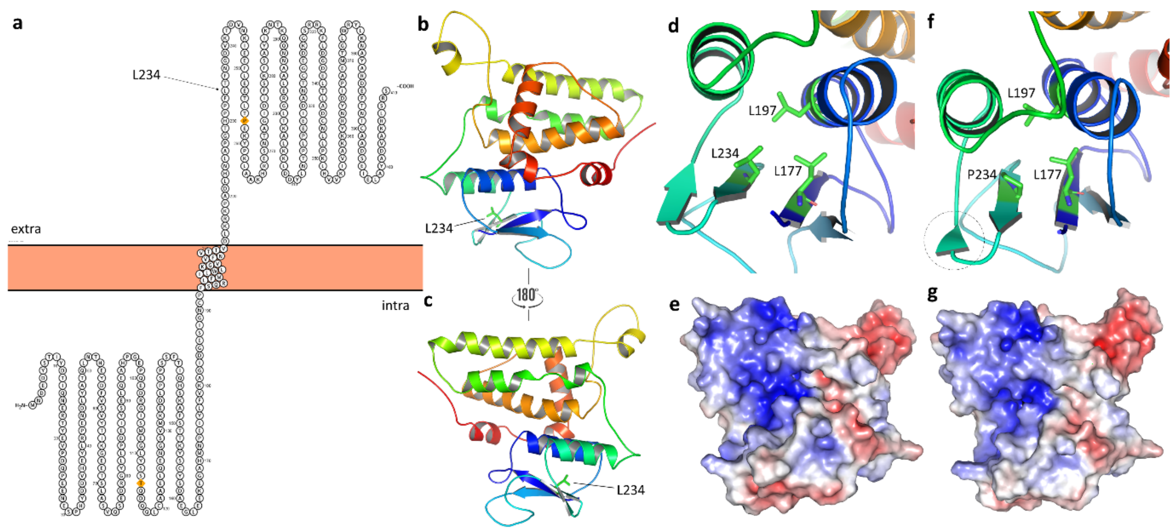

Figure 5.3: The residue Leu234 of NM_001114086.1:c.701T>C:p.(L234P), representing the long isoform of missense variant NM_016929.5:c.224T>C:p.(L75P) is located in the extracellular domain of the CLIC5 protein. (b, c) The overall structure of CLIC5 and the Leu234 residue (represented by a stick model). (d) Close-up view of the interaction pattern at position 234 of wild-type and mutant protein (f). Due to the mutation the shortness of β-strand observed in the mutant protein was highlighted by a dotted-circle. (e) The surface charge distribution of wild and (g) mutant CLIC5. Intra: intracellular; extra: extracellular.

5.1.4. Discussion

This study is, to our knowledge, the first report highlighting the association of HI with *CLIC5* variants in individuals of African ancestry, and the second to demonstrate this association globally. Thus, the data confirms *CLIC5* as a novel HI gene. Both pathogenic variants reported are novel: (NM_016929.5:c.224T>C) and splicing (NM_016929.5:c.63+1G>A), and were not found in 118 unrelated sporadic cases of NSHI cases, reinforcing the genetic and locus heterogeneity nature of HI, and the importance of investigating diverse populations, particularly the understudied African populations, that will help to enhance and refine HI disease-gene curation. The contribution of *CLIC5* to NSHI in humans was first described with the identification of a homozygous nonsense variant [NM_016929.5:c.96T>A; p.(Cys32Ter)] that abrogates the protein function and co-segregates with ARNSHI in a Turkish family [318]. The two affected individuals from the aforementioned Turkish family presented an early onset sensorineural HI which started mildly and progressed to severe-to-profound HI. This HI

phenotype is similar to that described in the present study, as our three affected participants described a history of prelingual HI, and presented with profound sensorineural HI at the time of the study [318]. The corresponding mutant mice model (*jbg* mice), which has a deletion in the *CLIC5* mice ortholog gene, resulting in impaired hearing and vestibular dysfunction [319]. *CLIC5* was also studied in 69 unrelated Spanish and 50 mainly Dutch patients with ARNSHI and no PLP variants were identified [318]. In the present study, we did not found any clinical evidence of vestibular or renal dysfunctions, unlike what was previously reported in the Turkish family [318], as well as in the corresponding mutant mice model (*jbg* mice) that were also shown to have abnormalities in the foot processes of the kidney podocytes leading to proteinuria [337,338]. Biological exploration of the kidney functions of Cameroonian affected individuals with PLP in *CLIC5* should be performed. In addition to the inner ear and kidney abnormalities, the jbg-mutant mice also exhibit emphysema-like lung pathology, hyperactivity, and gastric haemorrhage [318,339]. Additional studies on more families and populations worldwide are needed to refine the phenotype of *CLIC5*-induced HI in humans.

CLIC5 (mapped on 6p21.1 locus) encodes a protein that belongs to the chloride intracellular ion channels (CLICs) family [340]. The encoded protein (CLIC5) was shown to be highly expressed in the inner ear, and important for sensorineural hearing [319]. CLIC5 protein associates with actin-based cytoskeletal structures and may play a role in multiple processes, including hair cell stereocilia formation [319]. The main function of CLIC5A in the ear is the stabilization of membrane-actin filament linkages at the base of hair cell stereocilia [319]. Therefore, a variant that abrogates CLIC5A or destabilizes its activity would lead to destabilization of actin-based complexes, fusion, and elongation of hair cell stereocilia, and consequently impaired hearing [318,341]. The missense NM_016929.5(*CLIC5*):p.(L75P) variant, reported in this study is predicted to lead to the loss of the N-terminal GST domain. This is in turn expected to abrogate CLIC5's protein binding function (GO:0005515) and is therefore likely to affect binding to ERM proteins. Interaction of CLIC5 with actin-based cytoskeleton is dependent upon its protein-protein interaction with ERM proteins [341].

There are three isoforms of CLIC5 [342]: The canonical isoform CLIC5B (410aa), CLIC5A (251aa) and CLIC5C (205aa). All three isoforms show evidence of expression in the human inner ear, of which CLIC5A shows the highest expression (251aa) [343]. The splice site variant we identified in this study is predicted to affect two of these three isoforms [NM_016929.5:c.63+1G>A (251 aa; CLIC5A); NM_001256023.1:c.63+1G>A (205 aa; CLIC5C)], including isoform CLIC5A. This splice site variant is located at the 5' donor canonical splice site of exon 1 of these two isoform transcripts (position +1) and predicted to lead to a loss of the consensus 5' donor site. The missense variant reported in this study

[NM_016929.5: p.(L75P)] is predicted to affect all three isoforms of CLIC5 as a missense change.

Although the identified variants in the present study are predicted to be pathogenic (Table 5.2), and to affect the structure and the function of the protein (Figures 5.3-5.5), more studies in other populations, will likely inform and strengthened the HI disease gene-pair curation, globally, as illustrated with this case report.

5.1.5. Conclusions

We identified bi-allelic novel compound heterozygous pathogenic variants in *CLIC5* (MIM:607293), the missense [NM_016929.5:c.224T>C; p.(L75P)] and the splicing (NM_016929.5:c.63+1G>A), that co-segregated with non-syndromic autosomal recessive hearing impairment in three affected members of a non-consanguineous family from Cameroon. This study is the second report, worldwide, to describe the *CLIC5* -HI gene-disease pair in humans, and thus confirm *CLIC5* as a novel NSHI that should be included in targeted diagnostic genes panels. Our study emphasizes the urgent need of using WES to investigate hearing impairment in understudied African populations, in order to improve our understanding of hearing pathobiology.

Author Contributions: Conception of the project: A.W, SML; recruitment and molecular experiments: E.W.T.; exclusion of *GJB2* and *GJB6* variants: E.W.T. and S.M.A.; bioinformatics analysis: I.S., T.B., L.M.N.S., A.A.; in silico analysis of the pathogenicity of variants: E.W.T., I.S., K.K.E.; protein modelling: K.K.E., A.N.; issue of the first draft of the manuscript: E.W.T.; review and editing: all authors; supervision of the whole project: S.M.L. and A.W. All authors have agreed to the final version of the manuscript.

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Conflicts of Interest: The authors declare no competing interest.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1:

Table 5.1: Demographic and clinical characteristics of isolated NSHI cases screened for the identified *CLIC5* pathogenic variants. Mean age = 10.92 ± 4.84 (3 – 31) years.

Categories	**n/N**	**Frequency (%)**
Age range (years)		
<10	51/118	43.22
10 – 20	61/118	51.69
>20	6/118	5.08
Gender		
Male	63/118	53.39
Female	55/118	46.61
Ethnic group		
Bamileke (Semi-bantu)	49/118	41.53
Beti-fang (Bantu)	34/118	28.81
Bassa (Bantu)	11/118	9.32
Bamun (Semi-bantu)	9/118	7.63
Duala (Bantu)	7/118	5.93
Fulani (Sudanese)	4/118	3.39
Tikar (Semi-bantu)	3/118	2.54
Mbo (Bantu)	1/118	0.85
Age of onset		
Congenital/Prelingual (before 2 years old)	107/118	90.68
Perilingual (between 2 and 4 years)	9/118	7.63
Postlingual (after 4 years)	2/118	1.69
Degree of hearing impairment		
Moderate II	1/65	1.54
Severe I	4/65	6.15
Severe II	4/65	6.15
Profound I	14/65	21.54
Profound II	19/65	29.23
Profound III	12/65	18.46
Total	11/65	16.92
Type of hearing impairment		
Sensorineural	58/65	89.23
Mixed	7/65	10.77
Bilateral	65/65	100.00

Table 5.2: Description of pathogenic variants identified in *CLIC5*

	c.224T>C [p.(L75P)]	**c.63+1G>A**
Predicted effect	Missense	Splicing
Frequency in gnomAD	Absent	Absent
Frequency in UK10K	Absent	Absent
Frequency in GME	Absent	Absent
dbSNP rs number	Absent	Absent
GERP	5.73	4.72
PhyloP	9.29	4.49
PhastCons	1	1

SiPhy	15.30	12.06
SIFT	Damaging	NA
Polyphen2 HDIV	Probably damaging	NA
Polyphen2 HVAR	Probably damaging	NA
MutationAssessor	High	NA
LRT	Deleterious	NA
M-CAP	Damaging	NA
REVEL	Pathogenic	NA
MutPred	Pathogenic	NA
PROVEAN	Damaging	NA
MetaSVM	Damaging	NA
MetaLR	Tolerated	NA
MutationTaster	Disease causing	Disease causing
Eigen	Pathogenic	Benign
Eigen-PC	Pathogenic	Pathogenic
FATHMM-MKL	Damaging	Damaging
CADD	32	20.9
DANN	0.999	0.987
ACMG classification	Pathogenic	Pathogenic

NA, not applicable. RefSeq transcript used: NM_016929.5

Other variants that co-segregate with the hearing impairment within "Family 24"

Apart from *CLIC5*, only *CEP250* gene shows compound synonymous variants (i.e. NM_007186.5:c.1380T>C and NM_007186.5:c.1935C>T; Table 5.3) that co-segregate with hearing impairment, and was unlikely the cause of the disease. None of these synonymous variants is predicted to alter the splicing of the pre-mRNA. No homozygous variants segregate with the hearing impairment phenotype.

Table 5.3: Synonymous likely benign variants identified in *CEP250* gene

	c.1380T>C [p.(S460S)]	**c.1935C>T [p.(V645V)]**
Predicted effect	Synonymous	Synonymous
Frequency in gnomAD	0.0003	0.0005
Frequency in gnomAD_Afr	0.0009	0.0002
Frequency in GME	Absent	Absent
dbSNP rs number	rs142139756	rs370009858
ACMG classification	Likely benign	Likely benign

RefSeq used: NM_007186.5

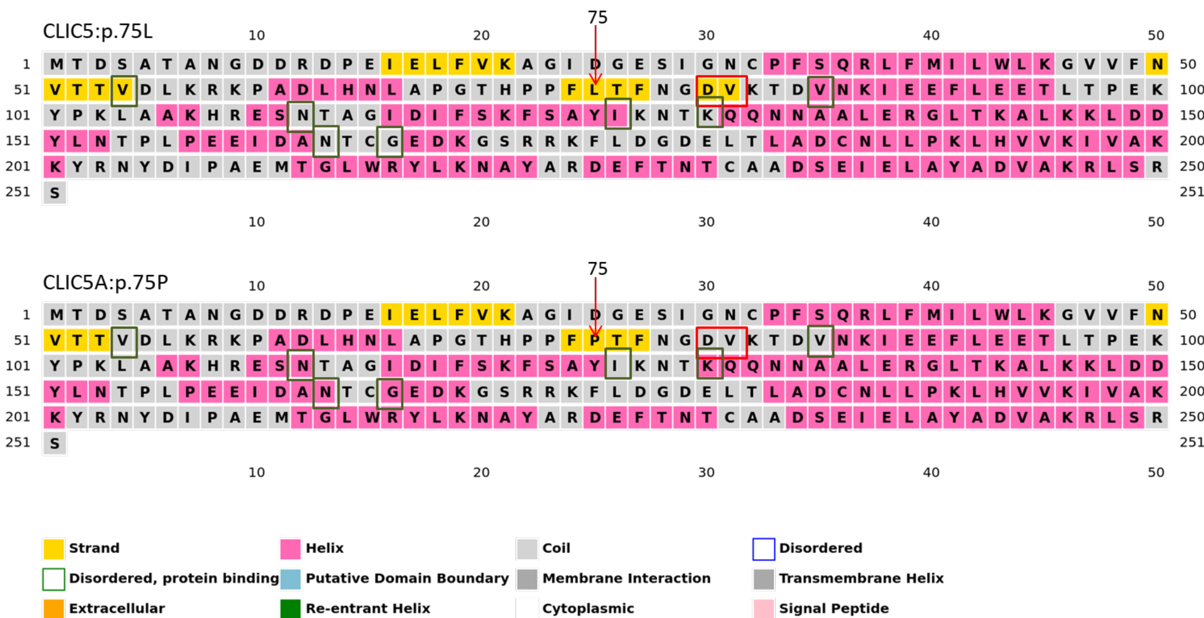

Figure 5.4: Secondary structure prediction of CLIC5 using the 251 amino acids isoform (NM_016929.5). Boxes indicate positions of difference between wild type (CLIC5A:p.75L) and mutant (CLIC5A:p.75P). Red boxes show loss of the fourth strand in the wild type while black boxes show changes in the lengths of strands and helices.

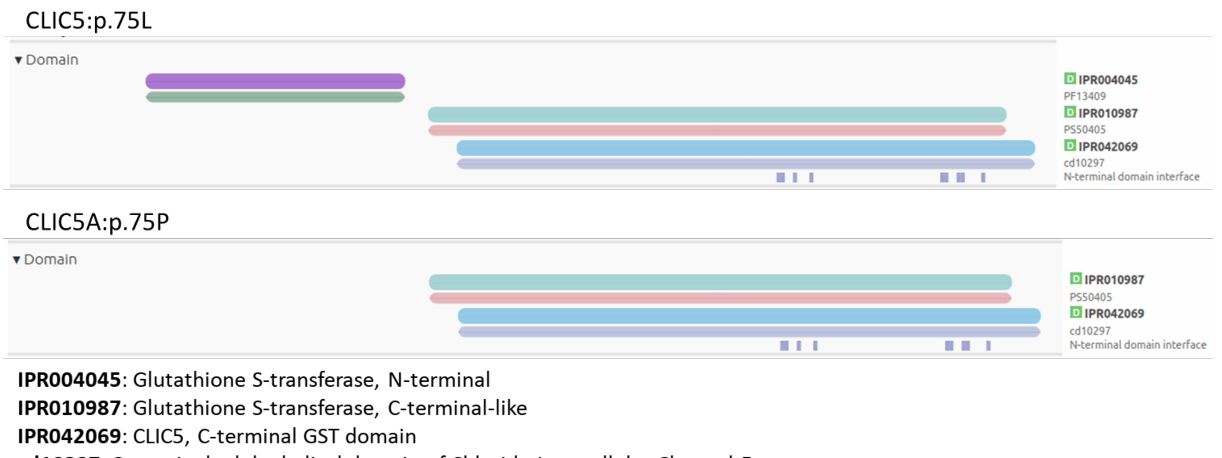

Figure 5.5: Domains of CLIC5A:p.75L (wild type) and CLIC5A:p.75P (mutant) predicted by InterPro, based on the 251 amino acids isoform (NM_016929.5). The GST N-terminal domain is lost in the mutant and its protein-binding activity is abolished.

CLIC5 physicochemical properties

Using ProtParam of the ExPASy Bioinformatics Resource Portal, CLIC5 wild type and mutant proteins were predicted to have similar physicochemical properties as expected including; pI

(5.70), molecular weight (MW = 28.2kDa) and extinction coefficients. However, they differed on instability classification. While the wild type protein was classified as stable (stability index = 39.34), the mutant protein was classified as unstable (stability index = 40.11). This was consistent with the I-MutantSuite (I-Mutant3.0) result which predicted the variant to impose a "Large Decrease" in protein stability.

CLIC5 Protein structure determination and analysis

Given the predicted secondary structural changes, it is conceivable that there would be a three dimensional (3D) structural change of the protein due to the mutation. Considering that the structure of CLIC5A has been recently solved (PDB: 6Y2H), we used a single template-based approach, utilizing this structure as template to model the structures of the wild type and mutant (CLIC5:pL75P) proteins. 6Y2H is a 236 amino acid (CLIC5A residues 16 - 251) X-ray crystallographic monomeric structure resolved to 2.15 angstroms with zero (0) Ramachandran outliers.

As expected, the global model quality estimation (GMQE) for the wild type (0.93) and mutant (0.93) models were comparable to the experimentally-determined structure (0.96; on a scale of 0 – 1) (Table 5.4). The model quality was generally better for the wild type as compared to the mutant, while both models received Swiss-Model "thumbs up" for QMEANs indicating high quality. Following refinement, a considerable increase in the quality of the models was achieved as was apparent by zero Ramachandran outliers and poor rotamers, lower MolProbity and clash scores, and high Ramachandran favored and favored rotamer scores. The relatively lower Galaxy energy for the wild type model indicates a higher stability as compared to the mutant model, consistent with earlier findings.

Table 5.4: Model parameters before and after refinement showing improvement in model qualities.

Parameter	Before Refinement		After Refinement	
	CLIC5A:p.75L	CLIC5A:p.75P	CLIC5A:p.75L	CLIC5A:p.75P
Galaxy energy	-	-	-6489.70	-6409.60
GMQE (0-1)	0.93	0.93	-	-
QMEAN (Goal: 0)	-1.45 (abs 1.45)	-1.53 (abs 1.53)	-	-
MolProbity (smaller is better)	0.85 (100th percentile)	1.02 (100th percentile)	0.50 (100th percentile)	0.75 (100th percentile)
Clash score (Goal: 0)	1.07 (99th percentile)	1.61 (99th percentile)	0 (100th percentile)	0.81 (99th percentile)
Ramachandran favored (Goal: >98%)	97.84%	97.41%	99.57%	98.28%
Poor rotamers (<0.3%)	0.50%	0.50%	0.00%	0.00%

Favored rotamers (>98%)	91.54%	92.04%	100%	99.50%
Ramachandran outliers (Goal: <0.05%)	0.43% (1)	0.86% (2)	0.00%	0.00%
Rama distribution Z-score (Goal: <2)	1.25 ± 0.53	1.22 ± 0.53	0.23 ± 0.50	0.31 ± 0.51
C-beta deviations (Goal: 0)	4	3	1	0
Bad bonds (Goal: 0%)	0.16%	0.16%	0.00%	0.00%
Bad angles (Goal: <0.1%)	0.90%	0.97%	0.47%	0.35%

GMQE, global model quality estimation; QMEAN, qualitative model energy analysis

Web Ressources

ANNOVAR	https://annovar.openbioinformatics.org/
Bureau international d'audiophonologie (BIAP)	https://www.biap.org/en/recommandations/recommendations/tc-02-classification
ClinVar	https://www.ncbi.nlm.nih.gov/clinvar/
dbNSFP (including dbscSNV)	https://sites.google.com/site/jpopgen/dbNSFP
dbSNP	https://www.ncbi.nlm.nih.gov/snp/
DRAGEN germline pipeline	https://emea.illumina.com/products/by-type/ informatics-products/basespace-sequence-hub/apps/edico-genome-inc-dragen-germline-pipeline.html
Ensembl	https://www.ensembl.org/index.html
Gene ontology (GO)	http://geneontology.org/
Genome aggregation database (gnomAD)	https://gnomad.broadinstitute.org/
Genome analysis toolkit (GATK)	https://gatk.broadinstitute.org/hc/en-us
Hereditary hearing loss homepage (HHL)	https://hereditaryhearingloss.org/
Human phenotype ontology (HPO)	https://hpo.jax.org/app/
Human splice finder (HSF)	https://hsf.genomnis.com/home

InterProScan	http://www.ebi.ac.uk/InterProScan/
MODELLER	http://www.salilab.org/modeller
NCBI-BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Online Mendelian inheritance in man (OMIM)	https://omim.org/
PDB	https://www.wwpdb.org/
PSIPRED	http://bioinf.cs.ucl.ac.uk/psipred/
PYMOL	http://www.pymol.org/
RefSeq	https://www.ncbi.nlm.nih.gov/refseq/
Swiss-Model	https://swissmodel.expasy.org/
Uniprot	https://www.uniprot.org/uniprot/Q9NZA1
UK10K	https://www.uk10k.org/
World Health Organisation	https://www.who.int/news-room/fact-sheets/ detail/deafness-and-hearing-loss

5.2. **Wonkam-Tingang, E.**, Schrauwen, I., Esoh, K. K., Bharadwaj, T., Nouel-Saied, L. M., Acharya, A., Nasir, A., Leal, S. M., & Wonkam, A. (2021). A novel variant in DXML2 gene is associated with autosomal dominant non-syndromic hearing impairment (DFNA71) in a Cameroonian family. Experimental Biology and Medicine, 153537022199974. https://doi.org/10.1177/1535370221999746.

Nature of Publication: Original Full Journal Article

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Candidate Contribution: Performed recruitment of participants, molecular experiments, in silico analysis of the identified candidate variant, and drafted the first version of the manuscript.

Co-Authors Contributions:

AW and **SML:** Conceived the project.

EWT: Performed the recruitment and molecular experiments.

IS, TB, LMNS, and **AA:** Performed the bioinformatics analysis.

KKE, EWT, AN, and **SM:** Performed the *in silico* analysis of the identified variant.

EWT: Issued the first draft of the manuscript and all the authors.

SML and **AW:** Supervised the entire project.

EWT, IS, KKE, TB, LMNS, AA, AN, SML, and **AW:** Reviewed and edited the manuscript, and read and agreed to the final version of the paper.

A novel variant in *DXML2* gene is associated with Autosomal Dominant Non-Syndromic Hearing Impairment (DFNA71) in a Cameroonian Family.

Short running title: *DMXL2* gene and Hearing Impairment

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Abstract

Approximately half of congenital hearing impairment (HI) cases are inherited, with non-syndromic HI (NSHI) being the most frequent clinical entity of genetic HI cases. A family from Cameroon with NSHI was investigated by performing exome sequencing using DNA samples obtained from three family members, followed by direct Sanger sequencing in additional family members and controls participants. We identified an autosomal dominantly inherited novel missense variant [NM_001174116.2:c.918G>T; p.(Q306H)] in *DMXL2* gene (MIM:612186) that co-segregates with mild to profound non-syndromic sensorineural HI. The p.(Q306H) variant which substitutes a highly conserved glutamine residue is predicted deleterious by various bioinformatics tools and is absent from several genome databases. This variant was also neither found in 121 apparently healthy controls without a family history of HI, nor 112 sporadic NSHI cases from Cameroon. There is one previous report of a large Han Chinese NSHI family that segregates a missense variant in *DMXL2*. The present study provides additional evidence that *DMXL2* is involved in HI aetiology, and we suggest *DMXL2* should be considered in diagnostic HI panels.

Keywords: non-syndromic hearing impairment; autosomal dominant inheritance; *DMXL2*; Africa

Impact Statement:

Prior to the present study, only one report from China has showed an association between a variant in *DMXL2* and HI in humans. Our study implicates for the first time the *DMXL2* gene in NSHI in Africans, and thus also confirms the contribution of *DMXL2* to HI in humans. The genetics of HI in Africans is not well elucidated beyond the study of pathogenic or likely pathogenic variants in connexin genes that were shown to be infrequent in most African populations. The present study therefore enriches the list of HI genes in Africans.

5.2.1. Introduction

Hearing impairment (HI) is, globally, a significant public health concern, with higher burden in lower income countries [3]. Approximately 3.6% of the general population and 14.8% of adults ≥50 years of age in sub-Saharan Africa live with disabling HI. [316] Identifying the underlying cause of HI is critical, as it allows for targeted therapeutic decision making [344]. About 30 to 50% of congenital cases of HI in sub-Saharan Africa are inherited, with non-syndromic HI (NSHI) representing approximately 86.1% to 92.5% of all genetic HI cases [5,316]. NSHI is mainly inherited in an autosomal recessive (AR) manner (~80% of cases), while autosomal dominant (AD) inheritance is less frequent but not negligible, as it accounts for ~18% of NSHI cases [8].

The genetics NSHI in Africans is unclear. Pathogenic variants in *GJB2* which constitute the major aetiology of genetic HI in Asian and European populations were found to be rare in Africans [345]. HI is highly genetically heterogeneous, as about 121 genes and 170 loci have been described to date (Hereditary Hearing Loss Homepage). However, targeted exome sequencing has also demonstrated a lower identification rate of pathogenic and likely pathogenic (PLP) variants in > 100 HI genes, amongst sporadic HI cases in populations of African ancestry i.e. Black South Africans, African Americans, and Nigerians, compared to Asians and Europeans [19,20]. Although, the HI PLP detection rate was highly improved with the targeted selection of multiplex families segregating NSHI, from Cameroon [58]. Moreover, the prevalence of PLP variants in AR NSHI genes, based on gnomAD, and selected from Deafness Variation and ClinVar databases [22], was evaluated at 5.2 per 100,000 persons for Africans or African Americans, which is lower than the 96.9 per 100,000 persons prevalence for Ashkenazi Jews [23,346]. Because of this low contribution of known variants in AR NSHI genes in African populations, next-generation sequencing (NGS) techniques have a high potential of identifying novel PLP variants, and novel HI genes in populations of African descent as was demonstrated for other populations [95–97].

In the present study, we have used whole exome sequencing (WES) and identified a variant in *DMXL2* (DFNA71; MIM:617605) in a multiplex family from the Bamileke tribe from

Cameroon, segregating autosomal dominant NSHI. We strengthen the evidence that *DMXL2* contributes to HI, as there is only a single report of a missense *DMXL2* variant segregating with NSHI in a large Han Chinese family [347].

5.2.2. Materials and Methods

5.2.2.1. Participants' Recruitment

Patients' recruitment procedure has previously been described [6]. Briefly, the proband of a Cameroonian family segregating HI (Family 23, Figure 5.6A) was selected via a school for HI individuals, and the other family members were recruited thereafter. Medical records of all our participants were analysed by a general practitioner, an ENT specialist, and a medical geneticist, and detailed personal and family histories were obtained through a rigorous clinical interview. For all HI participants, in addition to a systemic general examination, an otological assessment including pure tone audiometry (PTA) was performed. To classify hearing levels, we used recommendations from the Bureau International d'Audiophonologie (BIAP), Belgium [317]. Isolation of genomic DNA from peripheral blood was done within the division of Human Genetics at the University of Cape Town in South Africa, using the chemagic extraction protocol. All hearing-impaired participants were shown to not carry PLP variants in *GJB2* gene or the "*GJB6*-D13S1830" deletion, and the results were previously published [317].

Additionally, a total of 112 Cameroonian patients with isolated NSHI cases of presumed genetic aetiology (described in Table 5.5) were included in order to explore the frequency of the novel *DMXL2* variant in this group. Moreover, a total of 121 apparently healthy controls individuals from Cameroon, without a family history of HI, were chosen from randomly selected blood donors at Yaoundé Central Hospital and tested for the identified variant.

5.2.2.2. Whole Exome Sequencing and Data Analyses

WES was executed for DNA samples from two affected (I.2, and II.4) and one unaffected (II.3) (Figure 5.6A) members from Family 23 at Omega Bioservices (Norcross, GA, USA) company. Illumina instructions were followed to prepared samples, and an Illumina sequencer was used for the sequencing using 150bp pair-end reads. Low-quality reads were removed, and Burrows–Wheeler Aligner-MEM software (BWAv0.7.15) was used to align the filtered reads to the human reference genome hg19 [4,348]. After sorting and marking duplicate reads, the genome analysis toolkit (GATK) v4.0.6.0 was used to call single nucleotide variants (SNVs) and insertions/deletion (InDel) implementing base quality score recalibration [320]. Plink v1.9 was used to check the sex of each individual [321]. Via identify-by-descent sharing (plink v1.9) and the KING algorithm [321,322], familial relationships were verified.

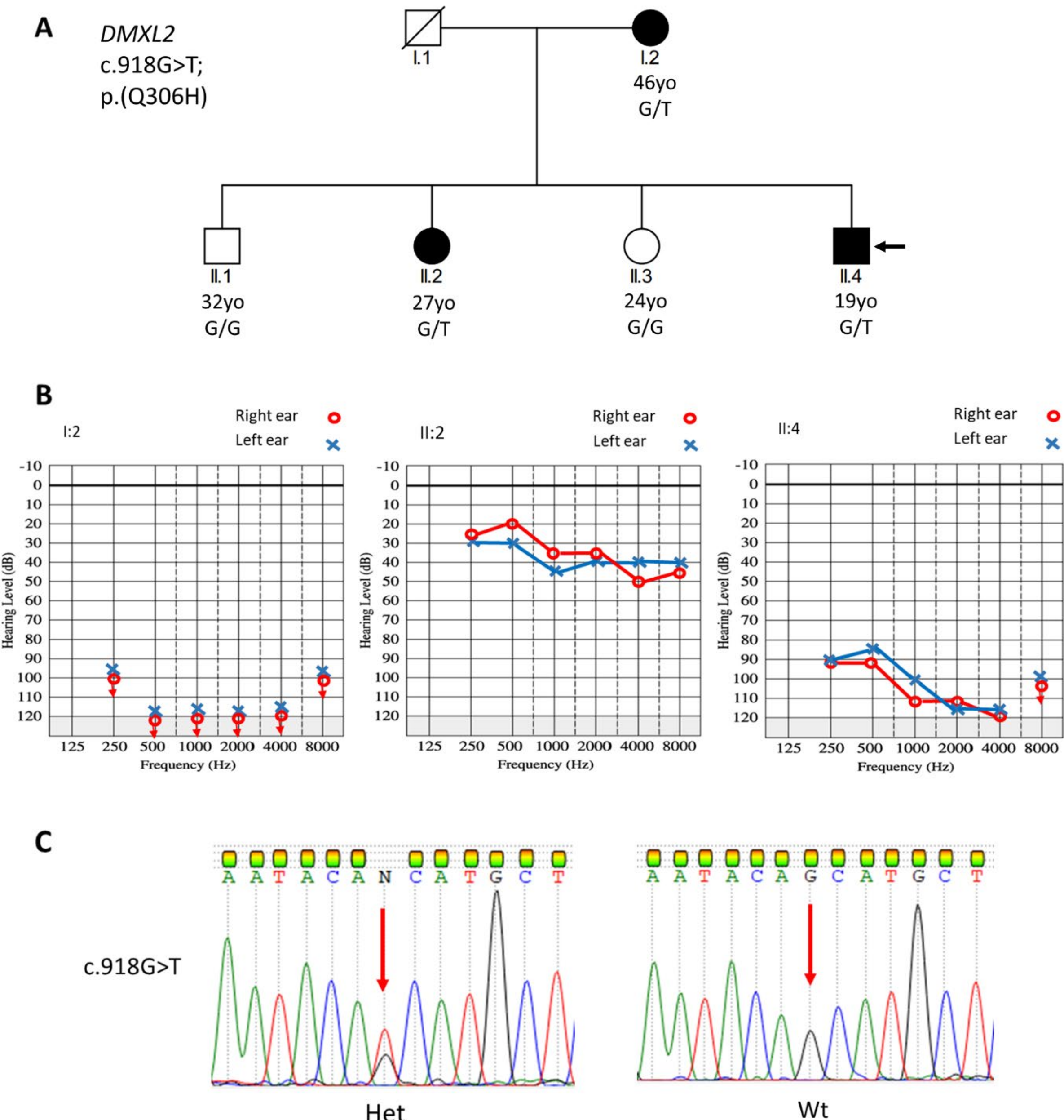

Figure 5.6: Pedigree, audiometry results, and sequencing chromatograms of family 23. (**A**) The family tree and co-segregation of the DMXL2 variant NM_015263.5:c.918G>T is compatible with AD mode of inheritance of the HI within the family. The proband is designated by the black arrow. (**B**) Pure tone audiometry (air conduction) of the three affected individuals, showing a bilateral profound hearing loss for the mother (I.2), a bilateral mild HI for child II.2, and bilateral profound HI for child II.4. (**C**) Sequencing chromatograms displaying the wild type and variant alleles. The red arrow indicates the nucleotide where the variant occurs. Het, heterozygous for the alternate allele; Wt, homozygous for the wild type; yo, years old.

5.2.2.3. Annotation and Filtering Strategy

ANNOVAR was used for filtering and variant annotation, [323] following methods described previously [4]. In brief for the analysis, 1) The AD mode of inheritance was considered; 2) Exonic and splice site variants were selected; 3) We prioritized variants with an expected effect

on pre-mRNA splicing or protein function (missense, start-loss, frameshift, splice site, nonsense etc.) with a minor allele frequency (MAF) of <0.0005 in all populations of the gnomAD database; and 4) Bioinformatics prediction scores were retrieved from dbNSFP and dbscSNV to assess the deleterious effect of missense and splicing variants respectively, including polymorphism phenotyping v2 (PolyPhen-2), SIFT, MutationTaster, deleterious annotation of genetic variants using neural networks (DANN), combined annotation dependent depletion (CADD), and Genomic Evolutionary Rate Profiling (GERP++) scores [349–354]. Previously reported association between our candidate variants/genes and HI was obtained from Human Phenotype Ontology (HPO) ClinVar, Online Mendelian Inheritance in Man (OMIM), and Hereditary Hearing Loss Homepage (HHL) databases, and genes expressed in the inner ear or genes known to be implicated in human/animal HI were retained [355]. The presence of known pathogenic HI variants from the ClinVar database was also assessed without any MAF cut-off.

5.2.2.4. Sanger Sequencing

The candidate variant obtained from filtering was validated using direct sequencing and its co-segregation with HI phenotype amongst the other family members that were available (I.2, II.1, II.2, II.3, and II.4; Figure 5.6A) was also evaluated. Additionally, a total of 112 individuals with sporadic NSHI of putative genetic origin, and 121 apparently healthy control individuals from Cameroon, without a family history of HI, were screened for the candidate variant. Primers to target our variant of interest in exon 8 (forward 5’-TCCAAAGCAGTTCATTTGTGTCT-3’; reverse 5’-CTGTGAACATCATAAGAACCGGG-3’) of *DXML2* gene were assessed through NCBI BLAST. PCR and Sanger sequencing reactions were both performed using the aforementioned primers within the Division of Human Genetics at the University of Cape Town in South Africa. FinchTV v1.4.0 software was used to manually check sequencing chromatograms, and the latter were aligned to the *DMXL2* reference sequence (ENSG00000104093; obtained through Ensembl server) using UGENE v34.0.

5.2.2.5. Conservation of Amino Acids and Protein Modelling Analyses

To assess how conserved the amino acid affected by the p.(Q306H) missense variant is, a multiple sequence alignment (MSA) of human DMXL2 with comparable proteins was performed. A BLASTp search (using the PSI-BLAST algorithm) [356] of the mutant protein against the non-redundant protein sequence database was realised, and the first isoform of the hits from each taxonomic group was retrieved for MSA. The MSA was performed with CLUSTAL O v1.2.4 [328] using custom scripts and the sequence trimmed to retain residues around the position of interest without gaps. Jalview v2.10.5 [329] was then used to visualize

the alignment. Additionally, the secondary structure of DMXL2 wild type and mutant proteins was predicted by using PSIPRED v4.0 [330].

The crystal structure of human DMXL2 protein was not available in the protein data bank (PDB) [357]. As such, comparative modelling methods were used to build the three-dimensional (3D) structure of human DMXL2. We obtained the amino acid sequence of DMXL2 through the NCBI database, and executed a pBlast search against PDB. We first built the homology model of the wild type using the PDB structure 2YMU (a highly repetitive propeller structure) as a template, and subsequently constructed the structure of the mutant by mutating the targeted residue using MODELLER [358]. PYMOL viewer was used to visualise the protein structure.

5.2.3. Results

5.2.3.1. Study Subjects Phenotypes

In this family, there was no history of ototoxic treatment, severe ear infections, head trauma, neonatal asphyxia, or was any other environmental factor identified as a possible cause of HI for any of the three HI members (I.2, II.2, and II.4; Figure 5.6A). None of them had a history of ophthalmological or neurological symptoms, and the physical examination did not reveal any vestibular, neurologic, or other systemic abnormalities. The segregation of NSHI for family 23 was compatible with an AD mode of inheritance, and both parents were unrelated (Figure 5.6A). Before our study, no formal otological evaluation was done for any of the HI individuals. The audiometry testing performed at the time of this study revealed that HI is bilateral and sensorineural for the three affected individuals. The mother (I.2) experienced prelingual and progressive HI in the past, and during the present recruitment, PTA revealed profound HI (Figure 5.6B). In one of the two affected children (II.4), HI was congenital, and the otological assessment at the time of the recruitment revealed a profound HI phenotype (Figure 5.6B). The other affected child (II.2), did not report any HI, until audiometric assessment for this study revealed a mild HI (Figure 5.6B).

5.2.3.2. Whole Exome sequencing and Identification of the Candidate Variant

WES was performed using DNA samples from three individuals; the average sequencing depth of the targeted region was 226X (I.2), 209X (II.3), 236X (II.4), and the fraction of the targeted region covered >10X was 96.27%. The mean base quality score across the samples was 36, implying high base call accuracy (>99.9%), while the mean ratio of reads that were mapped out of the target region (off-target reads) to the ratio of reads mapped unto the target region (on-target reads) was 2.7% (0.027), indicating high capture quality of the kit. Our filtering strategy described in the methods section identified a mono-allelic missense variant

[NM_015263.5:c.918G>T; p.(Q306H)] in *DMXL2*. This variant which leads to the substitution of glutamine by a histidine residue [NM_015263.5:p.(Q306H)] and co-segregates with HI following an AD mode of inheritance is expected to be damaging by MutationTaster (disease-causing, score: 0.937), Polyphen2 HDIV (possibly damaging, score: 0.895), CADD (score: 20.9), and DANN (score: 0.941). The variant is absent from gnomAD, trans-omics for precision medicine (TOPMed), Greater Middle East (GME) variome project, UK10K, and dbSNP databases, and 121 apparently healthy control individuals from Cameroon.

Sanger sequencing confirmed the occurrence of the variant in the heterozygous state in the affected mother (I.2) and children (II.2 and II.4), while it was absent in unaffected children (II.1 and II.3) (Figure 5.6C). This candidate variant was not identified in 112 isolated NSHI cases from Cameroon. The variant pathogenicity was categorised as of uncertain significance (PP1, PP3, and PM2) as referred to the American college of Medical genetics (ACMG) recommendations for variants interpretation.[335,336]

5.2.3.3. Analysis of NM_015263.5(*DMXL2*):p.(Q306H) variant

The 3036 amino acids protein (NP_056078.2) was retrieved from NCBI GenPept as a FASTA file and hereafter referred to as DMXL2.

Amino Acids Evolutionary Conservation

Performing an NCBI PSI-BLAST search of DMXL2 through the non-redundant protein database revealed the position of the variant (p.Q306H) to be conserved amongst all selected non-Homo sapiens species (Figure 5.7). This reflects a strong evolutionary conservation, typical of a significant functional role.

DMXL2 Secondary Structure Prediction

Helices and strands are secondary structural features that form motifs, which in turn constitute functional domains of proteins. Alterations of the secondary structural elements could thus affect the function of the protein. PSIPRED v4.0 server expects our candidate variant (NM_015263.5:p.Q306H) to have a significant impact on DMXL2 secondary structure. The variant was predicted to occur within a helix and its occurrence diminishes the helical propensity at several residues ($^{304}IH^{305}$ and K^{316}). In addition, the short helix at $^{346}RHI^{350}$ is abrogated (Red boxes in Figure 5.9). The variant further alters the β-strand propensity at several residues as indicated by the black boxes in Figure 5.9.

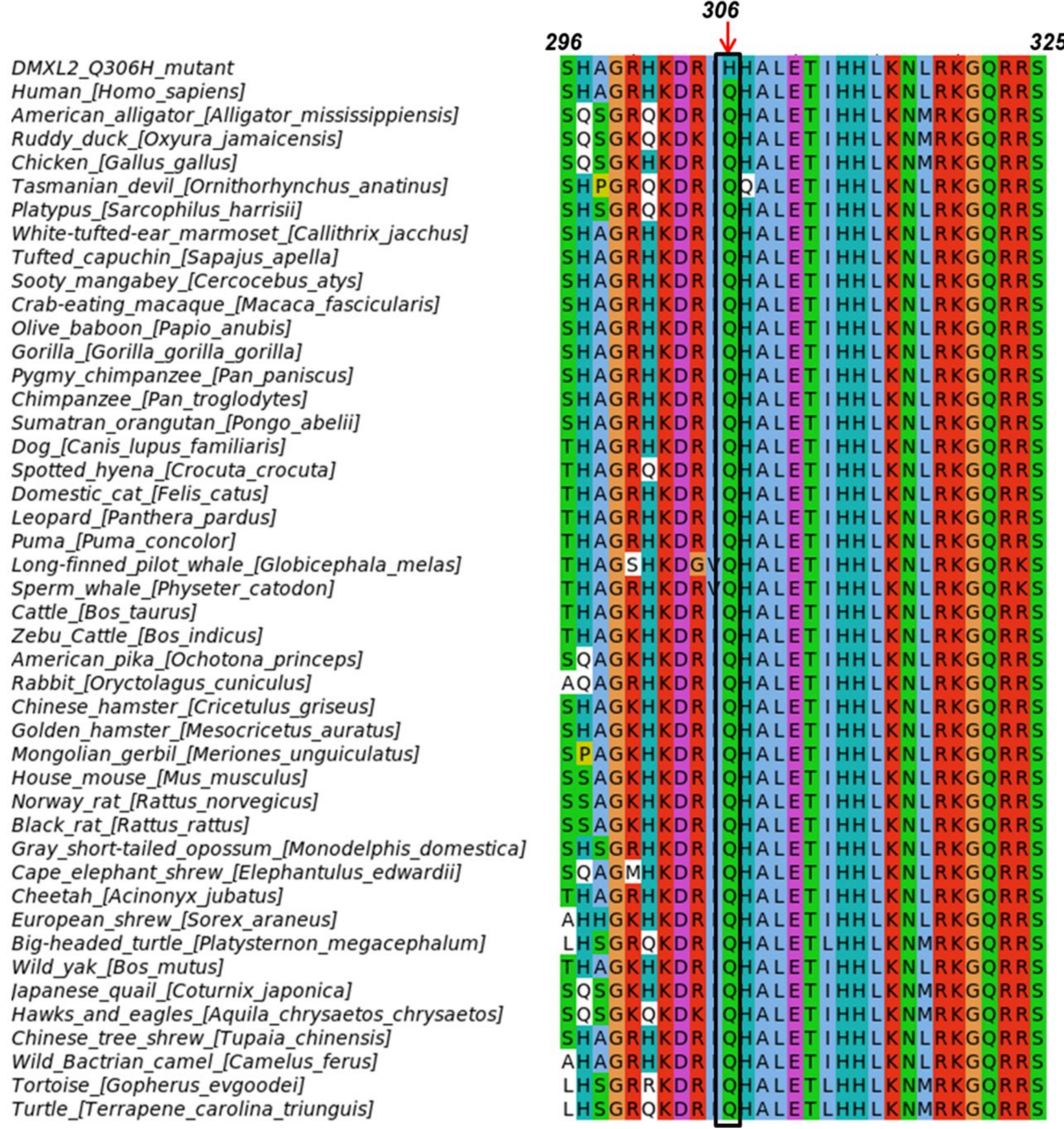

Figure 5.7: Conservation of the mutational position DMXL2:p.(Q306H) (designated by the red arrow) across species.

Protein Modelling

3D modelling of DMXL2 showed that the residue p.Q306 in DMXL2 exists in a β-sheet (Figure 5.8a). The p.Q306 in wild-type displays hydrogen bonding with T339 and L318 (Fig. 5.8b). The p.(Q306H) variant substituted the polar side chain with non-polar aromatic ring. As a consequence, a hydrophobic interaction is established with nearby residues as shown in figure 3c. As a result of these various contacts and different nature of amino acids, a slight shortening of the β-strand was observed in the altered protein. (Figure 5.8e).

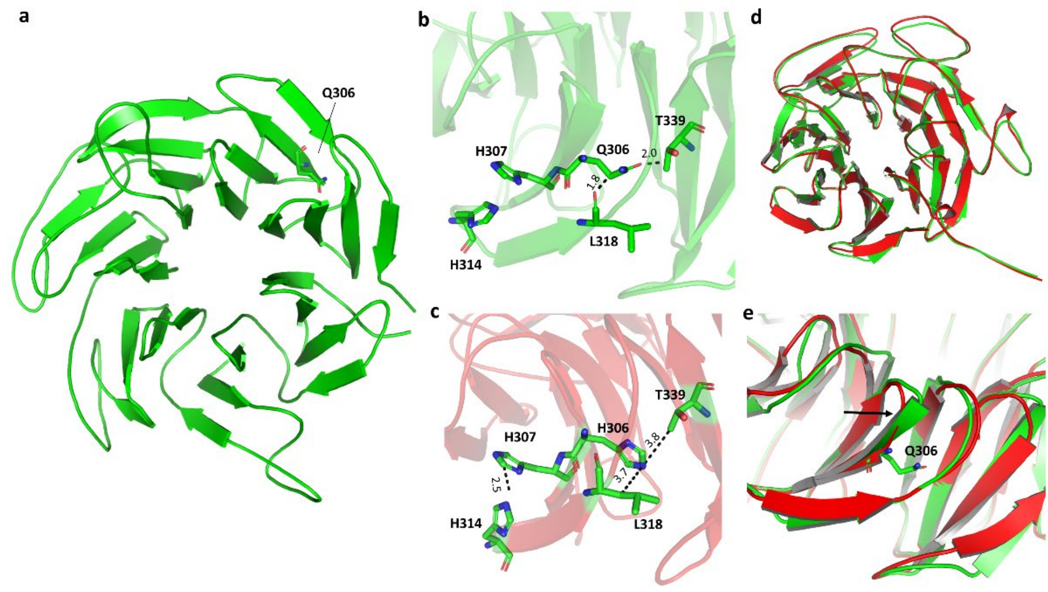

Figure 5.8: Model of the 3D structures of wild type DMXL2 and its Q306H mutant. (**a**) The overall structure of DMXL2 (**b**) Close-up view of the interactions at position 306 of wild and Q306H (**c**). The wild and mutant is represented by green and red colors, respectively. The superposed structure of wild and mutant type (**d**) shows the shortening of the β-strand in the mutant protein as indicated by an arrow in the close-up view (**e**).

5.2.4. Discussion

This study is to our knowledge only the second report on the implication of the *DMXL2* gene in AD NSHI. This study provides evidence that a missense variant [p.(Q306H)] in *DMXL2* is associated with NSHI in an African family from Cameroon and co-segregates with NSHI in an AD manner. This novel p.(Q306H) variant affects a conserved glutamine amino acid residue of DMXL2, is expected deleterious by various bioinformatics tools, and is predicted to significantly alter DMXL2 function and structure. The variant is likely to be private, as it was not detected in 112 sporadic NSHI cases, 121 apparently healthy control individuals without HI from Cameroon, and was absent from gnomAD, TOPMed, UK10K, GME, and dbNSP databases.

A missense variant in *DMXL2* was shown to be associated with HI for the first time in 2017 by Chen et al. [347], they reported on a large 7-generations Han Chinese family with AD HI (DFNA71), that segregated a mono-allelic missense variant p.(Arg2417His) in *DMXL2*. Chen et al. [347] also found *Dmxl2* gene to be expressed in the outer and inner hair cells of the mouse cochlea, and its ganglion neurons, suggesting an implication in the synaptic mechanism of hair cells. Moreover, orthologs of rabconnectin-3α were shown to be expressed in the cochlear of zebrafish and to be critical for hair cells innervation. Pathogenic nonsense

variants in the rabconnectin-3α gene of zebrafish led to vestibular dysfunctions and HI caused by abnormal acidification of synaptic vesicles [347,359].

The phenotype of patients included in the present study has some similarities with Han Chinese patients reported by Chen et al. [347], as they equally presented with bilateral and progressive sensorineural NSHI that was inherited in an AD manner, without clinical signs of vestibular dysfunction or any syndromic abnormality. Chen et al. [347] described in their study a late onset of the HI (occurring during the second decade of life), while two of the three patients reported here, present an prelingual onset of HI, with at least one patient presenting with congenital HI. Additionally, one of the three affected Cameroonian family members (II.2: 27 years old) presented with mild HI, while the two others (I.2: 46 years old, and II.4: 19 years old) presented profound HI, suggesting variable expressivity, a well-known feature of many AD traits [360]. It is also possible that II.2 could still expressed a more severe HI phenotype later in life, similar to what was reported for patients from China [347]. A regular assessment of hearing for individual II.2 is critical. Our results thus suggest that variants in *DMXL2* might lead to a NSHI phenotype that can occur in infancy and presents with variable expressivity. Studies of more individuals with HI due to *DMXL2* variants are needed to refine the phenotype.

Variants in *DMXL2* have been associated with diseases other than HI. A biallelic 15-bp in-frame deletion in *DMXL2* (p.1942_1946delSDGNG) was identified in a Senegalese family and led to polyendocrine-polyneuropathy syndrome (PEPNS; MIM:616113) [361]. PEPNS is a progressive neurodevelopmental and endocrine disorder occurring in early childhood and is characterized by hypoglycaemia, growth retardation, gonadotropic axis deficiency, peripheral demyelinating polyneuropathy, hypothyroidism, progressive non-autoimmune insulin-dependent diabetes mellitus, intellectual disability, pyramidal signs, and cerebellar ataxia [361]. Early infantile epileptic encephalopathy-81 (EIEE81; MIM:618663) which is also called Ohtahara syndrome was recently shown to be associated with variants in *DMXL2* [362]. EIEE81 is a developmental brain disorder that involves severe psychomotor development, intractable seizures, dysmorphic features (such as hypotonic facies and epicanthal folds), mild peripheral polyneuropathy, and sensorineural HI [362]. None of our patients or those previously described in the Han Chinese family NSHI presented with any neurological or endocrine phenotypes. Variants in *DMXL2* are thus associated with two clinical forms of sensorineural HI: 1) the non-syndromic form as that described in the present study and by Chen et al. [347], and 2) the form that occurs as part of a syndrome with neurological abnormalities, as described in the EIEE81 [362].

DMXL2 (on chromosome 15q21.2) encodes DmX-like protein 2 [DMXL2, also called rabconnectin-3α (RC3)] [362], the α subunit of the rabconnectin protein complex that

condenses on synaptic vesicles and is involved in neurotransmitters secretion [347,363]. DMXL2 is a vesicular protein made up of 3036aa (Mr 339,753 Da) and is involved in the Ca2+-dependent translocation of neurotransmitters across synaptic membranes [362,364] The expression of RC3 was found in many tissues, which include ear hair cells, and the brain [347,363]. The exact neurophysiological role of RC3 in the brain remains elusive [362]; however, studies on the rat brain suggest that RC3 may serve as a scaffold protein for Rab3A and its effector proteins. [365] Rab3A, which was shown to co-immunoprecipitate with RC3 in the rat brain, plays a role in the exocytosis of neurotransmitters across synaptic membranes by facilitating the translocation and docking of synaptic vesicles to presynaptic membranes and also preventing the fusion of the vesicles to the plasma membrane, a Ca2+-dependent process [365]. In mammalians, RC3 is also expressed in the brain and at synaptic terminals where it binds RAB3A interacting proteins as a dimer with Rabconnectin-3b (*DMXL1*), and is thought to be important for autophagy and brain development.[362] *Dmxl2* homozygous knockout mice were shown to be embryonic lethal, while heterozygous *Dmxl2* ($Dmxl2^{+/-}$) mice display corpus callosum dysplasia and macrocephaly, giving more evidence of the implication of this gene in the development of the brain [362,366]. The implication of RC3 in the hearing process was revealed by Einhorn et al. [359] when they showed that the zebrafish RC3 ortholog (Rbc3α) was expressed in the inner ear of zebrafish, and was concentrated at the basal region of hair cells. Also, mutant alleles of *rbc3α* selected from a cohort of zebrafishes were associated with HI and vestibular dysfunctions [359]. *rbc3α* mutants also lack light adaptation responses in melanocytes and spontaneous eye movements, suggesting a possible visual defect [359]. No clinical sign of vestibular or visual abnormalities was described in family 23, or in the Chinese family described by Chen et al. [347]; however, given the visual impairment observed in the *rbc3α* mutants, studies on the possible expression and function of DMXL2 in the retina are recommended. Last, Einhorn et al. [359] showed that the pH of the synaptic vesicles in the hair cells of the *rbc3α* mutant zebrafish was elevated, and the cytosolic unit of the V-ATPase was no longer concentrated in synaptic junctions.[359] Interestingly, deacidification of the synaptic vesicles was shown to reduce the loading of neurotransmitters in vesicles and thus negatively impact neurotransmission [367]. Based on these results, Einhorn et al.[359] proposed that Rbc3α regulates the synaptic activity in hair cells by allowing the concentration of the V-ATPase holoenzyme on synaptic vesicles, modulating thus the acidification of the vesicle and, ultimately, its neurotransmitters concentration.

To further support the linkage of HI in “Family 23” with *DMXL2* gene, genetic linkage analysis using the logarithm of the odds (LOD) score method could be useful. Also, functional analyses using cell lines and/or animal models are needed to further confirm the deleterious effect of the p.(Q306H) variant on DMXL2 function and structure. In addition to examining the exome,

identifying variations in parts of the genome not covered by WES might be of importance. Indeed, variants in noncoding DNA sequences including deep intronic variants were shown by previous studies to be associated with HI [368]. Although 10X depth of coverage may not be sufficient to detect small proportions of heterogeneous genetic variations in population-based studies involving large numbers of samples (coverage of at least 20X is generally ideal), family-based studies retain their power to detect such variants at 10X depth of coverage given that they generally look for segregation of sometimes private and/or rare variants within a family [369].

This study confirms the contribution of *DMXL2* to NSHI in humans and demonstrates for the first time such association in Africans. Our study thus enriched the list of HI-causing genes in humans and should consequently contribute to increasing the solving rate of genetic HI cases in clinical settings. The present study adds more evidence to the efficacy of WES in detecting causative variants in NSHI cases from sub-Saharan. Additional studies are necessary to assess the contribution of *DMXL2* gene to NSHI in other populations.

5.2.5. Conclusion

The present study reports a novel mono-allelic missense variant in *DMLX2* [p.(Q306H)], that segregates with NSHI in a Cameroon family with an AD mode of inheritance. This study provides additional support of the use of WES to identify variants that contribute to HI in sub-Saharan Africa. These data will complement and improve clinical diagnosis, and our knowledge of HI pathophysiology, globally.

Author contributions: A.W. and S.M.L. conceived the project; E.W.T. performed the recruitment and molecular experiments; I.S., T.B., L.M.N., A.A. performed the bioinformatics analysis; K.K.E., E.W.T., A.N. and S M. performed the *in silico* analysis of the identified variant; E.W.T. issue the first version of the paper and all the authors reviewed and edited it; A.W. supervised the entire project; all authors have read and agreed to the final version of the paper.

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Ethical Approval: All research procedures in the present study were authorised by the Columbia University's institutional review board (IRB-AAAS2343), the Institutional Research Ethics Committee for Human Health of the Gynaeco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon (No. 723/CIERSH/DM/2018), and the Human Research Ethics Committee of the University of Cape Town's Faculty of Health Sciences (HREC 484/2019). All participants were informed and provided written consent, including permission to publish data, in respect of the Declaration of Helsinki.

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Supplemental material: Supplemental material for this article is available online:

Table 5.5: Demographic and clinical characteristics of isolated NSHI cases screened for the missense variant.

Categories	n/N	Frequency (%)
Age range (years)		
<10	48/112	42.86
10 – 20	59/112	52.68
>20	5/112	4.46
Gender		
Male	59/112	52.68
Female	53/112	47.32
Ethnic group		
Bamileke (Semi-bantu)	48/112	42.86
Beti-fang (Bantu)	32/112	28.57
Duala (Bantu)	7/112	6.25
Bassa (Bantu)	10/112	8.93
Bamun (Semi-bantu)	7/112	6.25
Fulani (Sudanese)	4/112	3.57
Tikar (Semi-bantu)	3/112	2.68
Mbo (Bantu)	1/112	0.89
Age-of-onset		
Congenital/Prelingual (before 2 years old)	102/112	91.07
Perilingual (between 2 and 4 years)	8/112	7.14
Postlingual (after 4 years)	2/112	1.79
Degree of hearing impairment		
Severe I	5/61	8.20
Severe II	5/61	8.20
Profound I	12/61	19.67
Profound II	18/61	29.51
Profound III	10/61	16.39
Total	11/61	18.03
Type of hearing impairment		
Sensorineural	56/61	91.80
Mixed	5/61	8.20
Bilateral	61/61	100.00

Figure 5.9: DMXL2 secondary structure prediction. Boxes indicate positions of difference between wild type (DMXL2:p.306Q) and variant (DMXL2:p.306H). Red box shows loss of and attenuation of helixes, while black boxes display attenuation of β strands.

Web Resources

ANNOVAR — https://annovar.openbioinformatics.org/

Bureau international d'audiophonologie (BIAP) — https://www.biap.org/en/recommandation/recommendations/tc-02-classification

ClinVar — https://www.ncbi.nlm.nih.gov/clinvar/

dbNSFP (including dbscSNV) — https://sites.google.com/site/jpopgen/dbNSFP

dbSNP — https://www.ncbi.nlm.nih.gov/snp/

Ensembl — https://www.ensembl.org/index.html

Genome aggregation database (gnomAD) — https://gnomad.broadinstitute.org/

Genome analysis toolkit (GATK) — https://gatk.broadinstitute.org/hc/en-us

Hereditary hearing loss homepage (HHL) — https://hereditaryhearingloss.org/

Human phenotype ontology (HPO) — https://hpo.jax.org/app/

MODELLER — http://www.salilab.org/modeller

NCBI-BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
NCBI-GenePept	https://www.ncbi.nlm.nih.gov/protein
Online Mendelian inheritance in man (OMIM)	https://omim.org/
Protein databank (PDB)	https://www.wwpdb.org/
PSIPRED	http://bioinf.cs.ucl.ac.uk/psipred/
PYMOL	http://www.pymol.org/
Trans-Omics for Precision Medicine (TOPMed; BRAVO)	https://bravo.sph.umich.edu/freeze8/hg38
UK10K	https://www.uk10k.org/

Chapter 6: Results – Perception of Hearing Impairment, and Assessment of the Psychosocial Burden of Hearing Impairment in Cameroon.

6.1. Introduction

Hearing is a major sensation [370] in humans and it plays critical roles in interpersonal interactions as well as human interactions with their environment [371]. People with hearing impairment (HI) have partial or total inability to hear sound in one or both ears [370]. Globally, an estimated 466 million people are currently living with HI, the majority (over 80%) of which are based in low- and middle-income countries [372]. it is also anticipated that by 2050, the global estimates will be over 900 million [373]. HI can be due to genetic or environmental factors, and in many cases, it is not possible to establish a definite aetiology [33,34,316]. Environmental factors such as meningitis, measles, or ototoxicity are the leading causes of HI in low- and middle-income countries, while their burden is lower in high-income countries [5,34–36]. It is thought that approximately half of congenital HI cases are inherited [372]. Pathogenic variants in *GJB2* gene constitute the most common cause of non-syndromic HI in European and Asian populations [316], while their prevalence is close to zero in sub-Saharan Africa (SSA) [374]. Depending on whether there are additional clinical signs or not, genetic HI cases are classified as syndromic or non-syndromic.

Among parents and family members of people with HI, there is limited knowledge of the genetic causes of HI [372]. Most families, especially families without HI history do not however associate the aetiology of HI to genetic/inheritable factors [375]. Rather, they often cite causes such as premature birth, trauma at birth, infection, Rhesus incompatibility, drug intoxication, pregnancy complications, maternal rubella, and otitis [375]. This limited knowledge of the causes of HI may be more problematic in Africa, where perceived causes of deafness vary from environmental factors to mysterious ("evil forces") or superstitious beliefs [28,29]. For example, a study in South Africa reported that few parents associated their child's HI with genetic causes while most explained it to recurrent infections [372]. In the South African study, parents who reported genetic causes were likely to have been in contact with the genetics unit at their hospital as part of their child's healthcare [372]. In a review on HI in SSA, Kiyaga et al [376] stated that HI is often associated with mysterious fate, and, in some cases, "God's will". Similarly, studies in Nigeria and South Africa revealed that the majority of traditional healers ascribed HI to supernatural causes [29,377] and other superstitious beliefs [28].

In most cases, children with HI are born from hearing parents who have little or no experience with people with HI [378]. Thus, parents with children with HI tend to face challenges of parenting, some of which may affect the family structure especially in terms of communication

and social interaction [378]. Access to basic social services for children with HI and helping them interact with extended family members are additional challenges faced by parents of children with HI [378]. It is therefore essential to help parents and/or caregivers of children with HI to navigate some of these psychosocial challenges, especially in terms of sign language communication and interaction with children with HI, and adjusting to the new family dynamics brought about by the child with HI [379,380].

Previous studies from Cameroon assessed the challenges faced by persons with HI, and found that the attitude of the society towards persons with does not encourage their participation and involvement in the community, as they are often discriminated against [30]. Additionally, persons with HI were shown to have limited access to education in Cameroon, as they have few opportunities to further their education [381]. The understanding of people with HI of the causes of their condition, and their expectations towards policy makers in Cameroon, however, remain elusive. In the present study, we sought to revisit the challenges faced by persons with HI in Cameroon, explore their understanding of the causes of HI and how challenges can be remedied to improve the quality of life of persons with HI.

6.2. Methods

6.2.1. Ethical Considerations

Research ethics approval was obtained from the Institutional Research Ethics Committee for Human Health of the Gynaeco-Obstetric and Paediatric Hospital, Yaoundé, Cameroon (No. 723/CIERSH/DM/2018), and the University of Cape Town's Faculty of Health Sciences' Human Research Ethics Committee (HREC 484/2019). All participants provided written informed consent, including permission to digitally audio record interviews and to publish the findings of the study. In the case of minors (less than 21 years), informed consent was obtained from parents, with the verbal assent of the participant, all these in respect of the Declaration of Helsinki [382].

6.2.2. Study Sites and Population

We enrolled people residing in two rural (Far-North, and North) and one urban (Centre) regions of Cameroon, a Central African country with a population of approximately 24,053,727 [226], and with a HI prevalence ranging from 0.9% to 3.6% [316]. Of these, environmental factors such as meningitis, impacted wax, and age-related disorders contribute to more than half of all recorded HI cases, approximately 14.8% are due to hereditary causes, while in the remainder of cases, the cause is unknown [316].

This qualitative study is part of a bigger project (HI-GENES Africa) that aims to study the genetic aetiology of HI in Cameroon. The three aforementioned study sites were the starting points for the broader study. These regions have well-established schools and institutions for the deaf, thus presenting a good opportunity to recruit HI professionals, persons with HI, and their parents/caregivers. For example, of the 19 special schools for people with HI in Cameroon, nine are located in the study sites (three in the Far-North, three in the North, and three in the Centre regions) and are all privately run.

Potential study participants were divided into two groups: 1) professionals who provide health or educational services specific to HI and 2) families with a child with HI. The group of professionals (n=10) was made of three directors of schools, three specialised teachers, one audiologist, one audiology technician, one speech therapist, and one ear nose and throat (ENT) specialist. The group of families (n=10) consisted of three persons with, four parents, and three siblings. We distinguished two types of families: 1) multiplex families (n=6), in which at least two individuals in the same sibship were having HI, and the disease was inherited; and 2) simplex families (n=4), in which only one member with HI was found, and an environmental factor was identified as the cause of the disease. Participants with HI were selected through schools for the deaf, and family members via community engagement activities.

6.2.3. Interview Guides and Data Collection

Data were collected through one-on-one semi-structured interviews and participant observation [383]. In reporting the results, our participants' privacy and confidentiality, was protected through the use of pseudonyms.

A total of 20 interviews were conducted in a location convenient for the interviewee and in their preferred language (17 in French, and three in French sign language). A sign language interpreter assisted with the interviews (n=3) that involved persons with HI. A semi-structured interview guide was used to explore perceptions of HI including causes of HI, the psychosocial burden associated with HI based on their lived experiences, and means to overcome challenges. Before the interviews, participants completed a short questionnaire to collect socio-demographic information. All the interviews were digitally audio-recorded with consent, including those conducted with help from the sign language interpreter (the interpreter was recorded and considered as reflecting the participant’s responses).

6.2.4. Data Analysis

Audio-recordings were transcribed verbatim and translated from French to English. To familiarise ourselves with the data, each transcript was read several times. The transcripts

were imported into the qualitative data analysis software NVivo 12 Pro, and each of them was subjected to content analysis [384]. We used the thematic analysis method to identify themes within the data [385].

6.3. Results

6.3.1. General characteristics of our participants

A total of 20 participants were interviewed, including twelve males and eight females, with ages ranging from 16 – 54 years. The majority of our participants (13/20) lived in a rural area, while a few (7/20) were residing in urban settings. At the time of the interviews, most participants had secondary education (14/20), and the remaining 06 had obtained a tertiary degree (Table 6.1).

The results will be reported based on three themes: 1) perception of the causes of HI; 2) challenges faced by people with HI in Cameroon; and 3) expectations of people with HI to policymakers and researchers.

Table 6.1: Characteristics of participants

Category		
n	20	
Mean age (SD)	34.15 (11.36)	
Category of participant	n	%
Gender		
Male	12	60
Female	8	40
Home language		
French	17	85
FSL	3	15
Marital status		
Single	10	50
Married	8	40
Divorced	1	5
Widow	1	5
Area of residence		
Rural	13	65
Urban	7	35
Level of education		
Secondary	14	70

Higher	6	30
Relationship with HI		
Professionals	10	50
Families	10	50

FSL, French sign language; HI, hearing impairment; SD, standard deviation

6.3.2. Perception of the causes of HI

Our participants mentioned different factors as possible causes of HI. These factors were genetics, an ancestral or religious curse, environmental, or of unknown origins. The perception of the causes of HI in the group of families was influenced by how they are related to HI. Participants from multiplex families tend to associate the occurrence of HI to hereditary, while participants from simplex families mainly listed environmental aetiologies.

Defining HI

Participants in the group of families defined HI as a disability and referred to it as a disability that is linked not only to loss of hearing but also speech impairment.

> *"Basically, it is a disease that manifests through the inability of a person to hear or to talk" (Participant 008, brother of an individual with HI)*.

Participants in the group of professionals defined HI as a decrease in the hearing ability, and also further specified that depending on the degree of impairment, it can be classified as mild, moderate, severe, profound, or total.

> *"Hearing impairment means that there is a dysfunction of the ear. Hearing impairment can be total, moderate, severe or profound." (Participant 004, female, specialised teacher).*

HI as a cultural or religious curse

Most of our participants mentioned that some people in their communities associated HI with witchcraft or a curse (ancestral or religious). This was especially so when a family had many members with HI, in which case it was considered punishment or curse from a supreme being (God).

> *"Well, some of them think that deafness is a curse, you have been cursed, the person has been cursed. Some people think like that. And if there are several cases of deafness in a family, they will say that it is a curse" (Participant 008, brother of an individual with HI).*

A few interviewees reported that this misconception of HI was also common within their own families, and has, in some cases, led to family disputes and division.

> *"For them, it is like witchcraft, especially because I am a stranger, I am in a Bamiléké family. On my side, my family is saying Bamiléké put witchcraft on children. On my husband's side, they say that the witchcraft from my family has affected the children. It is very difficult."(Participant 019, mother of three children with HI).*

HI as an inherited disease

Participants from multiplex families mentioned genetics as a primary cause of HI and explained that when parents or grandparents have HI, children can inherit the condition.

> *"According to me, it is genetic. Because in my case, for example, the elder brother of my husband is deaf. So I think that these genes are in my husband. That is why my children took these genes." (Participant 019, mother of three children with HI).*

Interviews with the professional group also stated that HI can be inherited and when that is the case it is common to find several affected individuals in the same family. A few of them further stated that the gene that causes HI is recessive.

> *"We can also have congenital hearing impairment cases, which can be inherited. We often have some cases where we have a family with three deaf children while parents are not deaf. Since we know the gene for deafness is recessive, when it is present in both parents, then there is a risk for them to have deaf children." (Participant 015, male, audiologist)*.

HI as an acquired disease

Participants from simplex families frequently referred to environmental factors such as diseases, climatic conditions, and excessive noise as the main cause of HI. In the interviews, this group cited malaria, neonatal asphyxia, severe flu, headaches, exposition to the sun, or noise as possible aetiologies of HI. The most frequently cited disease was meningitis

> *"(sign language translation) When we are seriously sick, we can become deaf. There is the sun, there is meningitis." (Participant 012, male, student, person with HI)*.

In addition to these factors, persons in the professional group added that otitis, tumours, the use of ototoxic drugs, and infections such as mumps, measles, and rubella were also responsible for HI.

> *"Hearing impairment has many aetiologies. We have prenatal aetiologies, which are diseases that the mother can suffer from. We also have mismanagement of some diseases, by taking some antibiotics like paracetamol in excess, quinine; that is one of the prenatal aetiologies. We also have perinatal aetiologies. During delivery, the baby can have some trauma, such as anoxia so, the umbilical cord can surround the neck. And the third aetiology is postnatal causes. Here we have shocks. Here, the patient has already acquired speech. Diseases such as meningitis, or noise when wearing speakers on ears can alter hearing." (Participant 001, male, audiology technician)*.

HI as a mental disorder

A few participants from the group of professionals reported that some people in their communities conceptualised HI as a form of mental retardation or madness.

> *"As soon as they realise that a person is hearing impaired, they start treating him like a mentally disabled person. That is what we observe. They will say that it is madness (laughing). Yes, most of them will say that it is a disease that affects the brain, yes most of them" (Participant 020, male, speech therapist).*

Therefore they tend to consider people with HI as foolish or not intelligent, and always nervous.

> *"Most of the time they are called "foolish people", it means people who are not intelligent, people who are a little crazy, people who react quickly, people who are too stupid. This is not true." (Participant 002, male, director of a school for the deaf).*

6.3.3. Challenges faced by people with HI in Cameroon

In the interviews, we also asked participants about the challenges experienced by persons with HI. Our participants mentioned several challenges that could be grouped into four areas: socialization, management, employment, and autonomy issues.

Challenges with socialization

Our participants narrated how people with HI are often marginalized and discriminated against both by their own families and society at large, leading to their social withdrawal from their society. This is illustrated by the use of stigmatizing labels like "Moumou". In Cameroon, the

phrase “moumou” is often used to refer to persons who use hands to communicate, and what they say or express is construed by the community as mainly due to ignorance or stupidity. This discrimination issue was mostly reported by participants residing in rural areas. Some interviewees explained that children with HI are not respected at home, are treated differently as compared to hearing children, and are most of the time asked to do the most difficult duties.

> *"When you go to a remote area, they will say this is a “moumou”, since he is here, he is alone in his corner. But this is because people do not understand him." (Participant 015, male, audiologist)*.

Participants also explained that people with HI are systematically excluded from community activities.

> *“(sign language translation) There is somehow discrimination compare to people who can hear. They are not invited to some meetings; there are sidelined. They think that deaf people are not equal to normal people; they are always sidelined from some meetings. They say that he is deaf, what can he do? What can he say?” (Participant 012, male, student, person with HI).*

Some interviewees also stated how in communities where HI is associated with witchcraft, curse, or mental retardation, HI individuals experience heightened social exclusion (Figure 6.1).

> *“People who do not know deafness will reject them. Because they think that they are crazy, they are witches, they do not have all their senses, and they are not considered. But people who know deafness well accept them.” (Participant 003, male, specialised teacher).*

Overall, HI professionals emphasized the fact that the age of onset and the degree of HI impact on social integration of persons with HI in their communities. For example, a child who did not benefit from early management will have more communication and interaction issues compared to those who had early and appropriate management.

> *"A child who was born with hearing impairment has never heard anything, so he cannot speak. For people with acquired hearing impairment, they have few issues because, for someone who had meningitis at ten years old, he used to speak, the vocabulary is still there. However, if we do not do anything, the speech will deteriorate. Nevertheless, if he is brought to me, we will give him the appropriate hearing aid, and he will recover speech, he will recover hearing, and his social interaction will be easier." (Participant 015, male, audiologist).*

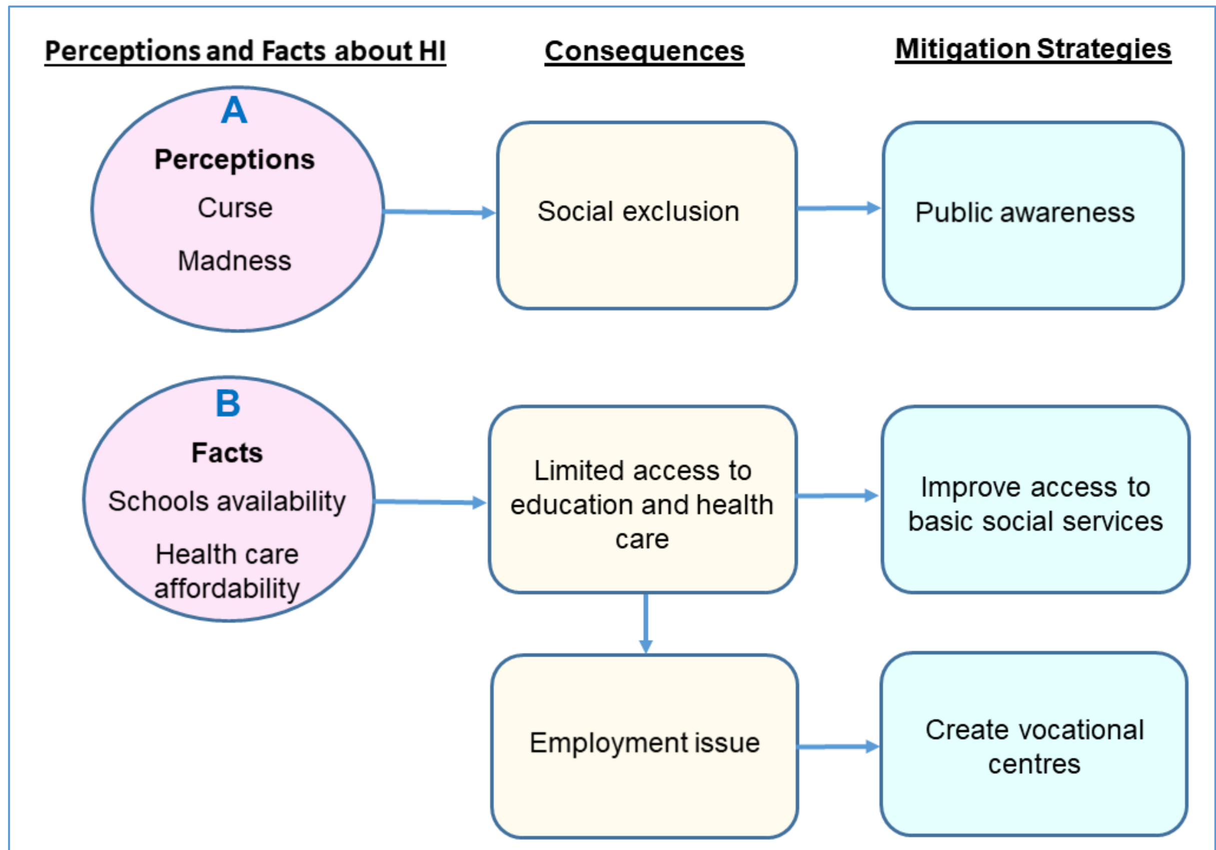

Figure 6.1: Summary of the main findings developed in our study. (**A**) The misunderstanding of the causes of HI will lead to the social exclusion of people with HI, which implies the need to educate the population about the aetiologies of HI. (**B**) The limited number of schools, and the high cost of tuition fees and health care services will favour the limited access to education and health care, which will ultimately be a barrier to the future employment of people with HI. This highlights the need to improve access to basic social services and to create vocational centres. HI, hearing impairment.

Challenges in accessing basic social services

The large majority of our participants highlighted that it is challenging for people with HI to have access to education and healthcare. This was mainly because of their need for sign language translation which is not available in most public and private services. Also, there are very few special schools for persons with HI and most of these schools are privately run not affordable. Some interviewees suggested that this challenge could be overcome through the provision of government subsidies to private institutions so as to reduce the cost for parents (Figure 6.1).

> *"Almost all specialised institutions are private and are therefore sometimes expensive. Parents have to pay tuition fees. Moreover, in addition to that, it is not enough; the government has to allocate funds, for the institution to work efficiently. So we really need funding to help everybody, because they are not*

a lot, but their management is expensive." (Participant 005, male, specialized teacher).

In terms of access to health care services, the main challenge was that audiology services are very expensive in Cameroon and many persons are unable to afford the cost. Therefore even when parents suspect that their child may be having challenges with hearing, they are still unable to seek care in a timeous and consistent manner.

"It is very expensive. First, diagnosing hearing impairment is a problem, especially in children. Apart from the eardrum assessment that we can do everywhere, the Auditory Brain Respond, a specialised exam, is not available everywhere, and it is costly. Moreover, even when the Auditory Brain Respond is done, we have to either operate the child to put a cochlear implant, and we cannot do it in Cameroon. A team has already started doing it, but it is a European team, and it is not common. Secondly, we can prescribe a hearing aid, which is expensive. One implant cost at least five hundred thousand francs that is the salary of four months for a civil servant. This is not affordable for a common citizen." (Participant 011, male, ENT specialist)

Challenges with gaining employment

Some interviewees reported that persons with HI have difficulties accessing gainful employment. This is primarily because of their inability to communicate easily with hearing-individuals thereby making job interviews particularly difficult, especially when sign language interpretation is not available as is the case in many institutions in Cameroon. Secondly, even operating within the workplace was considered to be a major challenge as they will find it difficult to communicate with colleagues.

"Because deaf people cannot hear, I do not see which area we can employ them. Because every working environment requests communication. I do not think that we can employ them in any area" (Participant 008, brother of an individual with HI).

Additionally, our interviewees estimate that society tends to exploit people with HI, as they are sometimes asked to do difficult jobs without any compensation in return.

"And when there is a deaf lady in the community, people tend to exploit her for work and so on. So exploit her in the sense that they don't want to compensate her for the work she did, they will say that she is deaf and she should work." (Participant 002, male, Director of a school for the deaf).

Lack of Autonomy

Participants who had a family member with HI expressed the heavy reliance of persons with HI on their family members for social and financial support. They explained that children with HI are often not able to travel alone or go to school unaccompanied, like other children. This according to our participants adds to the day-to-day workload of the parents. Children with HI are very dependent on their caregivers, which can have an impact on the morale of the caregiver as expressed by one mother of two children with HI who said the dependency of her children is so high that it seems she does not have a right to die.

> *"She is a big girl but she can't travel alone, she can't do anything alone, nothing….. it is difficult. You should always be with her, be her guardian angel, so I can't die? (laughing)" (Participant 016, mother of two children with HI).*

6.3.4. Expectations of people with HI

We also asked participants how the challenges they mentioned could be remedied. They highlighted the need for more specialised schools (from basic to tertiary) for persons with HI; public awareness activities on HI and the challenges; reducing the burden of inherited HI and implement a newborn screening program for HI; and the need for vocational centres.

Improving access to basic social services

The major expectation as emerged from the interviews relates to improved access and low-cost education and healthcare. In terms of access to education, they expected the government to fund tuition and training fees of people with HI, provide school supplies to existing schools, and create primary schools, high schools, and even universities for persons with HI (Figure 6.1).

> *"First of all, reduce our tuition fee. Secondly, they should build a high school for the deaf. Because I think there is no high school for the deaf. When they finish primary school, they have to attend a high school for hearing-people; I do not even know how they make it. So we have to build their high school, and if possible, their university." (Participant 018, mother of a child with HI).*

In terms of improving access to healthcare, some participants suggested the need for a comprehensive public health or national control program for HI, as is the case with HIV/AIDS, Malaria, and Tuberculosis. If that is done, they hope it will improve the affordability of audiology services.

> *"I would like their management to be funded just like the management of other diseases. They should consider deafness as a disease like AIDS, which we*

fight against, like cancer. So they should act in such a way that even poor parents can afford the treatment, so exams, hearing aids." (Participant 019, hairdresser, mother of three children with HI).

The professionals expressed a need for more healthcare workers who are specialised in the management of HI as well as improved infrastructure for their care.

"They should put in all major hospitals, the necessary infrastructures to diagnose HI, and also train more healthcare workers, in the proper management of hearing impairment. Fund hearing aid, train specialised doctors in the surgical management of hearing impairment. Moreover, have proper infrastructures in hospitals, to ensure the management of hearing impairment." (Participant 011, male, ENT specialist).

Create awareness on the causes of HI and challenges experienced by persons with HI

Participants highlighted the need for the government to raise awareness amongst the general population about HI, including the need not to discriminate against them in terms of access to education, employment, and basic social services. For some participants, public awareness could mitigate negative religious and cultural considerations surrounding HI, which will reduce the stigmatization and marginalization currently experienced by people with HI (Figure 6.1).

"The government should also raise awareness in the society so that when people will see individuals with hearing impairment, they will know exactly who they are; they will not stigmatise them, they will now know their psychology, they will understand that they react like that because of this or that situation. So the society will be informed, and they will have fewer problems." (Participant 002, male, Director of a school for people with HI).

Some participants mentioned how research programs could also be used as a platform for raising public awareness of HI.

"Now your work will help. It will help in the sense that when you publish, people will read it, and they will realise that there is an issue that is neglected. Furthermore, it will help people to understand better, as we said earlier, many people do not even know that it is a problem and that it is not madness (laughing). When I was younger, when I used to see people with HI, I did not use to consider them, I was asking: what can he do? I think people behave like that just because of their ignorance." (Participant 020, male, speech therapist).

Reduce the burden of inherited HI and implement newborn screening for HI

In addition to using research programs to raise public awareness on HI, interviewees from multiplex families expect researchers in the field of genetics to develop strategies to prevent future generations from having children with HI. Using HIV as an example, one participant narrated how with advances in HIV research it is now possible for a pregnant woman to take some medication to prevent the transmission of the infection to her child, and suggested that it could be the same for HI.

> *"I hope that genetics can prevent the next children not to undergo what our children are suffering from now. So I would like doctors to see whether they can help us so that our children do not give birth to deaf children in the future. So if during your research, you can act in such a way that our children do not give birth to deaf children. Just like with HIV, where the mother takes medicine for her baby not to be affected. That will be something great." (Participant 019, hairdresser, mother of three children with HI).*

Professionals expected genetic research to help implement premarital genetic testing to assess the risk of having children with HI. This, they suggested, could also help policymakers to implement legislation that would in the future make premarital genetic testing for HI compulsory. There was also the need for units within the hospital that could support newborn screening for HI.

> *"We want doctors to inform people of inherited hearing impairment. You should reach a point where every child who is born is brought to the ENT specialist, just like he is brought to the Paediatrician, before leaving the hospital, in order to diagnose early-onset hearing impairment. Moreover, why not at the level of the laws, we should reach a point that we recommend people to do premarital screening before getting married to not find themselves with children with hearing impairment anymore. So you should make publications, for people to be aware." (Participant 015, male, audiologist).*

Creation of vocational centres

Our interviewees, in general, expect the government to train people with HI and facilitate their employment. They suggest funding of self-employed projects, favouritism of people with HI as other disabled individuals when it comes to employment, creating training centres for the professional training of people with HI in areas such as sewing of dresses, carpentry, artwork, trading, and any manual works (Figure 1).

"The government should take care of these so disabled children. They should build a project for them. We have trading projects, a deaf person can easily do trading. If it is not the case, the government can create training centres for them to learn artwork, carpentry, or even dressmaking." (Participant 001, male, audiology technician).

6.4. Discussion

This study is, to our knowledge, one of the first reports from Cameroon to provide data on the perceptions of HI, the impact of the latter on the daily life of people with HI and their families, and their expectations towards policymakers and researchers. The findings are similar to studies reported in other parts of SSA [31,376].

Our data show that the occurrence of HI in Cameroon is sometimes associated with a curse; also, people with HI are often considered as being mentally disabled. This association of HI with supernatural forces has also been described in other African countries [376]. In Ethiopia, for example, some communities believe that people with HI are possessed by the devil and should be cured by witchcraft or purified water [376], while in Rwanda, some people perceive HI as foolishness [376]. This perception of HI is likely due to the non-understanding of the causes of the disease, and suggests thus, the need for more awareness on the causes of HI. Understanding the cause of the disease is critical for the wellbeing and social integration of people with HI, as our study found that in communities where HI is associated with supernatural beliefs, people with HI experience heightened social exclusion. The social exclusion of people with HI described by our interviewees was previously reported in Cameroon by De Clerck [30], and in Burundi [31]. Persons with HI interviewed in the study by De Clerck [30] reported discrimination against people with HI in that in social events such as family meetings their decision-making ability was sometimes questioned.

The findings of our study showed that persons with HI in Cameroon face challenges in accessing basic social services. Indeed, the few special schools for people with HI in Cameroon are all privately run and are expensive; also, there are currently no high schools for persons with HI in Cameroon. This undoubtedly contributes to the lack of education and ultimately future employment of people with HI. The high cost means that for families who are unable to afford tuition, there is the likelihood their child with HI may end up not being able to use sign language and by extension unable to access education and healthcare services even if sign language interpreters are available. The limited number of special schools currently available in Cameroon implies that most families likely stay far from schools, adding thus an additional difficulty in accessing education. The limited access to education observed in our study has been described in a study conducted previously in Cameroon [381]. With respect to

access to healthcare, there is no universal health insurance scheme in Cameroon, therefore, families pay for every treatment related to HI. Also, the lack of some therapeutic procedures such as cochlear implantation in Cameroon constitutes an additional barrier to the management of people with HI. Therefore, to improve the access of people with HI to basic social services, we recommend that policymakers should create more special schools for individuals with HI, aware families of the existence of these schools, train more qualified healthcare practitioners, establish well-resourced centres or services within hospitals for the management of persons with HI, and implement a health insurance scheme to financially support families. This difficulty for people with HI to have access to basic services was also been reported in Nigeria [386].

To reduce the burden of monogenic HI in Cameroon, our participants suggest the establishment of premarital genetic testing for HI. Coupled with genetic counselling, premarital screening is designed to determine whether individuals carry a genetic predisposition that may produce disease in their offspring, it includes premarital health counselling and a general medical examination, and is particularly important in the prevention of the spread of diseases [387]. The successful implementation of a premarital genetic screening programme is thus dependent on the existence of well-established genetic counselling services and also a good understanding of the genetic cause of the disease. However, there is currently to our knowledge no genetic counselling or medical genetics clinics in Cameroon. Also, the genetic aetiology of HI in SSA in general and in Cameroon particularly is not well known [316]. Indeed, variants in *GJB2* gene which are the major causes of HI in European, Asian, and Arab populations have a prevalence close to zero in Cameroon and in most SSA countries, suggesting that other genes are implicated in inherited HI in Africans [14,317,374]. It is therefore critical to train genetic counsellors and intensify genetic research on inherited HI, to identify the causative genes and variants, and to complement the genetic counselling of families with HI.

Our study confirms the difficult social interaction and access to education and health care faced by people with HI in Cameroon, and highlights the expectations of people with HI towards policy makers, especially in terms of access to basic social services.

6.5. Conclusions

There is a need to educate the general population on the causes of hearing impairment; this should mitigate negative thoughts surrounding HI, and allow a better acceptance and integration of people with HI in the community. This study shows that the cause of HI can be perceived as a curse similar to other communities, and people with HI are faced with stigma, discrimination, and difficult access to proper health care management. Additionally,

policymakers should invest in creating more special schools, training more teachers and healthcare practitioners, equipping healthcare services, and funding the management of HI to improve the access of persons with HI to basic social services. Lastly, our participants were concerned about the occurrence of inherited HI cases and recommended the implementation of a premarital screening to reduce its burden. This highlights the necessity to train genetic counsellors and intensify genetic research to identify the genes that contribute to inherited HI cases in Cameroon in order to provide appropriate genetic counselling to families.

Chapter 7: General Discussion and Conclusions

7.1. General Discussion

Epidemiology and aetiologies of HI in Cameroon

Disabling HI affects approximately 3.6% of the general population in Cameroon, and its prevalence rises to a level of 14.8% in people aged 50 years or more [316]. In contrast with high-income countries where HI is mainly inherited, the environment constitutes the major aetiology of HI in Cameroon (a low-income country), as it contributes to 52.6% to 62.2% of HI cases [6,57,316]. Diseases that are preventable by vaccination i.e. meningitis, measles, rubella, and mumps constitute the leading environmental aetiologies, as they are incriminated in 41.7% of HI cases in Cameroon [6]. These findings are consistent with studies performed in other SSA countries, including The Gambia and Sierra Leone, where infectious diseases that are preventable by vaccination represent 35.1% and 44.7% of HI aetiologies, respectively [35,67]. This reflects the weakness of health care systems in low- and middle-income countries, and emphasises the need to strengthen immunisation programs, and adequately equip health care facilities for proper prevention and management of HI-causing diseases [6,316]. Monogenic HI constitutes approximately 15% of all HI cases in Cameroon [6]. This lower contribution of heredity to HI was also reported in other African countries, including Nigeria (13.1%), The Gambia (8.1%), and Ghana (21.3%) [44,67,68]. However, the contribution of genetics to HI in Cameroon might be underestimated, as 32.6% to 37% of HI cases remain of unknown origin [6,57,316]. Indeed, in the absence of a molecular diagnosis, genetic HI cases that occur sporadically will be classified as being of unknown origin [6]. There is therefore the need to intensify genetic research in Cameroon to identify the most common genes and variants that contribute to hereditary HI [316]. This study has contributed to addressing such need in Cameroon, specifically with a recruitment strategy that covers all the 10 administrative provinces, and included participants from nearly all the ethnolinguistic groups of the Cameroonian populations.

Connexin genes do not contribute significantly to NSHI in Cameroon

As many other studies performed previously on African populations, our study confirmed that variants in connexin genes, especially *GJB2* gene do not contribute significantly to HI in the Cameroonian population. *GJB2* pathogenic and likely pathogenic (PLP) variants are known to be responsible for up to 50% of NSHI cases in Europeans and Asians, the deletion *GJB2*-c.35delG being the most frequent variant observed in these populations and accounting for up to 70% of all *GJB2* variants [7,42,316]. Other variants in *GJB2* gene were also found to be specific to some populations, including p.V37I and p.L79Cfs in Asians, p.W24X in Indians, and p.L56Rfs in Americans [388]. The founder variant *GJB2*-p.Arg143Trp was shown to be

frequent in Africans from Ghana, and accounts for approximately 25% of familial cases and 8% of isolated cases of HI from Ghana [44,388]. With the exception of Ghana, and as confirmed with the investigations of multiplex families segregating NSHI in Cameroon reported in the present thesis, *GJB2* PLP variants have a prevalence close to zero in most African populations (including Cameroonian, Nigerian, black South African, Ugandan, Kenyan, and Sudanese), and should not be screened routinely in clinical settings [14,15,93,94,317,345].

DMD burden in Cameroon is underestimated, and *DMD* is not associated with HI in humans

Our study has shown that patients with DMD in Cameroon experience first symptoms at an average age of 4.6 years, and include toe walking, difficulties climbing stairs, frequent falls, late walking, and difficulties running [389]. This age at onset is comparable to that of various populations around the world [253]. However, we noticed that diagnosis of DMD in our cohort was evoked later in adolescence at an average of 12.1 years, which is higher than that of most high-income countries [278,279]. This late diagnosis observed in our cohort suggests that the burden of DMD in Cameroon might be underestimated, and also highlighted the need to aware the general population and health professionals about the most frequent clinical signs of DMD i.e. calf hypertrophy, proximal and distal muscle weakness of the lower and upper limbs, Gowers' sign, muscle wasting, and flexion contracture of joints [282,283,291,389]. Variants associated with DMD in our cohort i.e. exon deletions and duplications are clustered between exon 45 and 50 of *DMD*, suggesting that screening of future patients from Cameroon with DMD should focus on the distal part of *DMD*. In contrast to studies performed on *mdx* mice which found a significant sensorineural HI associated with *DMD* gene [26], our study suggests that variants in *DMD* are not associated with HI in humans [389]. This study is one of the rare reports from Central Africa that describes the clinical and genetic profile of *DMD*-related muscular dystrophy. Despite the molecular testing been performed in a relatively limited number of patients (11/17), the data provide the much-needed insight into the features of this condition among black Africans.

NGS techniques are state of the art for the diagnosis of hereditary HI in Cameroon

Because of the high genetic heterogeneity of NHSI and the low pick-up rate when using targeted gene screening to solve HI cases in Cameroonians as shown by the present study, NGS appears as the most effective method to identify genes and variants that contribute to NSHI in Cameroon [5,316]. NGS offers the possibility of interrogating several genes at a time, which constitutes an advantage in using it in Cameroon, given the genetic diversity of the Cameroonian population (there are more than 200 distinct local languages in the country) [5,227]. Using WES, we successfully identified the candidate genes and variants in two unrelated Cameroonian families that were negative for PLP variants in *GJB2* gene, and

specifically contributed to confirm two candidates HI-associated genes, i.e. *CLIC5* and *DMXL2*, that deserve to be included in targeted diagnosis panels, since both of them were associated to HI only once, prior the present study. The efficacy of NGS techniques has been proven through various studies amongst populations where no candidate variants were found in common HI-genes [316]. In 2016, through the use of targeted genomic enrichment and massively parallel sequencing to interrogate a panel of 116 known HI-genes, Lebeko et al. [21] were able to find the candidate genes in 7 out of the 9 Cameroonian families investigated. The use of targeted exome sequencing has allowed the identification of variants that co-segregate with HI phenotype in 60% of Japanese families that presented with NSHI and that were negative for PLP variants in common NSHI-genes [96].

Our study adds more value to the use of NGS to solve HI cases amongst Cameroonians and other African populations. However, because of the associated high cost of the equipment and the computational challenges posed by the approach, NGS is not routinely used in developing countries [177]. There is therefore a need to build capacities to generate and analyze NGS data in Cameroon and Africa in general. Strategies to successfully implement the use of NGS technologies to solve hearing impairment cases and other genetic disorders in Africa should include:1) secure research grants to financially support the training of qualified scientists and the establishment of sequencing facilities; 2) take advantage of existing research networks like the human hereditary and health in Africa (H3Africa) [390] initiative and collaborate with other scientists on the continent and abroad to facilitate training and knowledge transfer; 3) establish continuing training programs for scientists in the analysis of NSG data; 4) establish and maintain high-performance computing and data storage facilities; 5) develop and implement a data management plan; 6) perform community and public engagement activities to engage policy makers and populations, and address ethical, legal, and social issues related to the handling of genomic data.

Variants in *CLIC5* contribute to HI in humans

Our study through the use of WES identified for the first time variants in *CLIC5* gene as candidates for HI in a multiplex Cameroonian family that segregates prelingual and progressive ARNSHI. The identified novel variants i.e the missense [NM_016929.5:c.224T>C; p.(Leu75Pro)] and the splicing (NM_016929.5:c.63+1G>A) occurred as compound heterozygous in the three affected family members. The contribution of *CLIC5* to NSHI in humans was originally demonstrated by a report from Turkey [318], through the combined use of homozygosity mapping and candidate gene sequencing. The authors of the aforementioned study identified a homozygous nonsense variant [NM_016929.5:c.96T>A; p.(Cys32Ter)] that abrogates the protein function and co-segregates with ARNSHI (DFNB103) in a Turkish family [318]. The two affected individuals from the original Turkish family presented an early onset

sensorineural HI which started mildly and progressed to severe-to-profound HI. This HI phenotype is similar to that described in the present study, as our three affected participants described a history of prelingual HI, and presented with profound sensorineural HI at the time of the study [318].

CLIC5 protein is implicated in chloride ion transport within various subcellular compartments [391], and has three isoforms: CLIC5A (251aa), CLIC5B (410aa), and CLIC5C (205aa). CLIC5A was initially isolated from placental epithelial cell microvilli as a component of the actin-based cytoskeletal complex, interacting with actin and ezrin at the cell cortex [391,392]. It was later shown to be highly expressed in the ear hair cell stereocilia, and important for sensorineural hearing (mice deficient for the protein exhibited impaired hearing and vestibular dysfunction) [319]. The main function of CLIC5A in the ear is the stabilization of membrane-actin filament linkages at the base of hair cell stereocilia [319]. In the ear, CLIC5A colocalizes with radixin (RDX), taperin (TPRN), and myosin VI (MYO6) at the base of hair cell stereocilia [341]. It was shown that RDX, protein tyrosine phosphatase receptor Q (PTPRQ), and TPRN (all associated with HI) are present at the base of hair cell stereocilia and were mislocalized in CLIC5-deficient mice.

Additionally, CLIC5A was shown to be particularly important for the compartmentalization/ localization of PTPRQ, RDX, and TPRN in the hair cell rootlet (stereocilia base) and CLIC5A-deficient hair cells showed diffusion of PTPRQ, RDX, and TPRN [341]. Therefore, a variant that abrogates CLIC5A or destabilizes its activity would lead to destabilization of actin-based complexes, fusion, and elongation of hair cell stereocilia, and consequently impaired hearing [318,341]. Our study is the second of its kind worldwide, and the very first one to show the contribution of *CLIC5* to NSHI in an African population. It is now clear that *CLIC5* is associated with HI in humans, and should thus be included in targeted diagnostic gene panels as a novel NSHI-gene.

DMXL2 is associated with HI in humans

A second Cameroonian family with NSHI was also investigated through the use of WES, and we identified an autosomal dominantly inherited novel missense variant [NM_001174116.2:c.918G>T; p.(Gln306His)] in *DMXL2* (MIM:612186) that co-segregates with mild to profound non-syndromic sensorineural HI. A missense variant in *DMXL2* was found to be associated with HI for the first time in 2017 by Chen et al. [347] in a large 7-generations Han Chinese family with AD HI (DFNA71) that segregated a mono-allelic missense variant p.(Arg2417His) in *DMXL2*. The phenotype of patients included in the present study has some similarities with Han Chinese patients reported by Chen et al. [347], as they equally presented with bilateral and progressive sensorineural NSHI that was inherited in an

AD manner, without clinical signs of vestibular dysfunction or any syndromic abnormality. Chen et al. [347] described in their study a late onset of the HI (occurring during the second decade of life), while two of the three patients reported here, present a prelingual onset of HI, with at least one patient presenting with congenital HI. Additionally, one of the three affected Cameroonian family members presented with mild HI, while the two others presented profound HI, suggesting variable expressivity, a well-known feature of many AD traits [360]. Our results thus suggest that variants in *DMXL2* might lead to a NSHI phenotype that can occur in infancy and presents with variable expressivity. Studies of more individuals with HI due to *DMXL2* variants are needed to refine the phenotype. Variants in *DMXL2* have been associated with diseases other than HI. A biallelic 15-bp in-frame deletion in *DMXL2* (p.1942_1946delSDGNG) was identified in a Senegalese family and led to polyendocrine-polyneuropathy syndrome (PEPNS; MIM:616113) [360]. Early infantile epileptic encephalopathy-81 (EIEE81; MIM:618663) which is also called Ohtahara syndrome was recently shown to be associated with variants in *DMXL2* [361].

DMXL2 (the protein encoded by *DMXL2*) is a vesicular protein made up of 3036aa and is involved in the Ca2+-dependent translocation of neurotransmitters across synaptic membranes [362,364]. RC3 was shown to be expressed in various tissues, including the brain, and ear hair cells [347,363]. The exact neurophysiological role of RC3 in the brain remains elusive [362]; however, studies on the rat brain suggest that RC3 may serve as a scaffold protein for Rab3A and its effector proteins [365]. In mammalians and mice, DMXL2 was shown to be essential for brain development and autophagy [362,366]. The implication of DMXL2 in the hearing process was demonstrated by studies performed in zebrafish and mice. Indeed, the zebrafish DMXL2 ortholog (Rbc3α) was shown to be expressed in the inner ear of zebrafish and was localized at the basal region of hair cells [359]. Mutant alleles of *rbc3α* isolated from a large cohort of zebrafish led to HI and vestibular dysfunctions [359].

Besides, expression studies have found the expression of Dmlx2 (the mouse DMXL2 ortholog) in mouse cochlea [347]. Dmxl2 in mice is expressed in the basal region of hair cells where synaptic vesicles are present, and the neurofilament extremity of spiral ganglion neurons projected into the hair cells, suggesting that DMXL2 may function on the presynaptic and postsynaptic sides of the hair cell innervations [347]. Our study demonstrates for the first time that *DMXL2* is a candidate gene for NSHI in an African population (Cameroon), and also confirms the contribution of *DMXL2* to HI in humans. *DMXL2* should therefore be considered in diagnostic HI panels. The present study enriches the list of HI-candidate genes in Africans and highlights the value of African populations for the identification of novel genes and variants associated with NSHI.

Perception of HI, and challenges faced by persons with HI in Cameroon

Our project revealed that perceived causes of HI in Cameroon include environmental factors, genetics, and a spiritual curse. Also, people with HI are often considered mentally disabled. Improving the knowledge of people about what HI is will contribute to ameliorate the social integration of people with HI, as our results showed that in communities where HI is associated with supernatural forces or intellectual disability, the marginalisation and social exclusion of people with HI is heightened. The wrong perception of the causes of HI and the stigmatisation thereof associated is also noticed in other SSA countries [31,376]. In Ethiopia, some communities believe that people with HI are possessed by the devil and should be cured by purified water [376]. A study from Burundi mentioned the family exclusion and social isolation of people with HI as some of the main challenges they face [31]. This perception of HI as resulting from supernatural forces is probably due to the non-understanding of the causes of the disease and emphasises the need to educate the general public on the causes of HI, which should, in turn, allow better acceptance of people with HI in the society.

In addition to the socialisation issue, people with HI are faced with challenges in accessing education and healthcare services. Indeed, the limited number of special schools for the deaf, the high cost of tuition, and the lack of secondary and tertiary institutions for persons with HI in Cameroon contribute to the education issue of persons with HI. Additionally, the lack of a universal health insurance scheme and some therapeutic procedures such as cochlear implantation constitute a barrier to the proper management of HI in Cameroon. A study from Nigeria also highlighted the challenges persons with HI face in accessing basic social services [386]. To improving the access of persons with HI to proper management in Cameroon, policymakers should therefore create more special schools, train more qualified healthcare workers, equip educational and healthcare facilities, and implement a health insurance scheme for people with HI. Prior to our study, the perceptions of the causes of HI, and the expectations of people with HI in Cameroon remained elusive. This study, therefore, provides a much-needed insight into the expectations of persons with HI in Cameroon i.e 1) improve access to basic social services, 2) create awareness on the causes of HI and challenges experienced by persons with HI, 3) reduce the burden of inherited HI and implement newborn screening for HI, and 4) create vocational centres.

7.2. Strengths and Limitations

Our attempt to determine the prevalence of HI in Cameroon through a systematic review of the available data was limited in that, the only two population-based studies that reported on the prevalence of HI in the Cameroonian population were both conducted in one of the 10 administrative regions of Cameroon [57,66]. However, the estimate of the prevalence (0.9 –

3.6%) obtained from this region gives us an idea of the public health burden of HI in Cameroon. Also, our study is the first one to summarise existing data on the aetiologies of HI in Cameroon.

The assessment of the hearing of the 17 patients with DMD included in our study was based on the conversational voice [389]; therefore, DMD patients with mild to moderate HI could have been missed. Besides, intellectual disability, which affects nearly one-third of DMD patients [315] was not formally assessed in our cohort. Despite these limitations, our study has significant clinical implications, as it described the most frequent clinical signs of DMD in Cameroon, as well as the most common associated variants. This should allow an early diagnosis of future patients.

Although the variants identified in *CLIC5* and *DMXL2* are predicted by various bioinformatics tools to be deleterious and alter the structure and function of the respective proteins [393,394], we did not explore through functional assays the definite impact of these variants on *CLIC5* and *DMXL2* functions and expressions. Also, we found the genetic aetiology of HI in only two families with NSHI, which is not enough to establish the panel of genes that contribute to NSHI in the Cameroonian population. The present study, however, has a major contribution to science, as it adds two more candidate genes to the list of HI-causing genes in Africans and highlights the value of African populations in identifying novel genes and variants associated with NSHI.

With regards to the qualitative part of our project, a major limitation is that participants were recruited from only three of the 10 administrative regions of Cameroon. Also, the three persons with HI included in our study were from well-established schools for the deaf, therefore, they had access to education and some awareness. However, this qualitative part of the study explores various aspects of HI and reports for the first time on the perceptions of the causes of HI, and the expectations of deaf individuals in Cameroon. This study also has a public health impact, as it will aware policymakers of the necessity to educate populations about the causes of HI and improve the access of persons with HI to basic social services.

7.3. Conclusions

Diseases that are preventable by vaccination such as meningitis, and those that can easily be managed by primary healthcare facilities such as impacted wax constitute the major aetiologies of HI in Cameroon. This suggests the need to improve the immunisation program, and to improve access to primary health care services. Our project confirms that variants in *GJB2* and *GJB6* genes do not contribute to NSHI in Cameroon as was the case reported in most African countries, except Ghana where the founder variant *GJB2*-p.Arg143Trp contributes to up to 26% of hereditary NSHI cases. Therefore, screening of connexin genes in African patients should not be conducted routinely. Additionally, our study suggests that

only *GJB2* and *GJB3* are recognized and validated HI genes. Variants in *DMD* that were shown to be associated with an increased hearing threshold in mouse, do not seem to be implicated in HI in Cameroonians. Despite the first symptoms of DMD occurring in infancy, the diagnosis is frequently made later in adolescence, indicating an underestimation of the number of cases of DMD in Cameroon, and suggesting the necessity to inform/educate the general population and health professionals of the common clinical signs of DMD. Future screening of deletions and duplications in patients from Cameroon should focus on the distal part of the *DMD*. Subsequently, this study successfully identified the candidate genes in two Cameroonian multiplex families with NSHI through the use of WES, and thus highlights the efficacy of NGS techniques in resolving HI cases in populations of African descent and in cases where no pathogenic variants are found in common HI-genes. Besides, our project which confirms that *CLIC5* and *DMXL2* genes are associated with HI in humans advocates for the inclusion of these two genes in diagnostic gene panels for NSHI in clinical settings. Lastly, this study shows that the cause of HI can be associated with a spiritual curse in some communities, and people with HI are faced with stigma, discrimination, and difficult access to proper management. It is thus a necessity to build facilities and capacities for proper education and management of persons with HI. Our report also highlights the need to educate populations on the causes of HI for a better acceptance of persons with HI in Cameroonian society.

7.4. Perspectives

The national prevalence and incidence of HI in Cameroon remains elusive; it is, therefore, necessary to conduct a country-wide study to determine the epidemiology of HI in Cameroon. The use of WES should be extended to (1) whole Genome Sequence to also capture possible effect non-coding and copy number variation variants, and (2) more Cameroonian families and additional African populations, in order to establish a comprehensive list of clinically actionable NSHI-genes specific to African populations. Building an African-specific deafness gene panel should allow an early diagnosis of HI, and increase the detection rate of monogenic HI in SSA, where about 33 to 60% of HI cases remain of unknown origin [316]. Also, gene expression and functional studies using cell lines and/or animal models should be performed on the newly identified (*CLIC5* and *DMXL2*) genes to assess the real impact of the candidate variants on the protein function and better understand the pathophysiology of HI in Africans, and contribute to improve the disease-gene pair curation, globally. Researchers and policy-makers should be involved in organising awareness campaigns to educate populations on the aetiologies of HI and mitigate stigma and negative considerations surrounding HI in some African communities.

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Appendices

Appendix 1: Supplementary materials

Connexin Genes Variants Associated with Non-Syndromic Hearing Impairment: A Systematic Review of the Global Burden

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Supplementary Materials

Table S1: Connexin genes primers sets used for targeted sequencing.

Gene	Forward/Reverse Primer	Frequency
GJB2	TTGGTGTTTGCTCAGGAAGA/GGCCTACAGGGGTTTCAAAT	8
GJB2	TCTTTTCCAGAGCAAACCGC/GACACGAAGATCAGCTGCAG	7
GJB2	CCTATGACAAACTAAGTTGGTTC/GACAGCTGAGCACGGGTTGCCTC	4
GJB2	GCTTACCCAGACTCAGAGAAG/CTACAGGGGTTTCAAATGGTTGC	4
GJB2	TCCGTAACTTTCCCAGTCTCCGAGGGAAGAGG/CCCAAGGACGTGTGTTGGTCCAGCCCC	4
GJB2	CCAGGCTGCAAGAACGTGTG/GGGCAATGCGTTAAACTGGC	3
GJB2	CTCCCTGTTCTGTCCTAGCT/CTCATCCCTCTCATGCTGTC	3
GJB2	TCTTTTCCAGAGCAAACCGC/GGGCAATGCGTTAAACTGGC	3
GJB2	AGTGGCCATGCACGTGGCCTA/TGATCTCCTCGATGTCCTTAA	2
GJB2	CCAGGCTGCAAGAACGTGT/ACAGCTGAGCACGGGTTG	2
GJB2	CCGGGAAGCTCTGAGGAC/GCAACCGCTCTGGGTCTC	2
GJB2	CTCCCTGTTCTGTCCTAGCT/AGCTGAGCACGGGTTGCCTCA	2
GJB2	CTGTCCTAGCTATGTTCC/TCAGCACGGGTTGCCTCAATC	2
GJB2	GAGGTTGTGTAAGAGTTGGTGTT/TCTTCTCATGTCTCCGGTAG	2
GJB2	GCCGCCCCCTCCGTAACTTTC/CGTGTGTTGGTCCAGCCCCCC	2
GJB2	GCTTACCCAGACTCAGAGAAG/CTTAATCTAACAACTGGGCAATGC	2
GJB2	GTCTCCCTGTTCTGTCCTA/TCTAACAACTGGGCAATG	2
GJB2	TCAAAGGAACTAGGAGATCGG/CAAGGACGTGTGTTGGTCCAG	2
GJB2	TCTTTTCCAGAGCAAACCGC/CTGGGCAATGCGTTAAACTGG	2
GJB2	TCTTTTCCAGAGCAAACCGCC/TGAGCACGGGTTGCCTCATC	2
GJB2	TGCTTACCCAGACTCAGAGAA/GACTGAGCCTTGACAGCTGAG	2
GJB2	AAGGGGCTGGCTCTTCA/GCACTTGGCCTGGGTAA	1
GJB2	AAGTCTCCCTGTTCTGTCCTAG/CCTCATCCCTCTCATGCTGTC	1
GJB2	ACACGTTCAAGAGGGTTTGG/CCTCATCCCTCTCATGCT	1
GJB2	ACACGTTCAAGAGGGTTTGG/GGGAAATGCTAGCGACTGAG	1
GJB2	ACTCATGGGGGCTCAAAGGA/GCAACCGCTCTGGGTCTCGCGGTCCCT	1
GJB2	AGAAGAAGAGGAAGTTCATCA/CCTCTCATGCTGTCTATTTC	1
GJB2	AGAAGAAGAGGAAGTTCATCAAGG/CTGAGGCCTACAGGGGTTTC	1
GJB2	AGACTCAGAGAAGTCTCCCTG/GACACGAAGATCAGCTGCAG	1
GJB2	AGACTCAGAGAAGTCTCCCTG/GCCAGTTTAACGCATTGCCC	1
GJB2	AGAGTGGTGTTTGCTCAGGA/GACTGAGCCTTGACAGCTGA	1
GJB2	AGCAAACCGCCCAGAGTAGAAG/TGATCTTCGTGTCCACGCCAG	1
GJB2	AGGCCGACTTTGTCTGCAACA/GTGGGCCGGGACACAAAG	1
GJB2	AGTCTCCCTGTTCTGTCCTA/TGAGCACGGGTTGCCTCATC	1
GJB2	AGTGCCTTTCAGCTAACGA/GTGGCATCTGGAGTTTCACC	1
GJB2	CACGCTGCAGACGATCC/GGTGGAGTGTTTGTTCAC	1
GJB2	CACTTTCCCAAGGCCTCTTCCAC/GTACGTCCACCACAGCGAC	1
GJB2	CAGTCTCCGAGGGAAGAGG/AAGGACGTGTGTTGGTCCAG	1
GJB2	CAGTCTCCGAGGGAAGAGG/GCAACCGCTCTGGGTCTC	1
GJB2	CCACGCCAGCGCTCCTAGTG/GAAGATGCTGCTGCTTGTGTAGG	1
GJB2	CCAGCATGACCTTTACCAG/ATCACTTGAATAAGAAGCCATTTG	1
GJB2	CCAGGCTGCAAGAACGTGTG/GACACGAAGATCAGCTGCAG	1
GJB2	CCAGGCTGCAAGAACGTGTG/RTGAGCACGGGTTGCCTCATC	1
GJB2	CCAGGCTGCAAGAACGTGTG/TGAGCACGGGTTGCCTCATC	1
GJB2	CCCTCCGTAACTTTCCCAGT/CCAAGGACGTGTGTTGGTC	1

GJB2	CCCTCCGTAACTTTCCCAGT/GCAACCGCTCTGGGTCTC	1
GJB2	CCTATGACAAACTAAGTTGGTTC/GCCTCATCCCTCTCATGCTGTC	1
GJB2	CCTCCGTAACTTTCCCAGT/AAGGACGTGTGTTGGTCCAG	1
GJB2	CCTGTGTTGTGTGCATTCGTC/CTCATCCCTCTCATGCTGTC	1
GJB2	CGAAGCCGCCTTCATGTACG/TTAGGGGAGCAGAGCTCCAT	1
GJB2	CGCACTATGCGGAGTACAGA/GGTGGCAGTGGGTCAAGTAG	1
GJB2	CGCGCTCCTCTCCCCGACT/TCCTTTGCAGCCACAACGAGGAT	1
GJB2	CGGAGACATGAGAAGAAGAGG/GATCTCCTCGATGTCCTTAA	1
GJB2	CGTAACTTTCCCAGTCTCCGAGGGAAGAGG/CCCAAGGACGTGTGTTGGTCCAGCC	1
GJB2	CGTAGCACACGTTCTTGCAGCCTG/CGATGCGGACCTTCTGGGTTTTG	1
GJB2	CGTCTTTTCCAGAGCAAACCG/AGCTCCATTGTGGCATCTGG	1
GJB2	CGTTCGTTCGGATTGGTGAG/CAGAAACGCCCGCTCCAGAA	1
GJB2	CTCATGGGGGCTCAAAGGAACTAGGAGATCGG/GGGGCTGGACCAACACACGTCCTTGGG	1
GJB2	CTCATGTCTCCGGTAGGCCAC/GCAGCATCTTCTTCCGGGT	1
GJB2	CTGGTGCTACGATCACTAC/TTCCAGACACTGCAATCATG	1
GJB2	CTTTTCCAGAGCAAACCGC/GGGCAATGCGTTAAACTGGC	1
GJB2	FTCTTTTCCAGAGCAAACCGCC/GACACGAAGATCAGCTGCAG	1
GJB2	GAAGTAGTGATCGTAGCACACGTTCTTGCA	1
GJB2	GAAGTCTCCCTGTTCTGTCCT/TCTAACAACTGGGCAATGC	1
GJB2	GAGCCTTCGATGCGGACCTT/TCATCCCTCTCATGCTGTC	1
GJB2	GCATTCGTCTTTTCCAGAGC/GGCCTACAGGGGTTTCAAAT	1
GJB2	GCATTCGTCTTTTCCAGAGCA/GAGCCTTCGATGCGGACCTT	1
GJB2	GCCAGGCTGCAAGAACGTGT/GGAGAAGCCGTCGTACATGA	1
GJB2	GCGCAAGCTTTATGGATTGGGGCACGCT/GCGCGGATCCCTAACTGGCTTTTTTGAC	1
GJB2	GCTTACCCAGACTCAGAGAAG/TGAGCACGGGTTGCCTCATC	1
GJB2	GGGCAATGCGTTAAACTGGC/CCAGGCTGCAAGAACGTGTG	1
GJB2	GGGGAGATGAGCAGGCCGAC/CGGCTGGTGAAGTGCAACGC	1
GJB2	GGGGCTCAAAGGAACTAGGA/AAGGACGTGTGTTGGTCC	1
GJB2	GGGGGCACTTGGGGAACTCA/GCAGAAACGCCCGCTCCAGAA	1
GJB2	GGTGAGGTTFTFTAAGAGTTGG/CTACGGTGTTACTCGAGACGA	1
GJB2	GGTGAGGTTGTGTAAGAGTTGG/TAGCGACTGAGCCTTGACAG	1
GJB2	GTCTCCCTGTTCTGTCCTAG/CTTCGAAGATGACCCGGAAG	1
GJB2	GTGCATTCGTCTTTTCCAGAGCA/TTGACAGCTGAGCACGGGTTG	1
GJB2	GTGGCCTACCGGAGACAT/CACTCTTTATCTCCCCCTTG	1
GJB2	GTGTGCATTCGTCTTTTCCAG/GCGACTGAGCCTTGACA	1
GJB2	GTGTTGTGTGCATTCGTCTTTTC/ACCTTCTGGGTTTTGATCTCCTC	1
GJB2	TACGATGGTTTTTCCTCTAATTCT/TTGCATAACTTAGTGAACTCAGAG	1
GJB2	TATGTCATGTACGACGGCT/TCTAACAACTGGGCAATGC	1
GJB2	TATGTTCCTGTGTTGTGTGC/CCTTCTGGGTTTTGATCTCC	1
GJB2	TCAAGGGGGAGATAAAGAGT/TGAGCACGGGTTGCCTCATC	1
GJB2	TCAAGGGGGAGATAAAGAGTG/TGAGCACGGGTTGCCTCATC	1
GJB2	TCAGAGAAGTCTCCCTGTTCTGTCC/TGAGGCCTACAGGGGTTTCAA	1
GJB2	TCCGTAACTTCCCAGTCTCCGAGGGAAGAGG/CCCAAGGACGTGTGTTGGTCCAGCCCC	1
GJB2	TCCGTAACTTTCCCAGTCTCCGAGGGAAGAG/CCCAAGGACGTGTGTTGGTCCAGCCC	1
GJB2	TCCGTAACTTTCCCAGTCTCCGAGGGAAGAGG/CCCAAGGACGTGTGTTGGTCCAGCCC	1
GJB2	TCCTGGGGGTGTGA/CCTGGGGGGTGTG	1
GJB2	TCGGCCCCAGTGGTACAG/CTGGGCAATGCGTTAAACTGG	1
GJB2	TCTTTTCCAGAGCAAACCGC/GTTGGGAAATGCTAGCGACT	1
GJB2	TCTTTTCCAGAGCAAACCGC/TGGGCAATGCGTTAAACTGGC	1
GJB2	TCTTTTCCAGAGCAAACCGC/TTGCCTCATCCCTCTCATGCTGT	1
GJB2	TCTTTTCCAGAGCAAACCGC3/CTGGGCAATGCGTTAAACTGG	1
GJB2	TCTTTTCCAGAGCAAACCGCC/CACGTGCATGGCCAGTAG	1
GJB2	TGCATCACCTCACATAGGTTA/TATTGGATACTTGAATCTGCTG	1
GJB2	TGCTTACCCAGACTCAGAGAA/CGACTGAGCCTTGACAGCTGA	1
GJB2	TGCTTGCTTACCCAGACTCA/CCTCATCCCTCTCATGCTGT	1
GJB2	TGGGGAACTCATGGGGGCTCAAAG/AGGTTCCTGGCCGGGCAGTCC	1
GJB2	TGGTGGCCATGCATGTGGCCTAC/CAGAAGTCTCCTTATGACGCAGC	1
GJB2	TGGTGTTTGCTCAGGAAGAG/TTGTGTAGGTCCACCACAGG	1
GJB2	TGTGCATTCGTCTTTTCC/CAGATCTTTCCAATGCTGGT	1
GJB2	TGTGCATTCGTCTTTTCCAG/CAGATCTTTCCAATGCTGGT	1
GJB2	TGTGCATTCGTCTTTTCCAG/GGGAAATGCTAGCGACTGAG	1
GJB2	TTGGGGCACGCTGCAGACGATCCTGGGGAG	1
GJB2	TTGGTGTTTGCTCAGGAAGA/GGCATCTGGAGTTTCACCTG	1
GJB2	TTTGCTCAGGAAGAGATTTAAGCA/GGGTTTTGATCTCCTCGATGTCC	1

GJB6	AGACTAGCAGGGCAGGGAGT/GGTTGGTATTGCCTTCTGGA	1
GJB6	GACTAGCAGGGCAGGGAGTT/CTCTTCTCTCCTCGCCTGAA	1
GJB6	TCCGTAACTTTCCCAGTCTCCGAGGGA AGAGG/CCCAAGGACGTGTGTTGGTCCAGCCCC	1
GJB6	GTCTGTAATATCACCGTGTCAC/CTCTTCAGGCTACAGAAGGAAC	1
GJB6	GGGCAGGGAGTTGAAGTTG/ACTCTTCAGGCTACAGAAGGAAC	1
GJB6	CTTTGCCCACTTTTGTCTGT/GACCCCTCTATCCGAACCTT	1
GJB6	TTTAGGGCATGATTGGGGTGATT/CACCATGCGTAGCCTTAACCATTTT	1
GJB6	CTCCTTTAGGGCATGATTGG/CCATGCGTAGCCTTAACCAT	1
GJB6	GACCACTTTTTCCCGGTGT/AGGTTGGTATTGCCTTCTGG	1
GJB6	CCTCCAGCTGATCTTCGTCT/GCAGCAGGTAGCACAACTCT	1
GJB6	TGCCCACCCCCCAAGTAGAG/TTTCGGTTTCATTCATTTTCCCTATT	1
GJB6	GCCCCAACCTTGTTGACTGC/GTTGGTATTGCCTTCTGGAG	1
GJB6	GGCAGGGAGTTGAAGTTGTA/ACGTTGTGTATGAATGGAGCA	1
GJB6	GAAAGGAAGGTCGGGCAAGG/CACAATCAAACCTCACTGCCATCTT	1
GJB6	GTTGCTTGTGCTTTTGGTGTCAT/AGCCCAGAAACAAACCCTTACATA	1
GJB3	GCAGGTAGGCAAGCCCCCACCAG/GCCACACTGCCCTGCATTTCCC	1
GJB3	ATTCTCTCAGGTAGGCACGG/TGGTGTTGCAGTCAAAGTCC	1
GJB3	CTATTCATTCATACGATGG/TCACTCAGCCCCTGTAGGAC	1
GJB3	CCCAGTTCCCAGTGTCAAA/TACGACAACGCAGGCAAGA	1
GJB3	TACGATGGTTTTTCCTCTAATTCT/TTGCATAACTTAGTGAACTCAGAG	1
GJB3	TATACGTGGTGGCTGCAGAG/CTGCGTTGTCGTACAGCTTG	1
GJB3	TAGGTCGGGCAATGTAGCA/GAACTCAGAACACTGCCTGGT	1
GJB3	CTCGCTGCTGGTCATCCT/CATATTGAAGCCATGCCAGA	1
GJB3	TTCCTCTACCTGCTGCACAC/GGCAGATGAGGTAGCAGAGC	1
GJB3	AGAGGGTCGTTGTGAGTATTG/AGAGGCGGATGTTGGAGATG	1
GJB3	TGCAGCTTGGGAGGAATAAC/CCCCTGTAGGACCTCTCCAC	1
GJB3	TGCTACGACAACTACTTCCCC/GGCGCCCACCATGAAGTAG	1
GJA1	GAAATACGTGAAACCGTT GG/ CCT GGT GCA CTT TCT ACA GC	1
GJA1	TGCGGTCTACACCTGCAAGA/ACCAAGGACACCACCAGCAT	1
GJA1	CGTGAAACCGTTGGTAGTATTT/CCTCCACCGGATCAAAATTA	1
GJA1	GGAGTTCAATCACCTTGGCGT/TCTTGATGCTTTCAAGCCTGT	1
GJA1	TTTGCAATCTGTGATCCTTGA/CCTGGTGCACTTTCTACAGC	1
GJB4	TCAATCGCACCAGCATTAAG/GGGGGACCTGTTGATCTTATC	1
GJB4	GCATTAAGGGTGCCCATCTC/TTTTCCTGGGTGGCCTCAT	1
GJC3	GCTCCCTCTGAAGGACAGTG /GGGAGGAGATCATCAGGACA	1
GJC3	TGGGTACGCACTGTGAAAAA/AGCTCCTCCTTGGACAGGAT	1

Table S2: *GJB2* variants with no clinical significance data from the three databases used.

Variant	Allele frequency [Reference]
IVS1+12G>A (NM_004004.6:c.+12G>A)	4/126 [1]; 1/1220 [2]
c.IVS1+27G>C	1/152 [3]
c.-3179G>A	1/218 [2]
c.23+G>A	8/90 [4]
51del12insA	2/72 [5]; 2/150 [6]
c.684C>A	1/300 [7]; 1/ 546 [8]; 1/209 [9]; 1/1220 [2]
p.His100ArgfsTer14/+ (c.299- 300delAT)	1/200 [10]
delE119 (c.355-357delGAG)	2/67[11]; 1/40 [12]; 3/150 [13]; 2/32 [14]
682C>T	1/114 [11]; 1/1220 [2]
c.964c>T	5/36 [15]
c.-31T>C	1/418 [9]
c.765C>T	3/113 [16]
p.P87del	1/112 [17]

Table S3: Eight commonly reported connexin 26 gene (GJB2) variants.

Country	p.Gly12 ValfsTer2 (c.35delG) rs80338939	p.M34T (c.101T>C) rs35887622	p.L79Cfs (c.235delC) rs80338943	p.V37I (c.109G>A) rs72474224	p.H100Rfs Ter14 (c.299_300 delAT) rs111033204	p.W24* (c.71G>A) rs104894396	p.L56Rfs (c.167delT) rs80338942	p.R143W (c.167delT) rs80338948	Reference
Japan								2/2	[18]
Japan			10/70	1/70				4/70	[19]
Morocco	61/162			3/162					[20]
Ghana								64/442	[21]
Israel	2/8								[22]
Malaysia				2/66		4/66			[23]
Syria	9/70								[24]
Syria	36/100	2/100					5/100		[25]

Saudi Arabia	14/118			1/118		2/118			[26]
Kuwait	21/200								[27]
Spain	4/4								[28]
Spain	5/29					23/29			[29]
Algeria	55/62								[30]
Algeria	36/50			3/50			2/50		[31]
Algeria	27/232								[32]
Venezuela	5/84								[33]
Greece	7/58								[34]
Turkey	11/58				2/58				[35]
Iran	12/90				2/90				[4]
Bangladesh						19/134			[36]
Morocco	107/304			10/304					[37]
Turkey	25/62								[38]
India						2/116			[5]
Yakutia	51/786			14/786			1/786		[39]
Turkey	96/470								[40]
USA	6/6								[41]
Brazil	74/600	8/600	1/600	1/600		1/600	2/600		[7]
Brazil	3/4								[42]
Slovenia	15/138								[43]
Turkey	2/40				2/40				[44]
Iran	547/4644		1/4644	2/4644	8/4644	31/4644	12/4644	7/4644	[45]
Germany	110/138	1/138		2/138					[46]
Tunisia	44/204								[47]
Brazil	14/66			1/66					[48]
Brazil	14/66			1/66					[49]
Tunisia	6/6								[50]
India	27/116					10/116			[51]
Russia	527/1410	12/1410	14/1410	4/1410		3/1410	12/1410		[52]
Russia	86/222	2/222	1/111						[53]

Germany	8/50								[54]
Iran	7/348		1/348						[55]
Iran	10/1016		2/1016	2/1016	1/1016	4/1016	2/1016	1/1016	[56]
Hungary	2/326								[57]
Romania	3/692								[57]
Ashkenazi							9/372		[57]
Turkey	8/154								[58]
Ghana								44/58	[59]
Pakistan	2/140					2/140			[60]
Germany	13/1012	1/1012					1/1012		[61]
Italy	164/752	5/752					2/752		[62]
Sweden	44/158	1/158						2/158	[63]
São Tomé and Príncipe		2/272							[64]
Guatemala		1/266							[65]
Brazil	8/154	1/154							[66]
China			27/228	84/228	3/228				[67]
China			7/1238						[68]
Iran	10/68		2/68			1/68			[69]
USA	28/146		3/146	47/146	2/146	2/146	1/146	2/146	[70]
USA	20/542	7/542	2/542	40/542	2/542	3/542	2/542	2/542	[71]
China			42/230	6/230	3/230				[72]
China			41/230						[73]
China					1/70				[74]
China			1/58						[75]
China				2/200					[10]
China			118/1402	101/1402					[76]
China	2/11600		356/11600	597/11600	45/11600				[77]
China			6/50	11/50	4/50				[78]
China	9/160		47/160		7/160				[79]
Italy	9/258	1/258				1/258	1/258		[80]

Korea			10/102	1/102					[81]
Brazil	24/98			2/98					[82]
Chile	28/226								[83]
Italy	310/12395	54/12395							[84]
USA	76/98						11		[85]
Brazil	12/154								[86]
Belgium	67/554			5/554			11/554	3/554	[87]
Australia	50/104	5/104		8/104		3/104	1/104	1/104	[88]
China	12/4126		510/4126	200/4126	97/4126			4/4126	[89]
China			721/6008						[90]
China			278/2348	88/2348	19/2348				[91]
Argentina	16/92	1/92							[92]
Argentina	85/952	13/952		7/952			11/952	6/952	[93]
Argentina	46/504	7/504		2/504			2/504	1/504	[94]
Belarus	407/782		5/782				6/782		[95]
Iran	1/2								[96]
Portugal	75/234			1/234		2/234			[97]
Netherlands	8/18								[98]
Brazil	12/154								[99]
Mexico	12/152						2/152		[100]
Brazil	84/414	6/414		4/414					2006
Romania	11/250					8/250			[101]
Romania	11/700								[102]
Turkey	2/2								[103]
Egypt	11/102								[104]
Egypt	13/72								[105]
USA	27/136	1/136	2/136	2/136			3/136		[106]
Iran	49/266								[107]
Brazil	8/202								[108]
Italy	83/120								[109]
Spain	18/120								[109]

Kuwait	18/200								[110]
France	10/376	12/376							[111]
Peru	16/266		1/266	2/266			3/266	21/266	[112]
USA	29								2004
Austria	2/90								[113]
Austria	50/204					3/204			[11]
Austria	20/86					2/86			[12]
Germany	38/268				2/268				[114]
China				15/1300					[115]
China			181/1390		63/1390			5/1390	[116]
Morocco	50/232								[117]
Egypt	14/206								[118]
Argentina	11/712								[119]
Argentina	26/188			2/188			2/188	4/188	[120]
Denmark	9/980	5/980		2/980					[121]
Italy	23/358								[122]
Germany	2/4								[123]
Iran	144/440						9/440		[124]
UK	9139/18404	9112/18404							[125]
Ghana								110/758	[126]
China			2709/142417						[127]
Iran	7/100								[128]
Japan			37/138					15/138	[129]
Sweden	34/4104								[130]
Turkey	101/126				1/126		1/126	1/126	[131]
Canada	12/114		1/114				2		[132]
UK	2/346	14/346							[133]
China			7/598	90/598	1/598				[134]
China			6/68	1/68	1/68				[135]
China			32/7728	38/7728	7/7728			5/7728	[136]
China			2/600	17/600	1/600				[137]

China	35/80								[138]
Taiwan			38/648	58/648	5/648				[139]
Greece	54/214								[140]
Saudi Arabia	2/160								[141]
Eastern Azarbaijan	27/258								[142]
Austria	44/408						1/408		[143]
China	1/352		53/352	23/352	6/352				[144]
China	1/120		12/120		2/120				[145]
China				3/6					[146]
China			26/310	28/310	2/310				[147]
China			1/2		1/2				[148]
India						28/172			[149]
Korea			1/56		1/56			2/56	[150]
Germany	44/674								[151]
Turkey	48/186					3/186			[152]
Turkey	32/130								[153]
Iran	29/206			1/206	1/206	2/206	3/206		[154]
Hungary	60/478	4/478		3/478		4/478			[155]
USA	137/225	22/225	8/225	23/225	1/225	2/225	22/225	1/225	[156]
USA	21/48	4/48					9/48	1/48	[157]
Qatar	9/252								[1]
India	21/100					8/100			[158]
Russia	51/100								[159]
Korea				1/6					[160]
Korea				2/192					[161]
Korea				2/4					[162]
Korea			16/1176	4/1176	3/1176			11/1176	[163]
Korea			3/320	1/320				2/320	[164]
Korea			1/206	2/206				1/206	[165]
Greece	1/3								[166]

Iran	25/280								[167]
Iran	30/68		1/68			1/68			[168]
Japan			7/78						[169]
Thailand			3/34			1/34			[170]
Germany	51/684								[171]
Portugal		2/4							[172]
France	172/1380	44/1380							[173]
Korea			4/44						[174]
Israel	8/14						4/14		[175]
Israel	9/54						43/54		[176]
China			10/432						[176]
China			60/250		14/250				[177]
China			2/2						[178]
USA	25/320	3/320	2/320	2/320					[179]
USA	14/308	3/308	1/302	7/302	3/302	1/302	3/302		[180]
China			9/254	2/254					[181]
China	2/236		48/236	5/236	10/236				[182]
China			2/6		1/6				[183]
Mexico	36/176	1/136							[184]
Finland	26/142	3/142		1/142					[185]
China			39/312	7/312	10/312				[186]
Jordan	40/304								[187]
Syria	8/140						1/140		[188]
Iran	3/124					2/124			[189]
Iran	20/458								[190]
Iran	15/228			1/228		1/228			[191]
Iran	17/154								[192]
India	1/90					1/90		1/90	[193]
China			23/270						[194]
India				2/1060		174/1060		1/1060	[195]
Brazil	41/162								[196]
France	287/414		1/414			4/414	6/414		[197]

France	62/192						2/192		[198]
Mexico	4/4								[199]
Tunisia	10/70								[200]
Portugal						2/12			[201]
Croatia	37/126		1/126					1/126	[202]
Croatia	3/18								[203]
Jordan	22/136								[204]
Gado Bravo	5/88					3/88			[205]
Queimadas	21/152								[205]
USA				2/16					[206]
Rhode Island New	9/84						2/84		[207]
Slovakia	9/108			1/108		25/108			[208]
Slovakia	122/546	6/546		6/546		15/546	3/546		[8]
India	1/30					6/30			[209]
Mauritania	5/278								[210]
Egypt	27/194								[211]
Brazil	6/142								[212]
Italy	40/106								[213]
Iran	25/262		4/262						[214]
Iran	11/336					1/336			[215]
India						7/54			[216]
Cyprus	49/60						1/60		[217]
Japan			96/2454	61/2454	8/2454			8/2454	[218]
Brazil	43/72			3/72					[219]
Iran	1/2								[220]
Iran	1/2								[221]
Italy	136/200	2/200					3/200	1/200	[222]
UK	4/4								[223]
France	3/10								[224]
USA		11/1042	1/1042	5/1042	1/102		24/1042	4/1042	[225]

Korea	1/294		15/294		1/294			1/294	[226]
Ecuador	2/222							3/222	[227]
China			52/180		6/180				[228]
Athens	1/2								[229]
Athens	4/6								[230]
Iran	8/84								[231]
Brazil	14/66			1/66					[48]
Italy	32/308	2/308		1/308			1/308		[232]
Poland	0	17/466	3/466	17/466			7/466		[233]
Bulgaria	45/102					1/102			[234]
Russia	18/152		6/152						[3]
USA	86/418	5/418		2/418					[235]
Italy				1/8					[236]
China	2/2328		290/2328	210/2328	55/2328				[237]
Romania	32/90				1/90	2/90			[238]
Austria	26/42								[239]
Italy	3/78	1/78		1/78					[240]
Iran	39/126								[241]
Tunisia	2/4								[242]
Tunisia	82/262		2/262	2/262					[243]
Tunisia				2/2					[244]
UK	3/102					2/102			[245]
USA	2/288	2/288		3/288					[246]
Colombia	1/10								[247]
France	31/60	5/60	1/60	3/60		1/60		1/60	[16]
Iran	10/106					1/10/6			[248]
Iran	7/80					1/80			[249]
Pakistan						43/340			[250]
Italy	147/204	3/204					12/204		[251]
Sicily	35/128								[252]
USA	1/218	2/218		12/218					[253]
Pakistan						9/392			[254]

Netherlands	24/444	1/444		1/444				1/444	[255]
USA	28/95	2/95	2/95	10/95			1/95		[256]
Czech	10/13								[257]
Czech	113/312					13/312			[258]
Pakistan	19/125					52/125			[259]
Palestin	11/96		2/96				7/96		[260]
USA	13/418	1/418		2/418			3/418	1/418	[9]
China			9/20		1/20				[261]
Korea			31/842		6/842			4/842	[262]
Norway	82/201					2/201			[263]
Austria	56/500	9/500	5/500	10/500		6/500	4/500	1/500	[264]
India	6/632			26/632		38/632			[265,266]
UK	22/48								[266]
Hungary						9/1982			[267]
Indonesia				4/240					[268]
Egypt	24/222			3/222					[269]
Israel	31/150						21/150		[6]
Brazil	47/360	3/360		2/360		2/360	1/360		[270]
Colombia	38/224	3/224					9/224		[271]
USA	120/1220	19/1220	1/1220	38/1220			6/1220	1/1220	[2]
Japan			8/1018	9/1018				/10181	[272]
Turkey	22/188						1/188		[273]
Turkey	33/42						1/42		[274]
Turkey	141/742				1/742	2/742	1/742	1/742	[275]
Mongolia			16/1068	6/1068	4/1068			1/1068	[276]
China			27/200						[277]
China	1/256		28/256		2/256				[278]
United Arab Emirates	14/100								[279]
Hungary	104/NA								2002

Japan	2/2686		142/2686	47/2686	11/2686			18/2686	[280]
Turkey	28/120					3/120			[281]
China			8/34		1/34				[282]
China			22/338		12/338				[283]
China			64/672		18/672				[284]
Thailand	0		10/332	37/332		3/332			[17]
China	2/1316		154/1316	23/1316	33/1316				[285]
USA	6/20								[286]
Austria	11/36	2/36		3/36			1/36		[287]
USA	71/648	28/648	2/648	11/648		1/648	24/648	1/648	[288]
Taiwan			38/840	129/840	8/840			1/840	[289]
Taiwan			25/2034	184/2034					[290]
China			1/4						[291]
China			47/470		6/470				[292]
Taiwan			12/506						[293]
UK						10/354			[294]
China	5/424		164/424		24/424				[295]
China			1/228	2/228	1/228				[296]
Malaysia		1/64		5/64		3/64			[297]
Iran	105/836		1/836	2/836				2/386	[298]
China	4/2134		298/2134	189/2134	27/2134			2/2134	[299]
China			188/2402	34/2402	35/2402				[300]
Azarbaijan	14/258		2/258						[301]
Ghana								37/40	[302]
India						30/430			[303]
Algeria	27/232								[304]
USA	54/238			1/238			3/238		[305]
Turkey	57/346								[306]
Malaysia				1/62		3/62			[307]
USA	301/434	31/434		18/434			22/434		[308]
Turkey	1/14	1/14	1/14				1/14		[309]
Turkey	4/24								[310]

Germany	102/670			4/670	1/670	3/670	3/670		[311]
Slovenia	115/436			5/436		4/436			[312]
Turkey	13/296								[313]
Italy	199/277	15/277		1/277		3/277	3/277	3/277	[314]
Russia	527/1410	12/1410	9/1410	4/1410		3/1410			[315]
Turkey	76/542		4/542			3/542		1/542	[13]
Balkan Gypsies						28/1206			[316]
Russia	29/54		1/54				1/54		[14]
Iran								3/76	[317]
Iran	15/200								[318]
USA	178/386	11/386	3/386	11/386		1/386	23/386	1/386	[319]
Korea			2/290		2/290				[320]
Brazil	12/77								[321]
USA	2/26	14/26							[322]
Brazil	43/370								[323]
Brazil	40/1200	2/1200		2/1200		1/1200			[324]
Iran	26/332				1/332	1/332			[325]
Iran	18/100				2/100				[326]
China			10/30						[327]
USA	7/38	2/38							[328]
Finland	14/184	3/184						2/184	[329]
Portugal	3/13	11/13							[330]
Portugal	19/178	6/178							[331]
China	2/1076		73/1076	24/1076	46/1076				[332]
China	24/4796		292/4796		57/4796			4/4796	[333]
China	1/566		29/566	10/566	6/566				[334]
China	2/968		46/968	10/968	20/968			2/968	[335]
Turkey	2/2								[336]
Egypt	24/102								[337]
Mongolia	6/378		8/378	4/378	4/378				[338]
Turkey	15/112					2/112			[339]

Iran	27/100							5	[340]
Brazil	16/100								[341]
Spain	3/76								[342]
Spain	5/8								[343]
Toronto (Canada)						4/4	8		[344]
Vietnam			2/174	54/174	1/174				[345]
China	2/142		5/142		2/142				[346]
Korea			26/4144	28/4144	3/4144				[347]
China	3/12		3/12						[348]
Iran								2/4	[349]
Iran	10/158								[350]
China	24/2114		340/2114		40/2114			3/2114	[351]
Mexico	7/156	1/156						1/156	[352]
China					3/60				[353]
Greece	111								[354]
Slovak Republic	13					1			[355]
China	2		105	29	25				[356]
Japan			1		1				[357]
USA	33	2					9		[358]
Russia	51								[359]
Korea			8	10					[360]
USA	32			1			2	1	[361]
USA	58								[362]
Japan			16/38	4/38				4/38	[363]
China			2/6		2/6				[364]
Romania	98/358					13/358			[365]
Romania	50/150					8/150			[366]
China	4/324		76/324	13/324	12/324				[367]
Slovenia	3/64								[368]
China			104/1268		10/1268				[369]

USA	47/64			2/64					[370]
China			26/171		2/171				[371]
China	1/420		67/420	1/420	1/420				[372]
China			10/86		2/86				[373]
Finland		19/30		2/30				1/30	[374]
France	14/512								[375]
China			108/1070	12/1070	27/1070			3/1070	[376]
Portugal	84/528	7/528		5/528		5/528	1/528		[377]
Mexico	3/22								[378]
Slovakia	9/108			1/108		25/108			[379]
Morocco	13/278								[380]
India						7/27			[381]
Italy	130/178								[382]
Iran						10/200	2/200		[383]
Hungary	69/1892								[384]
Iran	167/1328					11/1328	3/1328	1/1328	[385]
Cyprus	19/60								[386]
Italy	34/2080	1/2080		2/2080			8/2080		[387]
Poland	2/6								[388]
Japan	3/30	1/30							[389]
India			2/912			52/912	1/912		[390]
India						2/2			[391]
France	14/74	1/74		2/74					[392]
Greece	139/510					2/510			[393]
China	6/760		55/760	10/760	26/760				[394]
Brazil	15/82								[395]
Russia			5/440	2/440	2/440				[396]
Germany	313/NA								[397]
Italy	73/1468	11/1468		4/1468	1/1468		6/1468		[398]
USA	1001/14802	256/14802	36/14802	183/14802	3/14802	33/14802	83/14802	16/14802	[399]
China	1/358		57/358						[400]
India						14/26			[401]

Morocco	24/50								[402]
Croatia	28/54					1/54			[403]
Croatia	41/116			2/116		1/116			[404]
Iran	2/448					4/448			[405]
Czech Republic	113/312	2/312				13/312			[406]
Pakistan	4/300		2/300			9/300			[407]
Israel	2/2								[408]
Turkey	13/70								[409]
16 countries	2103/3062	70/3062	1/3062	56/3062		37/3062	79/3062	10/3062	[410]
Israel	31/150						21/150		[411]
Macedonia	15/66			2/66		4/66			[412]
Latvia	69/130	3/130	1/130						[413]
USA	48/204						9/204		[414]
Belgium	21/32								[415]
Japan			19/106					1/106	[416]
Iran	6/12								[417]
Algeria	34/160								[418]
Algeria	8/22								[419]
USA	1/20							1/20	[420]
Hungary	104/NA								[421]
Romania	17/174								[422]
Tunisia	45/190			1/190					[423]
Venezuela	13/80								[424]
Germany	2/46								[425]
China	24/194		4/194						[426]
Poland	73/204	1/204							[427]
USA			16/428	93/428					[428]
China	2/764		111/764		20/764			8/764	[429]
China			80/465	86/465	6/465				[430]
China			10/180	20/180					[431]

China			2/2						[432]
China			148/1012	33/1012	13/1012			2/1012	[433]
China	2/138		20/138						[434]
China	13/236		25/236						[435]
China			3/42						[436]
China	35/202						4/202		[437]
Australia			31/520		7/520				[438]
Turkey	51/302								[439]
China			222/2380		62/2380				[440]
China			140/678	17/678	38/678			2/678	[441]
China			6/472	12/472	1/472			1/472	[442]
China	1/636		50/636		13/636				[443]
Germany	54/456	9/456							[444]
France	69/280								[445]
USA	304/1474	11/1474		5/1474	4/1474				[225]
USA	33/768			2/768				1/768	[361]

The numerators in this column represent the number of mutated alleles, and the denominators the total number of screened alleles.)
NA, not applicable (the authors were not clear on the total number of alleles they have screened

Table S4: Known but rare PLP *GJB2* variants common in isolated populations.

	Variant	(c.IVS1+1G>A)		p.W172* (c.516G>A)		p.W172C (c.516G>C)		p.W172R (c.514T>A)		p.W44* (c.131G>A)	
	Variant reference number			rs1302739538		rs1302739538		rs770330002		rs104894407	
Country	Continent	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
Algeria	Africa	2/118	1.7%								
Ghana	Africa									2/162	1.2%
Egypt	Africa	1/222	0.5%								
Bangladesh	Asia	10/106	9.4%								
China	Asia	12/2502	0.5%								
India	Asia	8/1062	0.8%					4/1060	0.4%		
Iran	Asia	134/7842	1.7%								
Mongolia	Asia	42/1068	3.9%	1/1068	0.1%						
Palestin	Asia	1/96	1.0%								
Qatar	Asia	10/252	4.0%								

Russia	Asia	13/140	9.3%			52/592	8.8%		
Syria	Asia	14/140	10.0%						
Turkey	Asia	55/4814	1.1%	1/3062	0.03%				
Yakutia	Asia	382/702	54.4%						
Austria	Europe	3/398	0.8%						
Belgium	Europe	2/116	1.7%						
Czech	Europe	9/312	2.9%						
France	Europe	7/830	0.8%					1/318	0.3%
Italy	Europe	18/1338	1.3%						
Netherlands	Europe	3/488	0.6%						
Poland	Europe	24/466	5.2%						
Portugal	Europe	2/529	0.4%	3/528	0.6%				
Slovakia	Europe	3/546	0.5%						
USA	North America	14/4736	0.3%	2/15090	0.0%			6/2918	0.2%
Argentina	South America	1/188	0.5%						
Brazil	South America	5/1570	0.3%	3/632	0.5%				
Guatemala	North America							21/266	7.9%

n/N = number of affected alleles out of total alleles tested, % = allele frequency.

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DMD-related Muscular Dystrophy in Cameroon: Clinical and Genetic Profiles

Edmond Wonkam-Tingang, Séraphin Nguefack, Alina I. Esterhuizen, David Chelo, and Ambroise Wonkam

Table S5: Age ranges at onset of the disease

Age ranges (years)	n	Frequency (%)
[0 – 5[	9	52.9
[5 – 10[	8	47.1
Total	17	100

n, number of patients

Table S6: Ages ranges at diagnosis

Age ranges (years)	n	Frequency (%)
[0 – 10[	6	35.3
[10 – 20[	9	52.9
[20 – 30]	2	11.8
Total	17	100

n, number of patients

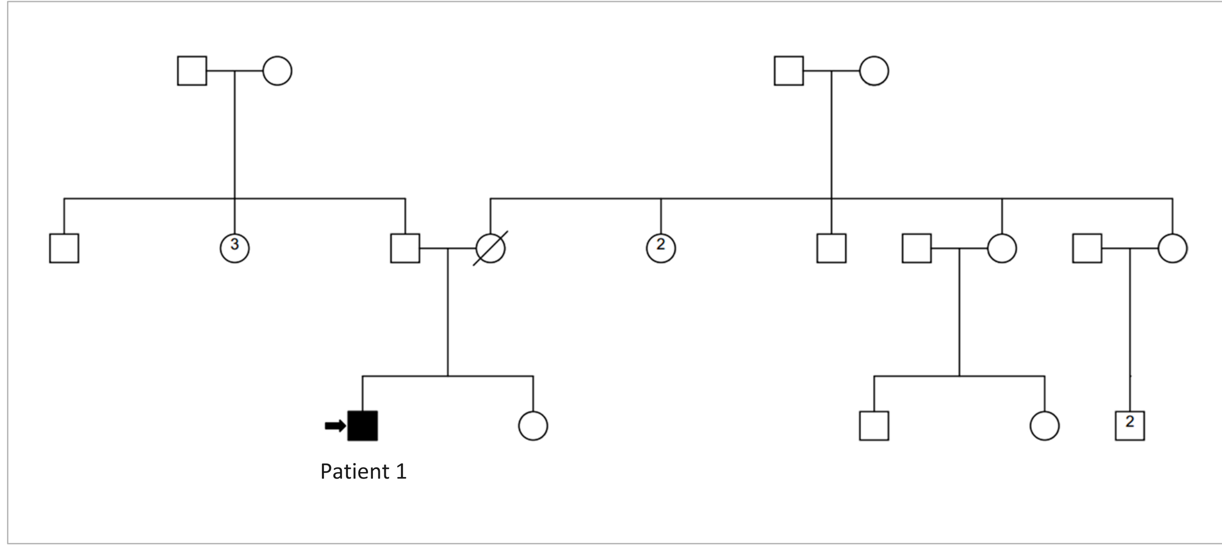

Figure S1: Pedigree of family 1

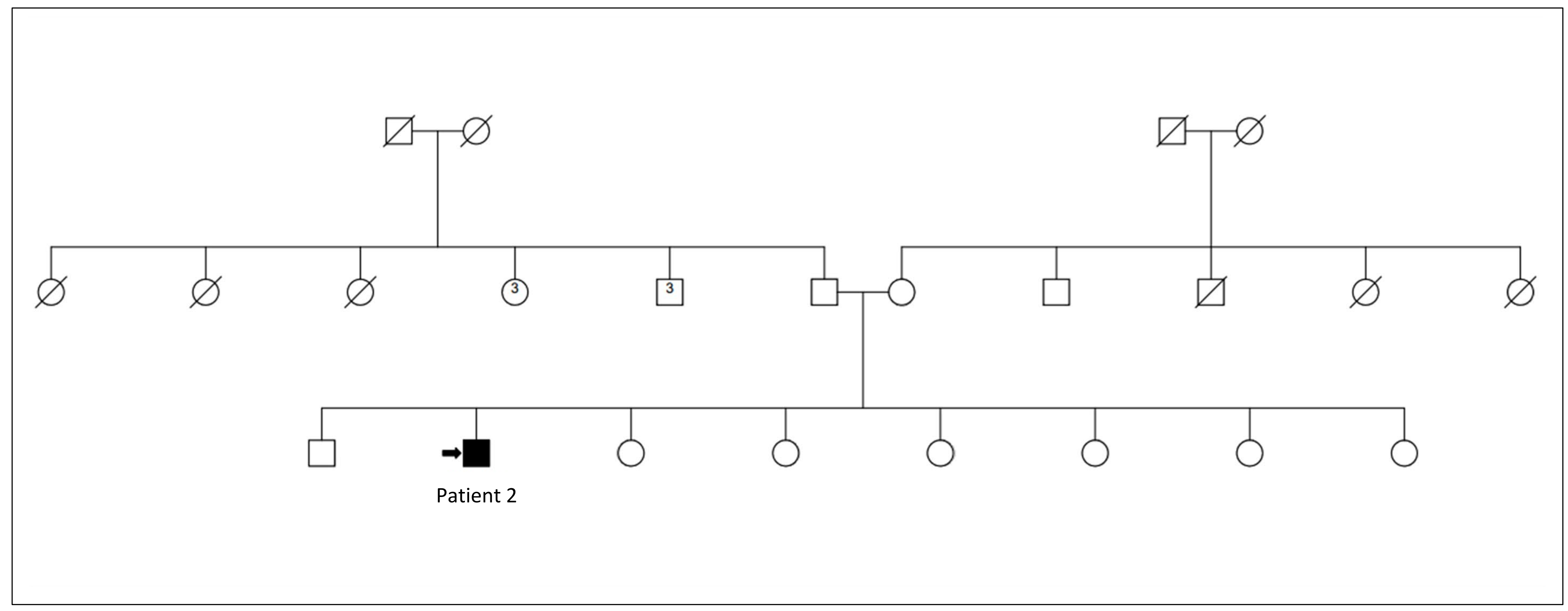

Figure S2: Pedigree of family 2

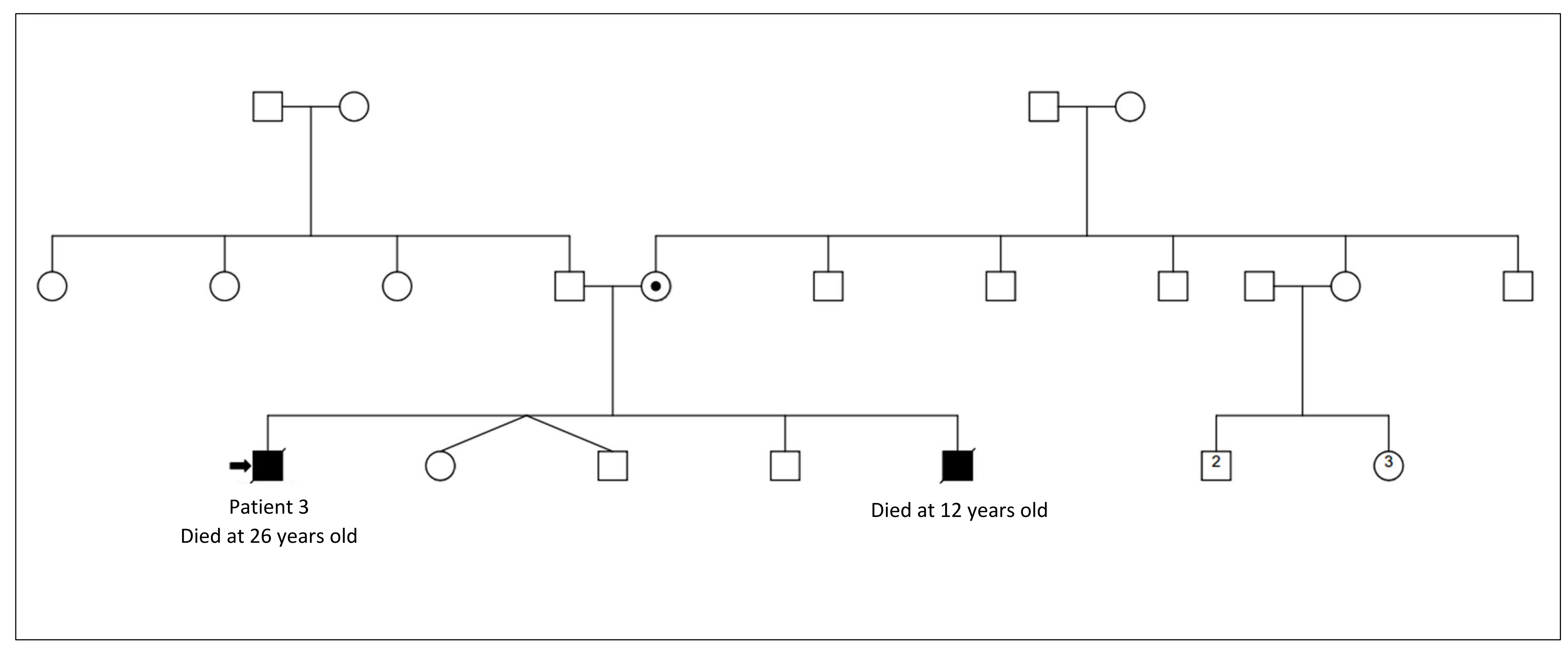

Figure S3: Pedigree of family 3

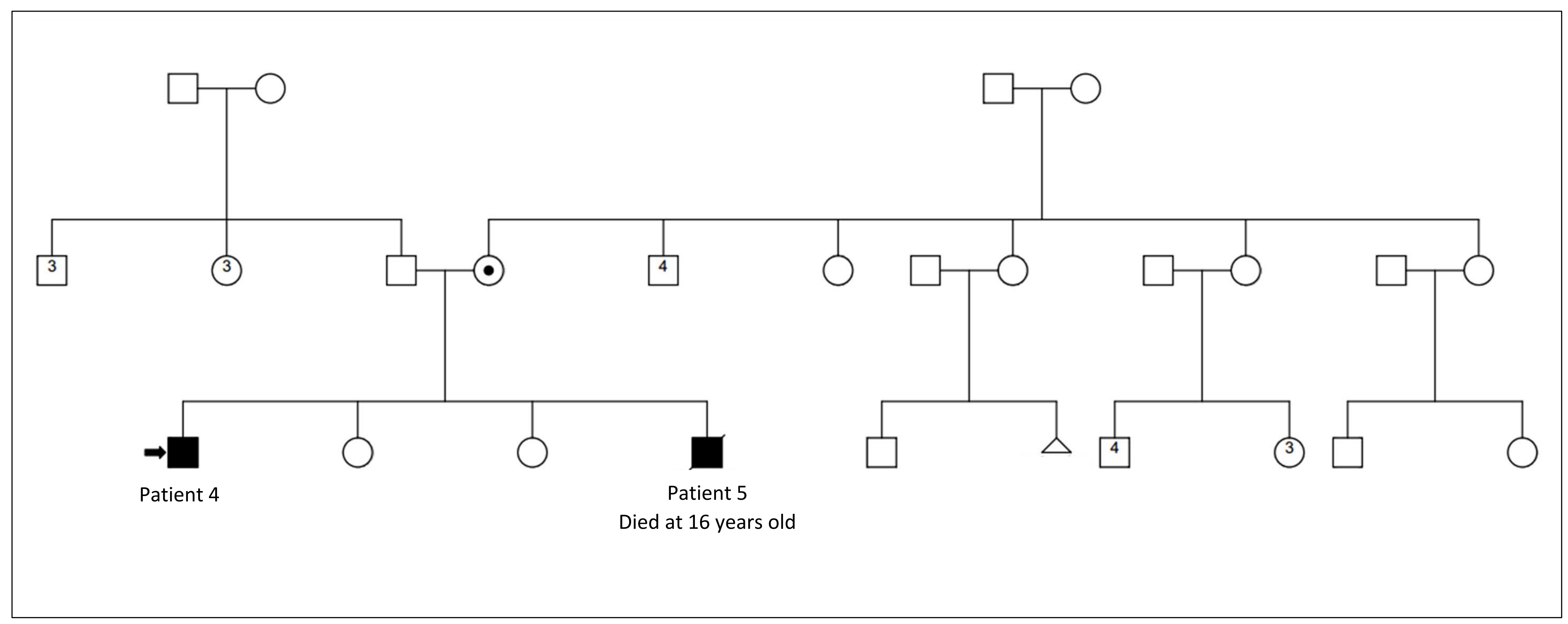

Figure S4: Pedigree of family 4

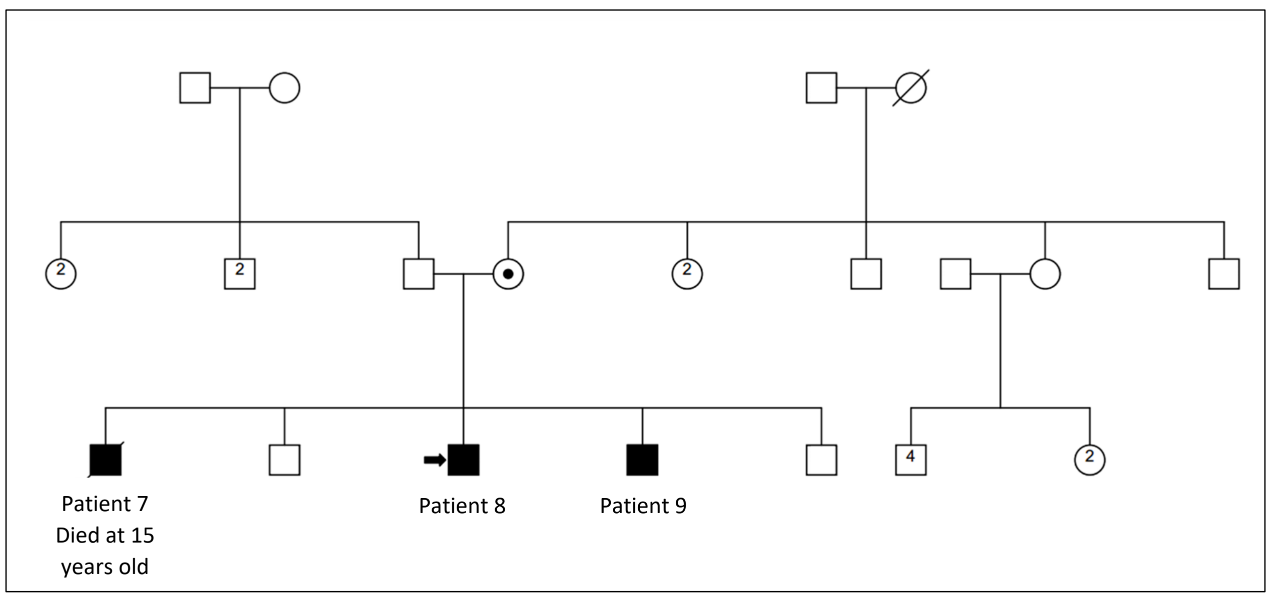

Figure S5: Pedigree of family 6

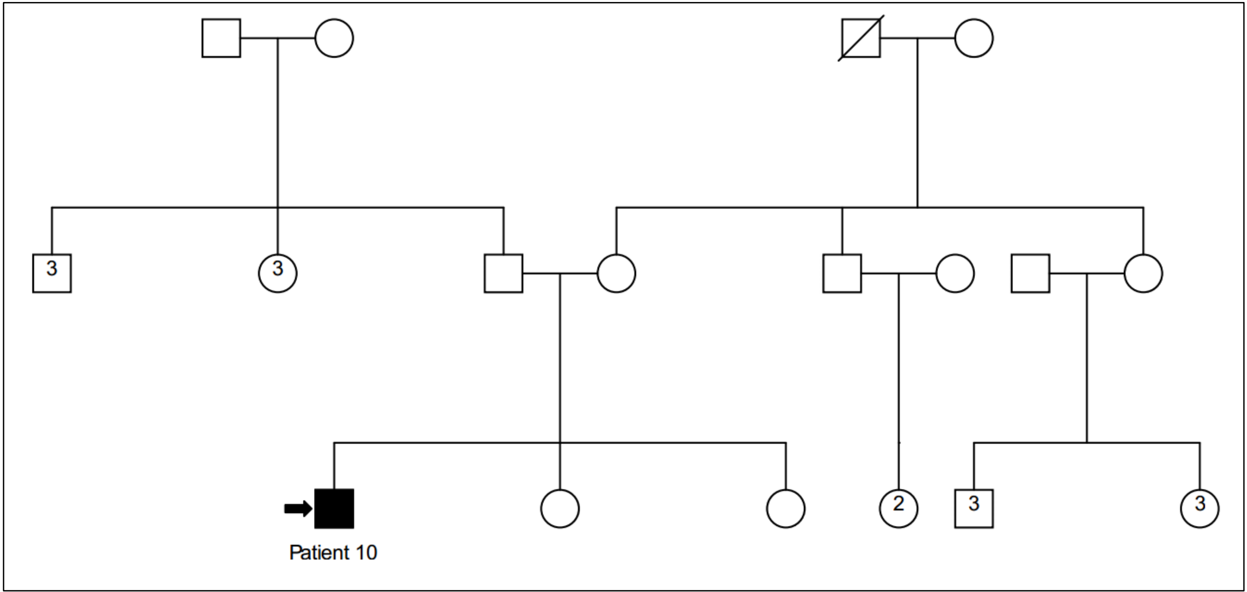

Figure S6: Pedigree of family 7

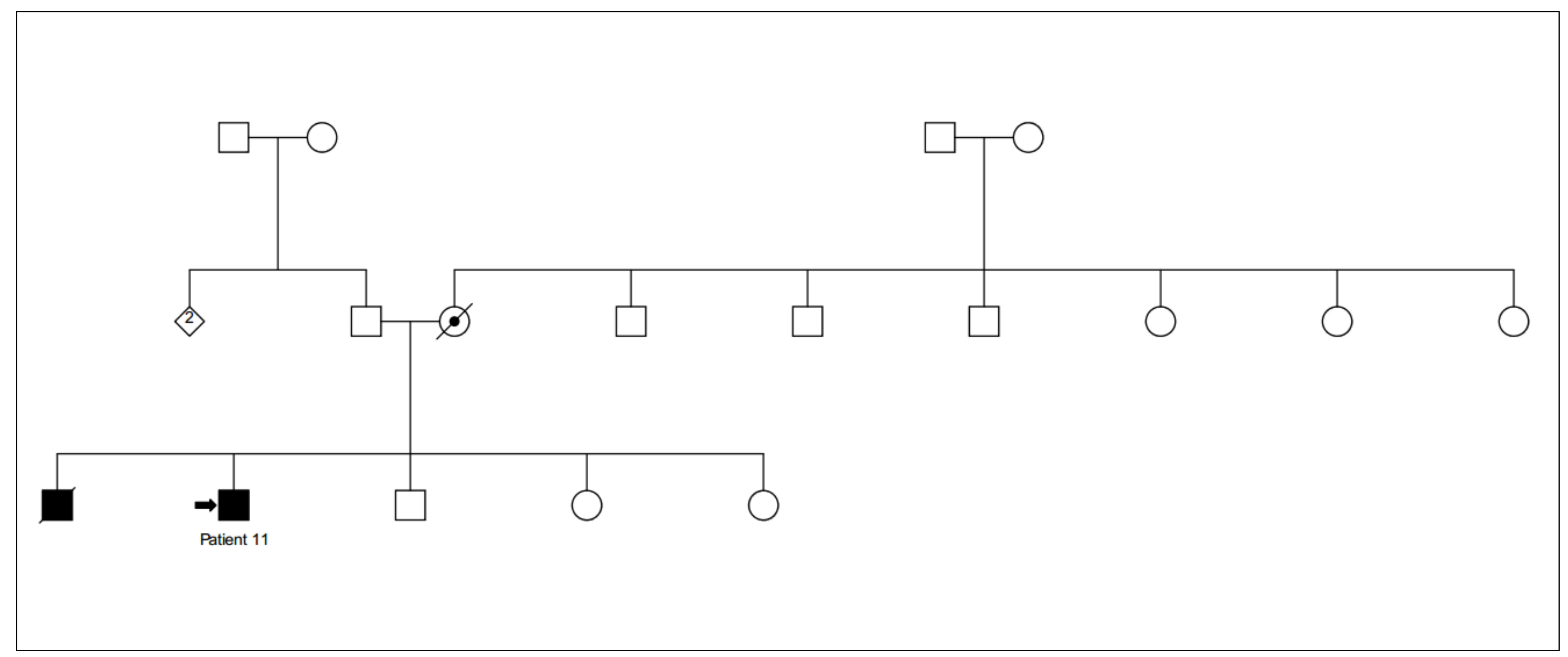

Figure S7: Pedigree of family 8

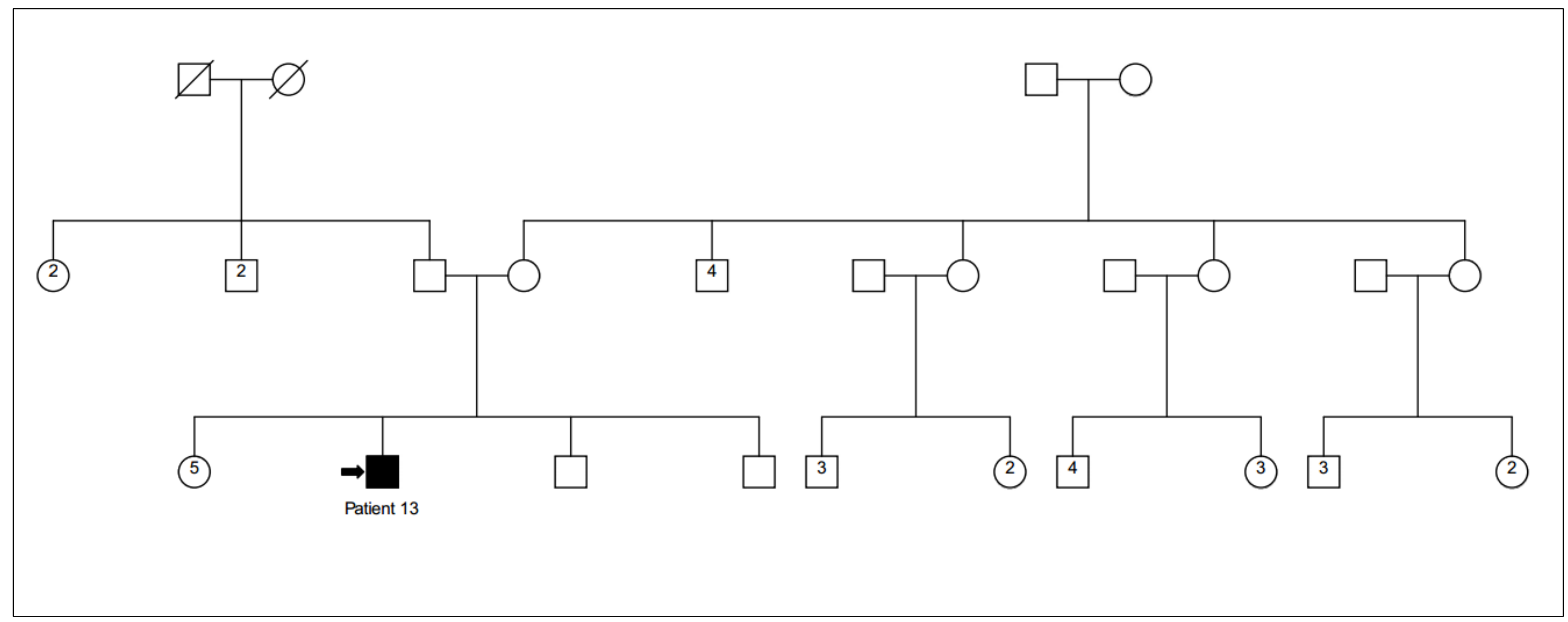

Figure S8: Pedigree of family 10

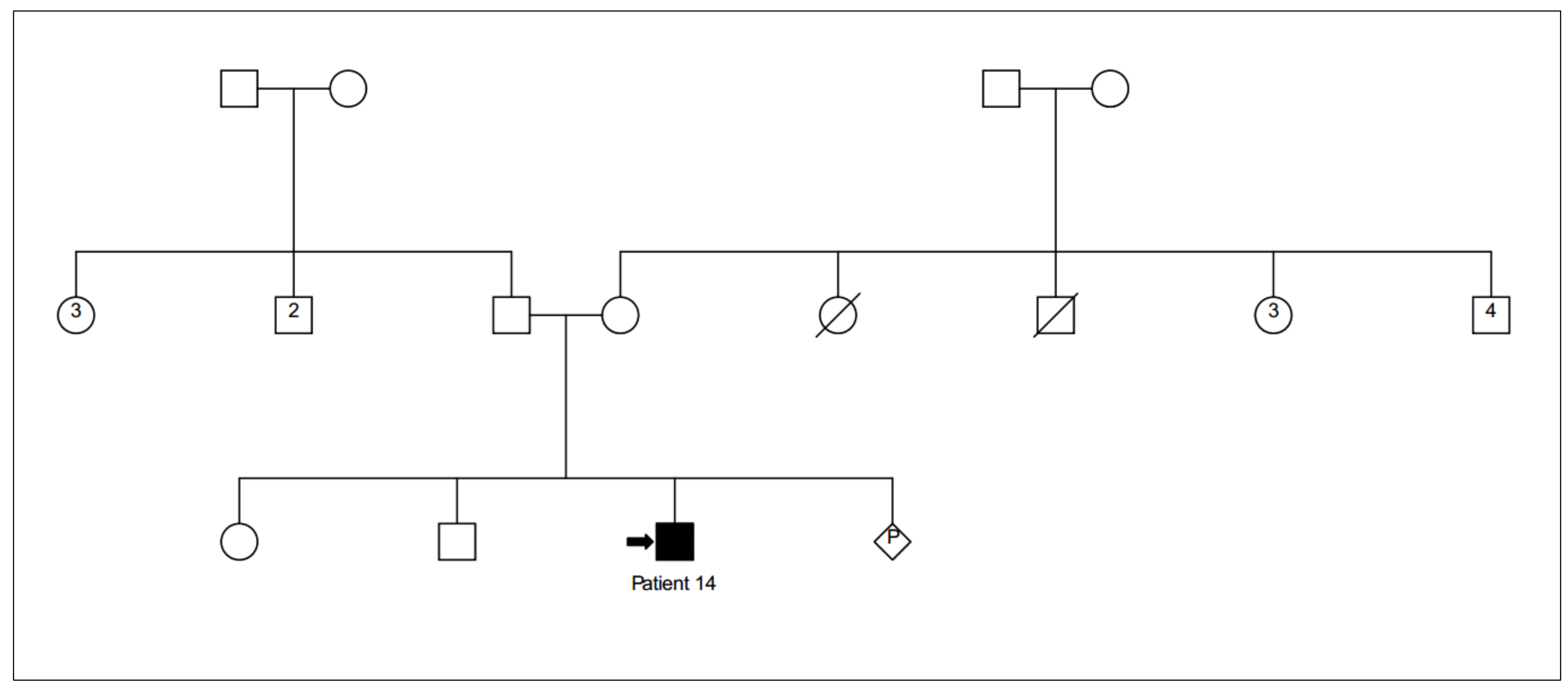

Figure S9: Pedigree of family 11

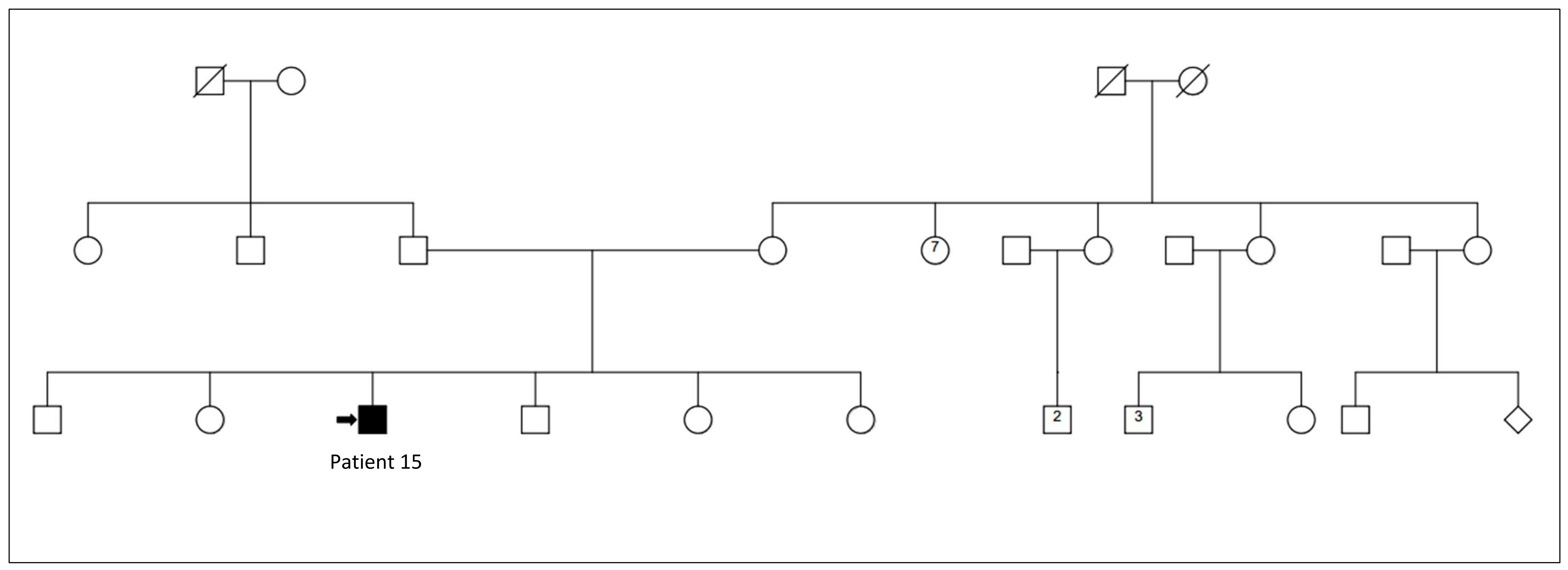

Figure S10: Pedigree of family 12

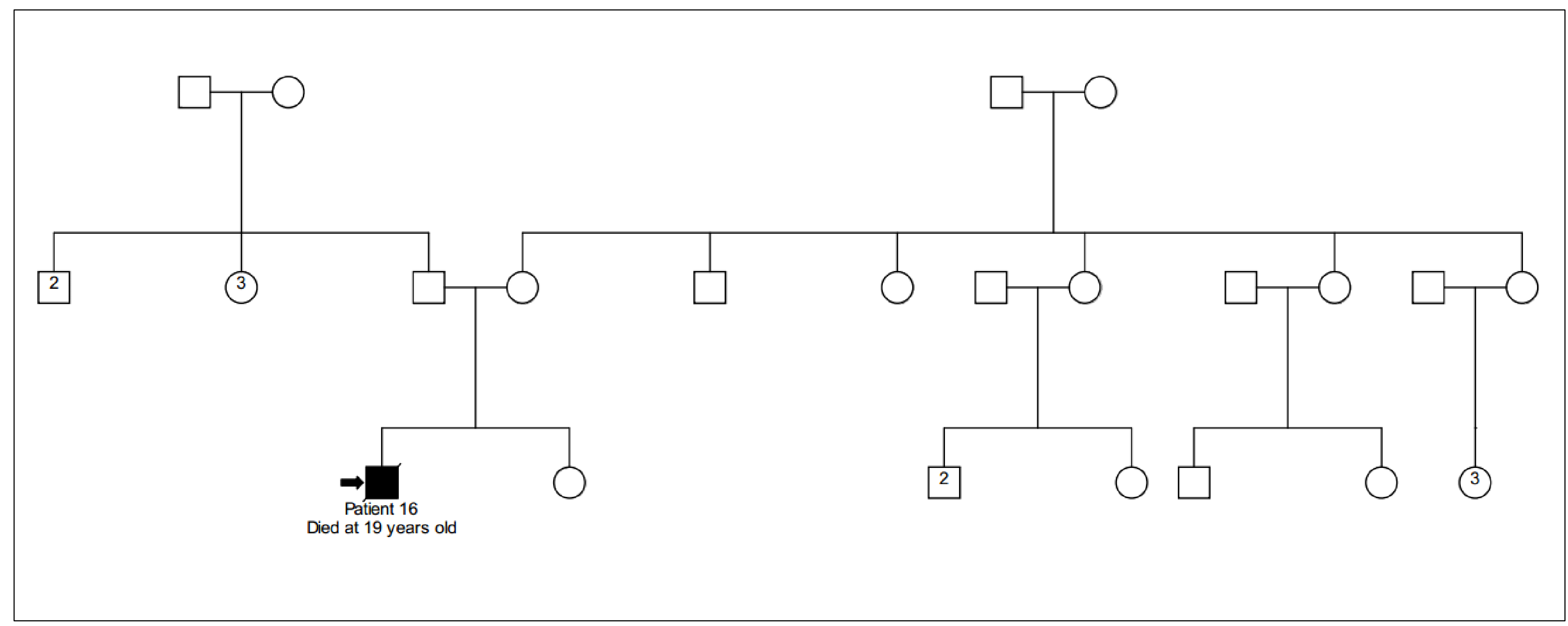

Figure S11: Pedigree of family 13

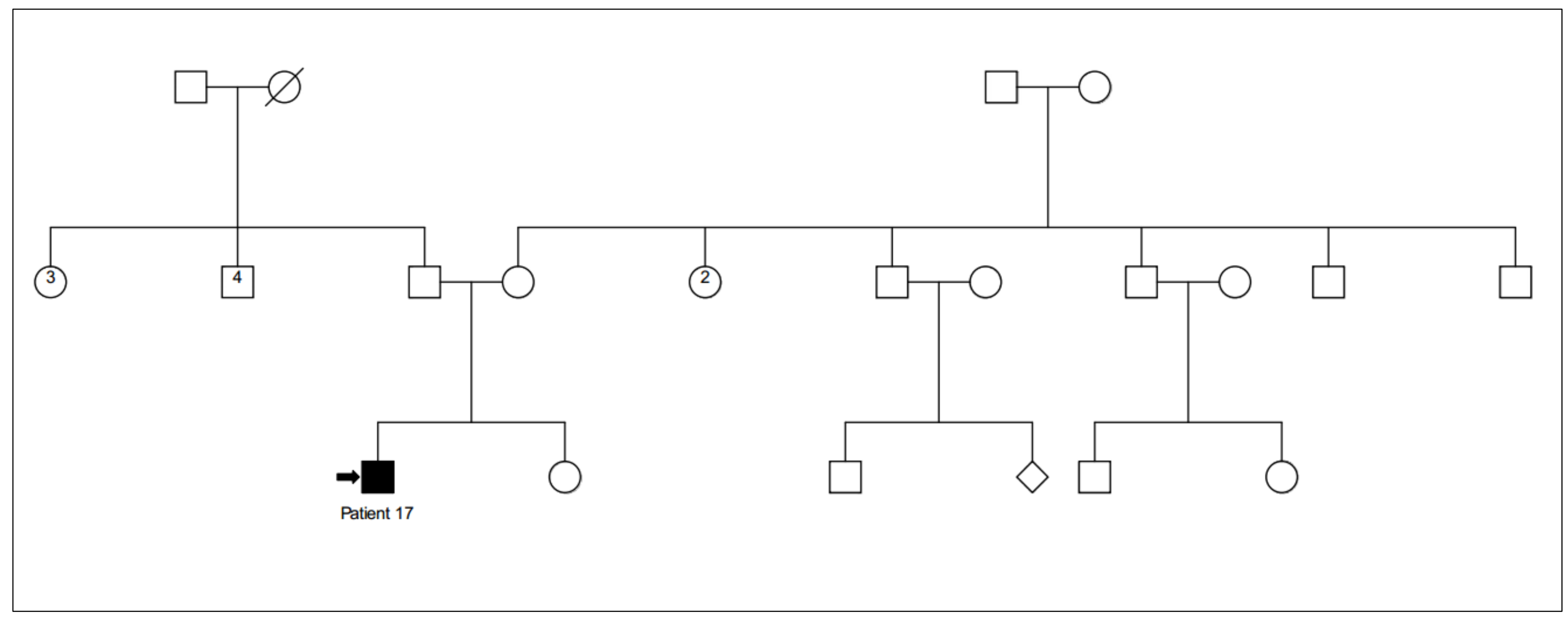

Figure S12: Pedigree of family 14

Appendix 2: Published papers excluded, with significant contribution of the candidate

1. Adadey, Samuel M., **Tingang Wonkam, E.**, Twumasi Aboagye, E., Quansah, D., Asante-Poku, A., Quaye, O., Amedofu, G. K., Awandare, G. A., & Wonkam, A. (2020). Enhancing Genetic Medicine: Rapid and Cost-Effective Molecular Diagnosis for a GJB2 Founder Mutation for Hearing Impairment in Ghana. *Genes*, *11*(2), 132. https://doi.org/10.3390/genes11020132. (Status: *published*)

Contribution to authorship

A.W., G.A.A., G.K.A., and S.M.A. conceptualized the idea.

S.M.A., **E.T.W. (Edmond Wonkam Tingang)**, E.T.A., and D.Q. developed the methodology.

A.W., G.A.A., G.K.A., and O.Q. validated the methodology.

S.M.A., **E.T.W. (Edmond Wonkam Tingang)**, E.T.A., and D.Q. performed the formal analysis.

A.W., G.A.A., G.K.A., A.A.-P., and O.Q. provided the resources.

writing—original draft preparation, S.M.A. wrote the first draft.

writing—review and editing, S.M.A., **E.T.W. (Edmond Wonkam Tingang)**, E.T.A., D.Q., A.A.-P., O.Q., G.K.A., G.A.A., and A.W. wrote, reviewed, and edited the manuscript.

A.W., G.A.A., G.K.A., and O.Q. supervised the project.

A.W. and G.A.A. secured funding.

Article

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

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Abstract: In Ghana, gap-junction protein β 2 (*GJB2*) variants account for about 25.9% of familial hearing impairment (HI) cases. The *GJB2*-p.Arg143Trp (NM_004004.6:c.427C>T/OMIM: 121011.0009/rs80338948) variant remains the most frequent variant associated with congenital HI in Ghana, but has not yet been investigated in clinical practice. We therefore sought to design a rapid and cost-effective test to detect this variant. We sampled 20 hearing-impaired and 10 normal hearing family members from 8 families segregating autosomal recessive non syndromic HI. In addition, a total of 111 unrelated isolated individuals with HI were selected, as well as 50 normal hearing control participants. A restriction fragment length polymorphism (RFLP) test was designed, using the restriction enzyme NciI optimized and validated with Sanger sequencing, for rapid genotyping of the common *GJB2*-p.Arg143Trp variant. All hearing-impaired participants from 7/8 families were homozygous positive for the *GJB2*-p.Arg143Trp mutation using the NciI-RFLP test, which was confirmed with Sanger sequencing. The investigation of 111 individuals with isolated non-syndromic HI that were previously Sanger sequenced found that the sensitivity of the *GJB2*-p.Arg143Trp NciI-RFLP testing was 100%. All the 50 control subjects with normal hearing were found to be negative for the variant. Although the test is extremely valuable, it is not 100% specific because it cannot differentiate between other mutations at the recognition site of the restriction enzyme. The *GJB2*-p.Arg143Trp NciI-RFLP-based diagnostic test had a high sensitivity for genotyping the most common *GJB2* pathogenic and founder variant (p.Arg143Trp) within the Ghanaian populations. We recommend the adoption and implementation of this test for hearing impairment genetic clinical investigations to complement the newborn hearing screening program in Ghana. The present study is a practical case scenario of enhancing genetic medicine in Africa.

Keywords: hearing impairment; *GJB2*-p.R143W; NciI-RFLP; rapid diagnostic test; Ghana

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1. Introduction

Globally, the most prevailing sensorineural disorder is hearing impairment (HI) [1], which accounts for about 466 million people worldwide [2]. According to the World Health Organization fact sheet, an estimate of 900 million people will be living with the condition by the year 2050 [2]. Over 119 genes [3] with more than 1000 mutations have been associated with hearing impairment of varied degrees in different populations [1]. Gap-junction protein β 2 (*GJB2*) and gap-junction protein β 6 (*GJB6*) are the most common genes associated with the condition globally, with high prevalence reported in the European and Asian populations. However, recent data including the use of mouse models has indicated that mutations in the coding region of the *GJB6* gene do not result in hearing impairment. The large genomic deletions in *GJB6*, especially *GJB6*-D13S1830, alters a *cis*-acting element and subsequently abolishes the expression of the *cis-GJB2* allele [4,5]. Thus, the *GJB6* gene itself plays no role in the development of hearing impairment but the surrounding sequences consisting of the *cis*-acting elements are responsible for the development of hearing impairment [5,6]. Nevertheless, in most African populations, *GJB2* and *GJB6* variants are rarely implicated in hearing impairment [7,8] with some *GJB2* cases found in Morocco [9,10], Sudan, and Kenya [11], yet an exceptionally high prevalence is found in Ghana [12–14]. Indeed, in Ghana, *GJB2* mutation (p.Arg143Trp) in the homozygous state accounts for 25.9% of cases in families segregating non-syndromic HI, as well as 7.9% of non-familial non-syndromic congenital HI cases (Adadey et al., 2019). This Ghanaian exception, in the African context, is predominantly due to a *GJB2* founder mutation (p.Arg143Trp), which was first reported in a village known as “the deaf village”, Adamorobe [13]. Adamorobe is a village located in the Eastern Region of Ghana and known to have a high hereditary hearing impairment incidence [15]. As of 2012, 41 people living with deafness were recorded among a population of 3500 in Adamorobe [16]. In this village, both the hearing and the deaf citizens interact and live together in one society.

The exceptionally high proportion of *GJB2* (p.Arg143Trp) variant in Ghana has created the need to develop a simple tool for testing in order to support appropriate informed counselling and planning for appropriate interventions. To develop molecular diagnostic tools for screening non-syndromic HI, there is a need for utilizing population and ethnic specific genetic markers due to the ethnically diverse nature of hearing impairment genes [17,18]. Recent clinical genetic testing efforts are centered around targeted genomic enrichment and/or massive parallel sequencing [18–20]. There are some efforts to develop polymerase chain reaction (PCR)-based diagnostic tools for screening for hearing impairment; however, most of these tools are in combination with DNA sequencing technologies [21–23], which are not easily implementable in low-income countries. To develop cheaper but effective diagnostic tools, mutations specific to populations have been considered, especially in populations where *GJB2* is prevalent. Specific genetic tests have been developed for carrier testing and prenatal diagnoses for *GJB2*-35delG variant in Caucasian populations [24,25]. In this study, we sought to design a restriction fragment length polymorphism test for *GJB2*-p.Arg143Trp genotyping in Ghana.

2. Materials and Methods

2.1. Ethical Approvals

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

2.2. Study Participants

Congenital hearing-impaired patients were recruited from schools for the deaf and from the Adamorobe community following procedures reported previously [12]. Briefly, all participants’ details,

as well as their personal and family histories, were obtained; medical records were reviewed by a medical geneticist and an ear, nose, and throat (ENT) specialist when possible; and relevant data were extracted, including three-generation pedigrees and perinatal histories, using a structured questionnaire to query possible environmental causes of hearing impairment. A general systemic and otological examination and audiological evaluation were performed, including a pure tone audiometric test, following the recommendation number 02/1 of the Bureau International d'Audiophonologie (BIAP), Belgium, to classify hearing levels [26,27]. The audiometric tests were conducted using KUDUwave portable audiometer (KUDUwave, Johannesburg, South Africa) in a quiet room. In bilateral octaves, the air conduction thresholds were from 250 H_Z through to 8000 H_Z and the bone conduction from 250 H_Z through to 4000 H_Z. The pure tone average was determined using thresholds at 500, 1000, 2000, and 4000 H_Z.

The study participants were categorized into three groups: (1) deaf community-based familial cases, (2) nation-wide isolated/non-familial cases, and (3) control individuals without a personal or family history of HI. The first group was made of families segregating HI, with at least two affected individuals and with evidence of non-environmental causes. In this group, 30 study participants from 8 families segregating hearing impairment were recruited from the Adamorobe community in the Eastern Region of Ghana. Out of the 30 participants, 20 were hearing-impaired and 10 participants had normal hearing. Apart from the families with putative genetic etiology of hearing loss, an additional family was found to have a putative environmental etiology of the condition and was excluded from the study. The second group of participants was made up of 111 isolated/non-familial cases of unrelated probands with putative genetic causes of hearing impairment and were recruited from 6 schools for the deaf across Ghana. All the affected individuals (familial and isolated cases) considered for the study had congenital non syndromic HI. The third group (the control group) was made of 50 normal hearing participants that were randomly recruited nationwide from the Ghanaian population.

2.3. Molecular Analyses

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

Polymerase chain reaction (PCR) and Sanger sequencing: At the Division of Human Genetics, University of Cape Town, specific primers (Table S1) were used to amplify the coding regions of *GJB2* (exon 2) and *GJB6*, as described by Bosch et al. in 2014. The annealing and extension temperatures for the PCR were 60 °C and 70 °C for 30 s and 1 min, respectively. The PCR amplicons were Sanger sequenced as described by Bosch et al. [28] using an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Screening for del(*GJB6*-D13S1830) was performed as previously described, using primers and methods by del Castillo et al. [29].

Restriction fragment length polymorphism (RFLP) technique: The p.Arg143Trp variant in the *GJB2* gene was investigated using RFLP technique designed as follows. *GJB2*-specific primers [28] were used to amplify exon 2 of the gene where the p.Arg143Trp variant is located. Carefully selected restriction enzyme NciI (supplied by New England Biolabs Inc., Massachusetts, MA, USA, through Inqaba biotec, Pretoria, South Africa) with the recognition site "CCSGG" was used to digest the PCR amplicons. The gene layout and the *GJB2*-p.Arg143Trp NciI-RFLP design including the cut sites is illustrated in Figure 1. The RFLP reaction consisted of 15 µL of the PCR product, 2 µL of 10X buffer, 0.25 µL of an NciI enzyme (20,000 units/mL), and 2.75 µL of nuclease-free water. The restriction reaction mixture was incubated overnight at 37 °C. The digested products were resolved on 2% agarose gel for 1.5 h. The accuracy, sensitivity, and specificity of the RFLP test was determined as described by Baratloo et al. [30] using sequencing as the gold standard.

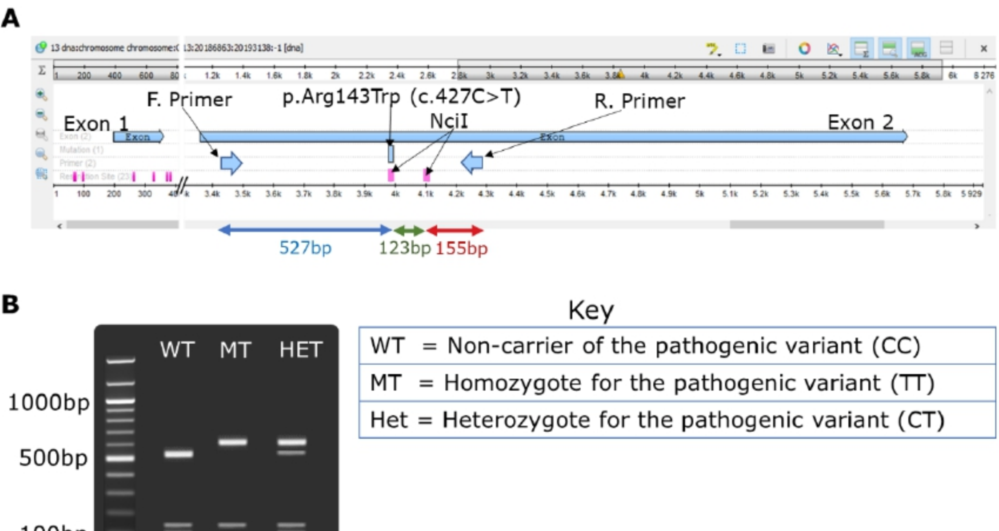

Figure 1. NciI restriction fragment polymorphism investigations for gap-junction protein β 2 (*GJB2*)-p.Arg143Trp (c.427C > T rs80338948) variant. (**A**) Unipro UGENE [31] map of *GJB2* exon 2 showing the primer binding sites (F. primer and R. primer) and the restriction sites (CCSGG) for the restriction enzyme NciI and the resulting DNA fragments. (**B**) Expected gel electrophoresis result.

2.4. Data Analysis

Data from the study was inputted into Microsoft Excel and analyzed with GraphPad Prism version 6. One-way analysis of variance (ANOVA) was used to determine the differences between the mean hearing measurements (pure tone average) of the different *GJB2*-p.Arg143Trp genotypes. Tukey's multiple comparisons test was used to compare between the *GJB2*-p.Arg143Trp genotypes. The specificity and sensitivity of the RFLP test were calculated as described by Schrauwen et al. [23].

3. Results

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

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

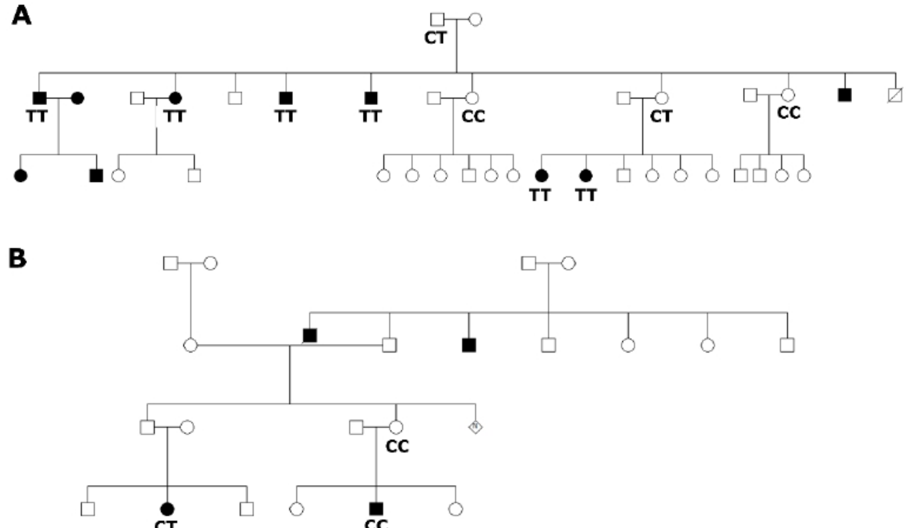

Figure 2. Pedigrees and genotypes of familial cases from Adamorobe. (**A**) Representative pedigree of families that segregate *GJB2*-p.Arg143Trp (c.427C > T rs80338948) variant with hearing impairment. (**B**) Pedigree of a family that did not segregate *GJB2*-p.Arg143Trp variant with the phenotype.

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

The target region of *GJB2*-p.Arg143Trp variant was PCR amplified for each participant (Figure S1). The NciI restriction enzyme had two restriction sites on the DNA amplified (Figure 1A) and cleaves the PCR amplicons of the wildtype (CC genotype) samples to give three products of the lengths 527, 123, and 155 bp. The NciI restriction digest of the homozygous mutant (TT) produced two fragments of the lengths 600 and 155 bp, with the enzyme cutting only once. The heterozygous carriers (CT) yielded four different fragments (600, 527, 123, and 155 bp) (Figure 1B). The NciI enzyme cleaved the PCR product in any of the above circumstances; this served as an internal control, and hence an invalid test was when there was no cleavage. The *GJB2*-p.Arg143Trp NciI-RFLP genotyping results of 20 selected samples from Adamorobe were validated using Sanger sequencing (Figure 3).

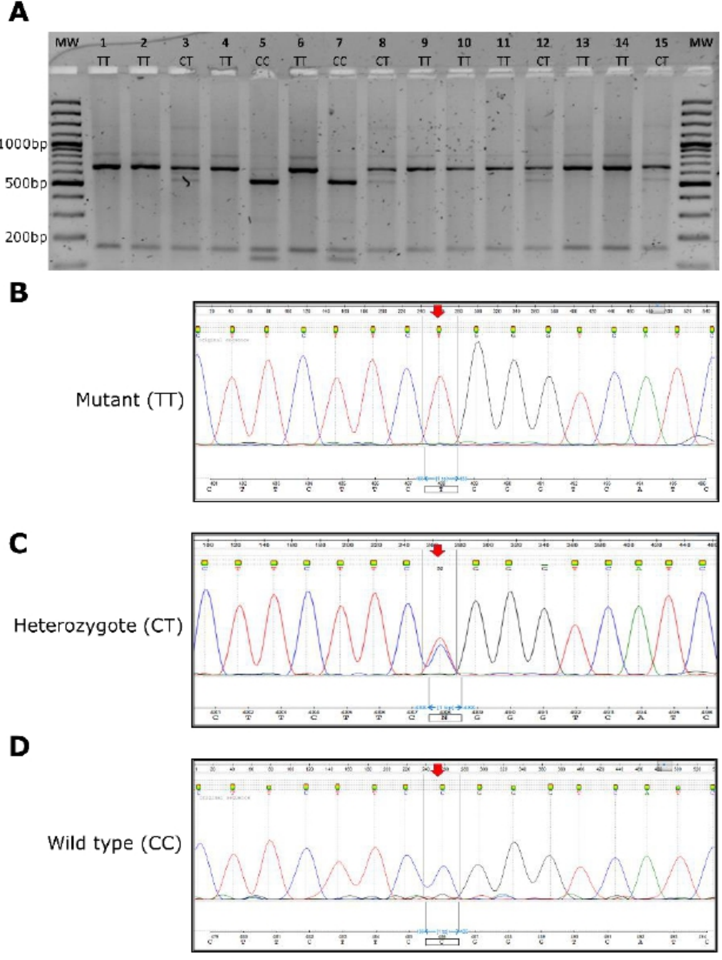

Figure 3. *GJB2*-p.Arg143Trp screening. (**A**) Representative gel of NciI-restriction fragment polymorphism (RFLP) test used to screen samples for *GJB2*-p.Arg143Trp variant. (**B**–**D**) Representative chromatograms of Sanger sequences validating the p.Arg143Trp NciI-RFLP results.

3.3. GJB2-p.Arg143Trp NciI-Restriction Fragment Polymorphism Investigations

The molecular analysis using *GJB2*-p.Arg143Trp NciI-RFLP test identified 18 out of the 20 hearing-impaired patients, from 7/8 families, to be homozygous for the p.Arg143Trp (TT) variant. In the eighth family were two individuals affected by HI, one was heterozygous (CT) and the other had the CC genotype (Figure 2B). In order to exclude *GJB6*-related HI in this family, we investigated variants in *GJB6*, and no variant was found. No other participant had a variant in the *GJB6* gene, (n = 20).

Seven (7) out of the 10 family members without hearing impairment were heterozygous (CT), thus having the p.Arg143Arg/p.Arg143Trp variant, while the rest had the p.Arg143 variant (Figure 3A).

A total of 111 individuals with non-familial isolated non-syndromic HI, whose samples were previously Sanger sequenced for *GJB2* variants [12], were analyzed using the developed *GJB2*-p.Arg143Trp NciI-RFLP test. Table 1 illustrates that the *GJB2*-p.Arg143Trp NciI-RFLP test was found to have 100% sensitivity compared to Sanger sequencing as the gold standard. To examine the clinical applicability of the test, 50 control participants with normal hearing were screened and found negative for the *GJB2*-p.Arg143Trp variant.

Table 1. Validation of *GJB2*-p.Arg143Trp NciI-restriction fragment polymorphism tests with Sanger sequencing.

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

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

3.4. Genotype to Phenotype Correlations

On the basis of *GJB2*-p.Arg143Trp genotypic classification of the familial cases from Adamorobe, the pure tone average of homozygous mutant (TT) ranged from 97 to 108 dB with a mean of 105.4 and 107.3 dB in the left and right ears, respectively. The pure tone average range for the heterozygote (CT) was from 17 to 108 dB, with a mean of 43.6 and 40.6 dB in the left and right ears, respectively. The range for the homozygous CC genotype (p.Arg143Arg) was from 18 to 108 dB, with the mean 53 and 46.5 dB in the left and right ears, respectively. There was a statistically significant difference between the audiometric measurements of the TT and CT genotypes in both ears. Similarly, in both ears, there was a statistically significant difference between the TT and CC genotypes (Figure 4).

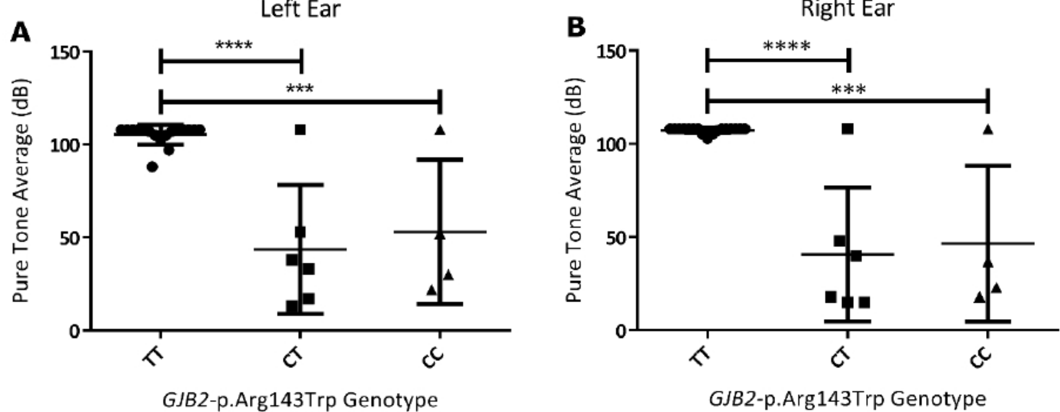

Figure 4. Audiological characterization of hearing-impaired participants from the deaf community of Adamorobe. (**A**) Left ear and (**B**) right ear pure tone average of participants according to their *GJB2*-p.Arg143Trp genotypes. The age range of the genotypes TT (n = 17), CT (n = 6), and CC (n =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

4. Discussion

This study designed a restriction fragment length polymorphism (RFLP) test for effective screening of *GJB2*-p.Arg143Trp (rs80338948). The *GJB2*-p.Arg143Trp variant results from a pathogenic point mutation (c.427C > T) in the exon 2 of the connexin 26 gene on chromosome 13 [13,14]. The drive for an efficient and cost-effective test was from the fact that the founder mutation, *GJB2*-p.Arg143Trp, is the most common variant associated with hearing impairment in Ghana [12–14].

The use of next generation sequencing (NGS) has been proposed as the best tool for the discovery of hearing impairment genes [32], especially in Africa because of the high diversity within the African population [33,34]. Due to ethical and social challenges, NGS needs to be carefully considered in clinical practice [35]. In developing countries, the clinical use of NGS is still a major challenge because of the associated high cost of the equipment and the computational challenges posed by the approach [36]. However, there were some attempts to develop relatively simple, low cost, and population-specific screening approaches for some of the major hearing impairment gene mutations [37–39].

For the first time, we designed and tested the effectiveness of RFLP, using the NciI enzyme, to screen for the founder mutation (*GJB2*-p.Arg143Trp) in Ghana. Accuracy, sensitivity, specificity, and predictive values are critical parameters considered for the clinical use of a test [30,40]. Our *GJB2*-p.Arg143Trp NciI-RFLP test had good positive and negative predictive values for genotyping of the *GJB2*-p.Arg143Trp variant in the Adamorobe participants from Ghana, and also in a nationwide sample of unrelated affected individuals. Nevertheless, the test cannot differentiate between other variants within the recognition site of the restriction enzyme; hence, similar results would be obtained for the following pathogenic mutations: p.Phe142Leu (c.426C > A), p.Y142del (c.424_426delTTC), and p.Arg143Gln (c.428G > A). To confirm the specific mutation at the enzyme restriction site, Sanger sequencing would be needed. However, the high prevalence of the *GJB2*-p.Arg143Trp variant within the Ghanaian population makes the NciI-RFLP test relevant. A 100% sensitivity was obtained for the *GJB2*-p.Arg143Trp NciI-RFLP test when compared with the gold standard, Sanger sequencing. Although a single gene test for hearing impairment is inefficient for many populations [39], the aforementioned qualities of the test would enable it to be used as a first-line diagnosis for hearing impairment genetics in the newborn hearing screening program, as well as for prenatal testing. The *GJB2*-p.Arg143Trp NciI-RFLP test would therefore be of great clinical value in Ghana.

The *GJB2*-p.Arg143Trp NciI-RFLP test identified the founder mutation in the eight Adamorobe families investigated. In all the families, the mutation segregated with the phenotype, and all affected individuals reported a homozygous variant (TT genotype), except in one family where one affected individual was heterozygous (CT) and the other without any variant (CC), suggesting that there are other genes still to be discovered to explain the HI in this family. Similar to a family from Japan [37], the heterozygous *GJB2*-p.Arg143Trp variant in the above family did not segregate with the HI phenotype (Figure 2B). Variants in the *GJB6* gene are no longer considered as causes of hearing impairment. However, the presence of *GJB6* variants affecting the *cis*-acting element upstream of both *GJB6* and *GJB2* in association with variants in *GJB2* (digenic inheritance) are now known to be pathogenic through the modification of *GJB2* expression [5,6]. Hence, we sought to exclude any *GJB6* variant that might disrupt the *cis*-acting element. We therefore investigated *GJB6* variants in particular; *GJB6*-D13S1830 and no *GJB6* pathogenic variant was identified in this family. Hence, we propose the use of whole exome sequencing (WES) in future, or targeted panel sequencing, which has been shown to be efficient in Cameroonian families [33], to further investigate this family, as well as any other family that is specifically negative for the *GJB2*-p.Arg143Trp variant in Ghana.

GJB2-p.Arg143Trp variant is known to be associated with profound HI [13,14,37]. The audiometric characterization of the *GJB2*-p.Arg143Trp homozygous individuals showed that they had profound HI. Previous studies by Brobby et al. from the same village indicated that *GJB2*-p.Arg143Trp homozygous individuals express profound hearing impairment [13]. Similar to the previous report [13], we found that there was no significant difference between the average hearing levels of the CT (heterozygote for

the pathogenic variant) and the CC (non-carrier of the pathogenic variant) genotypes. Our results and previous reports confirmed the autosomal recessive mode of inheritance of *GJB2*-p.Arg143Trp [12–14].

5. Conclusions

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

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/11/2/132/s1, Figure S1: Representative agarose gel picture of *GJB2* exon 2 PCR products, Table S1: Primer sequencing for *GJB2* and *GJB6* coding region amplification.

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

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Contribution to authorship

O.G.O. conceptualized the idea, performed parts of the experiments, analyses, prepared results, and drafted the manuscript.

K.K.E. and E.C. did parts of the analyses and results.

J.J.N., **E.W.T. (Edmond Wonkam Tingang)**, and N.M. diagnosed, recruited patients, and revised the manuscript.

AW is the project lead, supervised the recruitment, experiments, analyses, and revised the manuscript.

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Original Research — Highlight article

Whole exome sequencing identifies rare coding variants in novel human-mouse ortholog genes in African individuals diagnosed with non-syndromic hearing impairment

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Impact statement

Despite, human and murine hearing system being very similar, the contribution of variants in relevant mouse-ortholog genes to hearing impairment (HI) has not been fully investigated. The contribution of variants in the known non-syndromic hearing impairment (NSHI) genes among Africans is poorly studied, suggesting that the novel gene(s) and mutations are yet to be discovered in NSHI in the African populations. Using whole exome sequencing (WES), this study identified rare candidate pathogenic and likely pathogenic (PLP) variants in 3/34 novel human-mouse ortholog genes in 3/40 individuals, with one homozygous variant, *MCPH1*, c.2311C>G p.(Pro771Ala), likely to explain HI in one patient. *In silico* functional analyses suggest that these human-mouse ortholog genes could contribute to the understanding of the physiology of hearing in humans and thus the variants identified in those genes deserve additional investigations.

Abstract

Physiologically, the human and murine hearing systems are very similar, justifying the extensive use of mice in experimental models for hearing impairment (HI). About 340 murine HI genes have been reported; however, whether variants in all human-mouse ortholog genes contribute to HI has been rarely investigated. In humans, nearly 120 HI genes have been identified to date, with *GJB2* and *GJB6* variants accounting for half of congenital HI cases, of genetic origin, in populations of European and Asian ancestries, but not in most African populations. The contribution of variants in other known genes of HI among the populations of African ancestry is poorly studied and displays the lowest pick-up rate. We used whole exome sequencing (WES) to investigate pathogenic and likely pathogenic (PLP) variants in 34 novel human-mouse orthologs HI genes, in 40 individuals from Cameroon and South Africa diagnosed with non-syndromic hearing impairment (NSHI), and compared the data to WES data of 129 ethnically matched controls. In addition, protein modeling for selected PLP gene variants, gene enrichment, and network analyses were performed. A total of 4/38 murine genes, *d6wsu163e*, *zfp719*, *grp152* and *minar2,* had no human orthologs. WES identified three rare PLP variants in 3/34 human-mouse orthologs genes in three unrelated Cameroonian patients, namely: *OCM2*, c.227G>C p.(Arg76Thr) and *LRGI1*, c.1657G>A p.(Gly533Arg) in a heterozygous state, and a PLP variant *MCPH1,* c.2311C>G p.(Pro771Ala) in a homozygous state. *In silico* functional analyses suggest that these human-mouse ortholog genes functionally co-expressed interactions with well-established HI genes: *GJB2* and *GJB6*. The study found one homozygous variant in *MCPH1*, likely to explain HI in one patient, and suggests that human-mouse ortholog variants could contribute to the understanding of the physiology of hearing in humans.

Keywords: Hearing impairment, whole-exome sequencing, human-mouse ortholog genes, African populations

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Introduction

Hearing impairment (HI) is one of the leading causes of disability globally.[1] A disabling HI refers to hearing loss ≥40 decibels (dB) in adult (15 years or older) and ≥30 dB in children (0 to14 years). Congenital HI affects 1–2 in 1000 new-borns globally.[1] A higher prevalence is recorded in sub-Saharan Africa (about 6 out of 1000 live births) compared to the high income countries (about 1 out of 1000 live births).[2] In most African settings, the actual population prevalence of HI is not entirely known. Observational studies reported relatively high prevalence of 2.0% to 21.3% among children from the rural and urban settings in Nigeria, Ghana, Cameroon, Sudan, Kenya, Tanzanian, Zimbabwe, Uganda, Angola, and South Africa.[3]

In most populations, genetic factors contribute to about half of cases of congenital HI, of which about 70% are non-syndromic hearing impairment (NSHI).[4] In Cameroon, in a total of 582 patients, with limited molecular investigations, the genetic causes of HI were estimated at 14.8%, environmental factors at 52.6% and unknown causes estimated at 32.6%.[5] The inheritance pattern of NSHI of genetic origin is predominantly autosomal recessive (80%).[6] Among the populations of European and Asian ancestries, common mutations associated with NSHI are being identified in the *GJB2* and *GJB6* connexin genes.[6] But mutations in these connexins genes have been rarely found in HI in the populations of African ancestries.[7–9]

An exception is the founder mutation p.R143W in the *GJB2* gene reported in Ghana in 1998,[10] that accounts for nearly 25% of familial cases and 8% of isolated cases of NSHI in the Ghanaian populations.[11] HI is genetically highly heterogeneous with nearly 120 genes identified, to date.[12–14] Nevertheless, targeted panel sequencing has demonstrated a consistently lower pick-up rate in individuals of African ancestry.[15,16] Moreover, the prevalence of autosomal recessive non-syndromic hearing impairment (ARNSHI) due to pathogenic and likely pathogenic (PLP) variants, selected from the ClinVar and Deafness Variation Databases, and gnomAD database, was estimated at 5.2 per 100,000 individuals for Africans/African Americans, compared to the highest prevalence of 96.9 per 100,000 individuals for Ashkenazi Jews.[17] Therefore, there is an urgent need to investigate HI in populations of African ancestry in order to address this knowledge deficit that is likely to hinder our current understanding of the HI pathophysiology.

Physiologically, the human and murine hearing systems are very similar,[18] supporting the widely used approach of studying the genetics of HI through murine models. About 340 murine HI genes have been reported[18]; but whether all human-mouse ortholog genes have some roles in human HI remains to be fully investigated. A recent study identified 38 novel murine HI-associated genes in a cohort of newly generated mouse mutants and found among these genes, one human-mouse ortholog (*SPNS2*) that harbors PLP in childhood HI in humans.[19]

In the present study, taking into account the huge diversity of African populations,[20,21] and the low pick-up rate of known HI genes in African cohorts with HI,[15,16] we hypothesized that investigating novel human-mouse ortholog genes in African populations may reveal important findings that could contribute to the knowledge of NSHI genetic causes, and ultimately to genetic medicine practice.

We used whole exome sequencing (WES) to investigate PLP variants in selected novel human-mouse ortholog HI genes, associated with HI in an autosomal recessive manner, among individuals affected with NSHI from Cameroon and South Africa.

Materials and methods

Ethical approval

The study was performed following the Declaration of Helsinki. The study was approved in Cameroon by the Institutional Research Ethics Committee for Human Health of the Gynaeco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon (Ethics approval number N°723/CIERSH/DM/2018) and in South Africa by the Human Research Ethics Committee (Ethics approval number HREC Ref: 104/2018) of the University of Cape Town, South Africa. Written informed consent was obtained from all participants if they were 18 years or older, or from the parents/guardians with verbal assent from the children.

Participants

HI diagnoses and phenotypic classifications were done by audiologists and medical geneticists. Only individuals with NSHI and individuals with prelingual onset (0–3 years) were included. A total of 40 unrelated patients selected for WES were included: 18 from Cameroon and 22 from South Africa with Xhosa ethno-linguistic ancestry. A structured questionnaire to eliminate syndromic and environmental causes of HI was described previously.[7] A total of 28 patients were sporadic cases of HI, while 12 patients' clinical and pedigree profiles were compatible with ARNSHI. All the patients did not have PLP variants in *GJB2* and *GJB6* genes, as described previously.[7]

In addition to the WES study of the 40 NSHI individuals, 15 unrelated Cameroonians and 10 South Africans diagnosed with NSHI, and 26 ethnolinguistically matched control individuals with no personal or family history of HI were selected and investigated for the targeted mutations previously found in childhood HI in the human-mouse ortholog: *SPNS2*.[19]

Whole exome sequencing

Exome library preparation and WES were carried out by Omega Bioservices (Norcross, GA, USA). DNA concentration was measured using the QuantiFluor dsDNA System on a Quantus Fluorometer (Promega, Madison, WI, USA). An Illumina Nextera Rapid Capture Exome kit (Illumina, San Diego, CA, USA) was used for exome library preparation. Briefly, 50 ng of genomic DNA was fragmented using the Nextera transposomes. The resulting libraries were hybridized with a 37 Mb probe pool to enrich exome sequences. The libraries were then hybridized with a

37 M probe pool to enrich the sequences and were then sent for WES using the Illumina HiSeq 2500 (Illumina), 100 bp run format, with an average read depth of 30×.

Genotyping of targeted variants in SPNS2

For the selected 51 additional individuals (25 patients with NSHI, irrespective of the mode of inheritance, i.e. sporadic or autosomal recessive; and 26 ethnolinguistically matched controls), we used the Sanger sequencing technique to screen for the two deletion mutations (*c.1066_1067delCCinsT* and *c.955_957delTCC*) associated with childhood HI in the *SPNS2* human-mouse ortholog genes previously reported.[19] Specific primers targeting exons 5 and 6 were designed using Primer3 input and NCBI BLAST (Supplementary Table 1). The polymerase chain reaction (PCR) for the amplification of the specific exons was done using the PCR thermocycler machine. The optimal annealing temperature for the PCR was 60°C. The DNA sequencing was performed using the Sanger sequencing reaction on a BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Sequenced chromatograms obtained from the reaction were analyzed using the BioEdit software to identify the mutations.

Variants' annotation

The FASTQ files generated from the sequencer were processed to discard reads too short and the low-quality sequences. We checked for the high sequenced duplication rates, GC content, and Nextera adapter contaminations. Trimmomatic v0.39 was used for removing the Nextera adapters,[22] by trimming off three low-quality bases from the 5′ end and 30 low-quality bases at the 3′end of each read. Post sequencing reads were aligned to the build 37 of the human genome using the Burrows-Wheeler Aligner (BWA v0.7.15).[23] The sequencing quality check and data reports were done with the FastQC v0.11.5 and MultiQC v1.7 tools.[24] The GATKv4 MarkDuplicates command was used for marking and removing duplicate reads.[25] The GATKv3.8 IndelRealigner was used for the realignment of INDELs using the known sites on the 1000 Genomes Phase 1. The GATKv4 BaseRecalibrator and ApplyBQSR commands were used for the computational recalibration. The base quality score recalibration was done on the dbsnp 138. Variants were called from aligned sequence data using the GATK HaplotyperCaller to generate individual gVCF files. Supplementary Figure 1 illustrates the pipelines implemented for the variant calling. The variants were annotated with ANNOVAR,[26] SnpEff v4.3,[27] and ENSEMBL's variant effect predictor v98.2 [28] utility tools. The variants' rs IDs were then updated on the dbSNP151 database using the Bcftools v1.9 (SAMtools package).[29] The variant pathogenicity predictions were based on the *SIFT, PolyPhen2, mutation assessor, mutation taster, MetaLR, CADD,* and *LR* algorithmic scores.

From the initial predicted variants, we extracted those candidate variants predicted to be pathogenic in the list of 34 genes (Table 1) with a directed python command. Then, we excluded all the positions with the minor allele frequency (MAF) >5% in all the populations cataloged on the gnomAD database, and as well as the MAF with the AF ≤ 0.1% on the ExAC database. BEAGLEv5.0 was used for the data phasing for the site-specific heterozygosity, nucleotide diversity, and genotype frequencies computed using a python package, pyVCF. Selected candidate variants were queried against available WES data from Cameroonian control individuals ($n = 129$) without personal and family history of HI.

Table 1. Novel HI murine genes investigated.

Novel murine HI genes (Ingram *et al*., PLOS Biol 2019;17:e3000194)[19]	Chromosomal location in human	Mode of inheritance in human
acsl4	X	N/A
agap1	2	N/A
bai1	8	N/A
bhlhe40	3	N/A
brd2	6	AR
camsap3	19	N/A
cdk14	7	AR
csnk1g3	5	AR
cxcr2	2	N/A
duoxa2	15	AR
fads3	11	N/A
fam107b	10	N/A
fbxo33	14	N/A
gas2l2	17	N/A
herc1	15	AR
klc2	11	AR
klhl18	3	N/A
lrig1	3	AD
mcph1	8	AR
mir122	18	N/A
mkrn2	3	N/A
ocm	7	AR
pax9	14	N/A
pex3	6	N/A
phf20	20	N/A
selk	3	AR
setd5	3	N/A
spns2	17	N/A
srsf7	2	N/A
tram2	6	AR
usp42	7	N/A
wbp2	17	AR
ywhae	17	AR
zcchc14	16	N/A

N/A: not available; AR: autosomal recessive; AD: autosomal dominant.
Mode of inheritance was retrieved from OMIM database https://omim.org/ and GeneCards https://www.genecards.org/ based on clinical and functional information available on the condition/disorders associated with these genes.

Principal component analyses

The population structures of the cohorts were determined on the principal component analysis (PCA) chart using the smartpca tool.[30,31] The WES data (participants and controls) were merged and computed with data extracted from the 1000 genomes Phase3 [32]: 186 Yoruba (YRI) in Nigeria, 173 Esan (ESN) in Nigeria, 280 from Western Division in Gambia (GWD), 116 Luhya (LWK) in Webuye, Kenya, 112 from African ancestry in Southwest US (ASW), 128 Mende (MSL) in Sierra Leone and 123 from African Caribbean in

Barbados (ACB), and an available control dataset from the individuals from Democratic Republic of Congo. PCA was plotted and annotated using Genesis2 tool.

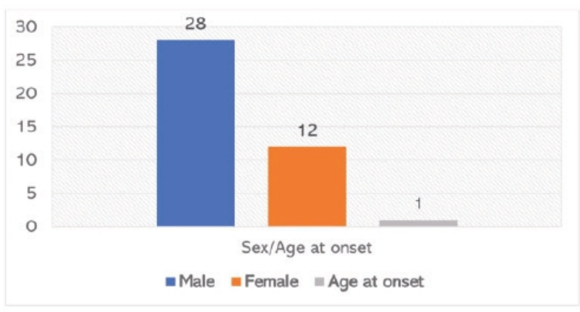
Protein modeling

Protein modeling of MCPH1 p.Pro771Ala. The protein sequence of MCPH1 isoform 1 was retrieved from NCBI and queried on the Protein Data Bank database. The 'A' chains of both templates were retrieved, using the PyMol's indicate chain command. The MCHP1 protein sequence was truncated to obtain the region covering the 'A' chains of the templates (including the mutated region). The rs369802722 (c.2311C>G, p.P771A) mutation was introduced into the appropriate position. A multiple template-based modeling strategy was used for the modeling of the 3D structures for the wild type (wt) and mutant (mt) using Modeller v9.23.[33] Top five models were selected based on their Discrete Optimized Protein Energy score evaluated on the Galaxy,[34] Rampage, and ProsA web services. The best model for both wt and mt structures was then retained based on its Galaxy energy and the proportion of residues in a Ramachandran plot, and ProsA Z-score.

RNA thermodynamic structure prediction of SPNS2, c867C>A p.(Pro289Gln). The DNA sequence of exon 6 of *SPNS2* was retrieved from Ensembl (www.ensembl.org). Positional substitution for *SPNS2,* c867C>A was done in the *FASTA* file. Using the Vienna RNA Package, the RNAfold server (http://rna.tbi.univie.ac.at/) was used to predict the minimum free energy structures and base pairing probabilities of the secondary structures of the single stranded RNA. The base pairing probability matrix in addition to the minimum free energy structure of the RNA for both the substituted and non-substituted *SPNS2,* c867C>A *DNA* sequences were generated and interactively studied.

Gene enrichment and network analyses

The gene enrichment analyses of the novel human-mouse ortholog genes were done using the *g.Proler* tool (https://biit.cs.ut.ee). *g.Proler* mapped the selected genes to their functional data sources and detected statistically significant enriched biological processes, pathways, regulatory motifs, and protein complexes.[35] Only the adjusted enrichment at threshold >16,000 for the biological terms, *P* values of 0.05, and Bonferroni correction threshold were considered. The gene network analyses were constructed, using STRING (v10), with the threshold at 0.4. The connected genes within the network were derived. All non-zero weighted edges were considered, and the fully connected components were visualized. Further analyses to understand the co-expression, physical interactions, shared protein domains, co-localization, and pathways shared by the selected genes *MCPH1*, *OCM* (i.e. *OCM1* and *OCM2*) and *LRIG1* and the well-established HI genes *GJB2* and *GJB6*; as well as gene-to-gene network interaction was analyzed using GENEMANIA.[36]



Average number	Cameroonians	Xhosa South Africa
Number of Familial Cases	6	6
Number of Sporadic Cases	12	16
Post lingual	0	2
Prelingual	18	20
Hearing perception left/right (db)	71–120	71–115
Other clinical signs	No	No

Figure 1. Study participants' demographic and phenotypic information. (A color version of this figure is available in the online journal.)

Results

Patients description

All the study participants had profound prelingual HI. The hearing acuities ranged from 71 to 120 dB. The percentage of male to female was 70% (28) to 30% (12). The median age at onset for HI was one year (range: 1–4 years). Twenty-eight individuals had isolated non-familial HI of likely genetic origin, and 12 had a familial history of HI inherited in an autosomal recessive non-syndromic pattern (Figure 1).

Identifications of sequenced variants

Up to 95% of the WES data were paired reads, and 97% of the targeted regions had $\geq$10× coverage. Up to 95% WES were paired reads, and 97% of targeted regions had $\geq$10× coverage. Four murine genes *d6wsu163e*, *zfp719*, *grp152*, and *minar2* have no human ortholog, therefore, 34/38 novel human-mouse ortholog genes were studied.

The average PHRED score for all the samples was 35 (Supplementary Figure 2). The percentage GC content of the coding regions was 45–50% (Supplementary Figure 3). The WES statistics showed that the quality was good (Supplementary Table 2).

In the Cameroonian cohort, a total of 4,490,688 single nucleotide variants (SNVs) were identified. Therefore, positions with read depth (DP) $<$ 8, and genotype quality (GQ) $<$ 20 were excluded resulting to a total of 152,289 SNVs (3.4% of the initial number) with a total genotyping rate of 99.8%. Of these, 143,489 (94.2%) were single nucleotide polymorphisms (SNPs), 3255 (2.1%) were insertions, and 5545 (3.6%) were deletions. The transition/transversion (Ti/Tv) ratio (2.46) of the variants fell within the expected range for good quality data. This ratio was even better for known variants only (3.50) which made up 1.5% (2279) of the total filtered variants based on dbsnp138. Most

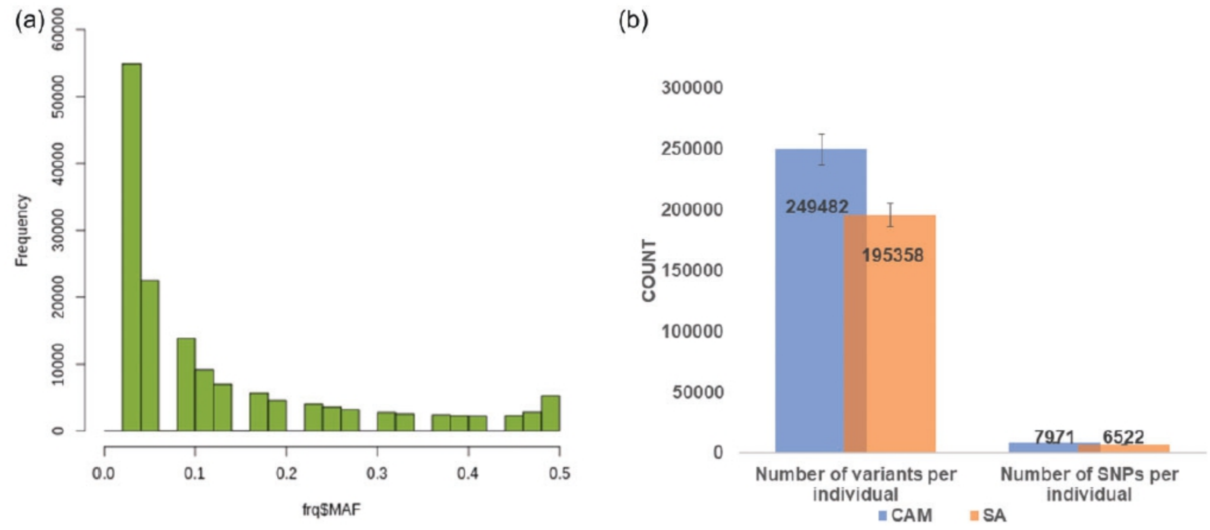


Figure 2. (a) Average number of variants and commonly reported SNPs identified per HI individuals (estimate of 40 samples) in the WES study. (b) The MAF distributions of all variants annotated on the gnomad database. (A color version of this figure is available in the online journal.)

of the variants observed were silent (98,220 – 51.3%), while missense variants made up 48.2% (92,151) and nonsense mutations constituted 0.52% (998).

In South African cohort, a total of 4,297,880 variants were called, 620 (1 multi-allelic) of which passed the following filters: minimum depth (DP) = 5, minimum GQ = 10. Of the 620 SNVs, 478 (77.2%) were SNPs. Then, 61 (9.85%) were insertions, while 81 (13.08%) were deletions. Missense mutations made up 44.83% of the variants while the remaining 55.17% were silent mutations. As expected, most of the variants (48.56%) were intronic, with the next most frequent category being intergenic variants (19.42%) which usually confer no significant effect. Also, as expected, exonic (1.68%), 5′ UTR (1.08%), and intragenic (0.78%) variants made up the least frequent categories.

Most of the annotated variants had the MAFs that ranged from 0.0 to 0.09 (Figure 2). The average number of annotated SNPs identified per patient was 7971 and 6522 in the Cameroonian and South African cohorts, respectively (Figure 2).

Population structure

The PCA analyses of common SNPs showed closer clusters between the HI samples and controls (PCA1), suggesting cases and controls population homogeneity. These clusters however differ with a significant distance from the other African populations (Figure 3). This result revealed unique genetic diversities in the African populations.

Pathogenic and likely pathogenic variants in WES. Three rare PLP variants were found in the WES studied in three human-mouse ortholog genes, among three unrelated Cameroonian patients (Table 2). Two PLP variants are in heterozygous state, i.e. *OCM2,* c.227G>C(p.(Arg76Thr), RNA not analyzed), *LRGI1,* c.1657G>A (p.(Gly533Arg), RNA not analyzed). In one patient, the variant *MCPH1,* c.2311C>G p.(Pro771Ala); RNA not analyzed) is in homozygous state. No PLP variant was found among the South African patients.

The patient identified with biallelic PLP variant *MCPH1,* c.2311C>G p.(Pro771Ala), was an eight-year-old girl, presenting with congenital prelingual bilateral and symmetrical sensorineural profound hearing loss (90–100 dB), from an non-consanguineous parents, with a family history compatible with ARNSHI (Supplementary Figure 4A). She did not specifically have microcephaly, described in the mouse model with mutation in *mcph1,*[19] or any learning difficulty. She attended a school for the deaf, with adequate academic progress. The two other Cameroonian patients who were monoallelic with variants in *LRGI1,* c.1657G>A p.(Gly533Arg) and *OCM2,* c.227G>C p.(Arg76Thr), respectively, also presented with congenital non-syndromic sensorineural HI with family histories compatible with autosomal recessive inheritance (Supplementary Figure 4B and C). We were unable to investigate the variants in the three genes among other family members because of the non-availability of the DNA samples.

Genotyping targeted variants in SPNS2

The previously reported *SPNS2* deletion mutations (*c.1066_1067delCCinsT*) and *SPNS2* (*c.955_957delTCC*) were not identified in the WES data, neither were they found in the 25 NSHI patients, nor in the 26 matched controls that were Sanger sequenced. One novel variant, *SPNS2,* c867C>A p.(Pro289Gln), was found in heterozygous state, in one Cameroonian patient.

MCPH1 (p.Pro771Ala) protein modeling

The protein modeling of the MCPH1 (p.Pro771Ala) showed that this variant occurs on the loop in the BRCT3 domain. Considering that MCPH1 chromatin (histone A2)-binding

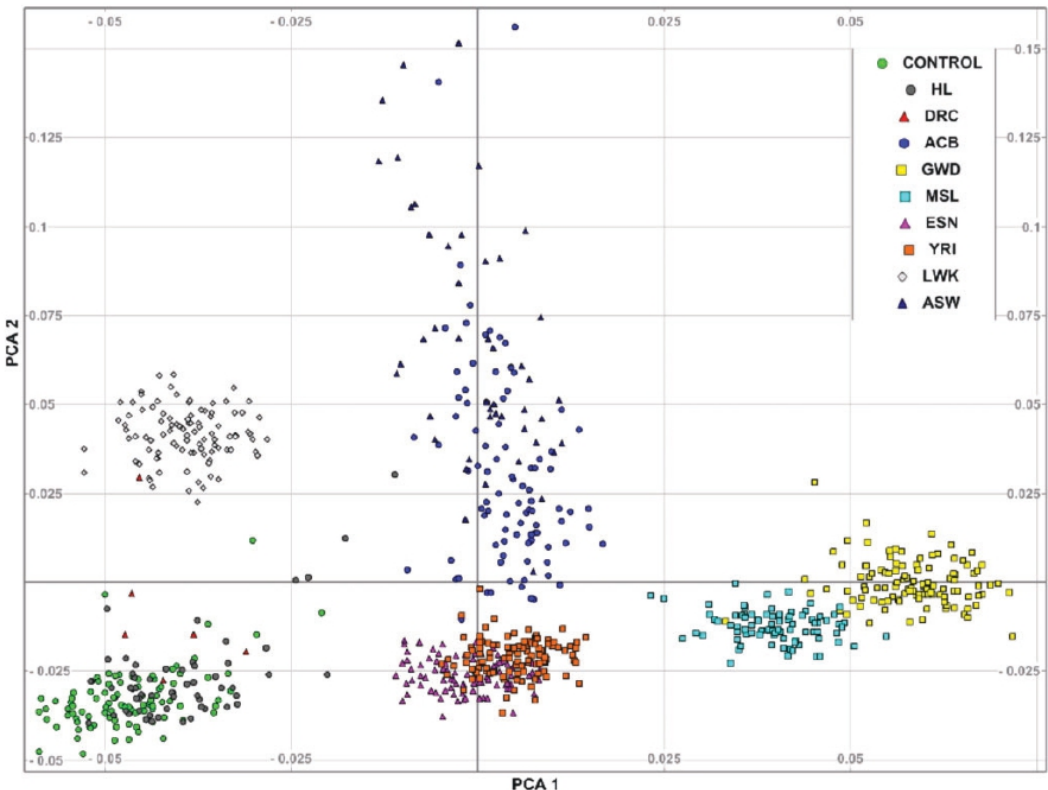


Figure 3. The PCA plot illustrating clusters distances of 0.075 to 0.15 that could be interpreted as disparity in allelic frequencies in the study cohorts (cases and controls) versus selected African populations in the 1000Genomes Phase 3 project.

Table 2. Candidate risk missense variants selected in this study.

dbSNP	Pos	Gene	Exon	OMIM	HGNC	NCBI	Coding	Amino acid change	Zygosity	Sift score	Polyphen2 score	Highest pop. AF	Country	No. of patient
rs139724916	7:97616436	OCM2	3	164795	34396	NM_006188.3	c.227G>C	p.R76T	Het.	0	0.998	0.0015	CAM	1
rs77775448	3:66436537	LRIG1	13	608868	17360	NM_015541.3	c.1657G>A	p.G553R	Het.	0.01	1	0.0242	CAM	1
rs369802722	8:6479071	MCPH1	13	607117	6954	NM_024596.4	c.2311C>G	p.P771A	Hom.	0.01	0.999	0.0002	CAM	1
Novel	7:4532615	SPNS2	6	612584	26992	NM_001124758.3	c.867C>A	p.P289Q	Het.	0.02	0.967	–	CAM	1

dbSNP: the single nucleotide polymorphism database; Pos: chromosomal position; highest pop AF: highest frequency of alternate allele in online databases; CAM: Cameroon; HGNC: The HUGO Gene Nomenclature Committee Identification number; NCBI: National Center for Biotechnology Information accession number; OMIM: Online Mendelian Inheritance in Man accession number; Het.: heterozygous; Hom.: homozygous.
All variants were validated on the Variant Validator database (https://variantvalidator.org).

site lies in the BRCT2 domain (residues 137–142), it is unlikely that the P771A mutation, which results in no visible change in the protein structure, would influence the overall protein function (Figure 4).

SPNS2, c867C>A p.(Pro289Gln) RNA thermodynamic structure. The *SPNS2,* c867C>A p.(Pro289Gln) RNA thermodynamic structure prediction showed that the variant is located at the D loop arm of tRNA and is likely to disrupt the intramolecular base-pairing which may in turn impair the tRNA metabolism as shown in Supplementary Figure 5.

Network interactions

The STRING network analyses showed functional interactions between the *MCPH1* gene and 10 non-HI genes (Figure 4). Of these, *NCAPG2, SMC2, NCAPHZ, NCAPD3,* and *SMC4* genes were derived in the weighted interactions, while *SET, ASPM, CENPJ, CDK5RAP2,* and *WDR62* genes were identified in the non-zero-weighted edges as shown in (Figure 4). The *in silico* functional interactions suggest a common biological pathway that represents systemic activities for hearing. Little is known about the functions of these associated non-HI genes in hearing. However, mutations in *NCAPG2*[37] and *NCAPHZ*[38] were previously associated

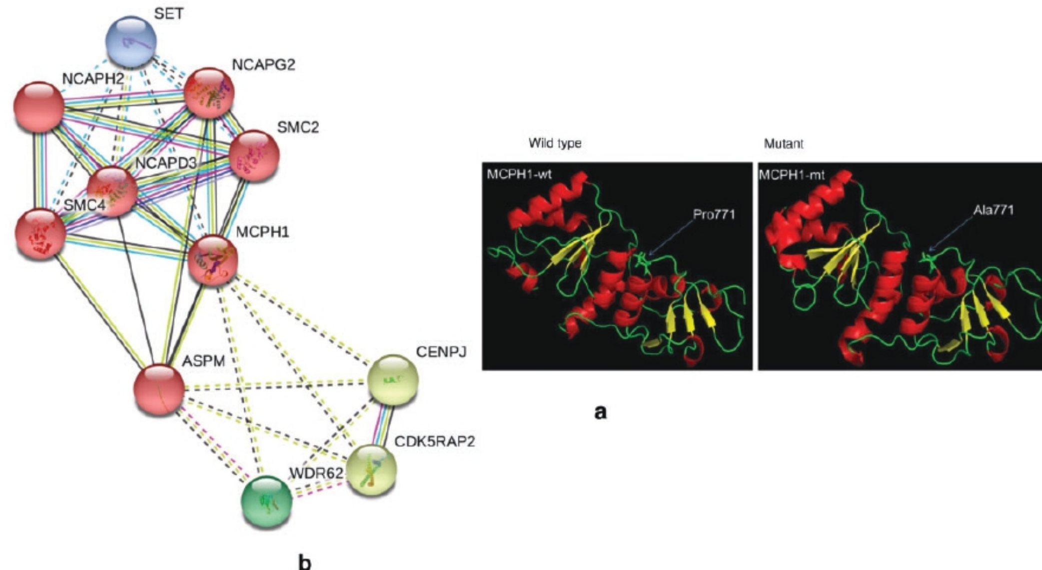


Figure 4. (a) Three-dimensional structures of both wild type (wt) and mutant (mt) of *MCPH1* P771A variant. The variant occurs on a loop in the BRCT3 domain. Considering that MCPH1 chromatin (histone A2)-binding site lies in the BRCT2 domain (residues 137–142), it is unlikely that the P771A mutation, which results in no visible change in the protein structure, would influence the overall protein function. (b) *MCPH1* STRING network.

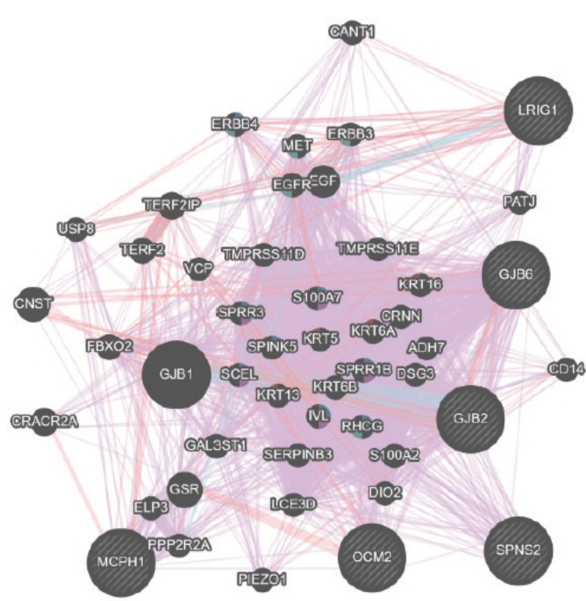


Figure 5. Illustration of functional co-expression interaction between the selected murine genes with the *GJB2* and *GJB6* genes. (A color version of this figure is available in the online journal.)

with a spectrum of neurodevelopment syndromes and pregnancy-associated abnormalities.

Furthermore, the GENEMANIA network analyses of these 34 human-mouse ortholog genes showed a biochemical association that functionally co-expressed and interacted with *GJB2* and *GJB6* (Figure 5). The gene enrichments results showed four significant systemic activities related to molecular function, nucleic acid binding, protein binding, and catalytic activities as shown in Figure 6. These four systemic activities representing the genes' functions pose active biological roles in the membrane-bonded organelles and intracellular/cellular compartments. Furthermore, CORUM significantly (P value 4.895×10^{-2}) identified six functional complexes (*CXCR2-NHERF1-PLC* beta-3 complex, *TS2-HERC1*, *RAF1-MAP2K1-YWHAE*, *PAX9-MSX1*, *HSF1-YWHAE* and *MLL1-WDR5*) which can be implicated in the various biological responses such as cell biogenesis and transcription factors (Figure 6).

Discussion

This is the first investigation of human-mouse ortholog genes in the African populations that found in a modest WES samples (n=40) a PLP variant, *MCPH1*, c.2311C>G p.(Pro771Ala), in a homozygous state (1/34) that could explain HI in a patient, and three additional PLP variants in 3 genes in heterozygous states. With a larger sample size, such an approach could lead to novel discovery of more novel HI PLP variants in these understudied populations, and possibly further our understanding of the pathophysiology of HI.

Indeed, the murine hearing system is very similar to that of humans[18] and had revealed important findings on the genetics of HI. Similar approaches previously identified two PLP variants in the human-mouse ortholog gene *SPNS2*,[19] that were not replicated in the present study. However, we found a novel heterozygous PLP variant, *SPNS2* c867C>A p.(Pro289Gln), in a single individual, which is predicted to disrupt protein functions (Supplementary Figure 5). The effects of mutations in this gene were previously associated with the transporters on the P13K/Akt pathway in lamellipodia formation in lung's

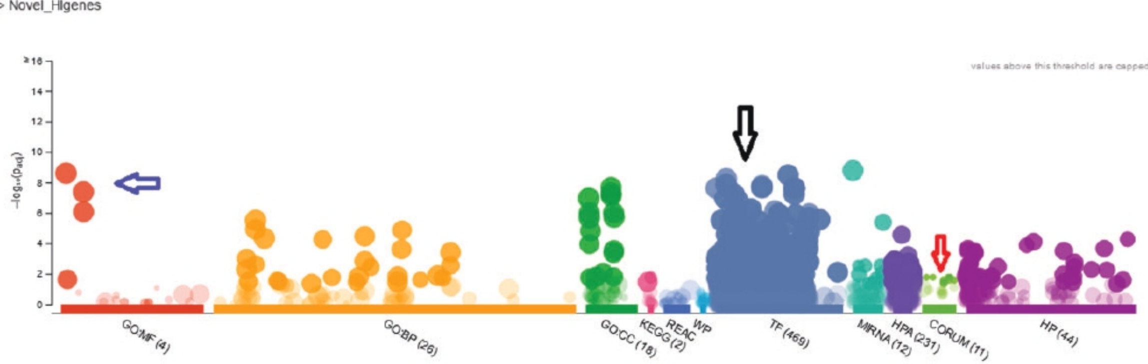


Figure 6. Multiquery Manhattan plot. X-axis shows the functional terms grouped and color-coded by data source. Functional enrichment terms for the 34 novel human genes. (1) Blue arrow shows four significant GO:MF terms namely (molecular function, nucleic acid binding, protein binding and catalytic activities), (2) black arrow shows strong transcriptional regulation and regulatory motif matches from TRANSFAC (TF), and (3) red arrow shows six significantly (*P* value 4.895×10^{-2}) identified functional complexes.

endothelium and reactive oxygen species generation.[39] However, there is no strong evidence to support the causative effects of this gene in our patient because the variant is in heterozygous state. The variant identified in the *MCPH1* gene in a patient in this study was in homozygous state. A few studies have been performed to understand the roles of the *MCPH1* gene: *MCPH1* has been categorized as a prominent DNA repair gene that works to prevent premature mitotic entry.[40] Its roles in HI in humans have not been previously reported. But, *MCPH1* is very close to the *DFNM2* locus on Chromosome *8*, with both *DFNM1* and *DFNM2* loci being hotspots for various NSHI genes.[41,42] Although the protein modeling of the variant identified *MCPH1*, c.2311C>G p.(Pro771Ala) did not show visible change in the protein structure (Figure 4(a)), the network interactions between the *MCPH1* gene and other genes as seen in the present study (Figure 4(b)) clearly showed *MCPH1* relevance in some biological activities. Furthermore, *OCM* (*OCM1* and *OCM2*) gene has not been the focus in human HI. It is noteworthy to state that *OCM* gene alias *ocm1* gene that was identified in the murine study[19] is an important paralog of *OCM2* with a similar function. Some studies have described the causative effects of *OCM2* gene, with a pathogenic mutation identified (c.227G>C(p.(Arg76Thr), RNA not analyzed) and associated with intellectual disability in a family-based study.[43] Also, in a genome-wide association study that found a significant variant in the *OCM2* gene associated with cardiac arrest in patients with coronary artery disease.[44] Nonetheless, there is no strong evidence to support the causative effects of this gene in our patient because the variant is in heterozygous state. In this study, we identified a PLP variant in the *LRIG1* gene. Unlike the previously discussed genes, genomic PLP variants in *LRIG1* were recently found in an adult onset of HI in humans.[45] Interestingly, the Cameroonian patient in which we found the monoallelic *LRIG1*, c.1657G>A p.(Gly533Arg) was the only adult patient of the cohort (Supplementary Figure 4B), although, the significance of the identified PLP variant in this gene in terms of the age at HI onset was not investigated. The LRIG1 protein is involved in the sequential developmental events that include axon guidance and synapse formation.[46] In addition, gain and loss of function assays in mice indicate that the LRIG1 protein restricts dendrite morphology.[47]

Taken together, the roles of mutations in these genes in HI will need further exploration in larger and multiple HI human populations and controls from multiple settings, to refine the disease-gene pair curation. Indeed, a high number of PLP variants in known HI-associated genes were found in the same hearing-impaired individuals, and the larger 1000 Genomes Project sample set, with unknown hearing phenotype or auditory function.[45] The population structure analysis of the WES dataset investigated in this study further highlights high genetic structure differences between the cohorts and across different African populations, a diversity associated with nearly 300,000 years of evolutionary changes.[21] This is an indication that future study on HI among Africans should recruit control individuals from the same regional and ethnolinguistic group as the patients. Indeed, in the African populations many variants may not be fully annotated because of the limited African genome data in the public genome databases.[48]

The major limitation to this study is the limited sample size of individuals with HI. Therefore, our findings cannot be generalized in the Cameroonians and South African populations. Future studies should include multiplex families with available biological samples and data that will afford the possibility of performing segregation analysis. Moreover, *in vitro* functional analysis could have complemented our finding because gene variants may affect protein expression or mRNA expression without changing the protein structure. The present study also revealed that not all murine genes have human ortholog (4/38), suggesting that numerous mutations in mice with HI will not necessarily apply to humans.

In conclusion, among the identified rare candidate PLP variants in 3/34 human-mouse ortholog genes, only one, the homozygous variant *MCPH1* p.Pro771Ala, could likely explain HI in a patient. *In silico* functional analysis revealed co-expression and interactions with well-established *GJB2*

and *GJB6* HI genes suggesting that the human-mouse ortholog genes investigated could coordinate important biological functions associated with the hearing system in humans. The identification of rare potentially pathogenic coding risk variants in these of patients of African ancestry could contribute to furthering our understanding of genotype-phenotype variations in NSHI.

AUTHORS' CONTRIBUTIONS: OGO conceptualized the idea, performed parts of the experiments, analyses, prepared results, and drafted the manuscript. KKE and EC did parts of the analyses and results. JJN, ETW, and NM diagnosed, recruited patients, and revised the manuscript. AW is the project lead, supervised the recruitment, experiments, analyses, and revised the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The study was approved in Cameroon by the Institutional Research Ethics Committee for Human Health of the Gynaeco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon (Ethics approval number Nº723/CIERSH/DM/2018) and in South Africa by the Human Research Ethics Committee (Ethics approval number HREC Ref: 104/2018) of the University of Cape Town.

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SUPPLEMENTAL MATERIAL

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Contribution to authorship

G.K.M., V.N., and A.W. are leading the H.I.O. implementation.

J.H., N.M., **E.W. (Edmond Wonkam Tingang)**, V.N., N.V., G.K.M., and A.W. curated the current H.I.O. content.

N.M., J.H., **E.W. (Edmond Wonkam Tingang)**, S.M.A., O.G.O., K.M., A.Y., and A.W. provided the H.I.O. content.

G.K.M., N.J.M., M.H., N.V., S.J. contributed to the ontology structure development and implementation.

G.K.M., J.H., N.M., and V.N. wrote the manuscript.

S.M.A., O.G.O., **E.W. (Edmond Wonkam Tingang)**, K.M., A.Y., M.H., N.V., N.J.M., S.J., and A.W. revised the manuscript.

Article

The Hearing Impairment Ontology: A Tool for Unifying Hearing Impairment Knowledge to Enhance Collaborative Research

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Abstract: Hearing impairment (HI) is a common sensory disorder that is defined as the partial or complete inability to detect sound in one or both ears. This diverse pathology is associated with a myriad of phenotypic expressions and can be non-syndromic or syndromic. HI can be caused by various genetic, environmental, and/or unknown factors. Some ontologies capture some HI forms, phenotypes, and syndromes, but there is no comprehensive knowledge portal which includes aspects specific to the HI disease state. This hampers inter-study comparability, integration, and interoperability within and across disciplines. This work describes the HI Ontology (HIO) that was developed based on the Sickle Cell Disease Ontology (SCDO) model. This is a collaboratively developed resource built around the 'Hearing Impairment' concept by a group of experts in different aspects of HI and ontologies. HIO is the first comprehensive, standardized, hierarchical, and logical representation of existing HI knowledge. HIO allows researchers and clinicians alike to readily access standardized HI-related knowledge in a single location and promotes collaborations and HI information sharing, including epidemiological, socio-environmental, biomedical, genetic, and phenotypic information. Furthermore, this ontology illustrates the adaptability of the SCDO framework for use in developing a disease-specific ontology.

Keywords: hearing impairment; hearing loss; ontology; data harmonization; meta-analysis

1. Introduction

Hearing impairment (HI), the partial or total inability to hear, is a communication barrier and language development impediment. It can thus have a huge effect on one's quality of life [1]. HI has the highest rate for age-standardized disability of life in the world [2,3]. According to the most recent World Health Organization (WHO) estimates [4], over six percent of the world's population, representing approximately 460 million individuals, are currently living with a disabling HI, of which 93% are adults and mostly males (242 million males vs 190 million females). The financial burden associated with HI, which includes costs for healthcare, education, social support, and loss of productivity [5], is estimated to be 750 billion US dollars annually [6]. Even though 60% of HI cases can be prevented [5], the number of cases is expected to significantly increase to over 900 million in 2050 [4] with huge negative economic implications, unless action is taken. As a matter of urgency, there is a need to strengthen collaborative HI research efforts aimed at curbing the projected increased burden of HI globally. The Global Hearing Loss project (https://thespindle.org/project/global-hearing-loss-database/) has highlighted that HI research data is commonly unstructured, stored in natural language format, and hardly shared. The general lack of standardization of research data on rare or neglected diseases across studies [7] hampers presentation, sharing, integration, and interoperability of important information, such as prevalence, socio-environmental, biomedical, and phenotypic information. The need for harmonized HI datasets motivated the World-Wide Hearing group to develop a standard platform, the Global Hearing Loss Database (GHLD). The GHLD is based on WHO protocols with a web portal and a smartphone application to ease HI data collection and sharing processes. However, given the complexity of HI etiologies and phenotypes, analyses of these datasets and inter-study comparability would require a standard knowledge representation of the HI knowledge domain [8]. A standard knowledge representation of the HI concepts or terms would include concise descriptions to ensure a common understanding of the domain and to enable automated reasoning and inferencing. Moreso, with the constant evolution of biomedical knowledge [7], a human- and machine-readable upgradeable system is needed for standardized and well-defined HI knowledge representation to enhance collaborative research in the field.

Recent advances in artificial intelligence have fostered the use of ontology models to represent knowledge and information-based systems [9] in a human- and machine-readable format to help process, reuse, and re-apply knowledge [10,11]. An ontology is useful in establishing a common and controlled vocabulary system, describing key concepts, properties, and hierarchical relationships between concepts [12], with precise definitions for clear and unambiguous communication. In the biomedical research context, several human disease-related ontologies have been introduced, including the Human Disease Ontology (DO), which consistently defines various concepts encountered in disease domains [13], the Mondo Disease Ontology, which provides a merged and comprehensive cross-species disease ontology (https://monarch-initiative.github.io/mondo/), and the Human Phenotype Ontology (HPO), providing controlled vocabularies of abnormal phenotypes encountered in human disease [14,15]. However, as previously argued in support of developing the Sickle Cell Disease Ontology (SCDO) [5], none of the existing ontologies comprehensively captures related concepts specific to HI due to the complex nature of HI etiologies and phenotypes.

We present the Hearing Impairment Ontology (HIO), built upon the SCDO framework. The HIO was compiled by a working group, which includes HI and ontology experts, who defined, in detail, essential aspects of the HI knowledge domain (e.g., phenotypes, genetics, therapeutics, diagnostics, etc.) and how these aspects are related. Similar to how the SCDO was built around the central concept, 'Hemoglobinopathy' [7], the HIO is built around the 'Hearing Impairment' concept. However, because HI can be associated with a myriad of phenotypes and/or syndromes, caused by various genetic, environmental, and/or unknown factors, the SCDO framework was adapted to account for the additional complexities of HI. To date, this developing HIO represents the most comprehensive standardized HI domain knowledge portal, which will allow for the application of ontology-driven mining approaches for the identification of pertinent research questions.

2. Materials and Methods

For this first version of the HIO, the working group consisted of experts from an existing Hearing Impairment Genetics Studies in Africa (HI-GENES Africa) project at the University of Cape Town, Division of Human Genetics in the Department of Pathology, and expert ontology developers to provide technical guidance. The experts from the HI-GENES Africa project included PhD students and Postdocs, with a varied range of expertise which included clinicians, biomedical scientists, geneticists, and bioinformatics experts. SCDO curators and developers led the design, following the published ontology development reporting guidelines [16] and best practice.

2.1. SCDO Model-Based HIO Development

After attempting to "fit" different forms of HI and their associated causes into the same upper classes used in the SCDO (except replacing 'Hemoglobinopathy' with 'Hearing Impairment'), it was apparent that the HIO model needed to be carefully adjusted to account for the marked differences in the causes and pathophysiology between these diseases. A schema for the HIO was drawn up to formalize how the HIO would be modelled (what main classes would be needed and what relationships would be described between these classes). It is worth noting that these two diseases have complex phenotypic expressions, influenced by several genetic and environmental factors. Since SCDO was built around the central concept 'Hemoglobinopathy' to include more factors influencing its phenotypes [7], likewise, HIO is built around the central concept 'Hearing Impairment' to ensure that all aspects influencing the disease outcome and phenotypic manifestations are captured. This is achieved by relating different HI concepts specified in the ontology to various factors, including genetic and environmental factors, that contribute to the disease outcome. The overview of different steps in the modeling, from populating the ontology, checking different concepts and relations to the release of the HIO by curators, domain and ontology experts via internal and external reviews, is described in Figure 1.

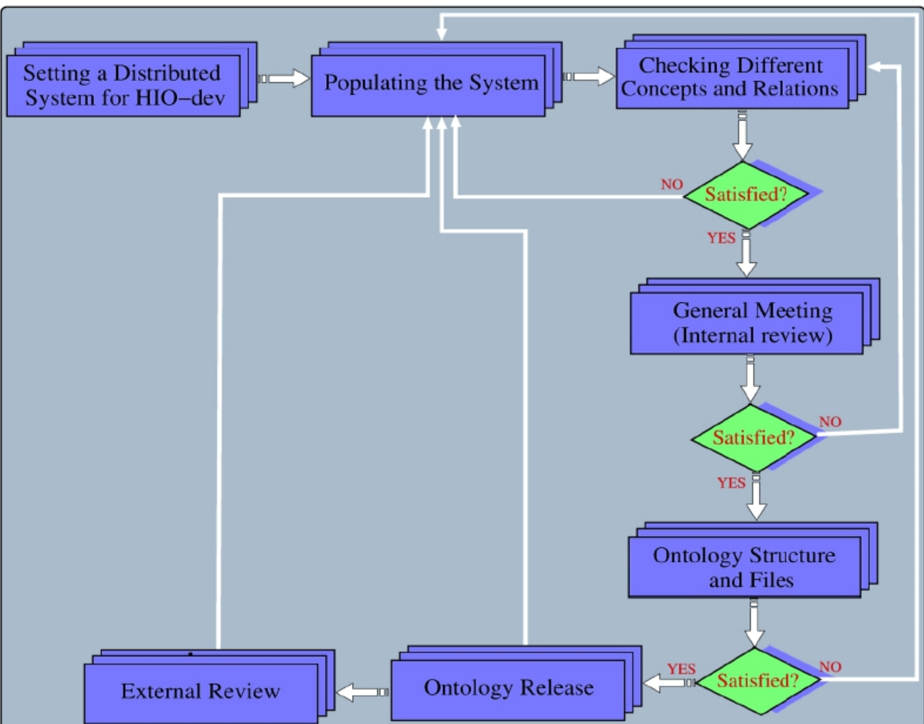

Figure 1. Flow chart of the dynamic and iterative ontology development process. It starts by setting up an online collaborative ontology development tool, WebProtege, which provides a highly distributed

ontology content management system, enabling domain experts, ontology curators, and developers to share and update information, and easily visualise the ontology classes and structure. A general discussion meeting (or internal review) is called to share a common understanding of existing Hearing Impairment (HI) knowledge currently included in the ontology and resolve any disagreement about a given concept.

2.2. Building Different HIO Objects

Annotation properties (both required and optional) were re-used from the SCDO. The additional annotation property, 'deprecated synonym', was included by the working group in order to indicate when a term has a synonym that is no longer acceptable. Terms to be included in the ontology were added and annotated in a shared online spreadsheet by the working group. The relationships between classes were also captured in shared online spreadsheets (each sheet dedicated to the relationships made by a certain object property). To keep track of terms reused from existing ontologies, the 'existence in other ontologies' annotation property was used to assign an 'existence status' to each term. The frequency of existence statuses was subsequently used also to evaluate ontology terms unique to the HIO and its contribution to updating HI terms in other ontologies.

2.3. Distributed Model-Based HIO Design

Coordinating an ontology development with groups of contributors from heterogeneous specialized backgrounds to derive a unified domain conceptualization is challenging. To ease the process, the online collaborative ontology development tool, WebProtege [17], was used to draft the skeleton structure (labels of terms only) of the HIO in Ontology Web Language (OWL) format. This tool provides a highly distributed ontology content management system, enabling domain experts, ontology curators, and developers to share and update information, and easily visualise the ontology classes and structure.

2.4. HIO File Refinement and Evolution

For quality control assurance of different concepts in the ontology and considering the dynamic evolution of the ontology structure, an iterative and collaborative process was used for refining different definitions and properties, as well as the topological structure. These concepts were validated by experts before being included into the hio-edit.owl file in WebProtege. Each term had to be checked by at least two members of the working group, including at least one HI expert. Once terms were validated during the general online discussion meeting (or internal review), curators added their annotations from the spreadsheet into the WebProtege project (recording in the spreadsheet which terms had been added). Thereafter, ROBOT, an open source tool for automating ontology development workflows and tasks [18], was used to compile the complete ontology release files, which are Web Ontology Language (OWL) and Open Biomedical Ontology (OBO) formats.

3. Results

3.1. HIO General Description

Other than the 'HIO' and 'deprecated terms' classes, the HIO currently consists of 10 upper classes (see Figure 2). It contains 495 terms that are topologically connected by 543 links (is_a relationships) and expected to be associated with each other by 45 object properties (excluding the owl:topObjectProperty). Figure 2 shows how the SCDO upper classes were adapted for the HIO. Figure 3 shows the number of concepts in the HIO's upper level classes (Graph A) and the number of relationships currently asserted between concepts via object properties (Graph B) in this first release version of the ontology.

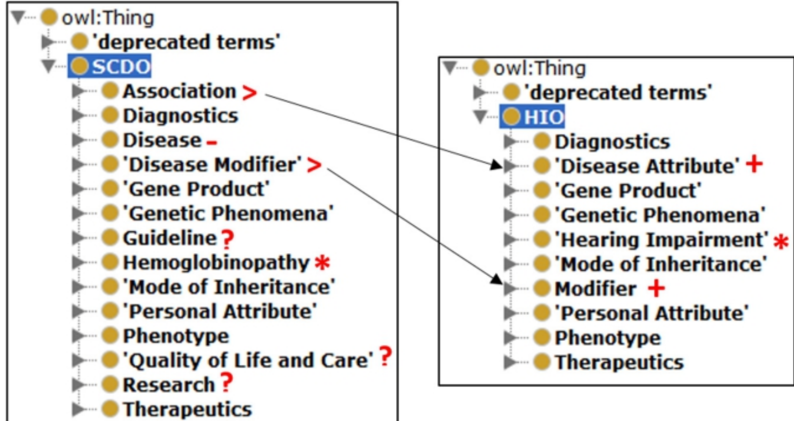

Figure 2. The upper classes of the Sickle Cell Disease Ontology (SCDO) and HI Ontology (HIO). (*) indicates the ontologies' central classes. (+) indicates classes in HIO but not in the SCDO. (-) indicates a class in SCDO but not in HIO. (>) indicates classes in SCDO that were incorporated in other HIO classes. (?) indicates SCDO classes that still need to be reviewed and adapted as necessary for inclusion into the HIO.

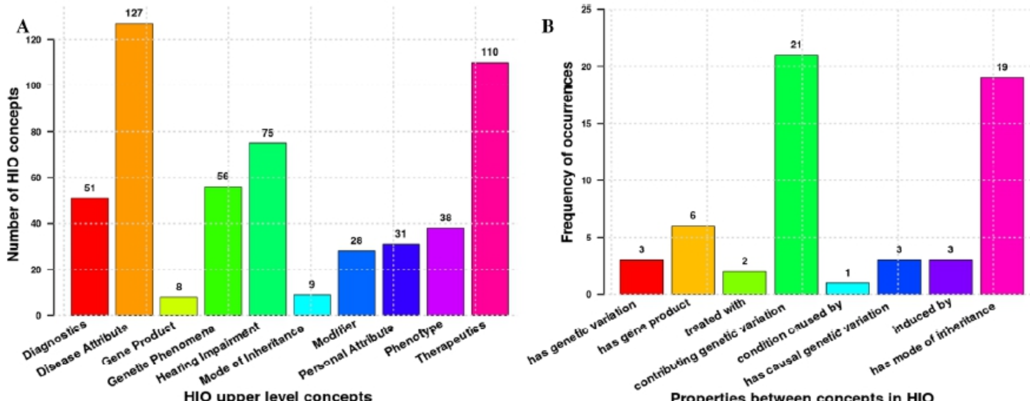

Figure 3. Summary statistics of current concepts and properties in the current HIO. Numbers at the top of bars represent the number of different HIO sub-classes topologically linked to upper level classes (**A**) and the occurrence frequency of a given property or association in the ontology (**B**). Note that 'contributing genetic variation' is used as the short hand label for the 'gene carrying contributing genetic variation' property and 'has causal genetic variation' for the 'condition has causal or contributing genetic variation' property.

The following upper classes from the SCDO are included in this first draft of the HIO: Diagnostics, Gene Product, Genetic Phenomena, Mode of Inheritance, Personal Attribute, Phenotype and Therapeutics. A new HIO identifier is assigned to each reused concept from other ontologies and attached to cross references to the source ontology. The SCDO's central 'Hemoglobinopathy' class has been replaced by a new central 'Hearing Impairment' class, which contains four main subclasses: 'Hearing Impairment by Cause', 'Hearing Impairment by Ear Affected', 'Hearing Impairment by Onset', and 'Hearing Impairment by Physiopathology Mechanism' (see Figure 4), which are comprehensively populated with the current HI domain knowledge, capturing various aspects associated with HI.

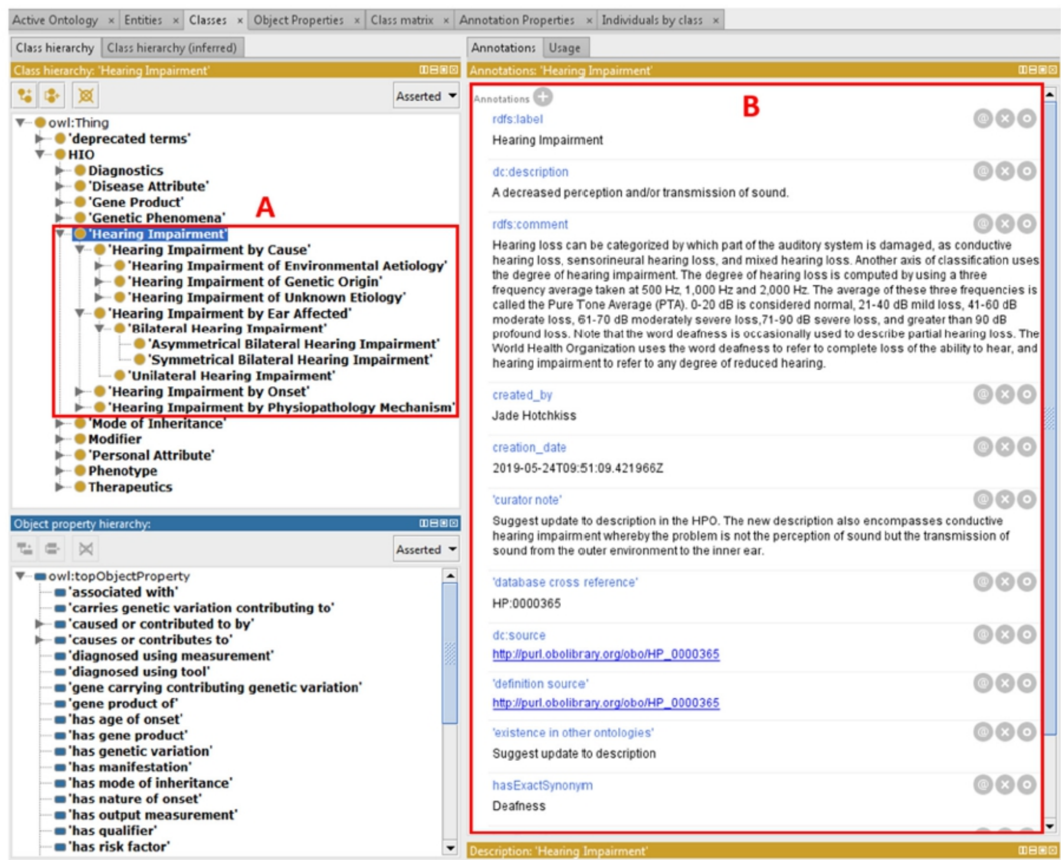

Figure 4. The 'Hearing Impairment' class within the HIO. (**A**) General categorization of hearing impairments in the 'Hearing Impairment' upper class and (**B**) annotations of the 'Hearing Impairment' class.

Two new (compared to the SCDO) upper classes have been included, namely: 'Disease Attribute' and 'Modifier' (see Figure 2). The 'Disease Attribute' class (see Figure 5) incorporates content similar to the SCDO's 'Association' class but notably also includes a 'Disease Cause' sub-class, which was found necessary due to the varied and often complex causes of hearing impairments [19–22]. The 'Disease Cause' class contains the term 'Unknown Etiology' and the two subclasses 'Environmental Disease Cause' and 'Intrinsic Disease Cause' [23–25], which are populated comprehensively with factors that cause or contribute in some way to HI.

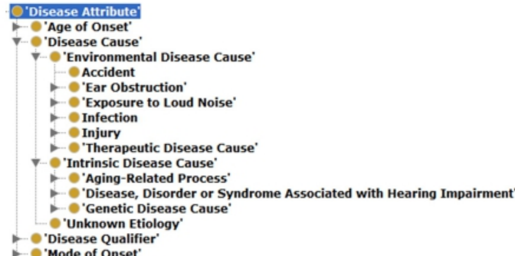

Figure 5. The 'Disease Attribute' class structure within the HIO. This hierarchy is intended to contain all possible features specific to or leading to HI.

The 'Modifier' class includes the SCDO's 'Disease Modifier' upper class as a sub-class, along with a new 'Disease Cause Modifier' class. This additional type of modifier was included because causes of hearing impairment sometimes have modifying factors that determine whether or not the disease

is in fact caused/present, e.g., ototoxicity induced by drugs (one cause of hearing impairment) has numerous modifying factors (e.g., dose, duration of therapy, concurrent renal failure, infusion rate, lifetime dose, coadministration with other drugs having ototoxic potential, genetic susceptibility) [26].

The HIO's central 'Hearing Impairment' class links, either directly or indirectly, to sub-classes of all other upper classes through numerous associations, as can be seen in Figure 6. For simplicity, associations made with terms in the 'Modifier' upper class are not shown (see these in Figure 7) and only associations with the 'Disease Cause' sub-class of the 'Disease Attribute' class are displayed. As shown by the large yellow c-shape in Figure 6, the 'Intrinsic Disease Cause' class encompasses portions of most of the other upper classes, namely 'Genetic Phenomena', 'Phenotype', 'Gene Product', 'Personal Attribute' and 'Therapeutics'.

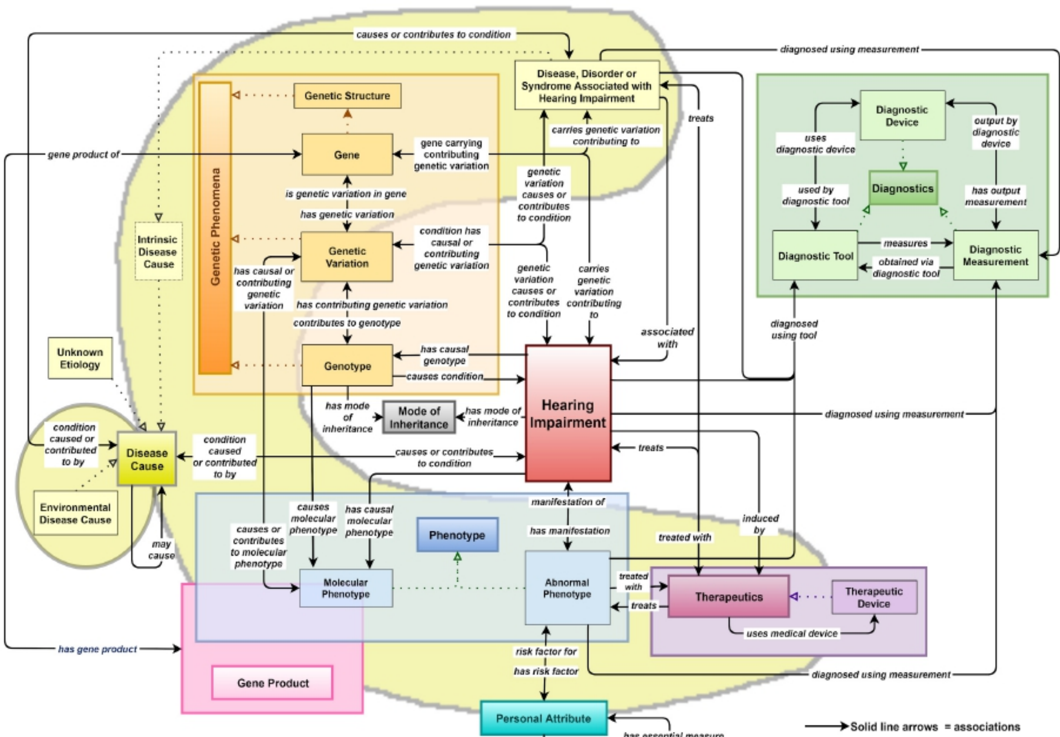

Figure 6. Associations made in the HIO between upper level (close to the root of the ontology) classes (excluding 'Modifier' class and only including 'Disease Cause' sub-class (yellow shapes) of the 'Disease Attribute' class). The 'Hearing Impairment' class is the central class.

The remaining associations made in the ontology, i.e., with the other disease attributes not in the 'Disease Cause' class (namely: 'Age of Onset', 'Mode of Onset', and 'Disease Qualifier' (which includes qualifiers such as 'Rare and 'Acquired')) and with the 'Modifier' class, are shown in Figure 7.

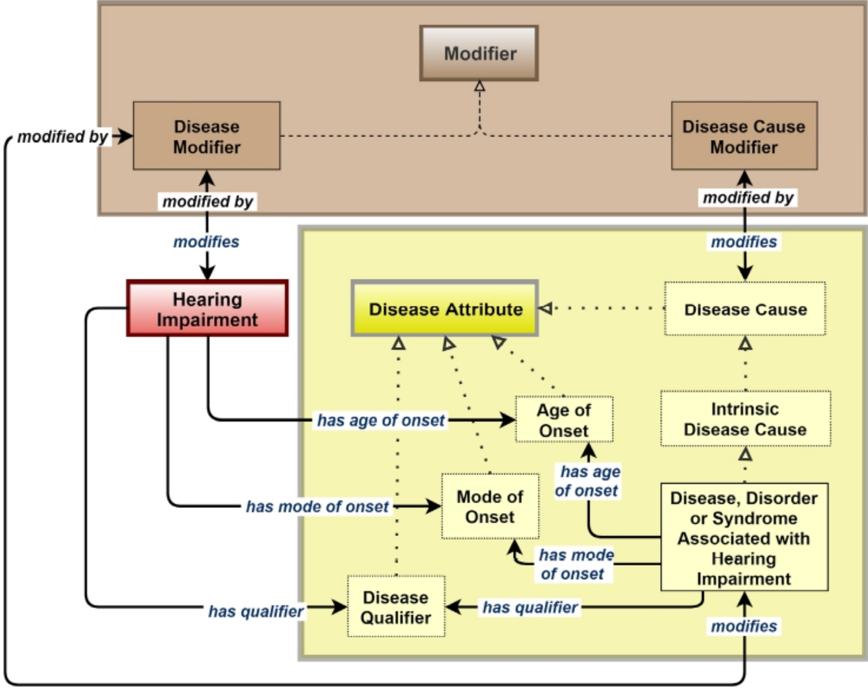

Figure 7. Associations made in the HIO to and from the 'Modifier' and 'Disease Attribute' classes, excluding those already shown in Figure 5 for the 'Disease Cause' class.

3.2. *Assessing the Relevance of HIO*

Of the 493 terms in the ontology (excluding 'deprecated terms' and upper 'owl:Thing'), at the time of writing this, 399 terms have descriptions and are considered as having all minimum required annotations (i.e., *rdfs:label*; *dc:description*; *definition source*, if relevant; *database cross reference*, if applicable; *dc:creator*, if the term was defined by an HIO curator using a source other than available ontologies; *existence in other ontologies*, used to record the existence status of the term prior to inclusion in the HIO; and *hasExactSynonyms*, to indicate synonyms, where relevant). Analysis of the existence statuses ascribed to these terms using the 'existence in other ontologies' annotation property (see Table 1) shows that the HIO, even in its first draft, is making contributions towards including and standardizing HI terms that were previously not included in other ontologies. Some of these terms are provided in Table 2 for illustration. Where applicable, terms unique to the HIO will be recommended for inclusion into other related ontologies, such as the HPO, Orphanet, DO, MESH, and NCIT.

3.3. *HIO Release and License*

The HIO is released every two months with possible special releases when there are significant incidental changes. It is freely available under the Creative Commons Attribution 4.0 Unported License (CC:https://creativecommons.org/licenses/by/4.0/legalcode) and further copyrighted to maintain the quality and integrity of the term vocabulary, meaning that any modification to the HIO can only be done by HIO developers and curators.

3.4. *Different HIO Access Platforms*

In order to foster the dissemination of and easy access to this novel ontology the latest OWL file produced has been uploaded to the NCBO BioPortal at https://bioportal.bioontology.org/ontologies/HIO. This also facilitates the searching and viewing of different HIO concepts. In addition, the OWL and OBO files can be accessible via the GitHub repository at https://github.com/hiodev/hi-ontology.

Table 1. Summary of terms' existence statuses prior to inclusion in the HIO.

Existence Status	Explanation of Status
Sufficient	Exists in other ontology and has appropriate description
Suggest update to description	Used term from existing ontology but will suggest they update their description to
Suggest update to label	Used term from existing ontology but will suggest they update their label to ou
Suggest update to label and description	Used term from existing ontology but will suggest they update their label and descriptio
Few but definitions not available	Term exists in a few ontologies but has not been given a description in any
Few but definitions not freely available	Term exists in a few ontologies but the description is not freely available
Few but definitions not specific enough	Term exists in a few ontologies but the definitions are not specific enough for the HIO
Not relevant to context of hearing impairment	Term exists in other ontologies but the definitions are not relevant to the HI fie
Negligible	No description or outdated ontology
None	Not in any existing ontology

Table 2. Some of terms that are unique to HIO.

Term Label	Term ID	Term Description
Symmetrical Bilateral Hearing Impairment	HIO:0000365	When the severity and configuration of hearing impairment is appr
Asymmetrical Bilateral Hearing Impairment	HIO:0000366	When each ear has a different severity and configuration c
Postlingual Hearing Impairment	HIO:0000475	Hearing impairment which develops after the acquisition of speech and
Prelingual Hearing Impairment	HIO:0000476	Hearing impairment which is either congenital or develops before the usually before the age of 6.
Temporal Bone Fracture with Otic Capsule Involvement	HIO:0000287	Traumatic injury to the temporal bone in which the continuity of the bo capsule is involved.
Temporal Bone Fracture without Otic Capsule Involvement	HIO:0000288	Traumatic injury to the temporal bone in which the continuity of the bo capsule is not involved.
Pseudo-Dominant Inheritance	HIO:0000228	When the inheritance of a recessive trait mimics a dominar
Cisplatin-Induced Hearing Impairment	HIO:0000215	Hearing loss caused by cisplatin (a chemotherapeutic
Neomycin-Induced Hearing Impairment	HIO:0000285	Partial or complete loss of hearing following ingesti
Maternal Medical History	HIO:0000362	A record of a patient's biological mother's background regarding health of the mother.
Hearing Impairment based on Immaturity	HIO:0000514	Hearing impairment that occurs due to premature birth (birth at or be

4. Discussion

Making use of ontological reasoning approaches may play a significant role in solving scalability and interoperability issues associated with current large-scale biological high-throughput datasets. This implies that building and maintaining biomedical ontologies is essential, especially in this current data rich era with an extensive consideration of big data analytics. With the contribution of HI domain experts, we have designed the HIO, which enables knowledge acquisition and harmonization, verification and validation of data available in different databases. This ontology is set to be the most comprehensive standardized HI domain knowledge portal, which will allow for the application of ontology-driven mining approaches for the identification of pertinent research questions. The HIO will foster clear and unambiguous communication and also facilitate sharing of information within the field.

4.1. HIO Structure, Other Disease Ontologies and HI Online Datasets

As pointed out previously, the HIO reuses concepts from other ontologies, including HPO, DO, and especially SCDO (see Table 1: summarizing the number of HI specific concepts vs reused concepts). These concepts were adjusted, where applicable, to incorporate new concepts specific to HI and relevant in various areas, such as HI subtypes, phenotypic expressions, genetic phenomena and different modes of inheritance. It is worth noting that, although we did not foresee all the adaptations that would be required, the use of the SCDO as a template for the HIO has been a very useful exercise. Whereas the general structure of the SCDO is more readily transferable to other monogenic diseases, we believe the HIO can be used as a template for designing disease-specific ontologies for diseases with a broader range of causes. Finally, note that there exist several online resources storing HI datasets and containing or enabling the retrieval of HI information. Table 3 lists some of these resources.

Table 3. Some existing online hearing impairment resources.

Scheme	Description	Types	
HHL	Hereditary Hearing Loss Homepage	An up-to-date overview of the genetics of hereditary hearing impairment for researchers and clinicians working in the field.	//heredita
SHIELD	The Shared Harvard Inner Ear Laboratory Database	An integrative gene expression database for inner ear research	https://shie
DVD	Deafness Variation Database	A comprehensive resource integrating available genetic, genomic, and clinical data together with expert curation to generate a single classification for each variant in 152 genes implicated in syndromic and non-syndromic deafness.	deafnessva
LOVD	Leiden Open Variation Databse	Retinal and hearing impairment genetic variant database	https://c share
NIDCD	National Institute on Deafness and Other Communication Disorders	A resource providing knowledge about Hearing, Ear Infections, and Deafness Diseases and Conditions. It also provides NIDCD Temporal Bone Registry at https://www.tbregistry.org/, a resource for learning about the pathology and pathophysiology of otologic disorders, which serves as a resource for scientists to analyze data from a collection of more than 12,000 temporal bone specimens.	https://w health/hea
gEAR	Gene Expression Analysis Resource	Visualization and analysis of multiomic data both in public and private domains.	https
OMIM	Online Mendelian Inheritance in Man	An Online Catalog of Human Genes and Genetic Disorders	https://

4.2. HIO Potential Future Applications

Even though we have shown the relevance of this new ontology by looking at how many of the classes are HI specific by querying against NCBO BioPortal, it should be noted that an ontology should be applied in order to appropriately assess its impact and suitability. We plan to use this ontology in data representation, which includes data harmonization, interoperability, and integration. For this, the HIO will be an essential resource in designing an ontology-based case report forms, providing essential data elements and controlled terminology. Different datasets can then be mapped to these data elements, making these datasets interoperable, thus easing the data integration process and meta-analysis. In the context of HI research, this will orient data analysis and enable the use of machine learning approaches with sufficient statistical power [31] for predicting disease clinical outcomes [32] and optimal therapeutic interventions, based on the disease pathophysiology mechanisms and other clinical parameters in patient records. It is expected that this HIO will contribute to fostering the subsequent HI research translation into healthcare, inferring knowledge based on patient clinical records, and the development of ontology-powered artificial intelligence medical tools helping in therapeutic interventions, prognosis, and diagnosis, as well as predictive models for an improved understanding of disease processes.

4.3. Challenges and Future Direction

Although the HIO has been designed in a manner that takes into account the various complexities of HI, there is admittedly still much content to be included, both with regards to terms and associations. New discoveries are also regularly being made in this field towards technological advances for diagnostics and therapeutic. This suggests that the ontology should be dynamic, in continuous evolution, keeping the HI knowledge up to date as new knowledge is accessible. There will thus be a need for ongoing input and maintenance of the HIO. For this, there is already a dedicated curation team aiming at assuring the quality and accuracy of the information contained in this ontology and also keeping it updated as HI knowledge evolves.

Going forward, these remaining upper classes of the SCDO will also be evaluated, and where necessary, adapted, for inclusion into the HIO: 'Research', 'Guidelines', and 'Quality of Life and Care'. We also plan to use competency questions defined by HI experts to evaluate the scope and domain coverage of the HIO. Beyond the use of competency questions, this ontology will also be assessed on its HI concept inclusion power, i.e., in terms of percentage of HI clinical terminologies from a given database, such as the GHLD database, or HI associated clinical reports or selected literature found in the HIO. This is particularly useful as it will provide an indication on the HIO ability in HI text mining tasks. Finally, the next critical challenge is to introduce this HIO into the dynamic clinical setting. This necessitates the development of testable and actionable health informatics applications to ensure clinical system-wide adherence. As indicated previously, HIO addresses the issue of unifying research clinical data from diverse sources. This ontology already paves the way towards the integration of clinical data into electronic medical records, which should facilitate the development of effective health informatics tools to potentially assist in the public and clinical management of hearing impairment conditions.

5. Conclusions

We have developed the HIO, a common controlled HI vocabulary, which is expected to enhance collaborative research. This ontology is currently the most comprehensive and standardized human- and machine-readable resource that unambiguously defines HI concepts and terminology for researchers, patients, and clinicians in order to help process, reuse, and re-apply existing HI knowledge in biomedical research and health-care systems. In the context of big data analytics, this ontology may facilitate retrospective data harmonization and contribute to mapping HI datasets to functional knowledge to enable the subsequent HI research translation into clinical applications and policy guidelines.

The HIO will allow researchers, clinicians, and patients to readily access standardized HI-related knowledge in a single location and promote HI data integration, interoperability, and sharing, including epidemiological, socio-environmental, biomedical, genetic, and phenotypic datasets.

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