Genomic Strategies for Diagnosis and Management of Congenital and Childhood Hearing Loss

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy
in the Faculty of Biology, Medicine and Health

2021

Leslie Patricia Molina Ramírez

School of Biological Sciences

List of Contents

Word count: 28,950

Figures

Tables

Abbreviations

ABR	Auditory Brainstem Response
ACGS	Association For Clinical Genomic Science
ACMG	American College of Medical Genetics
AD	Autosomal Dominant
AMP	Association For Molecular Pathology
AOM	Acute Otitis Media
AR	Autosomal Recessive
BAAP	British Association of Audiological Physicians
BAM	Binary Alignment Map Format File
BAPA	British Association of Paediatrician Audiologists
BOR	Branchio-Oto-Renal Syndrome
BWA	Burrow Wheelers Aligner
BWT	Burrow Wheelers Transform
CDC	Centers For Control and Disease Prevention
CES	Clinical Exome Sequencing
CGS	Clinical Genome Sequence
CHARGE	Coloboma, Heart Defects, Choanal Atresia, Growth Retardation, Genital Abnormalities, And Ear Abnormalities Syndrome
CHL	Conductive Hearing Loss
CI	Cochlear Implant / Cochlear Implantation
CMA	Chromosomal Microarray Analysis
CMG	*Centers For Mendelian Genomics*
CMV	Cytomegalovirus
CNV	Copy Number Variants
CRT	Cycle Reversible Termination
CSF	Cerebrospinal Fluid
CT	Computed Tomography
DDD	*Deciphering Developmental Disorders*
DFNA	Deafness, Neurosensory, Autosomal Dominant
DFNB	Deafness, Neurosensory, Autosomal Recessive
DFNX	Deafness, Neurosensory, X-Linked
DNA	Deoxyribonucleic Acid

DOORS	Deafness, Onychodystrophy, Osteodystrophy, And Mental Retardation Syndrome
DVD	Deafness Variation Database
ECG	Electrocardiogram
ENT	Ear, Nose and Throat
EP	Endocochlear Potential
ES	Exome Sequencing
EVA	Enlarged Vestibular Aqueduct
EVS	Exome Variant Server
ExAC	Exome Aggregation Consortium
FASTQ	Text-Based Format for Storing Both a Biological Sequence
FORGE	Finding Of Rare Disease Genes (Canada)
GATK	Genome Analysis Toolkit
GC	Guanine-Cytosine
gnomAD	Genome Aggregation Database
GS	Genome Sequencing
GTR	Genetic Testing Registry
HGMD	Human Gene Mutation Database
HL	Hearing Loss
HPO	Human Phenotype Ontology
IGF	Insulin-Like Growth Factor
IHC	Inner Hair Cells
JLN	Jervell-Lange Nielsen
KD	Kilodalton
LGS	Long-Read Sequencing Technologies
LOVD	Leiden Open Variation Database
MAF	Minor Allele Frequency
MDT	Multidisciplinary Team Meeting
MELAS	Myopathy, Encephalopathy, Lactic Acidosis and Stroke-Like Episodes
MIDD	Maternally Inherited Diabetes
MLPA	Multiplex Ligation-Dependent Probe Amplification
MRI	Magnetic Resonance Imaging
NGS	Next-Generation Sequencing
NHLBI	NIH National Heart, Lung, And Blood Institute
NHS	National Health Service

NICU	Neonatal Intensive Care Unit
NIH	National Institutes of Health
OAE	Otoacoustic Emissions
OHC	Outer Hair Cells
OME	Otitis Media with Effusion
OMIM	Online Mendelian Inheritance in Man Database
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RP	Retinitis Pigmentosa
SAM	Sequencing Alignment Map Format File
SBL	Sequencing By Ligation
SBS	Sequencing By Synthesis
SHIELD	Shared Harvard Inner Ear Laboratory Database
SIFT	Sorting Intolerant from Tolerant
SNA	Single Nucleotide Addition
SNHL	Sensorineural Hearing Loss
SNV	Single Nucleotide Variants
SOLiD	Sequencing By Oligonucleotide Ligation and Detection
TORCH	Toxoplasmosis, Rubella Cytomegalovirus, Herpes Simplex, And HIV
UDN	*Undiagnosed Diseases Network*
UHNS	Universal Hearing Newborn Screening
USH	Usher Syndrome
UTR	Untranslated Region
VCF	Variant Calling Format File
VRA	Visual Reinforcement Audiometry
VUS	Variant Of Uncertain Significance
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WHO	World Health Organisation
WS	Waardenburg Syndrome
ZA	Zonadhesin-Like Domain
ZP	Zona Pellucida Domain

Abstract

Genomic strategies for diagnosis and management of congenital and childhood hearing loss. Leslie Patricia Molina Ramírez, The University of Manchester, Doctor of Philosophy, 2021.

Hearing loss is the most common sensory disorder in children. Over 50% of these cases are genetic, and more than 100 genes have been associated with inheritance and different patterns of hearing loss. Identifying the underlying aetiology of hearing loss is paramount in the diagnosis and management of hearing loss, but the vast clinical and genetic heterogeneity poses a diagnostic challenge. This is problematic because if left untreated, hearing loss can have negative impact on quality of life, language, and social development. Genomic sequencing strategies have demonstrated clinical utility in many genetic conditions with similar diagnostic challenges. In this thesis, approaches were taken to study the diagnostic performance of genomic strategies currently used in the clinic, understand how genomic strategies perform when used with patients with hearing loss, and how integrated genomic and clinical data can reveal insights to inform personalised patient care.

Clinical exome sequencing (CES) analysis data and molecular results from 400 patients with rare disorders were analysed. CES results from a subset of 60 patients with ear abnormalities and hearing loss disorders were further studied. To obtain insights into the integrated use of clinical and genomic data, a phenotype-genotype correlation was conducted in patients with *USH2A*-related disease. Findings from this correlation were further validated using two external datasets. In addition, a review article highlights potential of genomic strategies in current management of patients with hearing loss and the potential benefit of obtaining a molecular diagnosis through genomic sequencing for cochlear implant candidates and recipients.

CES in rare disease patients was reported with an overall 24% diagnostic rate. Nervous system, head and neck, skeletal, ear and eye abnormalities were the most commonly reported clinical features in the patient cohort referred for CES. The use of different methods of phenotype-driven gene selection approaches for virtual panels clearly demonstrated a reduction of variant workload without compromising diagnostic rate. Sixty patients with ear disorders and hearing impairment underwent CES. Here, CES was reported with a diagnostic rate of 31%. CES results informed further diagnostic steps in 25% of patients with hearing impairment. The phenotype-genotype correlation in patients with *USH2A*-related disease identified the presence of specific alleles in patients with retinitis pigmentosa and unaffected hearing or late-onset, mild hearing loss. Furthermore, patients with congenital-onset and moderate-to-severe hearing loss were found to harbour protein truncating variants in their genotypes. Audiological surveillance could then be personalised based on the phenotype anticipated by the molecular diagnosis.

In conclusion, this study has demonstrated that clinical genomic sequencing can be a comprehensive and powerful tool in the investigation of genetic congenital and childhood-onset hearing loss. Integrated genomic and clinical data can enable precision medicine approaches in children with genetic hearing loss. This work adds to the increasing body of evidence that supports the use of genetic diagnosis as a potential prognostic factor to inform patient care and hearing habilitation and rehabilitation strategies.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

Acknowledgements

I would like to express my gratitude first and foremost to my supervisory team, Prof Graeme Black, Prof Iain Bruce, Prof William Newman. It was a privilege to learn from them, and their guidance and invaluable insights over the years were profoundly important to me.

I should also thank my collaborators and advisors at the University of Manchester and Manchester Centre for Genomic Medicine, who provided a rich, intellectually stimulating atmosphere which contributed greatly to my learning; Dr David Gokhale, Dr Emma Burkitt-Wright, Dr Jamie Ellingford, Dr Panagiotis S, Dr Eva Lenassi, Mr Haroon Saeed, Claire Kyle, Ronnie Wright, and Abigail Rosseau.

I am also very grateful for the opportunity to work with Lise Henderson, Christine Melling, and the entire Paediatric Cochlear Implant Team at MFT.

Navigating the NHS system was a challenge that I also had a deal of help and support with. As such, I also need to thank Karen Tricker, Michael Smith, Aleksandra Metryka, Stuart Ingram and Rahat Perveen.

To my PhD fellows and lab colleagues and friends, Elke, Charles, Katherine, Jonathan, I would like to sincerely thank you for your kind words and friendship over these last few years.

To Amanda Lurie, Maria Yoldi and Natasha Hood, many thanks for your support and advice through difficult times.
I am also grateful to CONACyT, Mexico, for my PhD scholarship.

To my family, Nicolás, Magdalena, Oscar, César and Tatiana; thank you for sending me your unconditional love and support across the globe.

To my husband, Gary, thank you for always being there for me. Life in Manchester would not have been the same without you.

Rationale for journal format

The body of work presented in this thesis was designed, conducted and written up as separate but connected sections of research, with each chapter presented as an individual paper suitable for submission to a peer reviewed publication. Chapter 2, Chapter 4, Chapter 5 have been published in the *Journal of Medical Genetics*, *Otology & Neurotology*, and *Cochlear Implants International,* respectively. Chapter 3 has just been accepted for publication in *Clinical Otolaryngology*. Chapter 6 is a co-authored publication also developed during this PhD and has been published in Trends in Hearing.

The chapters of this thesis present a unified study of the current use of genomic sequencing in the clinic, the utility in patients with hearing loss, the study of phenotype-genotype correlations and their potential use to stratify patients with syndromic hearing loss and finally, a discussion of potential applications of the use of genomic strategies in the assessment of cochlear implant patients. The different experimental approach for each chapter meant that the chapters can be thoroughly analysed on their own while maintaining a continuous and altogether, they represent a coherent representation of the research conducted in a more seamless way than the traditional format. Furthermore, having various aspects of the thesis scrutinised and reviewed by independent blind experts ensures that the individual chapters make substantive arguments.

Chapter 2 reviews the use of virtual gene panels in clinical exome sequencing conducted in patients with rare disorders. This chapter introduces the use of genomic sequencing currently applied to studying individuals with rare disorders, which at the moment of conducting the experiments of this PhD thesis, was the main genomic strategy utilised in patients with ear disorders and hearing impairment. Stemming from Chapter 2, Chapter 3 looks more closely at the use of clinical exome sequencing in patients with ear disorders and hearing impairment. Chapter 4 reviews a genotype-phenotype correlation in patients with syndromic hearing loss – Usher syndrome type IIA. This chapter analyses how integrated genomic and clinical data can identify patients with different audiological characteristics and prognosis, representing a potential prognostic factor that can be used in personalising patient care in *USH2A*-related disease, according to a patient's predicted audiological phenotype. Chapter 5

discusses the potential use of genomic sequencing to obtain genetic diagnosis in individuals with hearing loss, as part of the assessment in patients with cochlear implants. Chapter 6 is a co-author publication that reviews current and emergent genetic and genomic technologies in the study of hearing loss.

Author contributions

In Chapter 1: Cochlear implantation in the era of genomic medicine

Leslie P Molina-Ramirez, Iain A Bruce, Graeme C M Black

Cochlear Implants Int. 2020 Mar;21(2):117-120

Content: LPMR. Writing of the manuscript: LPMR. Revision of content and project supervision: IAB, GCMB. All the authors revised the manuscript for important intellectual content and approved the final version.

Chapter 2 Personalised virtual gene panels reduce interpretation workload and maintain diagnostic rates of proband-only clinical exome sequencing for rare disorders

J Med Genet. 2021 Apr 20;jmedgenet-2020-107303. doi: 10.1136/jmedgenet-2020-107303. Online ahead of print.

Leslie P Molina-Ramírez, Claire Kyle, Jamie M Ellingford, Ronnie Wright, Algy Taylor, Sanjeev S Bhaskar, Christopher Campbell, Harriet Jackson, Adele Fairclough, Abigail Rousseau, George Burghel, Laura Dutton, Siddharth Banka, Tracy A Briggs, Jill Clayton-Smith, Sofia Douzgou, Elizabeth A Jones, Helen M Kingston, Bronwyn Kerr, John Ealing, Suresh Somarathi, Kate E Chandler, Helen M Stuart, Emma MM Burkitt-Wright, William G Newman, Iain A Bruce, Graeme C Black, David Gokhale

Design and coordination of the study: LPMR, CK, JME, DG, GCMB. Data collection: LPMR, CK, AT. Analysis and interpretation: LPMR, CK, DG, GCMB. Bioinformatics and maintenance of bioinformatics pipeline: SSB and Bioinformatics department at Manchester Centre for Genomic Medicine. Virtual gene panel algorithm and development of clinical exome web referral system: AT. Clinical Exome Sequencing Experiments: LD. Contributing genetic and/or phenotypic data: CK, RW, SSB, CC, HJ, AF, AR, GeB, LD, SB, TAB, JCS, SD, EAJ, HK, BW, JE, SS, KC, HMS, EBW, WGN, GCMB. Project supervision: IAB, GCMB Writing of the manuscript: LPMR, CK, JME, DG and GCMB. All the authors revised the manuscript for important intellectual content and approved the final version.

Chapter 3 The diagnostic utility of clinical exome sequencing in 60 patients with hearing loss disorders: A single-institution experience.

Accepted for publication in Clinical Otolaryngology

Leslie P Molina-Ramírez, Emma MM Burkitt-Wright, Haroon Saeed, John H McDermott, Claire Kyle, Ronnie Wright, Christopher Campbell, Sanjeev S Bhaskar, Algy Taylor, Laura Dutton, Claire Forde, Kay Metcalfe, Audrey Smith, Jill Clayton-Smith, Sofia Douzgou, Kate Chandler, Tracy A Briggs, Siddharth Banka, William G Newman, David Gokhale, Iain A Bruce, Graeme C Black

Design and coordination of the study: LPMR, EMMBW, HS, IAB, GCMB. Data collection: LPMR, EMMBW, CK, AT. Analysis and interpretation: LPMR, EMMBW, HS, IAB, GCMB. Bioinformatics and maintenance of bioinformatics pipeline: SSB and Bioinformatics department at Manchester Centre for Genomic Medicine. Virtual gene panel algorithm and development of clinical exome web referral system: AT. Clinical exome sequencing experiments: LD. Contributing genetic and/or phenotypic data: CK, RW, EMMBW, SSB, CC, SB, AS, CF, JHMcD, TAB, JCS, SD, KC, WGN, GCMB. Project supervision: IAB, GCMB. Writing of the manuscript: LPMR, EMMBW, HS, IAB, GCMB. All the authors revised the manuscript for important intellectual content and approved the final version.

Emma MM Burkitt-Wright and I share first co-authorship

Chapter 4
Establishing Genotype-phenotype Correlation in USH2A-related Disorders to Personalize Audiological Surveillance and Rehabilitation

Leslie P Molina-Ramírez, Eva Lenassi, Jamie M Ellingford, Panagiotis I Sergouniotis, Simon C Ramsden, Iain A Bruce, Graeme C Black

Otol Neurotol. 2020 Apr;41(4):431-437.

Design and coordination of the study: LPMR, EL, JME, GCMB. Data collection: LPMR, JME. Analysis and interpretation: LPMR, EL, JME, GCMB. Clinical interpretation of variants: SCR and Eye Disease Clinical Scientists team at Manchester Centre for Genomic Medicine. Bioinformatics: JME and Bioinformatics Department at Manchester Centre for Genomic Medicine. Targeted NGS panel experiments were performed by clinical scientists at the Manchester Centre for Genomic Medicine. Contributing genetic and/or phenotypic data: PIS, SCR, GCMB. Project supervision: IAB, GCMB. Writing of the manuscript: LPMR, EL, JME, IAB and GCMB. All the authors revised the manuscript for important intellectual content and approved the final version.

Chapter 1. Introduction

1.1 Epidemiology and impact of hearing loss

Hearing loss is one of the most common congenital disorders in children worldwide. The World Health Organization (WHO) estimates that over 32 million children have a disabling, bilateral permanent hearing loss (1). The current incidence oscillates from 1 to 5 in 1000 newborns being detected with bilateral permanent hearing loss (2). Every year, over 750,000 children are born with hearing loss worldwide (3), with the prevalence increasing further with age (4). It is expected that more than 10% of the current worldwide population, including children and adults, will suffer disabling hearing loss by 2050 (1).

Unaddressed hearing loss in childhood can negatively affect speech and language development, causing a profound impact on communication, social and psychological development. Psychological disorders are more prevalent in children with hearing impairment, particularly in the presence of additional disabilities (5). Newborn hearing screening programmes provide early detection of congenital hearing loss to enable early intervention during the neonatal period; however, they fail at providing aetiological or prognostic information.

Member states of the WHO advocate for the introduction of mechanisms for detection of inherited causes, genetic counselling and ensuring information is available for populations at risk (6). However, under current diagnostic pathways, and despite the existence and availability of extensive clinical tests and investigations, different studies have reported that the identification of the cause of hearing loss remains unknown in 40% of cases.

1.2 Normal hearing

Our ability to hear relies on adequate morphological development and physiological function of the three anatomical parts of the ear: the external, middle and inner ear (Figure 1.1). The process begins as sound waves enter the external acoustic meatus of the ear and impact the tympanic membrane, producing sound-induced vibrations. These vibrations constitute a 'coded signal' that is then transmitted across the middle ear by a chain of three bones (ossicles). In anatomical order, the ossicular chain comprises: the malleus, incus and stapes. The main function of this mechanism is to further amplify the incoming acoustic stimuli. The footplate of the stapes, through a piston-like motion, transmits the sound vibrations into the sensory organ in the inner ear (cochlea), with the difference in cross-sectional area of the footplate as compared to the larger tympanic membrane providing additional amplification of the sound-induced vibrations.

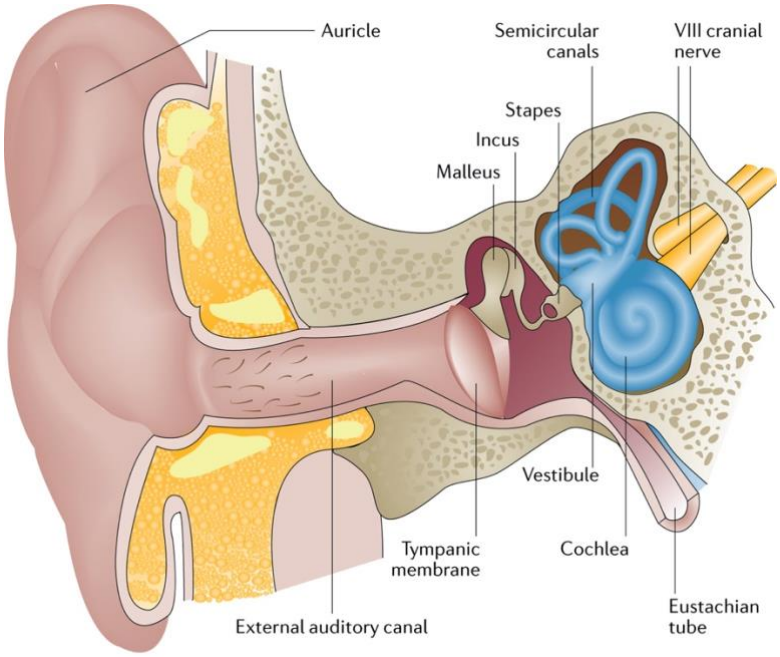


Figure 1.1 Schematic illustration of the ear. Adapted from (21)

1.2.1 The inner ear

The inner ear consists of a bony and a membranous labyrinth. The bony labyrinth has three cavities: the cochlea, the vestibule and the semicircular canals. The cochlea is subdivided in three liquid-filled compartments (channels): the scala vestibuli, the scala tympani and the scala media (Figure 2). The vibration of the stapes against the oval window of the cochlea causes a corresponding fluid waves to propagate along the perilymphatic fluid in the scala vestibuli, from the base to the apex of the cochlea (helicotrema). From the apex, the fluid wave then returns in the perilymph of the scala tympani to reach the base of the cochlea at the round window.

As the sound wave spreads (travelling wave) along the inside of the cochlea the movement of the perilymph stimulates the basilar membrane. The basilar membrane is wide at the base of the cochlea and narrows towards the apex. This characteristic confers a tonotopic organization that differentiates the sound signal into individual frequencies, with high-frequency sounds causing maximum displacement of the basilar membrane towards the base and low-frequency sounds at the apex. Through the movement of perilymph in the scala vestibuli, the sound signal passes through Reissner's membrane and propagates to the endolymph in the scala media. Movement of both the Reissner's membrane and the basilar membrane ultimately results in stimulation of the sensorial neuroepithelium, the organ of Corti (Figure 1.2).

The organ of Corti is a specialised neuroepithelium that contains hair cells which further amplify and transduce the acoustic stimuli to an electrical signal transmitted along the auditory pathway. The hair cells are divided into outer and inner hair cells. The outer hair cells (OHC) amplify sound by increasing the amplitude of the traveling wave while the inner hair cells (IHC) stimulate the synapses of the spiral ganglion, sending this afferent impulse via the cochlear nerve to the auditory brain.

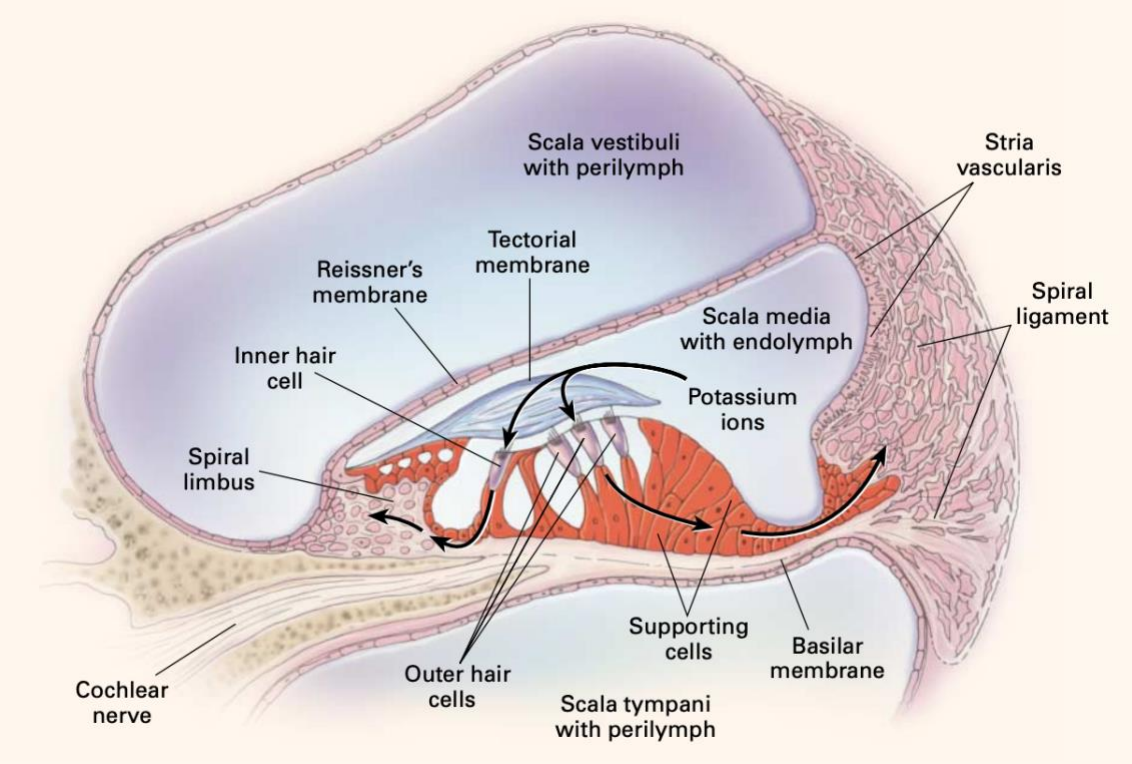


Figure 1.2 Schematic cross section of the human organ of Corti. Figure from (7).

1.2.2 Central auditory pathways

Up to 90-95% of ascending fibres that form the auditory nerve arise from inner hair cells whereas those from outer hair cells contribute only in 5-10% (8). Briefly, auditory information is transmitted by the auditory nerve to the auditory cortex via five central relays or nuclei: cochlear nucleus, superior olivary nuclei, lateral lemniscus, inferior colliculus, and the medial geniculate nuclei (Figure 1.3). Auditory information converges to the contralateral side of the brainstem and brain at each central relay; with the majority of fibres crossing at the superior olivary complex. Ascendent fibres synapse in the medial geniculate body of the thalamus before reaching the auditory cortex.

From this last precortical synapse, auditory information finally ascends towards the cerebral hemispheres. It is estimated that the auditory cortex represents 8% of the human brain cortex. At this point, auditory information is decoded into the core primary and secondary auditory cortices, Brodmann areas 41 and 42, respectively. Association cerebral cortices then receive input from the auditory cortices to the Wernicke area (Brodmann area 22) involved in speech perception, to Brodmann areas 39 and 40, involved in language and to the Broca area (Brodmann areas 44,45) involved in expressive language (9).

Efferent innervation is required for sound adaptation and three-dimensional perception of auditory stimuli (10). These mechanisms act as a protective mechanism for hair cells. The efferent auditory pathways emerge from the cerebral cortex and descend through each nucleus back to the hair cells in the cochlea. The largest proportion of fibres descend through the thalamus and the inferior colliculus. At the superior olivary complex, an estimated 2/3 of descending axons from the medial olivocochlear system project to the contralateral outer hair cells, whereas the 80-90% from the lateral olivocochlear system project to the ipsilateral cochlear inner hair cells (10). These pathways regulate the ascending auditory information through the regulation of sound amplification by outer hair cells.

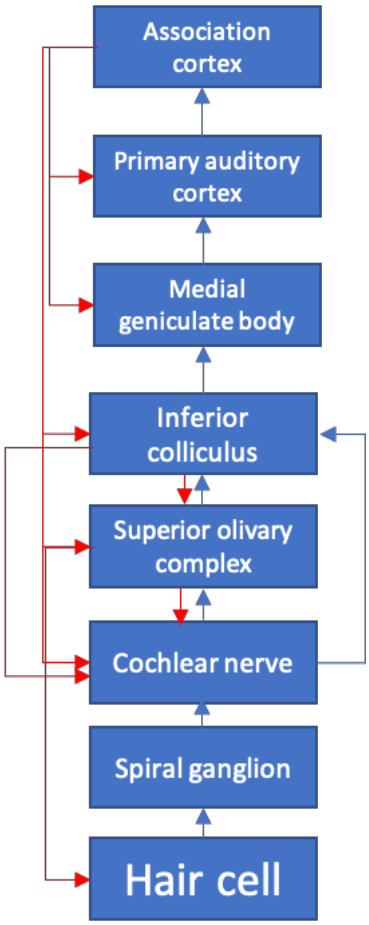
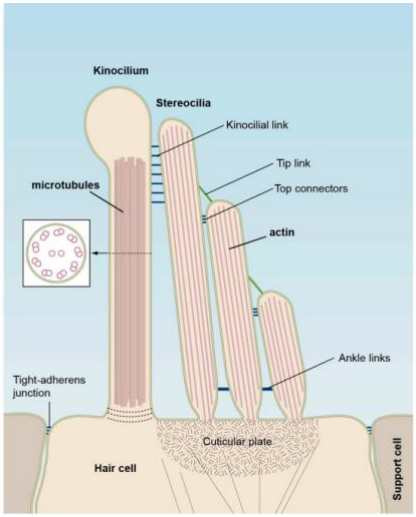


Figure 1.3 Schematic representation of afferent and efferent central pathways. Efferent pathways are shown in red. Afferent pathways are show in blue.

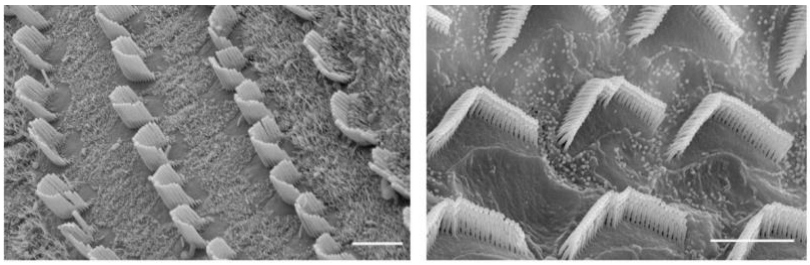
1.2.3 Molecular mechanisms of hearing

Sound mechanotransduction is the process by which mechanical energy is converted into electrical signals to be transmitted to the brain. Mechanotransduction is carried out by the hair cells in the organ of Corti (11). Vibration of the basilar membrane caused by movement of the perilymph elicits deflection of the stereocilia on the apical surface of IHC and OHC.

Stereocilia are actin-filled organelles organized in rows of decreasing heights, tethered by protein filaments that ensure a cohesive configuration (Figure 1.4). Different proteins have a key role in the development of stereocilia and maintenance of their unique configuration (12). In mechanotransduction, the hair cell bundle is deflected towards the tallest stereocilia. The tension created opens transmembrane channels, resulting in influx of K+ and Ca2+ during a phase know as fast adaptation (11). A slow adaption phase follows where movement of actin filaments lead to channel closure and myosin motor proteins restore tension (13). Increased intracellular Ca2+ levels trigger glutamate release at ribbon synapses which consequently conveys the signal to afferent nerve fibres. Deflection of the OHC stereocilia plays a role in sound amplification. OHC return energy into the system to enhance movement of the basilar membrane (13). This phenomenon increases low intensity signals, enhancing auditory sensitivity. Here, movement of the OHC hair bundle is coupled with movement of the tectorial membrane, a structured composed of collagen proteins and ear-specific glycoproteins. OHC depolarization stimulates voltage-induced motility by OHC-specific protein prestin (14) as well unconventional myosin proteins, producing changes in shape and length. This adaptation phase extends the dynamic range and allows for sound amplification. Feedback from efferent pathways assist in modulating cochlear sensitivity (15).



(a)



(b)

Figure 1.4 Organisation of hair cell stereocilia (a) Schematic illustration of hair cell stereocilia. These organelles taper at their insertion to the cuticular plate and are oriented towards the tallest stereocilia. (b) Hair bundle organisation. The unique morphology and organization are crucial to trigger the mechanotransduction response effectively. IHC stereocilia are organised by a U-shape (left) whereas OHC stereocilia are characterised by a W-shape configuration (right). Figure adapted from (16).

Different mechanisms and structures that maintain energy and ion homeostasis are crucial for adequate sensory transduction. The secretion of K, Cl-, Ca2+, HCO3- and absorption of K+, Na+ and Ca2+ must be balanced in order to regulate pH and sustain endolymph composition and volume (17). The endolymph exhibits a potential of +80mV, known as the endocochlear potential (EP). The EP drives the force of K+ influx and Ca2+ permeation which amplify hair bundle motility and therefore, increases hair cell sensitivity (18). In addition, K+ is an important charge carrier as cycling and buffering across K+ channels meet the low energy expenditure limit of the sensory cells. Different structures in the organ of Corti (such as Reissner's membrane, the spiral ligament and the stria vascularis) contain a vast number of proteins in tight and gap junctions that function as channels, pumps, transporters and regulatory signal pathways that play significant part in generating and maintaining the unique ionic composition of the endolymph in the scala media (17).

Notably, both sound mechanotransduction and amplification are controlled by a complex cellular and molecular machinery in the organ of Corti. It has been estimated that around 1% of human genes code for proteins with active roles in hearing biology (19). As illustrated in Figure 1.5, the majority of the molecules and cellular structures known to date that participate in hearing are crucial for developing and maintaining the essential physiological and morphological conditions for adequate mechanotransduction and ion homeostasis for energy supply as discussed above (20):

1) development and structure of hair cell stereocilia,
2) motility of adjacent membranes and supporting cells,
3) maintenance of ion homeostasis and
4) maintenance of the endocochlear potential.

Alteration in any of these mechanisms can result in loss of hearing.

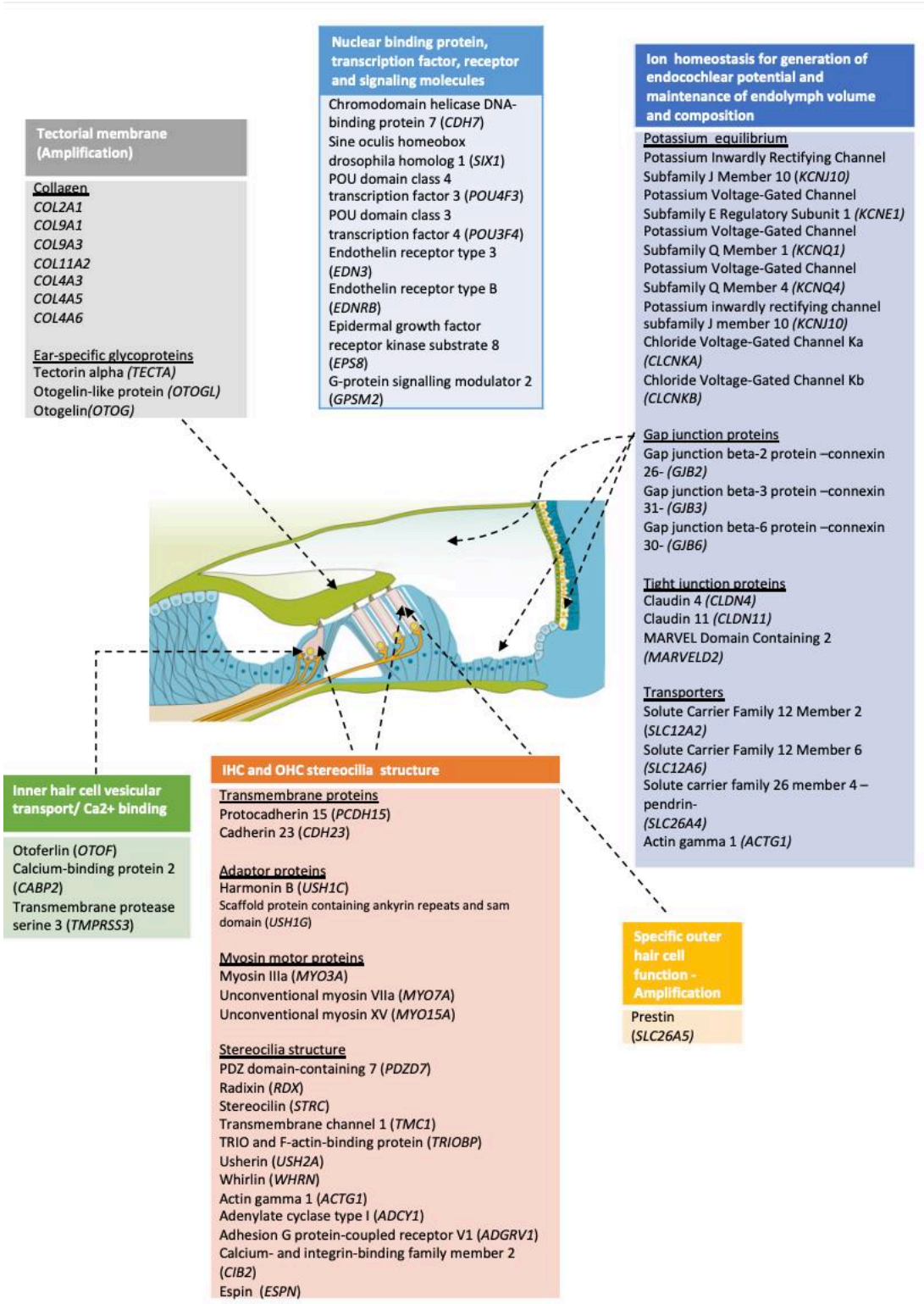


Figure 1.5 Representation of the organ of Corti and most studied proteins intervening in cochlear mechanotransduction and homeostasis. [Schematic of organ of Corti adapted from (21)]

1.3 Clinical characteristics of hearing loss

Hearing loss can be characterised based on onset, type, severity, laterality, symmetry and progression. These parameters can inform the need for further diagnostic evaluations and the type of initial auditory habilitation that is most appropriate for the patient.

The terms "congenital" and "prelingual" refer to the **onset** of hearing loss at birth (congenital) or before the acquisition of language skills (prelingual), respectively. Postlingual onset hearing loss may start late in childhood or during adulthood.

The **type of hearing loss** describes the site of primary lesion along the entire auditory pathway. Conductive hearing loss (CHL) occurs when the process of conduction of the external sound across the external and middle ear is affected (air conduction). Common causes of CHL include external or middle ear infections, foreign bodies in the ear canal or congenital external and middle ear malformations. The term sensorineural hearing loss (SNHL) corresponds to hearing loss where the mechanisms for sound mechanotransduction and perception in the inner ear, and subsequent transmission of the signal to the brain, are affected. Mixed HL is the term applied where HL results from a combination of both conductive and sensorineural HL.

The **decibel hearing level (dB HL)** scale is a logarithmic unit used in audiometric testing to represent hearing thresholds across frequencies (22). The average of the minimum sound pressure level perceived by normal hearing individuals is defined as 0 dB HL or audiometric zero (22,23). The **degree of hearing loss** can be classified as indicated in Figure 1.6. **Disabling hearing loss** is defined as a bilateral permanent hearing loss of more than 40 dB HL in the better ear(1).

Progression of hearing loss refers to the continuous deterioration in hearing thresholds. Stable hearing loss shows no changes whereas progressive hearing loss deteriorates over time. Fluctuating hearing loss indicates fluctuation of severity of hearing loss.

Frequency of sound is measured in Hertz (Hz) and indicates the number of vibrations (cycles) per second (e.g., 100 cycles in 1 second equals to 100 Hz). The human ear can detect frequencies between 20-20,000 Hz although it is more sensitive for frequencies between 500-2000 Hz (22).

Laterality refers to the affected ear(s); whether it is bilateral or unilateral.
Symmetry describes the similarity of the configuration of hearing loss between the two ears.

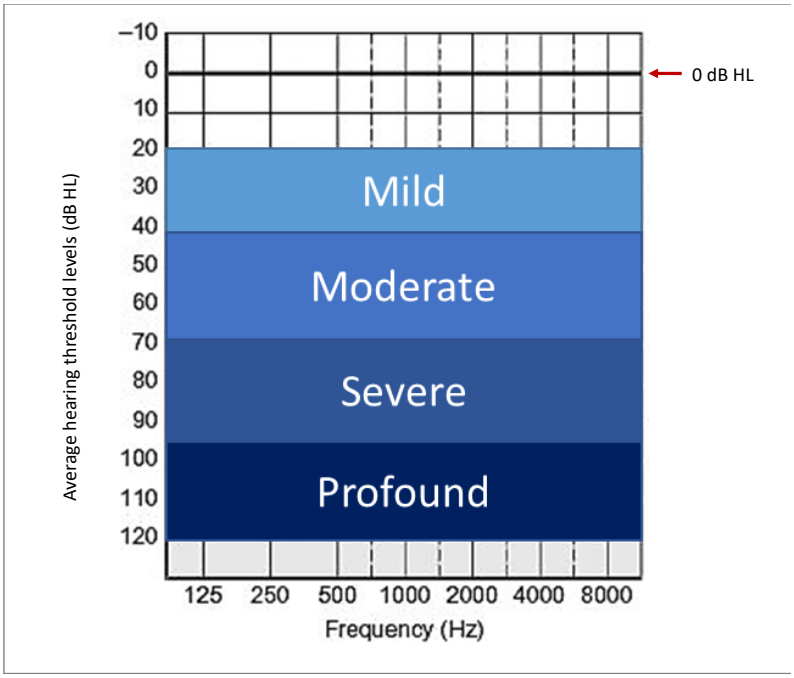


Figure 1.6 Classification of hearing loss severity according to the British Society of Audiology(https://www.thebsa.org.uk/public-engagement/faqs/).

1.4 Aetiologies of congenital and childhood hearing loss

Hearing loss can result from exposure to a single or multiple aetiologies. Aetiologies of hearing loss can be broadly divided in non-genetic and genetic (Figure 1.7). Generally, the causality of both types of aetiologies has been estimated in 50% for each category. Non-genetic aetiologies tend to be more common in developing countries whereas genetic aetiologies are more commonly reported in developed countries (1,24) (who.int/news-room/fact-sheets/detail/deafness-and-hearing-loss).

Non-genetic aetiologies are typically environmental and often preventable factors. Hearing loss can result from exposure to several risk factors before birth, during the neonatal period or during infancy. Risk factors during the antenatal period include: smoke exposure, maternal drug and alcohol use, and maternal infections (25,26). Perinatal risk factors include low weight, hyperbilirubinemia, prematurity, low Apgar scores, prolonged intensive care unit stay and hypoxia (27–29). Therapeutic interventions provided during intensive care such as prolonged mechanical ventilation or administration of ototoxic medication (such as aminoglycosides or loop diuretics) are also identified as important risk factors for hearing loss (30,31).

Prenatal viral infections are commonly associated with hearing loss. Up to 20% of congenital hearing loss has been associated with congenital cytomegalovirus (CMV) infection (32,33). Other viral infections that can cause hearing loss include rubella, measles and more recently, Zika virus (34). Causal bacterial infections include syphilis and toxoplasmosis. Rubella and bacterial meningitis caused by H. influenzae are infectious causes that have decreased in prevalence due to the implementation of vaccination strategies (35,36).

Hearing loss can result from bacterial meningitis caused by *Streptococcus pneumoniae* and *Neisseria meningitides* (37,38). Hearing loss results from spread of the infection from the subarachnoid space through the temporal bone via the cochlear aqueduct or through the vascular supply to the inner ear (39,40). Inflammatory changes in the cochlea induce fibrosis and consequently ossification (37). *S. pneumoniae* has been

identified as one of the most prevalent and harmful pathogens associated with hearing loss after meningitis (40,41).

In childhood, hearing loss results as a complication of acute otitis media (AOM) and otitis media with effusion (OME). OME is the leading cause of hearing loss in young children (42). Both AOM and OME usually lead to transient CHL although some cases may present further complications and damage in the inner ear resulting in SNHL. Different mechanisms have been proposed as precursors of sensorineural hearing impairment associated with OME (43). Due to the impact on Eustachian tube function, the presence of conditions such as cleft palate, craniofacial abnormalities and primary ciliary dyskinesia increases the risk of OME (44–46).

Germline variants contribute to congenital and childhood-onset hearing loss in 50-60% (4,21,47). This incidence has been estimated in up to 80% in developed countries. Hearing loss can be inherited as an autosomal recessive, autosomal dominant or X-linked trait. Variants in mitochondrial genes can predispose to hearing impairment (Bitner-Glindzicz & Rahman, 2007). There are over 150 currently known genes implicated and over 600 syndromes where hearing loss is a clinical component (48). This vast genetic and clinical heterogeneity poses a diagnostic challenge with potential impact on patient care.

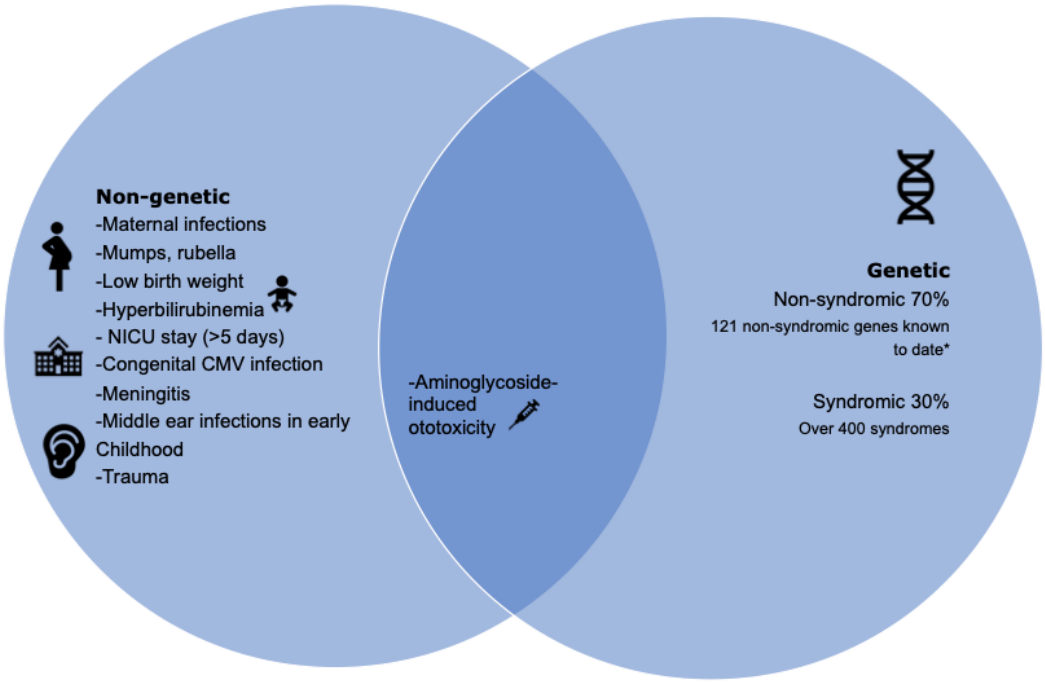


Figure 1.7 Overview of risk factors for congenital and childhood sensorineural hearing loss. CMV cytomegalovirus, NICU neonatal intensive care unit, SNHL sensorineural hearing loss.

* denotes non-syndromic hearing loss genes known to date reported on the Hereditary Hearing Loss Homepage (https://hereditaryhearingloss.org/, last accessed July 2020).

1.5. Genetic congenital and childhood-onset sensorineural hearing loss

Genetic congenital and childhood hearing loss is typically a single-gene (monogenic) disorder. Thus far, over 150 genes have been identified and associated with hereditary hearing impairment (49) [last accessed 11 February 2020]. The majority of these genes encode proteins with key roles in cochlear homeostasis and mechanotransduction (21,50,51). About 70-80% of the cases are autosomal recessive, 20% are autosomal dominant and 1% follow an X-linked inheritance (35). Variants in mitochondrial DNA can also lead to maternally inherited hearing impairment in 1% of congenital hearing loss and up to 5% of postlingual hearing loss (52,53).

Congenital and childhood hearing loss of genetic origin can be classified as non-syndromic or syndromic. Hearing loss manifested as the sole condition is defined as non-syndromic. In contrast, syndromic hearing loss presents with signs and symptoms in other organs. Non-syndromic hearing loss accounts for up to 70% while syndromic hearing loss is estimated in 30% of cases. Over 600 syndromes with congenital ear anomalies or hearing loss have been described (54).

1.5.1. Non-syndromic sensorineural hearing loss: Autosomal recessive

Over 70 genes have been identified associated with this type of inheritance (Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage (https://hereditaryhearingloss.org) (Table 1). Loci linked to non-syndromic autosomal recessive hearing loss are described using the prefix DFNB (Deafness, Neurosensory, autosomal recessive). This type of inheritance is commonly associated with early onset (congenital or prelingual) symmetric sensorineural hearing loss of severe to profound severity. Affected individuals typically have parents with normal hearing. Consanguinity is a significant risk factor for autosomal recessive disease.

Variants in *GJB2* are the leading genetic cause of non-syndromic autosomal recessive SNHL (DFNB1), responsible for up to 50% of cases worldwide with higher prevalence in individuals with European and Asian ancestry (55). *GJB2* encodes the gap junction beta 2 protein connexin 26 and was the first gene to be identified as being associated with non-syndromic autosomal recessive SNHL (56). Prevalence of *GJB2* variants show variation amongst ethnic group. For instance, the variant c.35delG is more frequently reported in individuals of European Caucasian ethnicity (57), whereas c.235delG is more commonly reported in East Asia (58,59) .

Hearing loss associated with *GJB2* presents more frequently at birth, is bilateral, and ranges from mild-to-profound severity. Phenotype-genotype correlations have been documented in *GJB2*-related SNHL (55). Truncating variants in *GJB2* resulting in aberrant non-functioning proteins are more prevalent in individuals with bilateral, severe to profound SNHL. Variants with mild single amino acid substitutions (missense variants), such as c.101T>C p.(Met34Thr), tend to be more frequently reported in individuals with mild *GJB2*-related SNHL. DFNB1 can also involve a deletion of *GJB6*. *GJB6* encodes for the gap junction protein connexin 30. Previous research suggests that deletions of *GJB6* in proximity to the regulatory region of *GJB2* result in SNHL due to the impact on *GJB2* expression at the transcriptional level (60,61). Biallelic *GJB6* deletions or single *GJB6* deletions in compound heterozygous with *GJB2* variants account for small proportion of DFNB1 cases. Variants in *GJB2* and *GJB6* are also reported in autosomal dominant inheritance, usually associated with skin abnormalities (62).

SLC26A4 encodes for pendrin, a transmembrane anion exchanger protein shown to transport chloride (Cl^-), iodide(I^-) and bicarbonate ($HCO3^-$) (63). Identified in 1997 (64), variants in *SLC26A4* have been reported in individuals with non-syndromic autosomal recessive SNHL (DFNB4) and syndromic SNHL (Pendred syndrome). Variants in *SLC26A4* are the second most frequent genetic aetiology associated with non-syndromic SNHL in children (65). Over 500 variants in this gene have been described (HGMD). DFNB4 is characterized by childhood onset sensorineural hearing loss, prelingual or early postlingual, starting as mild-to-moderate and progressing to severe or profound severity. Hearing loss is accompanied by the presence of bilateral enlarged vestibular aqueducts (EVAs). EVAs are the commonest inner ear malformation in children with sensorineural hearing impairment (66). Radiologically, EVAs dimensions reach a midpoint width of 1-1.5mm or an opercular width of 2 mm (67,68). EVAs in *SLC26A4*-related hearing loss present as Mondini's dysplasia, where the enlargement of the vestibular aqueduct is accompanied by a small 1.5-turn cochlea (69). Individuals with Pendred syndrome exhibit a similar audiological phenotype, with a higher frequency of EVAs and cochlear dysplasia (70). Up to a third of children with Pendred syndrome may show vestibular symptoms. In addition, patients with Pendred syndrome develop euthyroid or hypothyroid goitre during the second decade of life (71), as a result of the iodination defect caused by pendrin dysfunction in the thyroid follicular cells(72).

As expected for autosomal recessive inheritance, homozygous and compound heterozygous variants in *SLC26A4* are present in individuals with Pendred syndrome or non-syndromic SNHL with EVAs. Interestingly, the presence of monoallelic variants has also been documented although more frequently in patients with non-syndromic EVAs (73). It is estimated that 50-80% of individuals with EVAs have variants in *SLC26A4* (74). In some instances, it has been acknowledged as a confirmation of aetiological diagnosis of non-syndromic *SLC26A4*-related SNHL (75). The degree of hearing loss has also been correlated with the presence of biallelic or monoallelic variants (76,77). The identification of a haplotype of 12 upstream variants in *SCL26A4* in association with less severe audiological phenotypes has suggested the role of such variants as genetic modifiers when reported *in trans* with heterozygous *SLC26A4* variant (78). Interestingly, these variants have only been identified in individuals of Caucasian ethnicity. Digenic inheritance involving genes such as *KCNJ10* and the transcriptional

factor *FOXI1* has also been suggested in cases where no second *SLC26A4* variant is identified. While no strong evidence has supported a significant association (79), digenic inheritance involving other mechanisms is still being explored. A recent study by Li has revealed an interaction between the ephrin-B2 receptor (*EPHA2*) and *SLC16A4* that results in lack of pendrin in the apical membrane, implying the existence of possible synergistic mechanism that leads to SNHL and EVAs in individuals with monoallelic *SLC26A4* variants (80).

Variants in *STRC* are another major cause of non-syndromic SNHL (DFNB16) (81). *STRC* codes for stereocilin, a hair bundle protein found in the top connectors of OHC stereocilia and the tectorial membrane (82). Incidence of DFNB16 has been estimated to be of 1 in 16,000 (83). Biallelic variants in *STRC* are associated with prelingual-onset, mild-to-moderate SNHL (DFNB16) (84). Copy-number variants, in particular homozygous or compound heterozygous deletions, are commonly reported as causative variants in *STRC*. Onset later in childhood has also been reported (85). A recent report has documented the presence of abnormal vestibular responses in association with STRC-related hearing loss (86).

Variants in genes such as *MYO15A, MYO7A, CDH23, TMPRSS3, OTOF* have also been frequently reported associated with autosomal recessive non-syndromic SNHL. *MYO7A* and *CDH23* are also associated with a form of syndromic hearing loss, Usher syndrome type I. This type of Usher syndrome is an autosomal disorder characterized by congenital profound SNHL, early-onset vestibular imbalance and blindness caused by retinitis pigmentosa. Homozygous or compound heterozygous missense variants in *CDH23* have been associated with non-syndromic SNHL (87). *TMPRSS3* is associated with prelingual, and less commonly postlingual onset non-syndromic high-frequency hearing loss (88). *OTOF* encodes for otoferlin, a synaptic exocytosis protein mainly expressed in IHC and at the ribbon synapse. Variants in *OTOF* lead to prelingual severe SNHL and non-syndromic auditory neuropathy (89,90).

Table 1.1 Genes associated with autosomal recessive, non-syndromic SNHL

Locus (OMIM)	Cytogenetic Location	Gene symbol
DFNB1A	13q12	GJB2
DFNB1B	13q12	*GJB6*
DFNB2	11q13.5	*MYO7A*
DFNB3	17p11.2	*MYO15A*
DFNB4	7q31	*SLC26A4*
DFNB6	3p14-p21	*TMIE*
DFNB7/11	9q13-q21	*TMC1*
DFNB8/10	21q22	*TMPRSS3*
DFNB9	2p22-p23	*OTOF*
DFNB12	10q21-q22	*CDH23*
DFNB15/72/95	3q21-q25	*GIPC3*
DFNB16	15q21-q22	*STRC*
DFNB18	11p14-15.1	*USH1C*
DFNB18B	11p15.1	*OTOG*
DFNB21	11q	*TECTA*
DFNB22	16p12.2	*OTOA*
DFNB23	10p11.2-q21	*PCDH15*
DFNB24	11q23	*RDX*
DFNB25	4p13	*GRXCR1*
DFNB26	4q31	*GAB1*
DFNB28	22q13	*TRIOBP*
DFNB29	21q22	*CLDN14*
DFNB30	10p11.1	*MYO3A*
DFNB31	9q32-q34	*WHRN*
DFNB32/105	1p13.3-22.1	*CDC14A*
DFNB35	14q24.1-24.3	*ESRRB*
DFNB36	1p36.3	*ESPN*
DFNB37	6q13	*MYO6*
DFNB39	7q21.1	*HGF*
DFNB42	3q13.31-q22.3	*ILDR1*
DFNB44	7p14.1-q11.22	*ADCY1*
DFNB48	15q23-q25.1	*CIB2*
DFNB49	5q12.3-q14.1.	*MARVELD2/BDP1*
DFNB53	6p21.3	*COL11A2*
DFNB59	2q31.1-q31.3	*PJVK*
DFNB60	5q23.2-q31.1	*SLC22A4*
DFNB61	7q22.1	*SLC26A5*
DFNB63	11q13.2-q13.4	*LRTOMT / COMT2*

DFNB66	6p21.2-22.3	*DCDC2*
DFNB66/67	6p21.31	*LHFPL5*
DFNB68	19p13.2	*S1PR2*
DFNB73	1p32.3	*BSND*
DFNB74	12q14.2-q15	*MSRB3*
DFNB76	19q13.12	*SYNE4*
DFNB77	18q12-q21	*LOXHD1*
DFNB79	9q34.3	*TPRN*
DFNB84	12q21.2	*PTPRQ / OTOGL*
DFNB86	16p13.3	*TBC1D24*
DFNB88	2p12-p11.2	*ELMOD3*
DFNB89	16q21-q23.2	*KARS*
DFNB91	6p25	*SERPINB6*
DFNB93	11q12.3-11q13.2	*CABP2*
DFNB94	11q14.1	*NARS2*
DFNB97	7q31.2-q31.31	*MET*
DFNB98	21q22.3-qter	*TSPEAR*
DFNB99	17q12	*TMEM132E*
DFNB100	5q13.2-q23.2	*PPIP5K2*
DFNB101	5q32	*GRXCR2*
DFNB102	12p12.3	*EPS8*
DFNB103	6p21.1	*CLIC5*
DFNB104	6p22.3	*FAM65B*
DFNB106	11p15.5	*EPS8L2*
DFNB108	1p31.3	*ROR1*
DFNB107	17q25.1	*WBP2*
DFNB109	8q22.1	*ESRP1*
DFNB111	11q23.3	*MPZL2*
DFNB113	19q13.31-q13.32	*CEACAM16*
DFNB114	17p11.2	*GRAP*
DFNB115	17p13.2	*SPNS2*
-	16p13.3	*CLDN9*

From Hearing Loss Homepage (https://hereditaryhearingloss.org/): accessed 16 February 2020.

1.5.2 Non-syndromic sensorineural hearing loss: Autosomal dominant

Non-syndromic SNHL follows an autosomal dominant (AD) inheritance pattern in 20-30% of the cases. Over 45 genes have been identified (Table 2). AD SNHL loci are named with the prefix "DFNA" (Deafness, Neurosensory, autosomal dominant). Variants have been reported in genes that encode for structural proteins in the tectorial membrane (*TECTA, COL11A2*), the stereocilia (*DIAPH1*, *TMC1*), unconventional motor myosins (MYO7A, MYO6A), potassium channels (*KCNQ4*) and gap junction proteins (GJB6). Colocalized dominant and recessive loci are seen in SNHL associated with variants in *GJB2, GJB3, GJB6, TMC1, MYO7A, TECTA* (91).

Postlingual onset is the main clinical characteristic of AD SNHL. However, prelingual-onset AD SNHL is observed linked to genes such as *GJB2, GJB3, TECTA, SIX1* and *DFNA19*. AD SNHL associated with specific genes such as *TECTA, COCH* or *WSF1* exhibits distinctive audiometric configurations. *WFS1*-related hearing loss is characterised by a low-frequency pattern. Mid-frequency SNHL is associated with variants in *POU4F3, EYA4, TECTA, COL11A2* (92). AD SNHL is typically progressive, although velocity of deterioration may vary among genes and may be influenced by other external factors. AD SNHL associated with transcriptional factor genes such as *EYA4* and *POU4F3* has been reported with slower progression (less than one decibel per year) in comparison to that observed in patients with SNHL associated with variants in structural proteins such as *MYO6A* or *ACTG1*, which may range from 2 to 6 decibels per year respectively (93,94). There are no predominant genetic aetiologies associated with autosomal dominant SNHL; however, variants in the genes *TECTA, WFS1, KCNQ4, EYA4* are amongst the most frequently reported.

TECTA encodes the glycoprotein α-tectorin, which is a major structural component of the tectorial membrane (95). *TECTA* variants are estimated to occur in up to 5% of patients with AD SNHL (96,97). *TECTA* variants also cause AR SNHL, although in a minor proportion (92,95). Previous studies on *TECTA* genotype-phenotype correlations have identified the relationship between the localization of variants in either the zona pellucida domain (ZP) and zonadhesin-like domain (ZA) and the range of frequencies affected (98). Mid-frequencies are also affected in *TECTA*-associated AR SNHL, although SNHL across all frequencies can also be expected (99).

WFS1 encodes the membrane glycoprotein wolframin, which is localised in the endoplasmic reticulum (100). Previous research on the expression pattern of wolframin has indicated a possible role during inner ear development and ion (possibly K^+) homeostasis (101) . Heterozygous variants in *WSF1* can cause non-syndromic AD SNHL and syndromic AR SNHL (Wolfram syndrome). Progressive, low-frequency (below 2kHz) SNHL is a characteristic of the non-syndromic AD form. Patients with Wolfram syndrome present with SNHL, diabetes mellitus, diabetes insipidus and optic atrophy (102). Early childhood onset and high-frequency SNHL in Wolfram syndrome can be expected (103,104).

KCNQ4 encodes for a transmembrane voltage-gated potassium channel (105). In contrast to the impact on endolymph secretion underlying SNHL associated with other ion channel genes such as *KCNE1*, the mutated KCNQ4 channel causes SNHL by affecting the potassium current in OHC. A hotspot of missense variants is localised around the pore region of the protein (106). Variants in *KCNQ4* lead to progressive, high-frequency SNHL starting between the third and fourth decades of life. Over time, middle and low frequencies can also be affected (107). Genotype-phenotype correlation studies have shown a prevalence of missense variants in patients with earlier onset, mild severity across all frequencies (107,108). In contrast, patients who harbour frameshift variants tend to show more severe high-frequency SNHL of later onset (109).

Eye absent 4 protein, encoded by *EYA4*, is transcription factor with a presumed role in eye development and maturation of the organ of Corti (110). This protein also participates in the development of other organs such as the kidney and the pituitary gland (111). Variants in *EYA4* have been reported as cause of AD SNHL in different ethnicities (93). Patients with AD SNHL caused by variants in *EYA4* can present with slowly progressive HL starting at any point from childhood to adulthood. (112). Truncating *EYA4* variants have been correlated with SNHL affecting all frequencies whereas missense variants have been reported in patients with high-frequency SNHL (93). Large deletions in *EYA4* have been reported in patients with cognitive impairment, SNHL and cardiac phenotypes (113). The identification of a likely pathogenic synonymous variant has suggested haploinsufficiency as a likely mechanism associated with the pathogenesis of *EYA4*-related AD SNHL (114).

Table 1.2 Genes associated with autosomal dominant, non-syndromic SNHL

Locus (OMIM)	Cytogenetic Location	Gene symbol
DFNA1	5q31	*DIAPH1*
DFNA2A	1p34	*KCNQ4*
DFNA2B	1p35.1	*GJB3*
DFNA2C	1p36.11	*IFNLR1*
DFNA3A	13q11-q12	*GJB2*
DFNA3B	13q12	*GJB6*
DFNA4A	19q13	*MYH14*
DFNA4B	19q13.32	*CEACAM16*
DFNA5	7p15	*GSDME*
DFNA6	4p16.3	*WFS1*
DFNA7	1q21-q23	*LMX1A*
DFNA9	14q12-q13	*COCH*
DFNA10	6q22-q23	*EYA4*
DFNA11	11q12.3-q21	*MYO7A*
DFNA12	11q22-24	*TECTA*
DFNA13	6p21	*COL11A2*
DFNA15	5q31	*POU4F3*
DFNA17	22q	*MYH9*
DFNA20	17q25	*ACTG1*
DFNA22	6q13	*MYO6*
DFNA23	14q21-q22	*SIX1*
DFNA25	12q21-24	*SLC17A8*
DFNA27	4q12	*REST*
DFNA28	8q22	*GRHL2*
DFNA34	1q44	*NLRP3*
DFNA36	9q13-q21	*TMC1*
DFNA37	1p21	*COL11A1*
DFNA39	4q21.3	*DSPP*
DFNA40	16p12.2	*CRYM*
DFNA41	12q24-qter	*P2RX2*
DFNA44	3q28-29	*CCDC50*
DFNA48	12q13-q14	*MYO1A*
DFNA50	7q32.2	*MIRN96*
DFNA51	9q21	*TJP2*
DFNA56	9q31.3-q34.3	*TNC*
DFNA64	12q24.31-q24.32	*SMAC/DIABLO*
DFNA65	16p13.3	*TBC1D24*
DFNA66	6q15-21	*CD164*
DFNA67	20q13.33	*OSBPL2*

Locus (OMIM)	Cytogenetic Location	Gene symbol
DFNA68	15q25.2	*HOMER2*
DFNA69	12q21.32-q23.1	*KITLG*
DFNA70	3q21.3	*MCM2*
DFNA73	12q21.31	*PTPRQ*
DFNA71	15q21.2	*DMXL2*
DFNA74	7p14.3	*PDE1C*
DFNA75	7q22.1	*TRRAP*
DFNA76	3q23	*PLS1*
-	4q21.22	*SCD5*

From Hearing Loss Homepage: accessed 16 February 2020.

1.5.3 Non-syndromic sensorineural hearing loss: X-linked inheritance

X-linked inheritance accounts for approximately 1% of non-syndromic cases. The prefix designated for X-linked SNHL is "DFNX" (Deafness, Neurosensory, X-linked). Only five genes have been reported associated with this type of SNHL (Hearing Loss Homepage, last accessed 12 May 2020) (49). The most common gene associated with X-linked non-syndromic SNHL is *POU3F4.* Variants in this gene lead to mixed HL associated with cochlear hypoplasia and stapes fixation (115). Other genes such as *PRPS1*, *SMPX*, *AIFM1* and *COL4A5* have also been described following this type of inheritance.

1.5.4 Syndromic Hearing Loss

Approximately 30% of children born with permanent hearing impairment exhibit anomalies in another organ. The presence of facial dysmorphism or branchial cleft anomalies are often associated with malformations in the external and middle ear, which can lead to conductive hearing loss. Patients with CHARGE syndrome (coloboma, heart defect, choanal atresia, retarded growth, genital hypoplasia, ear anomalies or deafness) and BOR (Branchio-Oto-Renal) syndrome present with abnormalities in different parts of the ear, such as microtia and cochlear nerve aplasia, which results in CHL, SNHL or mixed HL. In some conditions such as Down syndrome or Stickler syndrome, mixed HL may be present, although it is more often associated with Eustachian tube dysfunction.

Over 600 syndromes involving SNHL have been described (48,52). The most frequent syndromic hearing loss conditions are summarised in Table 3. Typically, the onset of additional clinical features occurs in late childhood or adulthood, which creates an overlap between non-syndromic and syndromic SNHL. This is due to genetic variation that affects proteins expressed not just in the inner ear but also in other organs. The early clinical overlap can lead to a delay in establish an accurate early diagnosis.

Pendred syndrome is one of the most common syndromic hearing loss conditions in childhood deafness (52). As described in the previous section, this is an AR disorder caused by biallelic homozygous or compound heterozygous variants in *SLC26A4*, which encodes the ion exchanger pendrin (64). In addition to loss-of-function variants, missense variants leading to the addition or omission of a proline in the SLC26A4 protein sequence have been proposed as a mechanism associated with damaging function (116). Pendred syndrome has been more linked to the identification of biallelic *SLC26A4* variants whereas the non-syndromic SNHL and EVAs have been often reported in association with monoallelic *SLC26A4* variants (117). Pendrin is expressed in different organs such as the inner ear, the kidney and the thyroid gland (118). Patients with Pendred syndrome present with congenital or prelingual onset, bilateral, progressive SNHL and bilateral EVAs, accompanied by congenital or teenage-onset goitre (71). The thyroid phenotype is the result of an iodide organification defect (63); however, since the iodide organification does not entirely depend on pendrin function, patients may have normal thyroid function of subclinical hypothyroidism (71). The

partial role of pendrin in thyroid function is likely to be responsible of the phenotypic variability that leads to non-syndromic EVAs.

Usher syndrome (USH) is an AR condition characterized by congenital or prelingual SNHL and progressive blindness caused by retinitis pigmentosa (RP). Usher syndrome is the leading cause of genetic dual sensory impairment, and it is clinically and genetically heterogeneous. Genes associated with the different types of Usher syndrome are expressed in the cochlea, the vestibule and the retina. These genes encode for structural and motor USH proteins that connect the stereocilia and allow sound mechanotransduction. In the retina, the USH proteins have a role in phototransduction (119).There are three clinical types of Usher syndrome and 11 genes have been identified. Type I (USH1) is the most severe clinical type of Usher syndrome. Patients with USH1 present with congenital, severe-to-profound SNHL, vestibular dysfunction, with onset of night blindness at some point during the first decade of life (120). Usher syndrome type II is the most common clinical form. Patients with USH2 have congenital, slowly progressive, moderate-to-severe SNHL and RP onset late in the second decade of life (121). Patients with USH2 have normal vestibular function. Usher syndrome type III (USH3) is characterised by progressive SNHL, variable vestibular dysfunction and variable RP onset (122). This last clinical subtype is common in individuals of Finnish and Ashkenazi Jewish ancestry (123,124). There is an important clinical overlap between non-syndromic SNHL caused by variants in some USH genes and their respective syndromic counterparts. A recent meta-analysis has shown that approximately 7.5% of non-syndromic SNHL is associated with USH genes (125).

Table 1.3 Common forms of syndromic sensorineural hearing loss

Condition	Genes	Additional Clinical Features
Usher syndrome	USH1: Myosin VIIa (*MYO7A)* Harmonin *(USH1C)* Cadherin *23 (CDH23)* Protocadherin *15 (PCDH15)* Sans (Scaffold Protein Containing Ankyrin Repeats SAM Domain) *(USH1G)* Calcium and integrin binding family member 2 *(CIB2)* USH2: Usherin *(USH2A)* Adhesion G protein-coupled receptor V1 *(GPR98)* Whirlin *(WHRN)* USH3: Clarin *1(CLRN)* Histidyl-tRNA synthetase 1 *(HARS)*[a] Centrosomal protein 250-KD *(CEP250)*[b]	USH1: retinitis pigmentosa and vestibular dysfunction USH2: retinitis pigmentosa USH3: retinitis pigmentosa, variable vestibular dysfunction
Pendred syndrome	Solute carrier family 26 member 4 *(SLC26A4)* Forkhead Box I *(FOXI1)* Potassium voltage-gated channel subfamily J member 10 *(KCNJ10)*	Bilateral enlarged vestibular aqueduct, cochlear dysplasia and euthyroid goitre in late childhood
Jervell and Lange-Nielsen syndrome	Potassium voltage-gated channel subfamily E member 1 *(KCNE1)* Potassium voltage-gated channel subfamily KQT member 1 *(KCNQ1)*	Tachyarrhythmias associated with long QT interval
Waardenburg syndrome	Paired box protein Pax3 *(PAX3)* Microphthalmia- associated transcription factor *(MITF)* Endothelin 3 *(EDN3)* Endothelin B receptor *(EDNRB)*, Zinc-finger protein SNAI2 *(SNAI2)* Transcription factor SOX10 *(SOX10)*	Type 1: Pigmentary disorders of the iris, hair, and skin with dystopia canthorum; Types 2 with pigmentary disorders only; Type 3 with upper limb abnormalities and Type 4 with Hirschsprung disease
Branchio-oto-renal syndrome	Eyes absent homologue 1 *(EYA1)* Homeobox protein SIX1 (*SIX1*) Homeobox protein SIX5 (*SIX5*)	Ear malformations, branchial fistulae and cysts, and renal malformations
Treacher-Collins syndrome	Treacle protein *(TCOF1)* DNA-directed RNA polymerases I and III subunit RPAC1 *(POLR1C)* DNA-directed RNA polymerases I and III subunit RPAC2 *(POLR1D)*	Craniofacial hypoplasia, microtia, cleft palate, eyelid coloboma
Stickler syndrome	Collagen α1 (II) chain *(COL2A1)* Collagen α1 (IX) chain *(COL9A1)* Collagen α2 (IX) chain *(COL9A2)*	Spondyloepiphyseal dysplasia, myopia,

Condition	Genes	Additional Clinical Features
	Collagen α1 (XI) chain *(COL11A1)* Collagen α2 (XI) chain *(COL11A2)*	cataract, and retinal degeneration
Alport syndrome	Collagen α3 (IV) chain *(COL4A3)* Collagen α4 (IV) chain *(COL4A4)* Collagen α5 (IV) chain *(COL4A5)*	Nephropathy, lenticonus, maculopathy

*Candidate genes for USH3, as proposed by (a) (126), (b) (127).
Adapted from (21)and GeneCards (genecards.org, last accessed February 2020)

Waardenburg syndrome (WS) accounts for 1-3% of congenital SNHL. It is mainly characterised by congenital SNHL and pigmentation abnormalities in skin, the hair and the eyes. Depending on the clinical subtype, patients may also display a prominent broad nasal root (dystopia canthorum), synophrys, limb and intestinal abnormalities. The audiological phenotype may also vary depending on the clinical subtype (128). Patients with Waardenburg syndrome 1 (WS1) have a white forelock or premature grey hair, dystopia canthorum (90%), hypoplastic irides (10%) or heterochromia irides and skin hypopigmentation (50%). Approximately 20% of patients with WS1 experience SNHL (52). Patients with Waardenburg syndrome 2(WS2) have normal facial features and may show neurological features. About 40% of patients with WS2 have SNHL (52). Waardenburg syndrome 3 (WS3) is similar to WS1, although dystopia canthorum is less common and other clinical features include musculoskeletal abnormalities. Waardenburg syndrome 4 (WS4) is characterised by the presence of additional intestinal pseudo-obstruction.

All clinical subtypes of WS are genetically heterogeneous. Six genes have been identified across the 4 subtypes. Inheritance also varies across phenotypes. WS1 and WS2 follow AD inheritance whereas WS3 and WS4 have been reported following both AR and AD patterns. WS1 and WS3 are caused by variants in *PAX3* (paired box gene 3). *PAX3* is a transcription factor that participates in the development of tissues derived from neural crest (129). Heterozygous *PAX3* variants have been more reported in patients with WS1 whereas homozygous *PAX3* variants have been associated with WS3 (130). WS2 has been linked to variants in transcription factors *MITF* (microphthalmia-associated transcription factor) and *SOX10* (sex-determining region Y-box 10) (131,132). Heterozygous variants in *EDNRB* have also been reported in patients classified with WS2. Variants in *SOX10*, *EDNRB* and *EDN3* (endothelin 3) have been reported in patients with WS4 (133).

Jervell-Lange Nielsen (JLN) syndrome is a recessive condition characterised by profound SNHL in association with a prolonged QT interval (134). The frequency of this condition is relatively low, with an estimated incidence of 1 to 6 in 1,000,000 individuals (135). This disorder is caused by homozygous or compound heterozygous variants in potassium channel genes *KCNQ1* or *KCNE1* (136). Up to 90% of the cases are linked to variants in *KCNQ1* (137). JLN has a high mortality linked to the cardiac phenotype. Vestibular dysfunction may be present in a smaller proportion of patients (52).

1.5.4 Mitochondrial sensorineural hearing loss

Mitochondrial SNHL only accounts for less than 1% of the cases. The majority of variants are found in mitochondrial genes such as *MTRNR1* and *MTTS1* and are maternally inherited. Pathogenic variants such as m.1555A>G and m.1494C>T in *MTRNR1*, as well as m.7445A>G in *MTTS1* have been well correlated with early onset, progressive non-syndromic SNHL (138). Nuclear modifier genes and environmental factors such as exposure to ototoxic medication may influence clinical variability (139,140). Aminoglycoside-induced SNHL is caused by the variant m.1555A>G (141). A prevalence of 0.19% in European children has been estimated(142). Mitochondrial SNHL has also been reported in patients with no history of previous exposure to aminoglycosides (139).

Sensorineural hearing loss is also seen in patients with syndromic mitochondrial disease. Common mitochondrial disorders associated with hearing loss include maternally inherited diabetes (MIDD) and MELAS syndrome (myopathy, encephalopathy, lactic acidosis and stroke-like episodes). These two entities are associated with the variant 3243A>G in the tRNALeu gene (143). The prevalence of this variant in Caucasian population is 0.24% (144). Hearing loss is the consequence of atrophy of the stria vascularis due to the accumulation of abnormal mitochondria (145). MIDD is characterised by adult-onset, progressive SNHL and usually precedes diabetes onset (146). Velocity of HL progression may vary from 1-7dB per year and tends to be more rapid in men (147). Patients with MELAS experience progressive SNHL and vestibular dysfunction (148) which may present along with stroke-like episodes (149). Heteroplasmy (the proportion of mutant and wild-type mtDNA) has been proposed as an important factor of phenotypic variation in both mitochondrial syndromes (150,151).

1.5. Diagnostic investigations in congenital and childhood hearing loss

1.5.1 Newborn Hearing Screening

Initial detection of hearing loss is done through universal newborn hearing screening (UNHS). UHNS programmes were created to enable the early identification of children with bilateral permanent hearing loss. Early identification of hearing loss is the first step towards preventing the profound impact that unmanaged hearing loss can have on a child's future development. The implementation of UNHS has been considered a highly successful public health intervention over the past 20 years (4,152,153).

UHNS testing measures the electrophysiological response of the peripheral and central auditory pathways. This includes the measurement of the physiological activity of the outer hair cells of the cochlea (otoacoustic emissions) and the response in the cochlear nerve by measuring the auditory brainstem response. Otoacoustic emissions (OAEs) are sounds emitted by the outer hair cells of the cochlea in response to acoustic stimuli. OAEs are measured and recorded via a microphone placed in the external auditory canal. This response reflects the status of the cochlea. Auditory brainstem response (ABR) testing records the neural response of the cochlea, the cochlear nerve and the brainstem in response to auditory stimuli.

UHNS can be conducted as a hospital-based or community-based service. In line with the guidelines issued by the Joint Committee on Infant Hearing and the majority of professional bodies, screening should be performed during the first month of age (29). UHNS protocols vary depending on the underlying health conditions of the infant (154). In general, OAEs are indicated as the first test in well babies. Here, lack of a clear response prompts ABR testing. Patients with inconclusive ABR results are to be referred for early audiological assessment within the first month after completion of the initial UHNS test. A different protocol is recommended for babies who have been in neonatal intensive care units (NICU). The Joint Committee on Infant Hearing screening protocol also indicates the use of ABR as the only testing technique is recommended due to the higher exposure to hearing loss risk factors. The NICU screening protocol in

England recommends the use of both OAEs and ABR(155). ABR results with no clear response prompts early referral to Audiology within 4 weeks of screening. Audiological assessment at 7-9 months of age is indicated for all babies with important history of risk factors, even if ABR outcomes indicate a clear response. Infants with history of meningitis, congenital microtia or atresia or confirmed congenital CMV infection are not eligible for UNHS as referral for audiological assessments is mandatory as part of their diagnostic evaluations.

UNHS programmes create an opportunity for early identification of SNHL although it has shown limitations. Firstly, screening target the detection of hearing loss above 35dBHL and may not be able to detect cases with mild severity. Secondly, the timing of test hinders the detection of hearing loss where onset takes place outside the screening period (delayed onset prelingual hearing loss). A classic example is illustrated by the inaccuracy of detection of early-childhood onset of hearing loss in patients with genetic SNHL associated with variants in *SLC26A4* (156). Lastly, OAE-based UNHS protocols or where ABR testing is delayed, there is a higher rate of false negatives (up to 17%)(157). Due to the nature of the electrophysiological response measured in OAE testing, it can fail at detecting hearing disorders with normal outer hair cell activity such as auditory neuropathy (158).

1.5.2 Audiometric and physical examination

Results obtained through UNHS determine whether a referral to audiology services is required in order to provide auditory habilitation and ongoing support to the child and the family.

Audiological testing for children is selected based on the age of the patients. Behavioural testing consists of observing hearing behaviour to sound. This is used for babies under 6 months of age (159). Visual reinforcement audiometry (VRA) consists of using a child's natural response to sound localization by showing a visual "reward" in response to recognition of sound (160). VRA is the most used test in infants aged 6 months to 2.5 years old. Play audiometry testing is done for patients between 2.5 and 5 years old. In this test, the child is engaged in a game and asked to perform an action in response to sound (161). Older children (over 5-6 years old) can be evaluated using pure-tone audiometry. This test aims to determine the lowest intensity at which the patient is able to hear a pure tone sound. Both air and bone thresholds are measured across frequencies 250Hz to 8000Hz (158).

A complete audiological assessment should also include the evaluation of the middle ear compartment(158). A tympanometry measures the mobility of the tympanic membrane, the ossicles and the middle ear pressure (162). This is important as it is necessary to discard middle ear pathology and identify high or low-impedance abnormalities(163). The presence of high-impedance conditions, such as otitis media with effusion, may affect other audiological testing results when testing for SNHL. An impedianciometry can be used to measure the acoustic reflex, which is triggered in response to sounds above 75dBHL. The absence of this reflex may be suggestive of a severe hearing loss (164).

1.5.3 Aetiological diagnosis: clinical and laboratory evaluations in congenital and childhood hearing loss

The confirmation of the aetiological diagnosis has gained recognition over the years as it can provide guidance for management and counselling for patients and families. These investigations aim to recognise any factors that predict further progression of hearing loss, eventual presence of non-otological conditions or sensory impairment or (e.g. Usher syndrome) and determine whether there is any risk of hearing loss for family members (52).

Recommendation guidelines have been issued by different medical bodies with the aim to guide the approach for conducting aetiological investigations (29,165–167). In general, all these guidelines focus on 5 main aspects: (i) medical history, (ii)audiometric evaluation, (iii) family history, (iv) physical examination (ENT and other relevant allied health specialities) and (v) clinical laboratory and imaging testing. These evaluations (Figure 1.8) may be conducted following a stepwise approach. Table 4 shows the tiered approach recommendations prepared by the British Association of Paediatrician Audiologists (BAPA) and the British Association of Audiological Physicians (BAAP).

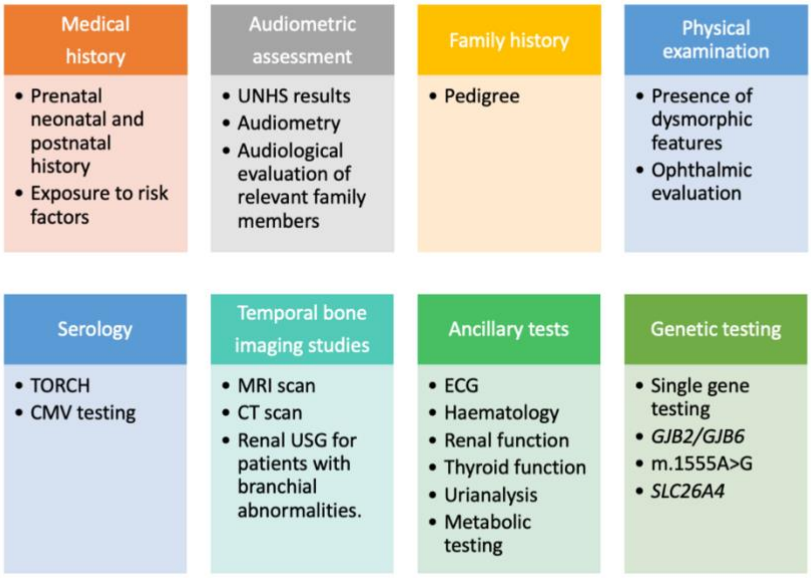


Figure 1.8 Summary of aetiological investigations in congenital and childhood hearing loss.

A comprehensive medical history at birth and during the prenatal and postnatal period may reveal information about exposure to risk factors, such as ototoxic medication or hyperbilirubinemia. It is also important to exclude acquired causes of congenital SNHL such as TORCH infections (Toxoplasmosis, Other (syphilis, varicella-zoster, parvovirus B19), Rubella, Cytomegalovirus, and Herpes). Screening for congenital cytomegalovirus (cCMV) is recommended as it is one of the leading causes of non-genetic congenital SNHL (168). While 90% of patients with cCMV infection remain asymptomatic, around 10-15% develop SNHL or other neurological abnormalities. cCMV screening is time sensitive and therefore should be conducted during the neonatal period (169). It can be done in urine or saliva or even umbilical cord blood samples using polymerase chain reaction (PCR) (170). Some protocols may complement with measurement of immunoglobulin M (IgM) levels.

The physical exam should include a detailed examination of the head and neck, the ear and the aerodigestive tract. It is important to exclude pathology of the ear that may alter results of audiometric evaluations (e.g., otitis media with effusion). The presence of cleft anomalies, abnormal skin and eye pigmentation, branchial anomalies or facial dysmorphism may suggest an underlying syndromic condition (170). Neurological and

motor milestones should also be evaluated. Delayed motor milestones; i.e. sitting, walking, are typical findings in infants with SNHL and impaired vestibular function (171). Vestibular testing in older children, in particular those with profound SNHL, may be beneficial and should also alert about the possibility of a syndromic condition (172).

The identification of clinical features such as congenital head and neck anomalies or EVAs via CT/MRI scans often prompts a referral for genetic consultation. Genetic testing is one of the aetiological investigations with highest yields in congenital hearing loss when compared to other clinical tests (173,174). An early referral for a consultation with the clinical geneticist may be more common for patients with likely syndromic conditions or positive family history(174). Genetic testing will be discussed in more detailed in the next section.

Ophthalmology assessments are crucial in the physical examination of children with SNHL. Vision is as important as hearing for adequate cognitive and social development. Hearing impaired children rely greatly on their vision for social interaction and developing non-verbal communication skills (175). The early recognition of a possible additional sensory impairment can make a significant different in the decision of future interventions for rehabilitation. The prevalence of abnormal ophthalmic findings in children with hearing impairment ranges between 20% to 60% (176–178). Refractive errors are the most common visual disorders, with a prevalence rate estimated around 40%(176). Another important goal of ophthalmic evaluations is the identification of non-refractive errors as they may enable the recognition of dual sensory syndromic conditions such as Usher syndrome. In this instance, conducting ophthalmic evaluations in very young children may not reveal abnormal findings as the onset of visual abnormalities occurs later in life.

Temporal bone imaging is an important part of the aetiological investigation algorithm. This primarily consists of magnetic resonance imaging (MRI) and/or high-resolution computed tomography (CT). Imaging tests, in particular MRI scans, are usually conducted during the evaluation for cochlear implant candidacy in order to identify labyrinth malformations that may need to be taken into account as part of the cochlear implant evaluation process. For instance, the identification of EVAs informs about the risk of cerebrospinal fluid (CSF) leak (Gusher) during surgery. This informs the medical team about the possible diagnosis of Pendred syndrome. MRI testing also identifies

brain or cochlear nerve pathology. CT scan testing has a low ratio of positive findings in patients with bilateral SNHL(174); as such, a CT scan of the temporal bone is a more appropriate test in the evaluation of congenital microtia atresia. Furthermore, bony anatomical abnormalities are more frequent in patients with unilateral hearing loss (179).

There is some discussion on the benefit-risk ratio in children undergoing imaging testing. Paediatric patients, especially those under age 3, require sedation due to the expected lack of compliance during testing. Exposure to anaesthetics may be more prolonged in MRI, which has raised concerns about the possibility of developing neurocognitive impairment as a side effect (180). Furthermore, it has been reported that children undergoing imaging testing may suffer anxiety and the lack of compliance may prompt the need for general anaesthesia, which increases the likelihood of adverse effects (181).

Other ancillary tests include electrocardiograms (ECG), renal and thyroid function tests, urine analysis and metabolic tests. Electrocardiogram testing in children can help identify cases with conduction disorders associated with congenital SNHL such as JLN. Thyroid function tests may be conducted in those patients where Pendred syndrome is suspected. Renal function tests and urine analysis may reveal renal conditions such as Alport syndrome. Laboratory tests have low diagnostic yields and therefore, their utilization is recommended to complement other main aetiological investigations (182).

Contrary to expectations, the overall yield of such comprehensive clinical and laboratory testing is low. It has been estimated that the aetiology in approximately half of children with SNHL remains uncertain (183–185). Complete follow up investigations are not conducted in approximately half of cases detected via UNHS (186). Another accountable factor may be the choice of timing and type(s) of testing. Consequently, there is a need to determine strategies that can improve diagnostic yield and therefore, inform patient management.

Table 1.4 BAPA/BAAP Recommendations for aetiological investigations for childhood hearing loss

Level 1
General history Details of pregnancy, maternal health, neonatal risk factors.
Family history Family audiograms If relevant, information from first degree relatives is recommended,
Physical examination ENT, evaluation of dysmorphic features.
Imaging MRI
Congenital infection CMV Rubella, toxoplasma, syphilis.
Ophthalmology assessment
Laboratory tests Urine analysis Blood, protein, metabolic screen
Genetic testing *GJB2/GJB6*
Autoimmune diseases [a]

Level 2
Imaging (Temporal bone CT scan)
Renal ultrasound In case of presenting with bilateral enlarged vestibular aqueducts, microtia, branchial cleft abnormalities
Electrocardiography
Haematology
Metabolic screening
Genetic testing m.1555A>G [a] (History of hearing loss through maternal inheritance) Chromosomal abnormalities *SLC26A4* [a] *EYA1/ SIX1* [b]
Vestibular function [b]

Adapted from "Guidelines for Investigating Infants with Congenital Hearing Loss Identified through the Newborn Hearing Screening" by the British Association of Paediatricians in Audiology (BAPA) and British Association of Audiological Physicians (BAAP).

* Usually during assessment for cochlear implantation.

[a] – Recommended as a Level 1 investigation in case of progressive hearing loss.

[b] – Recommended as a Level 2 investigation only in case of progressive hearing loss.

1.6 Standard genetic testing in congenital and childhood hearing loss

Genetic assessment and investigations were formally implemented in the early 2000s, as part of the aetiological workup of children with hearing loss recommended by the ACMG and the joint committee in infant hearing (187). A referral for evaluation by a clinical geneticist can facilitate the recognition of syndromic conditions. A comprehensive clinical genetic evaluation should entail a three-generation family pedigree and a detailed anamnesis including information on ethnicity, consanguinity, audiological and vestibular symptoms. Based on the clinical findings, molecular genetic testing can be subsequently offered. Currently, the most common and recommended initial genetic testing focuses on the identification of variants in *GJB2, SLC26A4* and the mitochondrial gene MT-RNR1.

1.6.1 *GJB2/GJB6* testing

Molecular genetic testing for hearing loss became available shortly after the identification of *GJB2* variants as a major genetic aetiology of SNHL worldwide. The rationale for focusing the initial genetic testing on analysis of *GJB2* is the high prevalence of SNHL associated with this gene. Screening for the common variant c.35delG using variant screening methods was used at some point (188); however, this is no longer effective for diagnostic purposes when applied to multi-ethnic populations (55,189).

The recommended protocol for *GJB2* diagnostic testing includes sequencing of exon 2, which contains the entire coding sequence, the splice sites near intron and detection of deletions del(GJB6-D13S1830) and del(GJB6-D13S1854) in *GJB6* (190). The detection of *GJB6* deletions can be beneficial for patients with negative results or those with *in trans* single heterozygous variants in *GJB2* (191–193). Reporting of homozygous or compound heterozygous variants in *GJB2* as well as compound *GJB2/GJB6* confirms a molecular diagnosis (190). Testing for *GJB2/GJB6* has shown a sensitivity and specificity of 97% and has been initially recommended for patients with apparent non-syndromic SNHL, regardless of having a positive family history (190). A diagnostic yield

of 20% has been reported for singleton cases (190), which is expected in keeping with the expected prevalence of *GJB2*-related SNHL (194). Carrier testing for parents and cascade testing may be considered on a case-by-case basis after identifying a causative variant, with prior consent of the parents or other relevant family members. Today, *GJB2/GJB6* remains as the standard "first-line" genetic investigation and it is recommended as a level 1 investigation as per the BAAP and BAPA guidelines (195).

1.6.2 *SLC26A4* testing

The identification of EVAs during imaging testing, abnormal thyroid function tests or thyroid enlargement, balance impairment and/or fluctuating SNHL highly suggests a likely diagnosis of Pendred syndrome and thus can prompt analysis of *SLC26A4* (196). Sequencing of the entire gene length is recommended as pathogenic variants have been reported located in 20 of the 21 exons (197). Current *SLC26A4* tests registered on the NIH Genetic Testing Registry (GTR) are available using methods such as Sanger sequencing, deletion/duplication through multiplex ligation-dependent probe amplification (MLPA) and targeted variant analysis assays (http://www.ncbi.nlm.nih.gov/gtr, last accessed May 2020).

SLC26A4 testing is recommended as a Level 2 investigation in the BAAP guideline for permanent childhood hearing impairment (2018), the BAAP/BAPA guidelines for congenital hearing loss identified via UNHS (2008) and the BAAP guidelines for severe to profound bilateral permanent childhood hearing impairment (2015) (195). One important issue to consider regarding *SLC26A4* testing is the degree of variability in *SLC26A4* genotype-phenotype correlations (77). Biallelic and monoallelic *SLC26A4* variants have been reported in only about 25% and 15% of cases with bilateral EVAs respectively (198). A report of biallelic *SCL26A4* variants can confirm a diagnosis of Pendred syndrome. However, biallelic *SLC26A4* variants in non-syndromic bilateral SNHL and EVAs have also been reported. The identification of a monoallelic *SLC26A4* variant is also more commonly found in patients non-syndromic bilateral SNHL and EVAs. This discrepancy may pose a challenge in assessing the diagnostic utility of the test, which may be even lower in patients with no family history and isolated bilateral EVAs, unilateral EVA or isolated bilateral or unilateral Mondini dysplasia (198–200). Analysis of a specific *SLC26A4* haplotype identified in patients with non-syndromic EVA

has been recently proposed as an alternative addition to diagnostic *SLC26A4* sequencing (78,201) in patients with monoallelic *SLC26A4* findings.

1.6.3 Mitochondrial genes

Mitochondrial SNHL occurs at a low frequency (1%) in countries like the United States or Germany, but it can account for up to 20% in nations like Spain, Japan, Korea and China. Testing for the variants m.1555A>G and m.1494C>T in the *MT-RNR1* gene and m.7445A>G in the *MT-RNR1* gene is recommended for patients with family pedigrees suggestive of mitochondrial inheritance (167,187,202). The variant m.1555A>G increases susceptibility to aminoglycoside ototoxicity and also contributes to development of SNHL later in life in the absence of exposure to ototoxic medication. m.1555A>G testing and other mitochondrial variants can be performed first by targeted variant analysis using PCR and Sanger sequencing. About 71% of SNHL-causing mitochondrial variants are located in Mt-RNR1 (202). As an alternative step, other protocols and laboratories offer sequencing the MT-RNR1 gene. Low-level heteroplasmic variants may pose a challenge for interpretation as these are not typically detected by some methods (203).

1.6.4 Additional genetic testing

Depending on the clinical characteristics of the patient, specific single-gene testing may be requested. For example, sequencing of genes *PAX3* or *SOX10* can be used for patients with likely Waardenburg syndrome. Sequencing of *EYA1* can be requested for those patients where Branchio-Oto-Renal syndrome is suspected. In presence of certain conditions such as neurodevelopmental disorders or multiple congenital anomalies, chromosomal microarray analysis (CMA) is recommended for initial genetic testing (204). CMA, also known as array comparative genomic hybridization, is a DNA hybridization method that can detect chromosomal copy number variation (e.g. deletions or duplications) throughout the entire genome by quantifying differences in hybridization signal between a patient's DNA sample and control DNA (205). All these methods of additional clinical genetic testing, as well as referral for evaluation by a clinical geneticist, are currently recommended as level 2 BAAP/BAPA investigations and are conducted when deemed appropriate (206).

Since the discovery of *GJB2* in the late 1990s until 2010, the vast majority of investigations in hearing loss in both research and clinical contexts were conducted using Sanger sequencing. The introduction of new DNA sequencing techniques coupled with the massive increase in the number of genes discovered using new technologies has opened up the possibilities of genetic testing in hearing loss to a high-throughput level. The use of these technologies in the study of Mendelian single gene disorders and in hearing loss will be discussed in a further section.

1.7. DNA sequencing technologies

1.7.1 Sanger sequencing

Sanger sequencing is a method developed by Frederick Sanger in 1977 (207). Also known as "dideoxy" sequencing, this method is based on the principle of DNA synthesis. Sanger sequencing relies on the selective incorporation of dideoxy-nucleotides (ddNTPs) by polymerase chain reaction (PCR) to seek out and amplify DNA strands (Sanger, Nicklen and Coulson, 1977; Gomes and Korf, 2018). The dideoxy-nucleotides (ddNTPs) are analogues of the four deoxyribonucleotide triphosphates (dNTPs) (208). Deoxyribonucleotide triphosphates are essential for DNA replication and repair. The original technique consisted of adding a small fraction of radio-labelled ddNTPs into a DNA reaction mixture, running four reactions in parallel, each corresponding to one of the four bases (207,209). Since dideoxy-nucleotides (ddNTPs) lack of the 3′ hydroxyl group required for elongation of DNA strands, the addition of ddNTPs inhibits chain termination in the reaction. Each reaction was visualized on polyacrylamide gel using autoradiography, allowing the read and interpretation of the nucleotide sequence (207). Further improvements of the original technique led to the substitution of radiolabelled ddNTPs for the method used today, which is automatized and uses fluorescently labelled ddNTPs and detection through capillary electrophoresis (210) (Figure 1.9).

Sanger sequencing was used to generate the first draft of the human genome from 1999 to 2003 (Lander *et al.*, 2001) and has been the gold standard for detection of genetic variation in clinical settings for many years. Today, Sanger sequencing is widely used for genetic investigations in clinical settings for single gene sequencing and as a confirmation method for validation of genetic variants identified through high-throughput DNA sequencing technologies.

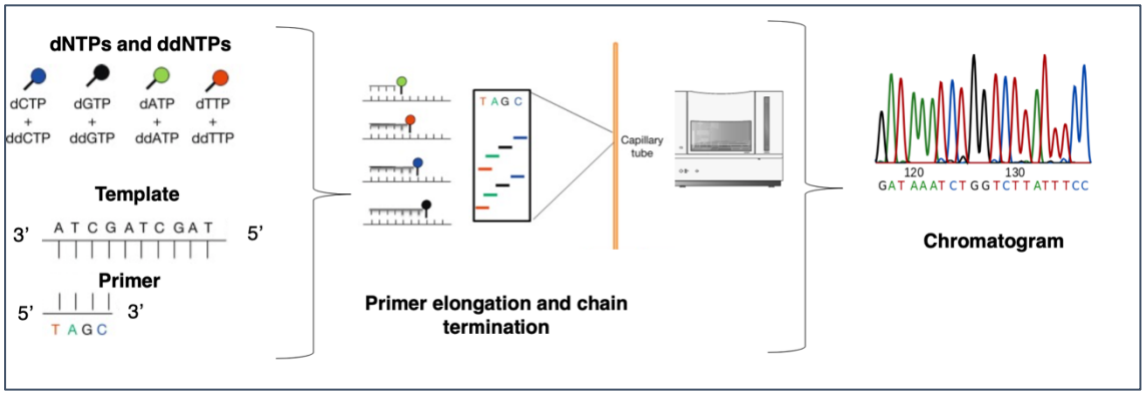


Figure 1.9. Sanger sequencing methods (211,212). A Sanger sequencing reaction requires: a DNA template, a primer, DNA (Taq) polymerase, deoxynucleotide triphosphates (dNTPs) for each base: arginine (A), cytosine (C), tyrosine (T), and guanine (G); fluorescent modified-dideoxynucleotides (ddNTPs) and buffer. The first step is denaturation of the double-stranded DNA. The resulting single-stranded DNA template is then annealed to the primer. The addition of fluorescent-labelled dNTPs by DNA polymerase elongates the primer, thus building a new double-stranded DNA. The sequence extends until fluorescent modified-dideoxynucleotides (ddNTPs) attach to their complimentary nucleotides, thus terminating the new strand. All reagents are combined in a single reaction and injected into a capillary. Extension products of different lengths are obtained and are separated using electrophoresis. Inside an automated DNA sequencing machine, a laser beam excites the dyed DNA strands, thus allowing the detection of the fluorescent dye intensity. Green fluorescence indicates ddATP, red indicates ddTTP, black indicates ddGTP and blue indicates ddCTP. The detected sequence is shown in a colour-coded chart representing the bases (chromatogram) (211,212). Figure adapted from (212). Chromatogram figure adapted under Creative Commons Attribution-Share A like 4.0 International license.

1.7.2 High-throughput sequencing technologies

Technological improvements in Sanger sequencing were made during the Human Genome Project (213,214). Nevertheless, the arduous task of sequencing the entire human genome called attention to the necessity to further improve methods of DNA sequencing that could reduce time, effort and laboratory costs.

A new generation of DNA sequencing technologies began to emerge shortly after the later improvements in Sanger sequencing (209,215). The development of sequencing technique called pyrosequencing marked a pivotal moment in the development of future DNA sequencing technologies. Pyrosequencing is a technique that determines nucleotide sequences by quantifying light emitted during pyrophosphate synthesis (bioluminescence) as a result of nucleotide incorporation (215,216). The main advancement brought into research by this method was the ability to run multiple sequencing reactions in parallel (209). Pyrosequencing was followed by the development of a new generation of DNA sequencing methods, which consequently demanded new design and development of machines that were capable to perform multiple sequencing reactions simultaneously (209). The term next-generation sequencing (NGS) was a term coined to describe all the upcoming massive parallel sequencing technologies. NGS technologies have massively increased throughput and reduced expenditure at a rate that surpasses Moore's law of technological development (217).

Next generation sequencing (**NGS**) platforms started becoming available in the mid 2000s (218). Each NGS platform is characterised by different length of DNA reads, output and run times, which are the result of differences in their protocols for preparation of DNA templates, sequencing chemistry, sequence visualisation and data analysis (219). NGS platforms can be broadly classified into **short-read** and **long-read** NGS. Short-read sequencing technologies produce reads between 30-700 base pairs (220). By contrast, long-read sequencing technologies (LGS) allow for sequencing of longer read lengths of up to 10kb (221). Long-read sequencing technologies have shown utility in transcriptomics and investigation of structural variants or other complex rearrangements. In the following section, I will discuss briefly the fundamental workflow of short-read sequencing techniques, with emphasis on

Illumina, one of the mostly used sequencing platforms. Sequencing results reported in this thesis were obtained through investigation performed using Illumina sequencing.

1.7.2.1 Short-read DNA sequencing platforms

Many breakthrough discoveries in human disease have been achieved as a result of the development of short-read sequencing. Since their initial development, short-read sequencing technologies have dominated the research and clinical landscape of massive parallel sequencing (220). Short-read sequencing technologies are characterised by a common workflow that consists of: (a) template preparation, (b) sequencing and (c) genome alignment and assembly for DNA sequencing analysis (219).

The **template preparation** consists of creating DNA libraries that are subjected to clonal amplification and cluster generation. The process begins with DNA fragmentation, which can be achieved by enzyme-based methods or mechanical methods (e.g. sonification) (220). Each fragment is then ligated to a custom adapter sequence for amplification and sequencing. For most short-read sequencing methods, templates are separated and attached onto a solid surface (219). Clonal amplification of the original templates is required to generate multiple DNA copies, which reinforces signal and reduces sequencing artifacts (222). Depending on the sequencing platform, different methods can be used for clonal amplification of DNA templates into clusters. Bead-based methods use emulsion PCR to amplify templates onto magnetic beads suspended in individual droplets in micellar solution (219). Each DNA template is amplified, generating multiple copies which are immobilised onto each bead. Beads are subsequently deposited onto an array or a glass surface for sequencing. Solid-phase bridge methods amplify templates directly onto a solid flow cell which contains covalently attached forward and reverse primers. Hybridised templates bridge over nearby primers, thus generating multiple clustered DNA copies. Here, control over the template-to-primer ratio is key to circumvent cluster overlapping (220). Patterned flow cells can be used to facilitate placement of primers on the slide. This ensures adequate spatial cluster localisation which in turn increases cluster density and therefore, augment sequencing throughput.

Sequencing. Based on their sequencing principle, short-read technologies can be classified into 2 main categories: **sequencing by ligation** (SBL) and **sequencing by synthesis** (SBS). In SBL, sequencing is achieved by oligonucleotide ligation mediated by DNA ligase. Fluorescent interrogation probes are ligated to an anchor sequence, which is complementary to the adaptor attached to the DNA template. By contrast, SBS platforms require multiple and continuous cycles of DNA polymerase to elongate nucleotides.

SBS technologies can be subdivided based on their sequencing chemistry: single nucleotide addition and cycle reversible termination(219). **Single nucleotide addition** (SNA), each nucleotide is added consecutively until their incorporation to the extending template. The addition of the complementary nucleotide emits a signal that is recorded by luminescence -i.e., pyrosequencing- (454Roche) or by change in pH after changes in H+ concentrations triggered by the addition of each nucleotide (Ion Torrent ™ /Thermo Fisher). The latter does not rely on an enzymatic reaction to emit the signal (220). **Cycle reversible termination** (CRT) is based on the principle of Sanger sequencing. DNA synthesis is terminated upon the incorporation of a single corresponding nucleotide. During each cycle, a complimentary primer binds to the adapter region of the DNA template. From this site, DNA polymerase incorporates fluorescently labelled deoxynucleotides on the 3'-end direction of the elongating strand. Laser-induced fluorescence emitted by the incorporation of the complementary deoxynucleotide is captured and imaged. The emitted signal indicates the identity of each nucleotide. Remaining unbound fluorophores are washed off and the 3'-OH is regenerated, restarting the cycle (219)

Cycle reversible termination is the sequencing technique used by **Illumina** platforms. Illumina is one of the most widely used platforms to date in pharmaceutical and academic research as well as clinical settings. It uses solid-phase clonal amplification by bridge PCR for cluster generation (219). Another characteristic of Illumina sequencing is that this is the only technology that can perform paired-end sequencing. Paired-end sequencing is a process that allows to obtain reads of higher quality as the DNA fragment is sequenced from both 5'-to-3' and 3'-to'5' directions. In keeping with the common workflow of short-read NGS technologies, the Illumina sequencing method consists of: (i) DNA sample preparation, (ii) cluster amplification by bridge PCR, (iii) cycle reversible sequencing and (iv) data analysis (Figure 1.10). After the

launch of the Genome Analyzer in 2006 by Solexa (later acquired by Illumina), Illumina sequencing instruments have been optimised to improve the utilisation of sequencing reagents, reducing number of sequencing cycles required and imaging, resulting in increased throughput and reduction of sequencing costs (223). The authorisation for clinical use granted to Illumina represented an important milestone in the transition of NGS from the research laboratory to clinical settings (224).

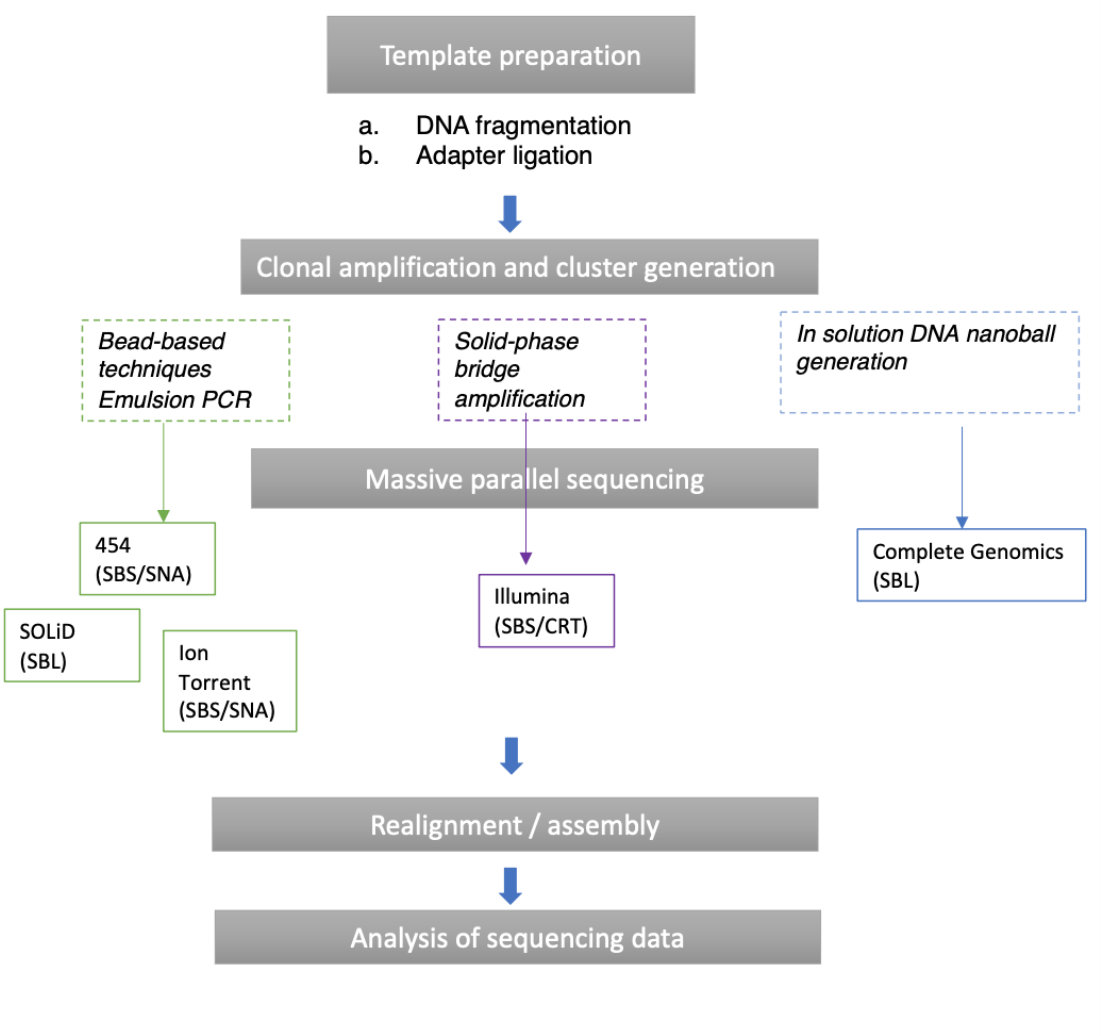


Figure 1.10. Workflow of short-read sequencing methods. Clonal amplification, cluster generation methods (dotted squares) and their respective sequencing chemistries are indicated by same colour. SBS: Sequencing by synthesis; SBL: Sequencing by ligation, SNA: Single nucleotide addition; CRT: cycle reversible termination; SOLiD: Sequencing by Oligonucleotide Ligation and Detection.

1.7.3 Bioinformatics analysis and interpretation of next-generation sequencing data

The analysis and interpretation of genomic data are some of the most complex steps in the application of NGS. Different types of computational tools are available for each step of the bioinformatics analysis (Figure 1.11). The type of sequencing technology and length of the DNA read dictates the type of software used for each step of the analysis. This section covers the analytic process of analysis of sequencing data from short-read technologies, taking the output of Illumina sequencers as an example.

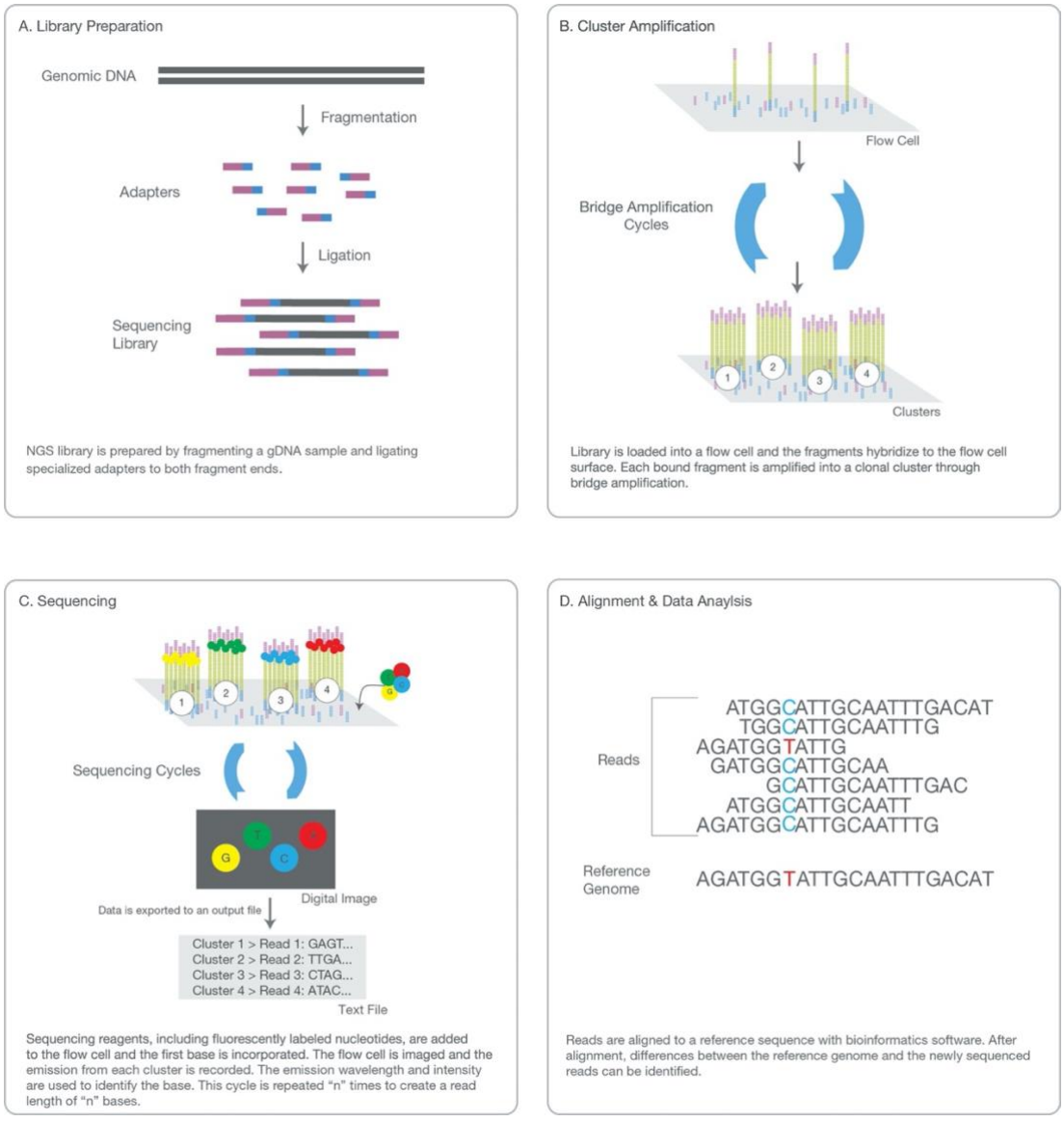


Figure 1.11 Illumina sequencing workflow. Image courtesy of Illumina, Inc.

Each NGS experiment generates millions of sequence reads. In order to obtain information about the biological impact of a genomic variant, all the raw DNA sequence data must be converted into different file formats as it undergoes each analytic step of the bioinformatics pipeline (Figure 1.12). Of note, sequencing can be performed by having one sample or a pool of samples, which are identified using bar codes added during template preparation. If multiple samples were pooled and sequenced at the same time, read **demultiplexing** is required to sort and separate reads by sample (225). After demultiplexing, base call data is converted into a file that contains a consensus evaluation of the sequence, the **FASTQ** file. The FASTQ file is a universally sequencing data format that contains the sequence and a quality score value assigned to each base call (226,227). The quality is expressed as a **Phred quality score**, which indicates the probability that a base call is incorrect (228). Quality control processing is required to remove low-quality reads and reduce error (229).

Following quality control, high quality reads are given a corresponding location in a process called **read alignment**. In this step, reads are mapped using the human reference genome as a template to determine their respective genomic location. Different mapping algorithms have been developed for this purpose, such as the Burrow Wheelers Transform (BWT) (230), which is one of the most accurate and effective algorithms for short sequence reads. Different programs have been based on the BWT, such as the Burrow Wheelers Aligner (BWA) (231) and Bowtie2. The output of this process is converted into a **SAM** file (sequencing alignment/map format) which contains mapped sequence reads with information on the location with respect to the reference genome (232). Typically, SAM files are compressed into a **BAM** (binary alignment/map format) file. The BAM file is the binary version of the same data contained in the SAM file (232). The use of the BAM file facilitates long-term data storage and manipulation. To ensure generation of high-quality data and increase the level of confidence in during variant calling, the resulting BAM file undergoes further processing to remove artifacts generated during the alignment, recalibrate base quality scores and realignment of insertions and deletions (233,234).

Variant calling is a key process of the bioinformatics analysis. Genomic variants are identified by detecting differences between the mapped reads against the reference genome. There is a plethora of different open-source and commercial computational algorithms for variant calling (235). Single nucleotide variants (SNVs) and small insertions and deletions (<50 base pairs) can be detected using widely known non-

commercial tools such as SAMtools (232) and the UnifiedGenotyper or the HaplotypeCaller from the Genome Analysis Toolkit (GATK) (236). SAMtools and GATK are also used for sorting BAM files prior variant calling (233). Alternatively, certain NGS companies such as Illumina provide with their own computational resources for SNVs variant calling (i.e. CASAVA, ISAAC) (237,238).

For detection of structural variants (SVs; >50 base pairs in length), computational algorithms for short-read sequencing are based on assessing distance and patterns between paired-end reads, detecting changes in read depth or detecting points of disruption when aligned to the genome (239,240). Software packages based on these analytical approaches can be added to the variant calling pipeline to extend the search for potential pathogenic SVs (241). Variant calling output data is deposited into a **VCF** file (variant calling format). This file contains aggregate information on the chromosomal coordinates of the variant, the reference allele (reference genome) and alternative allele (sample), quality scores.

Further information on the functional and biological impact of the genomic variant is retrieved during **variant annotation**. Ensuring correct annotations is key to distinguish pathogenic variants from benign variants during downstream analysis. This information can be obtained from a plethora of resources including publicly available databases, published scientific reports and computational tools that predict the impact of the variant on the protein. Different software tools; whether commercial or open-source, command-line or graphical user interface; can be used to gather information on the affected gene describing cDNA and protein changes, allele frequency, effects on predicted effect using sequencing ontology (242) , protein sequence conservation across species. Adequate transcript selection is crucial for variant annotation (243). A gene can produce different transcripts during transcription; therefore, variants may differ on the location in different transcripts. The selection of the transcript with more severe impact can reduce the possibility of excluding potentially pathogenic variants. However, it may increase false positives that pass to the next analysis step(244).

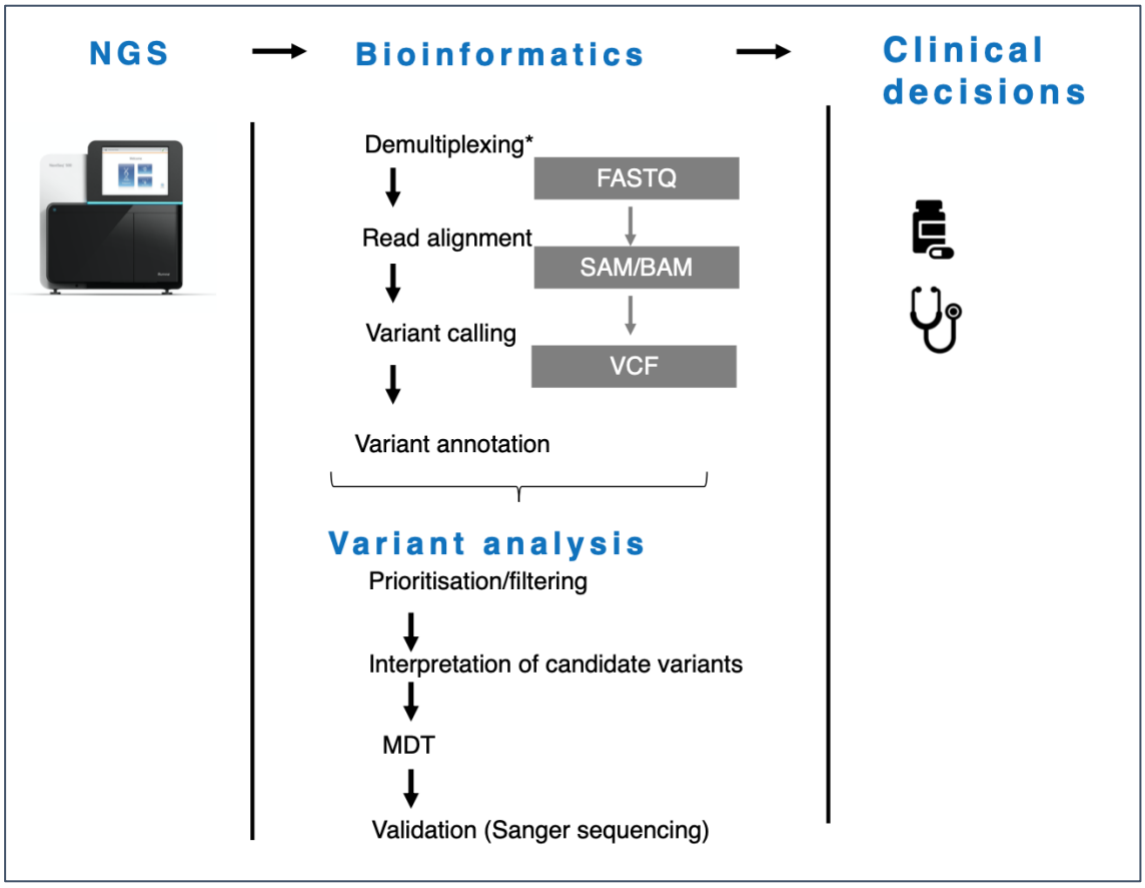


Figure 1.12 Bioinformatics workflow for processing NGS data. Grey boxes indicate file formats.
* demultiplexing step is performed when analysis includes multiple samples per sequencing run.
MDT: multidisciplinary team meeting.

1.7.4 Variant prioritisation and variant interpretation in Mendelian disorders

The goal of variant prioritisation is to reduce the number of candidate variants for downstream analysis and clinical interpretation. Variants are evaluated based on their specific annotations. This process is based on systematically adjusting different variant-based and gene-based parameters as per hypotheses constructed about inheritance, phenotype and biological mechanisms of disease.

Allele frequency and population stratification. Allele frequencies are used for prioritisation of rare variants (minor allele frequency [MAF] <1%), as these are often causal in Mendelian disorders (245). Filtering by using stringent allele frequency cut-offs eliminates common variants that are unlikely to be associated with rare disorders, or that are incompatible with the inheritance pattern(246). However, frequency thresholds require customisation according to disease and inheritance. For example, many disease-causing hearing loss variants may fall between a 1-5% MAF threshold (247). Another important element when estimating frequencies is ethnicity. It is not uncommon to identify certain disease-causing variants with higher prevalence in individuals of certain ethnicities. For instance, as described in previous sections, *GJB2* c.35delG is more observed in individuals of European Caucasian ethnicity whereas *GJB2* c.235del has been mostly reported in individual of Asian ethnicity. Allele frequencies can be determined using large control population databases such as 1000 Genomes Project (http://browser.1000genomes.org), NHLBI Exome Sequencing Project Exome Variation Server (http://evs.gs.washington.edu/EVS) or the Genome Aggregation Database (gnomAD, [https://gnomad.broadinstitute.org]). The latter, developed from Exome Aggregation Consortium (ExAC) (248), represents one of the most comprehensive catalogues of human variation. It contains sequencing data from 125,748 exomes and 15,708 genomes; including SNVs and structural variation including control individuals from six different ancestries (249).

Functional impact and conservation. The majority of variants in Mendelian disorders affect protein-coding regions. As such, variants affecting protein function or structure are predicted to be associated with disease. Variants that truncate protein-coding transcripts or remove exons, splice sites, change reading frames or that remove an entire gene (i.e. nonsense single-nucleotide substitutions, splice site variants and small indels) are commonly predicted to be deleterious and likely disease-causing in AR disorders (250). Missense single-nucleotide substitutions require more careful inspection as their impact varies depending on the evolutionary conservation of the amino acid or the changes in the protein's chemical properties induced by amino acid substitution (251). Several *in silico* tools (Table 5) have been developed to predict their pathogenicity based on: (i) the potential impact on protein chemistry and structure and/or (ii) conservation of sequence across species. Combined use of these tools or meta-predictors algorithms that integrate scores from multiple sources is recommended to increase the level of evidence towards assigning pathogenicity (251,252).

Table 1.5 Examples of commonly used *in silico* tools for missense variants

Missense *in silico* prediction tools [website/author(s)]	**Evolutionary conservation**	**Protein structure/function**
SIFT http://sift.jcvi.org (253)	✓	
Align GVGD http://agvgd.iarc.fr/agvgd_input.php	✓	✓
VariantTaster http://www.varianttaster.org	✓	✓
PolyPhen-2 http://genetics.bwh.harvard.edu/pph2	✓	✓
FATHMM http://fathmm.biocompute.org.uk	✓	

For splice site variants, various *in silico* tools have been developed to predict pathogenicity based on their potential consequences on RNA splicing; e.g. Gene Splicer (254), Spliceman (255), SPIDEX (256), Human Splicing Finder (257), MaxEntScan (258). It is recommended that potentially causal synonymous variants are also subjected to this assessment given their potential to alter mRNA structure, mRNA splicing and transcription factor binding (251,259). Many of these missense and

splicing prediction algorithms are web-based tools or have been incorporated in variant annotation software such as Alamut ® Visual (www.interactive-biosoftware.com) (260). With the introduction of genome sequencing, other tools are being developed for prediction of deleteriousness of variants in the non-coding genome (261,262).

Inheritance and segregation. The use of information about a patient's family history, including pedigrees and DNA sequencing data when available, is a key element in establishing segregation and inheritance (251,263). For instance, analysis of parental DNA, in unaffected parents, can be used to confirm *in trans* phase of variants in recessive disorders (264). For dominant conditions, the identification of heterozygous variants in similarly affected family confirms segregation of disease and dominant inheritance. It is important to consider that incomplete penetrance of disease, genetic modifiers and/or environmental factors can make difficult to distinguish segregation and inheritance patterns (264,265).

Phenotype. Many Mendelian conditions can be clinically distinguished by their characteristic set of signs and symptoms. However, it is not uncommon to find overlapping phenotypes or atypical clinical characteristics. The use of standard terminology (i.e., biomedical ontologies) has facilitated the development of algorithms that use these terms to infer gene-phenotype relationships. Phenotype and gene ontologies are of great utility particularly in the analysis of cases with atypical phenotypes. Widely used examples of these are the Online Mendelian Inheritance in Man (OMIM) database, which contains a comprehensive catalogue of Mendelian gene-disease associations (266); and the Human Phenotype Ontology (HPO), which provides with standard hierarchical terminology that describes clinical features of human disease (267). Genes and phenotypic features are designated with specific identifiers that allows algorithms to utilise the linkage between these terms to generate lists of disease for differential diagnosis, select potentially relevant genes (268), or rank genes according to the likelihood of association with disease(269).

The clinical significance of a variant can be determined by identifying whether altered gene function results in the expected phenotype. Phenotype-driven tools prioritise variants in genes whose product is part of the gene pathway of a similar phenotype (270). Other algorithms perform comparisons of gene-phenotype between humans and existing animal models. For example, Genomiser (non-coding variants) and Exomiser

(exome variants) rank variants by prioritisation of genes through cross-species phenotype comparisons, in addition to other variables such as predicted pathogenicity and minor allele frequency (271,272). PhenIX performs a similar prioritisation focused on genes with well-known association with human disease (270).

Biomedical databases. Publicly available databases such as ClinVar (273), the Human Gene Mutation Database (HGMD) (274) and the Leiden Open Variation Database (LOVD) (275) store information on previously identified variants submitted by clinical and research laboratories worldwide, many of which have been reported in the scientific literature. Depending on the database's system, variants are classified based on their clinical significance. While a certain level of discordance may exist across databases, particularly for missense, splice site or low penetrance variants (276), these databases are useful resources that facilitate sharing and access to variant-level evidence that can be assess to support variant interpretation.

Variant interpretation is one of the most complex and challenging steps of NGS experiments. All positive and negative variant-level and gene-level evidence is evaluated to establish the relationship between the candidate variants and the patient's phenotype. Consensus guidelines issued by the American College of Medical Genetics and the Association for Molecular Pathology (ACMG-AMP) provide a systematic framework for clinical interpretation of variants (251). This guidelines classify variants under a five-tier system that indicates their clinical significance based on parameters such as population frequency data, multiple computational predictions, functional evidence, family history and segregation data. Variants can be classified as: "pathogenic", "likely pathogenic", "variant of uncertain significance", "likely benign", and "benign". "Pathogenic" and "likely pathogenic" variants meet criteria that can support association (or likely association) with disease and thus, support further clinical decision-making. Variants with conflicting evidence that do not meet criteria in either pathogenic or likely pathogenic nor the benign or likely benign spectrum are designated by default as variants of uncertain significance (VUS). VUS may be subclassified by additional internal criteria as defined by the clinical laboratory or, for instance, the guidelines produced by the Association for Clinical Genetic Science (277). Internal VUS classifications can be used to determine whether a variant of uncertain significance should be reported back to the clinical team. For instance, heterozygous VUS identified *in trans* in a clinically relevant gene may require further discussion

between the clinical scientist and clinical genetics team in order to determine further actions. It is important to note that variant classification is not definitive, and variants may be subjected to reanalysis and be upgraded to a pathogenic or downgraded to benign category in light of new supporting evidence.

Despite advances and development of numerous computational tools, variant prioritisation and interpretation can be challenging and complex tasks. Sets of candidate variants are to be obtained from sifting through large volumes of variants identified per NGS experiment. From variant calling to variant prioritisation, bioinformatics pipelines and selection of computational tools require constant optimisation to ensure reducing false positives without compromising diagnostic power. Extensive time may be required for clinical interpretation of each variant, resulting in a time-consuming task if there are large amounts of prioritised variants (278). Longer interpretation times can affect turnaround times of results and increase the use of resources for interpretation, which in turn delays important clinical decision-making and affect the outcomes of a patient's diagnostic and therapeutic journey. As many healthcare systems require to apply cost-effective diagnostic interventions, there is a need to investigate pathways that can combine the comprehensiveness of genomic testing without increasing workload beyond a laboratory's capabilities.

1.8 Application of next-generation sequencing approaches to diagnosis of monogenic disorders and sensorineural hearing loss

The development of NGS marked the beginning of a new chapter in the study of rare monogenic disorders. From accelerating the pace of gene discovery to the implementation of massive parallel sequencing for clinical diagnosis, NGS approaches have changed our understanding of disease biology and paved the way to personalised patient care strategies (279).

NGS approaches can be classified according to the portion of the genome to be sequenced that will be the focus of investigation. (Figure 1.13). Whole genome sequencing is an untargeted approach that comprises sequencing of the entire genome, including both protein coding and noncoding regions. By contrast, targeted gene panels and whole exome sequencing are approaches that target a "region of interest". Genomic DNA of targeted regions is enriched by performing multiplex PCR amplification or hybridization-based methods at the template preparation stage (280). Each approach has strengths and limitations. While targeted NGS gene panels and WES currently dominate the clinical setting, large-scale initiatives are introducing the use of WGS through clinical research partnerships. In most instances, the choice of NGS approach is often based on the research or clinical questions to be addressed.

The use of NGS has evolved research and diagnostics of genetic hearing loss. It has tripled the rate at which new genes are identified (281) and it has introduced the use of targeted NGS gene panels in some centres (282). Some of these approaches have been used to explore the molecular genetic epidemiology of hearing loss across different populations (283,284), allowing the characterisation of more genotype-phenotype correlations and identifying some relationships between genetic diagnoses, course of hearing loss and response to auditory rehabilitation options (285,286).

1.8.1 Targeted next-generation sequencing gene panels

Targeted NGS diagnostic gene panels are designed to focus sequencing on a determined list of genes for a clinical condition or group of conditions (e.g. epilepsy or cardiomyopathy) (287,288). This type of NGS assay commonly used for diagnosing individuals with conditions characterised by a known genetic heterogeneity or conditions that have overlapping clinical features which require confirmation through molecular diagnosis (289).

Genes included in targeted NGS diagnostic gene panels are usually known genes with a well-characterised gene-disease association (290). The selection of genes should aim to provide the panel with high clinical sensitivity, clinical validity and clinical specificity (289). Genes of uncertain significance may be included at discretion of the laboratory depending on the level of evidence provided. Gene content and respective gene transcripts may vary depending on the laboratory and should be regularly reviewed and revalidated as more evidence for novel gene-disease associations appears over time (291).

A main advantage of targeted gene panels is their high coverage depth (number of reads that align to a reference sequence (292)), which is essential to maintain accuracy and a low false negative call rate (280). Due to the relatively smaller volume of sequencing data in comparison to exome or genome sequencing, targeted gene panels have shorter running times and reduced costs (293). This also allows to produce manageable sequencing data and facilitate downstream analysis, which in turn results in quicker turnaround times (280). The successful application has driven their implementation as clinical diagnostic tools in the early investigation of some monogenic conditions (289). Today, targeted gene panels have reported diagnostic utility in conditions such as neurodevelopmental disorders (287), primary immunodeficiencies (294), inherited retinal disease (295) and hearing loss (282).

1.8.1.1 Targeted next-generation sequencing gene panels for hearing loss

There are a number of hearing loss targeted NGS gene panels currently available in commercial or academically affiliated laboratories worldwide (Table 6) (296). A main difference among these gene panels is their gene content. This difference may depend on the clinical focus of the panel, for instance, whether it was developed for only testing non-syndromic genes only or for both syndromic and non-syndromic forms (297,298). Some gene panels only focus on specific conditions, for example, the Usher syndrome gene panel (University of Iowa). Gene panel content may also vary depending on the process of gene curation during panel design by the laboratory (297).

The OtoSCOPE gene panel (otologic sequence capture of pathogenic exons) , designed by the Molecular Otolaryngology and Renal Laboratory at The University of Iowa, was the first targeted NGS-based gene panel developed for investigation of genetic hearing loss (299). OtoSCOPE has been widely used for numerous studies. The diagnostic utility of this gene panel was notably demonstrated in the study of one of the largest multi-ethnic cohorts of patients with sensorineural hearing loss to date. Sloan-Heggen et. al. (2016) reported a genetic diagnosis in 39% of 1119 patients undergoing targeted NGS-gene panel testing using OtoSCOPE versions 4 and 5, which included 66 and 89 hearing loss genes respectively. Diagnoses were reported in 49 hearing loss genes, where *GJB2*, *STRC*, *SLC26A4*, *TMC1*, *GPR98*, *CDH23*, *USH2A*, *MYO15A*, *MYO7A* accounted for over 70% of the diagnoses. The up-to-date version of OtoSCOPE (version 8) currently includes 146 genes implicated in non-syndromic and syndromic hearing loss.

Table 1.6 List of targeted next-generation sequencing gene panels for hearing loss available worldwide.

Name of gene panel	Laboratory or academic affiliation	Number of genes in the panel	Additional information
OtoSCOPE	The University of Iowa| Molecular Otolaryngology and Renal Laboratory, USA	Version 8 152 genes, syndromic and non-syndromic.	Includes read depth analysis for CNV detection
Hearing loss	NHS Genomic Medicine Service, UK	117 diagnostic-grade genes	Includes genes associated with congenital hearing impairment and autosomal dominant deafness
OtoSeq	Cincinnati Children's Hospital Medical Center, USA	23 genes, non-syndromic and syndromic	------
OtoGenome	Harvard Medical School and Partners Healthcare, USA	110 genes, non-syndromic and syndromic.	Includes CNV analysis of *STRC* gene via droplet digital PCR
Otogenetics Comprehensive Deafness Gene Panel, Gx™ DA	Otogenetics | Emory University School of Medicine, USA	167 genes, syndromic and non-syndromic HL genes	Syndromic and non-syndromic HL genes https://www.otogenetics.com/products/clinical-genetic-testing/hearing-loss/
Hearing loss, nonsyndromic, autosomal recessive and X-linked	CeGat, Tübingen, Germany	71 genes	------
Hearing loss, nonsyndromic, autosomal dominant and X-linked	CeGat, Tübingen, Germany	39 genes	------
Syndromic hearing loss	CeGat, Tübingen, Germany	80 genes	------
Comprehensive Hearing Loss and Deafness Panel	Blueprint Genetics	239 genes, syndromic and non-syndromic	Includes CNV analysis
Deafness and hearing loss panel (autosomal dominant and recessive)	LifeLabs Genetics, Canada.	141 genes	------
Hereditary deafness ("*Sordera hereditaria*")	Instituto de Estudios Celulares y Moleculares, Spain	127 genes, non-syndromic and syndromic	------

Name of gene panel	Laboratory or academic affiliation	Number of genes in the panel	Additional information
Deafness panel ("Panel de sordera")	Bioarray, Spain	63 genes, non-syndromic and syndromic.	------

CNV- copy number variant; HL hearing loss

Within a range of 10-80%, the average diagnostic rate using targeted NGS gene panels is 40%.(282,297). It has been suggested that differences in gene panel content, cohort size and patient-specific characteristics such as inheritance, consanguinity, pre-screening as well as severity and onset of hearing loss may account for the variability in such diagnostic rates (282,284). Nonetheless, some of these studies have clearly demonstrated the robustness of performing targeted NGS based gene testing in individuals with hearing loss. Miyagawa et al. (2013) reported a genetic diagnosis rate of 31% in a 216-patient study utilising a 112-hearing loss gene panel (300). This NGS-based targeted approach was further used by Nishio and Usami (2015), reporting a genetic diagnostic rate of 40% after evaluating in 1120 Japanese patients with non-syndromic SNHL. The study revealed the diversity of genetic hearing loss in Japanese population, the majority of which was associated with genes such as *SLC26A4, USH2A, GPR98, MYO15A, COL4A5, CDH23, COL11A2, MYO7A, and OTOF, TECTA, MYH14*, and *WFS1* (283). The comprehensive analysis using targeted gene panels has also shown an increase in the number of aetiological diagnoses confirmed beyond common aetiologies such as *GJB2* or *SLC26A4.* Cabanillas et al (2018) obtained a 42% diagnostic rate in 50 patients undergoing testing using a 199-gene panel, where aetiologies such as *GJB2*, *OTOF* and *MT-RNR1* had been excluded (301).

1.8.2 Whole exome sequencing

Whole exome sequencing (WES or ES) is a method that sequences the whole protein coding region of the genome, the exome. The exome comprises only **~1-2% of the entire genome** but includes approximately 20,000 genes (302). *W*ES has been a valuable tool for gene discovery of rare monogenic disorders (303), as more than 80% of the variation associated with Mendelian disease is estimated to have a damaging impact in protein sequences (304). WES has facilitated the identification of genes for disorders where no loci had been previously identified (305). The accelerated expansion in gene identification and detection of most types of genomic variation reported in studies using WES has also contributed to the re-evaluation of phenotypes and correction of clinical diagnosis guided by molecular diagnosis ("reverse phenotyping") (305,306). WES has played a key role in the identification of *de novo* coding variants by facilitating trio analyses and sequencing of samples of multiple unrelated affected individuals (304,305). Large projects such as FORGE Canada (307), the Deciphering Developmental Disorders (DDD) in the UK (308) and the Centers for Mendelian Genomics (CMG) and Undiagnosed Diseases Network (UDN) initiatives in the USA (309,310) are examples of world-wide collaborative research initiatives that have used exome sequencing technology in the investigation of rare monogenic disorders (302).

1.8.3 Application of exome sequencing for diagnostics

Whole exome sequencing (WES) has gradually made the transition from research to diagnostics (290). The use of WES has been mostly indicated in patients who have undergone lengthy exhaustive laboratory testing ('the diagnostic odyssey') and still remain undiagnosed.

WES can be performed under a hypothesis-free analysis by covering the entire exome, but it also can be more clinically oriented under a phenotype-driven hypothesis. Some clinical laboratories have adopted a more affordable approach, clinical exome sequencing (CES), where only known genes associated with Mendelian disease are sequenced and analysed. The number of genes in a CES assay may range from 2,000 to 4,000, depending on the selected enrichment protocol (292). While CES protocols include only known coding genes associated with disease, CES still offers a comprehensive genomic testing option where both hypothesis-free and phenotype-driven analyses can be performed. Genes may be selected or prioritised based on their relevance to the patient's phenotype. This can be achieved in the form of virtual computational gene lists or exome "slices" (311,312). These gene lists may be curated in similar manner to the curation of genes for targeted NGS panels. An analytical advantage is that while targeted NGS gene panels are limited to the genes included in the original design, virtual gene lists in exome sequencing can be expanded or modified without incurring in panel design costs (313). Furthermore, CES data can be stored and be made readily available for reanalysis if deemed appropriate; for instance, in case no molecular diagnosis is confirmed during the initial analysis (291). As such, it provides an option to have DNA sequencing data with lifetime value from which the patient can potentially benefit at different points in life (314,315).

CES has become increasingly used particularly in the investigation of neurodevelopmental disorders, multiple congenital anomalies, congenital cardiovascular disease, growth abnormalities and /or dysmorphism (316,317). WES-based studies have demonstrated diagnostic utility in multiplex families (290) and for patients with conditions where sequencing a small number of genes is not cost-effective (e.g. intellectual disability, hearing loss) (Figure 1.13) (316). The majority of large-scale CES studies in adult and paediatric populations have reported molecular diagnoses in 20 to 30% of patients (318–323) (Table 7). Notably, there is an increased

likelihood of identifying a molecular diagnosis in patients with history of consanguinity and patients undergoing trio-based (parents and proband) CES analysis (324). Higher diagnostic rates (up to 50%) have also been reported in patients with neurological phenotypes, such as ataxia, epilepsy and profound developmental delay (325), sensory impairment (e.g. hearing disorders) (318,326); and skeletal abnormalities (327).

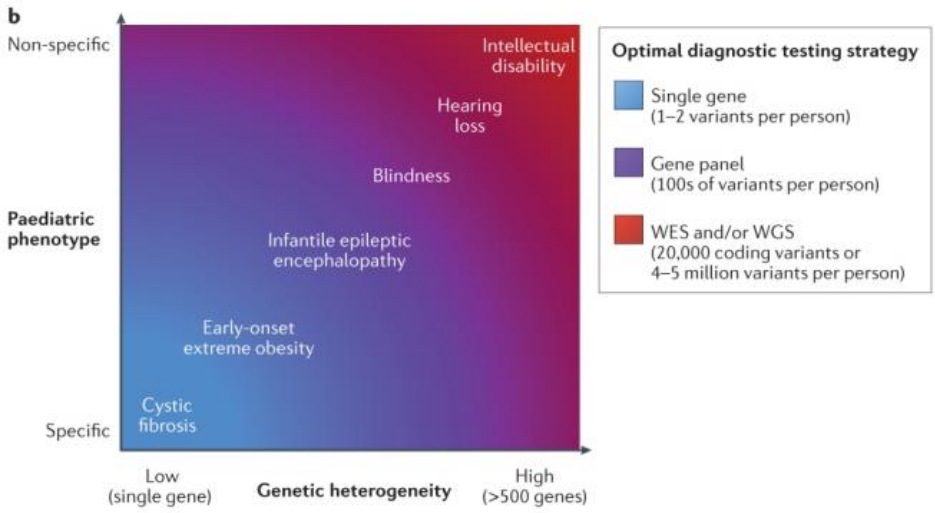


Figure 1.13 Schematic representation of the inverse relation between genetic heterogeneity and phenotype specificity, indicating NGS approaches that can suit the different degrees of genetic heterogeneity and clinical specificity. Figure adapted with permission from Springer Nature Customer Service Centre GmbH: Nature Reviews Genetics, (316).

Limiting disadvantages of WES/CES are the increased sequencing and data interpretation costs in comparison to targeted NGS gene panels. Increased number of genes for sequencing leads to increased volume of DNA sequenced data, which in turn can increase data storage needs and increase the number of variants filtered and retained for clinical interpretation. In addition, while CES analysis can be clinically oriented by focusing on genes relevant to a particular phenotype, it is recommended that laboratories report any findings in genes with possible identification of clinically actionable variants (**secondary findings**) (328–330). The increased number of retained variants and the increased likelihood of secondary findings can ultimately impact workload and costs of interpretation, especially for laboratories with limited resources. In addition, increased workloads may potentially delay turnaround times of results and any derived clinical decision.

Table 1.7 Summary of diagnostic rates and variants retained for interpretation of published studies on CES.

Author(s)/Year	Cohort size (number of cases)	Setting	CES strategy	Diagnostic rate	Retained variants	Reference
Lee H.,et al 2014	814 cases	Consecutive set of clinical cases, single university health system and outside referring physicians.	Trio and proband	Trio 31% Proband 22%	Range 0-50; both proband-CES and trio-CES	(320)
Posey J.,et al 2016	486 adult cases	Diagnostic WES at whole genome laboratory at Baylor College of Medicine	Proband only	17.50%	unspecified	(331)
Yang Y.,et al 2013	250 cases (80% paediatric)	Consecutive CES in accredited laboratory	Proband only	25%	Range 3-8 variants per case (including family members when available)	(321)
Yavarna T.,et al 2015	149 probands (74% with consanguinity)	Consecutive cases from Genetic Medicine clinic	Unspecified (likely trio based on methodology)	60%	unspecified	(324)
Taylor J.,et al 2019	76 cases	UK NHS tertiary referral hospital	Trio and proband	31.6%; Trio 34.9% Proband 22.2%	unspecified	(332)
Trujillano D.,et al 2017	2189 cases (82% trios)	Consecutive cases from 54 different countries, referred for diagnostic WES, accredited laboratory	Trio and proband	30.70%	unspecified	(318)
Hu X.,et al 2018	1323 patients (paediatric)	Cases referred for genetic testing at Shangai Children's Medical Center	Proband only	28.80%	unspecified	(333)
Iglesias A.,et al 2014	115 patients (79% paediatric)	Cases referred for WES with normal results from previous diagnostic	Unspecified	32.20%	unspecified	(322)

Author(s)/Year	Cohort size (number of cases)	Setting	CES strategy	Diagnostic rate	Retained variants	Reference
		evaluations; 4 laboratories, Ambry Genetics, GeneDx, Baylor College of Medicine and Columbia University.				
Retterer K.,et al 2016	3040 cases	Consecutive cases referred for CES, clinical diagnostic laboratory	Trio and proband	28.8.%, Trio 31% Proband 23.6%	Range 5-70	(327)
Maver A., et al 2016	405 cases	Retrospective study in cases referred for routine clinical diagnostic WES and Mendeliome.	Unspecified	39.20%	unspecified	(313)
Bergant G., et al 2018	1059 cases; paediatric and adult	Consecutive cases referred for diagnostic WES	Unspecified	38%-42%*	Average of 91 coding variants reviewed per case[(a)]	(334)
Stark Z.,et al 2017	80 cases	Single paediatric tertiary centre	Proband only	58%	107 variants per case for curation, range 70-168 variants.	(335)

CES – clinical exome sequencing

*Diagnostic rate when performing extensive analysis including copy number variant analysis, mitochondrial variant analysis and homozygosity mapping.

[(a)] without including copy number variant analysis, mitochondrial variant analysis and homozygosity mapping.

1.8.3.1 Use of exome sequencing in hearing loss

Whole exome sequencing has mainly played a pivotal role in understanding the genetic basis of hearing loss. Similar to the use of WES in rare Mendelian disorders, the clinical use of exome sequencing as a diagnostic tool for hearing loss has become a focus of research for the past three years. Key studies have reported diagnostic utility with identification of molecular diagnoses in 30 to 56% of patients (326,336–338). Higher diagnostic rates have been reported with concomitant use of other genetic testing methods. For instance, Downie et al 2020 reported one a diagnostic rate of 56%; where microarray analysis was concomitantly used along with CES and variants in *GJB2* accounted for 20% of their results (326). Higher diagnostic rates have also been observed in studies of multiplex families and autosomal recessive inheritance. Bademci et al (2016) identified a positive diagnosis in 56% of 160 negative-*GJB2* families with autosomal recessive non-syndromic hearing loss(339). When compared to targeted NGS gene panels, where gene panel content and clinical focus vary depending on the protocol and the laboratory, WES or CES-based testing for hearing loss offers the opportunity of providing comprehensive testing of genes through the selection of a single genomic test, where gene lists can be expanded without incurring in increased wet-bench costs. In the scenario of investigating a child with prelingual-onset, non-syndromic SNHL, selecting a CES-based approach would provide a cost-effective and comprehensive option, especially when considering the early clinical and genetic overlap between syndromic and non-syndromic SNHL conditions.

Whole and clinical exome sequencing in hearing loss have shown some limitations. For instance, despite that coverage of hearing loss genes is similar to coverage using targeted NGS gene panels (340), highly homologous sequences affect coverage of genes such as *STRC*, *OTOA* and *ESPN* during exome sequencing (337). This may limit the diagnostic utility of CES in patients whose hearing loss is caused by variants in any of these genes. Importantly, disease causing variants in *STRC* (particularly CNVs) are a recurrent genetic aetiology of SNHL in childhood (341). Nonetheless, similar exon-level coverage has been reported for targeted NGS gene panel and exome sequencing for the vast majority of known hearing loss genes (340). Use of supplemental genetic testing using microarrays or single-gene

sequencing for low-coverage genes can be used to ensure that variants in such genes are detected using more sensitive approaches (340).Since a considerable proportion of disease causing CNVs have been identified in genes such as *STRC* and *OTOA* in patients with SNHL (84), the use of microarray analysis for copy number variant detection can provide with an alternative to compensate the poor coverage of these regions (342) .

1.8.3 Next-generation sequencing for discovery of hearing loss genes

From the discovery of *GJB2* in the late 1990s until 2010, the vast majority of genetic research in hearing loss had relied on traditional gene identification methods and single gene testing. The adoption of NGS coupled with other gene identification methods, such as linkage analysis and homozygosity mapping, powered the discovery of genes associated with sensorineural hearing loss. Over 35 hearing loss genes have been identified since the adoption NGS-based approaches (Table 8). The majority of these have been reported in patients with non-syndromic hearing loss although a few genes associated with syndromic hearing loss have also been identified.

TPRN and *GPSM2* were the first two genes identified by incorporation of targeted NGS approaches. Rehman et al. (2010) performed targeted enrichment and massive parallel sequencing of 108 candidate genes mapped to an interval in chromosome 9 in a consanguineous family with segregation of prelingual, bilateral, severe non-syndromic SNHL. They identified the nonsense variant c.1056G>A, p.(W352Ter) in *TPRN.* Three pathogenic frameshift variants in *TPRN* were also identified in three other families in the same study. Rehman et al subsequently localised the *TPRN* product taperin in the taper region of inner hair cell stereocilia and supporting cells in mouse organ of Corti (343). Walsh et al. (2010) used exome sequencing and homozygosity mapping in a consanguineous family segregating prelingual, bilateral, severe to profound sensorineural hearing loss. By analysing within the linkage region in chromosome 1p13.3, they were able to identify the nonsense variant c.379C>T, p.(R127Ter) in the G protein signalling modulator 2 gene (*GPSM2*). The GPSM2 protein was localised in the apical surface of cochlear hair cells and pillar cells. In a later study by Doherty et al (2012), it was later

reported that one of the family members in the study by Walsh et al. also presented with brain abnormalities characteristic of findings associated with Chudley-McCullough syndrome (344).

Table 1.8 Hearing loss genes identified using NGS approaches.

Gene	Function	Phenotype	Study
ADCY1	Adenylate cyclase 1	AR, Prelingual (probably congenital), profound, mild-to-moderate mixed and SNHL, highly variable audiogram	(345)
BDP1	B double prime 1, subunit of RNA polymerase III transcription initiation factor IIIB	Prelingual, progressive, moderate-to-severe SNHL, down-sloping audiogram	(346)
*CABP2**	Calcium-binding protein 2	Prelingual, moderate-to-severe SNHL, flat or U-shaped audiogram	(347)
CEACAM16	Adhesion protein, interacts with tectorial membrane proteins	AD, sensorineural, late-onset, progressive HL	(348)
CLDN9	Tight junction protein claudin-9	AR, Moderate-to-profound SNHL	(349)
CLIC5	Chloride intracellular channel 5	AR, Prelingual, severe-to-profound, down-sloping audiogram, vestibular areflexia	(350)
COL4A6[(A)]	Collagen, type IV, alpha 6	Congenital, severe SNHL.	(351)
DCDC2	Doublecortin domain-containing protein 2	AR, isolated sensorineural HL	(352)
DMXL2	Rabconnectin-3α	AD, non-syndromic, severe-to-profound SNHL	(353)
ELMOD3	ELMO/CED12 domain-containing protein 3	Prelingual, severe-to-profound, mixed HL, variable audiogram	(354)
EPS8L2	Epidermal growth factor receptor Pathway Substrate 8 like 2, actin organization	AR, sensorineural, congenital or childhood-onset progressive hearing loss	(355,356)
ESRP1	Epithelial Splicing Regulatory Protein 1	AR, profound, bilateral, SNHL, rudimentary lateral semicircular canal	(357)
GPSM2	G protein signalling modulator 2, protein binding, mitotic spindle pole orientation	AR, sensorineural, prelingual, bilateral, severe to profound hearing loss	(358)
GRAP	Growth factor receptor-bound protein 2-related adaptor protein	AR, Congenital, profound, bilateral SNHL	(359)

Gene	Function	Phenotype	Study
*HARS2**	Mitochondrial histidyl-tRNA synthetase	Perrault syndrome	(360)
HOMER2	Intracellular Ca^{2+} homeostasis, cytoskeletal organization	AD, sensorineural hearing loss	(361)
HSD17B4	Hydroxysteroid 17-beta dehydrogenase 4 peroxisomal fatty acid beta-oxidation	Perrault syndrome	(362)
MCM2	Mini-chromosome maintenance protein, regulation of the cell cycle	AD, sensorineural, postlingual-onset, progressive, mild to profound hearing loss	(363)
MET	Mesenchymal epithelial transition factor,	AR, sensorineural, congenital-onset HL	(364)
MPZL2	Epithelial Junctional Protein Myelin Protein Zero-like 2	AR, childhood-onset, progressive, mild to moderate SNHL, more severe in high frequencies	(365)
NARS2	Mitochondrial asparaginyl-tRNA synthetase	AR non-syndromic sensorineural hearing loss, Leigh syndrome	(366)
OSBPL2	Oxysterol binding protein like 2, vesicle transport and lipid metabolism	AD, sensorineural, postlingual-onset, high frequency HL	(367)
OTOGL	Otogelin-Like Protein with presumed function in inner ear	AR, bilateral, congenital-onset, sensorineural, moderate HL	(368)
P2RX2	Regulation of excitatory postsynaptic responses in sensory neurons	AD, noise exposure exacerbated high frequency HL	(369)
PLS1	Fimbrin	AD, nonsyndromic, mid and high-frequency SNHL	(370)
PPIP5K2	Diphosphoinositol-pentakisphosphate kinase	AR, prelingual, profound SNHL	(371)
ROR1	Receptor tyrosine kinase-like orphan receptor 1, modulation of neurite growth	AR, sensorineural, congenital profound HL isolated and non-isolated with iris coloboma and pseudo cleft lip	(372)
S1PR2	Sphingosine-1-phosphate receptor 2, cell proliferation, survival, and transcriptional activation	AR, congenital-onset profound HL	(373)
SCD5	Stearoyl-CoA desaturase 5	AD, adult-onset, progressive SNHL	(374)
SLC12A2	Solute carrier family 12 (sodium/potassium/chloride transporter), member 2	AD, congenital, profound SNHL	(375)
SLC22A4	Solute career family 22, cation transporter of the stria vascularis endothelium	AR, sensorineural, bilateral, prelingual severe to profound HL	(376)

Gene	Function	Phenotype	Study
SMPX	Small muscle protein, likely function in inner ear morphogenesis and the IGF-1 pathway	X-linked sensorineural, postlingual, severe to profound progressive HL in affected males; moderate high frequency HL in female carriers	(377)
SPNS2	Sphingolipid transporter 2	Prelingual, moderate-to-severe SNHL	(378)
TBC1D24	TBC1 domain family, member 24	AD, adult-onset, progressive, down-sloping audiogram. AR, Prelingual, profound SNHL, flat audiogram. Other: -Epileptic encephalopathy, early infantile, 16. -DOORS syndrome -Myoclonic epilepsy, infantile, familial.	(379)
TMEM132E	Regulating the expression of certain ER stress-related genes in neuronal cells	AR, sensorineural, congenital-onset profound HL	(380)
TNC	Tenascin C	AD, postlingual, progressive, severe low-frequency SNHL, ascending audiogram	(381)
*TPRN**	Taperin, sensory epithelial protein	AR, sensorineural, prelingual, severe to profound HL featuring a down sloping or flat audiogram	(343)
WBP2	WW domain-binding protein-2	AR, prelingual, severe-to-profound SNHL	(382)

HL- hearing loss; AR- autosomal recessive; AD-autosomal dominant; All genes were identified using exome sequencing except those marked with *, which were discovered using NGS-based targeted enrichment.

(A) indicates gene identified using whole exome sequencing of chromosome X.

1.8.5 Whole genome sequencing

Whole genome sequencing (WGS) is a method that determines the sequence of most of the DNA present in an individual's genome, including coding and noncoding regions. Although it can be up to five more times more expensive than targeted NGS diagnostic gene panels and WES in terms of laboratory costs and data analysis, it represents the most comprehensive analysis of genomic variation (383). Since WGS does not require enrichment of regions of the genome, it offers an unbiased approach with more uniform coverage depth and genotype quality (384). WGS has facilitated the study of the role of structural variants (385) and noncoding elements in rare disease (272,386). The use of WGS has also led to an increase in diagnostic yield in the study of patients where other NGS approaches have not provided plausible diagnoses (385,387,388). WGS can identify variation often missed by NGS diagnostic gene panels or ES such as large structural variants, variants in regions with high or low GC content, deep intronic regions or regulatory regions (388). WGS can also be utilised for interrogation of the mitochondrial genome (383). The use of long-read sequencing techniques for WGS is increasing for improving the detection of structural variants in complex regions and to facilitate the analysis longer segments of the genome (383).

Whole genome sequencing (WGS) has recently started to be introduced in clinical settings for diagnostic purposes (319). The use of clinical genome sequencing (CGS) has been mostly documented in paediatric cohorts (389), more specifically utilised in patients with atypical, highly heterogeneous phenotypes and for the diagnosis of patients in paediatric and neonatal intensive care units (319,390). Diagnostic rates in large cohort studies have been reported around 20-30% (319,391). Higher diagnostic rates have been reported after using rapid genome sequencing in intensive care settings (390,392). The apparently limited diagnostic yield has been attributed to the fact that existing protocols for variants analysis focus primordially on coding variant regions, in similar to the exome analysis. It is expected that future utilisation of analysis tools and protocols in clinical diagnostic laboratories for allow interpretation of noncoding variants, deep intronic variants affecting slicing, this can lead to an increase in diagnostic yield when using GS (391). In addition, while some of these rates may be similar to clinical exome sequencing, clinical whole genome sequencing as shown to be a more

comprehensive tool for detection of different types of genomic variation. This attribute can be of diagnostic utility for detection chromosomal rearrangements that are not picked up by WES and that can also be missed by other testing like chromosomal microarrays (393).

Pioneering work from large-scale projects, such as Genomes Canada in Canada or the 100,000 Genomes Project in England (394), is establishing the initial framework for the implementation of whole genome sequencing as part of clinical routine care. The 100,000 Genomes Project was created to initiate hybrid diagnostic and research genomic sequencing within existing healthcare diagnostic pathways. This project focused initially on patients with cancer, infectious diseases and rare disease, where no genetic cause of disease was identified through previous genetic testing. Rare disease recruitment encompassed enrolling of patient with many monogenic, rare disease categories, including patients with hearing impairment and congenital ear anomalies– (395). It is expected that the 100,000 Genomes Project serves as a preamble for establishing a genomic medicine service (396). In some centres, the 100,000 is following up on previously established multidisciplinary genomic medicine projects, such as The WGS500 Consortium (397).

1.9 Cochlear implantation in the era of genomic medicine (Editorial Article)

Published in Cochlear Implants International. 2020

Taylor & Francis
Taylor & Francis Group

Cochlear Implants International
An Interdisciplinary Journal

ISSN: 1467-0100 (Print) 1754-7628 (Online) Journal homepage: https://www.tandfonline.com/loi/ycii20

Cochlear implantation in the era of genomic medicine

Leslie P. Molina-Ramirez, Iain A. Bruce & Graeme C. M. Black

To cite this article: Leslie P. Molina-Ramirez, Iain A. Bruce & Graeme C. M. Black (2020) Cochlear implantation in the era of genomic medicine, Cochlear Implants International, 21:2, 117-120, DOI: 10.1080/14670100.2019.1678895

To link to this article: https://doi.org/10.1080/14670100.2019.1678895

Commentary

Cochlear implantation in the era of genomic medicine

Leslie P. Molina-Ramirez[1,2], Iain A. Bruce[3,4], Graeme C. M. Black[1,2,5]

[a]Domain of Evolution, Systems and Genomics, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester M13 9PL, UK, [b]Manchester Centre for Genomic Medicine, St. Mary's Hospital, Manchester University NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester M13 9WL, UK, [c]Paediatric ENT Department, Royal Manchester Children's Hospital, Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, UK, [d]Division of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine and Health University of Manchester, Manchester, UK, [e]Manchester Royal Eye Hospital, Manchester University Hospitals NHS Foundation Trust, Manchester, UK

Hereditary hearing loss

Hereditary hearing loss is a sensory deficit characterised by a considerable genetic and clinical heterogeneity. At present, the estimated proportion of cases with a genetic cause ranges between 50 and 60% (Jayawardena *et al.*, 2015; Korver *et al.*, 2017). Traditionally, aetiological investigations in sensorineural hearing loss (SNHL) have been targeted at excluding known causal diagnoses, using a range of clinical (blood, urine, electrocardiography and ophthalmology) and radiological investigations. When non-genetic aetiologies have been eliminated, genetic testing has been available but focused in particular on the investigation of mutations in the connexin 26 gene, *GJB2*, a common cause of genetic hearing impairment worldwide (Snoeckx *et al.*, 2005). Guidelines in some countries have included evaluation by a clinical geneticist, in order to investigate other common genetic aetiologies such as mutations in the genes *SLC26A4 (encoding pendrin protein)* and the mitochondrial 1555A > G (predisposing to aminoglycoside-induced deafness) (Bitner-Glindzicz *et al.*, 2009; British Association of Audiovestibular Physicians, 2015; Mac Ardle and Bitner-Glindzicz, 2010). Despite such standard clinical and genetic testing, the cause of hearing impairment in more than half of cases still remains unexplained (Broomfield *et al.*, 2014; Petersen *et al.*, 2015) and most genetic causes remain unsought.

Correspondence to: L. P. Molina-Ramirez, Domain of Evolution, Systems and Genomics, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester M13 9PL, UK; Manchester Centre for Genomic Medicine, St. Mary's Hospital, Manchester University NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester M13 9WL, UK. Email: leslie.molinaramirez@postgrad.manchester.ac.uk

Genomic (DNA) sequencing

Due to decrease in costs and technological advances, next-generation genomic DNA sequencing (NGS) strategies can allow for simultaneous sequencing of: determined sets of disease-relevant genes (targeted NGS gene panels), the protein-coding part of the genome (whole exome sequencing, WES) or the entire genome (whole genome sequencing, WGS) (Figure 1). These strategies are now applied across many medical disciplines, continuously expanding the volume of genomic data (Metzker, 2010), shifting care strategies towards new avenues for personalised genomic-driven medicine. Through confirmation of genetic diagnosis, genomic medicine is transforming the cost-effectiveness of diagnostic and therapeutic journeys, steering further clinical diagnostic workup and management (Barwell *et al.*, 2018; Ellingford *et al.*, 2015).

The adoption of such sequencing strategies in inherited hearing impairment has expanded knowledge of molecular mechanisms underlying SNHL, through the discovery of new genes (Vona *et al.*, 2015) and the rapid identification of causative genomic variation (Shearer and Smith, 2015; Sloan-Heggen *et al.*, 2016). Today, there are over 100 genes (Van Camp and Smith, 2015) associated with inherited SNHL, with a wide range of types of genetic variation (Atik *et al.*, 2015; Azaiez *et al.*, 2018; Shearer *et al.*, 2014). The application of NGS for inherited SNHL remains largely applied in research settings (Korver *et al.*, 2017); however, it expands the range of options for clinical testing (Sloan-Heggen and Smith, 2016).

Genomic strategies allow an increased aetiological diagnostic rate for patients undergoing cochlear implantation. Confirmation of a genetic diagnosis

DOI 10.1080/14670100.2019.1678895

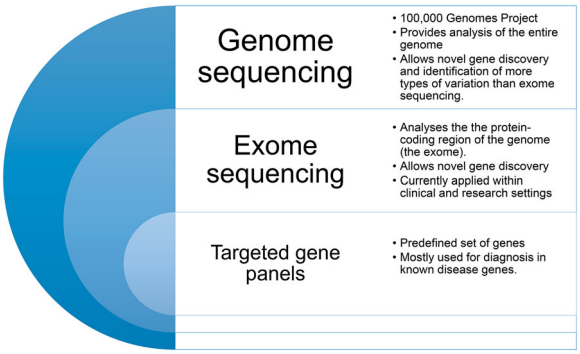


Figure 1 DNA next-generation sequencing approaches.

with consequent integration of genomic and clinical data can potentially enrich the wide spectrum of phenotype–genotype associations and allow the identification of points in the diagnostic pathway where genomic findings can be used to explore new alternatives for improving prognosis (e.g. prognostic modelling) and cost-effectively influence current practice.

Potential applications of genomic-driven medicine in hearing loss and cochlear implantation:

(1) *Rapid identification of aetiology in permanent SNHL*. It is accepted that traditional electrophysiological screening may fail to detect certain types of permanent childhood hearing impairment (PCHI), such as mild severity or cases with onset outside neonatal period. As such, it has been proposed that screening in early life through genomic sequencing could increase the detection rate when combined with existing universal newborn hearing screening (UNHS) programmes (Shearer *et al.*, 2019).

(2) *Timely prediction of the onset of a syndromic condition* through molecular diagnosis. Early confirmation of genetic diagnoses associated with additional disabilities, such as Usher syndrome, may prompt prioritised rehabilitation. Ensuring timely and optimal auditory rehabilitation is key for improving quality of life when facing additional disabilities (Damen *et al.*, 2006).

(3) *Predicting the clinical course of hearing loss (prognostication)*. Certain genetic aetiologies have been reported in association with progressive phenotypes and distinctive audiological phenotypes. A good proportion of these cases receive rehabilitation with hearing aids until these no longer offer benefit and they are ultimately assessed for CI (e.g. hearing loss associated with mutations in genes such as *LOXDH1* (Mori *et al.*, 2015), *SLC26A4* (Roh *et al.*, 2017), *USH2A* (Hartel *et al.*, 2017). Greater understanding of the likely mid- and long-term outcomes may allow clinicians and patients to determine personalised management plans, whilst removing some of the anxiety that accompanies uncertainty in clinical course.

Can molecular diagnosis offer personalised care for those undergoing CI?

Recent studies have started to provide some insight into other potential roles for molecular diagnosis in the assessment for cochlear implantation and (re)habilitation after surgery. It has been suggested that the identification of variants in genes with functional effects in certain parts of the auditory pathway may be used to predict speech outcomes (Eppsteiner *et al.*, 2012; Shearer *et al.*, 2017). For instance, mutations in *TMPRSS3* have been correlated with poorer speech outcomes in adult CI patients (Eppsteiner *et al.*, 2012; Shearer *et al.*, 2017). Nevertheless, while some functional evidence suggests a relationship between mutations in *TMPRSS3* and decreased protein function in the spiral ganglion (Shearer *et al.*, 2018), other studies report that satisfactory outcomes after CI and electroacoustic stimulation (EAS) can be expected (Miyagawa *et al.*, 2015). There has been some discussion with regards to other genetic aetiologies in CI cohorts and outcomes (Nishio and Usami, 2017; Wu *et al.*, n.d.). Good outcomes can be seen in the majority of individuals with confirmed genetic aetiologies undergoing CI; however, there remains a need to better understand the implications of identified genetic-phenotypic associations. Heterogeneity in the study population, outcome selection and outcome measure instruments risk bias when attempting to establish clear and reproducible phenotype-genotype hypotheses. Nonetheless, whilst the eventual outcome will continue to be influenced by multiple factors, the identification of any underlying molecular mechanism for the hearing loss can be potentially valuable in personalising aspects of the auditory rehabilitation process.

When inexorable progression in hearing loss is anticipated based on genotype, it is yet to be determined whether an 'early' or 'pre-emptive' CI with attempted hearing preservation is better functionally and emotionally than waiting until conventional hearing aids no longer provide benefit. Likewise, correlating genotype with other clinical, radiological or patient characteristics may provide important information about anticipated deterioration in preserved natural hearing after CI. For instance, several studies have explored the relationship between mutations in the gene *SLC26A4* and the presence of enlarged vestibular aqueducts (EVA) and cochlear dysplasia (King *et al.*, 2009). Thus far, there remains a lack of consistency in the association between the impact of the mutated protein, radiological findings and hearing loss severity (Chattaraj *et al.*, 2013; Liu *et al.*, 2016; Okamoto *et al.*, 2014; Zhao *et al.*, 2013), particularly in those cases where no mutations in *SLC26A4* are identified (Pryor *et al.*, 2005).

Table 1 Glossary of common terms in genomic medicine

Genomics	Study of all genetic material in an organism including all the genes and other genomic material that does not directly provide instructions for proteins.
Genome	Complete set of deoxyribonucleic acid (DNA) including all genes in an organism. More than 3.2 billion DNA base pairs are contained in the human genome. Around 98% of the genome is non-coding.
Reference genome	Representation of a 'normal' genetic sequence for a particular organism. Since the completion of The Human Genome Project in 2003, many versions have been curated by the Genome Reference Consortium [a].
Exome	Protein-coding portion of the genome that consists of exons, which are sequences that contribute to the final protein product of the gene. The exome constitutes less than 2% of the genome and is estimated to contain around 85% of disease-causing variants.
Gene	Region of DNA sequence that codes for a particular protein. There are an estimated 20,300 genes in the human genome
Phenotype	Measurable physical characteristics of an organism.
Variant	A difference between the DNA nucleotide sequence of two genomes or the reference genome. A variant may be disease-causing or not have any impact at all.

International collaboration between the Wellcome Sanger Institute (UK), the European Bioinformatics Institute, the National Centre for Biotechnology Information (NCBI) (USA) and The McDonnell Genome Institute at Washington University(USA).

Expanding the analysis using genomic sequencing could investigate other potential molecular genetic mechanisms involved in the development of cochleovestibular anomalies, which may have applications for electrode or device selection (Table 1).

Personalised genomic-driven assessment of cochlear implant candidates and greater understanding of relevant genotype-phenotype correlations could provide clinically useful diagnostic and prognostic information. Large-scale genomic medicine programmes such as *The 100,000 Genomes Project* (Caulfield *et al*., 2017), aim to provide information to support the future incorporation of genomic sequencing into routine patient care. The subsequent redesign and rationalisation of the diagnostic journey will likely translate into reduced healthcare costs through streamlining of diagnostics and selection of the most appropriate therapeutic interventions. Ultimately, genomic medicine may help to address many of the unanswered questions regarding *who to implant, how to implant, and when to implant*.

Acknowledgements

The principal author extends grateful thanks to Dr Jamie M. Ellingford, Health Education England Genomics Education Programme Postdoctoral Research Fellow, for his suggestions on the illustration and Table 1 of this editorial manuscript.

Funding

L. P. M. R is supported by the National Council of Science and Technology in Mexico (CONACYT).

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1.10 Current challenges

The increasing number of genes and gene-disease associations represents a challenge for diagnosis using single-gene sequencing. While disease-causing variants in *GJB2* are a major cause of genetic hearing loss in children, a considerable proportion of diagnoses associated with other hearing loss genes may be missed without the use of a more comprehensive approach.

NGS approaches allow massive parallel sequencing of multiple regions of the genome and therefore, it increases the probability of pinpointing an aetiological diagnosis. Despite the development in DNA sequencing technologies, the use of NGS approaches for diagnosis of hearing loss is still limited (398). Genetic evaluations, in particular comprehensive genetic testing, continue to being performed in late stages of the diagnostic journey, especially after many inconclusive laboratory tests(206). If a molecular diagnosis is confirmed, it may no longer inform therapeutic decisions. In addition, genetic investigations tend to be prioritised for patients with positive family history, clinical characteristics of syndromic conditions when, in fact, an important proportion of cases with genetic hearing loss are non-syndromic and have negative history of hearing loss in the family.

Despite the decreasing costs and enthusiastic attitude towards the use of NGS, significant challenges remain in regard to the adoption of NGS in the clinic (399). One major bottleneck pertains data management and interpretation (400). There is a need for identifying and sharing comprehensive, cost-effective methods that permit efficient variant analysis workflows. There is also a need for evaluating the use of NGS in current practice and the implications for diagnosis and clinical utility. Additionally, although there is emerging evidence on the use of molecular diagnosis for informing therapeutic decision-making in hearing loss (401), as more genotype-phenotype associations are identified, it is necessary to continue the research on their prospective utility in improving patient care and outcomes.

1.11 Hypothesis and research aims

This thesis will examine the hypothesis that **genomic sequencing can be an effective tool for improving diagnosis and management of genetic hearing loss.**

Based on the aforementioned current challenges, three key research aims are pursued:

(i): To evaluate variant prioritisation methods for clinical exome sequencing (CES) that improve cost-effectiveness in variant analysis in the clinical laboratory

The use of genomic sequencing strategies is rapidly increasing in clinical services. It is imperative to understand and evaluate how genomic sequencing strategies are currently applied for diagnosis of rare Mendelian disorders, evaluating their diagnostic utility, and identifying current strategies that can potentially further reduce costs of variant interpretation.

(ii): To evaluate the diagnostic utility of exome sequencing for genetic diagnosis of patients with hearing impairment.

Clinical exome sequencing is becoming a preferred diagnostic choice for the study of genetic hearing disorders. This second aim will focus on analysing the use of exome sequencing in a clinical context and evaluate: diagnostic rate, molecular results, and the clinical implications of undergoing CES in patients presenting with congenital or childhood-onset SNHL and/or congenital ear anomalies.

(iii): To integrate genomic and clinical data and analyse a proposed model of genotype-driven stratification for audiological surveillance

Many syndromic and non-syndromic SNHL conditions are indistinguishable during early childhood. Individuals with Usher syndrome (type I, type II and occasionally type III) present with SNHL of prelingual onset as the only clinical manifestation during the first decade of life. The clinical overlap with congenital and childhood

non-syndromic SNHL of genetic origin can lead to incorrect diagnoses and delay appropriate follow-up and rehabilitation.

This research aim focuses on a correlation of genomic and clinical data of patients with Usher syndrome type IIA and non-syndromic retinitis pigmentosa caused by variants in *USH2A*. A model has been formulated about the relationship between the presence of specific *USH2A* alleles and the variability of the presence of hearing loss in patients (402). Results from the genotype-phenotype correlation will be analysed under this model to determine whether it can provide prognostic information about hearing loss and potentially inform audiological surveillance for individuals with dual sensory impairment associated with *USH2A*-related disease.

Chapter 2. Personalised Virtual Gene Panels Reduce Interpretation Workload and Maintain Diagnostic Rates of Proband-Only Clinical Exome Sequencing for Rare Disorders

Published in J Med Genet. 2021

OPEN ACCESS

Original research

Personalised virtual gene panels reduce interpretation workload and maintain diagnostic rates of proband-only clinical exome sequencing for rare disorders

Leslie Patricia Molina-Ramírez ,[1,2] Claire Kyle,[2] Jamie M Ellingford ,[1,2] Ronnie Wright,[2] Algy Taylor,[2] Sanjeev S Bhaskar,[2] Christopher Campbell,[2] Harriet Jackson,[2] Adele Fairclough,[2] Abigail Rousseau,[2] George J Burghel ,[2] Laura Dutton,[2] Siddharth Banka ,[1,2] Tracy A Briggs,[1,2] Jill Clayton-Smith,[1,2] Sofia Douzgou ,[1,2] Elizabeth A Jones,[1,3] Helen M Kingston,[2] Bronwyn Kerr,[2] John Ealing,[3,4] Suresh Somarathi,[2] Kate E Chandler,[1,2] Helen M Stuart,[1,2] Emma MM Burkitt-Wright,[1,2] William G Newman,[1,2] Iain A Bruce,[5,6] Graeme C Black,[1,2] David Gokhale[2]

► Additional material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jmedgenet-2020-107303).

For numbered affiliations see end of article.

Correspondence to
Professor Graeme C Black; graeme.black@manchester.ac.uk

Received 29 June 2020
Revised 17 January 2021
Accepted 14 February 2021

To cite: Molina-Ramírez LP, Kyle C, Ellingford JM, *et al*. *J Med Genet* Epub ahead of print: [please include Day Month Year]. doi:10.1136/jmedgenet-2020-107303

ABSTRACT

Purpose The increased adoption of genomic strategies in the clinic makes it imperative for diagnostic laboratories to improve the efficiency of variant interpretation. Clinical exome sequencing (CES) is becoming a valuable diagnostic tool, capable of meeting the diagnostic demand imposed by the vast array of different rare monogenic disorders. We have assessed a clinician-led and phenotype-based approach for virtual gene panel generation for analysis of targeted CES in patients with rare disease in a single institution.

Methods Retrospective survey of 400 consecutive cases presumed by clinicians to have rare monogenic disorders, referred on singleton basis for targeted CES. We evaluated diagnostic yield and variant workload to characterise the usefulness of a clinician-led approach for generation of virtual gene panels that can incorporate up to three different phenotype-driven gene selection methods.

Results Abnormalities of the nervous system (54.5%), including intellectual disability, head and neck (19%), skeletal system (16%), ear (15%) and eye (15%) were the most common clinical features reported in referrals. Combined phenotype-driven strategies for virtual gene panel generation were used in 57% of cases. On average, 7.3 variants (median=5) per case were retained for clinical interpretation. The overall diagnostic rate of proband-only CES using personalised phenotype-driven virtual gene panels was 24%.

Conclusions Our results show that personalised virtual gene panels are a cost-effective approach for variant analysis of CES, maintaining diagnostic yield and optimising the use of resources for clinical genomic sequencing in the clinic.

INTRODUCTION

Exome (ES) and genome sequencing (GS) approaches are now commonplace in healthcare settings, enabling the identification and assessment of a broad spectrum of variants which may be causative of monogenic disorders. Clinical ES and GS strategies have demonstrated advantages over other diagnostic testing techniques, as they are capable of identifying previously undetected pathogenic variants, including those in genes not previously surveyed through custom gene panel or single gene approaches. Such findings can improve diagnostic yields and thereby guide appropriate patient management and therapeutic options. The diagnostic yield of clinical ES approaches is wide-ranging with reported rates between 20% and 50%,[1–9] impacted by cohort size and clinical characteristics. ES approaches have been shown to have reduced diagnostic yield compared with custom gene panel approaches dependent on patient recruitment criteria.[10]

The speed of variant interpretation remains an important challenge in the adoption of NGS as a clinical diagnostic test. NGS gene panels and, in particular, ES and GS generate large and complex volumes of data. The number of potentially pathogenic variants identified through ES and GS place a considerable burden on accredited medical genomic services in analysing and interpreting variants within a clinical setting, which may reduce accuracy and efficiency. The workload of interpretation is an important consideration when implementing genomic sequencing in the clinic. Cost-effective and accurate interpretation of genetic variants is fundamental to the widespread implementation of ES and GS in clinical settings, but currently, techniques to address this challenge in clinical contexts has been poorly assessed in current diagnostic practice.

In this study, we examined the use of a clinician-led and phenotype-driven approach to semi-automatic generation of personalised virtual gene panels. We evaluated the impact of this approach on diagnostic yield and interpretative workload, in the context of diagnosis of patients with presumed rare monogenic disease in an accredited clinical genomic medicine service.

METHODS

Cohort description and virtual gene panel generation

We conducted a retrospective survey of clinical exome variant results from patients with rare disease undergoing CES from October 2016 to January 2020. All CES data analyses and results described in this study were undertaken in a UK NHS Accreditation Service Clinical Pathology Accredited Medical Laboratory. Patients or guardians specifically consented for CES data analysis and data sharing as part of the diagnostic investigations and for results audit purposes. Written informed consent was obtained from patients or guardians explaining benefits and risks of CES testing.

Patients were referred by consultant clinical geneticists using a web-based referral system (WRS) to capture patient demographics, clinical features and/or Human Phenotype Ontology (HPO) terms to facilitate semi-automated generation of personalised virtual gene panels. Information entered into the WRS was used to create virtual gene panels using one or more of the following methods:

- Curated gene-disease panels: selection of curated gene lists from Genomics England PanelApp[11 12] and/or previous UK Genetic Testing Network panels.[13]
- HPO-based gene selection: selection of HPO terms[14] generates a list of candidate genes from OMIM[15] and Orphanet.[16]
- Customised selection of genes specified by clinicians, based on their clinical hypotheses.

HPO-based gene lists are created automatically on the WRS through a custom algorithm developed to use HPO terms intelligently, allowing additional terms entered to increase specificity of the panel rather than simply increasing in size (online supplemental methods figure S2). The inclusion of genes that are present on the American College of Medical Genetics and Genomics (ACMG) incidental findings list[17] was performed at the discretion of the clinician, with prior consent from the patient accordingly. Genes with low predicted coverage were flagged to the referrer during the submission process.

Targeted clinical exome sequencing

Sequencing, read mapping and variant calling

DNA was isolated from peripheral blood samples (n=393) and umbilical cord samples (n=7) and CES conducted using a custom-designed Agilent SureSelect XT Focused Exome capture library and the Illumina NextSeq 500 sequencing system with 75 bp paired end reads. Sequencing was conducted to a mean depth of 112 with >97% bases covered at 20× read depth. Reads generated by the Illumina NextSeq were aligned with BWA-MEM (V.0.6.2) to the human genome build GRCh37(hg19), with local realignment performed by ABRA (V.0.96). Variant calling was subsequently carried out using SamTools (V.0.1.18/gcc-4.4.6) for SNPs and small indels and Pindel (V.0.2.4.t) for indels >5 bp.

Variant prioritisation and pathogenicity evaluation

Sample-specific genome alignment (.BAM) and variant (.VCF) files were analysed using Golden Helix VarSeq software (V.1.4.4).[18] The VarSeq software interface allows users to customise configurable workflows for variant prioritisation. Annotation of variants was performed according to RefSeq: NCBI RefSeq Annotation Release 105[19] using the most clinically relevant GRCh37(hg19) transcript. The selection of the clinically relevant transcript by VarSeq is typically based on ACMG guidelines and ClinVar's algorithm for transcript selection. Only variants present within coding exons and +10 bp of the splice site junction were retained for analysis. Missense variants were analysed using a number of in silico predictors (eg, dbNSFP Function Predictions 3.0,[20] GHI (SIFT),[21] PolyPhen-2,[22] MutationTaster Mutation Assessor, FATHMM). Putative splicing variants were analysed using Alamut V.2.4.5, dbscSNV Splice Altering Predictions 1.1, GHI and SPIDEX.[23] Rare, high-quality, high-impact (genotype quality >20, read depth ≥10) single nucleotide variants and small indels with alternative allele frequency (AAF) <0.001 (gnomAD) were filtered. Variants listed as pathogenic or likely pathogenic on NCBI ClinVar were retained if present at 2% AAF. An example of our variant filtering is illustrated in online supplemental methods figure S1.

Filtered variants were analysed independently by two registered clinical scientists and classified according to ACMG guidelines[24] into one of five classes: (i) benign, (ii) likely benign, (iii) uncertain significance, (iv) likely pathogenic and (v) pathogenic. Variants were validated through Sanger sequencing. Segregation studies in parents of patients with possible compound heterozygous variants performed. Where needed, cases are reviewed at internal multidisciplinary team meetings or through internal communication between the clinical scientist and the clinical geneticist.

Retrospective evaluation of clinical characteristics and diagnostic rate of CES results

Clinical characteristics of the cohort were determined using the information available in their referrals (HPO terms, clinical descriptions). We used the phenotypic abnormality subontology of the HPO to classify the clinical characteristics of the cohort.

To determine the diagnostic rate, CES results were categorised as described in box 1. We analysed the diagnostic rate reported in relation to the main clinical referral indications for referral and the methods used for virtual gene panel generation.

Quantification of variant workload

We quantified the number of variants prioritised per sample. We then removed the virtual gene panel file and determined the number of variants obtained without the virtual gene panel and prioritising loss-of-function (LOF) and missense PP3 classified-variants (ACMG-AMP system[24]) . A comparison was made between variant workloads with and without use of virtual gene panels in order to determine the impact of using virtual gene panels in the clinical scientist's interpretative workload.

Statistical methods

Descriptive statistics (mean, median and SD) were used for phenotypic descriptions, virtual gene panel size and variant workload. One-sample binomial test was used to determine gender differences, χ^2 test was used for categorical variables where applicable, Spearman's rank correlation was used to determine the relationship between variant workload and total number of genes included in virtual gene panels. Statistical significance was denoted as $p<0.05$. R V.3.5 and IBM SPSS V.23 were used for analysis.

RESULTS

Clinical characteristics of referrals

A total of 400 patients with presumed rare disorders were referred as singletons from October 2016 to December 2019 for diagnostic targeted CES. A total of 273 cases were under age 18 years at the time of referral (69%, 273/394). Of these, 60% (164/273) were aged 5 years or less. Six referrals came from fetal samples. No significant difference in gender ratio was found

Box 1 Categorisation of CEs diagnosis

Diagnosis confirmed
In a clinically relevant gene, the presence of either:
1. A heterozygous class 4 or 5 variant in a dominant condition,
2. A homozygous/hemizygous class 4 or 5 variant in a recessive condition or
3. Two class 4 or 5 variants in the same gene in a recessive condition (potential compound heterozygote).

Diagnosis possibly confirmed
In a clinically relevant gene, the presence of either:
1. A homozygous/hemizygous class 3 variant* in a recessive condition,
2. A class 3 variant* and a class 4 or 5 variant in the same gene in a recessive condition (potential compound heterozygote) or
3. A heterozygous class 3 variant* in a dominant condition where parental studies suggest a possible de novo.

Diagnosis not confirmed
In a clinically relevant gene, the presence of either:
1. Any heterozygous class 3, 4 or 5 variant in a recessive condition,
2. A heterozygous class 3 variant* in a dominant condition where further parental testing has not been performed or
3. No plausibly causative variant identified.

*Report of variants of uncertain significance where further testing could be considered to reclassify the variant as likely pathogenic, as per the Association for Clinical Genomic Science Best Practice Guidelines for variant classification and interpretation in UK diagnostic genetic laboratories performing testing for rare disease. Variants considered to be causative of, or contributory to, the patient's clinical presentation were confirmed by Sanger sequencing prior to reporting.

(46.7% female vs 53.3% male; p=0.208, one sample binomial test).

Referred cases presented with a wide range of phenotypic characteristics. No significant difference was observed between the number of cases referred with one organ or system affected (50.5%, 202/400) in comparison to those presenting phenotypic abnormalities affecting two or more organs (49.5%, 198/400). Phenotypic abnormalities of the nervous system were present in more than half of the referrals (54.5%, n=218/400) (online supplemental figure 1). Of these, intellectual disability and/or developmental delay was present in 43.1% (94/218). Other major phenotypic abnormality categories were described by terms denoting conditions in head and neck (19%, 74/400), the skeletal system (16%, 65/400), ear (15%, 59/400), eye (15%, 58/400), and growth abnormalities (13%, 51/400).

Virtual gene panel generation

In more than half of the patients (57%, 226/400), virtual gene panels were generated by the clinical referrer combining gene selection methods. The use of curated panels (eg, PanelApp), either as a single method or in combination with one or more method(s), was the most common approach (73%, 291/400) followed by HPO-based gene lists (56%, 223/400) (online supplemental figure 2A). The combined selection of HPO-based gene lists and curated panels was mostly used in cases with two or more organs or systems affected. However, no significant difference was observed in the utilisation of a specific virtual gene panel method for the main clinical indications (ie, abnormalities in nervous system, ear, eye, head or neck, skeletal system and musculature abnormalities; χ^2 p=0.28,online supplemental figure 2B).

Virtual gene panels included a median of 107 genes (mean 233, range 1–1380, SD 296, 95% CI 204.30 to 262.49). Combining gene selection approaches generated larger virtual gene panels (n=226, median=165.5 genes) than those generated by one approach (n=174, median=64 genes, online supplemental figure 3A) (p<0.05, one sample Wilcoxon signed-rank test). When examining single methods, curated panels contained more genes in comparison to single HPO-based selection (p=0.0011, Wilcoxon signed-rank test). Virtual gene panels were significantly larger for cases with presence of more than two organs or systems affected (n=198, median 142 genes) in comparison to those with phenotypic abnormalities affecting only one single organ/system (p<0.05, Wilcoxon signed-rank test).

Impact of virtual gene panels on reduction of variant workload

The average number of filtered variants for interpretation per sample was 7.38 (median=5 variants), ranging from 0 to 61 variants (95% CI 6.58 to 8.18, online supplemental figure 3B). A significant correlation was observed between panel size and variant workload (r^2=0.76, p<0.05 Spearman's rank, online supplemental figure 4). We sought to compare the reported variant workload after using virtual gene panels with the variant volume produced by filtering LOF and missense with 4/6 in silico evidence of pathogenicity without phenotype-based targets. The latter prioritisation method led to an average of 45.3 variants, within a range between 18 and 125 (95% CI 44.01 to 46.68), showing a significant difference when compared with the variant workload obtained by using virtual gene panels (p<0.0001, unpaired t-test, figure 1).

Molecular results and diagnostic rate

A total of 180 variants across the 400 clinical exomes were identified and assigned ACMG classification scores[24] (online supplemental table 1). Pathogenic and likely pathogenic variants accounted for 62.2% of these (112/180). Sixty per cent were LOF (67/112) and 40% were missense (45/112). Only one variant was reported synonymous. Novel likely pathogenic and/or pathogenic variants at the time of original analysis accounted for 32.1% (36/112). Variants of uncertain significance accounted for 37.2% (68/180).

Overall, 24% of the patients received a confirmed and/or possibly confirmed molecular diagnosis (96/400). Seven additional cases (1.75%) had a genetic finding reported that confirmed only part of the phenotype—in one case with suspected digenic inheritance. The inclusion of these cases would increase the diagnostic rate to 26.75%. Findings associated with autosomal recessive inheritance were reported in 59.3% (58/96), autosomal dominant in 30% (32/96), X linked dominant in 5.2% (5/96) and X linked recessive inheritance in 1% (1/96). In two cases, likely pathogenic variants were identified in genes that have been associated with autosomal dominant and autosomal recessive inheritance. Single heterozygous variants in genes associated with autosomal recessive inheritance were identified in 2.5% of the cohort (10/400); these were reported in cases with presumed autosomal recessive conditions.

Variants of unknown significance identified in clinically relevant genes were considered for diagnosis in specific cases. Ten possibly confirmed cases were reported with a variant of unknown significance (VUS) in potential compound heterozygous state with a likely pathogenic or pathogenic variant. Other

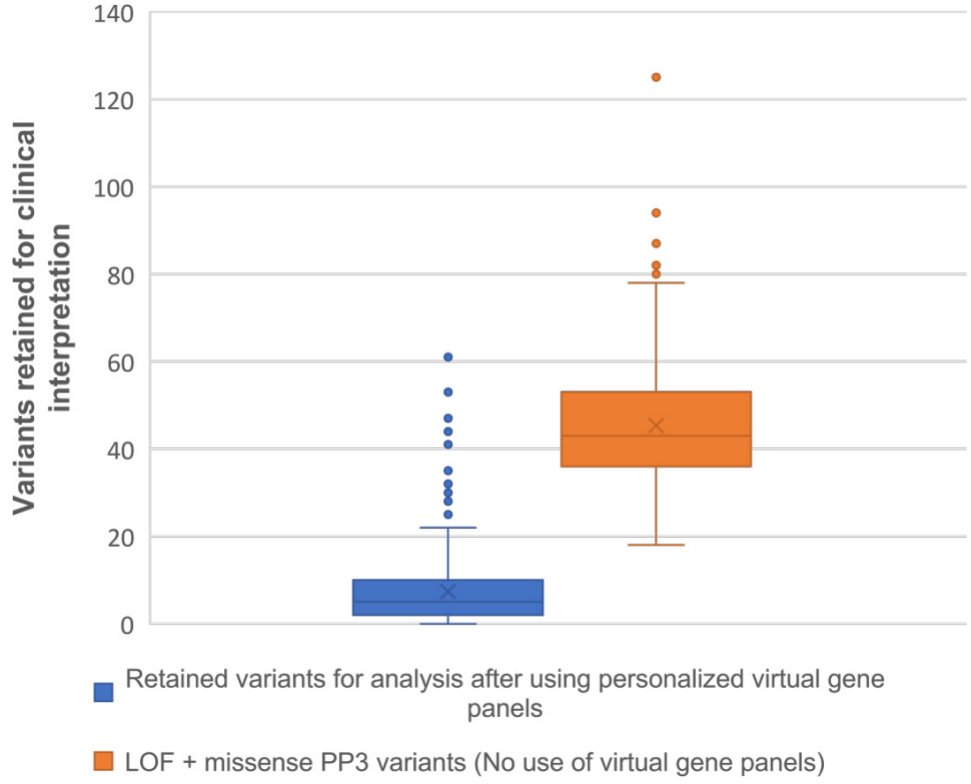

Figure 1 Comparison of variants retained for clinical interpretation between the use of personalised virtual gene panels and prioritisation of loss-of-function (LOF) and missense PP3 variants 20 without phenotype-based virtual gene panels.

nine possibly confirmed cases were reported with homozygous variants for disorders with autosomal recessive inheritance. Four cases were reported with confirmed de novo VUS in patients with autosomal dominant disorders. Cases with a reported heterozygous class 3 in an autosomal dominant condition where further parental testing has not been performed were not considered as plausible confirmation of diagnosis. No incidental findings were identified or reported, as expected by the gene selection approach.

The diagnostic rate for the most common phenotypic categories ranged between 21.5% and 32.7% (online supplemental figure 1B). No differences were observed in diagnostic yield when comparing the rate across the different methods for virtual gene panel generation (p=0.347) (online supplemental figure 5).

DISCUSSION

Identifying approaches to efficiently sift variants from ES and GS analyses for clinical interpretation can have considerable benefit in rationalising workstream flows in clinical diagnostic laboratories. Phenotype-driven approaches have become widely available tools for variant[25–27] and gene prioritisation[28 29] of genomic sequencing data. Therefore, incorporating detailed clinical phenotyping alongside CES and CGS offers an opportunity to develop personalised testing strategies for patients with rare disease through virtual gene panels.

Evidence has shown that CES using virtual gene panels can be an effective option for investigating individuals with rare Mendelian monogenic disorders.[29 30] In this study, we show that the availability of different phenotype-based approaches to gene selection can be beneficial for the design of personalised virtual gene panels. This is consistent with the increased sensitivity reported by Maver *et al* using phenotype-based virtual gene panels.[29] Notably, each virtual gene panel method is characterised by a set of features that can complement one another. Curated disease-gene panels (eg, PanelApp) are comprehensive, expertly curated evidence-based lists of genes. However, depending on the condition, they may contain genes that are irrelevant to the specific patient case. In this case, the sole or concomitant use of HPO-based gene selection may produce a more personalised selection that can be further improved as gene-disease associations increase over time.[31 32] Cases with atypical or unclear clinical diagnoses may certainly benefit from combined approaches. Similarly, clinical acumen can add sensitivity to panel design. Offering gene selection options based on different methods or algorithms facilitates the clinicians' choice of the most adequate approach for their patients and, if necessary, allows the combination of methods to increase the probability of inclusion of relevant genes in the panel.

We also show that the use of personalised virtual gene panels can increase the efficiency of clinical variant analysis strategies without compromising diagnostic yield. Our result is consistent with diagnostic rates reported to date for CES in clinical settings.[2 4 33 34] The diagnostic rates observed in the main five phenotypic categories (online supplemental figure 1) also highlight the utility of the singleton CES approach in the investigation of a breadth of frequent clinical indications for CES.[5 35] Furthermore, we expect that our diagnostic yield to be further improved following the introduction of parallel CNV analysis.[36] This addition would be particularly useful in cases where a heterozygous variant was detected in a phenotypically relevant recessive gene (3%, 10/400), where a second variant in trans is suspected beyond the detection of our current approach may be suspected.

Understanding the efficiency of variant analysis strategies is of paramount importance in clinical laboratories. A number of variant filtering and/or ranking strategies are available.[25 26 37]

Here, we show that variant analysis within a personalised gene selection is both a sensitive and more efficient technique to apply to CES datasets. This is evidenced by the reduced variant workload produced by using personalised virtual gene panels (median=5 variants) in comparison to the total variants retained after using the same filtering algorithm without virtual gene panels and prioritising LOF and missense variants with ACMG-criterion PP3 (median=43 variants). Our reported number of filtered variants per case (mean=7.38, median=5) is noticeably lower than the average reported in some studies that use phenotype-driven gene selection methods for ES analysis. Kernohan *et al* identified a minimum average variant burden ranging from 42 to 46 variants using Radboudumc and HPO-based panels in singleton-ES cases, respectively.[38] Bergant *et al* reported a total of 91 coding variants per case after initial ES analysis.[39] When searching for characteristics of our variant prioritisation workflow that could influence variant burden, we found that retaining very low frequency variants that occur in ≤0.1% of the population is an additional important factor for reduction of variant workload. Our findings suggest that the flexibility to choose the most adequate gene selection approach for virtual gene panel generation, in addition to filtering very low frequency variants, is an effective strategy that offers a deliverable variant analysis burden and maintains diagnostic efficacy in the clinical setting. Furthermore, more than half of the variants detected in our study were LOF and/or previously reported variants at the time of original analysis. It is possible to suggest that automated prioritisation of these variants could further expedite the variant sifting process. Future work incorporating variant zygosity and disease inheritance patterns, such as that developed by the Transforming Genetic Medicine Initiative[40] may further increase the sensitivity and efficiency of CES methodologies.

In summary, the utilisation of personalised virtual gene panels represents a sustainable approach for targeted clinical exome sequencing in patients with rare disease. It can reduce interpretative variant workload and preserve diagnostic yield and potentially maintain a deliverable timeframe for clinical laboratories. Importantly, the use of different phenotype-based strategies for gene selection plays a key role for optimal gene selection. In addition, semi-automated prioritisation of previously reported variants in addition to LOF variants could further expedite interpretative workload. These strategies altogether can potentially free up time for the investigation of more complex cases and to increase analysis throughput. The optimisation of approaches and resources for data analysis will allow a deeper adoption of genomic strategies as routine practice for personalised medicine in the clinic.

Author affiliations
[1]Division of Evolution and Genomic Sciences, School of Biological Sciences, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, UK
[2]North West Genomic Laboratory Hub, Manchester Centre for Genomic Medicine, Manchester University NHS Foundation Trust, Manchester, UK
[3]Manchester Centre for Genomic Medicine, Manchester University NHS Foundation Trust, Manchester, UK
[4]Department of Neurology, Salford Royal NHS Foundation Trust, Salford, UK
[5]Division of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, UK
[6]Paediatric ENT Department, Royal Manchester Children's Hospital, Manchester University NHS Foundation Trust, Manchester, UK

Twitter George J Burghel @BurghelG, Siddharth Banka @smbanka and Emma MM Burkitt-Wright @burkittwright

Acknowledgements We thank our patients, referring clinicians, bioinformatics and MCGM staff for their collaboration in this study.

Contributors Design and coordination of the study: LPMR, CK, JME, DG, GCMB. Data collection: LPMR, CK, AT. Analysis and interpretation: LPMR, CK, DG, GCMB. Bioinformatics and maintenance of bioinformatics pipeline: SSB. Virtual gene panel algorithm and development of clinical exome web referral system: AT. Contributing genetic and/or phenotypic data: CK, RW, SSB, CC, HJ, AF, AR, GeB, LD, SB, TAB, JCS, SD, EAJ, HK, BW, JE, SS, KC, HMS, EBW, WGN, GCMB. Writing of the manuscript: LPMR, CK, JME, DG and GCMB. All the authors revised the manuscript for important intellectual content and approved the final version.

Funding LPM-R is supported by the Consejo Nacional de Ciencia y Tecnología, México (CONACyT-392996). JME is funded by a postdoctoral research fellowship from the Health Education England Genomics Education Programme. The views expressed in this publication are those of the authors and not necessarily those of the Health Education England Genomics Education Programme. WGN is supported by the Manchester NIHR BRC (IS-BRC-1215-20007). GCB is supported by Wellcome Trust as part of the Transforming Genetic Medicine Initiative (TGMI).

Disclaimer The views expressed in this publication are those of the authors and not necessarily those of the Health Education England Genomics Education Programme.

Competing interests None declared.

Patient and public involvement statement This study was conducted as an audit of results and performance following changes to the standard procedures for the referral and analysis of the existing Clinical Exome service. Through the clinical exome informed consent process, patients consented to having their sample, genomic data and clinical information shared internally and with other researchers through scientific publications, controlled-access databases and open-access databases.

Patient consent for publication Not required.

Ethics approval This study was conducted as an audit of results and performance following changes to the standard procedures for the referral and analysis of the existing Clinical Exome service. Through the clinical exome informed consent process, patients consented to having their sample, genomic data and clinical information shared internally and with other researchers through scientific publications, controlled-access databases and open-access databases.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

ORCID iDs
Leslie Patricia Molina-Ramírez http://orcid.org/0000-0001-7573-6846
Jamie M Ellingford http://orcid.org/0000-0003-1137-9768
George J Burghel http://orcid.org/0000-0001-9360-8194
Siddharth Banka http://orcid.org/0000-0002-8527-2210
Sofia Douzgou http://orcid.org/0000-0001-8890-7544

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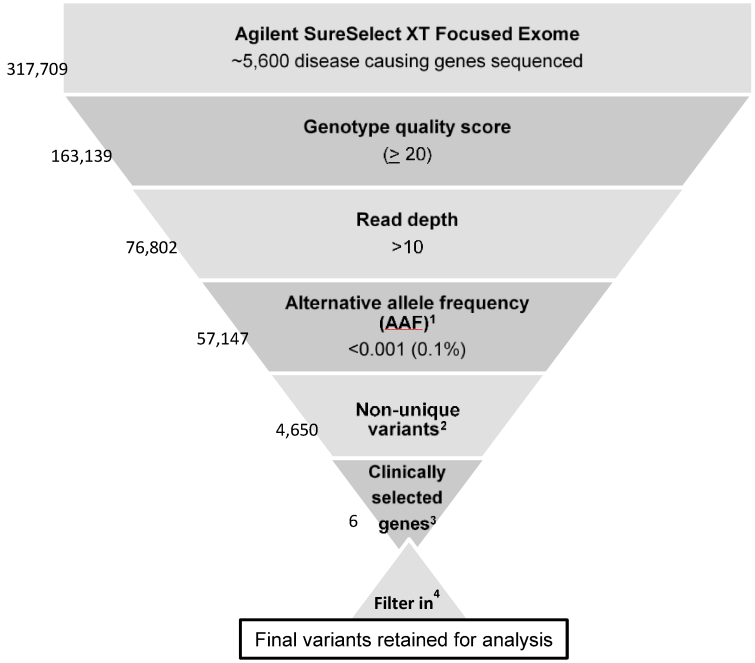
Personalised Virtual Gene Panels Reduce Interpretation Workload and Maintain Diagnostic Rates of Proband-Only Clinical Exome Sequencing for Rare Disorders.

Molina-Ramírez LP[1,2] , Kyle C[2], Ellingford JM[1,2], Wright R[2], Taylor A[2], Campbell C[2], Jackson H[2], Fairclough A[2] , Rousseau A[2], Burghel G[2], Dutton L[2], Banka S[1,2], Briggs TA[1,2], Clayton-Smith J[1,2], Douzgou S[1,2], Jones EA[1,2], Kingston H[2], Kerr B[2], Ealing J[2,3], Somarathi S[2], Chandler K[2], Stuart HM [1,2] , Burkitt-Wright E[1,2], Newman WG[1,2], Bruce IA[4,5], Black GC[1,2] (corresponding), Gokhale D[2].

1.-Division of Evolution and Genomic Sciences, School of Biological Sciences, Faculty of Biology, Medicines and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester, M13 9PL, UK
2.-NW Genomic Laboratory Hub, Manchester Centre for Genomic Medicine, St. Mary's Hospital, Manchester University NHS Foundation Trust, Oxford Road Manchester M13 9WL, UK
3.- Department of Neurology, Salford Royal Foundation Trust, Manchester Academic Health Sciences Centre (MAHSC), Manchester, UK
4.-Paediatric ENT Department, Royal Manchester Children's Hospital, Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre.
5.-Division of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine and Health University of Manchester, Manchester, UK.

Supplementary methods



Methods figure S1. Variant filtering and prioritisation strategy. Number of variants indicated on the left represents a common example of filtering for low frequency high impact variants. Number on the left indicates example of retained variants after each filter. 1 = gnomAD. 2 = non-unique variants defined as those present in other run samples (max 12 run samples). 3 = Virtual gene panels (Coding exons +/- 10bp of splice site junction). 4 = Variants listed as “Pathogenic” or “Likely pathogenic” on NCBI ClinVar with an AAF <2% are filtered in.

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$$D = \pi_{gene,} \\ \rho c_{COUNT(phenotype)}(P), \\ \rho p \frac{COUNT(phenotype)(P)}{COUNT(phenotype)C} \\ \sigma_{phenotype} P \in C \\ \gamma_{gene} P$$

$$S = \pi D, \\ \rho m_{MAX(c)}(D) \\ \sigma_{p>a} D, \\ \frac{c}{m} > r$$

$\sigma_{phenotype} P \in C$	Retrieve `(σ)` all genes that are linked to a phenotype `(phenotypeP)` that is in the list `(∈)` specified by the clinician `(C)`
$\gamma_{gene} P$	And for each `(γ)` gene `(geneP)` in the data
$D = \pi_{gene,}$	Get `(π)` the gene symbol `(gene)`
$\rho c_{(COUNT(phenotype)),}$	Get the total number of phenotypes `(COUNT(phenotype))` linked to the gene, and name it c `(ρc)`
$\rho p_{(COUNT(phenotype)}(P) / COUNT(phenotype)C$	Get the percentage of linked phenotypes `(COUNT(phenotype)(P))` relative to the number specified by the clinician `(COUNT(phenotype)C)` and call it p `(ρp)`

$S = \pi D,$	Get `(π)` all of the data we've calculated so far `(D)`
$\rho m_{max(c)}(D)$	Find the gene that's been linked to the largest number of phenotypes `(max(c)(D))`, and record how many phenotypes it's been linked to as "m" `(ρm)`
$\sigma_{p>a} D,$	Filter the list of linked genes `(σ),` where the number of phenotypes each gene is linked to, relative to the total specified by the clinician `(p)` is greater than the absolute threshold `(p > aD)`
$(c/m)>r$	And that the number of phenotypes it's linked to relative to the best-matched gene `(c / m)` is more than the relative threshold `(> r)`

Methods figure S2. Relational algebra formulas applied to the Human Phenotype Ontology (HPO) based gene selection for generating personalised virtual gene panels

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3.- Department of Neurology, Salford Royal Foundation Trust, Manchester Academic Health Sciences Centre (MAHSC), Manchester, UK
4.-Paediatric ENT Department, Royal Manchester Children's Hospital, Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre.
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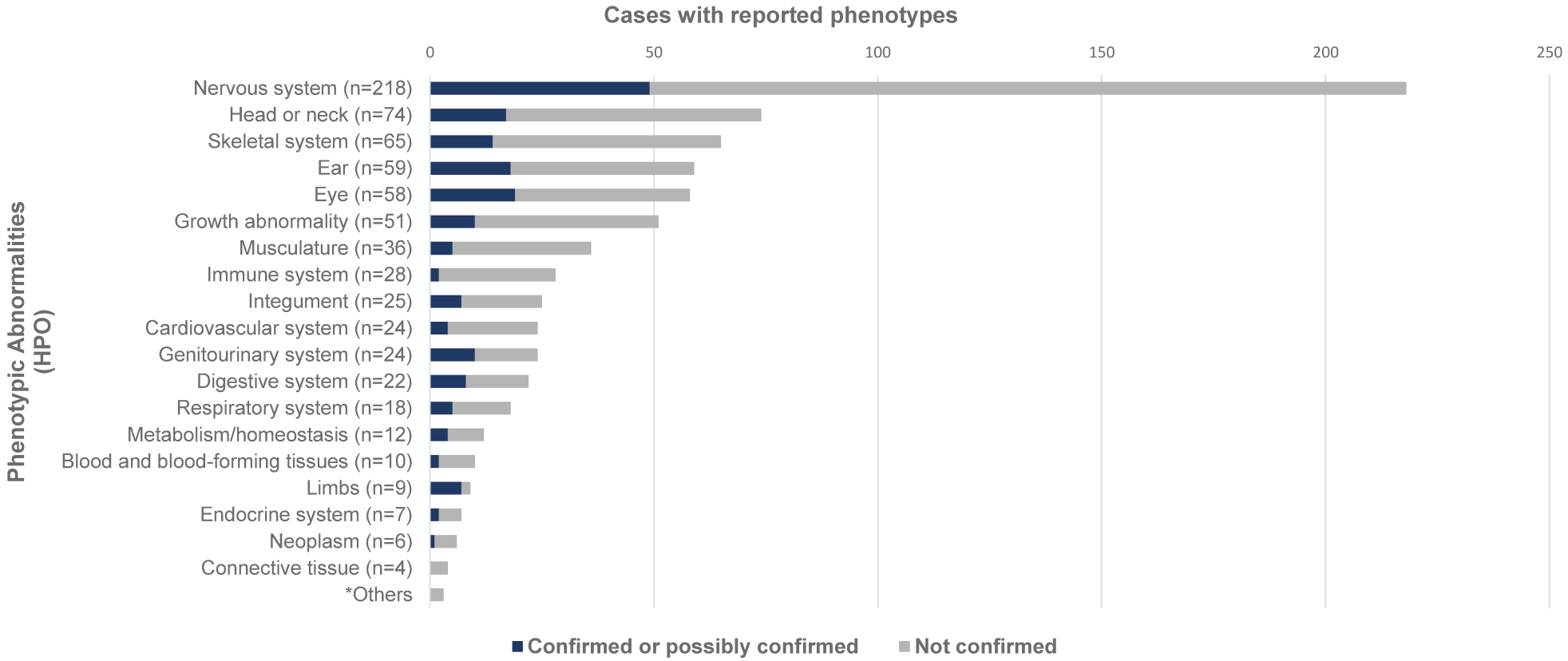
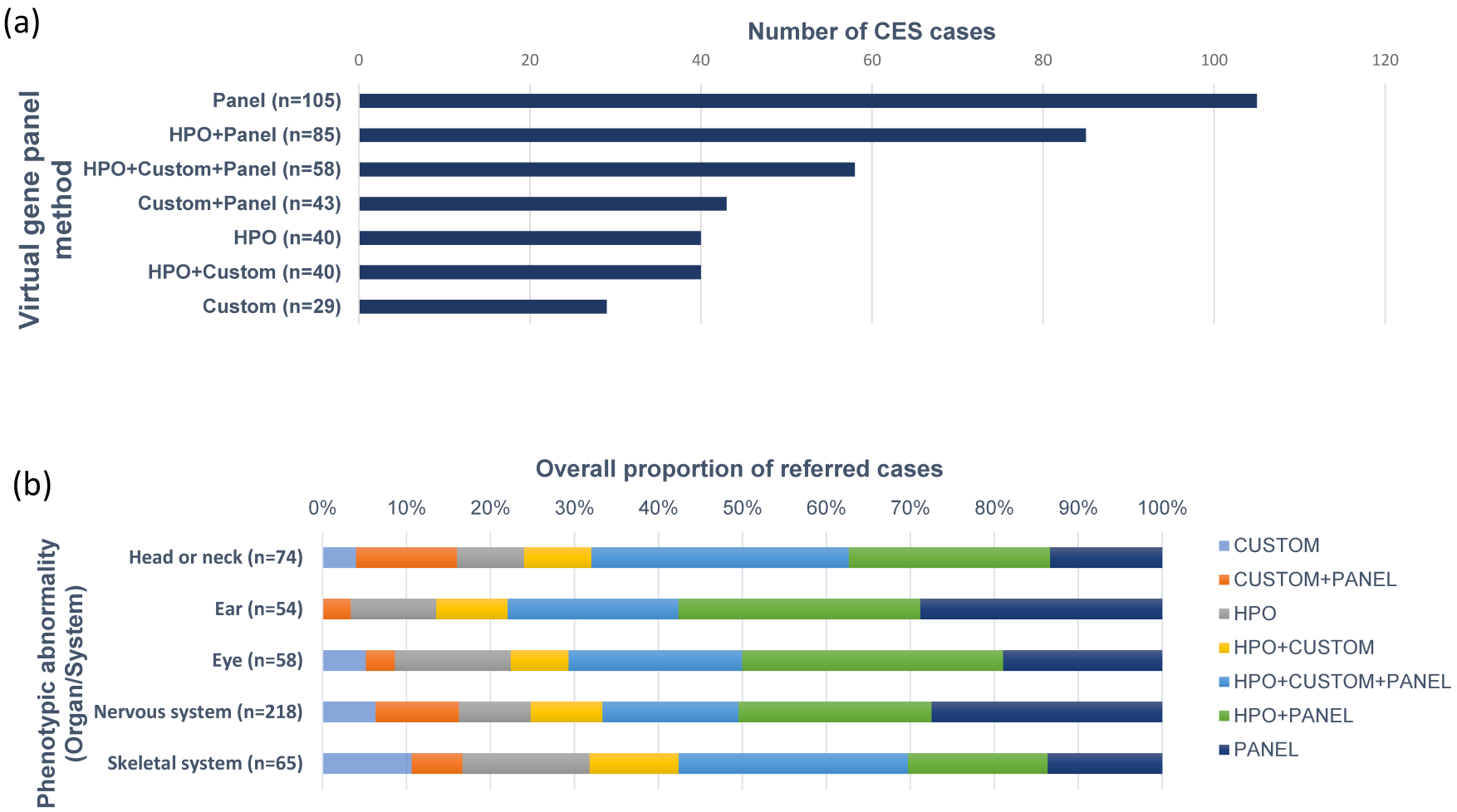
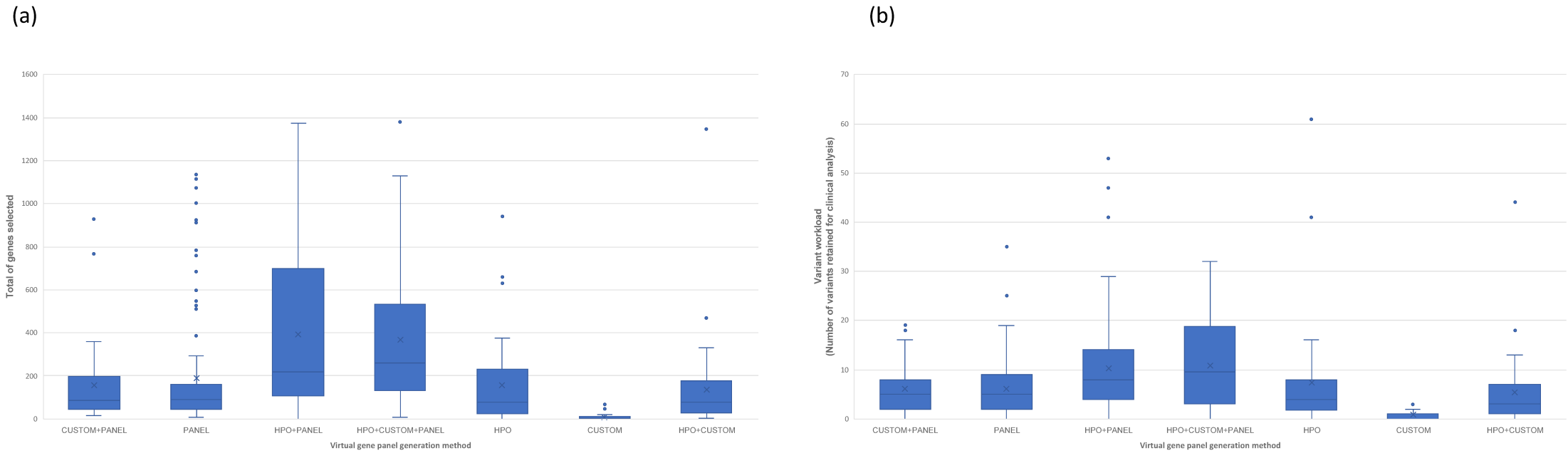
Supplementary figures



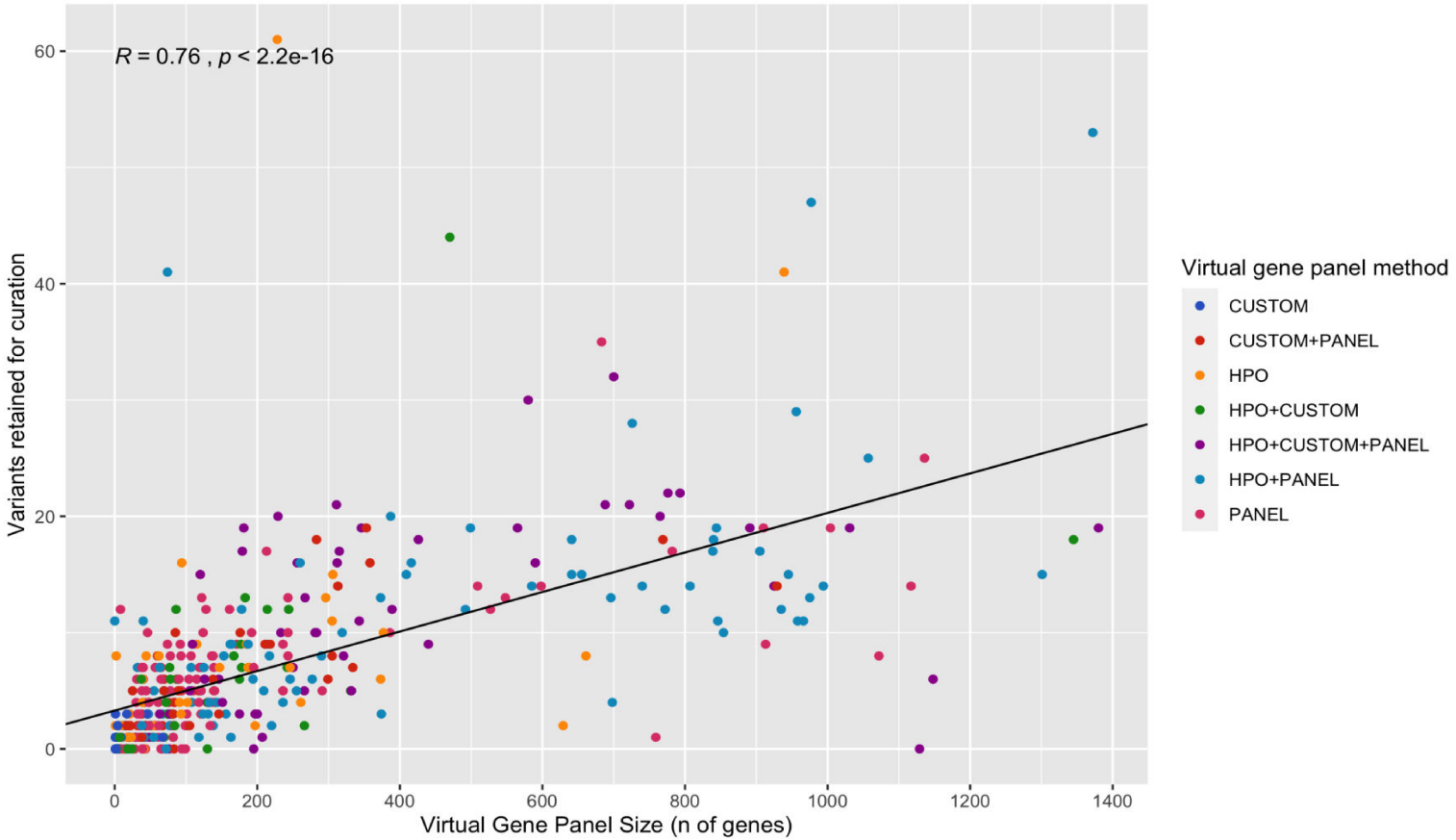
Supplementary Figure 1. Representation of phenotypic abnormalities reported in patients referred for targeted exome sequencing and proportion of cases where a molecular diagnosis was confirmed or possibly confirmed.



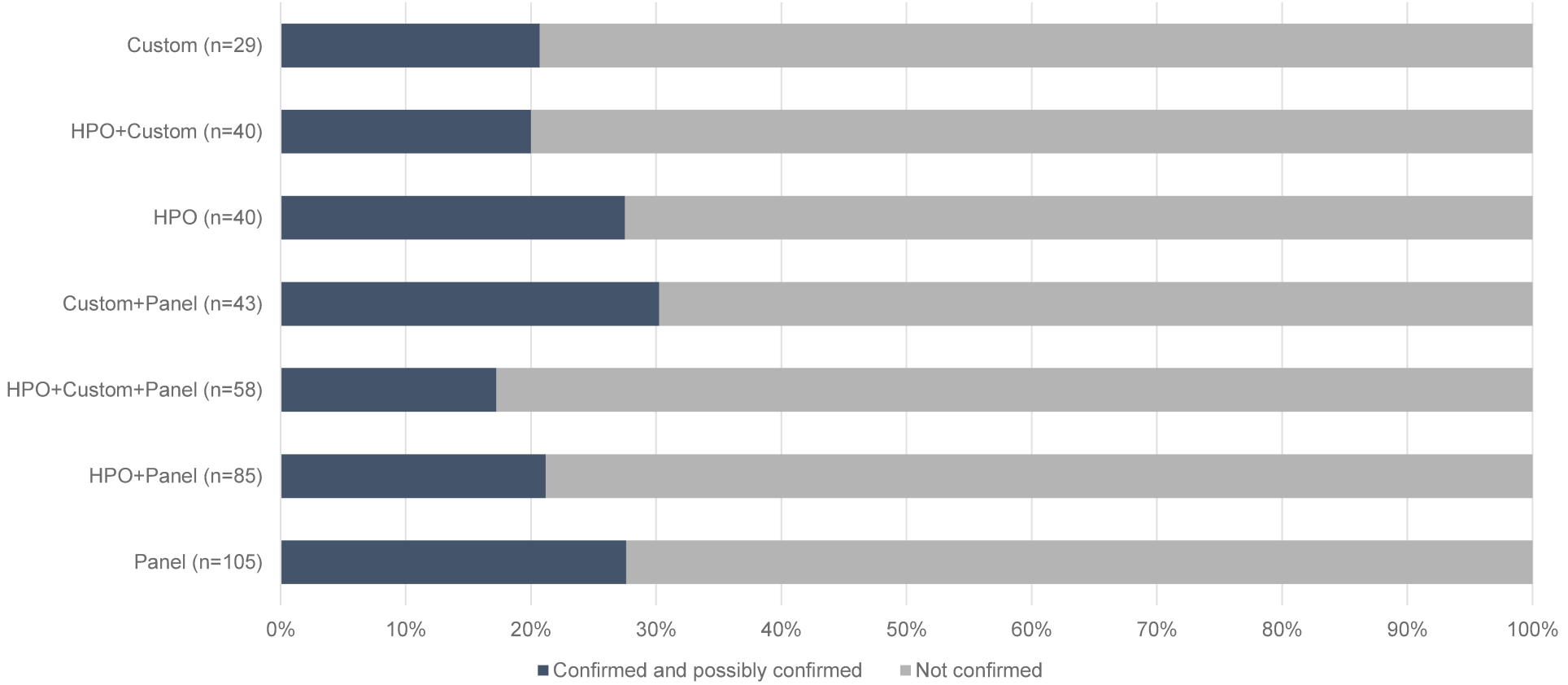
Supplementary Figure 2. (a) Total of cases per method(s) used for virtual gene panel generation. (b) Utilisation of method(s) for virtual gene panel generation across the main phenotypic categories.



Supplementary Figure 3. (a) Comparison of panel size and (b) variant workload across the different methods for virtual gene panel generation.



Supplementary Figure 4. Correlation between panel size and variants retained for clinical interpretation.



Supplementary Figure 5. Comparison of diagnostic rate among the different methods used for virtual gene panel generation. HPO- Human Phenotype Ontology

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE12015479	HP:0000316 - HYPERTELORISM HP:0001250 - SEIZURES HP:0001344 - ABSENT SPEECH HP:0010864 - INTELLECTUAL DISABILITY, SEVERE HP:0011081 - INCISOR MACRODONTIA HP:0012120 - METHYLMALONIC ACIDURIA	*ACSF3*	NM_174917.3	c.1075G>A	p.(Glu359Lys)	missense	Heterozygous	4	AR	NO	PMID: 21785126
				c.1672C>T	p.(Arg558Trp)	missense	Heterozygous	4		NO	PMID: 21841779
FE17012319	Late onset axonal neuropathy. Nerve conduction studies show axonal motor and sensory neuropathy.	*AMPD1*	NM_000036.2	c.133C>T	p.(Gln45Ter)	nonsense	Homozygous	3	AR	NO	PMID: 1631143 PMID: 14499869 PMID: 16021918 PMID: 21686757 PMID: 29095874 PMID: 10918252 PMID: 18855224 PMID: 23300193 PMID: 25525159
FE15007224	HP:0000252 - MICROCEPHALY HP:0000646 - AMBLYOPIA HP:0001263 - GLOBAL DEVELOPMENTAL DELAY	*ASPM*	NM 018136.4	c.7782_7783delGA	p.(Lys2595SerfsTer6)	frameshift	Homozygous	5	AR	NO	PMID: 19028728
FE17012524	HP:0002076 - MIGRAINE; HP:0040293 - RIGHT HEMIPLEGIA	*ATP1A2*	NM_000702.3	c.314G>A	p.(Gly105Glu)	missense	Heterozygous	3	AD	YES	_____
FE17012482	Changes suggestive of a macular dystrophy, central macular scars. Electroretinogram with no evidence of widespread retinopathy.	*BEST1*	NM_004183.3	c.97T>C	p.(Tyr33His)	missense	Compound heterozygous	4	AR	NO	PMID: 25489231
				c.152G>A	p.(Arg51Lys)	missense	Compound heterozygous	4		YES	_____
FE14001966	HP:0002721 - Oral ulcer HP:0004315 - IGG DEFICIENCY HP:0100633 - ESOPHAGITIS	*CARD11*	NM_032415.4	c.88C>G	p.(Arg30Gly)	missense	Heterozygous	4	AD,AR	YES	_____
FE11012584	Meckel-Gruber syndrome	*CEP290*	NM_025114.3	c.4825C>T	p.Gln1609Ter	nonsense	Homozygous	4	AR	YES	_____

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE16027107	HP:0000121 - NEPHROCALCINOSIS HP:0002150 - HYPERCALCIURIA HP:0002917 - HYPOMAGNESEMIA	*CLDN16*	NM_006580.3	c.445C>T	p.(Arg149Ter)	nonsense	Homozygous	5	AR	NO	PMID: 10390358 PMID: 25525159
FE16024957	Osteogenesis imperfecta	*COL1A2*	NM_000089.3	c.1378G>A	p.(Gly460Ser)	missense	Heterozygous	4	AD	NO	PMID: 8829649
FE16025631	Lethal skeletal dysplasia suggestive of osteogenesis imperfecta.	*COL1A2*	NM_000089.3	c.2845G>A	p.(Gly949Ser)	missense	Heterozygous	4	AD	NO	PMID: 8081394
FE16019022	HP:0000252 - MICROCEPHALY HP:0000518 - CATARACT HP:0000545 - MYOPIA HP:0002342 - INTELLECTUAL DISABILITY, MODERATE; HP:0003756 - SKELETAL MYOPATHY	*COL6A2*	NM_058174.2	c.2548_2549delCT	p.(Leu850AspfsTer48)	frameshift	Heterozygous	3	AR, AD	YES	_____
FE16017081	HP:0000006 - AUTOSOMAL DOMINANT HP:0001417 - X-LINKED HP:0003198 - MYOPATHY	*COL6A3*	NM_004369.3	c.3331G>A	p.(Ala1111Thr)	missense	Compound heterozygous	3	AR	NO	rs151021451
				c.1688A>G	p.(Asp563Gly)	missense	Compound heterozygous	3		YES	_____
FE15009211	HP:0000384 - PREAURICULAR SKIN TAG HP:0000639 - NYSTAGMUS HP:0000703 - DENTINOGENESIS IMPERFECTA HP:0000767 - PECTUS EXCAVATUM HP:0000846 - ADRENAL INSUFFICIENCY HP:0000953 - HYPERPIGMENTATION OF THE SKIN HP:0001010 - HYPOPIGMENTATION OF THE SKIN HP:0001510 - GROWTH DELAY HP:0001903 - ANEMIA HP:0007509 - PATCHY HYPO- AND HYPERPIGMENTATION HP:0012368 - FLAT FACE HP:0030021 - AURICULAR TAG	*COL7A1*	NM_000094.3	c.4867C>A	p.(Pro1623Thr)	missense	Compound heterozygous	4	AR	YES	_____
				c.4172dupC	p.(Gly1392ArgfsTer10)	frameshift	Compound heterozygous	3		NO	PMID: 10504458
FE17000123	HP:0000426 - PROMINENT NASAL BRIDGE HP:0001166 - ARACHNODACTYLY HP:0001762 - TALIPES EQUINOVARUS HP:0005684 - DISTAL ARTHROGRYPOSIS HP:0012745 - SHORT PALPEBRAL FISSURE	*DSE*	NM_013352.2	c.1763A>G	p.(His588Arg)	missense	Homozygous	3	AR	YES	_____

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE15019972	HP:0000175 - CLEFT PALATE HP:0000347 - MICROGNATHIA HP:0000973 - CUTIS LAXA HP:0001382 - JOINT HYPERMOBILITY HP:0002827 - HIP DISLOCATION HP:0007522 - INCREASED NUMBER OF SKIN FOLDS	*FKBP14*	NM_017946.3.	c.362dupC	p.(p.Glu122ArgfsTer7)	frameshift	Homozygous	5	AR	NO	PMID: 22265013 PMID: 27905128 PMID: 28617417 PMID: 24677762
FE15004412	Imperforate anus. Nerve conduction studies shows axonal motor and sensoty neuropathy	*GDAP1*	NM_018972.2	c.146T>C	p.(Leu49Ser)	missense	Homozygous	3	AR,AD	YES	_____
FE17007836	Mixed pattern of axonal degeneration and demyelination.	*GJB1*	NM_001097642.2	c.145T>C	p.(Ser49Pro)	missense	Hemizygous	4	XLD	NO	PMID: 12207932
FE16002937	HP:0000054 - MICROPENIS HP:0000175 - CLEFT PALATE HP:0000204 - CLEFT UPPER LIP HP:0000601 - HYPOTELORISM HP:0000871 - PANHYPOPITUITARISM	*GLI2*	NM_005270.4	c.922delT	p.(Ser308Glnfs)	frameshift	Heterozygous	4	AD	YES	_____
FE17003778	HP:0002342 - INTELLECTUAL DISABILITY, MODERATE HP:0007108 - DEMYELINATING PERIPHERAL NEUROPATHY	*HARS*	NM_002109.4	c.1190T>C	p.(Leu397Pro)	missense	Compound heterozygous	3	AR	YES	_____
				c.641G>A	p.(Arg214Gln)	missense	Compound heterozygous	3		YES	_____
FE16005088	HP:0000365 - HEARING IMPAIRMENT HP:0000635 - BLUE IRIDES HP:0001010 - HYPOPIGMENTATION OF THE SKIN HP:0001270 - MOTOR DELAY HP:0002211 - WHITE FORELOCK	*KITLG*	NM_000899.4	c.443T>C	p.(Ile148Thr)	missense	Homozygous	3	AR	NO	rs751013211
FE16010343	HP:0002415 - LEUKODYSTROPHY	*LAMA2*	NM_000426.3	c.1893_1897delCTTGA	p.(Asp631Glufs)	frameshift	Compound heterozygous	5	AR	NO	PMID: 11938437
				c.4307G>A	p.(Cys1436Tyr)	missense	Compound heterozygous	3		YES	_____
FE14009987	HP:0000609 - OPTIC NERVE HYPOPLASIA HP:0001332 - DYSTONIA HP:0006978 - DYSMYELINATING LEUKODYSTROPHY Profound bilateral SNHL	*LHFPL5*	NM 182548.3	c.395G>A	p.(Trp132Ter)	missense	Homozygous	4	AR	YES	_____

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE17015560	Hypertelorism, sclerocornea, sacral dimple with a small skin appendage. 3MC syndrome	*MASP1*	NM_139125.3	c.542G>A	p.(Cys181Tyr)	missense	Homozygous	4	AR	YES	_____
FE17012304	HP:0001629 - VENTRICULAR SEPTAL DEFECT HP:0001631 - ATRIAL SEPTAL DEFECT HP:0001719 - DOUBLE OUTLET RIGHT VENTRICLE HP:0006970 - PERIVENTRICULAR LEUKOMALACIA HP:0007229 - INTRACEREBRAL PERIVENTRICULAR CALCIFICATIONS	*MED12*	NM_005120.2	c.5941A>G	p.(Asn1981Asp)	missense	Hemizygous	3	XLR	YES	_____
FE17022800	Significant hypotonia, no spontaneous respiratory drive, paucity of movements, pleural effusions.	*MTM1*	NM_000252.2	c.1189dupT	p.(Tyr397LeufsTer2)	frameshift	Hemizygous	4	XLR	YES	_____
FE16024727	Congenital Microvillus Atrophy	*MYO5B*	NM_001080467.2	c.4739_4740delAC	p.(His1580LeufsTer3)	frameshift	Homozygous	4	AR	YES	_____
FE16008058	HP:0002652 - SKELETAL DYSPLASIA; HP:0004322 - SHORT STATURE	*MYO5B*	NM_001080467.2	c.673C>T	p.(Gln225Ter)	nonsense	Compound heterozygous	4	AR	YES	_____
				c.1355A>G	p.(Gln452Arg)	missense	Compound heterozygous	3		YES	_____
FE10011560	Polydactyly, short limbs, cleft lip, micrognathia, narrow thorax.	*NEK1*	NM_001199397.1	c.1014_1015delTA	p.(His338GlnfsTer22)	frameshift	Homozygous	4	AR	YES	_____
FE16022090	Multiple syndromes, nonrelated symphalangism spectrum disorder	*NOG*	NM_005450	c.124C>A	p.(Pro42Thr)	missense	Heterozygous	4	AD	NO	PMID: 23732071
FE15011757	HP:0004322 - SHORT STATURE	*OBSL1*	NM_015311.2	c.2100_2122del	p.(Gly701HisfsTer31)	frameshift	Homozygous	4	AR	YES	_____

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE16007307	HP:0000726 - DEMENTIA HP:0000727 - FRONTAL LOBE DEMENTIA HP:0001251 - ATAXIA HP:0002415 - LEUKODYSTROPHY	*PDGFB*	NM_002608.2	c.295A>G	p.(Thr99Ala)	missense	Heterozygous	3	AD	YES	_____
FE17012696	Large ventricles with cystic changes bilaterally, low signal thalami and basal ganglia, white matter abnormalities and small cerebellum.	*PDHA1*	NM_000284.3	c.999_1008+10del	p.(Glu334LysfsTer6)	frameshift	Heterozygous	4	XLD	YES	_____
FE17005848	HP:0000110 - RENAL DYSPLASIA HP:0000803 - RENAL CORTICAL CYSTS HP:0001562 - OLIGOHYDRAMNIOS	*PKHD1*	NM_138694.3	c.10315delG	p.(Asp3439MetfsTer6)	frameshift	Homozygous	5	AR	YES	_____
FE14022150	HP:0000256 - MACROCEPHALY HP:0001548 - OVERGROWTH	*PTCH1*	NM_000264.3	c.1067+1G>T	p.?	splicing	Heterozygous	5	AD	YES	_____
FE15024053	HP:0001251 - ATAXIA	*SACS*	NM_014363.5	c.10906C>T	p.(Arg3636Ter)	nonsense	Homozygous	4	AR	NO	PMID: 18465152
FE16021083	HP:0001263 - GLOBAL DEVELOPMENTAL DELAY HP:0001290 - GENERALIZED HYPOTONIA HP:0002789 - TACHYPNEA HP:0007308 - EXTRAPYRAMIDAL DYSKINESIA	*SLC16A2*	NM_006517.4	c.644C>T	p.(Pro215Leu)	missense	Hemizygous	4	XLD	NO	PMID: 27620904
FE16010798	Segmental, tremulous dystonia	*SLC20A2*	NM_006749.4	c.211C>T	p.(Arg71Cys)	missense	Heterozygous	3	AD	YES	_____
FE16019783	HP:0000776 - CONGENITAL DIAPHRAGMATIC HERNIA HP:0001338 - PARTIAL AGENESIS OF THE CORPUS CALLOSUM HP:0001511 - INTRAUTERINE GROWTH RETARDATION HP:0001643 - PATENT DUCTUS ARTERIOSUS HP:0002650 - SCOLIOSIS HP:0002937 - HEMIVERTEBRAE HP:0003808 - ABNORMAL MUSCLE TONE	*SMARCB1*	NM_003073.3	c.1129C>T	p.(Arg377Cys)	missense	Heterozygous	3	AD	YES	_____

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE10011847	HP:0001256 - INTELLECTUAL DISABILITY, MILD HP:0001332 - DYSTONIA	*SPG11*	NM 025137.3	c.3319_3322delTGTT	p.(Cys1107Ter)	nonsense	Homozygous	4	AR	YES	_____
FE16026183	HP:0001348 - BRISK REFLEXES HP:0002313 - SPASTIC PARAPARESIS	*SPTAN1*	NM_001130438.2	c.4737C>G	p.(Tyr1579Ter)	nonsense	Heterozygous	3	AD	YES	_____
FE16021580	Axonal neuropathy	*SPTLC1*	NM 001281303.1	c.859C>T	p.(Arg287Ter)	nonsense	Heterozygous	4	AD	YES	_____
FE16026525	HP:0008504 - MODERATE SENSORINEURAL HEARING IMPAIRMENT	*STRC*	NM_153700.2	c.4402C>T	p.(Arg1468Ter)	nonsense	Compound heterozygous	5	AR	NO	PMID: 26011646
				c.4517T>C	p.(Leu1506Pro)	missense	Compound heterozygous	3		YES	_____
FE16024864	HP:0000243 - TRIGONOCEPHALY HP:0004467 - PREAURICULAR PIT HP:0011218 - ABNORMAL SHAPE OF THE FRONTAL REGION	*TFAP2A*	NM_003220.2	c.746T>A	p.(Leu249Gln)	missense	Heterozygous	4	AD	YES	_____
FE16024987	HP:0001337 - AKINETIC RIGID HP:0003674 - ONSET	*TH*	NM_199292.2	c.440G>A	p.(Arg147Gln)	missense	Homozygous	3	AR	YES	_____
FE16010668	Meckel Gruber syndrome, multicystis dysplastic kidneys and posterior fossa abnormalities. Postmortem findings of meningocele, mild ventriculomegaly and some liver changes.	*TMEM67*	NM_153704.5	c.2522A>C	p.(Gln841Pro)	missense	Compound heterozygous	4	AR	NO	PMID: 19574260 PMID: 26092869 PMID: 28508964 PMID: 28497568 PMID: 27434533
				c.548C>A	p.(Ser183Tyr)	missense	Compound heterozygous	3		YES	_____
FE15010400	HP:0001251 - ATAXIA HP:0001270 - MOTOR DELAY HP:0002058 - MYOPATHIC FACIES	*TMEM67*	NM_153704.5	c.869+1G>C	p.?	splicing	Compound heterozygous	4	AR	YES	_____

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
	HP:0002419 - MOLAR TOOTH SIGN ON MRI HP:0011398 - CENTRAL HYPOTONIA			c.894T>G	p.(Phe298Leu)	missense	Compound heterozygous	3		YES	_____
FE16001356	HP:0000510 - ROD-CONE DYSTROPHY HP:0001513 - OBESITY HP:0001644-DILATED CARDIOMYOPATHY.	*ALMS1*	NM_015120.4	c.10769delC	p.(Thr3590Lysfs)	frameshift	Homozygous	5	AR	NO	PMID: 31810438
FE15001890	Dysplastic left cerebral hemisphere. Malformation of cortex and white matter. Absent corpus callosum and small cerebellum, interhemispheric cyst. Syndactyly left hand and toes. Cysts on tongue. Mild developmental delay.	*OFD1*	NM_003611.2	c.1411+1G>A het	p.?	splicing	Heterozygous	5	XLD	YES	_____
FE17027804	Ventriculomegaly, hypotonia, pachygyria and abnormal posterior fossa structures, small cerebellum and pons. Suspected dystroglycanopathy.	*POMT1*	NM_001080522.2	c.704G>T	p.(Gly235Val)	missense	Homozygous	3	AR	YES	_____
FE15011079	HP:0000175-CLEFT PALATE HP:0000280-COARSE FACIAL FEATURES	*CDKN1C*	NM_001362474	c.721C>T	p.(Gln241Ter)	nonsense	Heterozygous	4	AD	NO	PMID: 11414765
FE17012633	HP:0002804-ARTHROGRYPOSIS MULTIPLEX CONGENITA	*ECEL1*	NM_004826.2	c.1184+2T>A	p.?	splicing	Heterozygous	5	AR	YES	_____
				c.491T>C	p.(Leu164Pro)	missense	Heterozygous	3		YES	_____
FE17023866	Likely clinical diagnosis of Larsen syndrome.	*FLNB*	NM_001164317.1	c.535G>A	p.(Ala179Thr)	missense	Heterozygous	3	AD	YES	_____
FE17012318	Congenital myopathy and scoliosis	*SCN4A*	NM_000334.4	c.1342A>G	p.(Met448Val)	missense	Heterozygous	3	AD	YES	_____

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE17029030	Encephalopathic event with bilateral symmetrical basal ganglia signal change and brainstem haemorrhage/necrosis.	*RANBP2*	NM_006267.4.	c.1754C>T	p.(Thr585Met)	missense	Heterozygous	5	AD	NO	PMID: 19118815 PMID: 25522933
FE17013473	Nasal alae erythema, malar rash, laryngeal and supraglottic ulceration, two elevated interferon signatures. Normal development.	*PSTPIP1*	NM_003978.3	c.247C>T	p.(Gln83Ter)	nonsense	Heterozygous	3	AD	YES	_____
FE17030781	Hereditary Spastic Paraplegia	*SPAST*	NM_014946.3	c.1115G>C	p.(Arg372Thr)	missense	Heterozygous	5	AD	NO	_____
FE16000345	Early onset dementia, likely autosomal dominant.	*PSEN1*	NM_000021.3	c.737C>A	p.(Ala246Glu)	missense	Heterozygous	5	AD	NO	PMID: 7596406
FE17022955	Early onset epileptic encephalopathy, facial dysmorphisms, hypotonia and vacuolating leucodystrophy.	*ADSL*	NM_000026.2	c.1277G>A	p.(Arg426His)	missense	Heterozygous	5	AR	NO	PMID 10090474
				c.632T>A	p.(Leu211His)	missense	Heterozygous	3		YES	_____
FE18001063	Episodic ataxia. Probably type 2 with some chronic ataxia developing in later life. 3 affected individuals alive.	*CACNA1A*	NM_001127221.1	c.6047G>A	p.(Gly2016Glu)	missense	Heterozygous	3	AD	NO	rs16024
FE16026207	HP:0001251-CEREBELLAR ATAXIA	*AFG3L2*	NM_006796.2	c.2069G>A	p.(Ser690Asn)	missense	Heterozygous	3	AD	YES	_____
FE18002417	Clinical diagnosis of familial hemiplegic migraine	*ATP1A2*	NM_000702.3	c.2563G>A	p.(Gly855Arg)	missense	Heterozygous	4	AD	NO	PMID: 24921013

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE17027226	Severe demyelinating neuropathy. Presumed X linked CMT	*GJB1*	NM_000166.6	c.392T>C	p.(Leu131Pro)	missense	Hemizygous	4	XLD	NO	rs1555937166 PMID: 9818870 PMID: 27804109
FE17026201	Clinical diagnosis of primary ciliary dyskinesia.	*HYDIN*	NM_001270974.1.	c.10441C>T	p.(Arg3481Trp)	missense	Heterozygous	3	AR	NO	rs76078590
				c.9347C>G	p.(Thr3116Arg)	missense	Heterozygous	3		YES	_____
				c.6827A>G	p.(Gln2276Arg)	missense	Heterozygous	3		YES	_____
FE17017837	HP:0000252-MICROCEPHALY HP:0000518-CATARACT HP:0002197-GENERALIZED SEIZURES HP:0002342-INTELLECTUAL DISABILITY, MODERATE HP:0003508-PROPORTIONATE SHORT STATURE	*DYNC1H1*	NM 014946.3	c.7874C>G	p.(Ala2625Gly)	missense	Heterozygous	3	AD	YES	_____
FE17005729	Proximal muscle weakness. Query Limb Girdle muscular dystrophy	*ANO5*	NM_213599.2	c.692G>T	p.(Gly231Val)	missense	Compound heterozygous	4	AR	NO	PMID: 20096397 PMID: 23041008
				c.191dupA	p.(Asn64LysfsTer15)	frameshift	Compound heterozygous	5		NO	PMID: 23606453 PMID: 27708273
FE17014128	Joubert syndrome. Muscular hypotonia, Motor delay, Oculomotor apraxia. Metabolic screening revealed isolated Orotic aciduria, suggesting UMPS deficiency.	*UMPS*	NM_000373.3	c.928T>G	p.Phe310Val Hom	missense	Homozygous	3	AR	YES	_____
FE10005509	Profound generalised muscle weakness, severe cognitive impairment, inflammatory bowel disease, recurrent renal calculi.	*SLC5A7*	NM_021815.2	c.1240T>A	p.(Tyr4l4Asn)	missense	Homozygous	3	AR	YES	_____
FE17025791	End stage renal failure at about 6 months old. Appearance of small bright kidneys bilaterally (hypodysplasia). Previous likely unilateral hydronephrosis. Appearance of rhizomelia and brachydactyly. Cone shaped epiphysis. .Query Skeletal Ciliopathy	*WDR35*	NM_001006657.1	c.392G>T	p.(Cys131Phe)	missense	Homozygous	3	AR	YES	_____
FE17022845AV	Respiratory distress, hypoxia, required extensive resuscitation, multiple thromboses, acute kidney injury.	*WT1*	NM_024426.4	c.1096C>T	p.(Arg366Cys)	missense	Heterozygous	4	AD	NO	PMID: 9529364 doi.org/10.1002/ajmg.a.31924

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE18004080	Born with distal arthrogryposis. Normal IQ.	*PIEZO2*	NM 022068.2.	c.5951T>C	p.(Phe198Ser)	missense	Heterozygous	3	AD	NO	rs1408124992
FE17031083	Failure to thrive, hypotonia, global delay, microcephaly, telecanthus/epicanthic folds, long philtrum tented upper lip. High or undescended testes small genitalia, deep foot creases. Dystonia.	*GBE1*	NM_000158.4	c.721A>G	p.(Met241Val)	missense	Heterozygous	3	AR	NO	rs747155575
		GBE1	NM_000158.4	c.691+2T>C	p.?	splicing	Heterozygous	4		NO	rs192044702
FE15018880	HP:0000047-HYPOSPADIAS HP:0000316-HYPERTELORISM HP:0000358-POSTERIORLY ROTATED EARS HP:0002564-CONGENITAL HEART DISEASE HP:0008751-LARYNGEAL CLEFT	*MID1*	NM_000381.3	c.1286G>T	p.Ser429Ile	missense	Heterozygous	3	AD	YES	_____
FE18003789	Possible diagnosis of hereditary. Motor and sensory neuropathy. Pes cavu, nerve conduction study showed mixed motor and sensory axonal type polyneuropathy.	*MFN2*	NM_014874.3	c.838C>T	p.(Arg280Cys)	missense	Heterozygous	4	AD	YES	_____
FE18010010	HP:0002633-VASCULITIS HP:0002841-RECURRENT FUNGAL INFECTIONS HP:0010885-AVASCULAR NECROSIS	*TREX1*	NM_033629.4.	c.814delG	p.(Asp272IlefsTer5)	frameshift	Heterozygous	4	AD	YES	_____
FE14012423	HP:0000028-CRYPTORCHIDISM HP:0000187-BROAD ALVEOLAR RIDGES HP:0000252-MICROCEPHALY HP:0000586-SHALLOW ORBITS HP:0001272-CEREBELLAR ATROPHY HP:0002342-INTELLECTUAL DISABILITY, MODERATE HP:0003508-PROPORTIONATE SHORT STATURE HP:0008551-MICROTIA	*ORC6*	NM_014321.3	c.71C>T	p.Ala24Val	missense	Homozygous	3	AR	YES	_____
FE11700617	HP:0000347-MICROGNATHIA HP:0000494-DOWNSLANTED PALPEBRAL FISSURES HP:0000625-EYELID COLOBOMA HP:0000676-ABNORMALITY OF THE INCISOR HP:0011330-METOPIC SYNOSTOSIS HP:0040080-ANTEVERTED EARS HP:0200102-SPARSE OR ABSENT EYELASHES	*TCOF1*	NM_001135243.1	c.1444_1459dup	p.(Glu487ValfsTer4)	frameshift	Heterozygous	4	AD	YES	_____
FE16002792	Childhood onset, progressive sensorineural hearing loss, cochlear implant user. Family history of SNHL.	*TMPSSR3*	NM_024022.2	c.1276G>A	p.(Ala426Thr)	missense	Heterozygous	3	AR	NO	rs56264519 PMID: 21786053 PMID: 28566687
		TMPSSR3	NM_024022.2	c.208delC	p.(His70ThrfsTer19)	frameshift	Heterozygous	5	AR	NO	PMID 28566687 PMID 11907649 PMID 29293505

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE18013538	Possible Joubert syndrome. Hypotonia, disordered breathing, developmental delay, nystagmus and the appearance of a molar tooth sign on MRI scan.	*AHI1*	NM_017651.4	c.2296G>A	p.(Gly766Arg)	missense	Homozygous	4	AR	YES	_____
FE17024387	HP:0001188-HAND CLENCHING HP:0005484-POSTNATAL MICROCEPHALY HP:0007340-LOWER LIMB MUSCLE WEAKNESS HP:0008780-CONGENITAL BILATERAL HIP DISLOCATION HP:0010864-INTELLECTUAL DISABILITY, SEVERE	*CHD8*	NM_001170629.1	c.2493C>G	p.(Asn831Lys)	missense	Heterozygous	3		YES	_____
FE17020997	HP:0001093-OPTIC NERVE DYSPLASIA HP:0008619-BILATERAL SENSORINEURAL HEARING IMPAIRMENT	*CHD7*	NM_017780.3	c.3089A>G	p.(Asn1030Ser)	missense	Heterozygous	4	AD	NO	PMID 21041284 PMID 25077900
FE15011417	Multiple cavernous malformations, learning difficulties, previous absences, multiple naevi, multicystic dysplastic kidneys. Parental LD.	*PDCD10*	NM_145860.1.	c.496G>T	p.(Glu166Ter)	nonsense	Heterozygous	5	AD	NO	rs1559944602
FE16001689	Moderate sensorineural hearing loss.	*PDZD7*	NM_001195263.1.	c.1648C>T	p.(Gln550Ter)	nonsense	Homozygous	5	AR	NO	rs1554834161 PMID 26849169 PMID 20440071
FE9010089	HP:0005360-SUSCEPTIBILITY TO CHICKENPOX	*CTPS1*	NM_001905.3	c.1714_1724del	p.(Gly572ProfsTer2)	frameshift	Homozygous	3	AR	YES	_____
FE18010049	HP:0001040-MULTIPLE PTERYGIA	*CHRNA1*	NM_000079.3.	c.685C>T	p.(Arg229Cys)	missense	Homozygous	3	AR	NO	PMID 23037934
FE18001423	Global developmental delay Facial dysmorphic features ?Sotos syndrome	*C5orf42*	NM_023073.3.	c.98T>C	p.(Phe33Ser)	missense	Heterozygous	3	AR	YES	_____
				c.3341T>A	p.(Val1114Glu)	missense	Heterozygous	3		YES	_____

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FE17013163	HP:0000752-HYPERACTIVITY HP:0001007-HIRSUTISM HP:0010864-INTELLECTUAL DISABILITY, SEVERE San filippo syndrome	*HGSNAT*	NM_152419.2	c.744-2A>G	p.?	splicing	homozygous	4	AR	NO	PMID 19479962
FE18023423	Renal tubular acidosis, keratopathy	*SLC4A4*	NM_003759.3.	c.691_692delinsTA	p.(Met231Ter)	nonsense	Heterozygous	4	AR	YES	_____
FE18023071	Onset in teens of aching muscles after minor exercise. EMG reported borderline myopathic changes. Mild generalised weakness in all 4 limbs, creatine kinase levels normal.	*ANO5*	NM_213599.2	c.692G>T	p.(Gly231Val)	missense	Heterozygous	4	AR	NO	PMID 20096397
FE18032492	HP:0004345-GANGLIOSIDE ACCUMULATION	*PMM2*	NM_000303.2.	c.367C>T c.722G>C	p.(Arg123Ter) p.(Cys241Ser)	nonsense missense	Heterozygous Heterozygous	5 5	AR	NO NO	rs191295403 PMID 11058895 PMID 12705494 PMID 17166182 PMID 11715002 PMID 22012410 PMID 28566178 PMID 28425223 PMID 29482223
FE18021754	Severe global developmental delay, facial dysmorphism, congenital cataracts, epilepsy.	*MED25*	NM_030973.3.	c.518T>C	p.(Ile173Thr)	missense	Homozygous	4	AR	NO	PMID 30800049
FE18021035	Hereditary spastic paraparesis, positive family history.	*SPAST*	NM_014946.3	c.286delG	p.(Ala96ArgfsTer65)	frameshift	Heterozygous	5	AD	NO	PMID 11015453
FE11012997	Progressive relatively severe demyelinating CMT.	*MPV17*	NM_002437.5	c.122G>A	p.(Arg41Gln)	missense	Homozygous	5	AR	NO	PMID 26437932

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FE18011009	HP:0000196-LOWER LIP PIT HP:0000492-ABNORMAL EYELID MORPHOLOGY HP:0001849-FOOT OLIGODACTYLY HP:0009099-MEDIAN CLEFT PALATE HP:0009756-POPLITEAL PTERYGIUM	*IRF6*	NM_006147.4	c.250C>T	p.(Arg84Cys)	missense	Heterozygous	5	AD	NO	PMID 19036739
FE14014016	HP:0000717-AUTISM HP:0000824-GROWTH HORMONE DEFICIENCY HP:0002342-INTELLECTUAL DISABILITY, MODERATE HP:0010627-ANTERIOR PITUITARY HYPOPLASIA	*ASH1L*	NM_018489.2.	c.6826C>T	p.(Arg2276Ter)	nonsense	Heterozygous	3	AD	YES	_____
FE18018059	HP:0001332-DYSTONIA HP:0002180-NEURODEGENERATION HP:0002454-EYE OF THE TIGER ANOMALY OF GLOBUS PALLIDUS HP:0030890-HYPERINTENSITY OF CEREBRAL WHITE MATTER ON MRI	*PANK2*	NM_153638.3	c.629-2A>G	p.?	splicing	Homozygous	4	AR	NO	PMID 22930366
FE18024723	Prenatal pericardial effusion, fetal anaemia. respiratory distress at birth, seizures and abnormal EEG. MRI brain normal.	*RMND1*	NM_017909.3	c.87delG	p.(Met29IlefsTer2)	frameshift	Heterozygous	4	AR	YES	_____
FE18015666	HP:0001249-INTELLECTUAL DISABILITY HP:0001387-JOINT STIFFNESS HP:0012722-HEART BLOCK	*ANKRD11*	NM_001256182	c.7470+2T>A	p.?	splicing	Heterozygous	3	AD	YES	_____
FE18024073	HP:0000407-SENSORINEURAL DEAFNESS HP:0000545-MYOPIA HP:0003198-MYOPATHY	*CEP78*	NM_001098802.1	c.1450C>T	p.(Arg484Ter)	nonsense	Homozygous	4	AR	YES	_____
		ETFDH	NM_04453.3	c.1448C>T	p.(Pro483Leu)	missense	Homozygous	3	AR	NO	PMID 17584774 PMID 29530532
FE13004802	Severe bilateral sensorineural hearing loss.	*CABP2*	NM_001318496.1.	c.292C>T	p.(Arg98Ter)	nonsense	Homozygous	4	AR	NO	rs761766884
FE14007468	Moderate-severe learning disability, palmoplantar keratoderma, microcephaly	*PACS1*	NM_018026.3.	c.607C>T	p.(Arg203Trp)	missense	Heterozygous	5	AD	NO	PMID 23159249

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FE18029719	Congenital unilateral cleft lip and palate along with lower lip pits, likely diagnosis of Van der Woude syndrome.	*IRF6*	NM_006147.4	c.1169T>C	p.(Ile390Thr)	missense	Heterozygous	3	AD	YES	_____
FE18030683	Non-syndromic severe bilateral congenital hearing loss.	*LOXHD1*	NM_144612.6	c.6306T>G c.3727C>T	p.(Tyr2102Ter) p.(Arg1243Trp)	nonsense missense	Heterozygous Heterozygous	3 3	AR	YES NO	_____ rs369695848
FE18030610	Learning difficulties, thin corpus callosum.	*SPG11*	NM_025137.3	c.5399_5402delins8	p.(Gln1800LeufsTer31)	frameshift	Homozygous	4	AR	NO	PMID 28237315
FE18029416	HP:0000308-MICRORETROGNATHIA HP:0000581-BLEPHAROPHIMOSIS HP:0000647-SCLEROCORNEA HP:0001545-ANTERIORLY PLACED ANUS	*UBE3B*	NM_130466.3	c.469C>T c.730C>T	p.(Arg157Ter) p.(Gln244Ter)	nonsense nonsense	Heterozygous Heterozygous	4 4	AR	YES YES	_____ _____
FE18023357	Tylosis affecting his palmar and plantar surfaces, esophagitis.	*DSG1*	NM_001942.3	c.430A>T	p.(Arg144Ter)	nonsense	Heterozygous	5	AD	NO	PMID 19558595
FE18013680	Developmental delay , synophrys, patchy T2 white matter, hyperintensity in subcortical white matter.	*BCL11A*	NM_022893.3	c.192delC	p.(Ile64MetfsTer14)	frameshift	Heterozygous	5	AD	YES	_____
FE18031525	Low immunoglobulins, low vaccine response CVID, splenomegaly, coeliac, hyperthyroid, endometriosis and abdominal adhesions.	*STAT1*	NM 007315.3.	c.796G>A	p.(Val266Ile)	missense	Heterozygous	3	AD/AR	NO	PMID 26513235 PMID 23534974 PMID 26038974 PMID 29077208
FE18031312	HP:0001102-ANGIOID STREAKS OF THE FUNDUS	*ABCC6*	NM_001171.5	c.3490C>T c.3421C>T	p.(Arg1164Ter) p.(Arg1141Ter)	nonsense nonsense	Heterozygous Heterozygous	5 5	AR	NO NO	PMID 10835642 PMID 15086542 PMID 12714611 PMID 11179012 PMID 10954200

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE18000260	Macrophage activation syndrome, juvenile idiopathic arthritis.	*NLRP12*	NM_144687.3	c.2828_2829dupTC	p.(Arg944SerfsTer6)	frameshift	Heterozygous	3	AD	NO	rs533054990
FE14003054	Moderate sensorineural hearing loss.	*MARVELD2*	NM_001038603.2	c.1098G>A	p.(Trp366Ter)	nonsense	Homozygous	4	AR	NO	rs773552728
FE18030121	Multiple venous malformations	*GLMN*	NM_053274.2	c.157_161delAAGAA	p.(Lys53Ter)	nonsense	Heterozygous	5	AD	NO	rs762515373
FE13000175	HP:0001631-ATRIAL SEPTAL DEFECT HP:0001642-PULMONARY STENOSIS HP:0001647-BICUSPID AORTIC VALVE	*TAB2*	NM_015093.5.	c.1273C>T	p(Gln425Ter)	nonsense	Heterozygous	4	AD	YES	_____
FE18019948	Bilateral mild sensorineural hearing loss Bilateral enlarged vestibular aqueducts	*SLC26A4*	NM_000441.1	c.1151A>G c.1246A>C	p.(Glu384Gly) p.(Thr416Pro)	missense missense	Heterozygous Heterozygous	5 5	AR	NO NO	PMID 9618167 PMID 12788906 PMID 24224479 PMID 9618167 PMID 12788906 PMID 24224479
FE18026000	HP:0001903-ANAEMIA HP:0001943-HYPOGLYCAEMIA HP:0002242-ENTEROPATHY HP:0003256-COAGULOPATHY	*ALG8*	NM_024079.4	c.1579T>A	p.(Ter527Argext*?)	LOF	Homozygous	5	AR	NO	rs1346281230
FE18031582	HP:0002652-SKELETAL DYSPLASIA	*SOX9*	NM_000346.3.	c.343T>A	p.(Trp115Arg)	missense	Heterozygous	3	AD	YES	_____
FE19002770	Child of consanguinous roma gypsy couple (? relationship) Profound developmental delay, profound bilateral sensorineural hearing loss, progressive retinopathy based on worsening of ERGS and very limited vision, optic atrophy, seizures onset at 18 months, dystonic episodes, progressive scoliosis, marked hirsutism, hypoplastic basal ganglia, thin corpus callosum, decreased white matter.	*BSND*	NM_057176.2	c.23G>A	p.(Arg8Gln)	missense	Homozygous	3	AR	NO	PMID 29986705

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FE18021366	Possible Walker-Warburg, severe ventriculomegaly, delayed sulcal and gyral formation , cerebellar vermis hypoplasia.	*TUBA1A*	NM_006009.3	c.652G>A	p.(Asp218Asn)	missense	Heterozygous	4	AD	NO	PMID 20466733
FE19003044	Congenital agammaglobulinaemia and absent B cells, macrocytosis, recurrent rhinitis, lichen, sclerosis.	*TCF3*	NM_001136139.2	c.1663G>A	p.(Glu555Lys)	missense	Heterozygous	5	AD	NO	PMID 24216514
FE19001679	Microcephaly, learning difficulties, possible hearing loss, flat retina, chiasmal hypoplasia.	*KIF11*	NM_004523.3	c.1129-1G>C	p.?	splicing	Heterozygous	5	AD	YES	_____
FE19003614	HP:0005208-SECRETORY DIARRHEA	*GUCY2C*	NM_ 004963.3.	c.2536G>A	p.(Val846Met)	missense	Heterozygous	3	AD	YES	_____
FE19000072	HP:0001181-ADDUCTED THUMBS HP:0005684-DISTAL ARTHROGRYPOSIS HP:0010557-OVERLAPPING FINGERS	*ECEL1*	NM_004826.3.	c.589G>A	p.(Gly197Ser)	missense	Homozygous	4	AR	NO	rs1356377759 PMID 30131190
FE17004592	HP:0000007-AUTOSOMAL RECESSIVE, HP:0000365-HEARING LOSS, HP:0000407-SENSORINEURAL HEARING LOSS, HP:0011387-DILATED VESTIBULAR AQUEDUCT, HP:0012832-BILATERAL, HP:0031914-FLUCTUATING	*SLC26A4*	NM_000441.1	c.626G>T c.707T>C	p.(Gly209Val) p.(Leu236Pro)	missense missense	Heterozygous Heterozygous	4 5	AR	NO NO	PMID 9618166 PMID 11932316 PMID 24224479 PMID 26969326 rs80338848 PMID 9618166 PMID 20553101 PMID 10861298 PMID 26969326 PMID 12354788 PMID 18310264 PMID 20597900 PMID 15689455
FE18012228	HP:0000407-SENSORINEURAL HEARING LOSS HP:0000635-BLUE IRIDES HP:0012828-SEVERE HP:0012829-PROFOUND	*EDN3*	*NM_207034.2.*	c.476G>T	p.(Cys159Phe)	missense	Homozygous	4	AR	NO	PMID 8630503

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE87188	Congenital sensorineural deafness- progressive. Cochlear implant user. Mild learning disability.	*SLC26A4*	NM_000441.1	c.716T>A	p.(Val239Asp)	missense	Homozygous	4	AR	YES	PMID 12676893 PMID 12974744 PMID 25394566 PMID 30077349 PMID 16460646 PMID 22116360 PMID 27771369
FE19007612	HP:0012265-CILIARY DYSKINESIA	*CCDC40*	NM_017950.3.	c.1416delG	p.(Ile473Phefster2)	frameshift	Homozygous	5	AR	NO	PMID 23255504 PMID 23891469 PMID 30209139
FE19007613	HP:0000763-SENSORY NEUROPATHY HP:0001156-BRACHYDACTYLY HP:0001251-CEREBELLAR ATAXIA HP:0001260-DYSARTHRIA HP:0001337-TREMOR HP:0007256-PYRAMIDAL SIGNS	*POLR3A*	NM_007055.3.	c.1909+22G>A	p.?	splicing	Heterozygous	4	AR	NO	PMID 27029625 PMID 28459997 PMID 30564185 PMID 30323018 PMID 29691679 PMID 30847471
				c.3583delG	p.(Asp1195IlefsTer47)	frameshift	Heterozygous	4		NO	rs747683665
FE18013873	HP:0000365-HEARING LOSS HP:0012829-PROFOUND	*CDH23*	NM_022142.5	c.6133G>A	p.(Asp2045Asn)	missense	Homozygous	5	AR	NO	PMID 11090341
FE18018919	HP:0001272-CEREBELLAR ATROPHY HP:0002135-BASAL GANGLIA CALCIFICATION	*TREM2*	NM 018965.3.	c.549delT	p.(Leu184SerfsTer5)	frameshift	Homozygous	3	AD	YES	_____
FE19009211	HP:0000518-CATARACT HP:0000556-RETINAL DYSTROPHY HP:0001083-LENS DISLOCATION	*COL18A1*	NM_130445.2	c.2919dup	p.(Gly974ArgfsTer110)	frameshift	Heterozygous	5	AR	NO	rs768555371
				c.3514_3515delCT	p.(Leu1172ValfsTer72)	frameshift	Heterozygous	5		NO	rs398122391
FE16026130	HP:0000175-CLEFT PALATE HP:0000324-FACIAL ASYMMETRY HP:0000750-SPEECH DELAY HP:0001249-INTELLECTUAL DISABILITY HP:0001999-FACIAL DYSMORPHISM HP:0410030-CLEFT LIP	*CHD7*	NM_017780.3	c.5222G>T	p.(Arg1741Leu)	missense	Heterozygous	3	AD	YES	_____

Case ID	Phenotype*	Gene symbol	Transcript	Nucleotide change	Protein change	Variant consequence	Variant Zygosity	ACMG Classification(1)	Gene inheritance	Novel at time of analysis (2)	References(3)
FE19002968	HP:0000556-RETINAL DYSTROPHY	*SRD5A3*	M_024592.4.	c.57G>A	p.(Trp19Ter)	nonsense	Homozygous	5	AR	NO	rs398124401
FE18030615	HP:0000175-CLEFT PALATE HP:0000365-HEARING LOSS HP:0000823-DELAYED PUBERTY HP:0001263-DEVELOPMENTAL DELAY	*CHD7*	NM_017780.3	c.4353+4A>G	p.?	splicing	Heterozygous	3	AD	YES	_____
FE18032769	HP:0000252-MICROCEPHALY HP:0000280-COARSE FACIES HP:0000996-FACIAL CAPILLARY HEMANGIOMA HP:0001508-FAILURE TO THRIVE HP:0002247-DUODENAL ATRESIA HP:0002509-LIMB HYPERTONIA HP:0009062-INFANTILE AXIAL HYPOTONIA HP:0100704-CORTICAL VISUAL IMPAIRMENT	*GRIN2B*	NM_000834.3	c.2011-1G>A	p.?	splicing	Heterozygous	5	AD	NO	PMID 27605359
FE18018608	Postaxial polydactyly both hands and feet, global developmental delay, echogenic kidneys, possible BBS.	*BBS7*	NM_176824.2.	c.712_715delAGAG	p.(Arg238GlufsTer59)	frameshift	Heterozygous	5	AR	NO	PMID 19402160 PMID 26518167 PMID 20177705
				c.1967_1968delinsC	p.(Leu656ProfsTer18)	frameshift	Heterozygous	4		NO	
FE19009692	HP:0001181-ADDUCTED THUMBS HP:0001290-HYPOTONIA HP:0001762-TALIPES EQUINOVARUS	*CHST14*	NM_130468.3.	c.652C>A	p.(Arg218Ser)	missense	Heterozygous	3	AD	NO	PMID 25703627
FE14012166	HP:0000365-HEARING IMPAIRMENT HP:0000729-AUTISTIC BEHAVIOR HP:0007483-DEPIGMENTATION/HYPERPIGMENTATION OF SKIN	*CRYM*	NM_001888.3	c.703G>C	p.(Glu235Gln)	missense	Heterozygous	3	AD	YES	_____
		FGFR2	NM_000141.4.	c.1032G>A	p.(Ala344Ala)	synonymous	Heterozygous	5	AD	NO	PMID 7987400 PMID 8957519 PMID 7558045 PMID 16158432
FE15003583	Bilateral moderate-to-severe sensorineural hearing loss, cochlear implant user.	*LOXHD1*	NM_144612.6.	c.2295G>A	p.(Trp765Ter)	nonsense	Compound heterozygous	4	AR	YES	_____
				c.6368_6369delCA	p.(Thr2123ArgfsTer30)	frameshift	Compound heterozygous	4		YES	_____
FE18011361	HP:0000252-MICROCEPHALY HP:0002617-ANEURYSM	*ESCO2*	NM_001017420.2.	c.1489A>T	p.(Ile497Phe)	missense	Homozygous	3	AR	YES	_____

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FE18007064	likely retinal dystrophy	*AHI1*	NM_017651.4	c.2988delA	p.(Val997SerfsTer20)	frameshift	Heterozygous	3	AR	NO	PMID 28041643
FE85265	congenital myopathy	*AMPD1*	NM_000036.2	c.133C>T	p.(Gln45Ter)	nonsense	Homozygous	3	AR	NO	PMID 1631143 PMID 21343608
FE15019977	Moderate to severe cerebellar atrophy, brisk lower limb reflexes, clonus, slow speech, learning difficulties.	*KCNA1*	NM_000217.2	c.884G>A	p.(Arg295His)	missense	Heterozygous	3	AD	YES	_____
FE17000781	Bilateral sensorineural hearing loss, bilateral enlarged vestibular acqueduct.	*SLC26A4*	NM_000441.1	c.578C>T	p.(Thr193Ile)	missense	Heterozygous	4	AR	NO	rs111033348 PMID 23273637 PMID 26752218
FE17005641	Bilateral sensorineural hearing loss and bilateral enlarged vestibular aqueduct.	*SLC26A4*	NM_000441.1	c.349C>T	p.(Leu117Phe)	missense	Heterozygous	4	AR	NO	PMID 26969326 PMID 10700480 PMID 11932316
FE17002610	HP:0000294 - LOW ANTERIOR HAIRLINE HP:0000582 - UPSLANTED PALPEBRAL FISSURE HP:0000664 - SYNOPHRYS HP:0000885 - BROAD RIBS HP:0001607 - SUBGLOTTIC STENOSIS HP:0002208 - COARSE HAIR HP:0002786 - TRACHEOBRONCHOMALACIA HP:0003016 - METAPHYSEAL WIDENING HP:0003300 - OVOID VERTEBRAL BODIES HP:0004322 - SHORT STATURE HP:0005280 - DEPRESSED NASAL BRIDGE HP:0008430 - ANTERIOR BEAKING OF LUMBAR VERTEBRAE	*GNPTAB*	NM_024312.4	c.3503_3504delTC	p.(Leu1168GlnfsTer5)	frameshift	Heterozygous	4	AR	NO	PMID 16465621 PMID 20880125 PMID 25788519
FE18017011	Severe Combined Immunodeficiency	*LRBA*	NM_006726.4	c.6584+1delG	p.?	splicing	Heterozygous	4	AR	NO	rs1320366310
FE18032044	Moderate, bilateral sensorineural hearing loss	*OTOA*	NM_144672.3	c.828delT	p.(Ser277ValfsTer3)	frameshift	Heterozygous	4	AR	NO	rs751447996

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	Moderate, bilateral sensorineural hearing loss	*MY015A*	NM_016239.3	c.5866C>T	p.(Argl956Trp)	MIssense	Heterozygous	3	AR	NO	PMID 26969326
FE14008583	HP:0000407-SENSORINEURAL HEARING IMPAIRMENT HP:0001263-DEVELOPMENTAL DELAY HP:0002066-ATAXIC GAIT HP:0002072-CHOREA HP:0100022-MOVEMENT DISORDER	*CDH23*	NM_022142.5	c.6133G>A	p.(Asp2045Asn)	missense	Heterozygous	5	AR	NO	PMID 11090341
FE14011757	Absent gag reflex, hirsuitism, Mongolian blue spot, dysmorphic features including microcephaly and epicanthic folds, previous flexion contracture of finger	*DHCR7*	NM_001360.2	c.964-1G>C	p.?	splicing	Heterozygous	5	AR	NO	rs138659167
FE15018778	Progressive, profound sensorineural hearing loss	*TMC1*	NM_138691.2	c.1165C>T	p.(Arg389Ter)	nonsense	Heterozygous	4	AR/AD	NO	rs151001642

AR - autosomal recessive, AD: autosomal dominant.
* Phenotypes are described using main clinical description or Human Phenotype Ontology terms entered into the Web Referral System (WRS).
(1): Richards, Sue, et al. "Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology." Genetics in medicine 17.5 (2015): 405-423.
(2):CES cases analysed between September 2016- December 2019
(3): Reference in published literature (PMID PubMed identifier) and/or reference ID number

Chapter 3. The diagnostic utility of proband-only clinical exome sequencing in 60 patients with hearing loss disorders: A single-institution experience

Published in Clinical Otolaryngology. 2021

Received: 21 December 2020 | Revised: 8 April 2021 | Accepted: 8 May 2021

DOI: 10.1111/coa.13826

CLINICAL EXPERIENCE

WILEY

The diagnostic utility of clinical exome sequencing in 60 patients with hearing loss disorders: A single-institution experience

1 | INTRODUCTION

Congenital ear anomalies and hearing impairment are often present in patients with underlying genetic disorders. Germline genomic variants are responsible for at least 50% of congenital and/or childhood-onset sensorineural hearing loss (SNHL). Furthermore, 20%–60% of patients with bilateral microtia and congenital aural atresia may have an identifiable genetic syndrome.[1]

Standard diagnostic approaches involving single gene testing and chromosomal microarrays have limited utility in diagnosing monogenic conditions with high degrees of genetic and phenotypic heterogeneity.[2] Delay in confirming a genetic diagnosis can lead to incorrect diagnostic workup, uncertain prognosis, inadequate treatment, delayed referral to relevant medical subspecialties and lack of anticipation of potential additional comorbidities.

The development of next-generation DNA sequencing (NGS) has contributed significantly to the diagnosis, study and care of Mendelian monogenic disorders. The capacity to perform simultaneous sequencing of multiple genomic regions makes NGS particularly appropriate for the investigation of genetically and clinically heterogeneous conditions, such as hereditary hearing loss. NGS-based–targeted gene panels and exome sequencing have become increasingly available for monogenic disorders. Reported diagnostic rates range between 20% and 50%, depending upon the patient cohort and the chosen testing platform.[3]

Next-generation sequencing approaches can lead to molecular diagnoses which can inform clinical decision-making. Clinical exome sequencing (CES) is therefore increasingly being used for testing patients with congenital ear anomalies and hearing disorders.[4–6] As the clinical availability of NGS increases, there is a need to evaluate its impact in routine practice. This study reports experience with CES in 60 consecutive patients with congenital ear or hearing disorders with a suspected genetic aetiology, to determine diagnostic yield and document the clinical implications.

2 | PATIENTS AND METHODS

We conducted a retrospective survey of clinical and molecular results from 60 consecutive patients with ear and hearing disorders, referred for proband-only clinical exome sequencing (CES) to the NHS North West Genomic Laboratory Hub. CES was performed for diagnostic purposes, with prior consent by the patients and/or their guardian(s). All patients were evaluated and referred for CES by a consultant clinical geneticist. CES experiments were conducted using a custom-designed Agilent SureSelect XT Focused Exome capture library and the NextSeq 500 sequencer (Illumina, Inc). A phenotype-driven virtual gene panel was generated per patient, customised based on their clinical features, as described previously.[7] Following bioinformatics analyses (supplemental material), the clinical significance of candidate variants was interpreted independently by two registered Clinical Scientists as per the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP) guidelines.[8] These guidelines assign genomic variants identified into a five-tier system based on different types of evidence (population data, computational and predictive analysis, functional criteria and segregation data). As per these criteria, genomic variants are classified as pathogenic, likely pathogenic, variant of unknown significance, likely benign and benign.[8] Where needed, cases were further reviewed at internal Multidisciplinary Team meetings or through internal communication between the consultant clinical geneticist and clinical scientists from the Rare Disease Clinical Exome team.

3 | EVALUATION OF CES DIAGNOSTIC UTILITY

A search was conducted in the internal referral database identifying patients with phenotypes matching query terms (Table S1, supplemental material) that indicated the presence of hearing loss and/

or abnormalities of the external, middle or inner ear, either isolated or as part of their phenotype. Gender, age at referral, clinical features, previous genetic testing, CES results and actions prompted after CES were obtained from available medical records, the laboratory's internal database. Finally, to determine the diagnostic rate, CES results were categorised as follows: (1) confirmed, (2) possibly confirmed and (3) not confirmed (Table S2).

4 | RESULTS

4.1 | Cohort clinical characteristics

Seventy-eight per cent (47/60) of patients were under age 18 (median 6, range 0–73 years, 95% CI 7.51–14.73). A 40:60 female: male ratio was observed. Twenty patients (33%) had apparently isolated sensorineural hearing loss (19 bilateral and 1 unilateral SNHL). Twenty-seven patients (45%) had SNHL as part of a complex phenotype (25 bilateral and 2 unilateral SNHL). Ten (17%) had a degree of microtia and/or atresia accompanied by other clinical features. Three (5%) were referred with mixed hearing loss with additional clinical features, most commonly neurological or ophthalmic disorders (Table 1).

Key points

- Genetic aetiologies are an important cause of congenital ear anomalies and hearing impairment.
- Next-generation sequencing (NGS) strategies, such as targeted gene panels or clinical exome sequencing (CES), are effective tools in the diagnosis of patients with inherited hearing impairment, with clear advantages over previous genetic testing approaches.
- Clinical exome sequencing in our cohort shows the genetic heterogeneity of syndromic and non-syndromic congenital ear and hearing disorders, highlighting the clinical utility of undergoing genomic investigations.
- Multidisciplinary decision-making for diagnostic workup and management, including close collaboration between genetics, otolaryngology, audiology and other allied specialties, is key in the investigation of congenital ear and hearing disorders.
- Timely molecular genetic diagnosis can streamline patient care and potentially improve clinical outcomes.

4.2 | Genetic investigations prior to CES

Thirty-six patients (60%) had some genetic testing prior to undergoing CES (Table 2). No variants of clinical significance were reported in 32 patients (88%), and inconclusive findings were reported in the remaining four: two had chromosomal microarray variants of uncertain significance (VUS), namely a balanced rearrangement of chromosome 10 and a deletion on the X chromosome. Two patients had single heterozygous variants in *GJB2*; c.306G>C, p.(Lys102Asn) and c.101T>C, p.(Met34Thr), but no second GJB2 variant was found *in trans* in either patient, rendering these findings unlikely to account for their auditory phenotype.

TABLE 1 Additional phenotypes in patients with syndromic SNHL and syndromic microtia-atresia ($n = 41$)

Category	No. of patients with reported abnormality (%)
Neurodevelopmental delay	16 (39%)
Ophthalmic disorders	14 (34%)
Central nervous system malformation	7 (17%)
Other neurological abnormalities	6 (15%)
Oral cleft	6 (15%)
Congenital cardiovascular defect	5 (12%)
Limb abnormalities	4 (10%)
Facial dysmorphology	3 (7%)
Skin disorder	3 (7%)
Renal abnormalities	3 (7%)
Abnormal thyroid physiology/ morphology	2 (5%)
Retrognathia/micrognathia	2 (5%)
Upper aerodigestive tract abnormality	2 (5%)
Gastrointestinal abnormality	2 (5%)
Early-onset obesity	1 (2%)
Growth abnormality	1 (2%)

Abbreviation: SNHL, sensorineural hearing loss.

4.3 | Variant spectrum and diagnostic yield

Forty distinct variants were identified in 24 genes (Table S3). Genes most frequently harbouring variants were *SLC26A4*, *LOXHD1*, *CDH23* and *CDH7*. Eleven variants (27%) were novel at time of analysis. Predicted loss-of-function and missense variants were reported in equal proportions ($n = 13$, respectively). Twenty-six variants (65%) were found as pathogenic or likely pathogenic and fourteen variants were classified as variants of uncertain significance (VUS) (35%) according to ACMG guidelines.[8]

Clinical exome sequencing resulted in an overall diagnostic yield of 31% (19/60). This was higher in patients with sensorineural hearing loss: 60% (12/20) in the non-syndromic 22% (6/27) in the syndromic SNHL groups (Figure 1). Only one case with syndromic microtia-atresia, was categorised as "possibly confirmed", with a homozygous class 3 variant in *ORC6* (Meier-Gorlin syndrome type 3). Autosomal recessive inheritance underpins 85% of diagnoses in this cohort without a family history. Inconclusive findings were reported in 11 patients (18%): Seven presented with complex phenotypes

TABLE 2 Genetic testing prior to CES

Genetic investigation	No. of cases	Clinical category
GJB2/GJB6 testing	19	Non-syndromic SNHL (*n* = 14)
		Syndromic SNHL (*n* = 4)
		Syndromic mixed HL (*n* = 1)
Chromosomal microarray	12	Syndromic SNHL (*n* = 9)
		Syndromic microtia-atresia (*n* = 3)
Fragile X syndrome	3	Syndromic SNHL (n = 2)
		Syndromic microtia-atresia (*n* = 1)
m.1555A>G	2	Syndromic SNHL (n = 1)
		Non-syndromic SNHL (*n* = 1)
NGS gene panel[a]	2	Syndromic SNHL (*n* = 2)
Specific single gene testing[b]	1	Syndromic SNHL (*n* = 1)

Abbreviations: NGS, next-generation sequencing; SNHL, sensorineural hearing loss.

[a]Leeds leukodystrophy and mitochondrial leukodystrophy panels; Newcastle panel of genes for complex I deficiency.

[b]*SCA17* gene.

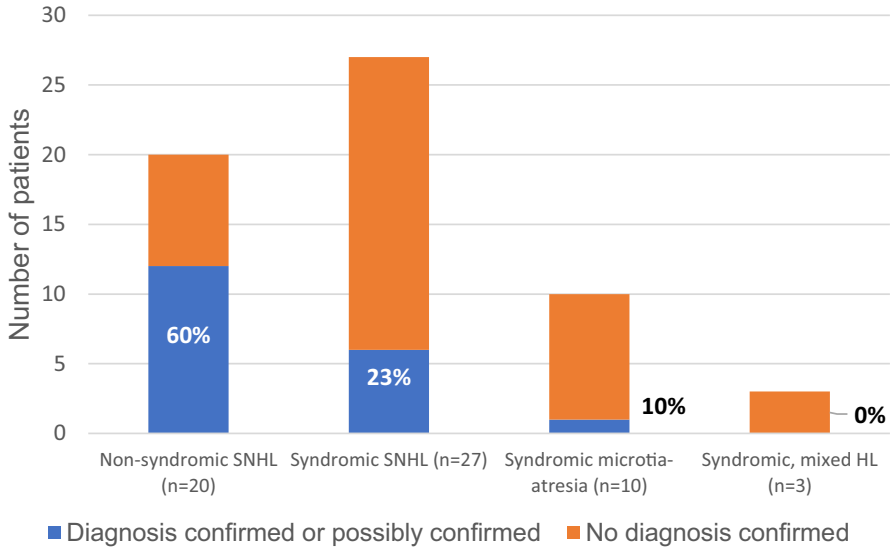

FIGURE 1 Diagnostic rate of clinical exome sequencing (CES) per clinical hearing loss category. Percentages indicate proportion of cases with confirmed and/or possibly confirmed genetic diagnosis. HL, hearing loss; SNHL, sensorineural hearing loss

including developmental delay, learning disability, myopathy and/or dual sensory impairment (Table S3). No plausibly pathogenic variants were identified in 30 patients (50%).

4.4 | CES directs clinical care

Clinical exome sequencing results directly informed clinical decision-making in 15 patients (25%). Five were referred for assessment or reassessment by other specialists. One patient referred with non-syndromic SNHL, had a homozygous likely pathogenic variant in *CDH23*, prompting reassessment by ophthalmology to assess for any evidence of Usher syndrome. Two patients were referred to specialist multidisciplinary clinics for inherited cardiac anomalies and CHARGE syndrome respectively. Two patients were referred back to audiology for further detailed phenotypic evaluation. In five cases, molecular findings prompted genetic investigations in similarly affected family members. Results in a patient with bilateral SNHL and myopathy with a possible dual diagnosis involving *CEP78* and *ETFDH* enabled testing and early diagnosis in a similarly affected daughter. Another patient was found to be a carrier for Becker muscular dystrophy, which enabled cascade testing and clinical investigations for family members. Finally, nine patients without confirmed diagnosis underwent whole genome sequencing as part of the 100 000 Genomes Project.[9]

5 | DISCUSSION

The implementation of genomic sequencing approaches has demonstrated diagnostic utility in the context of SNHL.[10] Here, we report our experience with CES in a cohort of patients presenting with a variety of ear and hearing loss phenotypes. Our diagnostic rate of

31% is broadly comparable to the available literature for this patient group[4,5] and shows an increased diagnostic utility in comparison with previously available genetic testing. In settings where patients present with a diverse range of genetically and clinically heterogeneous conditions, CES can offer effective diagnostic rates and represent a long-term, more cost-effective and suitable choice compared with targeted NGS approaches (i.e., targeted diagnostic gene panels).[2,11] While a few technical limitations still exist,[5] CES clearly can increase genetic diagnostic rates in comparison with previously available standard genetic testing for hearing loss.

Timely identification of a genetic diagnosis can have strong implications in the context of ear and hearing disorders. Firstly, the early confirmation or exclusion of syndromic conditions can inform future diagnostic workup and promote more cost-effective use of healthcare resources.[12] Compared with standard of care, CES can reduce unnecessary diagnostic interventions through the increased identification of molecular diagnoses. It has been shown that, while CES incurs additional costs, it can be cost-effective in hearing loss due to the increase in diagnostic yield.[13] Results can streamline diagnostic interventions and treatment options. In a hearing loss context, timing of testing can be key in assessing the clinical utility and consequent economic impact of genomic sequencing. It is possible that redefining the hierarchy of diagnostic testing battery is required to balance the number of interventions needed to obtain an adequate amount of clinical data to be used for clinical interpretation of genomic sequencing variants. Further health-economics research should be conducted to validate this across different payer healthcare systems.

Secondly, the increasing integration of genomic, clinical and laboratory data, including outcomes, can be used to develop prognostic models that inform management decision-making. Although it is highly unlikely that genomic diagnosis would preclude cochlear implantation or any other type of hearing habilitation or rehabilitation, molecular diagnosis can facilitate the identification of patients in need of targeted rehabilitation due to a predicted risk of poor performance. There is a growing body of evidence on the use of genetic diagnosis for prediction of cochlear implantation outcomes,[14,15] and in SNHL associated with enlarged vestibular aqueducts, the presence or absence of key genomic variants may also be of prognostic value for hearing loss severity and/or progression.[16] Early genetic diagnosis can inform clinical care teams of an increased risk of hearing loss progression, warranting closer surveillance that prompts early consideration of cochlear implantation. Finally, genomic findings are clinically relevant to family members, permitting confirmatory testing and accurate counselling about reproductive risks and choices.

While this study is limited by a relatively small sample size and its retrospective design, it confirms the current diagnostic capability of CES and offers some insights into real-life, clinical use of genomic sequencing. It highlights the heterogeneity in patients currently referred to NHS Genomic Medicine services. It is also important to note that the diagnostic capability of CES is enriched by good phenotyping. Analysis of CES data is clinically driven and thus requires detailed phenotyping. The need for phenotype reassessment in some patients denotes the importance of pursuing further integration between clinical and genomic services. Multidisciplinary team evaluation by ENT surgeons, audiologists and clinical geneticists can enhance the quality of phenotype data and reduce referral delays between specialities. Consequently, this can facilitate interpretation of CES data, shorten turnaround times and expedite molecular diagnosis, allowing for a timely optimisation of individualised hearing and disease surveillance.

In summary, CES is a powerful tool in the diagnostic investigation of patients with ear and hearing disorders, with direct implications for patient care. Due to the prevalence and diversity of genetic aetiologies for congenital ear anomalies and hearing impairment, incorporating early molecular diagnosis into existing comprehensive multidisciplinary care has the potential to improve both patient counselling (regarding recurrence risks and disease prognostication) and, in turn, clinical outcomes.

ACKNOWLEDGEMENTS

LPMR was sponsored by Mexico's National Council of Science and Technology, (CONACyT)-392996. HS is supported by Peter Mount award (Manchester University NHS Foundation Trust)-G70658; Data Driven Award (Manchester University NHS Foundation Trust)-NH_SY_020419 and the Royal College of Surgeons. WGN is supported by Manchester NIHR BRC IS-BRC-1215-20007. GCB is supported by the Wellcome Trust (200990/Z/16/Z, Transforming Genetic Medicine Initiative). The authors would like to thank Jonathan Edgerley, clinical scientist at the Manchester Centre for Genomic Medicine, for his support and assistance with data collection for this study.

CONFLICT OF INTEREST

We also hereby declare that none of the authors have any disclosures to share, nor any conflict of interest.

AUTHOR CONTRIBUTIONS

LPMR, EMMBW, IAB and GCMB designed and coordinated the study. LPMR, EMMBW, CK and AT collected the data. LPMR, EMMBW, HS, IAB and GCMB analysed and interpreted the data. SSB contributed to bioinformatics and maintenance of bioinformatics pipeline. AT contributed to virtual gene panel algorithm and development of clinical exome web referral system. LD contributed to exome sequencing experiments. CK, RW, EMMBW, SSB, CC, SB, AS, CF, JHMcD, TAB, JCS, SD, KC, WGN and GCMB contributed to genetic and/or phenotypic data. LPMR, EMMBW, HS, IAB and GCMB wrote the manuscript. All the authors revised the manuscript for important intellectual content and approved the final version.

ETHICAL APPROVAL

This study was conducted as an audit of results and performance following changes to the standard procedures for the referral and analysis of the existing Clinical Exome service. Through the clinical exome-informed consent process, patients consented to having their

sample, genomic data and clinical information shared internally and with other researchers through scientific publications, controlled-access databases and open-access databases.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Leslie P. Molina-Ramírez[1,2]
Emma MM. Burkitt-Wright[1,2]
Haroon Saeed[3]
John H. McDermott[1,2]
Claire Kyle[2]
Ronnie Wright[2]
Christopher Campbell[2]
Sanjeev S. Bhaskar[2]
Algy Taylor[2]
Laura Dutton[2]
Claire Forde[2]
Kay Metcalfe[1,2]
Audrey Smith[1,2]
Jill Clayton-Smith[1,2]
Sofia Douzgou[1,2]
Kate Chandler[1,2]
Tracy A. Briggs[1,2]
Siddharth Banka[1,2]
William G. Newman[1,2]
David Gokhale[2]
Iain A. Bruce[3,4]
Graeme C. Black[1,2]

[1]*Division of Evolution and Genomic Sciences, Manchester Academic Health Science Centre, Faculty of Biology, Medicine and Health, School of Biological Sciences, University of Manchester, Manchester, UK*
[2]*Manchester Centre for Genomic Medicine, St. Mary's Hospital, Manchester University NHS Foundation Trust, Manchester, UK*
[3]*Paediatric ENT Department, Manchester Academic Health Science Centre, Royal Manchester Children's Hospital, Manchester University Hospitals NHS Foundation Trust, Manchester, UK*
[4]*Division of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine, Health University of Manchester, Manchester, UK*

Correspondence
Iain A Bruce, Manchester Academic Health Science Centre, Royal Manchester Children's Hospital, Manchester University Hospitals NHS Foundation Trust, Oxford Road Manchester, M13 9WL, UK.
Email: iain.bruce@manchester.ac.uk

Leslie P. Molina-Ramírez and Emma Mm Burkitt-Wright have co-first authorship.

ORCID

Leslie P. Molina-Ramírez https://orcid.org/0000-0001-7573-6846
Haroon Saeed https://orcid.org/0000-0002-2624-0518
John H. McDermott https://orcid.org/0000-0002-5220-8837
Jill Clayton-Smith https://orcid.org/0000-0002-9602-2321
Sofia Douzgou https://orcid.org/0000-0001-8890-7544
Tracy A. Briggs https://orcid.org/0000-0001-5208-2691
Siddharth Banka https://orcid.org/0000-0002-8527-2210
William G. Newman https://orcid.org/0000-0002-6382-4678
Iain A. Bruce https://orcid.org/0000-0003-0831-4760
Graeme C. Black https://orcid.org/0000-0001-8727-6592

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Molina-Ramírez LP, Burkitt-Wright EMM, Saeed H, et al. The diagnostic utility of clinical exome sequencing in 60 patients with hearing loss disorders: A single-institution experience. *Clin Otolaryngol*. 2021;00:1–6. https://doi.org/10.1111/coa.13826

3.1 Supplemental Materials

Supplementary methods: Generation of virtual gene panels

Clinical characteristics and/or Human Phenotype Ontology (HPO) terms (1) were used to perform semi-automated generation of personalised virtual gene panels. Clinicians selected one or more of the following methods:

A. Selection of hearing loss gene panel Genomics England PanelApp (2)
B. Selection of HPO to generate a list of candidate genes from OMIM (3) and Orphanet (4);
C. Customised selection of genes as specified by clinicians, provided on a case-by-case basis and according to their clinical hypotheses.

Genes present on the American College of Medical Genetics and Genomics (ACMG) incidental findings list (5) were included only at the discretion of the clinician, with prior consent from the patient and/or the parent/guardian.

Clinical Exome Sequencing: Sequencing and Bioinformatics

DNA was isolated from peripheral blood samples (n=60). Clinical exome sequencing (CES) was conducted using a custom-designed Agilent SureSelect XT Focused Exome capture library. Sequencing was done using the NextSeq 500 system (Illumina, Inc.) with 75-bp paired end reads, to a mean depth of 112 with >97% bases covered at 20x read depth. Reads were aligned with BWA-MEM (version 0.6.2) to the Human Genome build GRCh37(hg19). Local realignment performed using ABRA (version 0.96). Variant calling was carried out using SamTools (0.1.18/gcc-4.4.6) for SNPs and small indels and Pindel (v0.2.4.t) for indels >5bp. Sample-specific genome alignment (.BAM) and variant calling files (.VCF) and their associated virtual gene panel lists (.BED file, coding exons +/- 10bp of splice site junction) were imported and analysed using Golden Helix VarSeq® software (v1.4.4) (6). Variants were annotated according to RefSeq: NCBI RefSeq Annotation Release 105, selecting the most clinically relevant GRCh37(hg19) transcript. Missense variants were analysed in silico prediction tools: dbNSFP Function Predictions 3.0 (7), SIFT (8), PolyPhen-2 (9), MutationTaster(10), MutationAssessor (11), FATHMM (12). Prediction of splicing effects was performed using Alamut ® version 2.4.5 (Interactive Biosoftware), dbscSNV Splice Altering Predictions 1.1, GHI and SPIDEX (13). Variant prioritisation was performed considering variants with

quality scores of >20 and minimum coverage depth of x10. Variants with a frequency above 0.1% (0.001) in gnomAD (14) and those present in other run samples were discarded. Alternatively, for cases with no retained variants after prioritisation, variants listed as "Pathogenic" or "Likely pathogenic" on NCBI ClinVar (15) with an AAF <2% reported in genes contained in the virtual gene panel were filtered in. Variants considered to be causative of, or contributory to, the patient's clinical presentation were confirmed by Sanger sequencing prior to reporting.

Supplementary Table S1. Query terms

Query terms
Deafness
"Hearing loss"
"Sensorineural hearing loss"
"Hard of hearing"
"Ear anomaly"
"Inner ear"
Microtia
Anotia
"Cochlear implant"
"Cochlear implantation"

Supplementary Table S2. Categorisation of CES diagnosis

Diagnosis confirmed*.* In a clinically relevant gene, the presence of either: i. A heterozygous class 4 or 5 variant in a dominant condition, ii. A homozygous/hemizygous class 4 or 5 variant in a recessive condition or, iii. Two class 4 or 5 variants in the same gene in a recessive condition (potential compound heterozygote).
Diagnosis possibly confirmed**.** In a clinically relevant gene, the presence of either: i. A homozygous/hemizygous class 3 variant* in a recessive condition or, ii. A class 3 variant* and a class 4 or 5 variant in the same gene in a recessive condition (potential compound heterozygote), iii. A heterozygous class 3 variant* in a dominant condition where parental studies suggest a possible *de novo*.
Diagnosis not confirmed**.** In a clinically relevant gene, the presence of either: i. Any heterozygous class 3, 4 or 5 variant in a recessive condition, ii. A heterozygous class 3 variant* in a dominant condition where further parental testing has not been performed, iii. No plausibly causative variant identified.

*Report of variants of uncertain significance where further testing could be considered to re-classify the variant as likely pathogenic, as per the Association for Clinical Genomic Science (ACGS) Best Practice Guidelines for variant classification and interpretation in UK diagnostic genetic laboratories performing testing for rare disease.

Supplementary table S3. Cases for confirmed and possibly confirmed genetic diagnoses obtained by clinical exome sequencing.

Subject ID	Gender	Age at referral (years)	Clinical category	Phenotype	CES results (gene, transcript, cDNA change, protein change, zygosity)	Variant classification (ACMG guidelines)	Inheritance	Reference (dbSNP, DVD or PMID)
ES-ENT-1	xx	4	Syndromic SNHL	Congenital, progressive SNHL Hypothyroidism, Bilateral enlarged vestibular aqueduct	*SLC26A4* NM_000441.1c.260A>G, p.(Asp87Gly), heterozygous	4	AR	rs111033344
					SLC26A4 NM_000441.1c.1151A>G, p.(Glu384Gly), heterozygous	5		PMID 9618167 PMID 12788906 PMID 24224479
ES-ENT-11	xy	8	Non-syndromic SNHL	Bilateral, moderate SNHL detected at age 5.	*STRC* NM_153700.2c.4402C>T p.(Arg1468Ter), heterozygous	4	AR	PMID 26011646
					STRC NM_153700.2c.4517T>C p.(Leu1506Pro), heterozygous	3		Novel
ES-ENT-24	xy	6	Syndromic microtia atresia	Short stature. Microcephaly. Moderate to severe developmental delay. Microtia. Cerebral and cerebellar atrophy. Cryptorchidism. Thick alveolar margins.	*ORC6* NM_014321.3.c.71 C>T p.(Ala24Val), homozygous	3	AR	Novel
ES-ENT-25	xy	46	Non-syndromic SNHL	Childhood-onset, progressive SNHL. Hypermetropia	*TMPRSS3* NM_024022.2 c.1276G>A p.(Ala426Thr), heterozygous	3	AR	PMID 21786053 PMID 28566687
					TMPRSS3 NM_024022.2c.208delC p.(His70ThrfsTer19), heterozygous	5		PMID 28566687 PMID 11907649 PMID 29293505
ES-ENT-27	xy	14	Non-syndromic SNHL	Bilateral, moderate sensorineural hearing loss.	*PDZD7* NM_001195263.1 c.1648C>T p.(Gln550Ter), homozygous	5	AR	rs1554834161 PMID 26849169 PMID 20440071
ES-ENT-30	xy	15	Non-syndromic SNHL	Bilateral, severe sensorineural hearing loss.	*CABP2* NM_001318496.1. c.292C>T p.(Arg98Ter), homozygous	4	AR	DVD ID 247342
ES-ENT-34	xy	18	Non-syndromic SNHL	Bilateral, severe SNHL	*LOXHD1* NM_144612.6c.6306T>G p.(Tyr2102Ter), heterozygous	3	AR	Novel
					LOXHD1 NM_144612.6 c.3727C>T p.(Arg1243Trp), heterozygous	3		DVD ID 408260

Subject ID	Gender	Age at referral (years)	Clinical category	Phenotype	CES results (gene, transcript, cDNA change, protein change, zygosity)	Variant classification (ACMG guidelines)	Inheritance	Reference (dbSNP, DVD or PMID)
ES-ENT-36	xx	6	Non-syndromic SNHL	Bilateral, moderate SNHL.	*MARVELD2* NM 001038603.2 c.1098G>A p.(Trp366Ter), homozygous	4	AR	DVD ID 627065
ES-ENT-38	xy	8	Non-syndromic SNHL	Bilateral, severe-to-profound SNHL.	*CDH23* NM_022124.5 c.7814A>G p.(Asn2605Ser), homozygous	4	AR	PMID 27573290 PMID 30303587
ES-ENT-41	xy	9	Non-syndromic SNHL	Congenital, bilateral, progressive, mild-to-moderate SNHL Bilateral enlarged vestibular aqueducts.	*SLC26A4* NM_000441.1c.1151A>G p.(Glu384Gly), heterozygous	5	AR	PMID 9618167 PMID 12788906 PMID 24224479
					SLC26A4 NM_000441.1c.1246A>C p.(Thr4l6Pro), heterozygous	5		PMID 9618167 PMID 12788906 PMID 24224479
ES-ENT-43	xx	26	Non-syndromic SNHL	Bilateral, fluctuating SNHL. Bilateral enlarged vestibular aqueducts. Positive vestibular dysfunction symptoms. CSF Gusher during CI. No goitre or other thyroid problem.	*SLC26A4* NM_000441.1c.626G>T p.(Gly209Val), heterozygous	4	AR	PMID 9618166 PMID 11932316 PMID 24224479 PMID 26969326
					SLC26A4 NM_000441.1c.707T>C p.(Leu236Pro), heterozygous	5		rs80338848 PMIDs PMID 9618166 PMID 20553101 PMID 10861298 PMID 26969326 PMID 12354788 PMID 18310264 PMID 20597900 PMID 15689455
ES-ENT-44	xy	10	Syndromic SNHL	Long segment Hirschsprung's disease Bilateral severe-to-profound SNHL. Blue irides. query Waardenburg syndrome type 4.	*EDN3* NM_207034.2c.476G>T p.(Cys159Phe), homozygous	4	AR	PMID 8630503
ES-ENT-45	xx	11	Non-syndromic SNHL	Congenital, bilateral SNHL. Mild learning disability.	*SLC26A4* NM_000441.1 c.716T>A p.(Val239Asp), homozygous	5	AR	PMID 12676893 PMID 12974744 PMID 25394566 PMID 30077349 PMID 16460646 PMID 22116360 PMID 27771369

Subject ID	Gender	Age at referral (years)	Clinical category	Phenotype	CES results (gene, transcript, cDNA change, protein change, zygosity)	Variant classification (ACMG guidelines)	Inheritance	Reference (dbSNP, DVD or PMID)
ES-ENT-46	xy	3	Non-syndromic SNHL	Bilateral, profound SNHL.	*CDH23* NM_022124.5.c.6133G>A p.(Asp2045Asn), homozygous	4	AR	PMID 11090341
ES-ENT-51	xy	29	Syndromic SNHL	Bilateral, progressive SNHL. Lax skin with hyperextensibility and paper-thin scars, joint hyperlaxity. Aneurysmal atrial septum. High myopia, bilateral retinal detachments and scleral rupture with minimal trauma Possibly Ehlers-Danlos syndrome. Undervirilised habitus. Learning disability	*PLOD1* NM_000302.3. c.1257G>A p.(Trp419Ter), homozygous	5	AR	Novel
ES-ENT-53	xy	34	Syndromic SNHL	Bilateral SNHL. Bilateral optic nerve dysplasia, probably colobomatous in nature.	*CDH7* NM_017780.3c.3089A>G p.(Asn1030Ser), heterozygous	4	AD	PMID 21041284 PMID 25077900
ES-ENT-55	xy	5	Syndromic SNHL	Bilateral SNHL. Microcephaly. Learning difficulties. Flat retina. Chiasmal hypoplasia	*KIF11* NM_004523.3 c.1129-1G>C, p.?, heterozygous	5	AD	Novel
ES-ENT-57	xx	2	Non-syndromic SNHL	Bilateral, moderate-to-severe SNHL	*LOXHD1* NM_144612.6c.2295G>A p.(Trp765Ter), compound heterozygous	4	AR	Novel
					LOXHD1 NM_144612.6 c.6368_6369delCA, p.(Thr2123ArgfsTer30), compound heterozygous	4		Novel
ES-ENT-60	xx	1	Syndromic SNHL	Unilateral, left SNHL. White, midline forelock. Cutaneous hypopigmentation involving all distal extremities and symmetric distribution. Motor delay	*KITLG* NM_000899.4c.443T>C p.(Ilel48Thr), homozygous	3	AR	rs1461795798

SNHL: sensorineural hearing loss, AR: autosomal recessive, AD: autosomal dominant, dbSNP: National Center for Biotechnology Information (NCBI) and National Human Genome Research Institute (NHGRI) Single Nucleotide Polymorphism Database number, PMID: PubMed unique identifier number, DVD: Deafness Variation Database ID number.
Novel indicates variants reported as novel at the time of original analysis. 5= pathogenic variant, 4 =likely pathogenic variant, 3=variant of uncertain significance (VUS) as indicated in Richards S, Aziz N, Bale S, et al., Genetics in medicine. 2015 May;17(5):40

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Chapter 4. Establishing Genotype-Phenotype Correlation in *USH2A*-related Disorders to Personalize Audiological Surveillance and Rehabilitation

Published in Otol Neurotol. 2020

OPEN

Establishing Genotype–phenotype Correlation in *USH2A*-related Disorders to Personalize Audiological Surveillance and Rehabilitation

*†Leslie P. Molina-Ramírez, *†‡Eva Lenassi, *†Jamie M. Ellingford, *†‡Panagiotis I. Sergouniotis, †Simon C. Ramsden, §||Iain A. Bruce, and *†‡Graeme C. M. Black

*Division of Evolution and Genomic Sciences, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester, UK; †North West Genomics Laboratory Hub, Manchester Centre for Genomic Medicine, St Mary's Hospital, Manchester University NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, UK; ‡Manchester Royal Eye Hospital, Manchester University Hospitals NHS Foundation Trust; §Paediatric ENT Department, Royal Manchester Children's Hospital, Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre; and ||Division of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine and Health University of Manchester, Manchester, UK

Objective: *USH2A*-related disorders are characterised by genetic and phenotypic heterogeneity, and are associated with a spectrum of sensory deficits, ranging from deaf blindness to blindness with normal hearing. It has been previously proposed that the presence of specific *USH2A* alleles can be predictive of unaffected hearing. This study reports the clinical and genetic findings in a group of patients with *USH2A*-related disease and evaluates the validity of the allelic hierarchy model.

Patients and Intervention: *USH2A* variants from 27 adults with syndromic and nonsyndromic *USH2A*-related disease were analyzed according to a previously reported model of allelic hierarchy. The analysis was replicated on genotype–phenotype correlation information from 197 individuals previously reported in 2 external datasets.

Main Outcome Measure: Genotype–phenotype correlations in *USH2A*-related disease.

Results: A valid allelic hierarchy model was observed in 93% of individuals with nonsyndromic *USH2A*-retinopathy (n = 14/15) and in 100% of patients with classic Usher syndrome type IIa (n = 8/8). Furthermore, when two large external cohorts of cases were combined, the allelic hierarchy model was valid across 85.7% (n = 78/91) of individuals with nonsyndromic *USH2A*-retinopathy and 95% (n = 123/129) of individuals with classic Usher syndrome type II ($p = 0.012$, χ^2 test). Notably, analysis of all three patient datasets revealed that USH2A protein truncating variants were reported most frequently in individuals with hearing loss.

Conclusion: Genetic testing results in individuals suspected to have an *USH2A*-related disorder have the potential to facilitate personalized audiological surveillance and rehabilitation pathways. **Key Words:** Personalized medicine—Hearing loss—Retinitis pigmentosa—*USH2A*-related disease—Usher syndrome.

Otol Neurotol **41:**431–437, 2020.

Defects in the Usher syndrome type IIa (*USH2A*) gene are an important cause of visual and auditory sensory impairment (1). To date, more than 1,050 disease-causing DNA variants (Human Gene Mutation Database, accessed July 16, 2019) have been reported. These are associated with significant clinical heterogeneity: biallelic *USH2A* mutations have been linked to combined congenital sensorineural hearing loss (SNHL) and

Address correspondence and reprint requests to Graeme C.M. Black, O.B.E., D.Phil., F.R.C.Ophth., F.MedSci., Manchester Centre for Genomic Medicine, Manchester University NHS Foundation Trust, St Mary's Hospital, Manchester M13 9WL, UK; E-mail: graeme.black@manchester.ac.uk

L.P.M.-R is supported by National Council of Science and Technology in Mexico (CONACYT).

J.M.E. is funded by a postdoctoral research fellowship from the Health Education England Genomics Education Programme.

The views expressed in this publication are those of the authors and not necessarily those of the Health Education England Genomics Education Programme.

Disclosure of funding received for this work from any of the following organizations: National Institutes of Health (NIH), Wellcome Trust, Howard Hughes Medical Institute (HHMI), and other(s).

E.L. and G.C.M.B. are supported by the Wellcome Trust as part of the Transforming Genetic Medicine Initiative grant 200990/Z/16/Z.

The authors disclose no conflicts of interest.

Supplemental digital content is available in the text.

DOI: 10.1097/MAO.0000000000002588

retinitis pigmentosa (USH2), nonsyndromic retinitis pigmentosa (nsRP), (2) and nonsyndromic SNHL (3).

Current understanding of the molecular mechanisms that underpin this variability remains incomplete despite the fact that recent observations on large cohorts of patients with *USH2A*-related disease have provided important insights (4–6). Notably, a model aiming to predict the audiological phenotype from the *USH2A* genotype has been previously proposed (4). This allelic hierarchy model suggests that genotypes including at least one ''nsRP-enriched'' allele are significantly more prevalent in individuals with nsRP than in individuals with USH2. Preservation of hearing function has also been attributable to the predicted variant consequence at the protein level: *USH2A* missense variants have shown a tendency to occur in association with development of retinopathy with normal hearing or less severe SNHL (5,6). Conversely, *USH2A* protein truncating variants have been consistently linked to the development of more severe SNHL (6). Overall, this emerging evidence may suggest that genotypic information can be used to personalise audiological surveillance of individuals with *USH2A*-disease.

In this study, we assessed the impact of *USH2A* genotypes on hearing function by investigating genotype–phenotype correlations and assessing the validity of the *USH2A* allelic hierarchy model. These results were then compared and combined with previous findings from two large external cohorts of patients with *USH2A*-related disease (5,7).

SUBJECTS AND METHODS

Study Subjects

Unrelated patients with a diagnosis of nsRP or USH2 were ascertained retrospectively through the database of the Manchester Regional Genetic Laboratory Service, Manchester, United Kingdom. Individuals with presumed biallelic disease-causing variants in *USH2A* were selected. A total of 27 unrelated individuals with *USH2A*-related disease were included in the study: 15 had nsRP, 8 had classic USH2, and 4 had atypical USH2 (please see below for relevant definitions). DNA analysis was performed using gene panel testing in all 27 cases. Fourteen of these patients were tested on a 105-gene panel and 13 samples were tested on a 176-gene panel using previously described methods (8,9). A single proband also underwent genome sequencing as previously described (10). Variants were analyzed and reported in accordance with the American College of Medical Genetics and Genomics guidelines for variant interpretation (11).

Collection and retrospective analysis of available clinical data such as visual acuity, fundoscopic findings, and, in selected cases, optical coherence tomography findings was performed. Age of onset for visual and hearing symptoms, pure-tone audiograms, and/or notes with information on previous audiological evaluations were extracted from the medical records. For the purposes of this study, patients were classified as follows: 1) nsRP, patients with retinitis pigmentosa and no complain of hearing loss, 2) classic USH2, patients with congenital/prelingual SNHL and retinitis pigmentosa, and 3) atypical USH2, patients with retinitis pigmentosa manifesting SNHL later in life.

Characterization and Classification of *USH2A* Variants From This Cohort and the Biomedical Literature

The variants identified with this study were combined with previously reported *USH2A* variants present in the LOVD-USHBases (12) and the Human Gene Mutation Database (13). To reduce the risk of including misclassified variants and to avoid the significant effects of sampling variance at very low allele counts, we classified the following variants as ''unknown/novel'': 1) all changes for which their phenotypes were not obtainable and/or 2) all changes present in less than two probands. All other variants were classified as ''nsRP-enriched'' or ''nonspecific.'' The distinction between these two categories was based on the following ratio: [probands with USH2 carrying a specific *USH2A* variant]/[probands with nsRP carrying this *USH2A* variant]. To determine a cut-off value we evaluated the c.2276G > T variant, one of the most common mutations identified in patients with nsRP and a change repeatedly reported to be enriched in individuals with nonsyndromic disease (2). Following extensive curation of the literature, the c.2276G > T variant was found in 96 individuals with nsRP and in 20 individual with Usher syndrome (4). Thus, all alleles with a ratio of 1/5 or less were considered ''nsRP-enriched'' while alleles exceeding this ratio were considered ''nonspecific.'' Importantly, the above definitions suggest that nsRP-enriched alleles are expected to have a significantly smaller effect on hearing but they might still be encountered in patients with USH2.

Assessment of the *USH2A* Allelic Hierarchy Model

Following classification of each allele, the *USH2A* allelic hierarchy model was evaluated. We considered the model to be valid in two instances: 1) an individual with nsRP carrying at least one nsRP-enriched variant, 2) an individual with Usher syndrome that has no nsRP-enriched alleles (Table 1).

In addition to testing the *USH2A* allelic hierarchy model in our cohort, we tested it in external datasets obtained from two studies reporting findings in large cohorts of individuals with *USH2A*-related disease: Pierrache et al., 2016 (148 patients; 33 nsRP, 73 USH2) and Carss et al., 2017 (49 patients, 34 nsRP, 15 USH) (5,7). These studies were selected as they contain significant numbers of patients with *USH2A*-related disease (i.e., both Usher syndrome and *USH2A*-associated nsRP) and they report their clinical and genetic findings in detail in the corresponding supplementary material sections.

Statistical analyses were performed using R version 3.5.0. Fisher's or χ^2 tests were used for categorical variables where applicable. A p value < 0.05 was considered statistically significant.

TABLE 1. *USH2A allelic hierarchy model hypothesis*

	USH2A Allele Combination	
USH2A-Phenotype	Allele 1	Allele 2
nsRP (patients with nsRP and no complaint of SNHL)	nsRP-enriched	Any[a]
Classic Usher syndrome type IIa (RP and congenital SNHL)	Nonspecific	Nonspecific

[a]Any with either an nsRP-enriched, unspecific, unknown/novel allele.

nsRP indicates nonsyndromic retinitis pigmentosa; RP, retinitis pigmentosa; SNHL, sensorineural hearing loss.

TABLE 2. *Summary of clinical findings and molecular results in patients with USH2A-related disease*

Patient-ID	Year of Birth	Sex	Clinical Diagnosis	Onset of SNHL (Decade)	Onset of Visual Symptoms (Yrs)	VA OD	VA OS	Main Ophthalmology Examination Findings	*USH2A* Allele 1	*USH2A* Allele 2
11012656	1984	M	USH2	1st	13	0.6	0.5	Blunted foveal reflexes, attenuated vessels, peripheral pigmentum	c.1558delT p.(Cys520Alafs*71)	c.1558delT p.(Cys520Alafs*71)
12000462	1967	M	USH2	1st	42	0.04	0.02	Bone spicule pigmentary changes and attenuated retinal arterioles	c.1859G > T p.(Cys620Phe)	c.2139C > T p.(Gly713Gly)
0087001	1983	F	USH2	1st	20	1.1	1	Profoundly reduced peripheral vision, peripheral pigment, attenuated vessels	c.2299delG p.(Glu767Serfs*21)	c.4321G > T p.(Glu1441X)
11013807	1971	F	USH2	1st	20	1	0.48	NA	c.4474G > T p.(Glu1492*)	c.5614delins12 p.(Ala1872Leufs*64)
13008753	1986	F	USH2	1st	19	0.3	0	ERG widespread photo receptor dystrophy, bone spicule pigmentation in mid retinal periphery	c.4645C > T p.(Arg1549*)	c.3158−2A > G p.?
15014727	1990	F	USH2	1st	23	0.1	0.1	Mid-peripheral pigmentum, ERG extinguished pattern	c.6862G > T p.(Glu2288*)	c.3407G > A p.(Ser1136Asn)
10003406	1994	F	USH2	1st	24	0.02	0	ERGs extinguished	c.5776+1G > A p.?	Exon 57 to 60 3 copies p.?
16017684	1967	F	USH2	1st	22	0.32	0.32	Peripheral pigmentum, cystoid changes	c.2299delG p.(Glu767Serfs*21)	c.6446C > A p.(Pro2149Gln)
14014093	1979	F	Atypical USH2	4th	33	0	0.2	Bone spicule pigmentation in nasal retina	c.11699A > G p.(Tyr3900Cys)	c.9371+1G > C p.?
10004715	1970	M	Atypical USH2	5th	19	0.2	0.1	Macule hole dry, severe peripheral pigmentum	c.2276G > T p.(Cys759Phe)	c.1256G > T p.(Cys419Phe)
13007042	1944	M	Atypical USH2	7th	65	0.3	0	Retinal pigmentary changes, peripheral vision severely affected; ERGs significant receptor dystrophy	c.2276G > T p.(Cys759Phe)	c.2276G > T p.(Cys759Phe)
12008422	1964	M	Atypical USH2	5th	20	0.3	0.5	Lens opacities	c.6446C > A p.(Pro2149Gln)	Ex33−34del
14020775	1972	F	nsRP	No complaint	18	0.4	0.4	Bilateral RPE changes, macular cystic changes, dry fovea	c.10073G > A p.(Cys3358Tyr)	c.1111_1112delAT p.(Ile371Phefs*)
0070378	1973	F	nsRP	No complaint	31	1	HM	ERG widespread retinopathy, peripheral pigmentum	c.10342G > A p.(Glu3448Lys)	c.2276G > T p.(Cys759Phe)
13015666	1956	M	nsRP	No complaint	17	NA	NA	NA	c.13441A > G p.(Arg4481Gly)	c.7595−3C > G, p.(Pro2533Asnfs*5)
12011272	1965	F	nsRP	No complaint	46	0.1	0.1	Focal areas of bone spicules, nasal pigmentum	c.2276G > T p.(Cys759Phe)	c.13283G > A p.(Gly4428Asp)
15002225	1960	M	nsRP	No complaint	40	0.3	0.3	Peripheral pigmentum, preservation of central area	c.14791+2T > A p.?	c.2276G > T p.(Cys759Phe)
13004912	1974	F	nsRP	No complaint	26	1.4	1.5	Bone spicules, attenuated vessels§, cystoid macular edema	c.2276G > T p.(Cys759Phe)	c.2299delG p.(Glu767Serfs*21)
12008423	1987	M	nsRP	No complaint	18	0	0.2	NA	c.4222C > T p.(Gln1408*)	c.4222C > T p.(Gln1408*)
11006504	1962	M	nsRP	No complaint	47	0.9	0.8	Peripheral pigmentum, epiretinal membranes with retinal thickening in the left eye and left cystoid macular edema	c.9413G > A p.(Gly3138Asp)	c.1804G > A p.(Gly602Arg)

TABLE 2 *(Continued)*

Patient-ID	Year of Birth	Sex	Clinical Diagnosis	Onset of SNHL (Decade)	Onset of Visual Symptoms (Yrs)	VA OD	VA OS	Main Ophthalmology Examination Findings	*USH2A* Allele 1	*USH2A* Allele 2
15008755	1951	F	nsRP	No complaint	NA	0.1	0.2	RP changes	c.4645C > T p.(Arg1549*)	c.4106C > T p.(Ser1369Leu)
15008804	1966	M	nsRP	No complaint	48	NA	NA	NA	c.10342G > A p.(Glu3448Lys)	c.10342G > A p.(Glu3448Lys)
15011185	1973	M	nsRP	No complaint	25	0.2	0.2	Peripheral pigmentum and atrophy, registered partially sighted	c.2276G > T p.(Cys759Phe)	c.9371+1G > C p.?
15017064	1945	M	nsRP	No complaint	20	0.8	0.8	Bony spicules and bilateral atrophic maculae	c.2276G > T p.(Cys759Phe)	c.12574C > T p.(Arg4192Cys)
15021183	1946	F	nsRP	No complaint	56	0.2	0.2	Limited RP	c.2276G > T p.(Cys759Phe)	c.13331delC p.(Pro4444Glnfs*17)
15005941	1985	F	nsRP	No complaint	NA	-0.1	-0.1	Bilateral dry macula	c.9974G > A p.(Gly3325Glu)	Exon10 to14 del p.(Cys549Metfs*5)
16003144	1946	F	nsRP	No complaint	20	NA	NA	Severe RP	c.3485C > A p.(Ser1162*)	c.2276G > T p.(Cys759Phe)

Atypical USH2 indicates retinitis pigmentosa and hearing loss complaint in adulthood; ERG, electroretinogram; HM, hands movement; NA, not available information; nsRP, nonsyndromic retinitis pigmentosa; OD, right eye; OS, left eye; RP, retinitis pigmentosa; RPE, retinal pigmentary epithelium; SNHL, sensorineural hearing loss; USH2, Classic Usher syndrome type IIa; VA, visual acuity.

RESULTS

Clinical Findings

Ophthalmic findings were in keeping with *USH2A*-related disease in all study subjects (Table 2). The documented mean age of onset for eye disease in the 15 individuals with nsRP and 8 with classic USH2 was 31.69 and 22.88 years, respectively. The mean age of onset for the atypical USH2 subjects was 34.25 years.

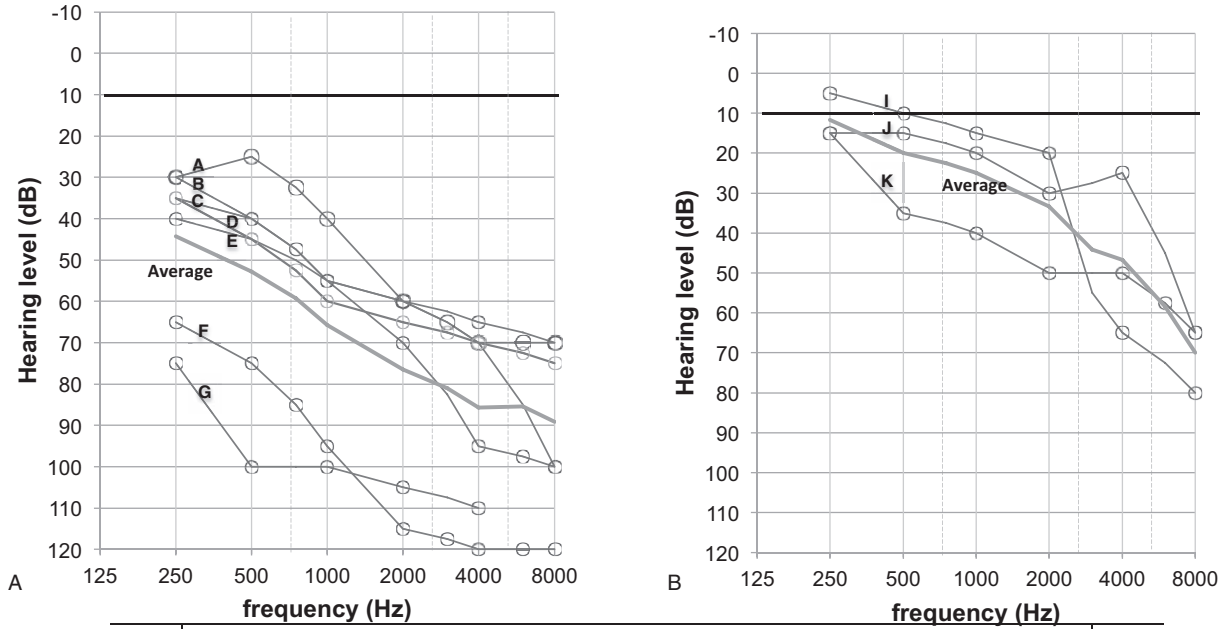
All the eight individuals with classic USH2 presented with congenital-onset SNHL. Atypical USH2 patients complain of postlingual SNHL (Table 2). Pure-tone audiometry data were available in seven of eight patients with classic USH2 and in three of four patients with atypical USH2. These audiometric findings revealed bilateral, downward-sloping, moderate-to-severe SNHL patterns across frequencies 0.5, 1, 2, 4, and 8 kHz. (Fig. 1A). The mean pure-tone average hearing threshold among individuals with classic USH2 was 59.7 dBHL (SD 23.8) for low frequencies (0.25 kHz–2 kHz), 70.1 dBHL (SD 24.7) for extended mid-frequencies (0.5 – 4 kHz) and 80.9 dBHL (SD 20.7) for high frequencies (2–8 kHz). Patients with atypical USH2 showed better hearing thresholds in comparison to the individuals with classic USH2 (Fig. 1B). For this group, the mean pure-tone average was 22.5 dBHL (SD 12.4) for low frequencies (0.25–2 kHz), 31.9 dBHL (SD 16.1) for extended mid-frequencies (0.5–4 kHz), and 50.5 dBHL (SD 17.5) for high frequencies (2–8 kHz). Six of the eight classic USH2 patients received hearing amplification with hearing aids and in one case, rehabilitation with unilateral cochlear implantation was required.

Molecular Results in *USH2A*-related Disease

Genetic analyses of the 27 patients in our cohort identified a total of 35 likely pathogenic and pathogenic variants (Fig. 2). Of these 35 variants, 19 (54.28%) were previously identified in the literature and 15 (42.85%) were novel at the time of analysis. The most prevalent mutation was the c.2276G > T p.(Cys759Phe) missense change; this was identified in 10 study subjects with nsRP. The second most prevalent change was the c.2299delG p.(Glu767Serfs*21) variant, identified in three individuals with Usher syndrome type II. Copy-number variants were detected in three cases: 1) a deletion of exons 10 to 14 in compound heterozygous state with the c.9974G > A p.(Gly3325Glu) variant in an individual with nsRP, 2) a deletion of exons 33 to 34 in a compound heterozygous state with the c.6446C > A p.(Pro2149Gln) variant in a patient with atypical USH2, and 3) a duplication of exons 57 to 60 in a presumed compound heterozygous state with the c.5776+1G > A variant in a patient with classic USH2.

Replication of the *USH2A*-Allelic Hierarchy Model

From the variants reported for the present cohort, we categorized 4 of 35 as nsRP-enriched (11%), 11 of 35 variants as nonspecific (31%), and 20 of 35 variants as



C

	Audiogram ID	Case ID	Frequency (Hz) 250	500	750	1000	2000	3000	4000	6000	8000	PTA 0.5-4	
Usher syndrome type IIa	A	12000462	30	25	32.5	40	60	65	70	70	70	45	Hearing level (decibels)
	B	16017684	30	40	47.5	55	70	82.5	95	97.5	100	58	
	C	13008753	35	40	47.5	55	60	62.5	65	67.5	70	51	
	D	10003406	35	45	52.5	60	65	67.5	70	72.5	75	55	
	E	11012656	40	45	50	55	60	65	70	85	100	54	
	F	11013807	65	75	85	95	115	117.5	120	120	120	94	
	G	87001	75	100	100	100	105	107.5	110	NA	NA	98	
Atypical USH type IIa	I	10004715	5	10	12.5	15	20	55	65	72.5	80	23	
	J	14014093	15	15	17.5	20	30	27.5	25	45	65	21	
	K	12008422	15	35	37.5	40	50	50	50	57.5	65	38	

FIG. 1. Pure-tone audiogram of patients with classic USH2 *(A)* and patients with atypical USH2 *(B)*.

unknown/novel (57%). The localization of these variants in the respective protein domains is shown in Figure 2. We found that the *USH2A*-allelic hierarchy model was valid in 14 of 15 cases (93%) with nsRP. Also, all the eight cases with classic USH2 (100%) had nonspecific alleles in keeping with the model. Of note, the four cases with atypical USH2 carried either an nsRP-enriched allele or an unknown/novel allele.

We then combined our cohort with two previously reported, large external datasets (Table S2, http://links.lww.com/MAO/A933) (5,7). These 3 cohorts altogether included 220 individuals with *USH2A*-related disease: 91 with nsRP (41.3%) and 129 individuals with classic USH2 (54.5%); the 4 cases with atypical USH2 from the present cohort were excluded from this analysis. A total of 172 *USH2A* alleles were reported accounting for disease in all these individuals. Twelve *USH2A* variants (14%) were categorized as nsRP-enriched alleles, 61 (32%) as nonspecific alleles, and 99 (57%) remained in the unknown/novel category (Table S1, http://links.lww.com/MAO/A932). The allelic hierarchy model was valid in 86% (n = 78/91) of individuals with nsRP and 95% (n = 123/129) of individuals with USH2 across all cohorts.

USH2A Variants and Predicted Consequence at Protein Level

After examining the proportion of *USH2A* variants based on their predicted consequence at protein level, we observed a tendency for two missense *USH2A* variants to be found in probands with nsRP in comparison with those with classic USH2:

These were observed in 42% (n = 38/91) of the nsRP cases and in 11% (14/129) of the USH2 cases. In contrast, presumed two protein truncating variants, in homozygous or compound heterozygous state, were reported in 6% (6/91) of nsRP cases and in 48% (62/129) of cases with classic USH2.

DISCUSSION

Personalized medicine proposes to optimize patient care based on individual conditions and molecular diagnoses. Confirmation of a molecular diagnosis in individuals with inherited SNHL is swiftly gaining a role in clinical care as it has the potential to enable prompt, accurate, and personalized diagnosis and prognosis (14) as well as a personalized decision-support for rehabilitation strategy planning (15). Furthermore, presymptomatic, newborn, and preimplantation genomic testing are

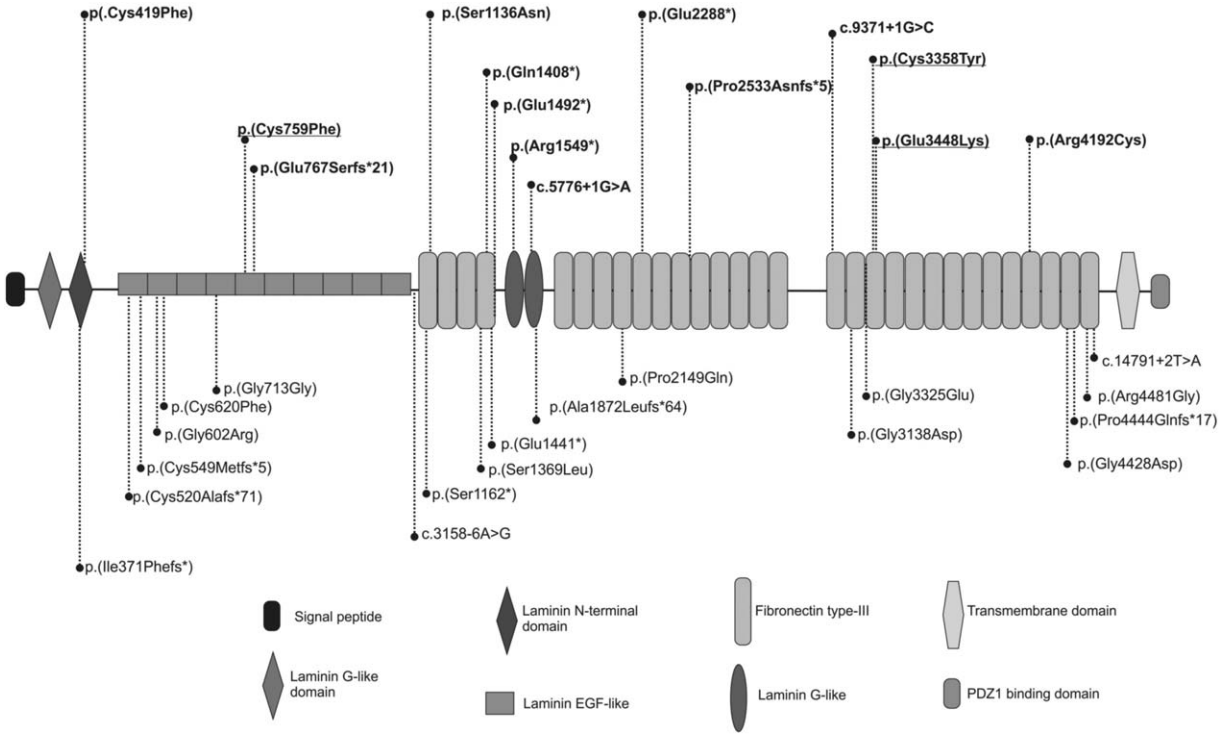


FIG. 2. Schematic illustration of USH2A protein domains and localization of *USH2A* alleles identified and characterized in the Manchester Centre for Genomic Medicine patient group. Mutations shown above schematic are nonspecific (bold) and nsRP-enriched (underlined) *USH2A* alleles. Unknown *USH2A* alleles are displayed below schematic. EGF indicates epidermal growth factor.

gaining momentum (16) and, as a result, predicting the natural history of a disorder from a specific genotype is becoming increasingly more relevant.

The present study illustrates the importance of establishing a molecular diagnosis in *USH2A*-related disease, a clinically and genetically heterogeneous condition frequently associated with SNHL. The presence or absence of congenital hearing loss is a key clinical feature that impacts management and quality of life of patients affected by this disorder. Aiming to evaluate the extent to which the audiological phenotype can be predicted by the *USH2A* genotype, we performed a detailed genotype–phenotype correlation study and assessed the validity of a previously proposed *USH2A*-allelic hierarchy model (4). This model classifies *USH2A* disease-causing alleles into three categories (nsRP-enriched, nonspecific, and unknown/novel) according to each allele's prevalence in each phenotype. The model is an extension of a concept first described by Rivolta et al. (2) who identified that the c.2276G > T variant is not significantly associated with hearing loss. Overall, we observed that patients harboring at least one nsRP-enriched or unknown/novel allele (presumed to be nsRP-enriched) were consistently reported not to have prelingual-onset SNHL. The allelic hierarchy model was valid in 86% for individuals with nsRP in a combined dataset including the present cohort and two external cohorts (5,7). The common c.2276G > T allele was identified in 42 (38/91) of cases with nsRP and only 4.6% (6/129) of cases with classic USH2. We speculate that nsRP-enriched alleles allow complete or partial preservation of USH2A protein function in outer hair cell stereocilia leading to normal hearing or mild SNHL. As a result, affected individuals may develop normal speech and require fewer audiological assessments. Notably, individuals presumed to have *USH2A*-related nsRP may manifest SNHL later in life. While it is important to consider that the presence of SNHL in nonsyndromic retinopathy may be linked to additional extrinsic (e.g., infection, trauma, etc.) or intrinsic mechanisms (e.g., patient 13007042 manifesting likely age-related hearing loss), one cannot exclude the possibility of this being associated with underlying defects in *USH2A*. Future functional investigations of mutant *USH2A* variants (and their interaction with the rest of the Usher syndrome complex) are expected to provide important insights into the role of *USH2A* in photoreceptors and stereocilia. The identification of biallelic nonspecific alleles in an individual should alert the clinician to conduct a closer and detailed audiological evaluation. Notably, progression of hearing loss has been documented in *USH2A*-related disorders (17–19). A recent study reported individuals carrying nonspecific alleles, such as c.1256G > T or c.2299delG, to have more rapid progression and more severe hearing thresholds (6,19). It can be speculated that faster progression is associated with the presence of nonspecific alleles or protein truncating variants. As a result, there might be a

link between the need and timing of cochlear implantation and the *USH2A* genotype. Further studies should determine whether early intervention in patients with specific genotypes would be beneficial.

The *USH2A* allelic hierarchy model has a number of limitations. First, many individuals affected by *USH2A*-related disorders carry at least one unknown/novel variant. This reflects the frequency of previously unreported *USH2A* alleles and, to a lesser extent, the scarce phenotypic information available in some scientific reports. The value of the model is limited in such cases as an accurate prediction can only be made when a previously unreported change is combined with nsRP-enriched alleles. We believe that this issue may be partly addressed with the increasing availability of well-phenotyped cohorts of patients with *USH2A*-related disease. Second, the allelic hierarchy model is probabilistic: even if someone carries an nsRP-enriched allele, they might still present with childhood-onset SNHL—it is just that the likelihood of developing more severe hearing deterioration is significantly reduced. Outcome prediction based on genotype is a clearly complex and multidimensional task, even for a monogenic disorder such as *USH2A*-related diseases. The clinical presentation is likely to be due to a complex interplay of the inherited *USH2A* variants, changes in other genes related with SNHL, and/or environmental factors. Wider adoption of genomic testing in clinic will enable the identification of more patients with *USH2A*-related disorders, enabling more refined/accurate models to be developed. Lastly, due to the retrospective design of the study, data from the three datasets were combined without accounting for the methodological heterogeneity of the ascertained groups.

In summary, our findings replicate the *USH2A*-allelic hierarchy model and propose that careful analysis and classification of variants in *USH2A*-related disorders can guide targeted audiological surveillance. Detailed audiological phenotyping in large genotyped cohorts, functional work on the effect of variants in cochlear hair cell function, and study of the interaction between Usher syndrome associated proteins are expected to provide important insights. Finally, further research should be undertaken to determine whether *USH2A* genotype can predict the need to perform cochlear implantation in individuals with *USH2A*-related disorders.

Acknowledgments: The authors are grateful to Andrew Causon, from the Richard Ramsden Centre for Hearing Implants, Manchester University NHS Foundation Trust, for assistance with the audiological data presented in this article. We would also like to acknowledge the following sources of funding: Retina UK and Fight for Sight (GR586, RP Genome Project), the National Institute for Health Research (NIHR) Manchester Biomedical Research Centre, the UK Inherited Retinal Disease Consortium (UK IRDC) funded by Retina UK and Fight for Sight and the NIHR Clinical Lecturer Programme (CL-2017-06-001, PIS).

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Supplementary data

Molina-Ramírez LP, Lenassi E, Ellingford JM, Sergouniotis PI, Ramsden SC, Bruce IA, Black GCM. Establishing Genotype-phenotype Correlation in USH2A-related Disorders to Personalize Audiological Surveillance and Rehabilitation. Otol Neurotol. 2020 Apr;41(4):431-437.

Table S2. Genotype-phenotype correlation and allelic hierarchy model classification in *USH2A*-related disease.

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
Carss et al 2017	Classic USH2	c.3831_3834delACTAinsG, p.Leu1278del	c.920_923dupGCCA, p.His308GlnfsTer16	Unknown	non-specific	1	Y
Carss et al 2017	Classic USH2	c.2299delG, p.Glu767SerfsTer21	c.2299delG, p.Glu767SerfsTer21	non-specific	non-specific	1	Y
Carss et al 2017	Classic USH2	c.12819T>A, p.Tyr4273Ter	c.1055C>T, p.Thr352Ile	Unknown	non-specific	1	Y
Carss et al 2017	Classic USH2	c.12954C>A, p.Tyr4318Ter	c.5603T>G, p.Phe1868Cys	non-specific	Unknown	1	Y
Carss et al 2017	Classic USH2	c.2299delG, p.Glu767SerfsTer21	c.2081G>A, p.Cys694Tyr	non-specific	Unknown	1	Y
Carss et al 2017	Classic USH2	c.14426C>T, p.Thr4809Ile	c.2299delG, p.Glu767SerfsTer21	non-specific	non-specific	1	Y
Carss et al 2017	Classic USH2	c.7595-3C>G, p.?	c.2299delG, p.Glu767SerfsTer21	non-specific	non-specific	1	Y
Carss et al 2017	Classic USH2	c.10561T>C, p.Trp3521Arg	c.7595-2144A>G, p.?	non-specific	non-specific	1	Y
Carss et al 2017	Classic USH2	c.12309delC, p.Phe4103LeufsTer11	c.12309delC, p.Phe4103LeufsTer11	Unknown	Unknown	1	Y
Carss et al 2017	Classic USH2	c.2299delG, p.Glu767SerfsTer21	Deletion 1:216240159-222780953	non-specific	Unknown	1	Y
Carss et al 2017	Classic USH2	c.2299delG, p.Glu767SerfsTer21	c.820C>G, p.Arg274Gly	non-specific	non-specific	1	Y
Carss et al 2017	Classic USH2	c.3395G>A, p.Gly1132Asp	c.2994A>T, p.Arg998Ser; c.895delC, p.Gln299AsnfsTer37	non-specific	Unknown	1	Y
Carss et al 2017	Classic USH2	c.11694delC, p.Asn3899ThrfsTer34	c.3158-6A>G, p.?	Unknown	Unknown	1	Y

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
Carss et al 2017	Classic USH2	c.9860_9873delATGATGGCCATGGC, p.His3287ProfsTer54	c.9860_9873delATGATGGCCATGGC, p.His3287ProfsTer54	Unknown	Unknown	1	Y
Carss et al 2017	nsRP	c.6050-1G>A, p.?	c.2299delG, p.Glu767SerfsTer21	Unknown	non-specific	1	Y
Carss et al 2017	nsRP	c.7334C>T, p.Ser2445Phe	c.3902G>T, p.Gly1301Val	Unknown	nsRP-enriched	1	Y
Carss et al 2017	nsRP	c.9882C>G, p.Cys3294Trp	c.653T>A, p.Val218Glu	nsRP-enriched	non-specific	1	Y
Carss et al 2017	nsRP	c.10342G>A, p.Glu3448Lys	c.6670G>T, p.Gly2224Cys	nsRP-enriched	unknown	1	Y
Carss et al 2017	nsRP	c.10073G>A, p.Cys3358Tyr	c.920_923dupGCCA, p.His308GlnfsTer16	nsRP-enriched	non-specific	1	Y
Carss et al 2017	nsRP	c.10073G>A, p.Cys3358Tyr	c.2299delG, p.Glu767SerfsTer21	nsRP-enriched	non-specific	1	Y
Carss et al 2017	nsRP	c.13576C>T, p.Arg4526Ter	c.4222C>T, p.Gln1408Ter	unknown	unknown	1	Y
Carss et al 2017	nsRP	c.13335_13347delGAACATGGACTCTinsCTTG, p.Glu4445_Ser4449delinsAspLeu	c.11864G>A, p.Trp3955Ter	nsRP-enriched	non-specific	1	Y
Carss et al 2017	nsRP	c.12575G>A, p.Arg4192His	c.10073G>A, p.Cys3358Tyr	nsRP-enriched	nsRP-enriched	1	Y
Carss et al 2017	nsRP	c.5012G>A, p.Gly1671Asp	c.5012G>A, p.Gly1671Asp	unknown	unknown	1	Y
Carss et al 2017	nsRP	c.6446C>A, p.Pro2149Gln	c.2276G>T, p.Cys759Phe	unknown	nsRP-enriched	1	Y
Carss et al 2017	nsRP	c.4510dupA, p.Arg1504LysfsTer26	c.2276G>T, p.Cys759Phe	unknown	nsRP-enriched	1	Y

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
Carss et al 2017	nsRP	c.7358T>A, p.Val2453Asp	c.4027A>C, p.Asn1343His	unknown	unknown	1	Y
Carss et al 2017	nsRP	c.10073G>A, p.Cys3358Tyr	c.2276G>T, p.Cys759Phe	nsRP-enriched	nsRP-enriched	1	Y
Carss et al 2017	nsRP	c.13335_13347delGAACATGGACTCTinsCTTG, p.Glu4445_Ser4449delinsAspLeu	Deletion 1:216259365-216318209	nsRP-enriched	unknown	1	Y
Carss et al 2017	nsRP	c.10073G>A, p.Cys3358Tyr	c.2276G>T, p.Cys759Phe	nsRP-enriched	nsRP-enriched	1	Y
Carss et al 2017	nsRP	c.12145G>A, p.Ala4049Thr	c.9785G>T, p.Gly3262Val	unknown	unknown	1	Y
Carss et al 2017	nsRP	c.5012G>A, p.Gly1671Asp	c.5012G>A, p.Gly1671Asp	unknown	unknown	1	Y
Carss et al 2017	nsRP	c.2276G>T, p.Cys759Phe	c.2276G>T, p.Cys759Phe	nsRP-enriched	nsRP-enriched	1	Y
Carss et al 2017	nsRP	c.13126T>G, p.Trp4376Gly	c.12874A>G, p.Asn4292Asp	nsRP-enriched	nsRP-enriched	1	Y
Carss et al 2017	nsRP	c.2802T>G, p.Cys934Trp	Deletion 1:216009683-216011948	non-specific	unknown	1	Y
Carss et al 2017	nsRP	c.13396C>T, p.Pro4466Ser ; c.13396C>T, p.Pro4466Ser	Deletion 1:215836170-215851932	Unknown	unknown	1	Y
Carss et al 2017	nsRP	c.13331C>T, p.Pro4444Leu	c.8223+1G>C, NA	nsRP-enriched	Unknown	1	Y
Carss et al 2017	nsRP	c.2299delG, p.Glu767SerfsTer21	c.2276G>T, p.Cys759Phe	non-specific	nsRP-enriched	1	Y
Carss et al 2017	nsRP	c.9571-2A>G, p.?	c.2299delG, p.Glu767SerfsTer21	nsRP-enriched	non-specific	1	Y
Carss et al	nsRP	c.13331C>T, p.Pro4444Leu	c.13126T>G, p.Trp4376Gly	nsRP-	nsRP-	1	Y

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
2017				enriched	enriched		
Carss et al 2017	nsRP	c.11507C>T, p.Pro3836Leu	c.9571-2A>G, p.?	unknown	nsRP-enriched	1	Y
Carss et al 2017	Classic USH2	c.2299delG, p.Glu767SerfsTer21	c.2276G>T, p.Cys759Phe	non-specific	nsRP-enriched	1	N
Carss et al 2017	nsRP	c.13274C>T, p.Thr4425Met	c.8981G>A, p.Trp2994Ter	non-specific	non-specific	1	N
Carss et al 2017	nsRP	c.13316C>T, p.Thr4439Ile	c.12574C>T, p.Arg4192Cys	non-specific	non-specific	1	N
Carss et al 2017	nsRP	c.11156G>A, p.Arg3719His	c.920_923dupGCCA, p.His308GlnfsTer16	non-specific	non-specific	1	N
Carss et al 2017	nsRP	c.12505A>G, p.Thr4169Ala	c.12505A>G, p.Thr4169Ala	non-specific	non-specific	1	N
Carss et al 2017	nsRP	c.926C>T, p.Pro309Leu	c.100C>T, p.Arg34Ter	non-specific	non-specific	1	N
Carss et al 2017	nsRP	c.15433G>A, p.Val5145Ile	c.2522C>A, p.Ser841Tyr	non-specific	non-specific	1	N
Carss et al 2017	nsRP	c.13274C>T, p.Thr4425Met	c.11875_11876delCA, p.Gln3959AsnfsTer53	non-specific	non-specific	1	N
Pierrache et al 2016	Classic USH2	c.187C>T, p.(Arg63*)	c.949C>A, p.[(Tyr318CysfsX17)]	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.486-14G>A, p.?	c.12729G>A, p.(Trp4243*)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.653T>A, p.(Val218Glu)	c.6967C>T *, p.(Arg2323*)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.785-?_+5572+?dup deletion exon 5-27	c.1256G>T, p.(Cys419Phe)	unknown	non-specific	2	Y
Pierrache et	nsRP	c.917_918insGCTGp.(Ser307Leufs*	c.11007C>A, p.(Ser3669Arg)	unknown	unknown	1	Y

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
al 2016		17)					
Pierrache et al 2016	Classic USH2	c.920_923dup, p.(His308fs)	c.7950dup, p.(Asn2651Glnfs*10)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.949C>A, p.[(Tyr318CysfsX17)]	c.4773del, p.(Val1592*)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.949C>A, p.[(Tyr318CysfsX17)]	c.11676del, p.Lys3892Asnfs*41	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.949C>A, p.[(=,Tyr318CysfsX17)]	c.11864G>A, p.(Trp3955*)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.1036A>C, p.(Asn346His)	c.4810G>A, p.(Asp1604Asn)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.1036A>C, p.(Asn346His)	c.7301-?_10939+?39-55indel	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.1036A>C, p.(Asn346His)	c.10525A>T **, p.(Lys3509*)	non-specific	non-specific	1	Y
Pierrache et al 2016	nsRP	c.1036A>C, p.(Asn346His)	c.14545T>C, p.(Trp4849Arg)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.1039G>C, p.(Asp347His)	c.1039G>C, p.(Asp347His)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.1227G>A, p.(Trp409*)	c.1227G>A, p.(Trp409*)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.1227G>A, p.(Trp409*)	c.1256G>T, p.(Cys419Phe)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.1227G>A, p.(Trp409*)	c.11875C>T, p.(Gln3959*)	non-specific	unknown	1	Y
Pierrache et al 2016	nsRP	c.1227G>A, p.(Trp409*)	c.12575G>A, p.(Arg4192His)	non-specific	nsRP-enriched	1	Y
Pierrache et	Classic	c.1227G>A, p.(Trp409*)	c.13262T>C, p.(Leu4421Pro)	non-	unknown	1	Y

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
al 2016	USH2			specific			
Pierrache et al 2016	Classic USH2	c.1256G>T, p.(Cys419Phe)	c.1606T>C, p.(Cys536Arg)	non-specific	non-specific	5	Y
Pierrache et al 2016	Classic USH2	c.1256G>T, p.(Cys419Phe)	c.4056G>A, p.(Trp1352*)	non-specific	unknown	1	Y
Pierrache et al 2016	nsRP	c.1256G>T, p.(Cys419Phe)	c.4106C>T, p.(Ser1369Leu)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.1256G>T, p.(Cys419Phe)	c.7121-8313_11048-962delins12	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.1256G>T, p.(Cys419Phe)	c.8079G>A, p.Trp2693X	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.1256G>T, p.(Cys419Phe)	c.10387+2T>C	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.1256G>T, p.(Cys419Phe)	c.10525A>T, p.(Lys3509*)	non-specific	non-specific	4	Y
Pierrache et al 2016	nsRP	c.1256G>T, p.(Cys419Phe)	c.10901A>C, p.(His3634Pro)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.1256G>T, p.(Cys419Phe)	c.11864G>A, p.(Trp3955*)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.1256G>T, p.(Cys419Phe)	c.14545T>C, p.(Trp4849Arg)	non-specific	unknown	2	Y
Pierrache et al 2016	Classic USH2	c.1328+1G>A, p.?	c.2140C>T, p.(Gln714*)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.1606T>C, p.(Cys536Arg)	c.1606T>C, p.(Cys536Arg)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.1606T>C, p.(Cys536Arg)	c.6658-2A>G, p.?	non-specific	unknown	1	Y
Pierrache et	Classic	c.1606T>C, p.(Cys536Arg)	c.10525A>T, p.(Lys3509*)	non-	non-	1	Y

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
al 2016	USH2			specific	specific		
Pierrache et al 2016	Classic USH2	c.1606T>C, p.(Cys536Arg)	c.14131C>T, p.(Gln4711*)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.1808G>A, p.(Gly603Glu)	c.7501C>T, p.(Gln2501*)	unknown	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.1876C>T, p.(Arg626*)	c.1876C>T, p.(Arg626*)	non-specific	non-specific	1	Y
Pierrache et al 2016	nsRP	c.1965T>G, p.(Cys655Thr)	c.6926G>T, p.(Cys2309Phe)	unknown	non-specific	1	Y
Pierrache et al 2016	nsRP	c.1978G>A, p.(Gly660Arg)	c.9258G>T, p.Gln3086His	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.2014C>T, p.(Gln672*)	c.2014C>T, p.(Gln672*)	unknown	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.2242C>T, p.(Gln748*)	c.4405C>T, p.(Gln1469*)	non-specific	unknown	1	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.486-14G>A, p.?	nsRP-enriched	non-specific	4	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.949C>A, p.[(Tyr318CysfsX17)]	nsRP-enriched	non-specific	1	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.1256G>T, p.(Cys419Phe)	nsRP-enriched	non-specific	1	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.2276G>T, p.(Cys759Phe)	nsRP-enriched	nsRP-enriched	2	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.2299del, p.(Glu767Serfs*21)	nsRP-enriched	non-specific	6	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.5516T>A, p.(Val1839Glu)	nsRP-enriched	unknown	1	Y
Pierrache et	nsRP	c.2276G>T, p.(Cys759Phe)	c.5576T>G, p.(Phe1859Cys)	nsRP-	unknown	1	Y

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
al 2016				enriched			
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.7121-?_11047+?, p.?	nsRP-enriched	unknown	1	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.7950dup, p.(Asn2651Glnfs*10)	nsRP-enriched	non-specific	1	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.8723_8724del, p.(Val2908Glyfs*29)	nsRP-enriched	unknown	1	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.10073G>A, p.(Cys3358Tyr)	nsRP-enriched	nsRP-enriched	1	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.10525A>T, p.(Lys3509*)	nsRP-enriched	non-specific	1	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.10689T>A, p.(Tyr3563*)	nsRP-enriched	unknown	1	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.14174G>A *, p.(Trp4725*)	nsRP-enriched	unknown	1	Y
Pierrache et al 2016	nsRP	c.2276G>T, p.(Cys759Phe)	c.14803C>T, p.(Arg4935*)	nsRP-enriched	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.920_923dup, p.(His308fs)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.1036A>C, p.(Asn346His)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.1039G>C, p.(Asp347His)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.1227G>A, p.(Trp409*)	non-specific	non-specific	2	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.1256G>T, p.(Cys419Phe)	non-specific	non-specific	5	Y
Pierrache et	Classic	c.2299del, p.(Glu767Serfs*21)	c.1606T>C, p.(Cys536Arg)	non-	non-	4	Y

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
al 2016	USH2			specific	specific		
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.2168-1G>C, p.(Leu724Valfs*31)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.2299del, p.(Glu767Serfs*21)	non-specific	non-specific	15	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.4732C>T, p.(Arg1578Cys)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.4957C>T, p.(Arg1653*)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.5516T>A, p.(Val1839Glu)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.6722C>T, p.(Pro2241Leu)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.7121-8313_11048-962delins12	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.7187G>A, p.(Trp2396*)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.9815C>T, p.(Pro3272Leu)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.10525A>T, p.(Lys3509*)	non-specific	non-specific	2	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.13316C>T, p.(Thr4439Ile)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.13508_13523delinsAGp.(Val4503Glufs*54)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.2299del, p.(Glu767Serfs*21)	c.15053-2A>T *, p.?	non-specific	unknown	1	Y
Pierrache et	Classic	c.2299del, p.(Glu767Serfs*21)	deletion exon 12-13	non-	unknown	1	Y

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
al 2016	USH2			specific			
Pierrache et al 2016	Classic USH2	c.2391_2392del, p.(Cys797X)	c.7595-2144A>G, p.(Lys2532Thrfs*56)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.2555-1G>C, p.?	c.2710_2720dup, p.(Leu908Profs*63)	unknown	unknown	1	Y
Pierrache et al 2016	nsRP	c.3045C>G, p.(His1015Gln)	c.12394del, p.(Leu4132Trpfs*35)	unknown	unknown	1	Y
Pierrache et al 2016	nsRP	c.3368A>G, p.(Tyr1123Cys)	c.7054C>T, p.(Pro2352Ser)	non-specific	unknown	1	Y
Pierrache et al 2016	nsRP	c.4362_4367delinsACTC	c.13274C>T, p.(Thre4425Met)	unknown	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.4645C>T, p.(Arg1549*)	c.4645C>T, p.(Arg1549*)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.6544_6548dup, p.(His2183fs)	c.14289del, p.(Ile4764Serfs*42)	unknown	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.6722C>T, p.(Pro2241Leu)	c.13316C>T, p.(Thr4439Ile)	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.6722C>T, p.(Pro2241Leu)	c.7121-?_11074+?p?	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.7121-8313_11048-962delins12	c.7121-8313_11048-962delins12	non-specific	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.7121-8313_11048-962delins12	c.8954delG, p.(Gly2985Alafs*3)	non-specific	unknown	1	Y
Pierrache et al 2016	nsRP	c.7853G>A, p.(Trp2618*)	c.9258G>T, p.? [p.Gln3086His]	unknown	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.7931G>A, p.(Trp2644*)	c.11819A>C, p.(Tyr3940Ser)	unknown	non-specific	1	Y
Pierrache et	Classic	c.8079G>A, p.(Trp2876*)	c.12806C>A, p.(Pro4269His)	non-	unknown	1	Y

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
al 2016	USH2			specific			
Pierrache et al 2016	Classic USH2	c.8628G>A, p.(Trp2876*)	c.14977_14978del, p.(Phe4993Profs*7)	unknown	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.8723_8724del, p.(Val2908Glyfs*29)	c.10525A>T, p.(Lys3509*)	unknown	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.9372-?_9570+?	c.9372-?_9570+?	unknown	unknown	1	Y
Pierrache et al 2016	nsRP	c.9433C>T, p.(Leu3145Phe)	c.9815C>T, p.(Pro3272Leu)	unknown	non-specific	1	Y
Pierrache et al 2016	nsRP	c.10073G>A, p.(Cys3358Tyr)	c.13274C>T, c.(Thr4425Met)	nsRP-enriched	non-specific	1	Y
Pierrache et al 2016	Classic USH2	c.10561T>C, p.(Trp3521Arg)	c.14802C>G, p.(Tyr4934*)	non-specific	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.12992A>G, p.(Tyr4331Cys)	c.12992A>G, p.(Tyr4331Cys)	unknown	unknown	1	Y
Pierrache et al 2016	Classic USH2	c.14803C>T, p.(Arg4935*)	c.15089C>A, p.(Ser5030*)	non-specific	non-specific	1	Y
Pierrache et al 2016	nsRP	c.1256G>T, p.(Cys419Phe)	c.13274C>T, c.(Thr4425Met)	non-specific	non-specific	1	N
Pierrache et al 2016	Classic USH2	c.2276G>T, p.(Cys759Phe)	c.1206G>T, p.(Lys402Asn)	nsRP-enriched	unknown	1	N
Pierrache et al 2016	Classic USH2	c.2276G>T, p.(Cys759Phe)	c.1256G>T, p.(Cys419Phe)	nsRP-enriched	non-specific	2	N
Pierrache et al 2016	Classic USH2	c.2276G>T, p.(Cys759Phe)	c.7132_7133del, p.(Tyr2378Hisfs*39)	nsRP-enriched	unknown	1	N
Pierrache et al 2016	Classic USH2	c.2276G>T, p.(Cys759Phe)	c.14803C>T, p.(Arg4935*)	nsRP-enriched	non-specific	1	N
Pierrache et	nsRP	c.2296T>C, p.(Cys766Arg)	c.4732C>T, p.(Arg1578Cys)	non-	non-	1	N

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
al 2016				specific	specific		
Pierrache et al 2016	nsRP	c.2299del, p.(Glu767Serfs*21)	c.4133T>C, p.(Leu1378Pro)	non-specific	non-specific	1	N
Pierrache et al 2016	nsRP	c.2299del, p.(Glu767Serfs*21)	c.15433G>A, p.(Val5145Ile)	non-specific	non-specific	1	N
Pierrache et al 2016	nsRP	c.10525A>T, p.(Lys3509*)	c.13274C>T, c.(Thr4425Met)	non-specific	non-specific	1	N
This study (MCGM)	nsRP	c.10073G>A p.(Cys3358Tyr)	c.1111_1112delATp.(Ile371Phefs*)	nsRP-enriched	unknown	1	Y
This study (MCGM)	nsRP	c.10342G>Ap.(Glu3448Lys)	c.2276G>Tp.(Cys759Phe)	nsRP-enriched	nsRP-enriched	1	Y
This study (MCGM)	nsRP	c.13441A>G p.(Arg4481Gly)	c.7595-3C>G, p.(Pro2533Asnfs*5)	Unknown (novel)	non-specific	1	Y
This study (MCGM)	nsRP	c.2276G>T p.(Cys759Phe)	c.13283G>A p.(Gly4428Asp)	nsRP-enriched	Unknown (novel)	1	Y
This study (MCGM)	nsRP	c.14791+2T>A p.?	c.2276G>T p.(Cys759Phe)	Unknown (novel)	nsRP-enriched	1	Y
This study (MCGM)	Classic USH2	c.1558delTp.(Cys520Alafs*71)	c.1558delTp.(Cys520Alafs*71)	unknown	unknown	1	Y
This study (MCGM)	Classic USH2	c.1859G>T p.(Cys620Phe)	c.2139C>T, p.(Gly713Gly)	Unknown (novel)	unknown	1	Y
This study (MCGM)	nsRP	c.2276G>Tp.(Cys759Phe)	c.2299delG, p.(Glu767Serfs*21)	nsRP-enriched	non-specific	1	Y
This study (MCGM)	Classic USH2	c.2299delG p.(Glu767Serfs*21)	c.4321G>T (p.Glu1441X)	non-specific	unknown	1	Y
This study (MCGM)	Classic USH2	c.4474G>T p.(Glu1492*)	c.5614delins12 p.(Ala1872Leufs*64)	non-specific	unknown	1	Y
This study	Classic	c.4645C>Tp.(Arg1549*)	c.3158-2A>G p.?	non-	unknown	1	Y

Study	Diagnosis	Allele 1	Allele 2	Allele 1	Allele 2	Total of cases	Model Y/N
(MCGM)	USH2			specific			
This study (MCGM)	nsRP	c.9413G>A, p.(Gly3138Asp)	c.1804G>A, p.(Gly602Arg)	unknown	Unknown (novel)	1	Y
This study (MCGM)	nsRP	c.4645C>T p.(Arg1549*)	c.4106C>T, p.(Ser1369Leu)	non-specific	unknown	1	Y
This study (MCGM)	nsRP	c.10342G>A p.(Glu3448Lys)	c.10342G>A, p.(Glu3448Lys)	nsRP-enriched	nsRP-enriched	1	Y
This study (MCGM)	nsRP	c.2276G>T p.(Cys759Phe)	c.9371+1G>C p.?	nsRP-enriched	non-specific	1	Y
This study (MCGM)	Classic USH2	c.6862G>T p.(Glu2288*)	c.3407G>A p.(Ser1136Asn)	non-specific	non-specific	1	Y
This study (MCGM)	nsRP	c.2276G>T p.(Cys759Phe)	c.12574C>T p.(Arg4192Cys)	nsRP-enriched	non-specific	1	Y
This study (MCGM)	nsRP	c.2276G>T p.(Cys759Phe)	c.13331delC p.(Pro4444Glnfs*17)	nsRP-enriched	Unknown (novel)	1	Y
This study (MCGM)	Classic USH2	c.5776+1G>A p.?	exon57to60 3 copiesp.?	non-specific	unknown	1	Y
This study (MCGM)	nsRP	c.9974G>A p.(Gly3325Glu)	exon10to14 del hetp.(Cys549Metfs*5)	Unknown (novel)	unknown	1	Y
This study (MCGM)	nsRP	c.3485C>Ap.(Ser1162*)	c.2276G>Tp.(Cys759Phe)	Unknown (novel)	nsRP-enriched	1	Y
This study (MCGM)	Classic USH2	c.2299delG p.(Glu767Serfs*21)	c.6446C>Ap.(Pro2149Gln)	non-specific	unknown	1	Y
This study (MCGM)	nsRP	c.4222C>Tp.(Gln1408*)	c.4222C>Tp.(Gln1408*)	non-specific	non-specific	1	N

nsRP non-syndromic retinitis pigmentosa, USH2: Usher syndrome type IIa; Y valid model, N not valid model; MCGM Manchester Centre for Genomic Medicine

Table S1. Allele classification as per the *USH2A* allelic hierarchy model

	Nucleotide change	Amino acid change	Key references
nsRP-enriched *USH2A* alleles	c.9882C>G	p.(Cys3294Trp)	(1,2)
	c.9571-2A>G	p. ?	(2,3)
	c.4027A>C	p.(Asn1343His)	(2,4)
	c.3902G>T	p.(Gly1301Val)	(2,5)
	c.2276G>T	p.(Cys759Phe)	(4,6–19)
	c.13335_13347 delGAACATGGACTCTinsCTTG	p.(Glu4445_Ser4449delinsAspLeu)	(2,4,20)
	c.13331C>T	p.Pro4444Leu	(2)
	c.13126T>G	p.(Trp4376Gly)	(2)
	c.12874A>G	p.(Asn4292Asp)	(2,21)
	c.12575G>A	p.(Arg4192His)	(4,5,11,20,22,23)
	c.10342G>A	p.(Glu3448Lys)	(2,3,20,24–26)
	c.10073G>A	p.(Cys3358Tyr)	(2,5,11,19,20,22,23,27–29)
non-specific *USH2A* alleles	c.9815C>T	p.(Pro3272Leu)	(18,26,30–35)
	c.949C>A	p.(Tyr318Cysfs*17)	(6,18,32,33,36–40)
	c.9371+1G>C	p.(?)	(5,22,33,41)
	c.920_923dupGCCA	p.(His308Glnfs*16)	(9,12,17,18,33,36,39,42–46)
	c.8981G>A	p.(Trp2994*)	(2,47)
	c.820C>G	p.(Arg274Gly)	(2,18,19)
	c.8079G>A	p.(Trp2876*)	(27,48)
	c.7950dup	p.(Asn2651Glnfs*10)	(27,33)
	c.7595-2144A>G	p.(Lys2532Thrfs*56)	(2,18,22,27,33,38,44,49–52)
	c.7595-3C>G	p.(Pro2533Asnfs*5)	(5,18,22,50,53–55)
	c.7121-8313_11048-962delins12	p.?	(27)
	c.6926G>T	p.(Cys2309Phe)	(4,27,33)
	c.6862G>T	p.(Glu2288*)	(22,27,33,47)
	c.6722C>T	p.(Pro2241Leu)	(27,56)
	c.653T>A	p.(Val218Glu)	(2,4,6,18,40,46,50,53)
	c.5776+1G>A	p.(?)	(4,5,7,29,32,33,39,45,49)
	c.4957C>T	p.(Arg1653*)	(18,23,27,33,39)
	c.486-14G>A	p.?	(18,23,27,53,57)
	c.4732C>T	p.(Arg1578Cys)	(18,22,27)
	c.4645C>T	p.(Arg1549*)	(7,18–20,22,27,33,38)
	c.4474G>T	p.(Glu1492*)	(16,18,19,33,52)

	Nucleotide change	Amino acid change	Key references
	c.4222C>T	p.(Gln1408*)	(17,33,58)
	c.4133T>C	p.(Leu1378Pro)	(18,22,27)
	c.3407G>A	p.(Ser1136Asn)	(22,33), this study
	c.3395G>A	p.(Gly1132Asp)	(2,59)
	c.3368A>G	p.(Tyr1123Cys)	(27,53,59)
	c.280T>G	p.(Cys934Trp)	(2,5,33,35,60,61)
	c.2522C>A	p.(Ser841Tyr)	(2,18,19,22,45)
	c.2391_2392del	p.(Cys797*)	(18,27)
	c.2299delG	p.(Glu767Serfs*21)	(5,7–9,12,13,15–19,22,31–33,36–38,40,42,45,46,49,50,52,59,62–72)
	c.2296T>C	p.(Cys766Arg)	(4,27,33,52)
	c.2242C>T	p.(Gln748*)	(27,36)
	c.2168-1G>C	p.(Leu724Valfs*31)	(18,38,71)
	c.1978G>A	p.(Gly660Arg)	(18,27,33)
	c.187C>T	p.(Arg63*)	(10,12,22,48,49,59,71)
	c.1876C>T	p.(Arg626*)	(12,17,18,27,33,39,46,48,50,61,65,66,73)
	c.1606T>C	p.(Cys536Arg)	(11,12,22,27,36,39,40,59)
	c.15433G>A	p.(Val5145Ile)	(2,4,18,20,22,27)
	c.15089C>A	p.(Ser5030*)	(27,33,40,50)
	c.14977_14978del	p.(Phe4993Profs*7)	(18,27,33,49)
	c.14803C>T	p.(Arg4935*)	(6,8,18,20,27,33,50,53,65)
	c.14426C>T	p.(Thr4809Ile)	(2,5,18,33,40,65)
	c.14131C>T	p.(Gln4711*)	(7,27,33,44)
	c.13316C>T	p.(Thr4439Ile)	(2,5,18,22,27,33,39)
	c.1328+1G>A	p.?	(19,27)
	c.13274C>T	p.(Thr4425Met)	(2,23,27,33,37,50)
	c.12954C>A	p.(Tyr4318Ter)	(2,20,33,39)
	c.12574C>T	p.Arg4192Cys	(2,6,33,34,43,74), this study
	c.1256G>T	p.(Cys419Phe)	(5,17,18,22,27,31,36,42,75)
	c.12505A>G	p.(Thr4169Ala)	(2,18,22)
	c.1227G>A	p.(Trp409*)	(27,36)
	c.11875_11876delCA	p.(Gln3959Asnfs*53)	(2,18,22,33,39,47,48)
	c.11864G>A	p.(Trp3955*)	(2,27,48)
	c.11819A>C	p.(Tyr3940Ser)	(4–7,17,18,20,22,30,32,33,37–40,44,47,49,59)
	c.11156G>A	p.(Arg3719His)	(2,5,20,33,76)
	c.10561T>C	p.(Trp3521Arg)	(2,4,20,22–24,27,33,39,48)
	c.10525A>T	p.(Lys3509*)	(23,27,48)

	Nucleotide change	Amino acid change	Key references
	c.1055C>T	p.(Thr352Ile)	(2,6,32,33,39,40,50)
	c.1039G>C	p.(Asp347His)	(27)
	c.1036A>C	p.(Asn346His)	(12,18,22,27,33,36,39,44,50,66)
	c.100C>T	p.(Arg34*)	(2,12,16,22,33,44,45,77)
Unknown and/or novel *USH2A* alleles	Exon 57 to 60 duplication		(5)
	Exon 33-34 deletion		-
	Exon 12-13 deletion	p.(Ile658Phefs*23)	(27)
	Exon 10-14 deletion	p.(Cys549Metfs*5)	-
	Deletion 1:216259365-216318209		-
	Deletion 1:216240159-222780953		-
	Deletion 1:216009683-216011948		-
	Deletion 1:215836170-215851932		-
	c.9974G>A	p.(Gly3325Glu)	-
	c.9860_9873delATGATGGCCATGGC	p.(His3287ProfsTer54)	-
	c.9785G>T	p.(Gly3262Val)	(2)
	c.9433C>T	p.(Leu3145Phe)	(26)
	c.9413G>A	p.(Gly3138Asp)	(56)
	c.9372-?_9570+?		-
	c.926C>T	p.(Pro309Leu)	(2,33)
	c.9258G>T	p.(Gln3086His)	(27)
	c.917_918insGCTG	p.(Ser307Leufs*17)	(23)
	c.8954delG	p.(Gly2985Alafs*3)	(19)
	c.8723_8724del	p.(Val2908Glyfs*29)	(27,37)
	c.8628G>A	p.(Trp2876*)	(27,49)
	c.8223+1G>C	p.(?)	-
	c.7931G>A	p.(Trp2644*)	(18,27)
	c.7853G>A	p.(Trp2618*)	(27)
	c.785-?_+5572+?dup deletion exon 5-27		(27)
	c.7501C>T	p.(Gln2501*)	(27)
	c.7358T>A	p.(Val2453Asp)	(2)
	c.7334C>T	p.(Ser2445Phe)	(2)
	c.7301-?_10939+?39-55indel		(27)
	c.7187G>A	p.(Trp2396*)	(27)
	c.7132_7133del	p.(Tyr2378Hisfs*39)	(27)
	c.7121-?_11047+?	p.(?)	(27)
	c.7054C>T	p.(Pro2352Ser)	(27,56)

	Nucleotide change	Amino acid change	Key references
	c.6967C>T *	p.(Arg2323*)	(27)
	c.6670G>T	p.(Gly2224Cys)	(2,78)
	c.6658-2A>G	p.?	(27)
	c.6544_6548dup	p.(His2183fs)	(27)
	c.6446C>A	p.(Pro2149Gln)	(2); this study
	c.6050-1G>A	p.?	(2)
	c.5614delins12	p.(Ala1872Leufs*64)	-
	c.5603T>G	p.(Phe1868Cys)	(2,22)
	c.5576T>G	p.(Phe1859Cys)	(23,27)
	c.5516T>A	p.(Val1839Glu)	(27)
	c.5012G>A	p.(Gly1671Asp)	(2,22)
	c.4810G>A*	p.(Asp1604Asn)	(27)
	c.4773del	p.(Val1592*)	(27)
	c.4510dupA	p.(Arg1504Lysfs*26)	(2,22)
	c.4405C>T	p.(Gln1469*)	(36)
	c.4362_4367delinsACTC		(27)
	c.4321G>T	p.(Glu1441*)	-
	c.4106C>T	p.(Ser1369Leu)	(27)
	c.4056G>A	p.(Trp1352*)	(27)
	c.3831_3834delACTAinsG	p.(Leu1278del)	-
	c.3485C>A	p.(Ser1162*)	-
	c.3158-6A>G	p.(?)	(2)
	c.3045C>G	p.(His1015Gln)	(27)
	c.2994A>T	p.(Arg998Ser)	(2)
	c.895delC	p.(Gln299Asnfs*37)	(2)
	c.2710_2720dup	p.(Leu908Profs*63)	(27)
	c.2555-1G>C	p.?	(27)
	c.2140C>T	p.(Gln714*)	-
	c.2139C>T	p.(Gly713Gly)	-
	c.2081G>A	p.(Cys694Tyr)	(2,5)
	c.2014C>T	p.(Gln672*)	(27)
	c.1965T>G	p.(Cys655Thr)	(27)
	c.1859G>T	p.(Cys620Phe)	-
	c.1808G>A	p.(Gly603Glu)	(27)
	c.1804G>A	p.(Gly602Arg)	-
	c.1558delT	p.(Cys520Alafs*71)	-

	Nucleotide change	Amino acid change	Key references
	c.15053-2A>T *	p.(?)	(27)
	c.14802C>G	p.(Tyr4934*)	(27)
	c.14791+2T>A	p.(?)	-
	c.14545T>C	p.(Trp4849Arg)	(27)
	c.14289del	p.(Ile4764Serfs*42)	(27)
	c.14174G>A *	p.(Trp4725*)	(27)
	c.13576C>T	p.(Arg4526*)	(2,79)
	c.13508_13523delinsAG	p.(Val4503Glufs*54)	(27)
	c.13441A>G	p.(Arg4481Gly)	-
	c.13396C>T	p.(Pro4466Ser)	(2)
	c.13283G>A	p.(Gly4428Asp)	-
	c.13262T>C	p.(Leu4421Pro)	(27)
	c.12992A>G	p.(Tyr4331Cys)	(27)
	c.12819T>A	p.(Tyr4273*)	(2)
	c.12806C>A	p.(Pro4269His)	(18,27)
	c.12729G>A	p.(Trp4243*)	(27)
	c.12394del	p.(Leu4132Trpfs*35)	(27)
	c.12309delC	p.(Phe4103Leufs*11)	(2)
	c.12145G>A	p.(Ala4049Thr)	(2,18)
	c.1206G>T	p.(Lys402Asn)	(27)
	c.11875C>T	p.(Gln3959*)	(27)
	c.11699A>G	p.(Tyr3900Cys)	-
	c.11694delC	p.(Asn3899ThrfsTer34)	(2)
	c.11676del	p.Lys3892Asnfs*41	(27)
	c.11507C>T	p.(Pro3836Leu)	(2)
	c.1111_1112delAT	p.(Ile371Phefs*)	(7,80)
	c.11007C>A	p.(Ser3669Arg)	(23)
	c.10901A>C	p.(His3634Pro)	(27)
	c.10689T>A	p.(Tyr3563*)	(27)
	c.10387+2T>C p.(?)	p.?	(27)
	c.13331delC	p.(Pro4444Glnfs*17)	-

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Chapter 5. Discussion

At the time of commencing this PhD, NGS testing in hearing loss had been mostly applied for clinical research and gene discovery purposes. Interestingly, despite the growing use of NGS testing, traditional single-gene testing has continued to dominate routine clinical practice. The vast genetic heterogeneity of hearing loss poses a challenge for diagnosis. As such, robust and comprehensive diagnostic testing methods are needed to overcome the low yield achieved by conventional aetiological investigations.

The central theme of this thesis was to determine whether genomic sequencing can effectively improve diagnosis and management of patients with congenital and childhood-onset genetic hearing loss. For this purpose, this study set out with the aim to evaluate the diagnostic capabilities of clinical genomic sequencing in monogenic disorders, to analyse the current status of genomic sequencing offered in the clinic to patients with hearing loss, and to analyse a phenotype-genotype correlation to identify how molecular diagnosis can inform patient prognosis and further management.

Research presented in Chapter 2 of this thesis demonstrates that personalised virtual gene panels are an effective approach to filtering variants from CES data in patients with monogenic disorders. Personalised virtual gene panels are created with input from predetermined gene lists, physician's choice and/or use of phenotype-ontologies, which is a novel approach to tailor CES analysis to a patient's clinical needs. Since the start of this thesis, other groups have reported use of virtual gene panels in monogenic disease, with a variant burden ranging from 42 to 90 coding variants per case (312,403). Here, there is a notable reduction of variant burden, with a median of 7.38 variants per case. These encouraging results suggest that personalised virtual gene panels can yield manageable variant datasets for filtration and further clinical interpretation, without compromising the diagnostic strength/capabilities.

Findings from Chapter 3 show the diagnostic rates and the clinical benefit of CES in patients with ear and hearing disorders. Before the start of this PhD project, genomic sequencing testing in hearing loss had been more applied in controlled research settings. Here, the study provides with a real-world picture of the current application of CES in hearing loss. The study illustrates how CES data analysis, which was made under the personalised virtual gene panel approach, can be adapted to different types of ear and hearing disorders. In terms of diagnostic utility, CES identified a molecular diagnosis in 30% of patients. Notably, a great proportion of diagnoses were associated with genes that are not part of routine genetic investigations in patients with hearing loss. CES results informed clinical decision making in 25% (n=15/60) of patients. Of these, molecular diagnosis prompted additional diagnostic investigations for early diagnosis in family members of only 5 patients. Notably, the number of actions triggered by molecular diagnoses remains small and is mostly consists of referrals to other specialists' clinics.

Results from Chapter 4 show that the identification of genetic diagnosis can potentially inform about hearing loss progression in patients affected by *USH2A*-related disease. Retinitis pigmentosa specific alleles (e.g., c.2276G>T) and non-truncating *USH2A* variants are more prevalent in patients with visual phenotypes only and in less proportion affected by mild sensorineural hearing loss. In contrast, truncating variants or nonspecific *USH2A* alleles (c.2299delG) are more commonly associated with more severe sensorineural hearing loss. This supports previous reports of patients with c.2299delG who have even undergone cochlear implantation (404). Timely detection of these specific variants may help predict which patients will present with more severe hearing loss and which patients are likely not to exhibit any degree of hearing loss. As such, it could allow to personalise audiological monitoring based on genotypes and identify patients who are likely to more severe hearing impairment and therefore, more likely to require the evaluation of other hearing rehabilitation strategies, for instance, cochlear implantation.

Overall, the findings of these chapters support the fact that genomic sequencing can improve diagnostic investigations in hearing loss, which can be done in an effective manner in clinical settings. In terms of management and patient care,

however, while genotype phenotype correlations clearly indicate patterns that give more information about prognosis of hearing loss, it does not trigger clinical actions yet and more evidence is needed to support an objective use of molecular diagnosis for precision medicine in patients with hearing loss.

5.1 Molecular diagnosis for precision medicine in hearing loss.

Precision medicine is an approach that enables the efficient and optimal course of patient care by incorporating individual genetic, clinical, environmental and/or demographic data. Defining with precision an aetiological diagnosis is a critical component of this approach to personalised patient care. The identification of a confirmed genetic diagnosis in inherited conditions facilitates access to genetic counselling for patients and their family members. For individuals with hearing loss, it can also inform decisions around diagnosis and management. The early confirmation of a genetic diagnosis can enable the timely differentiation between non-syndromic and syndromic hearing loss, allowing for the subsequent anticipation of additional medical needs prior to the onset of signs and symptoms of non-otological pathology. Moreover, it has been proposed that molecular diagnosis could inform prognosis on the course of hearing loss (i.e., severity and velocity of deterioration), thus possibly influencing therapeutic decision-making based on prediction of outcomes. As such, this precision medicine approach has the potential to shift care and surveillance from being 'reactive' to 'dynamic', thus removing the reliance upon the early identification of children with failing hearing.

Chapter 4 shows how the identification of a genetic diagnosis can inform the course of hearing loss, which can potentially be used to inform a 'dynamic' and personalised audiological surveillance model. The investigation of the *USH2A* allelic hierarchy model (402) in the cohort studied in Chapter 4 revealed differences in the genotype of patients with *USH2A*-related disease. There was a consistent identification of 'retinal-specific' *USH2A* variants in patients with unaffected hearing whereas 'unspecific' *USH2A* variants were mostly found in patients with more severe SNHL. Protein truncating *USH2A* variants were also found to be enriched in patients with more severe SNHL.

The findings described in Chapter 4 corroborate previous research on the phenotypic variability in *USH2A*-related disease. Hartel et al (2016) et al identified a

correlation between the presence protein truncating *USH2A* variants in association with more severe hearing impairment (405). Hartel et al (2016) found that genotypes consisting of any protein truncating variant (either in homozygous or compound heterozygous state) or the common c.2299delG *USH2A* allele are more associated with severe hearing phenotypes in comparison to those of patients harbouring non-truncating *USH2A* variants (406). Findings from Lee et. al. (2020) further reinforce this correlation, identifying protein truncating variants in patients with Usher syndrome IIA who presented with rapid deterioration of hearing loss in early adulthood (407). This existing evidence and the results from Chapter 4 support the characterisation of genotypes as a clinical prognostic factor, where either by applying the *USH2A* allelic hierarchy model or identifying protein truncating variants, it can help distinguish patients with different audiological needs and plan their audiological surveillance accordingly. As a result, this can allow clinicians to anticipate and monitor closely the progression of audiological phenotypes where protein truncating variants or 'non-specific' *USH2A* alleles are identified.

It is important to acknowledge limitations of the use of the *USH2A* allelic hierarchy model. Firstly, ethnic differences in the *USH2A* variant spectrum should be considered in the interpretation of variants in individuals from specific ethnic background (408). Secondly, there is a level of uncertainty as to the pathogenicity of *USH2A* variants reported in databases. This may be partly explained based on the fact that *USH2A* is a gene postulated to have with significant tolerance to variation (409). Thirdly, the classification of many variants requires detailed otological and audiological information about onset, progression, and severity of hearing loss, which is not very well documented in many studies. Finally, while we can observe a higher prevalence of truncating variants and non-specific USH2A alleles in patients with more severe SNHL, it is still uncertain what are the implications for our patients. The findings in the study confirm the phenotype-genotype correlation but it cannot yet be used to prognosticate with absolute certainty. The diagnosis will be informative for patients and clinicians in terms of genetic counselling and to get a deeper understanding of their condition, but it is still unlikely to change clinical management as there is still need for further

experimental research to confirm underlying mechanisms of specific *USH2A* variants at protein level.

The use of genetic diagnosis to inform prognosis and clinical management could benefit patients with other overlapping non-syndromic and syndromic SNHL, for instance, those affected by variants in *CDH23*, *MYO7A* and *USH1C*. The study of the genetic and clinical heterogeneity in these conditions has identified comparable trends to *USH2A*-related disease. By studying two cohorts of individuals with Usher syndrome type 1D (USH1D) and non-syndromic *CDH23*-related SNHL DFNB12 respectively, Pennings et al., detected missense *CDH23* variants enriched in patients with DFNB12 whereas splice-site variants were found in patients with USH1D (410), who were also reported with more severe SNHL. Schultz et al., identified that while missense CDH23 variants are found *in trans* in patients with non-syndromic CDH23 SNHL, patients with Usher syndrome type 1D have at least one truncating CDH23 variant in their genotype (87). A recent meta-analysis identified a total of 19 variants (predominantly missense) in *CDH23* as well as *MYO7A* and *USH1C* consistently reported in homozygous state associated with non-syndromic SNHL (125). Nonetheless, all this evidence suggests that early identification genetic diagnosis in any Usher syndrome gene is of huge clinical significance given the high prevalence and clinical overlap found in patients with congenital or prelingual hearing impairment. This information can help prioritise hearing habilitation, particularly in patients who will inevitably present with visual impairment.

Genetic diagnosis can contribute to distinguishing patients with different prognosis and potentially inform decision making for cochlear implant candidacy screening. Genetic diagnosis has been recognised as a potential predictor of postoperative outcomes in cochlear implant recipients. In the editorial article included in Chapter 1, I discussed potential applications of the implementation of genomic-driven patient care in hearing loss and cochlear implantation. Previous studies that evaluated cochlear implant recipients with confirmed genetic diagnoses have noted that the location of the defect in the auditory pathway could influence cochlear implant outcomes (285,286,411–414). Some studies have reported CI patients with variants in genes expressed in the spiral ganglion (e.g., *TMPRSS3*, *PJVK*) with poor post-CI outcomes whereas studies in patients with variants in genes with mostly

intracochlear expression (e.g., *GJB2*, *SLC26A4*, *OTOF*) have shown better post-CI outcomes. These findings strengthen the role of genetic diagnosis as a potential prognostic factor, but also highlight the need to investigate in more detail this relationship, considering the many external factors that can influence outcomes. A noteworthy example of this is the findings by Eppsteiner (2012) and Shearer (2017) on poor speech outcomes in CI patients with *TMPRSS3* variants, which are contrary to findings by Nishio and Miyigawa (2015), which argue that *TMPRSS3* patients are good candidates for CI and electro-acoustic stimulation, despite previous findings. A possible explanation could be the lack of uniformity in these studies. Genetic information as a prognostic factor should be examined across multiple studies to understand how it can be applied to tailor patient care and improve clinical outcomes. Longitudinal and solid prognostic studies are needed, taking into account environmental factors that can possibly influence phenotypic variability. Future studies correlating genetic diagnosis and clinical characteristics of cochlear implant recipients should include the use of standardised sets of outcomes (415,416).

5.2 Use of clinical exome sequencing as a tool for genomic diagnosis

Being the most comprehensive approach to the identification of molecular diagnosis, genomic testing strategies (i.e., targeted gene panels, whole exome or whole genome sequencing) play a key role in the establishment of precision medicine. However, the adoption of NGS testing in the clinic has faced some challenges. Genomic testing, in particular exome and genome sequencing, can generate large datasets that require significant work sifting through data to identify candidate variants. This can cause significant impact on turnaround time and ultimately, delay identification of molecular diagnoses. Timing is paramount in clinical decision making and a delayed diagnosis can hinder further preventative actions and shape the prognosis of the patient.

There is a constant need to identify methods to further increase efficiency in analysis of genomic data, in particular methods applicable where analysis can represent a major burden, such as the clinical diagnostic laboratory. Chapters 2 and 3 illustrate the usage and diagnostic value of CES in patients with rare monogenic disorders, and in patients with ear abnormalities and hearing impairment, respectively. The diagnostic rate (24% in rare disease) reported in Chapter 2 is consistent with those reported in published studies on CES (318,322,327,336,417). The findings in Chapter 2 reflect the efficiency at maintaining a clinical focus of variant filtering through clinically driven virtual gene panels to produce reduced and manageable variant workloads without having a negative impact on current diagnostic yield.

There are clear advantages that illustrate the clinical diagnostic utility and cost-effectiveness of the personalised virtual gene panel approach. Historically, targeted disease NGS gene panels have proven diagnostic utility for a wide range of medical conditions, including retinal dystrophy, immunodeficiencies, and epilepsy (282,287,293–295). However, regular modification, updates and validation of a targeted disease NGS panel may result in long-term increased laboratory costs (289). Investing in an exome capture kit and performing *in silico* targeted gene

analysis represents a more cost-effective alternative. With the personalised virtual gene panel approach, the analysis can be adapted to a patient's phenotype and, since the panel is created with input from different sources, it increases the probability of selecting the correct gene and obtaining a diagnosis.

For patients with inconclusive results, sequencing data can be stored and periodically reanalysed as indicated by the clinician. If needed, CES data can be reanalysed by modifying the virtual gene panel adjusting or reselecting new genes *ad hoc* based on any new change or progression of clinical features. As such, there would not be any need to process additional blood samples. Avoiding the need for retesting relieves patients' discomfort and burden caused by phlebotomies and transportation to hospital. In addition, it can reduce overutilization of laboratory services caused by sample processing and wet NGS laboratory costs. Archiving sequencing data represents a minimum cost when compared to NGS costs. Nonetheless, while reporting of results represents only about 4% of the total cost of processing of sequencing testing (418), clinical interpretation of CES data can be lengthy and costly. Furthermore, each case has a different level of complexity and thus a variable time dedicated to clinical interpretation. Personalised virtual gene panels significantly reduce the number of variants filtered for interpretation in comparison with other virtual gene panels reported in the literature (312,403) and compared with not using any phenotype-driven approach at all. As such, the reduced variant workload can potentially reduce analysis time spent per sample. Consequently, it simplifies CES analysis and allows clinical scientists to dedicate more time to more complex cases. Finally, conducting proband-only CES represents a safe strategy that can minimise costs. While trio analysis increases the possibility of diagnosis and determines phasing of compound heterozygous variants; the identification of potential compound heterozygous or *de novo* variants using proband-CES can be alternatively validated using Sanger sequencing in parental samples.

An important limitation of the study is that, due to the retrospective nature of the study and the use of historical cohorts, it was not possible to compare between personalised gene panels against the use of phenotype-driven variant prioritisation tools, for example, Exomiser (270) or against using available targeted NGS gene panels. A comparison would have been beneficial to quantify with more precision

the clinical utility across the different types of CES analysis, as it would have tested whether gene panels created from multiple sources can achieve higher diagnostic yields in comparison with a predetermined targeted NGS gene panel.

5.2.1 Clinical exome sequencing in hearing loss

Results from chapter 2 allowed me to deepen my understanding of the methodology and diagnostic implications of CES. In Chapter 3, I dive into the use of CES in the context of patients with hearing impairment and/or ear abnormalities. The use of CES in patients with hearing impairment reported an overall diagnostic rate of 31%, with the identification of 40 distinct variants in 24 genes. With the exception of *SLC26A4*, variants were identified in genes that are not typically recommended in guidelines as part of standard genetic investigations for SNHL. The diagnostic rate obtained specifically in the subset of patients with SNHL (38%) is consistent with other studies on the use of CES in SNHL (where *GJB2/GJB6* diagnoses are excluded) (326,336–338). The diagnostic utility obtained through CES is comparable to that of targeted NGS gene panels (284,340), with the advantage of being able to reanalyse data and modify the original gene selection without incurring in additional wet laboratory costs should initial analysis not produce any potential findings.

A limitation of the study here presented is that it was not possible to determine whether the clinical actions prompted by CES findings resulted in clinical benefit for the patients. Results of 25% of patients with positive diagnosis prompted referral to specialty clinics, audiology reassessments and/or further testing for family members. Early diagnosis in an asymptomatic family member was enabled by the molecular diagnosis identified in only one patient via CES. However, it was not possible to access follow-up information. It is proposed that a precise genomic diagnosis can optimise patient care and improve clinical outcomes. This can only be evaluated by following up on the number of interventions, diagnostic or therapeutic, actioned by a molecular diagnosis and quantify their clinical benefit in order to determine the clinical value of conducting genomic investigations.

There are still some previously mentioned limitations when using CES in patients with SNHL. Previous research has shown that CES testing has inadequate read depth of genes such as *STRC* and *OTOA* due to poor coverage of highly homologous sequences (337,340). Pre-screening for variants in these genes using single gene testing before CES has been proposed as an alternative for circumventing this limitation (340). Of note, two heterozygous variants in *STRC* were identified in one patient in our study. Although there was no record of parental testing that could confirm phasing and compound heterozygous state, this finding reflects the diagnostic resolution of CES in the laboratory. Nonetheless, given that disease-causing variants in *STRC* are a common cause of childhood-onset, moderate and progressive SNHL, it would be reasonable to consider *STRC* pre-screening in patients whose hearing impairment fits into this description (342). In addition to this, it is important to acknowledge a limitation in the bioinformatics pipeline applied to the present study. Analyses were confined to the identification of single nucleotide variants and small insertions and deletions. CES analysis in hearing loss could benefit from including CNV detection, as this type of genetic variation has shown to play an important role in the pathogenesis of genetic hearing impairment (339,419). Studies on the use of read-depth algorithms for CNV identification in exome sequencing data have reported increase in diagnostic yield (420,421). Rajagopalan et. al., (2020) validated a modified workflow for the CNV detection algorithm ExomeDepth (421), which allowed the successful identification of CNVs in *STRC* in exome sequencing data (422). Future incorporation of promising modified strategies for CNV identification applied to current bioinformatics pipelines may potentially maximise the diagnostic capability of CES in hearing loss.

5.3 Looking ahead to genomic-driven healthcare in hearing loss

As discussed earlier, since the beginning of this Ph.D., significant developments have taken place in the genomic diagnostic arena. Genetic testing is transitioning from the analysis of specific variants and/or genes to whole genome sequencing. Large-scale whole genome sequencing studies worldwide have enabled the transition of such genomic strategies from research laboratories into routine healthcare. One of the most important accomplishments of the 100,000 Genomes Project (394) is the establishment of a framework for the implementation of genomic medicine services within the UK National Health Service (NHS). Within the Rare Disease chapter, the 100,000 Genomes Project created a research and diagnostic opportunity for individuals with hearing loss disorders including congenital hearing impairment, auditory neuropathy spectrum disorder and ear malformations. This represented an unprecedented opportunity for researchers, laboratory scientists, geneticists, cochlear implant surgeons and audiologists to initiate an ongoing collaboration with the aim to improve the discovery of genomic variants associated with the development of hearing impairment, understand their association with disease progression and personalise therapeutic approaches and improve outcomes.

In parallel to the work conducted in this PhD and the research outputs presented in this thesis, I participated in the selection, recruitment, phenotyping of patients with hearing impairment and deafness to the 100,000 Genomes project at the North West Genomic Laboratory Hub in Manchester. A total of 103 families (231 individuals: 38 singletons, 65 families) were recruited to the project under the hearing disorders category. Fifty-eight percent of probands were identified and recruited from the Cochlear Implant Programme and 42% were referred from clinical genetics service. Following the protocols established by the 100,000 Project, I identified eligible participants by assessing phenotype information through the paediatric cochlear implant clinics. Recruitment of participants included a wide range of activities such as the development of visual materials, informative talks (given to health care professionals, audiologists, consultant CI surgeons, speech therapist scientists, teachers of the deaf), patient engagement activities during CI family information sessions and paediatric CI clinics, obtaining informed consents,

liaising with surgical teams and research staff for sample collections, as well as the capture of demographic and clinical data. Through these activities I was able to act as a facilitator between clinics and genomic services, establishing connections within clinical services and raising awareness of the value of genomic investigations and the potential utility of these in clinical decision-making. My background as an ENT surgeon and previous clinical experience allowed me to communicate the close relationship between the clinic and genomic molecular strategies. Where applicable, I provided input at 100,000 Genomes Project MDT meetings where challenging rare disease and hearing loss cases were reviewed. I believe that these activities have set the precedent to continue a collaborative effort that in the future could facilitate multidisciplinary and integrated patient care between ENT/Audiological and Genomic medicine services.

The 100,000 Genomes Project is now continuously delivering outputs. As analyses continue, confirmed genetic diagnoses are being returned to the NHS for feedback to patients and families while increasing the discovery of pathogenic variants associated with hearing impairment. In addition to genetic counselling, these results will determine the need to conduct further clinical tests, foresee any potential complications and highlight the potential need for preventive clinical actions in patients with dormant phenotypes. The vast amount of genomic and clinical data generated through the 100,000 Genomes Project will undoubtedly provide further therapeutic options for individuals with congenital hearing loss and will continue to arise as WGS is being rolled out more widely in the NHS over the coming years. Furthermore, collaborations and recruitment activities conducted for the 100,000 Genomes Project are closing the gap between genetic and ENT/cochlear implantation services, having illustrated and refined the 'roadmap' for the evaluation of patients with hearing impairment of likely genetic origin. Genomic variant analysis, delivery of results and integration of clinical and genomic data are evolving processes that in time will demonstrate a level of cost-effectiveness that justifies the integration of such services into routine NHS practice.

5.4 Future work

Genomic sequencing provides an unprecedented research arena to underpin yet unknown molecular causes of hearing loss and continue increasing the discovery of pathogenic variants. The 100,000 Genomes Project represented the first step towards a mainstream genomic medicine and set the basis for the launch of the Genome UK strategy. With the Genome UK programme moving forward, it is of huge importance to continue improving the application of genomic testing in patients with hearing impairment. In this context, there is still a gap in understanding of the relationship between gene expression/impact of the variant on protein function, hearing loss pattern, affected frequencies, progression, velocity of deterioration and post-CI outcomes. There is still a need to fully comprehend how molecular mechanisms of disease can best inform clinical decision making and evaluate the clinical added value of such diagnostic interventions.

Increasing the number of patients undergoing genomic sequencing will increase the number of genotype-phenotype correlations and as a result, better define the molecular aetiology underlying the development hearing impairment. Importantly, to allow for further reduction of costs, sequencing must be done at scale (418). Whilst large datasets pose an analytical challenge, further development of current bioinformatic solutions and, streamlining of analysis through virtual gene panels can facilitate analysis in a timeframe of clinical utility, with the potential to reduce time-related costs of interpretation. The current approach for variant prioritisation could be adjusted for the analysis of exomes/genomes of individuals with hearing loss. With the aim of further improving efficiency in variant interpretation, it would be valuable to investigate the performance of the virtual gene panel approach shown in this thesis with cross-referencing of specific databases such as the Deafness Variation Database (DVD). The Deafness Variation Database (DVD) (http://deafnessvariationdatabase.org/) is an open-access comprehensive resource that collects genetic variants in known hearing loss genes (https://hereditaryhearingloss.org/) from major public databases (423). Genetic variants in this database are expertly annotated, curated and evidence-based classified with the aim to unify variant information and facilitate variant interpretation. Additionally, it would be worth considering the application of AMP-

ACMG hearing loss variant interpretation guidelines (247) for hearing loss patients, as an alternative to using standard guidelines (AMP-ACMG (251) and ACGS guidelines (Ellard et al., 2020)) and to determine the effect of a tailored interpretation on diagnostic rate. Close collaboration between ENT/Audiology and genomic medicine services will be pivotal in the collection of relevant data utilised as supporting evidence in variant interpretation.

Through WGS, research and diagnostic possibilities expand through the creation of a unique genomic hearing loss dataset that enables the study of non-coding regions. Whilst most currently known clinically relevant variants are located in the exome, approximately 50-60% of hearing loss cases still remain without confirmation of diagnoses, thus posing the question and need to explore beyond exome covered regions. RNAseq data can be used to determine the functional consequence at the transcript level for all rare synonymous, splice region, untranslated region (UTR), and deep intronic variants. Resources containing genomic, transcriptomic and proteomic information of the inner ear such as the Shared Harvard Inner Ear Laboratory Database (SHIELD) can be used for variant annotation and interpretation. Skin biopsies as fibroblasts have typically the largest number of genes expressed and as such, these may be considered to determine the presence of aberrant splicing events and establish potential disease-causing roles.

Genotype-phenotype correlations are key in strengthening the clinical value of molecular diagnosis. Data from the 100,000 Genomes Project can be further used to create a robust genotype-phenotype dataset. A retrospective evaluation of audiological tests could reveal insights into the association between genetic diagnosis, hearing loss patterns and velocity of deterioration. In addition to congenital SNHL, another patient population of interest is those with adult-onset monogenic hearing loss. In these patients, it will be important to identify the presence of external factors that could worsen level of hearing in order to avoid bias. Additionally, as previously discussed, there is a need to gain a deeper understanding of how to integrate genetic diagnosis in CI decision making. A starting point could be by conducting a longitudinal, observational, prospective study, with a reasonable follow-up period (e.g., 2 years), that includes the use of a standardised set of outcomes in CI patients with a confirmed genetic diagnosis. Analyses could focus on the presence of residual hearing, degree of hearing loss,

velocity of hearing loss, post-CI outcomes, genetic diagnosis including gene and type of variant, together with the assessment of other prognostic factors, for instance, age at implantation in paediatric CI recipients. Resulting genotype-CI phenotype/outcome correlations should facilitate to classify patients by prognosis. Results that can be replicated by multiple and independent studies will then corroborate the status of genetic diagnosis as a prognostic factor and eventually enable personalised integrated genomic-clinical patient care in cochlear implantation.

Another avenue for future research is the evaluation of the cost-effectiveness and clinical value of using genomic testing at different points in the diagnostic journey. Initial evidence has suggested that early genomic sequencing may improve the cost-effectiveness of the diagnostic pathway (323). Moreover, it has been proposed that performing genomic sequencing in newborns, along with universal hearing and metabolic screening programmes, would provide information with lifetime value (48). However, ethical issues have surfaced concerning the identification of medically actionable secondary findings in babies and children (unbiased GS), in particular adult-onset conditions (424). Interestingly, results from the Baby Beyond Hearing Project in Australia revealed a positive parental response towards obtaining more information for their children based on additional findings (425). Certainly, the delivery of such screening interventions should be conducted within a supportive environment for patients and their families. In the current pathway of diagnostic investigations, NGS testing usually takes place once the patient has undergone several laboratory tests and possibly therapeutic procedures. Nonetheless, it is important to acknowledge the value of prior physical examinations, laboratory, electrophysiological and/or imaging tests, as these may provide information that aids interpretation of genetic variants. Conversely, it could guide the appropriate selection of genetic testing. It would be worth studying the clinical added value of obtaining molecular diagnosis in patients with common genetic hearing loss aetiologies such as *SLC26A4*. Here, the identification of bilateral enlarged vestibular aqueducts via MRI in a patient with bilateral SNHL could automatically narrow the search down to testing for variants in *SLC26A4*. On the other hand, early molecular diagnosis may potentially inform the most appropriate time to conduct additional diagnostic interventions. Predicting the future need for cochlear implantation via genetic diagnosis could enable timely access for preoperative cochlear implant

assessments and enable a multidisciplinary plan of hearing habilitation. Importantly, a balance must be struck between the amount of clinical diagnostic testing prior and the timing of NGS testing in order to obtain the highest possible level of clinical utility from both without incurring unnecessary procedures. Findings from such evaluations could be used to further refine timing of testing to achieve maximum clinical benefit, giving further evidence to stakeholders to continue supporting the integration of genomic strategies in patient care.

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