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Identification of Mutations Causing Congenital Nonsyndromic Hearing Loss in 29 Families in West Bank and Gaza

By
Issam T. Farrah

In Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biotechnology

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I

The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

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"Identification of Mutations Causing Congenital Nonsyndromic Hearing Loss in 29 Families in West Bank and Gaza "

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ABSTRACT

Background: Hereditary hearing loss is a common human disease in the countries of the third world and it is widespread worldwide, as these countries have a high percentage because of endogamy. So, the high prevalence of hearing loss has attracted the interest of investigators in the medical field. Many factors are contributing to this disease, one of them called genetic factors. However, it is hard for researchers to specify all the genetic factors associated with hearing loss due to high heterogenicity in individuals with this disorder. In addition, there is difficulty in collecting data from families of deaf patients and unveiling the disorder history in these families. This study investigates the genetic causes of mutagenic deafness in children born with nonsyndromic hearing problems in the West Bank and Gaza.

Methods: Genetic analysis was conducted on 29 participating children and their close relatives. An initial assessment was done using gene panel testing. Validation of genetic identification was carried out using PCR technology. Sanger sequencing was then done on the families to search for the genetic mutation that we found in the affected child within the family to confirm the segregation of the mutation.

Results: At the Hereditary Research Lab, Bethlehem University, DNA was extracted and sequenced for several genes known to cause nonsyndromic hearing loss in the Palestinian population. Segregation analysis was performed on all the identified mutations, in 29 participant families. The results detected the segregation of mutations in 18 families. The testing did not detect the genetic segregation in the remaining families either because Sanger sequencing (the specific procedure used) is not sufficiently specific or because insufficient material was collected to confirm genetic segregation.

Conclusion: considering the confirmation of segregation of the mutation in the family as solved cases and not confirming the segregation as not solved cases, we notice that every family has a known mutation/s in their genes. Hence, the results show that genetic factors play an important role in causing hearing loss among children in West Bank and Gaza.

التعرف على الطفرات التي تسبب فقدان السمع الخلقي اللا متلازم في 29 عائلة من الضفة الغربية وقطاع غزة
عصام تيسير فرّاح

ملخص

يعتبر فقدان السمع الوراثي أحد الأمراض الوراثية المعقدة والمنتشرة بشكل كبير في العالم بالشكل العام ودول العالم الثالث (النامي) بشكل خاص. تتصدر ظاهرة "زواج الأقارب" المشهد في كونها أحد المسببات الرئيسية لمرض فقدان السمع الوراثي والذي يعود تشكّله الى عوامل عدّة أهمها "العامل الجيني الوراثي". لذلك أظهر الباحثون اهتماماً واضحاً بدراسة هذه الطفرات والجينات عن طريق التقنيات والفحوصات الوراثية التقليدية التي تحدد الجينات المسؤولة عن فقدان السمع. بالرغم من ذلك، فقد واجهوا العديد من الصعوبات أهمها عدم القدرة على الوصول الى عائلات التي لها تاريخ وراثي للمرض، الأمر الذي أضعف من قدرتهم على تحديد جميع الجينات المسببة لمرض فقدان السمع.

لذلك، يُسلّط بحثنا الحالي الضوء على دراسة الأسباب الوراثية (الطفرات) المتعلقة بفقدان السمع الوراثي غير المتلازم لعدة أطفال كانوا قد ولدوا في مناطق الضفة الغربية وقطاع غزة ويعانون من مشاكل في السمع. بحيث تمت مراسلة عدد من العائلات التي يعاني اطفالها من مشاكل في السمع وأخذ الموافقة بحسب الأصول وإرسال نموذج لموافقة الأهل على مشاركة أبنائهم في البحث، الأمر الذي سهّل عملية أخذ عينة دم بهدف التشخيص الجيني لفقدان السمع. وبعد أن تمت جمع الموافقات المطلوبة، تم مقابلة كل من الأهل والمصابين بهدف التعرف الى تاريخ العائلة ورسم الشجرة العائلية الوراثية والتطرق لبعض الاسئلة التي ترتبط بحالة فقدان السمع، بالإضافة الى أخذ عينة دم من المصابين والوالدين والأخوة إن وجدوا والموافقين بطبيعة الحال.

بعد الانتهاء من هذه الاجراءات، تم الانتقال الى الخطوة التالية وهي عملية استخراج المادة الوراثية DNA داخل المختبرات في جامعة بيت لحم وعمل فحص جيني للعينات لمعرفة الجين والطفرة المسببة لكل عائلة على حدا، كما وتم تأكيد وراثة الطفرة عن طريق دراسة الحمض النووي للعائلة إن وجد، وتم تقديم استشارة للوالدين وتوضيح نوع الطفرة وماهيتها وفرصة ولادة طفل اخر يحمل الطفرة ذاتها.

ومن المهم ذكره أخيراً بأن بحثنا الحالي شمل 29 عائلة من مناطق الضفة الغربية وقطاع غزة. تم افتراض أن العائلات التي أكدت نتائج البحث أن الطفرة موروثة من الأهل الحصول على نتيجة إيجابية وعددهم 18 عائلة, بينما العائلات التي لم تؤكد نتائج البحث على أن الطفرة موروثة من الأهل حصلت على نتيجة سلبية وعددهم 11 عائلة, بالرغم من أن جميع العائلات تبين أن المسبب للمرض فيها هو عامل جيني, لكن بسبب قصور البحث على استخدام التشخيص بطريقة (sanger sequencing) أو عدم وجود عينات كافية, لا يمكننا الجزم بأن الطفرة في العائلات الحاصلة على نتيجة سلبية موروثة من الأهل.

DECLARATION

I declare that the Master Thesis entitled "dissertation title" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: ____Issam Tayseer Farrah_____

Date_____

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Abbreviations:

AD	Autosomal Dominant
ABR	Auditory Brainstem Response
ANSD	Auditory Neuropathy Spectrum Disorder
AR	Autosomal Recessive
ASSR	Auditory Steady-State Response
BOA	Behavioral Observation Audiometry
BOR	Branchio-Oto-Renal Syndrome
CHL	Conductive Hearing Loss
CMV	Cytomegalovirus
CPA	Conditioned Play Audiometry
CSF	Cerebrospinal Fluid
DFN	X- Linked
EOAEs	Evoked Otoacoustic Emissions
ESRRB	Estrogen Related Receptor Beta
HL	Hearing Loss
HRL	Hereditary Research Lab
IHC	Inner Hair Cell
NSHL	Nonsyndromic Hearing Loss
OHC	Outer Hair Cell
PCR	Polymerase Chain Reaction
PGD	Preimplantation Genetic Diagnosis
SHL	Syndromic Hearing Loss
SNHL	Sensorineural Hearing Loss
VRA	Visual Reinforcement Audiometry

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CHAPTER 1

Introduction:

Hearing loss (HL) is the most prevalent form of sensory lack of communication. However, genetic factors are the most important factors causing hearing loss in approximately half of the cases (CDC, Center of Disease Control, Genetics of Hearing Loss). It is classified into two types according to clinical manifestations, namely syndromic hearing loss (SHL) and Nonsyndromic (NSHL) (Gao et al., 2015). It is also subdividing according to many factors such as phenotypic manifestation, severity age of onset, audiometric profile, and etiology. Moreover, there are three types of hearing loss according to their place of origination in the ear, namely sensorineural, conductive, and mixed hearing loss (Gao & et al., 2015). The severity of the hearing loss is classified as mild, moderate, and severe. All of the classifications have possible reasons including environmental factors or a combination of genetic and environmental factors such as some drugs, peri-natal infections, and exposure to strong noises for long periods.

HL is considered a highly heterogeneous disease; thus, different molecular events leading to deafness arise. Among hundreds of possible genes, recently, a total of 36 autosomal recessive nonsyndromic hearing loss (ARNSHL) genes and 24 autosomal dominant nonsyndromic hearing loss (ADNSHL) genes have been described (Dror and Avraham, 2010). In addition, scientists mapped 45 loci for recessive and 34 loci for dominant deafness to specific chromosomal regions (Dror and Avraham, 2010).

Nowadays, gap junction beta 2 (GJB2) mutation is known as the primary mutation that causes hearing loss. The corresponding gene encodes typically Connexin 26 (CX26). The primary mutations in GJB2 are population-specific; attributed to founder influences, and include c.35delG affecting Caucasians (Wonkam et al., 2013), c.167delT affecting those of Ashkenazi Jewish ancestry (Liu et al., 2002), and c.235delC affecting East Asians (Pandya et al., 2003).

In this study, we study the segregation of mutations causing congenital nonsyndromic hearing loss among 29 families with hearing loss history in the West Bank and Gaza.

1.1 Deafness:

Deafness involves damage to the hearing sense, caused by a reduction in the reception of sound, and it can be a partial or total loss. Loss of volume is measured in decibels (dB) and can vary according to frequencies (0.25, 0.5, 1, 2, 4, and 8) in kHz. In measuring hearing loss, each frequency has a sound presented at a specific intensity to a subject and the response of all frequencies is recorded graphically in an audiogram. A loss in hearing indicates if the threshold for perception of sound for any frequency elevates by 10 dB or greater than the defined expected value for each frequency. (Essammak, 2014).

Partial or complete damage in one part or more of the ear could occur in the ear's outer, central, or internal parts. Deafness could be considered a common disorder. Congenital hearing damage occurs approximately in 1:1000. (Egilmez & Kalcioglu, 2016)

Deafness is subdivided into nonsyndromic hearing loss and syndromic hearing loss, according to the clinical manifestations. The syndromic one has malformations in the body's organs and it represents about 30% of hereditary hearing loss. In contrast, nonsyndromic one has no signs which represent about 70% of hereditary hearing loss (Jaraysa, 2016).

Bosch (2013) viewed that deafness reduces a person's ability to hear for any reason. It has far-reaching effects on both persons and their families, leading to stress, and reducing educational performance. In the nonsyndromic hearing loss, most patients have an autosomal recessive inheritance and they represent up to 80% of all cases, while others have an autosomal dominant mode of inheritance. Furthermore, about 30 genes are suspected to cause hearing loss as Bosch mentioned. The 70 loci identified from these 30 genes encode several proteins that have a vital function in the hearing sense and are responsible for autosomal dominant deafness. These genes have the main form of hair bundle and components of the extracellular matrix. They play a role in cochlear ion balance or act as transcription factors (Bosch, 2013).

With high and quick development in sequencing technology, new genes were identified in hereditary hearing loss, such as TBC1D24, P2RX2, and TNC. (Schrijver, 2004)

1.2 Anatomy and physiology of the inner ear:

There are two functions for the inner ear: hearing and balance. Hearing is related to cochlea (inner ear) function, and the balance is related to semicircular canals and the vestibule. The two parts of the inner ear together are known as the vestibular system. Our ears catch the sound waves from the environment and pass them to the brain to analyze them. The ear contains three components: outer, middle, and inner parts. Any defect of one of these parts may affect the hearing or cause hearing loss. It could be conductive, sensorineural, or a mix of both. Each part of the ear has its unique function. The outer ear starts the hearing process by catching the sound waves and passes them through the ear canal to the eardrum membrane, then on to the oval window of the fluid-filled inner ear past the middle ear. The middle ear consists of three tiny bones located in the tympanic cavity and assists as a link between the eardrum and the oval window of the fluid-filled inner ear. (Vona, 2014) The inner ear, which is located in a bony cavity, is a fluid-filled organ consisting of two central regions, namely the cochlea, which processes the auditory signal, and the vestibular apparatus (utricle, saccule, and ampullae which are semicircular canals), which helps preserve balance by responding to gravity and acceleration. The processing of auditory signals occurs in the cochlea, a bony canal that surrounds the central nucleus, called modiolus (Essammak, 2014).
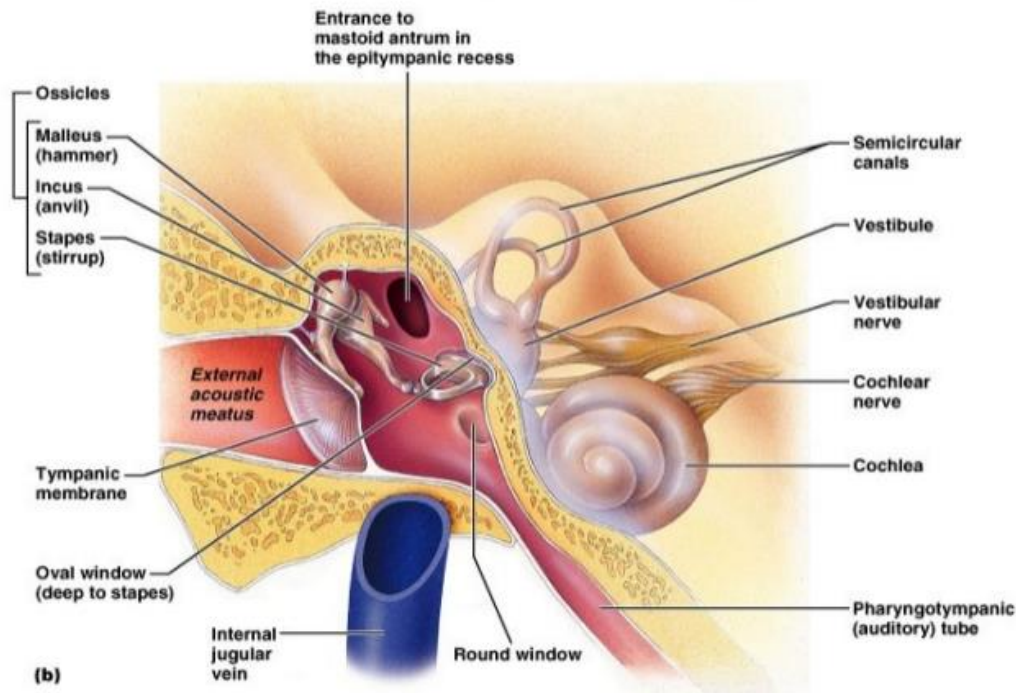
Figure 1: Structure of the inner ear.

Three large cavities are divided into the cochlea, the scala vestibule, and Scala Timpani. Inside them, there is a liquid called perilymph. The third cavity, the scala media (cochlear canal), is located between the vestibular scala and scala drum. The Scala media is a triple membranous channel that is restricted to the Reissner's membrane (top), the basilar membrane (bottom), and stria vascularis (stria abneural side) and contains the endolymph fluid. (Davis, 2003)

The cochlear duct differs according to length and reflects differences and gradients of gene expression. These differences make the molecular physical and biological characteristics. The stria vascularis is an ion transport epithelium consisting of three cell types (as shown in Figure 2): marginal, intermediate, and basal. Together, they produce high endolymphatic potassium ion concentration and the possibility of positive endocochlear. The cochlear transduction is a sensory organ called the Corti apparatus and it is located on the top of the basal membrane throughout the canal. It is made of hair cells, supporting cells, neurons, blood vessels, and bronchial membrane. (Alberti, 2004)
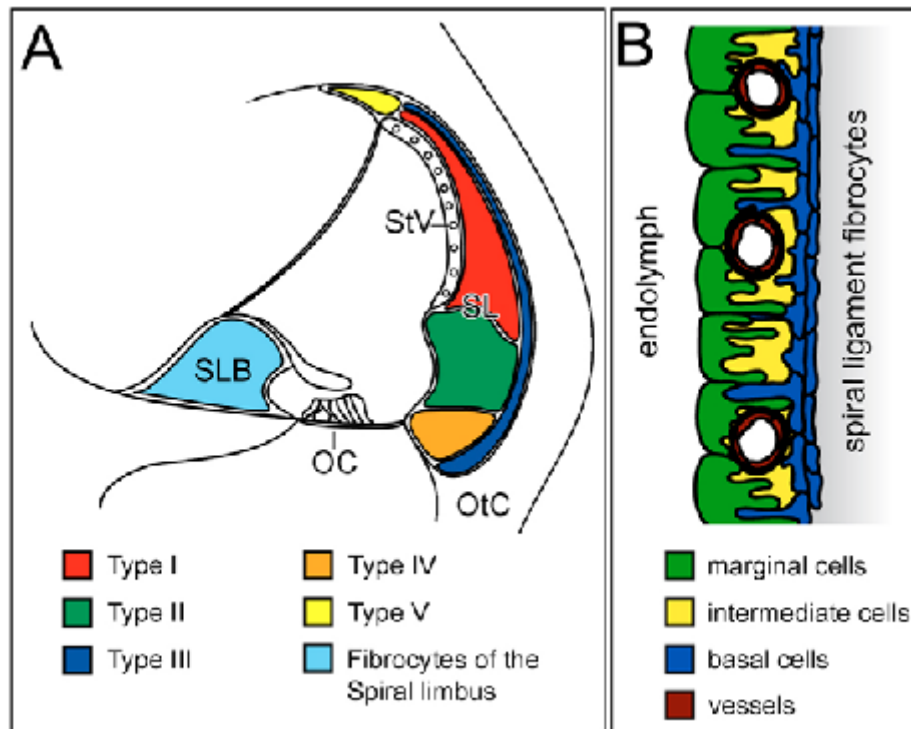
Figure 2: Diversity of fibrocytes in the cochlea and structure of the stria vascularis. (A) Illustration of the distribution of subtypes of otic fibrocytes on a midmodiolar cochlea section. Subtypes are as indicated in the figure. Based on location, cell morphology, and marker gene expression, five major types of fibrocytes can be distinguished in the spiral ligament. (B) Scheme of the cellular structure of the stria vascularis. Cell types are as indicated in the figure. Stria vascularis integrity relies on a three-layered tissue architecture of marginal, intermediate, and basal cells enclose a dense capillary network. The formation of long cell processes by all three cell types and a high degree of interdigitation characterizes strial architecture. OC, Organ of Corti; OtC, otic capsule; SL, spiral ligament; SLB, spiral limbus; StV, stria vascularis (Mark-Oliver Trowe, 2008).

As shown in Figure 3, the supporting cells contain various types of cells, most of which have a poorly understood function. The supporting cells function to recycle the potassium ions, form an impenetrable barrier between the endolymph and perilymph filled spaces, preserve the hair cell function and support the Corti organ structurally, specifically as the hair cells connected with supporting cells. Gap junctions connect the supporting cells (comprising connexins 26, 30, 31, and 43, in the cochlea, 26, 30, and 43, in the vestibular system), allowing them to act as a functional unit. The cochlea's hair cells are the sensual cells at their apical surface in bundles of stiff-actin-filled microvilli, named stereocilia. (Essammak, 2014)
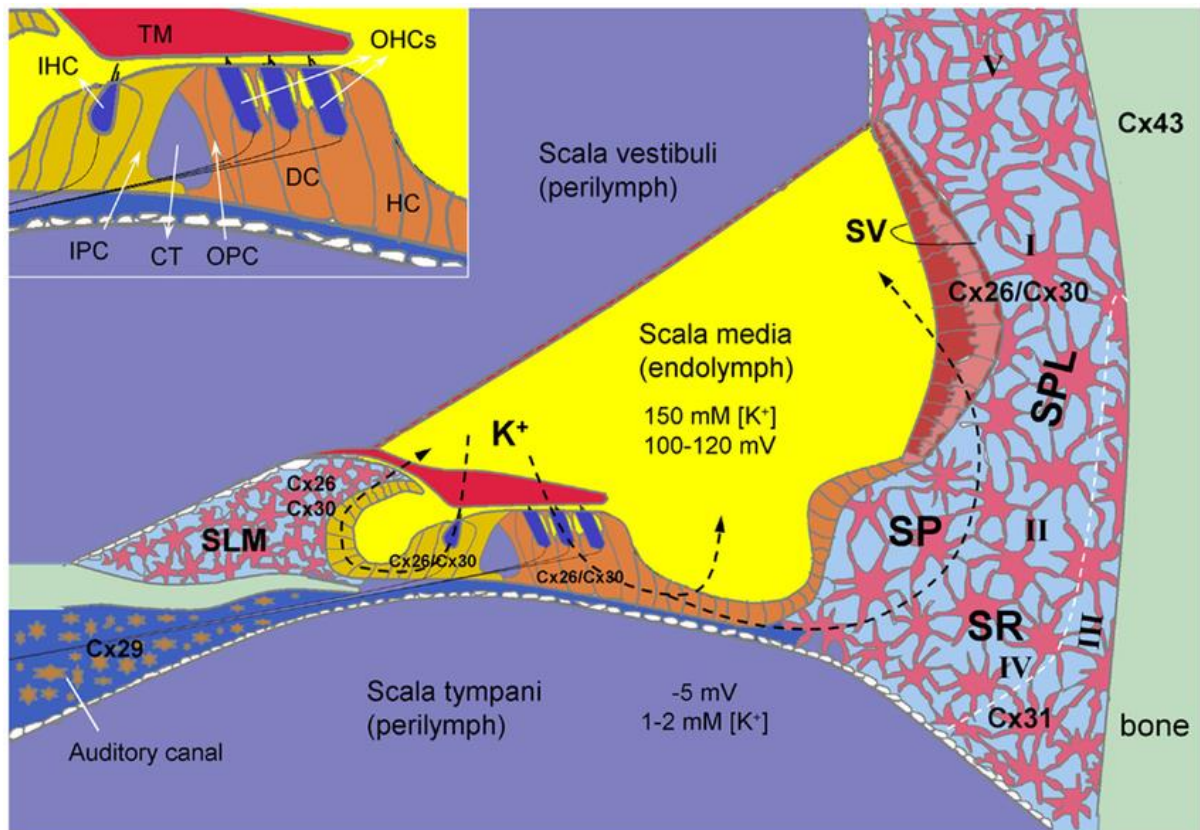
Figure 3: Connexin expression and hypothesized K+-recycling in the cochlea. Cx26 and Cx30 co-localized in supporting cells of the organ of Corti, the spiral limbus (SLM), the stria vascularis (SV), and fibrocytes of the spiral ligament (SPL). Cx31 is localized at type II and IV fibrocytes in the subcentral region (SR) below the spiral prominence (SP). Cx43 is expressed in the bone of the otic capsule. Cx29 is localized only at the Schwann cells wrapping the spiral ganglion neurons in the auditory canal. However, hair cells have no connexin expression. Inset: the organ of Corti. CT, cochlear tunnel; DC, Deiters cell; HC, Hensen cell; IHC, inner hair cell; IPC, inner pillar cell; OHCs, outer hair cells; OPC, outer pillar cell; TM, tectorial membrane; I–V, type I–V fibrocytes (Jeffrey C. Wingard,2015).

As shown in Figure 4, the cochlea's hair cells are subdividing into inner hair cells (IHCs) and outer hair cells (OHCs) that vary in structure, innervation pattern, and function. The outer hair cells (OHCs) transfer the changes in membrane potential into macroscopic changes in the length of their cell bodies located in the middle of Prestin, a transmembrane protein located in the outer hair cell membrane. This situation creates the mechanical energy needed to amplify the sound-induced vibrations in the cochlea, mainly responsible for the ear's hearing sensitivity and frequency resolving capability. But, the inner hair cells (IHCs) function as the hearing sensual receptors and transfer all auditory information to the brain (Dallos & Fakler, 2002).

Inner Hair Cell

Outer Hair Cell

MET channel

MET channel

PMCA2

PMCA1

Ca^{2+} channel

Mitochondria

Afferent nerve

● MET channel
● nAChR channel
○ Ca^{2+} channel
○ CaATPase pump

Sub-synaptic cisterna

nAChR

Efferent nerve
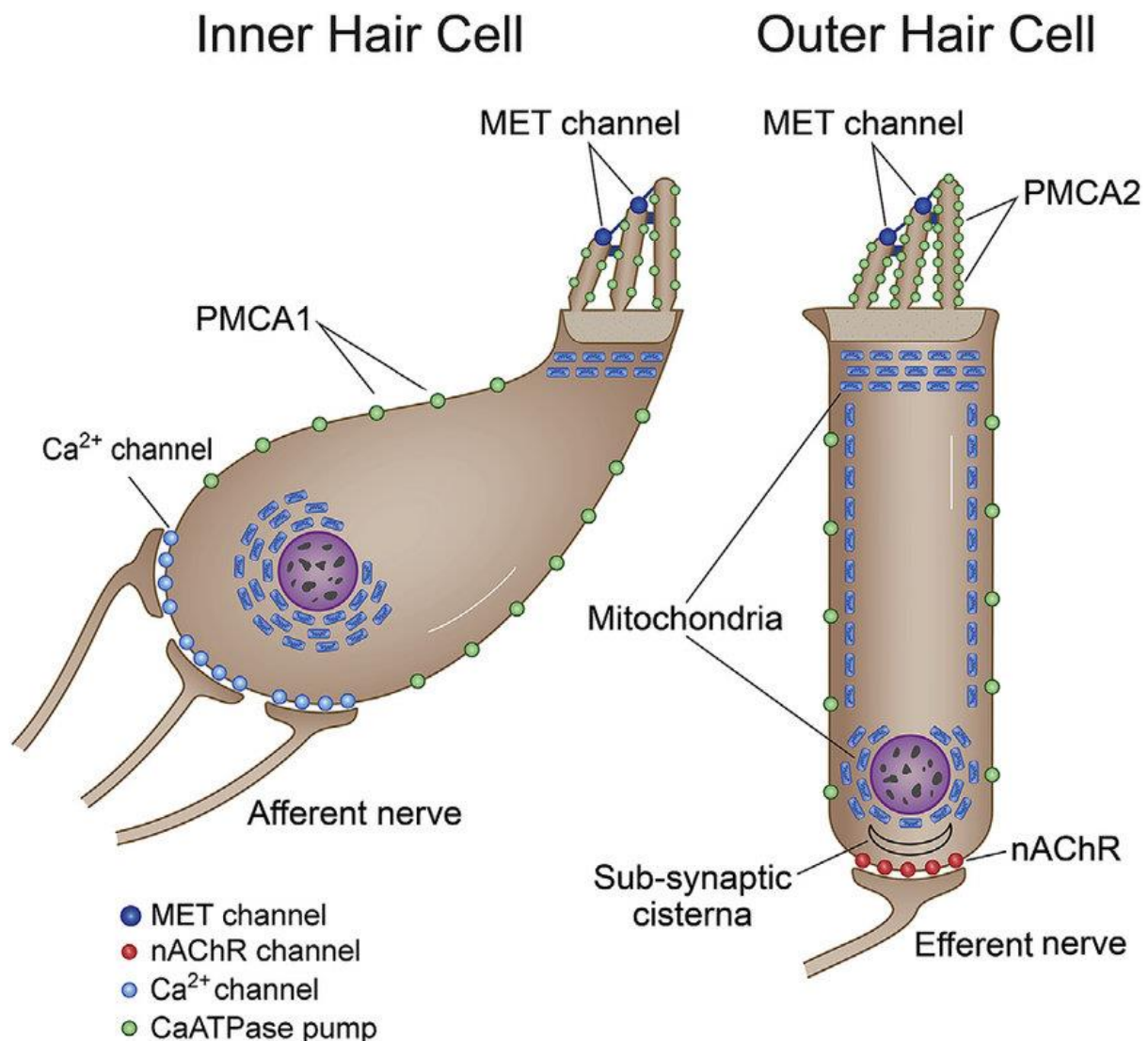
Figure 4: Factors involved in Ca 2þ homeostasis in cochlear inner hair cells (IHCs) and outer hair cells (OHCs). Ca 2þ enters via mechanotransducer (MET) channels in the stereocilia of both hair cell types, and also via voltage-dependent Ca 2þ channels (mainly IHCs) and nicotinic acetylcholine receptors (nAChR) in OHCs. Ca 2þ is buffered by cytoplasmic calcium-binding proteins and by fixed organelles, mitochondria, and sub-synaptic cisternae. Ca 2þ is extruded by a plasma membrane CaATPase pump, PMCA2 in the OHC and IHC stereocilia, and PMCA1 in the IHC soma (Robert Fettiplace,2018).

1.3 Classification of Hearing Loss (HL):

Hearing loss is classified by degree and type. The degree depends on the pure tone average, or the average of pure tone in testing at the "speech" frequencies (500, 1000, and 2000) in Hz. Bess and

McConnel (1981) defined it as "audiometric classification." People who lose about 35-69 dB are called hard of hearing, which those who lose above 70 dB are called deaf. (Paul, 2007)

The three primary classifications of hearing loss are sensorineural, conductive, and mixed. There is another classification, which some people consider the fourth one, and some consider it separately like another classification because the problem of the hearing loss in this classification is not in the ear rather in the brain. This type of hearing loss is called central (Burkey, 2006).

- Sensorineural:

Sensorineural is a nerve hearing loss that occurs when the hair cells within the cochlea are damaged, so there is no more sense of the fluid movement in the inner ear and there is no signal sent to the brain. Even a minimal amount of damage to few nerve cells can cause mild hearing loss (Burkey, 2006).

Maximal damage that affects more cells, caused a more significant hearing loss. This damage can make a deformed sound. For example, it could cause a loss of tonality for listening to music, and it could cause problems with understanding speech. Noise exposure and aging are typical causes. Unfortunately, unlike conductive hearing loss, sensorineural hearing loss is not treatable by medication (Shemesh, 2010).

- Conductive:

Hearing loss is classified as conductive when a barrier prevents sound from being delivered through the middle or outer ear; thus, the sound block. Ear wax could be the most common cause of conductive hearing loss because it blocks the ear canal. Other causes of conductive hearing loss are an infection in the outer or central ear, and rupture of the eardrum, or damage of ossicles (Burkey, 2006).

Conductive hearing loss is treatable often and its treatment would be effective most of the time because the causes are always physical problems. Infection can be treated with medication, the ear wax can be cleaned and removed, and a rupture of the eardrum can often be repaired. Rarely cases cannot be completely corrected or treated (Shemesh, 2010).

- Mixed:

In cases of simultaneously both conductive and sensorineural hearing loss, hearing loss is called a mixed situation. Because it is a combination of them, the conductive could be treated, but the sensorineural could not. So, the hearing could be improved but not fully treated (Essammak, 2014).

- Central:

When the hearing problem originates in the auditory areas of the brain or affects sound pathways, it is called central hearing loss. Unfortunately, it is not like the other classifications because there is a clear sound signal, but the brain cannot understand or analyze it (Burkey, 2006).

Some people could be born with a central hearing loss. As well, accidents can cause a central hearing loss problem, like brain damage from stroke, trauma, or car accident (Essammak, 2014).

1.4 Epidemiology of Congenital Hearing Loss:

Hearing loss is the most common sensorial defect in humans. It affects approximately 1:1000 newborns. Genetic factors and environmental factors could cause it or both. The main environmental factors that have been implicated are maternofetal infections such as cytomegalovirus (CMV), toxoplasma and rubella, perinatal complications, maternal diabetes, infections, and ototoxic drugs. Genetic factors could be a single gene mutation or a genetic abnormality that plays a multifactorial process (Coffey & Brumback, 2006).

The incidence of congenital hearing loss in Europe is about 1.47:1000 births. A review of children screened for hearing loss in the United States in 2004 showed that 1.1:1000 infants had lasting hearing loss. In China, a 2010 study stated the prevalence of congenital deafness in the Hubei province to be 0.5:1000 births, and a trial in Shanghai found a rate of 1.5:1000 births. Although Japan is a developed country, it has a higher hearing loss than Europe and the USA; 3:1000 births suffer from congenital hearing loss in Japan. The prevalence of congenital and early-onset hearing loss in South Africa has been estimated at 5.5:1000 individuals, much higher than in Europe and America and more in line with other Sub-Saharan countries. This rate most likely relates to inadequate health care as the spread rate reported in the private health sector is 3:1000, compared to 6:1000 in the public health sector. A community screening program in the Western Cape found 4.5:1000 infants screened during vaccination (Bosch, 2013).

1.5 Detection of Hearing Loss:

The following tests can detect hearing loss:

- Physiologic tests:

Physiologic tests objectively determine the functional status of the auditory system and can be performed at any age. Hildebrand and Shearer explained the most popular physiologic tests in (2008):

> ➢ Auditory brainstem response test (ABR, also known as BAER, BSER): ABR uses a stimulus (clicks) to arouse electrophysiological responses, originating in the eighth cranial nerve, auditory brainstem, and record with surface electrodes. ABR "wave V detection threshold" correlates best with hearing sensitivity in the 1500-4000 Hz region in neurologically normal individuals; ABR does not assess low frequency (<1500 Hz) sensitivity.

> ➢ Auditory steady-state response test (ASSR): ASSR is an electrophysiological measurement of hearing severity used extensively in Australia, Asia, Canada, and now frequently in the United States and Europe. Skin electrodes measure whether the auditory response is phase locking to changes in a continuous tonal stimulus. Because the stimulus is a continuous signal, the average sound pressure level that can be delivered is higher; than ABR, which uses click stimuli. This difference means that ASSR can often supply an estimate of hearing sensitivity in children who demonstrate no response to ABR testing.

> ➢ Evoked otoacoustic emissions (EOAEs): EOAEs are sounds produced inside the cochlea, measured in the external auditory canal using a probe with a microphone and transducer. The EOAEs primarily reflect the activity of the cochlea's outer hair cells across a broad frequency range and are present in ears with hearing sensitivity better than 40-50 dB HL.

> ➢ Immittance test (tympanometry, acoustic reflex thresholds, acoustic reflex decay): Immittance audiometry evaluates the peripheral auditory system, including middle ear pressure, tympanic membrane mobility, Eustachian tube function, and mobility of the middle ear ossicles.

- Audiometry:

Audiometry is the assessment of how the person processes auditory information, i.e., hears. Audiometry consists of behavioral testing, pure tone audiometry, air conduction audiometry, conditioned play audiometry, and conventional audiometry.

> ➢ Behavioral testing: includes behavioral observation audiometry (BOA) and visual reinforcement audiometry (VRA). The BOA, applied on infants from birth to six months old, is highly dependent on the tester's skill and is subject to error. VRA is applied on children from six months old to 2.5 years and can give a reliable and complete audiogram, but the accuracy of results is dependent on the child's maturational age and the tester's skill (Roeser & Marion, 2011).

> ➢ Pure-tone audiometry: (air and bone conduction) includes defining the lowest intensity at which an individual "hears" a pure tone as a function of frequency (or pitch). Octave frequencies (250) (close to middle C) to 8000 Hz tested using earphones—intensity or loudness measure in decibels (dB), known as the ratio between two sound pressures. (0) dB

HL is the average threshold for a regular hearing adult; (120) dB HL is so loud as to cause pain (Kutz, 2016).

➢ Air conduction audiometry: provides sounds through earphones; thresholds rely on the external ear canal, middle ear, and inner ear status.

➢ Conditioned play audiometry: (CPA) is applied to children from 2.5 years old. Each ear should obtain a complete frequency-specific audiogram from a cooperative child.

➢ Conventional audiometry: applies to children 5 years and older; the child indicates when he can hear the sound (Hildebrand & Shearer, 2008).

• Screening:

Screening programs to reveal hearing loss have mainly targeted infants and children. Hearing screening in adults has typically been done when noise exposure or normal hearing is essential for performing the implicated tasks. Screening of an older population is not generally applied. However, due to the high spread of hearing impairment and the benefits rehabilitation has to provide, screening could be acceptable and effective in 55-74 years, where the hearing loss in the better ear is at least (35dB) HL (White, 2004).

CHAPTER 2

Literature Review

2.1 The Heterogeneity of Hearing Loss:

Normal hearing requires about 300 to 500 necessary genes (Rita Teek et al., 2007). A mutation in a single gene that affects or causes hearing loss could lead to extreme genetic heterogeneity. Genotype-to-phenotype has an essential correlation because different phenotypes could result from different mutations in the same gene. Moreover, different mutations in a single gene can cause syndromic, nonsyndromic, recessive, or dominant hearing loss. (Friedman & Griffith, 2003).

Mutations in the GJB2 gene cause approximately 60% of nonsyndromic autosomal recessive disorders (NSARD) cases. This percentage is high and it is thought to be ever higher from 2% to 5% depending on the ethnic group (Gasparini et al., 2000).

2.2 Genetics of Hearing Loss:

Till now (as Van Camp mentioned in his paper), there are about 100 to 150 genes that have a relationship with hearing physiological processes. Scientists identified about 146 alleles that cause deafness, 57 dominant, 77 recessives, 8 X-linked, 1 Y-linked, 2 modifiers, and one auditory neuropathy. (Van Camp & Smith, 2009)

Allelic mutations in some genes cause recessive and dominant hearing loss, whether syndromic or nonsyndromic. About 70% of the cases with inherited hearing loss are nonsyndromic, and 80% are autosomal recessive (AR) (Li & Friedman, 2002).

Most nonsyndromic hearing losses are considered to be sensorineural, and it can be autosomal recessive deafness (DFNB), autosomal dominant deafness (DFNA), mitochondrial deafness, and X-linked deafness (DFN) (Van Laer et al., 2003).

The common genes and proteins that are responsible for nonsyndromic hearing loss are:

- Motor Proteins

Two motor proteins related to the myosin family are Myo7A and Myo6. These proteins are good examples of motor proteins. The Myo7A is located in stereocilia and any loss of this protein will cause loss of stereocilia structure and disorganization of the remaining hair bundle. (Kadir & Kalcioglu, 2016). The second is Myo6 which travels toward the actin base of stereocilia. Any mutation in the Myo6 gene will cause defective protein that could stop the movement along the actin. Mutation in this gene could be autosomal recessive or autosomal dominant (Ahmed et al., 2003).

- Cell-Cell junction proteins

There are many crucial cell-cell junction proteins, such as Cadherin-related 23 (CDH23), Connexin 26 (GJB2), and Otoancorin (OTOF). The CDH23 protein is essential for preserving hair bundle development. However, mutations in the CDH23 gene impact cell polarity and lead to the disorganization of stereocilia and the tip links (Liu et al., 2012). Changes in the OTOF gene cause auditory neuropathy spectrum disorder (ANSD), leading to damage of the inner hair cell. Mutations in the GJB2 gene account for more than 50% of the NSHL cases. This gene in its typical version is responsible for cochlear amplification by intercellular communication that allows ions pathway and cell signaling molecules (Wingard & Zhao, 2015).

- Transporter proteins

There are many essential transporter proteins, such as SLC26A4, the second most common gene that causes hearing loss after GJB2. The SLC26A4 encodes an anion transporter in the hearing pathway (chloride and iodide), mutations in this gene lead to the enlargement of the vestibular aqueduct and sacs, followed by head trauma.

Other mutations in genes involved in the hearing pathway participate in the hearing loss phenotype. Bi-allelic mutation in the TMPRSS3 gene decrease hearing at the high frequencies compared to the low ones. Severe mutations in this gene lead to prelingual hearing loss, whereas mild alterations lead to post-lingual hearing loss. GPSM2 gene encodes G-protein signaling modulator expressed in the hair cells. Mutations in the GPSM2 gene will affect cell polarity and the asymmetric organization of the apical (organ of the Corti). Another example of these genes is the TECTA gene which codes for an alpha-tectorin protein of the tectorial membrane that surrounds the outer hair cells and is responsible for amplifying the sound. Mutation in this gene leads to hearing loss as well (Verhoeven et al., 1998).

- Adhesion Proteins:

There are many adhesion proteins and they link the stereocilia of hair cells in the cochlea to the tectorial membrane. Also, hair bundles stabilize by a set of temporary links by these proteins, such as transient lateral links and ankle links. These proteins have other functions like induction of growth and maturation with signaling complexes (Kadir & Kalcioglu, 2016, p.2).

- Proteins of synapses:

Synapse proteins play a role in the inner hair cells, like VGLUT3, a vesicular glutamate receptor encoded by SLC17A8 in the DFNA25 locus and related to autosomal recessive hearing loss. This protein regulates both the exocytosis and the endocytosis of glutamate.

The role of glutamate excites auditory neurons to send a signal to the brain, a digital signal known as a spike. Without glutamate there are no spikes, so no information travels from the ear to the brain (Kadir & Kalcioglu, 2016).

- Electromotility:

The process of passing the sound from the outer hair cell to the cochlea, which is sensitive and selective to sounds, is called electromotility. There is a protein called Prestin that is responsible for this electromotility. It changes the membrane's potential and enables the outer hair cell to alter its length. When this occurs, the outer hair cell becomes longer upon hyperpolarization and shorter upon depolarization, amplifying its sensitivity to the sound (Kadir & Kalcioglu, 2016).

- Cytoskeleton:

Any mutation in the cytoskeleton regulating genes can cause NSHL (nonsyndromic hearing loss). Common examples of these genes are ESPN (espin), RDX (radixin), TRIOBP (trio-binding protein), ACTG1 (γ-actin), TPRN (taperin), DIAPH1 (diaphanous), and SMPX (small muscle protein, X-linked). Each protein product of these genes has its role. For example, protein espin provides stability to the stereocilia cytoskeleton. A mutation in ESPN can cause autosomal recessive and autosomal dominant hearing loss (Kadir & Kalcioglu, 2016).

- Ion Homeostasis:

There are two types of fluids in the cochlea: perilymph and endolymph. Perilymph is high in sodium and low in potassium. Endolymph is low in sodium and high in potassium; this condition makes a highly positive potential (+80mV) called endo cochlear potential (Kadir & Kalcioglu, 2016).

Many proteins are involved in the ion homeostasis process. They are related to hereditary hearing loss. Examples include tight junction protein 2 (TJP2), tricellulin (MARVELD2/TRIC), claudin 14

(CLDN14), KCNQ4 (KCNQ4), Barttin (BSND), ATP2b2 (ATP2b2/PMCA2), some connexins (GJB2s), and pendrin (SLC26A4) (Stelma, 2014).

2.3 Nomenclature of nonsyndromic hearing loss:

The abbreviation (DFN) refers to "Deafness" related to the field of nonsyndromic genetics problems that are X-linked. Furthermore, the abbreviation (DFNA) refers to "autosomal dominant deafness, where one mutation in one allele is enough to manifest the disease'. (DFNB) is an abbreviation for "autosomal recessive nonsyndromic deafness, where the mutation should be on two alleles to manifest the disease". These abbreviations with suffixes match the order in which they were explained. These names match with the loci and do not necessarily match different genes (Tang, 2006).

2.4 Types of Nonsyndromic hearing loss:

2.4.1 Autosomal Recessive:

Most of the hearing loss cases are considered recessive and they account for about 75- 80% of cases, which makes it very significant in hearing loss. There are two types of recessive cases. They could be homozygous or compound heterozygous in one gene who have two different alleles that arose independently, but both alleles (of the same gene) are defective. Also, if the parents are either heterozygous or carriers, there is a 25% chance to inherit a recessive hearing loss case (Modell & Darr, 2002).

In the areas where consanguineous marriages prevail, the autosomal recessive conditions are at high levels as well, such as the Middle East, South Asia, Sub-Saharan Africa, and Southeast Asia (Modell & Darr, 2002).

As Liu noted (2001), there are eighteen genes responsible for autosomal recessive deafness; the most common gene being GJB2 (Connexin 26). The mutations of this gene account for about 30-50% of recessive nonsyndromic deafness worldwide. The gap junction contains Connexin that attaches to cells. Many types of Connexin can also associate with each other to configure mixed gap junction channels (Cohen & et al., 2002). Moreover, many connexin genes when mutated are responsible for autosomal recessive deafness, including Connexin 30 (GJB6 gene) and Connexin 43 (GJA1 gene) (Liu & et al., 2001).

2.4.2 Autosomal Dominant:

The autosomal dominant (AD) genetic disorder is hard to track or isolate because it is found in late-onset and progressive deafness families. There are many types of it; they are distinguished clinically or phenotypically based on the pattern of inheritance, age of onset, the severity of the hearing loss, type of audiogram, and progression of the hearing loss (Taylor et al., 2013).

Autosomal dominant nonsyndromic hearing loss can be due to mutations in about 25 genes and more than 60 loci (Egilmez et al., 2016). The mutation in the Diaphanous (DIAPH1) gene is dominant (Goldfarb et al., 2002). The DIAPH1 mutation is a splice mutation that leads to protein truncation. Moreover, most autosomal dominant loci cause post-lingual hearing impairment with an exception in which hearing loss is prelingual gene include GJB2 and GJB6 (DFNA3), TECTA (DFNA8/12), and DFNA19 (Taylor et al., 2013). The genes implicated in autosomal dominant nonsyndromic hearing impairment and their clinical manifestations are summarized in Table 1.

Table 1: Autosomal Dominant Nonsyndromic Hearing Impairment: Genes and their Clinical Manifestations (Van Camp & Smith, 2017).

Gene	Locus	Onset/Decade	Audio profile
ACTG1	DFNA20/26	Post lingual	High frequency; progressive
CCDC50	DFNA440	Post lingual	Low to mild frequencies; progressive
CD164	DFNA66	Post lingual	Flat or mid-frequency; progressive
CEACAM16	DFNA4B	Post lingual	Flat; progressive
COCH	DFNA9	Post lingual	High frequency; progressive
COL11A2	DFNA13	Post lingual	Mid-frequency loss
DFNA5	DFNA5	Post lingual	High frequency; progressive

Gene	Locus	Onset/Decade	Audio profile
DIAPH1	DFNA1	Post lingual	Low frequency; progressive
DMXL2	-	Post lingual	Flat; progressive
DSPP	DFNA39	Post lingual	High frequency; progressive
EYA4	DFNA10	Post lingual	Flat/gently downsloping
GJB2	DFNA3	Prelingual	High frequency; progressive
GJB3	DFNA2B	Post lingual	High frequency; progressive
GJB6	DFNA3	Prelingual	High frequency; progressive
GRHL2	DFNA28	Post lingual	Flat/gently downsloping
HOMER2	DFNA68	Post lingual	High frequency; progressive
KCNQ4	DFNA2	Post lingual	High frequency; progressive
MIR96	DFNA50	Post lingual	Flat; progressive
MCM2	DFNA70	Post lingual	High frequency; progressive
MYH14	DFNA4	Post lingual	Flat/gently downsloping
MYH9	DFNA17	Post lingual	High frequency; progressive
MYO1A	DFNA48	Post lingual	Progressive
MYO6	DFNA22	Post lingual	High frequency; progressive

Gene	Locus	Onset/Decade	Audio profile
MYO7A	DFNA11	Post lingual	Flat/gently downsloping
OSBPL2	DFNA67	Post lingual	High frequency; progressive
P2RX2	DFNA41	Post lingual	Flat; progressive
POU4F3	DFNA15	Post lingual	High frequency; progressive
SIX1	DFNA23	Prelingual	Down sloping
SLC17A8	DFNA25	Post lingual	High frequency; progressive
TBC1D24	DFNA65	Post lingual	High frequency; progressive
TECTA	DFNA8/12	Prelingual	Mid-frequency loss
TJP2 & FAM189A2	DFNA51	Post lingual	High frequency; progressive
TMC1	DFNA36	Post lingual	Flat/gently downsloping
WFS1	DFNA6/14/38	Prelingual	Low frequency; progressive

Post lingual is defined as hearing impairment after the acquisition of speech and language, often after the age of six, whereas prelingual is defined as hearing impairment before the acquisition of speech and language.

2.4.3 X and Y linked hearing loss:

X- Linked hearing loss is rare. It has few forms of hearing loss, unlike autosomal recessive and autosomal dominant. It is distinguished by profound sensorineural and conductive hearing loss with stapes fixation. It is also variable. It is defined as a syndrome because of its relation to stapes fixation,

leading to a perilymphatic gusher during surgery (gusher- deafness syndrome). The Y- Linked hearing loss mutation is rare, found at only one locus in seven generations of a Chinese family (wang et al., 2004).

2.4.4 Mitochondrial hearing loss:

The function of the human mitochondrial chromosome involves the extraction of chemical energy by oxidative phosphorylation. Because of this, scientists formerly did not expect that mutations in mitochondria could be related to hearing loss. Later, many mitochondrial DNA mutations were discovered and these mutations interfere with energy production and cause systemic neuromuscular disorders. Also, inherited mitochondrial mutations can cause nonsyndromic hearing loss and predispose to aminoglycoside-induced hearing loss, while acquired mitochondrial mutations are thought to be one of the causes of presbycusis (Flexer & Madell, 2008).

Hearing loss could be a component of many mitochondrial syndromes. Some of them are related to neuromuscular disease. Two forms of mitochondrial hearing loss are nonsyndromic: mutations in MT-RNR1 and MT-TSI (encodes 125rRNA and tRNAser) in mitochondrial genes are responsible for hearing loss. These mutations and their location in the mitochondrial DNA show that this hearing loss is maternal in families. Pathogenic variants in MT-RNR1 can be associated with a predisposition to aminoglycoside ototoxicity or late-onset sensorineural hearing loss. Pathogenic variants in MT-TS1 are usually related to childhood-onset of sensorineural hearing loss. Moreover, hearing loss associated with aminoglycoside ototoxicity is bilateral and severe to profound, occurring within a few days to weeks after administering any amount of aminoglycoside antibiotics such as gentamycin, tobramycin, amikacin, kanamycin, or streptomycin. Although hearing loss is associated with pathogenic variants in MT-TS1, it is considered nonsyndromic and the m.7445A>G substitution is also associated with palmoplantar keratoderma (Pandya A, 2004).

2.5 Syndromic genetic Deafness:

Genetic heterogeneity does not play the leading role in the syndromic type of hearing loss. Most syndromes with developmental disorders are genetically homogeneous and can be easily diagnosed with DNA through mutation analysis or linkage analysis.

Usher syndrome is the syndrome that has the most heterogeneous of genetics related to hearing loss. It is characterized by additional retinitis pigmentosa. Usher syndrome has three types, type I has six different loci, type II has three loci, and type III has one. The most common one is type I, which accounts for about 60% of cases.

About 30% of prelingual deafness is caused by syndromic hearing loss. These disorders are monogenic and they are dominant as they need one mutated gene to be inherited in the genome to cause the disorder (Friedman et al., 2003).

- Waardenburg Syndrome

This syndrome, called by the name of the geneticist who described it, Petrus Johannes "Waardenburg" is characterized by developmental anomalies of the eyelids, nasal root, eyebrows, and other findings of heterochromia irides, white forelock, and sensorineural deafness. Molecular methods characterize four types. The primary differentiating aspect is the presence (type 1) or absence (type 2) of the lateral displacement of the medial canthus and lacrimal puncta. Waardenburg syndrome type 3 (Klein—Waardenburg) is similar to type I but is also characterized by musculoskeletal anomalies (an ortho-osteomyomo dysplasia of the upper limbs or abnormalities of the arms). Waardenburg type 4 (Shah—Waardenhurg syndrome) is the association of Waardenburg syndrome with congenital ganglionic megacolon (Hirsch sprung disease) (Spiegel & Wright, 2003).

- Branchio- Oto- Renal Syndrome

Branchio- Oto- Renal Syndrome is rare; the possibility to occur is about 1/40,000 (Fraser & et al., 1978). The term branchio-oto-renal syndrome (BOR) was first used in 1975 by Melnick, 1976 to refer to patients with branchial cleft, fistulas, or cysts; otological anomalies, including malformed pinnae, preauricular pits or sinuses, and hearing loss in addition to renal anomalies of various types.

This syndrome includes branchial and kidney malformations as well as hearing loss, a genetic disorder—a highly variable expression of the disease, even among members of the same family. Malformations of the branchial arches can include cupping of the outer ear, ear pits in front of or on the outer ear, tags of skin in front of the ear, and cysts or fistulas on the neck. Renal abnormalities can range from mild renal hypoplasia to bilateral renal agenesis; however, many individuals with BOR have either no renal disease or do not experience symptoms of their renal anomalies. On rare occasions, individuals with BOR may also have blocked tear ducts that interfere with tear flow and require surgical repair. Most individuals with BOR (more than 90%) have some degree of hearing loss, and some also have radiologic abnormalities such as enlarged vestibular aqueducts. The type of loss can be mixed (52%), conductive (33%), or sensorineural (29%) with severity ranging from mild to profound, and the loss can either be non-progressive (about 70%) or progressive (about 30%). Approximately 40% of individuals have a mutation in EYAI, less than 1% have a mutation in S1XI. Moreover, others likely have mutations in additional genes yet to be discovered (Flexer & Madell, 2008).

- Usher Syndrome

When a human has both blindness and deafness, he or she could carry a genetic mutation causing Usher Syndrome. As many as 50% of the cases of individuals with loss of hearing and blindness are

affected by Usher Syndrome. An autosomal recessive mutation causes this syndrome, which means that the affected individual must have two mutated genes. Most cases are inherited evidently from parents, who carry one mutated copy but are not affected. These mutations cause degeneration of hair cells in the cochlea (inner ear), resulting in deafness. They also destroy the rod cells in the retina. Vision loss in Usher Syndrome usually begins as night blindness and loss of peripheral vision (tunnel vision) and progresses to blindness (retinitis pigmentosa)(Matthew et al., 2007).

Usher syndrome type 1 is characterized by profound hearing loss present at birth, which does not worsen over time. Until now, five genes and two loci are known to cause (Usher syndrome type 1. Affected people also have balance problems resulting from changes to their inner ear (vestibular ataxia). The onset of vision loss begins in childhood. People with Usher syndrome type 2 generally have milder hearing loss than in type 1. These people do not have the balance problems experienced in type 1 and vision loss usually occurs only in adolescence, but vision problems progress with age. The hearing loss in Usher Syndrome type 3 worsens with time and generally starts after children have begun speaking (post-lingual hearing loss). Balance problems of varying severity may be present. The onset of vision loss ranges from adolescence into adulthood. Diagnosis of Usher syndrome is usually done on people who have combined deafness and blindness. Because vision loss occurs later in life, most individuals with Usher Syndrome are first diagnosed as having nonsyndromic hearing loss (hearing loss not associated with other features) (Bork et al., 2001).

CHAPTER 3

3.1 Problem statements and objectives:

In Palestine, marriage between related families is widespread. Therefore, it is likely that children inherit diseases and disorders resulting from the same and convergent genome that is carried by both parents. One of these inherited disorders is hearing loss.

The main work of this study is to screen the mutated genes in the families that have these disorders and compare them to candidate genes that are known previously. This will enable us to recognize the cause of hearing loss, improve proper diagnosis, and provide proper genetic counseling for affected families.

The current study aims to achieve the following objectives:

1. Validation of hearing loss mutations in the family members by PCR.
2. Investigate candidate mutation/s using the Sanger sequencing method.
3. Confirm the mutation/s by testing the segregation of the variant/s in affected families.

3.2 Subjects and Methods

3.2.1 Study Location:

Experimental work was done at the Hereditary Research Laboratory (HRL) at Bethlehem University.

3.2.2 Study Population:

The study population consisted of 29 families diagnosed with prelingual bilateral hearing loss, 29 children, and their family members for a total of 135 samples obtained.

The hereditary research lab staff contacted many organizations and institutions for hearing loss care. The staff sent a form to the parents requesting the participation of their children in the research. After collecting the approvals, the lab assistants interviewed parents and students to identify the family history, constructed genetic pedigrees, and asked some questions about relatives in the family. A blood sample was taken from the patients and their families who were accepted to join the study.

3.2.3 Ethical Consideration:

The study is part of a research work approved by the ethical review board of Bethlehem University. First informed consent assent from each patient or his\her parents before enrolling in this study, then staff interviewed children, their parents, other informative relatives.

3.2.4 Blood Sample:

The staff collected five to ten milliliters of peripheral blood samples in EDTA vacutainer tubes from the 29 families, for a total of 135 patients diagnosed with hearing loss.

3.2.5 Study Design:

After confirming many solved probands through Connexin gene mutation GJB2 (in which mutations primarily cause hearing loss) by a colleague at Hereditary Research Laboratory (HRL) in Bethlehem University, the remaining 29 Probands were not solved. The 29 Probands went through the Comprehensive Hearing Loss and Deafness Panel to check which mutations have each. After knowing and defining the mutations for each proband, the lab assistant took blood from their families to study the segregation and designed the primers. Each proband and his or her family members that gave a DNA sample had amplified by their specific primers and sequenced to confirm the segregation of the mutation.

This project studied the segregation of the 29 probands mutation in their families members (135 patients) after defining the mutation and designing the specific primers for each one.
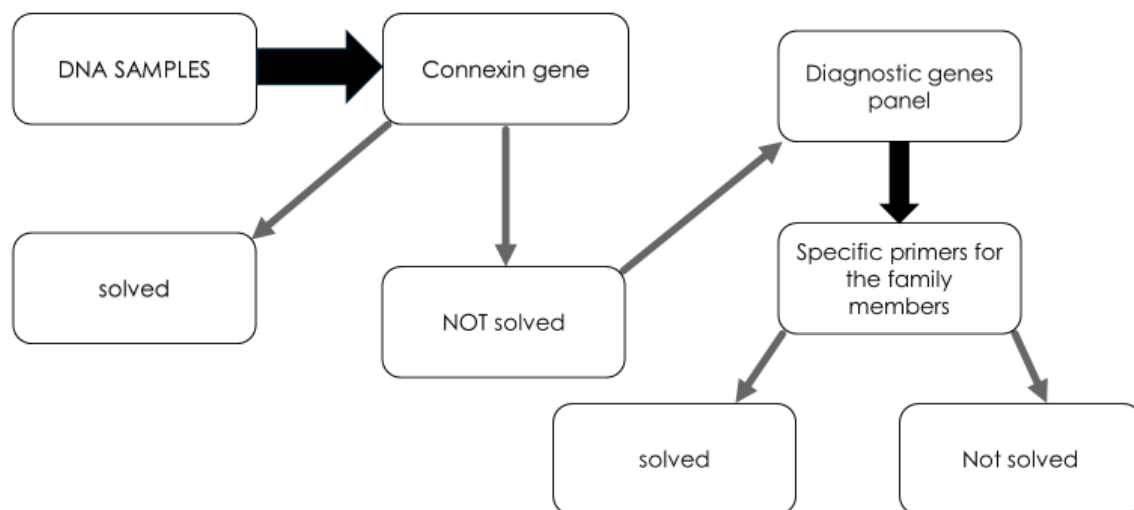
Study design

Figure 5: Study design: to define the mutation in hearing loss cases, first we assessed Connexin gene mutation if it is found. After that we do a diagnostic hearing loss gene panel for the cases that don't have the connexin gene mutation, to know which mutation each case does indeed have.

3.2.6 Genetic analysis

3.2.6.1 Genes selection:

There are 48 common genes recorded responsible for hearing loss among Palestinian populations, as shown in Table 2 (A.A Rayyan, 2020). However, because there is no deafness genetic panel for the Palestinian population, the Comprehensive Hearing Loss and Deafness Panel containing 181 known and candidate genes for hearing loss was done for the probands we have. Specific mutation for each family is shown in Table 3.

Table 2: Genes associated with HL among the Palestinian population.

Genes responsible for hearing Loss in Palestinian families			
ADGRV1	ESPN	MYO6	MYO3A
CACNA1D	ESRRB	MYO7A	RDX
CDC14A	GIPC3	MYO15A	SLC26A4
CDH23	GJB2	OTOA	STRC
CLDN14	GPSM2	OTOF	TBC1D24
CLPP	HSD17B4	PAX3	TECTA
COCH	ILDR1	PCDH15	TMC1
COL11A2	LARS2	PJVK	TMPRSS3
COL4A6	LHFPL5	POU3F4	TRIOBP
DFNA5	LOXHD1	POU4F3	USH1C
EDNRB	LRTOMT	PTPRQ	USH1G
EPS8L2	MARVELD2	PTRH2	USH2A

Table 3: Mutations of the sample's family members and their genes.

Family code	HL phenotype	gene	Mutation
AA-A	Severe to profound	MYO7A	R895C
AA-G	Severe to profound	CLDN14	P28L
AA-L	moderate	SLC26A4	G334V

AA-O	Severe to profound	CLDN14	P28L
AA-K	Severe to profound	CLDN14	P28L
GA-A	profound	LARS2	N153H
GA-C	severe	ADGRV1	V617M\I2332F
GA-D	moderate	CDH23	P559S
GA-F	Severe to profound	TMC1	Q395fs
GA-G	Severe to profound	POU4F3	E18fs
GA-H	Severe to profound	CLPP	Donor: +: I:3 (8.69>1.83)
GA-M	profound	SLITRK6	R635H
GA-N	Severe to profound	GPSM2	W326X
GA-O	profound	ILDR1	E98fs
GA-P	profound	TMC1	R34X
IA	No audiograms	USH2A	I4996F\V4868M
IB	Severe to profound	MYO15A	S222W\P2500L
IE	Severe to profound	MYO15A	R2298Q
IH	Severe to profound	RDX	R171fs
II	Severe to profound	ILDR1	Q185X
IJ	moderate	ADGRV1	S4772X
IM	Severe to profound	OTOF	E57X
IP	Moderate to severe	CDH23	P2205L
IT	Severe to profound	MYO7A	Q234X\Q1661X
IW		EPS8L2\MYO6	p.S50fs\D382N
AA-E	profound	ESPN	S694fs of 854
GA-E	Moderate to severe	SLC22A4	S66T
IO	profound	MYO15A	MYO15A-Donor: +: I:5 (9.25>2.65) G> A
AA-X	Severe to profound	TMPRSS3	Splice mutation, c.323-6G>A

3.2.6.2 DNA extraction by salting–out technique:

Five to ten milliliters of whole blood were collected in sterile EDTA vacutainer tubes. Four times the volume of the red blood cells lysis buffer, as H. Nasiri,2005 technique, samples were added to each sample and mixed gently. Tubes containing samples with lysis buffer were kept on ice for 30 minutes and then centrifuged at 2000 rounds per minute (2000 rpm) for 10 minutes at 4°C. After centrifugation, the supernatant was carefully discarded and the pellet was re-suspended in 3 ml of red blood cells lysis buffer and centrifuged again. The conserved nucleated cell pellet was then suspended in a mixture of 3 ml of 1X lysis buffer, 100µl of 20% SDS (Amresoc-Cat# M112), and 100µl of 5mg/ml Proteinase K (Amresco-Cat# E115) and incubated overnight at 37°C. After incubation, 1ml

of 6M sodium chloride (NaCl) was added to the lysate, vortexed vigorously, and then centrifugated at 3000 rpm for 20 minutes at room temperature. The clear supernatant containing genetic material is carefully isolated and transferred gently into a 15 ml tube while avoiding the salt protein deposit.

The nucleic acid is precipitated from the supernatant by adding absolute cold ethanol (EtOH) two times the supernatant volume and mixed gently by inverting the tube. Using a glass Pasteur pipette, DNA was removed, washed in 70% ethanol, and dried for few minutes. The extracted DNA was then dissolved in (200-1000μl) of 0.02% sodium azide depending on the amount of DNA and left overnight at room temperature.

3.2.6.3 Polymerase Chain Reaction (PCR):

The extracted DNA was used as a template for PCR reaction for hearing loss genes, in general, using specific primers. In general, PCRs were done using 6.25μl of 2X PCR Ready-mix (Abgene _Cat# AB-0575-DC-LD), 0.25μl of 10 pmol of each primer (forward and reverse), 0.5μl from 100ng\μl extracted DNA template, and 5.25 μl doubled distilled water (DDH2O) in a total volume of 12.5 μl.

Each primer has a primary PCR program depends on the design and the instructions for the manufacturer.

3.2.6.4. Electrophoresis:

Depending on the amplified DNA fragment, 1% agarose gel prepared using 1X TAE buffer containing 0.01% ethidium bromide (Amresco-Cat # E406) was used to separate the amplicon by electrophoresis. Three μl of each PCR product along with a DNA ladder (Thermo Scientific-Cat# SM0241) were loaded into the gel for 30-40 minutes at 100V. DNA fragments were observed and visualized using Bio-Rad ultraviolet imaging system.

3.2.6.5. Exo-SAP PCR cleanup:

Polymerase Chain Reaction (PCR) products were cleaned up using Exo-SAP kit (Thermo Scientific-Cat# 78200.200.UL) to remove residual nucleotides as indicated below:

Component	Volume per one sample
dd H2O	1.5 ul
Exo	0.25 ul

SAP	0.25 ul
PCR products	5 ul

3.2.6.6 Big Dye Terminator Cycle Sequencing:

A cycle sequencing reaction was performed using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Cat#4337450) similar to a PCR reaction except for single primer either forward or reverse used as the following:

Component	Volume per one sample
Dd H2O	11.5 ul
buffer	1.5 ul
BD 64 buffer	2 ul
Big Dye	2 ul
F or R primer	0.5 ul
Cleaned DNA	2 ul

3.2.6.7 Cleaning of the PCR product using EDTA/Ethanol precipitation method for sequencing:

Precipitation and cleaning of each PCR reaction were carried out by adding 100 ul of Absolute Ethanol and five μl of 125mM EDTA (Amresco-Cat # 0720) followed by incubation at 20° for 30 minutes and then centrifugation for 30 minutes at 3800 RPM at 4°C. The supernatant was discarded, and 60 μl of 70% ethanol was added to each reaction and centrifuged again for 20 minutes. The samples were then put on tissue paper for 1 minute at 500 RPM and dried at 95°C for 5 minutes. 16 μl of Hi-Di Formamide (Applied Biosystems, Cat # 4311320) were then added to the samples before drying again at 95°C for 2 minutes. Finally, the reaction products were put on ice for 5 minutes before the following sequencing steps.

3.2.6.8 Sequencing of the purified PCR Product:

The sequencing plate was prepared by adding 12 ul high dye, incubating at 95 ℃ for 5 minutes, then over ice for 5 minutes after this sequencing was done using ABI 3130 DNA Sequencer (Applied Biosystems, S/N:20355-023).

3.2.6.9 Sequencing analysis:

Chromatogram files were manually checked using Chromas 2.6 and then analyzed and aligned using UCSC Genome Browser. Sequencing analysis for many genes that may affect the hearing process was done. All positive results for any candidate mutation were targeted for segregation analysis in family members to confirm the results.

CHAPTER 4

Results:

A significant way to diagnose hearing loss genetically is to look for the Connexin gene mutation (GJB2) because it is the most frequent mutation. If the Connexin gene mutation is not found, then a genetic panel must define the other hearing-related gene and the specific mutation in the gene (De Keulenaer, 2012).

Twenty-nine probands did not have the Connexin gene mutation, so the genetic panel defined the mutations for hearing loss. Blood samples were taken from the families, then the DNA was extracted, then amplified by PCR techniques for not only the 29 probands but also for their family members, so we got 135 total samples to solve.

First of all, as shown in Table 3, the mutation for each family member was not validated, so PCR amplification was done to validate these mutations. After the amplification, the study ensures that the mutations are existing, so the mutations are validated.

Table 3: Mutations of the sample's family members and their genes.

Family code	HL phenotype	gene	Mutation
AA-A	Severe to profound	MYO7A	R895C
AA-G	Severe to profound	CLDN14	P28L
AA-L	moderate	SLC26A4	G334V
AA-O	Severe to profound	CLDN14	P28L
AA-K	Severe to profound	CLDN14	P28L
GA-A	Profound	LARS2	N153H
GA-C	Severe	ADGRV1	V617M\I2332F
GA-D	moderate	CDH23	P559S
GA-F	Severe to profound	TMC1	Q395fs
GA-G	Severe to profound	POU4F3	E18fs
GA-H	Severe to profound	CLPP	Donor: +: I:3 (8.69>1.83)
GA-M	Profound	SLITRK6	R635H
GA-N	Severe to profound	GPSM2	W326X
GA-O	Profound	ILDR1	E98fs
GA-P	Profound	TMC1	R34X
IA	No audiograms	USH2A	I4996F\V4868M

IB	Severe to profound	MYO15A	S222W\P2500L
IE	Severe to profound	MYO15A	R2298Q
IH	Severe to profound	RDX	R171fs
II	Severe to profound	ILDR1	Q185X
IJ	moderate	ADGRV1	S4772X
IM	Severe to profound	OTOF	E57X
IP	Moderate to severe	CDH23	P2205L
IT	Severe to profound	MYO7A	Q234X\Q1661X
IW		EPS8L2\MYO6	p.S50fs\D382N
AA-E	Profound	ESPN	S694fs of 854
GA-E	Moderate to severe	SLC22A4	S66T
IO	Profound	MYO15A	MYO15A-Donor: +: I:5 (9.25>2.65) G> A
AA-X	Severe to profound	TMPRSS3	Splice mutation, c.323-6G>A

As considering the confirmation of segregation of the mutation in the family as solved cases and not confirming the segregation as not solved cases. After sequencing, alignment, and checking the segregation of the mutation between the family members, 18 families had confirmed the segregation of mutations in the family members. In comparison, 11 families did not confirm that; for many reasons that will be discussed in the discussion chapter.

29 families

- 11 families not solved due to various reasons 38%
- 18 families solved as segregated known mutations 62%

■ 11 families Not solved due to several reasons   ■ 18 families solved as segregated known mutations
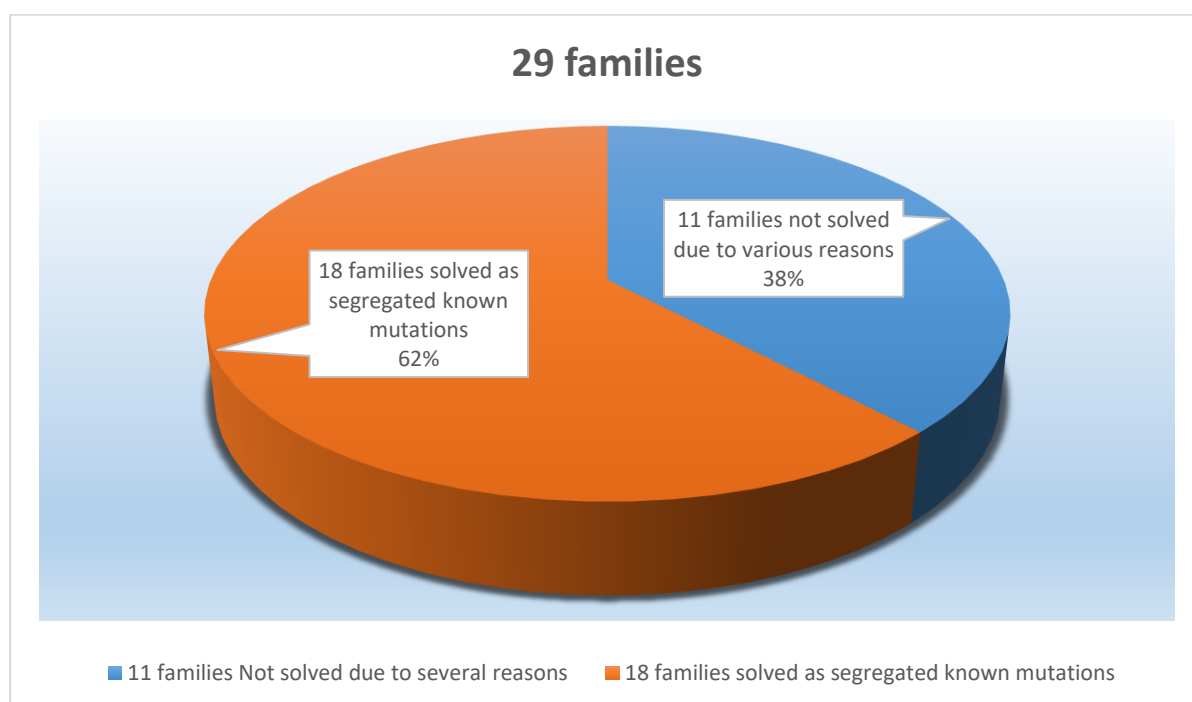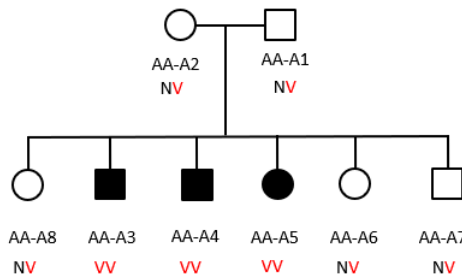
Figure 6: Diagram showing number of families included in this study and percentages of solved and unsolved cases.

For example, these are family pedigrees for four families that confirmed the segregation.
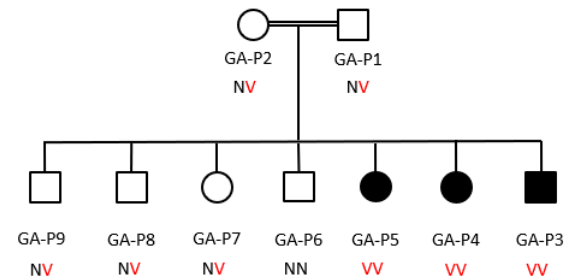
Family AA-A

Family GA-P

Mutation:
chr11:76891516
MYO7A_R895C ,c.C2683T (exon22), gerp 4.3, ExAC=2

Mutation:
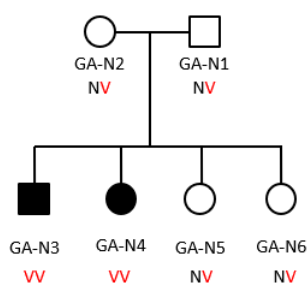TMC1_R34X (c.C100T (exon7)), chr9:75309494

Family II
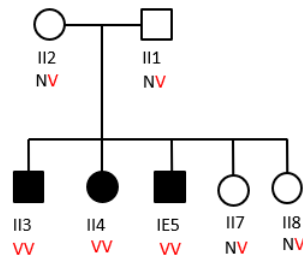
Family GA-O

Mutation:
LDR1-Q185X, c.C553T (exon5),chr3: 121712776

Mutation:
ILDR1_E98fs(c.294delA (exon3)), chr3:121724176

Figure 7: Illustrates four-family pedigrees as examples that indicate a possible autosomal recessive pattern of inheritance. (V: mutant allele, N: normal allele).

In Figure 7, we can notice that parents are carriers Also, some sons are heterozygous, and not affected by hearing loss. Affected offspring are homozygous recessive, as shown in the figure.
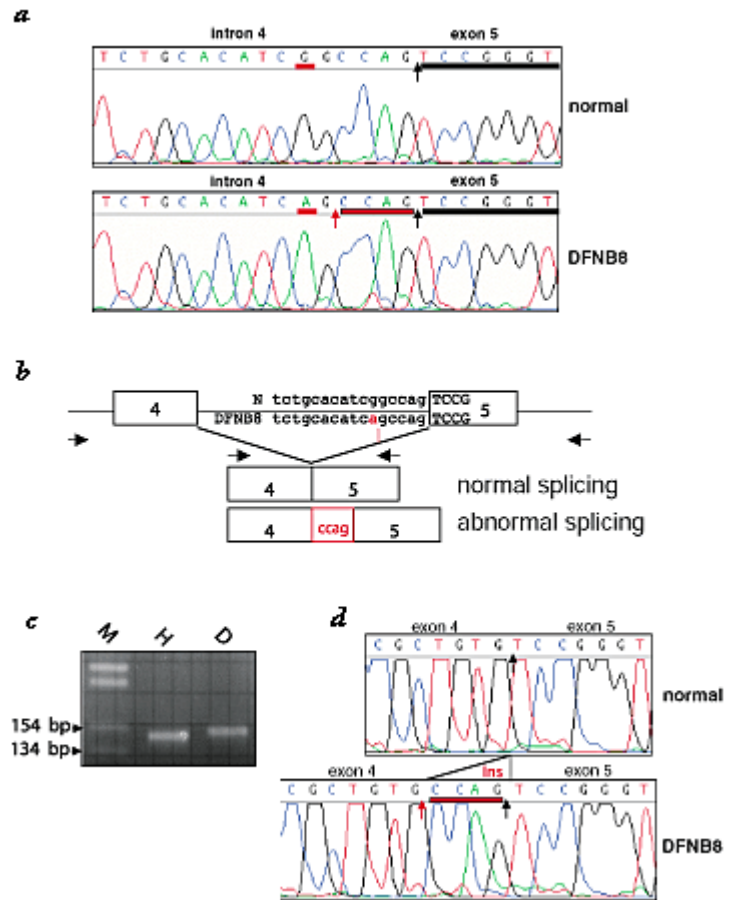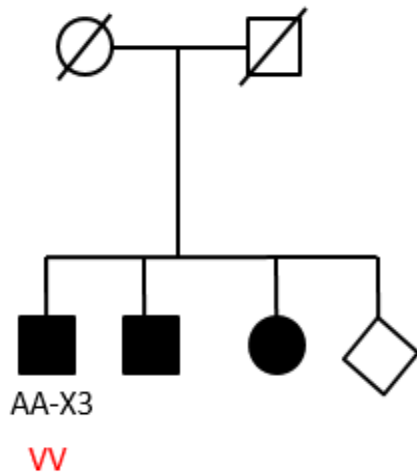
Family AA-X

AA-X3

VV

Figure 8: Pedigree of AA-X family and TMPRSS3 splice mutation, (a) Chromatograms of normal and affected individuals for the TMPRSS3 gene showing the G→A substitution. (b) A genomic fragment containing exons 4 and 5 of TMPRSS3 was inserted into an exon 4. (c,d) PCR for exon 4 identified a 4-bp insertion between exons 4 and 5.

Family AA-SX confirms the previous novel mutation validated by Scott and his group, 2001.

This mutation in TMPRSS3 is a splice mutation that makes a splice terminator not in the same place as typically known. Because of this mutation, there are 4 bp added to mRNA, and a frameshift that makes the splicing after them, this frameshift is a result of a substitution of G base to A. The result from this frameshift is addition of 25 amino acids.

CHAPTER 5

Discussion and Conclusion

5.1 Discussion

5.1.1 Hearing loss is highly heterogeneous:

Hearing loss is considered the most common congenital sensory impairment worldwide, as it affects 1 of 650 newborns (Mehl &Thomson, 2002), with nonsyndromic accounting for about 70% of hereditary hearing loss (Kemperman et al., 2002). Hearing Loss is an even more common and serious health problem in the Middle East. The high consanguineous marriage rate in Middle Eastern societies is a significant contributor to autosomal recessive hearing loss.
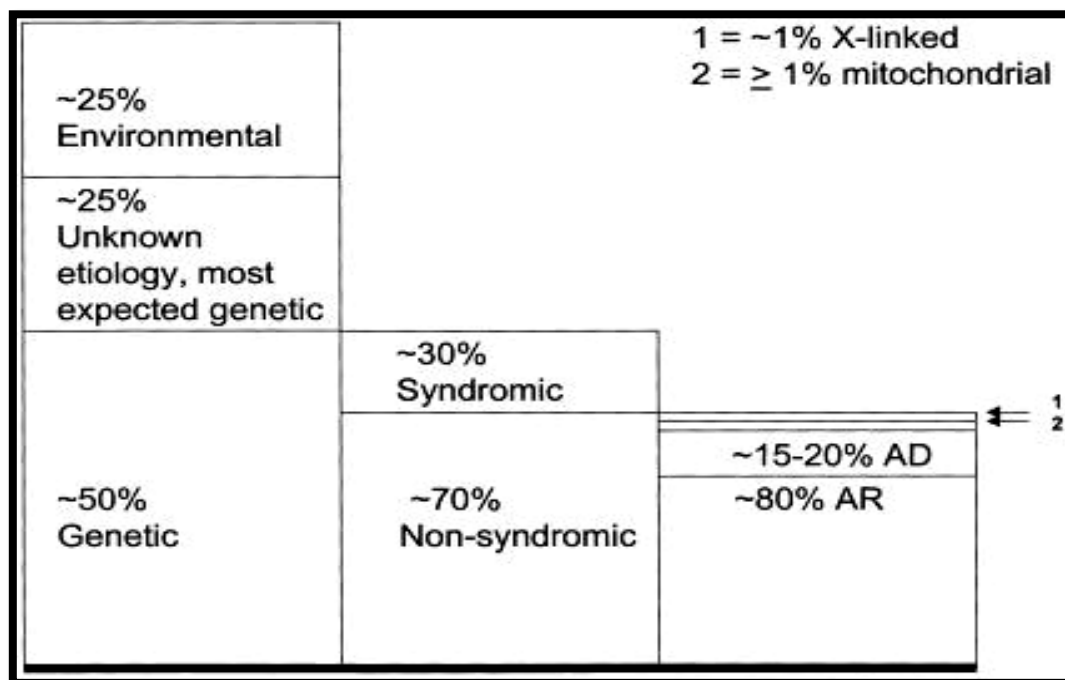

Figure 9: (column 1) causes of hearing, (Column 2) syndromic versus nonsyndromic HL, (Column 3) inheritance pattern in the nonsyndromic group. (Schrijver, 2004)

Some scientists argue about the percentage of X-linked mutations, such as Virgina Corvino and her team, they mentioned in their paper (2018) that X-linked mutation percentage up to 2%, and more than mitochondrial mutations.

5.1.2 Importance of the study:

Because of the high consanguinity marriage rate in the Palestinian population that may cause children's disorders including hearing loss which results from combining recessive mutations, Palestinians need to know the genetic factors that cause this disease, and consequently be aware that consanguineous marriage, and its potential detrimental effects in case of family history. We cannot change unreasonably long-term traditions and religion. As well most mutations are recessive. Therefore, testing can help. A priority for public health services should be premarital genetic. Counseling and testing to prevent passing genetic mutations to the next generation.

Our results indicate that the genetic factor is the principal factor causing hearing loss in the Palestinian population, whatever the percentage of confirmation of the segregation of the mutation, all the samples show that they have at least one mutation.

5.1.3 Limitations of the study:

Despite the mutations defined and validated for each family, there are many reasons for the families that did not confirm the segregation of the mutation. One of them is sample size as there were not enough members to check the segregation of the mutation, and there were many families that we did not get samples from the parents due to refusing to join the study, and in many families, we only had one sample from one of the parents, not both.

There are many limitations to this project. First of all, at the level of PCR, false positives are due to nonspecific annealing, and polluted DNA by another DNA, but can potentially be overcome by more stringent PCR conditions. Second, at the level of Sanger sequencing, dye effects due to differences in incorporating the dye-labeled chain terminators into the DNA fragment, resulting in unequal peak heights and shapes in the electronic DNA sequence trace chromatogram after capillary electrophoresis. These limitations are common in genetic studies, and they are not restricted to this study.

We sought to reveal the mutations in the blood samples as they have existed in the tissue, but it is not accurate. Genetic mutation could exist in the tissue samples but not in the blood sample.

The most important reason for this limitation is the methodology of the study. The study was done at the level of sequencing. Nevertheless, we do not know if the genes are transcript cis or trans.

The genetic causes of gene expression changes can be classified into two categories: changes in cis-acting elements (e.g., promoters and enhancers), which are on the same chromosome of the gene they affect, and changes in trans-acting factors (e.g., transcription factors and chromatin modifiers), which are diffusible and can influence the expression of genes on other chromosomes. How gene expression is changed can affect its inheritance pattern and evolution (Ronald and Akey 2007). Thus, it is important to distinguish between these two types of change to understand the causes of intraspecific variation and interspecific divergence in gene expression.

For example, Figure 10 shows the family pedigrees for four families that did not confirm the segregation of the mutation from the parents to the children.
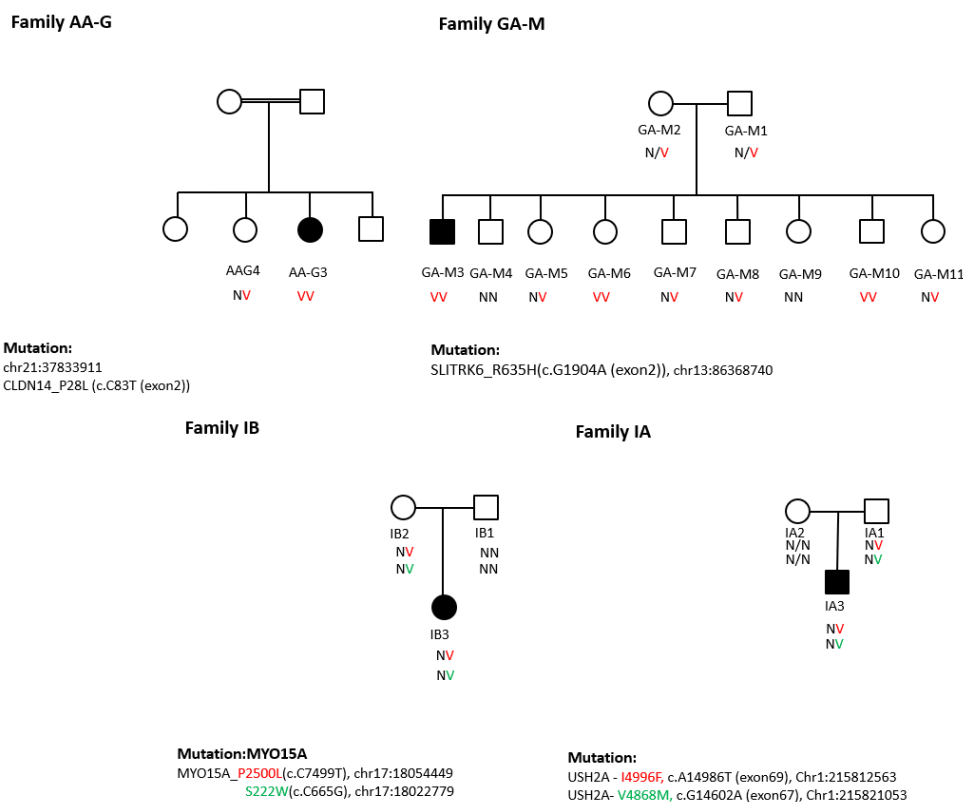

Figure 10: Four family pedigrees as examples that did not confirm the segregation of the mutation, despite of knowing the mutations in these families.

Figure 10 illustrates the three possible reasons why we were not able to confirm segregation for the 11 families. In the first scenario, such as family AA-G, we don't have DNA samples from the parents because they didn't participate in the study, so we can't study the segregation. However, if we study the CLDN14 mutation, we notice that this mutation is autosomal recessive, so we can conclude that the mutation is likely, but not certainly inherited from the parents.
In the second scenario, such as family GA-M, the parents are carriers (heterozygous) for the mutation. However, the son and daughter who are homozygous for the mutation are not affected.

The reason could be at the level of the expression of the gene. Perhaps if we study the SLITRK6 mutation, we can see that this mutation affects people in old age, not early age, so we can assume that this mutation is segregated from the parents, but we don't have enough details about their age to confirm or disprove this hypothesis.

In The third scenario, such as families IB and IA, is that one of the parents is the carrier for two mutations but not affected, while the other parent is healthy. The offspring of these families have the same alleles for the same mutations as the carrier parent, but the offspring are affected. In this situation, we can't know if the mutation inherits independently or not. Further studies must be done on these families to know the type of inheriting for these mutations if they are cis or trans to confirm the segregation of the mutations.

5.1.4 Genetic counseling and pre-implantation genetic diagnosis (PGD):

Genetic counseling advises parents on how can genetic factors could affect their family and sons. The counselor studies the family history to detect disorders abnormalities and advises them to do some tests before marriage, like implantation genetic diagnosis (PGD).

The PGD is a test for embryos or oocytes before the stage of pre-implantation for genetic defects. It is suitable for parents who have a high risk of severe Mendelian disorders, structural chromosome abnormalities, or mitochondrial disorders. This test needs in vitro fertilization (IVF) after screening the oocytes and the sperms, it can help avoid specific syndromes or disorders (Geraedts, 2009).

This test should be enclouded in a health care program for the people who have hearing loss or carrier of the mutation. It will help the parents avoiding the disorder for future children.

5.2 Conclusion and Recommendations:

Many factors play a role or contribute to hearing loss, such as environmental factors and genetic factors. The genetic factor is the most interesting factor for investigators because this factor gives us the specific cause for hearing loss for each individual. This study aims to define the specific genetic factors (mutations) for each individual and ensure segregation. Further studies must be done about hearing loss at the level of sequencing and the level of expression.

We hope to reach the stage where every human has a hearing loss problem, or family history will be able to check genetic factors for hearing loss before family planning so that the percentage of affected individuals can decline.

CHAPTER 6

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