ABSTRACT

The aetiological diagnosis of recessive non-syndromic hearing loss poses a challenge owing to marked heterogeneity and the lack of identifying clinical features. The finding that up to 50% of recessive non-syndromal genetic hearing loss among Caucasians was due to mutations in *GJB2*, the gene encoding Connexin 26 (Cx26) was a breakthrough, whose value as a diagnostic tool has been limited by the significant variation in the prevalence of deafness genes and loci among population groups. The significant association of the *GJB6*-D13S1830 deletion among individuals with one mutant *GJB2* allele highlighted the need to explore population specific genetic mutations for NSHL. Although data from Sub-Saharan Africa is limited, reported studies found a high prevalence of R143W *GJB2* mutation among Ghanaian, the 35delG mutation in 5 out of 139 Sudanese and a low prevalence of *GJB2* variations among 385 Kenyan deaf children. The mutation spectrum of Waardenburg Syndrome (WS) in Africans has not been documented.

During a visit to a School for the Deaf in the Limpopo Province of South Africa in 1997, it was noted that a high number of students came from Nzhelele sub-district. All had childhood onset hearing loss with no associated anomalies or disorders. The question arose as to whether there was a high-risk area for deafness in the Limpopo Province and what the aetiology of this hearing loss was.The main aim of this study was to investigate the role of *GJB2*, the *GJB6*-D13S1830 deletion, and the four common mitochondrial mutations, A1555G, A3243G, A7511C and A7445G, in the African hearing-impaired population of Limpopo province in South Africa, and to identify the mutation spectrum of the deafness genes found. The type and degree of hearing loss in this hearing impaired population would also be assessed. Secondly,

this study sought to identify the mutations in a sibling pair with clinical WS and to use the findings in a future study to establish the mutation spectrum of WS in the African population of the Limpopo province and of South Africa in general.

The study was designed as a two phase study, in which phase 1 was used for hypothesis formulation and phase 2 was for hypothesis testing. While phase 1 was a descriptive retrospective case study, phase 2 was a combination of sample survey and prospective descriptive case study. In phase 1, demographic data of 361 students in two schools of the deaf in the Limpopo province was analyzed for evidence of areas of high risk populations for deafness in the province. In phase 2, a group of 182 individuals with genetic non-syndromic hearing loss (NSHL) and two siblings with clinical WS from two schools for the Deaf in the Limpopo Province of South Africa were investigated. A thorough clinical examination, audiological evaluation and urinalysis were done. Mutational screening was carried out in all 184 subjects using genomic DNA using single-strand conformation polymorphism (SSCP), multiplex polymerase chain reaction (PCR), and direct sequencing for *GJB2,* and Restriction Fragment-Length Polymorphism (PCR–*RFLP)* analysis for *GJB6*, and SSCP, hetero-duplex analysis, and direct sequencing of the first 8 exons of *PAX3* and all of *MITF* for Waarenburg syndrome. Data analysis was by geographical mapping, frequency tables, tests of association with calculation of odds ratios, and binary logistic regression analysis using STATA and GIS mapping systems.

The results indicate that there seem to be areas of genuine populations at risk for hearing loss in the Limpopo province of South Africa, namely Mutale and parts of Makhado and Thulamela municipalities. In Thulamela (NP343) wards 11-15, 26-30

and 31-35, and in Mutale (NP 344) wards 6-10, together accounted for 67 (18%) of participants in phase 1, and 33 (18%) of the participants in phase 2 of the study. Mutale municipality in the Vhembe district gave with a projected prevalence of at least 13.14 deaf children per 100,000 African population attending the local school for the deaf.

The observed hearing loss is a genetic, non-syndromic form, which is mainly severe and severe to profound, although without any clear defining configuration or shape. It is a stable, non-progressive and prelingual form of hearing loss, implying that this may be a recessive form of deafness. No identifiable environmental confounding factors or associations were identified. The deafness is not linked the common known auditory gene mutations in *GJB2,* the *GJB6*-D13S1830 deletion, or the common mitochondrial mutations A1555G, A3243G, A7511C and A7445G. Severe and profound levels of hearing loss were found in 22.8% and 75% of the cohort respectively, with the majority exhibiting flat (70.1%) or sloping (23.4%) audiograms that were commonly symmetrical (81.5%). However, as indicated, there was no clear pattern in the audiological findings overall.

None of the 184 hearing impaired individuals exhibited any of the reported disease causing mutations of *GJB2*, including 35delG. There was, however, a high prevalence of two variants, the C>T variant at position g.3318-15 and the C>T variant at position g.3318-34, occurring in 21.4% and 46.2% of the deaf cohort respectively. The same variants were found to occur in 35% and 42.6% of a normal hearing control group (n = 63) respectively, indicating that these variations are polymorphisms. In three subjects (1.63% of the cohort), a T>A homozygous variation at position g.3318-6 was

detected. Its significance in the causation of NSSNHL is yet to be determined. The *GJB6*-D13S1830 deletion was not detected in any of the participants. None of the four mitochondrial mutations screened for were found.

These results indicate that *GJB2* is not a significant deafness gene in the African population of the Limpopo Province of South Africa and that significant genes for non-syndromic recessive hearing loss in this population are yet to be found. The geographical clustering of deafness found in this study, combined with the lack of identifiable common associated clinical features among the subjects of this study (excluding the WS sibling pair), suggests that these subjects have a genetic recessive non-syndromal type of hearing loss. In the context of historical and cultural evidence of consanguinity in this population, a founder effect cannot be ruled out.

A rare mutation, R223X, previously identified only once out of 470 WS patients, was identified in the *PAX3* gene among the WS sibling pair. A novel silent change GGG>GGT at amino acid 293, was also identified. These identical findings document, for the first time, a molecular defect in WS in an African sibling pair, and confirm WS Type I in this family, which could be found in other WS type I South Africans in the Limpopo Province of South Africa.

The current study demonstrated that parents of genetically hearing impaired children in these areas are able to detect hearing loss at an early age, with over 60% suspecting their children's hearing loss below 6 months of age. A child-centered management model encompassing all the areas relevant to childhood deafness/hearing impairment, which takes into consideration the prevailing logistical and financial constraints of the

available healthcare system, is proposed. The implementation of this model requires a paradigm shift from the current fragmented model of service delivery to a cohesive patient-centered approach, based on concrete data from appropriate community based research, in which all the relevant parties communicate and share resources.

It would achieve the goals of early detection and intervention, as well as inclusive education for all. The relevant health and education policies are already in place and the posts funded. Equitable implementation of these policies would require appropriate community based research, as well as improved communication and consultation between the various stakeholders to ensure an efficient and affordable quality healthcare service for all hearing impaired South Africans.

Key words

Recessive, non-syndromic hearing loss, Connexin 26 (Cx26), Sub-Saharan Africa, *GJB2*, *GJB6*-D13S1830 deletion, Waardenburg Syndrome (WS) type I, mitochondrial mutations A1555G, A3243G, A7511C and A7445G, Limpopo Province of South Africa, *PAX3* R223X mutation, *PAX3* silent change GGG>GGT at amino acid 293, prevalence, high-risk area for deafness, single strand conformation polymorphism (SSCP) assay, direct sequencing for *GJB2*, Restriction Fragment-Length Polymorphism (PCR–*RFLP)* analysis, hetero-duplex analysis, direct sequencing, enzyme digest, congruent choropleth maps, computer-generated cluster-display map, populations-at-risk, geographical displays, consanguinity, risk factors for hearing loss, audiogram configuration, polymorphisms, congenital hearing loss, childhood hearing loss, Universal Neonatal Hearing Screening, EDHI, early detection of hearing impairement programmes

CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION

Summary of the chapter

This chapter i**ntroduces** hearing loss and its classification, and lays a background of the expression of genes in populations. The chapter also paints a picture of the study area and study population, and concludes with the rationale for this study.

"Few areas of audiology have advanced as rapidly as cochlear physiology and biophysics have over the past decade. The advance began with the shock realization that existing knowledge and accepted concepts could not explain the response of the cochlea to sound and in particular otoacoustic emissions. Our very understanding of both the physical basis of hearing and the nature of hearing impairment was challenged."

Grandiori, Cianfrone and Kemp, 1990 VIII

Hearing impairment is a complex but common disorder worldwide. Its prevalence increases with age, affecting 10% to 15% of the population (Hotchkiss 1989; Gorlin et al., 1995; Willems, 2000; Liu et al., 2001). The estimated incidence of childhood hearing loss is approximately 1 to 3 in 1000 (Davidson, Hyde, & Alberti, 1989), with the incidence of deafness at birth (pre-lingual) at about 1 in 1000 live births (Morton, 1991; Gorlin et al 1995). About 1 in 1000 children becomes deaf before adulthood in developed countries (Morton, 1991; Gorlin et al 1995). It is thought that figures in the developing world could be about twice this due to the impact of environmental factors especially infections (Davidson et al., 1989).

Because of the combined impact of environmental factors and genetics, as well as the interaction with an individual's genetic predisposition, there is a progressive increase in the prevalence of hearing loss within the general population over time in relation to age (Smith & van Camp 2005). An example of the impact of environmental interaction with an individual's genetic susceptibility is the aminoglycocide-induced

ototoxicity in individuals carrying the mitochondrial mutation 1555 A>G (Prezant et al., 1993). One study estimated that overall, 0.3% and 2.3% of the population manifest a hearing loss greater than 65 dB HL between ages 30 to 50 years and 60 to 70 years respectively in the United Kingdom (Davis et al., 1990).

The aetiologies of hearing loss (fig. 1.1) include both genetic and environmental factors, with genetic factors accounting for over 50%. In 30% of these, a syndrome is implicated. The rest, 70%, are believed to be non-syndromic, with up to 80% autosomal recessive, 20-25% autosomal dominant, and 1-1.5% X-linked, (Morton, 1991; Gorlin et al 1995; Fraser, 1964; Rose et al., 1977; Frazer, 1978; Parving, 1984; Newton 1985; Parving 1996; Petit et al., 2001; Rabionet et al., 2000; Smith & van Camp, 2005). The positions of genes within a genome are known as loci. Nomenclature for dominant loci are denoted with the prefix "DFNA", recessive loci with "DFNB", X-linked loci with "DFN", and modifying loci with "DFNM".

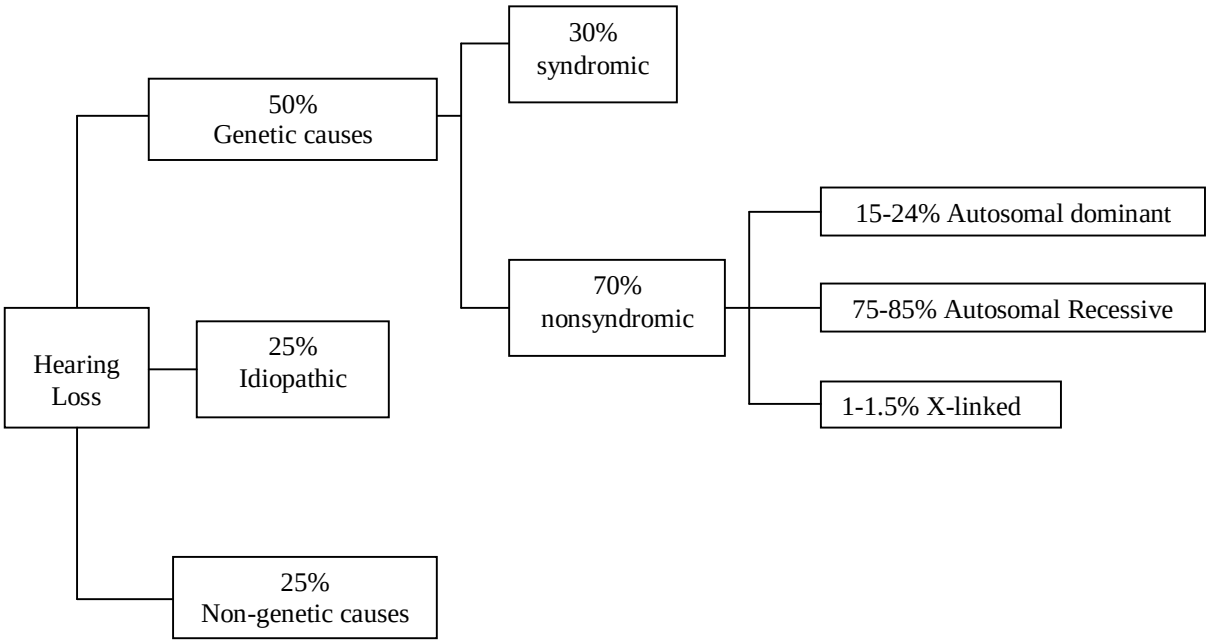


Figure 1.1: Aetiological classification of genetic hearing loss

(adopted from Fraser 1964, Rose et al 1977, Frazer 1978, Parving, 1984, Newton, 1985, Morton, 1991, Parving, 1996; Petit et al., 2001; Rabionet et al., 2000; Smith and van Camp, 2005.)

The majority of genetic hearing loss in humans is non-syndromic, that is, it is not associated with any visible abnormality of the ear or other organs, nor are there any related medical conditions. There may, however, be middle or inner ear abnormalities. Syndromic hearing loss on the other hand may be associated either with malformations of the external ear or other organs, or with other medical abnormalities (Smith & van Camp, 2005).

In children, the causes of acquired hearing loss include prenatal infections such as toxoplasmosis, rubella, cytomegalovirus, and herpes (the TORCH organisms), or postnatal infections such as bacterial meningitis (Smith & van Camp, 2005). Unrecognized asymptomatic congenital cytomegalovirus infection has been shown to cause variable, fluctuating, sensorineural hearing loss in children (Harris et al., 1984; Hicks et al., 1993; Schildroth et al., 1994).

Hearing loss is phenotypically and genetically heterogeneous (Konigsmark, 1969; Konigsmark & Gorlin, 1978, Fraser, 1976; Gorlin et al., 1995; Keats & Berlin, 1999; Steel & Kros, 2001; Resendes et al., 2001). This was underscored by Gorlin et al. (1995) when he listed 427 different forms of hereditary hearing impairment, both syndromic and non-syndromic (Gorlin et al., 1995). Fraser, in a visionary moment earlier (Fraser, 1976), had declared that **"within the foreseeable future it will be possible to define each type of genetically determined deafness by other criteria, such as can be employed at present, for example, for defining galactosemia and phenylketonuria, on biochemical grounds as two specific forms of mental**

subnormality.". This is the place reached in the later part of the 20th century and has been instrumental in driving the work presented in this thesis.

Major advances have been made in the study of genetic hearing loss in the past twenty years. Initially, the pathology of non-syndromic recessive hearing loss was confirmed mainly by microscopy (Ormerod, 1960; Schuknecht, 1967; Michaels et al., 1983; Schuknecht, 1987; Smith et al., 1992). The findings often showed evidence of degeneration or loss of sensory hair cells and sometimes a dysfunction of the sensory hair cells was implied (Ormerod, 1960; Schuknecht, 1967; Michaels et al., 1983; Schuknecht, 1987). This, however, did not adequately reveal the key processes leading to hair cell death or apoptosis. Diagnosis is known to be more accurate when studied at the molecular level (Steel & Bock, 1983, Steel & Brown 1994; Steel & Brown, 1996). It is therefore no longer adequate to determine the aetiology of hereditary hearing loss on clinical findings alone (Bussoli & Steel, 1998).

The advent of the mouse as a model for human deafness unlocked the molecular basis of hearing loss (Steel, 1995). Various studies of hereditary hearing loss revealed the molecular basis of normal auditory development and function (Petit, Levilliers, & Hardelin, 2001; Mustapha et al., 2001; Rabionet et al., 2000). It has been clearly shown that some of the genetic forms of hearing loss involve early developmental defects that interfere with normal hair cell function, even before cell death, while others have normally developing sensory hair cells which degenerate through apoptosis at sound presentation in the immediate post-natal period (Cohen-Salmon et al., 2002).

Studies also show that different mutations in the same gene may cause either syndromic or non-syndromic deafness, or even dominant or recessive types of deafness. Examples of these genes include *MYO7A* implicated in syndromic and non-syndromic hearing loss (Liu et al., 1997c), *PDS* implicated in both syndromic and non-syndromic hearing loss (Everett et al., 1997), as well as in a recessive type of hearing loss (Li et al., 1998), and the α-tectorin gene, *TECTA*, which has been shown to cause both dominant (Verhoeven et al., 1998) and recessive (Mustapha et al., 1999) forms of deafness. Some syndromes are caused by mutations in different genes. These include Waardenburg syndrome caused by mutations in *PAX3* (Tassabehji et al., 1992), *MITF* (Tassabehji et al., 1994), *SOX10* (Pingault et al., 1998) and *EDNRB* (Attie et al., 1995). Stickler syndrome is caused by mutations in *COL2A1* (Williams et al., 1996), *COL11A1* (Richards et al., 1996) as well as *COL11A2* (Vikkula et al., 1995). Alport syndrome is caused by mutations in *COL4A5* (Barker et al., 1990), *COL4A3* (Mochizuki et al., 1994a) and *COL4A4* (Mochizuki et al., 1994b), while Jarvel and Lange Nielsen syndrome is caused by mutations in *KVLQT1* (Neyroud et al., 1997) and *KCNE1* (Tyson et al., 1997). Details of some of these genes are found in table 3.1a & b.

These findings have opened up avenues for exploration of treatment modalities that could halt or reverse the process of hair cell degeneration or apoptosis, raising hopes to the possibility of gene therapy. It is now possible to screen for mutations in specific genes implicated in the causation of genetic hearing loss, by using the candidate gene approach. The opening quote by Grandiori, Cianfrone and Kemp, though made in reference to the physiology and acoustics of hearing, could just as easily apply to the molecular basis of hearing loss. Our understanding of the hearing mechanism has

been challenged by the findings of the studies on the molecular basis of hearing loss. Just as soon as key questions have been answered, more questions have been raised. Although many genes expressed in the ear have been discovered, for many, their specific roles in the hearing process, as well as the products they code for, have not been identified. The collaboration between research teams worldwide and the pooling and sharing of information on the molecular level as it unfolds is narrowing the gap in our understanding of the hearing process and causation of hearing loss.

1.2 GENES AND POPULATIONS

In an extensive anthropological review MacEachern (MacEachern, 2000) noted that it is generally accepted that there are groups of individuals who are genetically more similar to each other than to other groups. This is believed to be due to a greater degree of inbreeding, as a result of geographical isolation or specific cultural practices. These groups have been termed demes. He notes that whereas this concept has been easily applied to plant and animal population genetics, it is not practical for the study of human genetic variation. He also notes that although individuals may be identified according to groupings such as villages, towns, ethnic groups and nations, there are still challenges to these groupings. As such, studies that assess human genetic variability are challenged by the difficulty of associating genetic variability with identifiable modern populations. However, Carvalli-Sforza (Carvalli-Sforza et al., 1994) used a set of assumptions to group individuals into related or endogamous groups using language and other factors. As noted by MacEachern, these groups were assumed to have *"a relatively predictable drop-off in genetic interchange across reasonably well-defined group boundaries, and thus to be reasonable approximations to genetic population units" (sic).* Their assumptions that ethnicity, language and

genetic inheritance are shared characteristics of clearly defined human populations are noted by MacEachern (MacEachern, 2000) to be essential for any analysis of genetic data, even though they result in distortion of human genetic research.

In Africa for example, the Lembas are considered to be an endogamous group, though they speak a number of African languages. They share a number of common customs and taboos, such as those relating to food and circumcision of the males, with the Jews. According to oral tradition, they migrated from Sena in the north and after a few stops on the way settled in southern Africa among various tribal groups they found in South Africa, Zimbabwe, Malawi and Mozambique (Ruwitah, 1997; Thomas et al., 2000). They traded widely in the region, and also worked as copper and ironworkers. Although they belong to different Christian groups and some are Muslims (in Zimbabwe), they always saw themselves as different from the other African tribes. Sena is believed to be 'Sanaa' in Yemen, Judea, Egypt or Ethiopia (Ruwitah, 1997; Thomas et al., 2000).

One Lemba leader, Raulinga Hamisi, who was a speaker at the burial of Maanda William Mawela Ratshilingana Mhani in July 1996, is recorded to have said "the Senas left Judea under the leadership of Buba and settled in Yemen where they built their city of Sena, hence Senas". Another Lemba leader (Mathivha, 1992) in a private publication states "the Bhuba lineage (one of the Lemba clans) came down from Judea as the leading lineage of the Basena when they left Judea in their early migration to the Yemen where they settled and built the city of Sena. They ruled over all the lineages in good manner".

The results of a genetic study among the Lembas (Thomas et al., 2000) give support to the Lemba oral tradition. The study found a high frequency of CMH (0.088 of the entire population) among the Lemba, a Y chromosome marker that has been suggested as the signature haplotype for the ancient Hebrew population. The study concludes that the CMH found in the Lemba may have an exclusive Judaic origin. This is a clear example of how the advances in genetic analysis, in this case use of the paternally inherited nonrecombining portion of the Y chromosome, has become a powerful tool for investigating and establishing beyond reasonable doubt common origins between geographically distant populations.

It has been demonstrated that, sometimes, knowledge of ancestry may be important in pointing to or predicting the location of the disease gene (Keats & Berlin, 1999). With regard to hearing loss, an example is Usher syndrome type 1 where all the families of an Acadian ancestry mapped to a narrow region on the short arm of chromosome 11. The majority of the affected family members exhibited a single homozygous mutation over a 6-cM interval, a haplotype that was not found on Acadian chromosomes not carrying the disease allele (Keats et al., 1994). The conclusion was that for all profoundly deaf children of Acadian ancestry, marker typing could be used to differentiate between Usher syndrome type 1C and other forms of nonsyndromic hearing impairment (Keats et al., 1994).

Endogamous populations originating from Pakistan, India, Israel, Bali, Tunisia, Lebanon, Syria and Palestine have been instrumental in mapping genes for nonsyndromic hearing loss (Keats & Berlin, 1999). Results have also consistently shown specific genetic defects or mutations to occur more commonly in specific

population groups. An example is the finding of *GJB2* population-specific mutations among Jewish, Chinese and European populations. These include 167delT among Ashkenazi Jews, 235delC among the Chinese and 35delG among Caucasoids, often with demonstration of a founder effect (Gasparini et al., 2000; Van Laer et al., 2001; Del Castillo et al., 2003; Yan et al., 2003).

In Ghana, it was found that *GJB2* variants in the studied deaf population had more mutations in the C-terminus of the gene than the variants found in other parts of the world (Hamelmann et al., 2001). On the other hand, a study of a deaf population from the Sudan and northern Kenya (Gasmelseed et al., 2004) found that there was a very small percentage of carriers of *GJB2* variants within the coding region of the gene, yet there were many variants in the region just upstream of the coding region. They conclude in their study that for most of the variants found in this study population, an association with non-syndromic autosomal recessive hearing loss could not be made. They suggest that the cause of this type of genetic deafness in Kenya and Sudan could be due to other genes.

After taking all the above into consideration, the current study chose to explore the contribution of known genes to deafness in the South African setting.

1.3 THE LIMPOPO PROVINCE

1.3.1 The Land.

The Limpopo Province, formerly the Northern Province, is the northernmost province of South Africa, covering an area of 123,910 square kilometres, about 10% total

surface area of South Africa. It borders on Botswana in the west, Zimbabwe in the north, and Mozambique in the east (**www.golimpopo.com*)***. The province is divided into six main districts, with each district further subdivided into a number of municipalities (figures 1.2 and 1.3).

The Limpopo province has varied and breathtaking scenery and landscapes (figures 1.5-1.8). With over 3.6 million hectares of national parks, and game and nature reserves, the province holds custody to 70% of South Africa's protected land. The varied landscape consists of semi-arid regions near the border with Botswana and mountainous, forested regions in the east, with a scattering of baobab trees, the icon of the Limpopo province (figs. 1.4-1.8) in the north (**www.golimpopo.com*)***. It is often referred to as a land of myths and legends. One myth is that drinking a solution in which the seeds have been soaked will protect one against crocodile attacks. The Limpopo province has held onto its traditional cultures, and is believed to hold the largest areas of rural communities in South Africa who are still living as they have had to for centuries as shown in figures 1.9 – 1.11 (**www.golimpopo.com*)***.

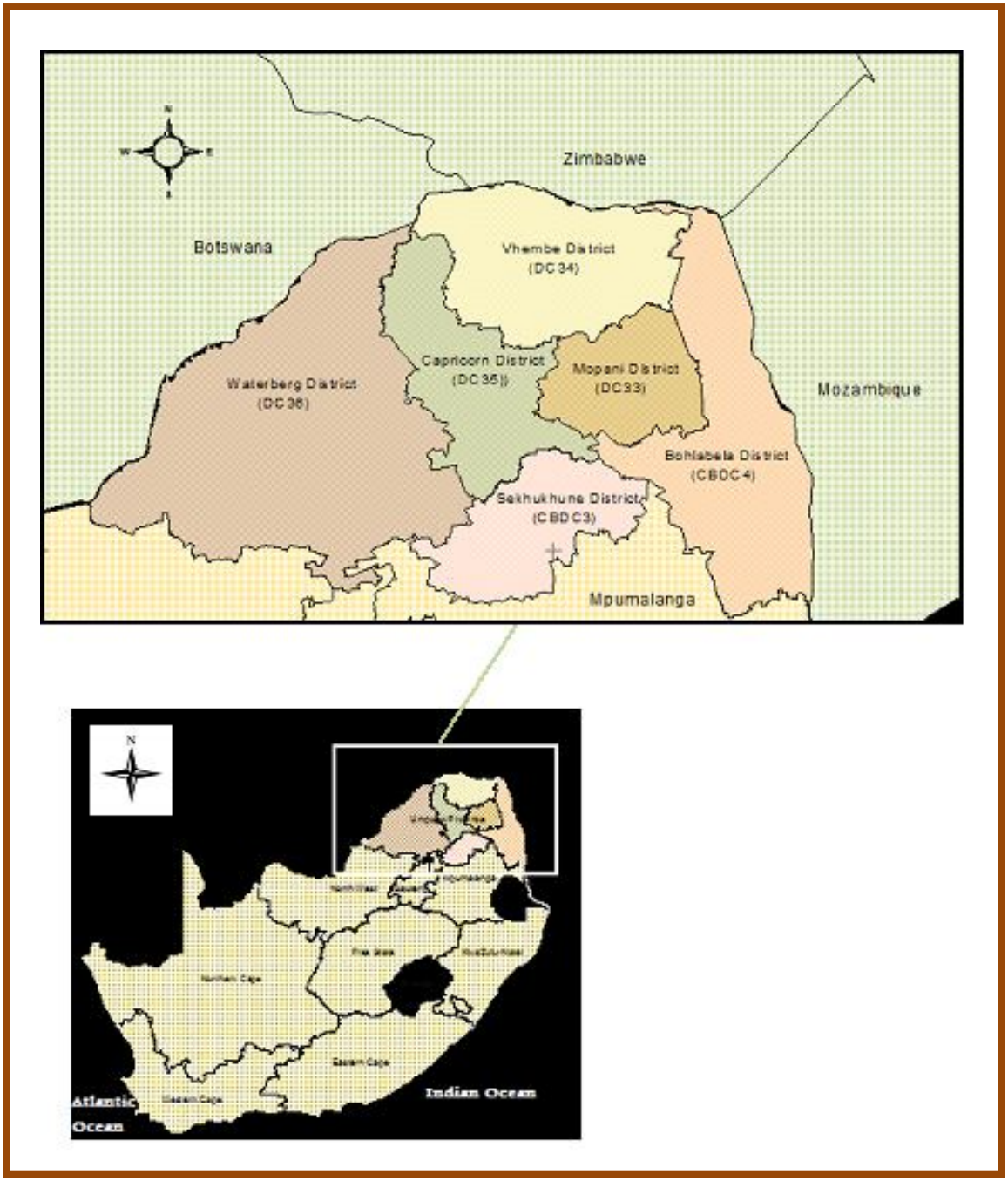


Fig. 1.2: Location Map of the Study area (Limpopo Province) within the map of South Africa

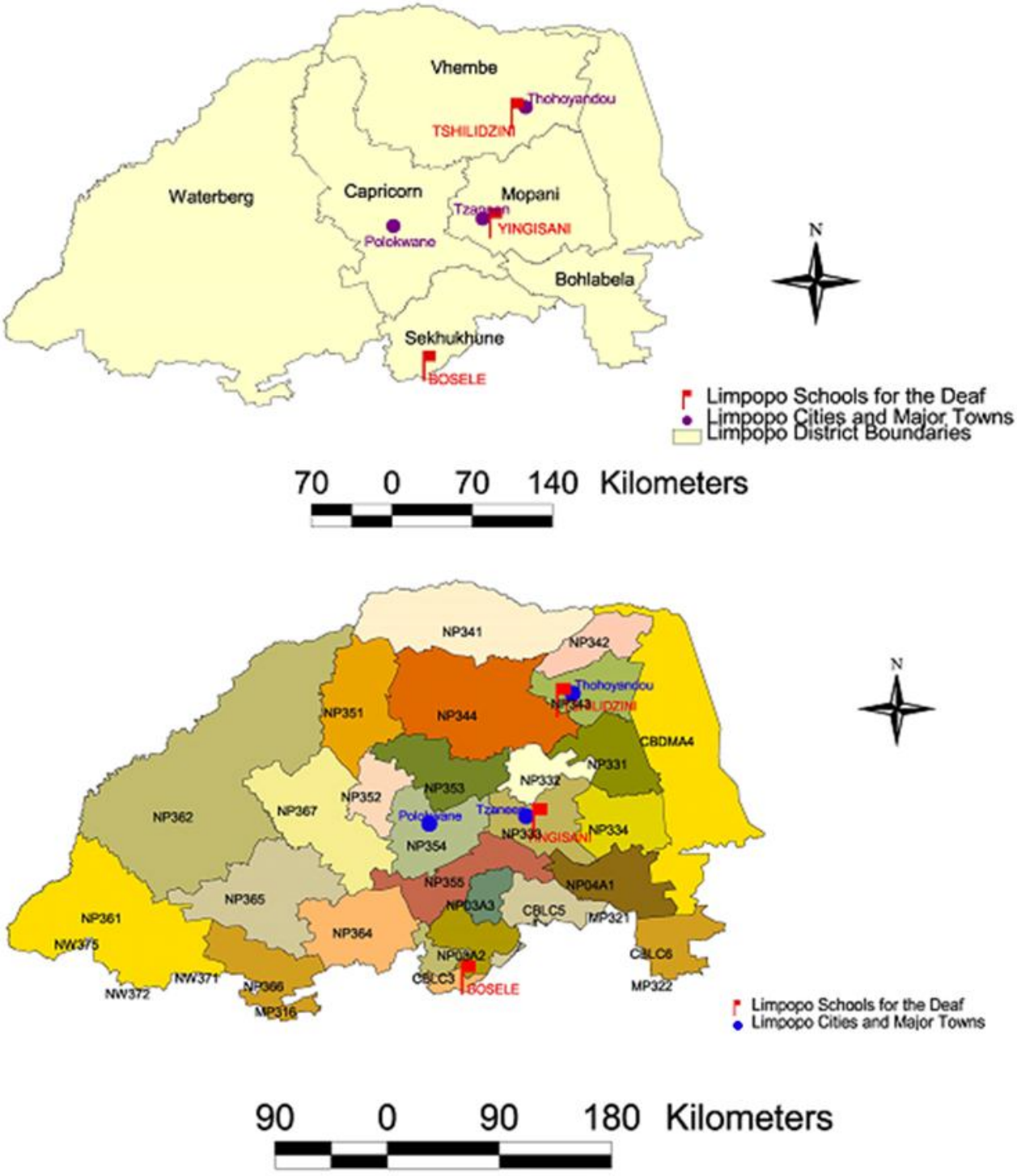


Fig. 1.3: Map of the Limpopo province showing the districts and municipal boundaries

Fig. 1.4: The Baobab tree, the Icon of Limpopo Province

Fig. 1.5: The Land of the legends, Lake Fundudzi, Venda

Fig 1.6: The arid landscape of No-Body and Moria regions, with the ZCC church headquarters star logo etched in the mountainside

Fig 1.7: A group following a climbing trail, in the Agatha mountains

Fig 1.8: The Tzaneen Dam, with the Drakensberg mountain with range in the background

Fig 1.9: Polishing the homestead floor fresh cow dung in a Giyani village

Fig 1. 10: Sharing a meal, the typical homestead arrangement seen in the background

Fig 1.11: A Shangaan girl greeting visitors to the homestead in a Giyani village

1.3.2 Population characteristics

According to the 2004 mid-year estimates of the Department of Health and Social Services, Limpopo Province Population and Development Unit (Limpopo Dept Of Health, 2005*)*, the province had an estimated population of 5,273,642 inhabitants, up from the 4,929,368 inhabitants reported in the 2001 census (StatsSA, 2002) (Tables 1.1-1.3). More data can be found in appendix 8.

Table 1.1: Population of Limpopo province by home language and district in 2004

Language	Sekhukhune	Bohlabela	Mopani	Vhembe	Capricorn	Waterberg	Limpopo
Afrikaans	1,419	3,331	18,200	13,734	33,689	52,365	**122,738**
English	1,024	1,327	6,018	4,549	10,139	5,837	**28,894**
IsiNdebele	20,246	151	332	1,755	26,343	29,719	**78,546**
IsiXhosa	1,029	474	831	678	1,213	10,073	**14,298**
IsiZulu	8,409	16,977	2,136	864	2,998	2,997	**34,381**
Sepedi	685,607	214,239	446,978	27,904	1,018,183	357,104	**2,750,015**
Sesotho	5,068	23,150	13,153	7,716	8,649	11,728	**69,464**
Setswana	1,369	811	1,071	831	3,337	75,744	**83,163**
SiSwati	13,211	38,226	1,989	354	2,057	1,796	**57,633**
Tshivenda	610	611	4,497	818,836	10,613	4,442	**839,609**
Xitsonga	6,781	295,443	468,127	316,721	35,955	57,538	**1,180,565**
Other	695	459	906	5,937	1,516	4,812	**14,325**
Total	**745,468**	**595,199**	**964,238**	**1,199,879**	**1,154,693**	**614,154**	**5,273,631**

Source: Department Of Health, Limpopo Province 2005

Table 1.2: Limpopo province population in five – year age groups according to race in 2004

Age group (yrs)	Black African	Coloured	Indian/Asian	White	Total
0 - 4	592486	1069	861	8105	**602521**
5 - 9	709197	1118	735	8931	**719981**
10 - 14	741575	1139	705	10824	**754243**
15 - 19	682598	1139	645	11178	**695560**
20 - 24	467756	970	810	7375	**476911**
25 - 29	356512	886	1035	9010	**367443**
30 - 34	285270	777	818	9905	**296770**
35 - 39	264103	739	628	9609	**275079**
40 - 44	214706	574	532	9710	**225522**
45 - 49	180353	463	447	8642	**189905**
50 - 54	142087	372	466	7871	**150796**
55 - 59	103248	270	312	6714	**110544**
60 - 64	106207	195	229	5654	**112285**
65 - 69	88140	183	157	4475	**92955**
70 - 74	84691	121	100	3669	**88581**
75 - 79	42552	69	51	2377	**45049**
80 - 84	40916	41	30	1425	**42412**
85+	26217	37	24	802	**27080**
Total	**5128614**	**10162**	**8585**	**126276**	**5273637**

Source: Department Of Health, Limpopo Province 2005

Table 1.3: Disabled population by district in the Limpopo province in 2004

Disability	Sekhukhune	Bohlabela	Mopani	Vhembe	Capricorn	Waterberg	Limpopo
No disability	705,931	567,502	914,193	1,145,233	1,091,685	580,188	**5,004,732**
Sight	9,997	7,367	13,401	13,727	16,256	8,891	**69,639**
Hearing	6,416	4,559	9,074	9,675	9,134	5,658	**44,516**
Communication	1,591	1,149	1,633	2,051	2,225	1,171	**9,820**
Physical	8,211	5,734	9,805	10,831	12,201	7,223	**54,005**
Intellectual	3,864	2,737	4,352	5,664	6,550	2,985	**26,152**
Emotional	5,213	3,461	6,669	7,452	8,358	3,824	**34,977**
Multiple	4,245	2,693	5,107	5,253	8,276	4,213	**29,787**
Total	**745,468**	**595,201**	**964,235**	**1,199,886**	**1,154,685**	**614,155**	**5,273,630**

Source: Department Of Health and Social Services, Limpopo Province 2005

1.3.3 Employment

According to the Labour Force Survey of March 2004 (StatsSA, 2004), unemployment, defined as '*people who have not had a job for the past month, who are actively looking for work, are available to work and are eligible for work'*, in the province was 30.8%, compared to the national average of 27%. The figures for the rest of South African provinces were: Western Cape 17%, Northern Cape 22%, Gauteng 28%, Eastern Cape 32% and Kwazulu Natal 32%. Unofficially, if one were to include people who are not actively looking for work but are willing to work, the figures could range from 40% upwards for the province, depending on the area surveyed. However, apart from employment in mines, the public sector and retail, there is both commercial and subsistence farming.

1.3.4 Health profile of the people in the Limpopo Province

The 2004 mid-year estimates *(dept Health and Social Services, Limpopo Province population and development unit 2005)*, indicated an estimated 47,061 disabled HI individuals in the province, of whom 45,757 were classified Black African. The total population of the province was estimated at 5, 273,642 inhabitants with 5,128,614 Black Africans. This gives an overall estimated prevalence of disabling hearing

impairment of 8.9 per 1000 among Black Africans, and 8.92 per 1000 inhabitants for the Limpopo province. Statistics for congenital hearing impairment were not available (Appendix 8).

The HIV and AIDS epidemic has affected the province, with the overall prevalence rate rising from an estimated low 1.9% in 1995 to 7.1% in 1999 (Department of Health, South African. 2006). The HIV prevalence leveled off to 7.3% in 2008 but remained high at 12.5% among the 19-49 year age-group (Health Systems Trust (HST), November 2008 www.hst.org.za/healthstats). The concomitant tuberculosis is also on the rise but effective treatment is readily available nationally and provincially.

On top of the common ailments affecting rural African communities, Limpopo Province is endemic for malaria and bilharzia (bladder schistosomiasis), especially in the Lowveld region. Bilharzia is caused by a parasite that spends some if its life cycle in fresh water snails. This parasite infects man when he swims or wades through infected water. The parasite ultimately settles in the urinary bladder wall where it provokes a chronic inflammatory reaction that leads to symptoms of painless terminal haematuria. Many young boys in the province contract the disease when they swim in the infected water of rivers, streams and dams. They present at the local clinics or hospitals with terminal haematuria. Praziquantel or its equivalent, an effective bilharzia treatment, is available in all government run clinics in the endemic areas of South Africa as part of the Primary Health Care package. Although urine samples are sent out to any of the NHLS laboratories in the province for confirmation of the diagnosis, treatment is often started on clinical history alone. Primary health care nurses are trained and competent in diagnosing and treating this disorder.

1.3.5 Access to health care in the Limpopo Province

South Africa is believed to have some of the world's best health policies, although there have been noted challenges with the implementation, especially at provincial, district, municipal and community levels (Department of Health, South Africa. July 2000). Starting with the Restructuring and Development Programme (RDP) of 1995 and followed by the White Paper for the Transformation of the Health System in South Africa (Department of Health, South Africa. April 1997), the post apartheid government and the Department of Health sought to restructure the health system.

This was based on the finding that although South Africa was classified as a middle-income country, spending 8.5% of its gross domestic product (GDP) on healthcare, the former apartheid policies had ensured major disparities on the basis of racial, gender and provincial status. The majority of South Africans had inadequate access to all the basic services including health, clean water and sanitation, with an estimated 35-55% of the population living in poverty (Department of Health, South Africa. April 1997). These two documents underpinned the need of building a single, equitable, and unified health system for all South Africans, in which the Primary Health Care approach and the District Health System would play an integral role. Out of this came The Primary Healthcare Package for South Africa (Department of Health, South Africa. March 2000).

The goals and objectives of the White Paper for the Transformation of the Health System in South Africa (Department of Health, South Africa, April 1997), were founded on the RDP and can be summarized (for further details refer to appendix 10) as follows:

1. To unify the fragmented health services at all levels into a comprehensive and integrated National Health Service (NHS)
2. To promote equity, accessibility and utilization of health services
3. To extend the availability and ensure the appropriateness of health services
4. To develop health promotion activities
5. To develop the human resources available to the health sector
6. To foster community participation across the health sector and
7. To improve health sector planning and the monitoring of health status and services.

The government and department of Health committed to maximizing the effectiveness and efficiency of all health care resources, ensuring effective referral systems at primary, secondary and tertiary levels, establishing health care financing policies that promote equity between people utilizing the health services, as well as equitable distribution of health personnel throughout the country. The government also committed to the recruitment and development of competent health personnel who could respond appropriately to the people they serve while at the same time building capacity to prioritise all identified issues at provincial, district, local and community levels, ensuring that interventions were appropriate and cost-effective. To this effect, the national treasury faithfully budgeted and gave funds to all provinces, leaving the provincial governments free to utilize the funds according to locally identified needs (Department of Health, South Africa. July 2000).

The Limpopo provincial department of health adopted the mission and goals set out in the RDP and White Paper for the Transformation of Health, while also developing a

District Health System based on the Primary Health Care service model. The province is divided into six health districts (figure 1.3), Capricorn, Vhembe, Mopani, Waterburg, Bohlabela and Sekhukhune. By 2005, there are 42 hospitals in the province, one of which, Pietersburg Mankweng Hospital Complex, a twinning of Pietersburg Provincial Hospital (figs 1.11 –1.12) and Mankweng Hospital, is the tertiary referral hospital. There were also over 421 clinics in the province, including fixed and mobile facilities. There are also two major private hospitals in the province, one in Polokwane and the other in Tzaneen.

Fig 1.12: One of the reception areas inside the Pietersburg Provincial Hospital

The official figures (Dept of Health and Social Services, Limpopo Province Population and Development Unit, 2005) indicate that nationally, the proportion of the South African population with medical insurance or medical aids was 18%. In the

Limpopo Province, the figure was much lower, with estimates indicating that about 5.237 million of the population were dependent on the public sector health services, with less than 40,000 inhabitants on medical aid.

The South African National Department of Health goal is that all people should live within 5-kilometre radius of a primary health care facility. The Limpopo province has provided fixed primary health care facilities in many areas, but where this has not been possible, mobile clinic services are used, running once or twice a week. Unfortunately there are still areas in the province that do not have adequate health care coverage.

Fig 1.13: Ear Nose and Throat outpatient clinic at the Pietersburg Provincial Hospital

The South African National Department of Health goal is that all people should live within 5-kilometre radius of a primary health care facility. The Limpopo province has provided fixed primary health care facilities in many areas, but where this has not been possible, mobile clinic services are used, running once or twice a week. Unfortunately, there are still areas in the province that do not have adequate health care coverage, and where the health care worker ratio per person in the province was still low (table 1.4).

Table 1.4: Public sector human resource data, Limpopo Province in 2004

Human resource category (Public service)	Number per 100,000 of the population
Medical officers	14.3
Specialist doctors	1
Dental practitioners	1
Pharmacists	22
Professional nurses	119
Speech and Hearing therapists	6

Source: Department of Health , Limpopo Province, 2004

The implementation of compulsory community service in the public health sector, for example doctors in 1999, speech therapists and audiologists in 2003, has helped patients access the health services. Some hospitals however, such as the Letaba Hospital in the Limpopo province, did not receive the community service speech and hearing therapists until 2005. Due to the junior nature of these community service personnel, and the lack of senior staff to train and supervise them in many of the hospitals, the intended impact still has to be felt. There has, however, been an improvement in the screening and referral of cases to secondary and tertiary level health care facilities that could not be handled at level one and two health care

facilities where these personnel are stationed. The trend of recruitment of nurses to work overseas in more lucrative jobs has negatively impacted of availability of experienced nurses, especially in the public healthcare facilities.

1.4 THE PEOPLE AND THEIR CULTURE

This section is limited to the history and culture of three language groups, namely the Venda, Pedi/Northern Sotho and Shangaan/Tsonga. The decision to assess only these language groups was dictated by the research participants' demographics.

1.4.1 The Venda people and politics

According to one anthropological study on Venda history, religion and tribal ritual (van Warmelo, 1963), in the past, the whole of Venda initially consisted of mutually independent kingdoms polarized around a few powerful kingdoms. These people were mainly traditional Bavhenda (Venda speaking people), with some Sotho-speaking immigrants from the west, other immigrants from Zimbabwe in the north, as well as Tsonga people from the east and the south. A modern day Venda woman in traditional dress is depicted in figure 1.14.

The major part of the Bavhenda are believed to have been originally concentrated in the mountains of Zoutpansberg for a long time (van Warmelo, 1963) and this is still the case. Anthropologists believe that the Bavhenda in the nineteenth century were still sheltered from foreign influences in several quarters by natural isolation, such as the tsetse-ridden country to the south east, and the flat arid country to the north of the Limpopo River. Other reports state that another reason for the relatively superficial

European influence on the Bavhenda was that up to the end of the nineteenth century, the Sibasa district was not yet a seriously depressed area economically, as its adequate water and fertile soils allowed the men to work from home (sapir.ukc.ac.uk.).

According to the same document, the Bavhenda submitted to the rule of the Transvaal republic in 1899 and they were the last Bantu speaking people to be seriously affected by the European influence. The government set up agricultural enterprises and forestry in the area and also subsidized the work of the missionaries who were already in the region providing schools and hospital services. By the late 1950s, it is noted that about 275,000 Venda speaking people lived in the Republic of South Africa, mainly in reserves, or European-owned farms in Louis Trichardt, in Sibasa and in the Zoutpansberg mountains (sapir.ukc.ac.uk.).

Fighting the apartheid policies of the time, the homeland of Venda declared independence from the Transvaal in 1979 although no other country recognized it as an independent country. In the 1980s its economy was based on farming, forestry, small scale industries and coal mining. By the late 1980s the Venda population was estimated at 700,000. Many men, some estimate up to 70%, had left for work elsewhere in South Africa, contributing 40% of the homeland's GDP (www.gaabomotho.co.za). It is stated that by the time the Venda homeland applied to be reincorporated into the Republic of South Africa in 1991, it was facing economic collapse. At the same time, there were ongoing political negotiations for the new post-apartheid South Africa in the 1990s, which resulted in the dissolution of all homelands in 1994 (www.gaabomotho.co.za).

The Bavhenda were socially organized along small kinship groups dispersed among many households. These were then arranged into chiefdoms but without a paramount chief. The tribe was considered a political and territorial unit, with people choosing allegiance to a particular dynasty. In the Sibasa District for example, there were twelve chiefs (www.gaabomotho.co.za). The Bavhenda were traditionally divided into two classes, the commoners and the royalty. Royalty had dominion over the land while commoners dwelt on the land but never owned it. There are many cultural differences between these two groups.

1.4.1.1 Betrothal and marriage amongst the Bavhenda

Consanguinity was a common practice among the Bavhenda, especially the royals. According to the Venda Law, Part 1 (van Warmelo, 1963), consanguinity was well accepted. For example, among the Vhailafuri clan from the Nzhelele area, marriage was only allowed within the clan. To this end, the royal kraal even allowed sister and brother to marry, as long as they had different mothers. If the chief died leaving many children, the offspring of the wife from the royals took precedence in inheritance especially as heir to the chiefdom, even if they were younger than their siblings from a commoner wife (private communication). There were even occasions where a son was passed over in favour of a daughter as heir to the chiefdom on grounds of the mother's ancestry.

When talking to the local people (private communication), there appears to be a perceived high incidence of congenital anomalies among the royals, but this is not openly discussed. Among the Lemba, the men did not marry Bantu speaking women, nor did they give their daughters in marriage to any but their own (van Warmelo,

1963). The men practiced circumcision, unlike the rest of the Venda people. Since the end of apartheid in 1990, and with it the breakdown of the barriers protecting *'separate development'*, there has been increasing intermarriage between the different ethnic groups within the Province, which would tend to affect the genetic pool isolate of this population.

1.4.1.2 Attitudes towards disability amongst the Venda

Disability, or Vhuhole as it is known among the Bavhenda, refers mainly to visible physical disability. The term may also be used to refer to mental disabilities. Because of the nature of hearing loss as a hidden disability, it is often accepted in its moderate form. Consequently, the hearing impaired persons do not seem to be as shunned as for example the epileptics and severely physically or mentally disabled people are. Culturally, most disabilities are believed to be due to witchcraft (van Warmelo, 1963).

1.4.1.3 Ear disease and traditional healing amongst the Venda

Traditional healers are approached for most illnesses including hearing loss. For acute earache, various substances, whose nature is not very clear, are applied or instilled into the ear. These are likely to be plant extracts, including leaves, bark and roots. The recipe of traditional treatments, though kept secret, is often herbal. Sometimes animal fat is used in conjunction with herbal mixtures. For ear toilet, a feather is used traditionally.

1.4.2 The Shangaan (Tsonga) people and politics

The Shangaan people are found in Mozambique and Zimbabwe, but also in the Limpopo Province of South Africa where they are addressed as the Tsonga.

Historically, they once ruled the Gaza Empire, which at one time extended from the southeastern part of Zimbabwe, down to southern Mozambique and some areas of South Africa. The Zulus under Soshangane conquered them. The divide and rule tactics of apartheid conferred on them the name Shangaan. Because the Shangaans were immigrants and refugees without political heads such as headmen and chiefs, they more easily accepted European influence compared to the Vendas. They accepted employment in the police and in government service. They have however retained their lifestyle and culture in the rural villages (figs 1.9, 1.10, 1.16).

1.4.2.1 Betrothal and marriage among the Shangaan

Consanguinity was discouraged among the Shangaans, unlike between the Venda and Pedi. They tended to marry within the population group. Their men practiced circumcision. Since the end of apartheid in 1990, and with it the breakdown of the barriers protecting *'separate development'*, there has been free movement of the peoples and presumably increasing intermarriage between the different ethnic groups.

1.4.3 The Pedi people and politics

The Pedi forum section, edited by Deborah James, in the World Culture Encyclopaedia (www.everyculture.com) gives interesting facts about the Pedi. The term Pedi was in the past used to describe all the people speaking the entire dialects of Sotho who lived in the Northern Transvaal. These people are now referred to as the Northern Sotho. These people form a large congregation of groups including Batlokwa, Dikgale, Gananwa (Mmalebogo), Kone (Matlala), Kone, Mathabathe, Mmamabolo, Molete, Mphahlele, Ntwane, Roka, Thwene, and Tau, all who form the high-veld Sotho. The lowveld Sotho group includes Kgakga, Kone, Kutswe, Lobedu,

Mogoboya, Narene, Pai, Phalaborwa, and Pulana. These different groupingss are identifiable not only from their dialect but also from their traditional wear (fig 1.16).

The Pedi were socially organized along small kinship groups called Kgoro where membership was given on condition that one accepted the head of the Kgoro's authority. Royal or chiefly dikgoro also existed. Politically, this group included all of people living within the area ruled over by the Maroteng dynasty established during the eighteenth century. During and after apartheid, the migrant labour practices have scattered these people (www.everyculture.com).

Sekhukhuneland is considered the present day location of the Pedi. It is situated between the Olifants River and its tributary, the Steelpoort River (Tubatse) and the Drakensberg range. Under Thulare (1790-1820) Pedi territory stretched up to present day Rustenburg and as far south as the Vaal River. Defeated by the British in 1879, the Pedis were gathered into the Geluks Location, created for them by the Transvaal Republic's Native Location Commission. Further reserves were created for the Pedis over the next hundred years, which culminated in the formation of the Lebowa Homeland (www.everyculture.com).

Many Pedi chose to move to farms and to townships adjoining Pretoria and to Johannesburg, dispersing this population group. In 1961 the total Pedi population had come down to an estimated 118,743 but after 1950, forced relocations from rural areas and cities by the apartheid machinery increased the Pedi numbers within the area. The political negotiations for the new post-apartheid South Africa in the 1990s

resulted in the dissolution of all homelands in 1994 including Lebowa (www.everyculture.com).

1.4.3.1 Betrothal and marriage among the Pedi

The preferred marriage partner, especially among the chiefly or ruling families, was the close or classificatory cousins, and especially for a man, his mother's brother's daughter (www.everyculture.com). This was apparently to have the two prospective in-laws closely related before the marriage. The bride-wealth *(bohadi)* of a daughter's marriage was kept in the family to pay for her brother's bride and he (the brother) would in turn repay his sister by offering a daughter to her son in marriage. This is still practised today but to a lesser extent.

Fig 1.14: A Venda woman in full traditional attire

Fig 1.15: Shangaan women dance group

Fig 1.16: Pedi women's dance group from Mashashane

1.5 RATIONALE FOR THIS STUDY

In August 1997, a visit was made to the Tshilidzini School for the Deaf, located in the Limpopo Province of South Africa, as part of the outreach services for the department of Otorhinolaryngology, Pietersburg Provincial Hospital, Limpopo Province. While looking through the student records, it became clear that a large percentage of students in the school came from one geographical area, Nzhelele sub-district, an area mainly populated by Venda speaking people (table 1.5).

Table 1.5: Home area of students at Tshilidzini School, August 1997

Blind or Partially Sighted			**Hearing impaired**		
Area of origin	Number of students	Percentage of the school Blind Section (approximately)	Area of origin	Number of students	Percentage of school Deaf Section (Approximately)
Nzhelele	35	67	Nzhelele	96	47
Louis Trichardt	4	8	Sibasa	47	23
Lwamondo	3	6	Louis Trichardt	25	12
Vuwani	2	4	Lwamondo	9	4
Mutale	1	2	Vhufuli	8	<4
Mukula	1	2	Tshakuma	6	3
Ngudza	1	2	Mutale	5	2
Fondwe	1	2	Mashau	2	1
Tshandama	1	2	Siloam	1	<0.5
Tshaulu	1	2	Vuwani	1	<0.5
Muledane	1	2	Muila Thotholo	1	<0.5
Tshifudi	1	2	Mukula	1	<0.5
			Masia	1	<0.5
			Dzimauli	1	<0.5
			Rudzani	1	<0.5
Total	**52**		**Total**	**205**	

Source: Field Survey, Dept of Otorhinolaryngology, Pietersburg Provincial Hospital, August 1997

Informal inquiries seemed to point to a genuinely high incidence of deafness and blindness among the population of Nzhelele, who also practiced the traditionally accepted close cousin marriages. Consanguinity and the closed nature of the society

could theoretically have led to the concentration of a defective gene in this population, leading to a recessive form of genetic hearing loss.

These findings triggered a number of questions. Could it be that there was a genuine high incidence of deafness and blindness in the Nzhelele region? What other factors could be contributing to the apparent high incidence of deafness and blindness in the area? Could it perhaps be due to selective admission of students to the school?

There has been much valuable work done by researchers (Sellars & Beighton, 1983b; Sellars & Beighton, 1983a; McPherson & Holborow, 1985; Seely, Gloyd, Wright, & Norton, 1995) investigating the aetiology of hearing loss among the hearing impaired children in Africa. This laid groundwork on which further research into childhood hearing loss could be based.

Due to the incomplete, and in some cases absent, medical records in most of Africa, many of these studies concluded by inference as to the aetiologies involved. For example, a history of a 'rash' during pregnancy reported as rubella without laboratory confirmation could have been due to many other causes. This leaves questions as to the validity of such a conclusion. Thus this study intended lay groundwork that would eventually help to establish the various aetiologies of genetic hearing loss among the people of the Limpopo Province of South Africa, through scientifically acceptable methods.

Secondly, this research sought to determine the level of consanguineous mating and its possible impact on the aetiology of genetic hearing loss in this population, with the

purpose of educating families and the communities about the consequences of consanguineous mating.

Thirdly, this study intended to provide basic data on hearing loss in the province that could be used for improving and boosting the secondary preventative rehabilitation measures. For example, the results of this study could lay a foundation for **early identification** and **early appropriate rehabilitation** of significant childhood hearing loss to be instituted in the province. Through these measures, individuals affected by significant hearing impairment would be assisted to become empowered, self-sufficient, and productive members of their communities. As one of the poorer provinces of South Africa at the time of this study, Limpopo Province lacked basic data as to the magnitude and impact of hearing impairment in the province, affecting budget allocation and the structuring of services in both the education and the health sectors.

Hypothesis

The indigenous people of some areas of Limpopo, such as in Nzhelele sub-district, widely practice consanguineous marriage. This is likely to predispose them to recessive hereditary disorders. The reported high rate of congenital and progressive hearing loss/deafness in these communities could be due to the concentration and segregation of recessively inherited gene variants.

CHAPTER 2: LITERATURE REVIEW AND BACKGROUND INFORMATION - PART I

Summary of this chapter

*This chapter gives an **overview** of the genetics of hearing loss, including the modes of inheritance, **outlines** the history of research into deafness genes and the epidemiology of hearing loss, and provides a **background** on the anatomy, physiology and pathology of hearing loss. It concludes by **outlining** the molecular basis of auditory function and hearing loss.*

2.1 OVERVIEW OF GENETICS OF HEARING LOSS

2.1.1 Disease Inheritance

During gamete formation, chromosomes recombine creating new chromosomes that are a mixture of maternal and paternal DNA. The chromosomes stick together via their homologous (same sequence) alleles. If successful, the exchange of material will be equal and opposite, resulting in normal functional chromosomes. Mistakes can occur during replication (formation of bivalents) or during recombination itself. Duplication of a whole chromosome can also occur. Mistakes during recombination generally lead to gross chromosomal abnormalities. These comprise deletions, translocations, duplications and inversions, which may lead to disruption or deletion of many genes. Mistakes during replication can cause small duplications, base pair deletions, substitutions and insertions, often leading to disruption of the colon frames (Strachan and Read, 2007, ch. 2, pg 33-58).

Mutated genes may lose their function altogether or may gain a harmful function. Generally, recessive phenotypes are due to **loss of function** mutations. This is because

most of the cells can function on half a dose of gene product (Strachan and Read, 2007 ch. 2, pg 33-58). This is clearly demonstrated in USH1B affected families. Heterozygous individuals who had no auditory, vestibular or visual symptoms are predicted to have more than 50% of the normal myosin-VIIA (Weil et al., 1997). On the other hand, a condition of **haploinsufficiency** has also been described such as in Waardenburg syndrome type I (Tassabehji et al., 1992). In this condition, there is failure of neural crest cells to migrate and function normally due to the presence of only half the normal dose of protein encoded by the PAX3 gene (Tachibana et al., 1996; Bondurand et al., 2000). In other conditions still, mutant versions of proteins exert a **dominant negative effect**, in which the remaining normal proteins that are part of a multicentric assembly line fail to function normally (Weil et al., 1997). Thus changes in the genetic code – mutations – can cause many diseases including hearing loss. When the mutation is inherited by an offspring, it can cause disease.

2.1.2 Modes of Inheritance

There are four main modes of inheritance:

1. Autosomal dominant,
2. Autosomal recessive,
3. X-linked and
4. Mitochondrial.

Autosomal dominant affected individuals usually have one affected parent since each child has 50% probability of being affected (figure 2.1). Some conditions in this category show variable penetrance or non-penetrance of an expected characteristic. An example is Waardenburg syndrome in which the phenotype presents with varying degrees of hearing loss and clinical signs (Liu et al., 1995). In the adult or delayed onset forms of genetic hearing loss, penetrance is often age related.

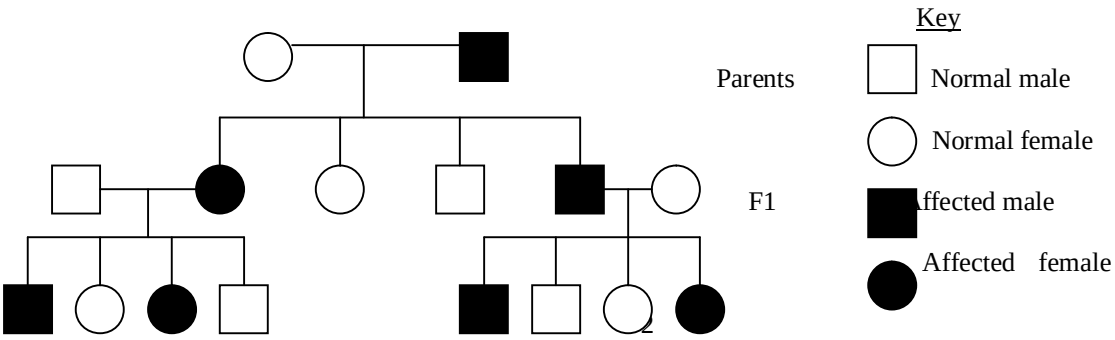


Figure 2.1: Pedigree showing autosomal dominant inheritance

Autosomal recessive disorders show individuals who are homozygous for the abnormal gene, while their parents are usually phenotypically normal carriers (figure 2.2). These conditions are frequent in multiply inbred genetic isolates where many people carry a particular abnormal allele. These communities played a crucial role in the mapping of genes responsible for recessive profound hearing loss (Guilford et al., 1994; Lalwani et al., 1994; Weil et al., 1995; Liu et al., 1997; Kelsell et al., 1997; Leon, Raventos, Lynch, Morrow, & King, 1992; Steel et al., 1996; de Kok et al., 1995; Lalwani et al., 1994; Reid, Vernham, & Jacobs, 1994a; Tiranti et al., 1995; Tassabehji et al., 1995; Prezant et al., 1993).

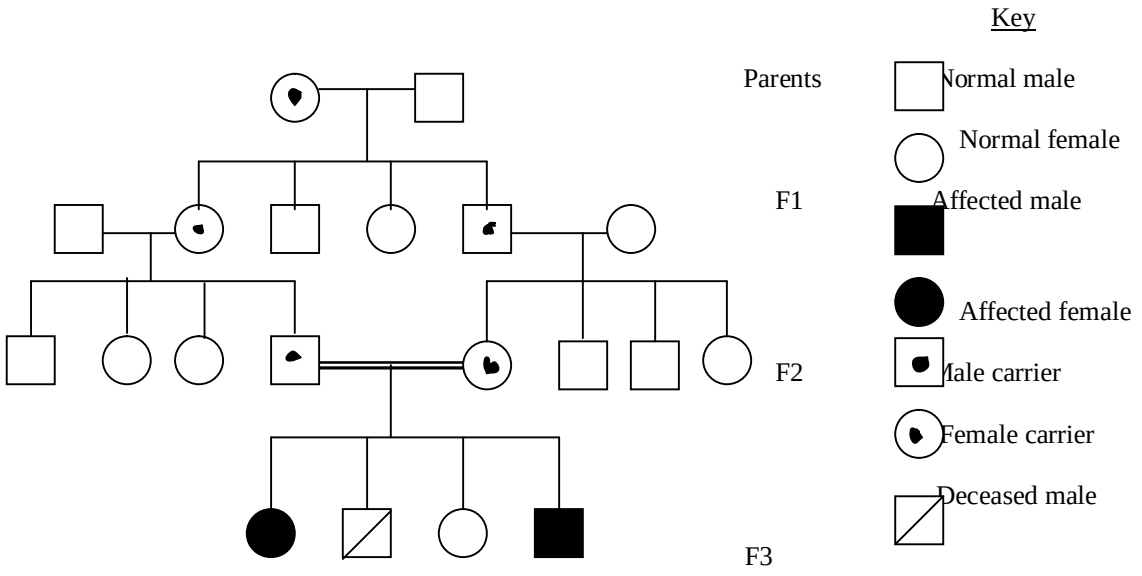


Figure 2.2: Pedigree showing autosomal recessive inheritance

In X-linked disorders, the defective gene is carried on the X sex chromosome, giving a characteristic mode of inheritance. It is not always easy to differentiate between recessive and dominant forms because of the lack of a heterozygous state among the males, as well as the phenomenon of X-inactivation among the females. The two forms can be distinguished if a good family tree is drawn (figures 2.3, 2.4).

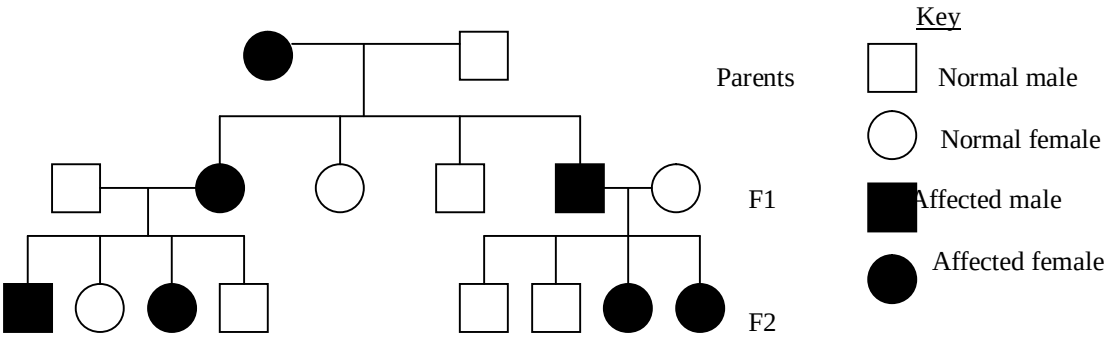


Figure 2.3: Pedigree showing dominant X-linked inheritance

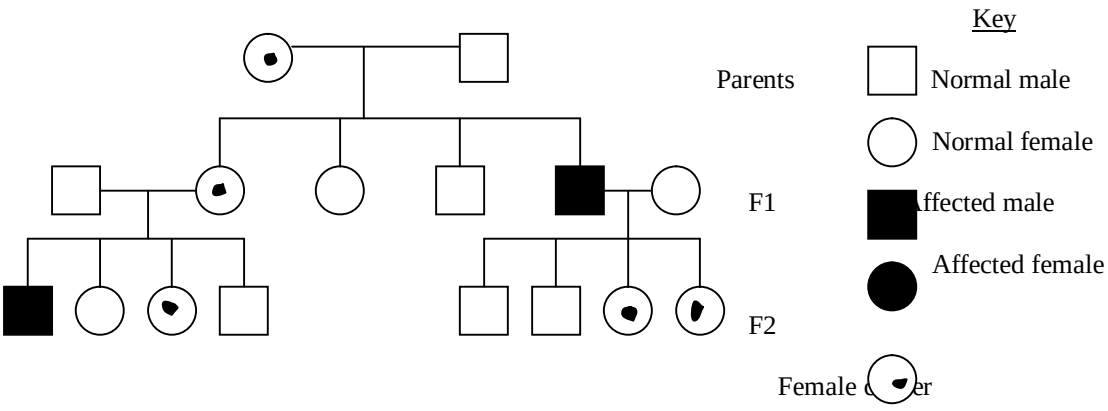


Figure 2.4: Pedigree showing recessive X-linked inheritance

Mitochondrial inheritance (figure 2.5) has grown in importance in many genetic disorders (Reardon, et al., 1995; Reid et al., 1994a; Reid, Vernham, & Jacobs, 1994b; Tiranti et al., 1995). These range from the rare neuromuscular syndromes such as MERRF (Shoffner et al., 1990) and MELAS (Goto et al., 1990) to mutations causing

nonsyndromic hearing loss (Prezant et al., 1993; Reid et al., 1994) and syndromic hearing loss (Hao et al., 1995; Sevior et al., 1998). All mitochondria in the cell cytoplasm contain DNA. The Mitochondrial genome has been shown to contain 16,569 bases, encoding 37 genes. Since sperm has no mitochondria, all mitochondrial genes are inherited from the mother. Likewise, mutations in mitochondrial genes can only be of maternal origin. About 18 mutations in mitochondrial DNA were recognizable by 1997. Point mutation A1555G in protein coding gene 12SrRNA for example has been shown to predispose to amino glycoside cochlear damage (Hu et al., 1991; Fischel-Ghodsian et al., 1993).

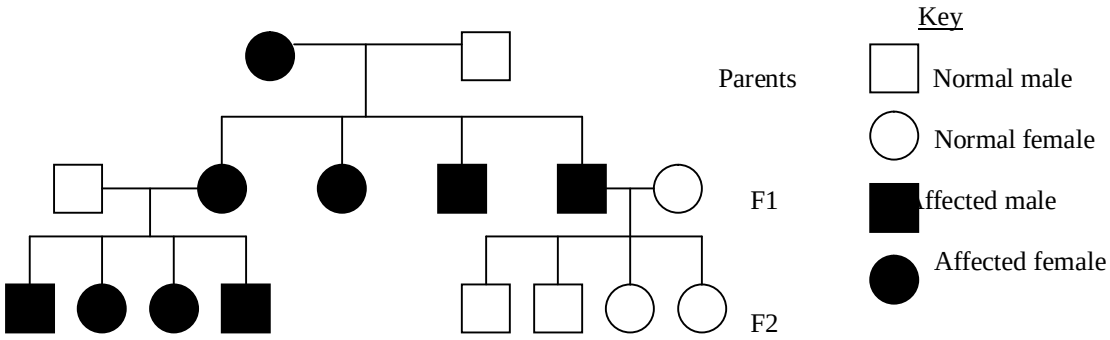


Figure 2.5: Pedigree showing mitochondrial inheritance

2.1.3 Research into Genes for Hearing Loss

Until 1994, the mapping of genes for autosomal recessive hearing loss was considered too difficult for a number of reasons. First, non-syndromic hearing loss exhibited unparalleled heterogeneity, that is, mutations in many genes resulting in the same clinical phenotype, meaning that there could be more than one deafness-causing gene in the same affected family (Van Camp, et al., 1997). Secondly, due to the assortative (non-random) mating whereby deaf individuals tend to marry one another, there is an introduction of a second deaf family history. Thirdly, the occurrence of deafness

resulting from non-genetic causes such as from oto-acoustic trauma, infection, and chemical toxicity further compound the issues. Fourthly, the absence of clinical identifying features/characteristics distinguishing between the various forms of sensorineural deafness was a challenge.

The three approaches used to map the genes responsible for autosomal recessive forms of hearing loss included the functional approach, the candidate gene approach and the positional approach. Conventional LOD scores were calculated to detect genetic linkage (Stopps & MacDonald, 1998; Chen et al., 1997; Sheffield et al., 1994). The functional approach involved identification of altered protein, establishment of its protein amino acid sequence and the use of reverse genetic coding to design a probe to isolate the gene encoding the protein. With the candidate gene approach on the other hand, a previously characterised gene was tested for mutations among individuals with hearing loss (Weil et al., 1995). In positional cloning, the chromosomal location of a postulated defect was mapped and the genes in that region screened for mutations in the deaf individual (Robertson et al., 1994, Sheffield, 1994; Chen et al., 1997, Robertson et al., 1998).

The discovery of the mouse as a model for human deafness was the key that unlocked the genetics of human deafness (Steel & Brown 1994). Research teams studied deaf mice using a positional cloning approach, identifying the genes involved in deafness (Steel & Brown 1994; Steel, 1995). It was not until 1994 that the first successful linkage study of autosomal recessive non-syndromic hearing loss was reported (Gibson et al., 1995; Weil et al., 1995). Three years later, in 1997, the first recessive deafness genes, *MYO7A* and *GJB2,* were identified (Liu et al., 1997; Kelsell et al.,

1997). The screening of DNA from a large consanguineous family from Tunisia with profound non-syndromic profound hearing loss led to the mapping of the DFNB2 locus on chromosome 11q13.5 (Guilford et al., 1994). The researchers proposed that a defective Myosin VIIA might also be responsible for DFNB2 (Weil et al., 1995). Sequence analysis of the coding axons of myosin VIIA gene (*MYO7A*) was undertaken in the DFNB2-affected family. These results showed that different mutations in *MYO7A* result in either an isolated (non-syndromic) or a syndromic form of deafness. Details of these studies are mentioned elsewhere (section 3.1.6)

2.2 EPIDEMIOLOGICAL PERSPECTIVES OF HEARING LOSS

2.2.1 General Considerations in the Aetiology of Hearing Loss

Prevalence estimates for childhood hearing loss differ between countries and between studies. There are noted inconsistencies in the areas of diagnosis, classification, and methodology. Factors such as inclusion criteria for hearing impairment, the degree of patient and family investigation, the actual population and ascertainment, and various environmental factors including the degree of medical care have been found to influence results.

Hearing loss can lead to delayed and defective language, speech, cognitive and psychosocial development, the impact of which is mainly determined by age at onset and degree of hearing loss (Denoyelle et al., 1999). Most published work deals with prelingual forms, which are often severe in degree, with fewer studies published on the late onset forms (Davidson et al., 1989). The difficulty of separating the

interaction between environmental and genetic aetiologies cannot be underestimated, and may account for the scarcity of studies in these age groups.

Adult onset hearing loss was shown to occur in 14% of individuals aged 45-64 years and in 30% of individuals over 65 years of age in the United States of America (Hotchkiss 1989). The results of the extensive population data collection and analysis in one study in the US aimed at establishing the causes of later-onset hearing loss (Sill et al., 1994), although suggesting a genetic aetiology in a large proportion of the participants, failed to provide or estimate the percentage. It has been shown that age-dependent penetrances and phenocopies should be taken into account when analysing family data, whatever the reported age of onset, as there may be inter-familial heterogeneity (Vahava et al., 1998).

It is important to determine the aetiology of deafness, for the management of both the patient and the patient's family. The two main factors known to increase homozygosity among individuals and the emergence of autosomal recessive traits are small size isolates and high frequency of marriage between relatives (consanguinity). Because of this, large consanguineous families from isolated populations were instrumental in the mapping of recessive deafness loci. These endogamous populations have been identified from Bali, India, Israel, Lebanon, Palestine, Pakistan, and Tunisia. For example, a large deaf Tunisian family living in an isolated village led to the identification of the DFNB1 gene to chromosome 13 by linkage analysis (Kelsell et al., 1997). Likewise in a small village in Bali, where 2.5% of the population was deaf, DFNB3 was mapped to chromosome 17. No marriage had occurred between relatives in this geographical isolate, and all the couples had normal hearing parents. They all had deaf children. Using the technique of homozygosity

mapping, a single gene mutation was identified (Leon et al., 1992; Lalwani et al., 1994).

Most of these families have demonstrated severe-profound pre-lingual sensorineural hearing loss, the onset usually occurring before 12 months of age. One exception was consanguineous kindred from Pakistan who exhibited normal hearing until 10 years of age, followed by rapidly progressive sensorineural hearing loss to profound levels within 4-5 years (Veske et al., 1996).

The mapping of most of the deafness loci in autosomal-dominant hearing loss was mainly in single large pedigrees (Verhoeven et al., 1998; van Camp et al., 1997; Talebizadeh et al., 1998; O'Neil et al., 1996). These families characteristically demonstrate post-lingual hearing loss with minimal inter- and intra-familial variability in affected frequencies (Keats & Berlin, 1999). This does not, however, hold true for the auditory-pigmentary syndromes, such as Waardenburg Syndrome (WS), which characteristically exhibit variable penetrance and expression (Tassabehji et al., 1993; Farrer et al., 1994; Liu et al., 1995).

2.2.2 Epidemiological Models of Hearing Research.

There are six different groupings of epidemiological methods in common use today including:

- Census and list compilations,
- Cross-sectional sample surveys,
- Longitudinal sample surveys,
- Case-control studies,

- Clinical trials/observational studies and
- Screening and intervention studies.

Each of these methods has advantages and disadvantages (table 2.1). Choice of method therefore depends very much on the design of the study as well as the information sought.

Among published literature, epidemiological research into hearing disorders shows marked inconsistencies in diagnosis, classification, and methodology, making it impossible to compare studies for lack of standardization (Davidson et al., 1989). Unlike the clinical perspective, the epidemiological perspective deals with hearing in the population and in specific sub-groups of the population rather than the individual. Domains and measures of auditory function as defined by Davis et al. (1992) are summarized in table 2.2. Assessment of the domains is completed using different measures and criteria. A summary of the key features of each epidemiological method in use, the measures of auditory function, as well as the relationship between genetic and environmental factors in relation to age in the causation of hearing loss are summarized in tables 2.1, 2.2 and figure 2.6 respectively.

Three of these study designs are useful in epidemiological research establishing the aetiology of hearing loss. Case-control studies are useful for assessing the importance of aetiological factors in epidemiological studies. They have the advantage of being powerful, quick, economic, and reliable, and a small sample size is usually adequate for hypothesis testing. Their main drawbacks include a need for sophisticated statistics, heavy reliance on collating history of exposure, resulting in unobtainable,

biased or wrong information. It may also be difficult to ensure adequate control groups since some population characteristics cannot be controlled for (Davis & Wood, 1992).

Table 2.1: Features of some epidemiological methods in use

Study method	Uses	Advantages	Disadvantages	Example of publication
Census and list compilations	Ascertaining the population with a rare disorder	Provides useful scientific data. With a survey reduces overall variance. Used in social welfare and administration. Appealing to respondent	Only used for rare conditions e.g. <2% prevalence	Thornton (1986) Scheim and Delk (1974) Waksberg (1961)
Cross-sectional sample surveys	Ascertain prevalence and aetiological factors, evaluate success of preventive and/or rehabilitative measure	Relatively inexpensive, Good estimates with known bounds. Exclude crude forms of bias that may build up in non-random surveys. Allow calculation of the predictive values and probabilities	Indirect approach to aetiology and natural history. Unable to disentangle age, time and cohort. Broad incidence data only by inference from age difference.	*Bastos et al (1994)* McPherson and Holborow (1985)
Longitudinal sample surveys	Study of natural history and aetiology, estimate incidence.	Allows close study of natural history and aetiological factors.	Very expensive, less reliable if attrition is high. Take long =>? Relevance of information	Gordon and Kannel (1970) Dawber (1970) Davis (1983a)
Case-control studies	Assess importance of aetiological factors in epidemiological studies	Powerful, quick, economic, reliable Small sample size usually adequate for hypothesis testing	Need sophisticated statistics; heavy reliance on collating history of exposure- giving unobtainable, biased or wrong information; Difficult to ensure adequate control groups	*Seely et al 1995*
Clinical trials observational studies	Assess outcome of preventive measures	Useful for rehabilitation strategies	In Audiology of limited use	
Screening and intervention studies	Ascertain prevalence, assess outcome of rehabilitation measures	Essential for formulating secondary preventive measures	No ideal screen yet available, problems with sensitivity and specificity. Need to manage condition identified	*Watkins (1996)* *White et al (1993)*

Adopted (with personal modifications in italics) from Davis & Wood, 1992***Studies dated later than 1992 personal modifications intended to provide more recent examples of relevant epidemiological studies.***

Cross-sectional sample surveys, which are useful for ascertaining prevalence and aetiological factors and for evaluating the success of preventive and/or rehabilitative measures, have the advantages of being relatively inexpensive, and giving good estimates within known bounds. Cross-sectional sample surveys also provide for exclusion of crude forms of bias that may build up in non-random surveys, allowing for the calculation of the predictive values and probabilities. The main drawbacks are their indirect approach to aetiology and natural history, their inability to disentangle age, time and cohort, and the fact that broad incidence data can only be obtained by inference from the age difference (Davis & Wood, 1992).

Longitudinal sample surveys on the other hand are of great advantage in that they allow for close study of the natural history and the aetiological factors of a disease. But because they are long term studies, often running for up to and beyond 20 years, the results may be rendered unreliable in the presence of high levels of attrition (Davis & Wood, 1992).

There is a recognized contribution of genetic factors and environmental factors to the aetiology of hearing loss at all age groups (figure 2.6), with the genetic component high at birth in comparison to environmental factors, gradually reducing as environmental factors increase with age (Bussoli and Steel, 1998, Davis et al 1983a). A deaf neonate, for example, though more likely to have hearing loss of genetic origin, could have an acquired, and therefore environmental, aetiology, such as cytomegalovirus or rubella infection. The converse is true for adults. Whereas their hearing loss is most likely to be from environmental causes, it could still be due to a genetic cause. This has to be taken into consideration when designing a diagnostic

algorithm for assessing and investigating a hearing impaired person, be it a neonate or an adult (Bussoli & Steel, 1998; Smith & van Camp, 2005).

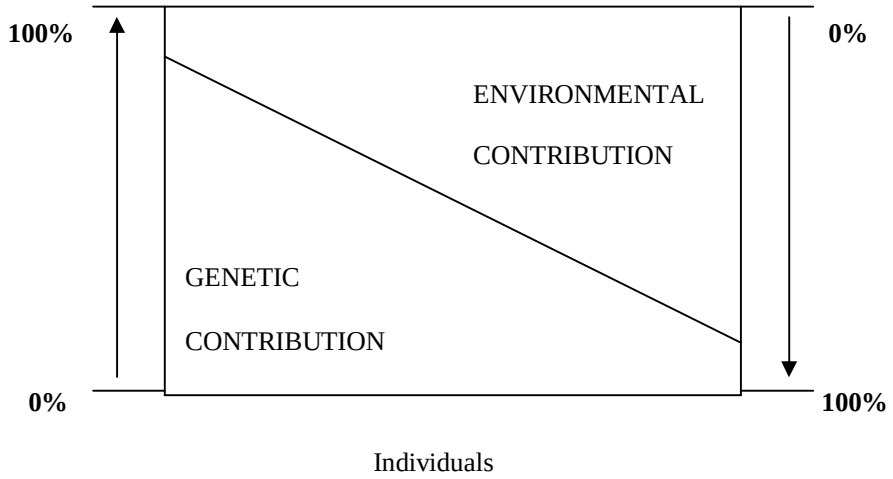


Figure 2.6: The relationship between genetic and environmental factors in causation of hearing loss as a function of age (adapted from Bussoli and Steel, 1998, Davis et al 1983a)

The complexity of the hearing mechanism, the number of structures or components and cell types that make up the hearing organ all combine to make the investigation of hearing loss a complicated issue as the defect could lie at any level. A correct diagnosis must be made, not only of the degree but also of the type of hearing loss. Ultimately, the aetiological diagnosis is required for proper management of the patient, especially in relation to counseling and prognosis, and rehabilitation of the patient according to the level of handicap (Smith & van Camp, 2005; Kenneson et al., 2002).

No single tool can be used to fully evaluate and rehabilitate a hearing impaired person. A diagnostic test battery and team management approach are needed (HPCSA, 2007, Smith & van Camp, 2005; Moodley et al 2000). The quality of the results will be dictated not only by the availability of resources but also by the quality

of health care personnel involved (HPCSA, 2007; Smith & van Camp, 2005; Kenneson et al., 2002; Moodley et al 2000)

Table 2.2: The domains and Measures of Auditory dysfunction

Auditory Function Domain (Definition)	Areas affected	Type of test	Areas covered by test/ Comments	Class of index
Pathology (Disorder of the hearing organ)	Middle ear Inner ear Hair cells Auditory nerve Brainstem Auditory cortex	Clinical examination Radiology Pure tones (Bone and air conduction) Evoked otoacoustic emissions (EOAEs) Auditory steady state response (ASSR) Immittance testing	External and middle ear Whole auditory Pathway Middle ear disorder	Medical Evaluation
Impairment (Abnormal function of the auditory system)	Auditory sensitivity Temporal processing Frequency resolution Auditory localization Binaural integration	Hearing threshold – pure tones, ECoG, ERA including EOAEs, ASSR, ABR Psychoacoustics tuning curves Gap detection tests	Parts of Auditory pathway Frequency resolution Temporal resolution	Physiological Electro-Physiological
Disability (Reduced abilities of the individual)	Environmental awareness Orientation Speech perception in noise Speech perception in quiet Group conversation TV, Radio, telephone use	Hearing threshold Speech perception Hearing thresholds with psychoacoustics and gap detection tests	By inference Especially for rehabilitation Good prediction of scores on speech tests though indirect	Behavioural
Handicap (Adverse effects on life)	Psychological effects: suffering, anxiety, need for extra effort Psychosocial effects: restrictions on independence, personal relationships, employment, remuneration	Curtailed activities Legislation	Patient questionnaires Major difficulties with quantification	Perceptual measures

Source: Adapted and modified from Davis et al 1983

In the ideal situation, a good team should have adequate knowledge and understanding of the hearing mechanism, the evaluation tests used and the foundation for accurate interpretation of results and application of interventions (HPCSA, 2007; Smith & van Camp, 2005; Kenneson et al., 2002). Table 2.2 cross-tabulates auditory function disorders with the possible levels of occurrence of the hearing defect, giving a selection of possible evaluation techniques to choose from when considered together with table 2.1. The addition of disability and handicap completes the management spectrum, a useful tool for the management of HI persons.

2.2.3 Epidemiological Models for SNHL

A number of epidemiological models for sensorineural hearing loss have been proposed. Davidson (Davidson et al., 1989) postulated that if the exact distribution of hearing level at birth and how this level changed over time were known, any data could be compared with ease. They compared different studies on hearing loss based on four main assumptions:

1. Attrition through death was ignored
2. SNHL was considered irreversible
3. All children were considered to have received equivalent level of health care
4. All audiological tests were assumed to have been reliable and sensitive for the level of hearing loss evaluated

Two major observations were made in this study. First, that at any given age, the prevalence of SNHL is a monotonically decreasing function of hearing loss criterion. Second, that for any specified hearing loss criterion, the prevalence of SNHL is a monotonically increasing function of age.

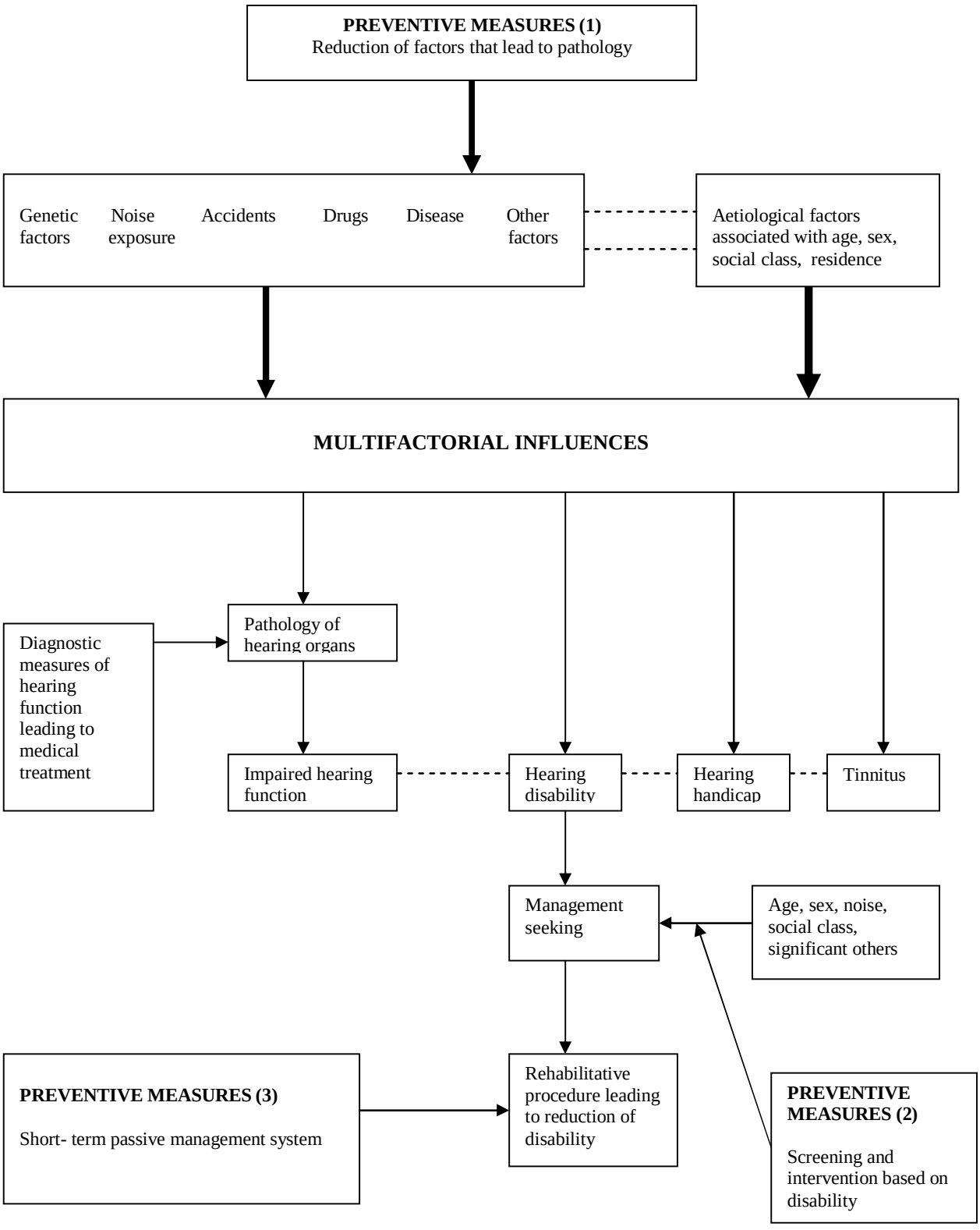


Figure 2.7: Epidemiological model of hearing function (after Davis et al 1989; Davis et al., 1990)

The authors concluded that with such knowledge of hearing loss distribution, one could identify optimal time(s) for hearing loss detection, help determine the adequacy of a particular test, or facilitate monitoring of hearing loss prevention techniques/programmes.

The model proposed by Davis et al. (1990) is more inclusive, showing the interaction of pathology, impairment, disability and handicap (figure 2.7). It also allows for the fitting in of the three measures of prevention (primary, secondary and tertiary). From this model, the greatest indicators of pathology (and so of hearing impairment and disability) were found to be demographic factors, especially age. This study shows up the limitation, in effectiveness, of the primary preventive measures, and the weakness of tertiary preventive measures, which seek to limit disability in a responsive manner.

The choice of the model used depends on the study design, the financial implications, as well as the advantages and disadvantages of the particular method. The UK national study showed that it is not possible to design a single step study that will cover all aspects of even prevalence estimation. A multi-staged study is best suited for this.

2.2.4 Epidemiology of Hearing Loss in Africa

African studies on hearing loss vary greatly in methodology and study design. As such they are not comparable because the data cannot be standardized. This is not unique to studies in Africa however, it is a worldwide problem. This is highlighted in the findings of Davidson et al (1989)**,** summarized in table 2.3 below.

Table 2.3: Examples of studies on the Prevalence of Hearing Loss in Childhood

First Author	Date	Country	Age	Method	Hearing level	Prevalence
Scheim	1974	USA	3 years 19 years	Questionnaire	'Deaf' 'Deaf'	1.0 2.3
Pal	1974	India	5 years	Distraction or pure Tone	60 dB 60 dB	2.0 4.2
Fein Messer	1982	Israel	5 years	Modified Ewing Strycar test or play	55 dB	1.7
Kankkunen	1982	Finland	6 years	VRA, play or respiration	70 dB	1.3
Martin	1982	UK, Belgium Denmark France Ireland Italy Netherlands	8 years	Questionnaire and review of medical records	50 dB	1.0 0.74 1.48 0.56 0.92 0.92 0.91 0.93
Upfold	1983	Australia	7-17 years	Pure tone	60 dB 90 dB	1.1 0.48
Parving	1983	Denmark	2-12 years	Pure tone ERA or ECoG	35 dB 55dB 75 dB	1.4 0.92 0.60
Thringer	1984	Sweden	5 years	Play	40 dB 60 dB	1.4 0.9
McPherson	1985	West Africa (Gambia)	10 years	Play, distraction, Play or pure tone	70 dB 95 dB	2.7 2.2

Source: Davidson et al., 1989

The key challenges facing researchers in Africa include high attrition, especially due to migrant labour practices, major differences in provision of health care in different areas, unreliable collating history due to illiteracy, as well as the need to know and understand the culture of the community under study. Then there is the question of funding. With a large part of the population still unable to access basic health care,

research funding would seem like a luxury. The result has been a paucity of prevalence and aetiology data from Africa, where possibly up to two-thirds of the world's hearing impaired children are believed to be found (Olusanya et al., 2004b; Swanepoel et al., 2005b).

The Sierra Leone study (Seely et al., 1995) is one of the better designed studies. This population based case-control study analysed risk factors for hearing loss. The subjects were aged 5 to 15 years. The results showed that 9.1% subjects exhibited moderate or greater hearing loss, while 4 per 100 had bilateral hearing loss. The risk factor in the study found to correlate most strongly with hearing loss was otorrhoea lasting longer than one month (Seely et al., 1995). The researchers had difficulty in ascertaining age in 9.8% of the subjects. This is a real problem for studies conducted in rural African settings where birth registration is not mandatory, and where home birthing is frequent.

In South Africa, it is estimated that about 30% of children are not born in health facilities (Statistics SA, 2002). In Tambo district for example, almost half the deliveries are home births, with an estimated rate of 47% home deliveries, 2% clinic and 51% hospital deliveries (Statistics SA, 2002). Birth registration in South Africa, though, is better than most other African countries. It is ultimately mandatory for each individual to obtain an identity document, and for this birth registration must first take place.

Less work has been done on late childhood onset and adult onset forms of non-syndromic hearing loss in Africa (Olusanya et al., 2004b; Swanepoel et al., 2005b).

This may be due to a combination of factors, such as the challenges of separating environmental and genetic aetiologies at this age (figure 2.6), lack of funding and skilled personnel. These all hinder data collection through formal research. For example, in the Limpopo province, South Africa, three centres were set-up for a pilot project aimed at establishing the incidence and prevalence of congenital hearing loss. The protocol included screening a six-month cohort of all newborns birthed at three centres, namely, Mankweng, Pietersburg Provincial and Mapulaneng Hospitals, from April to September 1999, using TEOAEs, and to then follow up this cohort for three years. The project had to be abandoned due to lack of funds and the work-overload for the few speech and hearing therapists in the province that could not allow them to diligently screen and follow-up all the children screened (Department of ENT outreach report, 1999, the Pietersburg-Mankweng Hospital Complex, Limpopo province, South Africa).

In another part of the country, steps towards early identification of at-risk infants were started with a proposal for a multidisciplinary management approach by researchers at the University of Pretoria, South Africa (Moodley et al 2000). In Nigeria, Olusanya was challenging developing African countries to come on board in recognizing that hearing impairment and its effects in Africa could be minimised through appropriate preventative mechanisms as well as through early identification and rehabilitation (Olusanya, 2000, Olusanya et al, 2006c).

In a series of research projects, Olusanya has spearheaded infant hearing screening on the African continent and suggested unique remedial measures to challenges identified as hindering neonatal and infant hearing screening within cash-strapped,

illiterate or underdeveloped communities (Olusanya, 2001, Olusanya et al., 2004a & 2004b, Olusanya, 2005, Olusanya et al., 2005, Olusanya et al., 2006a). Olusanya has, for example, shown that, by screening the infants at the immunization clinics, universal infant hearing screening can be achieved in Africa by linking it to the already well established primary healthcare programmes (Olusanya & Okolo, 2006). Together with South African counterparts, key review papers have come out of the research projects centered on infant hearing screening (Olusanya et al., 2006b, Olusanya et al., 2007).

In South Africa, this work has been spearheaded by Swanepoel and others (Swanepoel et al., 2004, Swanepoel et al., 2005a, Swanepoel et al., 2005c, Swanepoel, 2006, Swanepoel et al., 2006, Swanepoel et al., 2007)) culminating in a position statement by the Professional Board for Speech and Language and Hearing Professionals, under the auspices of the Health Professions Council of South Africa, on early hearing detection and intervention (EHDI) programmes in South Africa (The Health Professions Council of South Africa, 2007). This statement, while accepting and incorporating the Joint Committee for Infant Hearing (JCIH) position statement (JCIH, 2000), and the American Academy of Paediatrics (AAP) statement (AAP,1999), recognizes the unique challenges facing developing countries, including South Africa, and is structured to address some of these challenges (HPCSA, 2007).

In summary, the position statement (HPCSA, 2007) recommends EHDI programmes through service delivery mechanisms that already exist on the ground, namely district and provincial health systems, involving both public and private partners, and including NGOs. Universal neonatal and infant hearing screening is to be carried out

using objective physiologic measures such as otoacoustic emissions (OAEs), both transient (TEOAEs) and distortion product (DPOAEs), as well as automated auditory brainstem response (AABR). Furthermore, confirmatory diagnostic audiological and medical assessments should be completed by the age of 4 months, and interventions initiated by the age of 8 months. All at risk infants are to be followed up through ongoing monitoring by the relevant healthcare professionals trained and experienced in infant hearing. Key medical professionals in all EHDI programmes identified include paediatricians, otorhinolaryngologists and other medical specialists including geneticists. Further details are contained in section 3.3.1.2 on universal hearing screening of this thesis.

The prevalence figures on deaf and hard of hearing persons in the Limpopo province based on the department of health and social services 2004 mid-year estimates (Department of Health, Limpopo Province, 2004) indicated that about 44,516 persons in the Northern Province, now Limpopo province, were deaf of hard of hearing (table 1.3). However, the 2002 census had indicated that 47,061 persons had severe hearing loss in the Limpopo Province (SatsSA, 2002 Census). It is unclear how deafness or hearing loss was defined in the census or the survey. If the EHDI programmes are established and run diligently, then, presumably, data will come out of them to give a clearer indication of the incidence and prevalence of congenital and early onset hearing loss within the South African context.

2.2.5 Epidemiological Studies on Genetic Hearing Loss

All the epidemiological surveys among communities of people with inherited deafness have repeatedly shown great genetic heterogeneity (Van Camp et al., 1997).

This is in terms of the numbers of genes and mutations, as well as in the prevalence of types of mutations in different communities and population groups.

There is now evidence to show that *GJB2* mutations are a common cause of genetic hearing impairment, changing our perception of deafness (Morton &Nance, 2006; Smith & van Camp, 2005; Del Castillo et al., 2003; Kenneson et al., 2002; Estivill et al., 1998). From the assumption that genetic hearing loss was caused by a large number of rare genotypes, it has now became clear that genetic hearing loss is a more homogeneous disease in many populations. It is now understood that although non-syndromic deafness is an extremely heterogeneous disease, it often segregates as a monogenic trait. This has significance for studies conducted in countries with a paucity of research and data on hearing impairment as will be clarified below.

Mutations in *GJB2* have been shown to account for up to 50% of cases of non-syndromic genetic hearing loss among populations in Europe, North America and Asia (Pandya et al., 2003; Arnos et al 2003; Liu et al., 2002). There are population differences in the distribution of *GJB2* alleles in all described populations. The 35delG allele of *GJB2*, found to range from 10% to 20% among Caucasians of northern European descent, was as high as 30% to 40% in the Mediterranean regions (Gasparini et al., 1997; Green et al., 1999). In other population groups, 167delT, 235delC and R143W are the most common alleles associated with *GJB2* hearing loss among Ashkenazi Jewish, Japanese/Chinese, and Ghanaian populations respectively (Rabionet et al., 2000; Zelante et al., 1997; Hamelmann et al., 2001). The evidence for a founder effect in *GJB2* related deafness among Ashkenazi Jews and in Caucasian

populations in Western Europe has been overwhelming (Del Castillo et al., 2003; Kenneson et al., 2002).

The *GJB6*-D13S1830 deletion was identified as the accompanying mutation in ~50% of the deaf *GJB2* heterozygotes in Caucasian populations (Pallares-Ruiz, Blanchet et al. 2002; Del Castillo, Moreno-Pelayo et al. 2003; Gunther, Steiner et al. 2003). It is the commonest mutation in *GJB6*, and it is associated with NSHL when homozygous, or when present on the opposite allele of a *GJB2* mutation. The *GJB6*-D13S1830 mutation has been shown to occur in up to 20% of the hearing-impaired USA population and is estimated to account for ~10% of all DFNB1 alleles with an extremely wide range based on ethnic origin (Del Castillo, Moreno-Pelayo et al. 2003).

In other studies among African Americans, the 35delG mutation was not found among 173 (Morrel et al 1998), and 190 (Gasparini et al., 2000) deaf individuals. Neither was it detected in 365 profoundly deaf students in Ghana (Hamelmann et al., 2001)**.** The finding of a very low frequency of *GJB2* variants among the deaf populations of Sudan and Kenya further highlights this trend (Gasmelseed et al., 2004). Because the rate of non-syndromic hearing loss is not lower among African Americans compared to their Caucasian counterparts, these are significant findings.

Genetic research is costly. When studying genetic defects in virgin populations, initially investigating for reported known genes causing the defect makes economic sense. For the Limpopo study, the results of the above reported studies were especially significant, and posed key questions. The current study therefore chose the

GJB2 gene as a starting point in investigating the observed non-syndromic genetic hearing loss in the study population. The Lemba, who are considered to be of Jewish descent (Thomas et al., 2000; Mathivha 1992), are a special group among the Vendas in the Limpopo Province of South Africa. Since mutations in *GJB2* have been reported among people of Jewish descent, similar mutations could be found among the deaf Lemba. But it is also possible that, like other studies on deaf people of African descent, genetic analysis would reveal no mutations in the *GJB2* gene.

The full implication of these findings will only become clear after assessment of *GJB2* variants among non-Caucasian genetically hearing impaired populations and their corresponding control populations. Data from these epidemiological studies will yield valuable information for the design of molecular diagnostic protocols that are appropriate for different populations.

2.3 THE EAR IN GENETIC HEARING LOSS

2.3.1 Development of the Ear

Ear development occurs at different times for the different structures within the ear. As such abnormalities in one part of the ear may not necessarily occur with abnormalities in the rest of the ear. However, in 2 to 12% of cases, abnormalities occur concurrently in the external, middle and inner ears (Naunton & Valvassori, 1968; Potter, 1969).

Ear development in man starts as an otic placode made up of thickened ectoderm opposite the neural fold of the hindbrain. The otic placode first appears around the

21st embryonic day, induced by the notoderm and further influenced by the migrating neurocrest cells. The placode invaginates to form the otic cup, subsequently closing off at the surface and detaching to form the otic vesicle by the 28th embryonic day. This in turn undergoes a period of cell proliferation, neurogenesis, programmed cell death and differentiation, to form the mature ear (Swanson, Howard, & Lewis, 1990; Sanz, Leon, Troppmair, Rapp, & Varela-Nieto, 1999; Fekete & Wu, 2002; Shnerson, Lenoir, van de Water, & Pujol, 1983).

Table 2.4: Table depicting time of appearance of ear features

Feature	mm	2		3		4	5	6	8	10		15		20		25	30
	Weeks	4							5			6			7		8
	Stage	6	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
..Optic placode																	
..Pharyngeal pouch 1																	
..Pharyngeal cleft																	
..Otic pit																	
..Otic vesicle																	
..Otic vesicle closed from surface; Otic Capsule																	
..Endolymphatic appendage tapered, cochlear duct beginning																	
..Auricular hillocks beginning																	
..Utriculosaccular diverticulum																	
..Tubotympanic recess, 6 auricular hillocks																	
..1-3 semicircular ducts, stapes, and stapedius, auricular hillocks merging																	
..Cartil. otic capsule; malleus, incus																	
..Tensor tympani																	
..Cochlear duct nearly 2 and ½ turns																	

Source: O'Rahilly, Muller *Human embry. & terat.3rd edition*

The otic vesicle is the primordium for the formation of the membranous labyrinth, giving rise to the semicircular ducts, cochlear ducts, endolymphatic ducts, the utricle and the saccule. Mesenchyme surrounding the otic vesicle condenses to form the otic

capsule which gives rise to the bony labyrinth. Neurocrest cells derived from the medial wall of the vesicle form, in part, the cochleovestibular ganglion cells. They proliferate and differentiate into neurons whose processes extend into the vestibular and cochlear nuclei in the midbrain (Hemond & Morest, 1991; Rubel & Fritzsch, 2002; Alsina et al., 2003). The middle ear develops from the first pharyngeal pouch, and the first and second branchial arches (Naunton & Valvassori, 1968; Potter, 1969). The external ear develops from the first and second pharyngeal arches and the first pharyngeal cleft (table 2.4). The structure of the fully formed ear is depicted in figure 2.9.

Correct development of the ear is regulated by intrinsic and extrinsic factors. These include the growth and neurotrophic factors from the developing spinal cord and brain. As such, abnormalities of the spine are often associated with ear malformations (Hemond & Morest, 1991; Rubel & Fritzsch, 2002; Alsina et al., 2003). The major processes of inner ear development including induction, proliferation and morphogenesis, neurogenesis, programmed cell death and differentiation are summarized in figure 2.8.

Functional development of the cochlea continues into the 2^{nd} and 3^{rd} trimesters. Melanocytes derived from the neural crest are found in the stria vascularis, where they play an important role in maintenance of the endocochlear potential of the endolymph, and in the utricle. Cochlear axons have been demonstrated to enter the brain stem in the second trimester. Myelination of the auditory pathway, however, does not commence until after the onset of hearing (Hemond & Morest, 1991; Rubel & Fritzsch, 2002; Alsina et al. 2003).

Apoptosis and programmed cell death are essential to the development of the ear (O'Rahilly and Muller in *Human embryology & teratology 3rd edition*). By 29 weeks of intrauterine life, the fetus can hear and respond to sounds. The fetal response to sound includes increased fetal heart rate, a startle response, discrimination between frequencies and speech sounds.

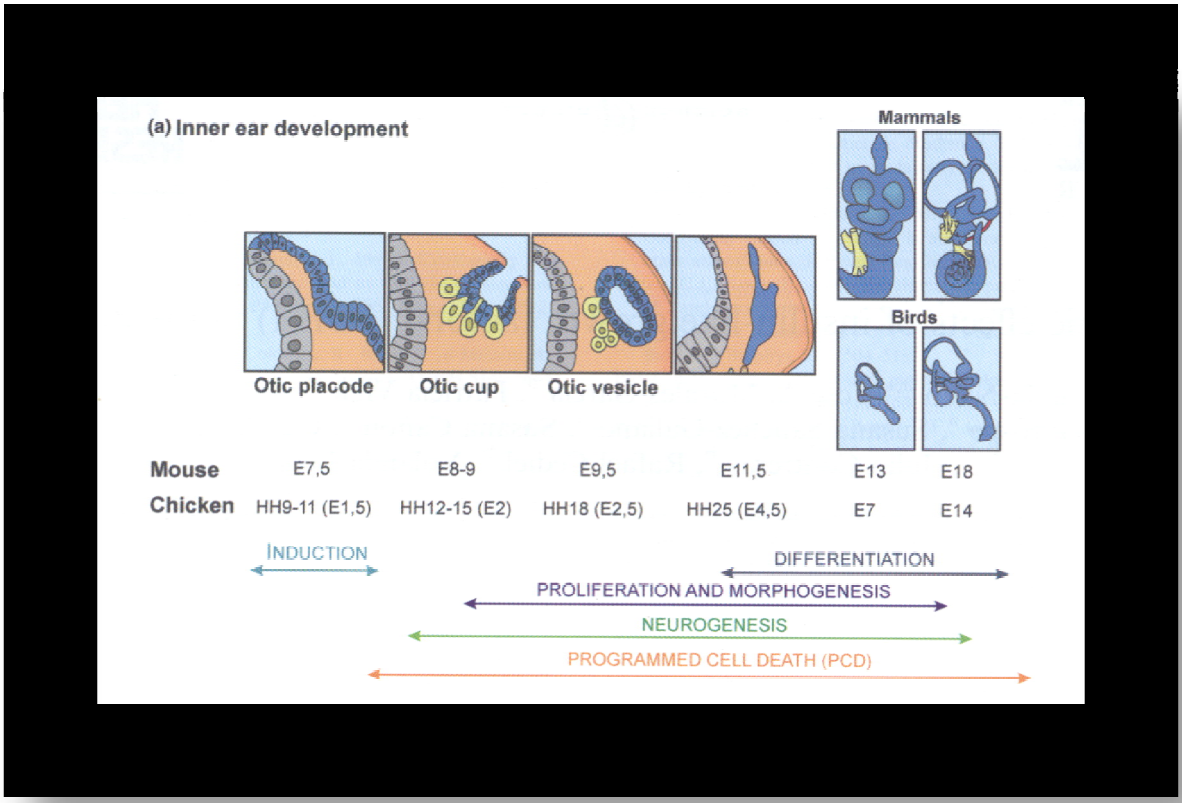


Fig 2.8: Schematic drawing of inner ear development in mammals (Varela-Nieto et al., 2004)

2.3.2 Overview of the Anatomy of the Mature Inner Ear

The sensory epithelium in the organ of Corti is contains two types of receptor cells: the outer hair cells (OHC) and the inner hair cells (IHC). Both types of cells carry highly organized stereocilia on their apical surfaces which are held in place by supporting cells.

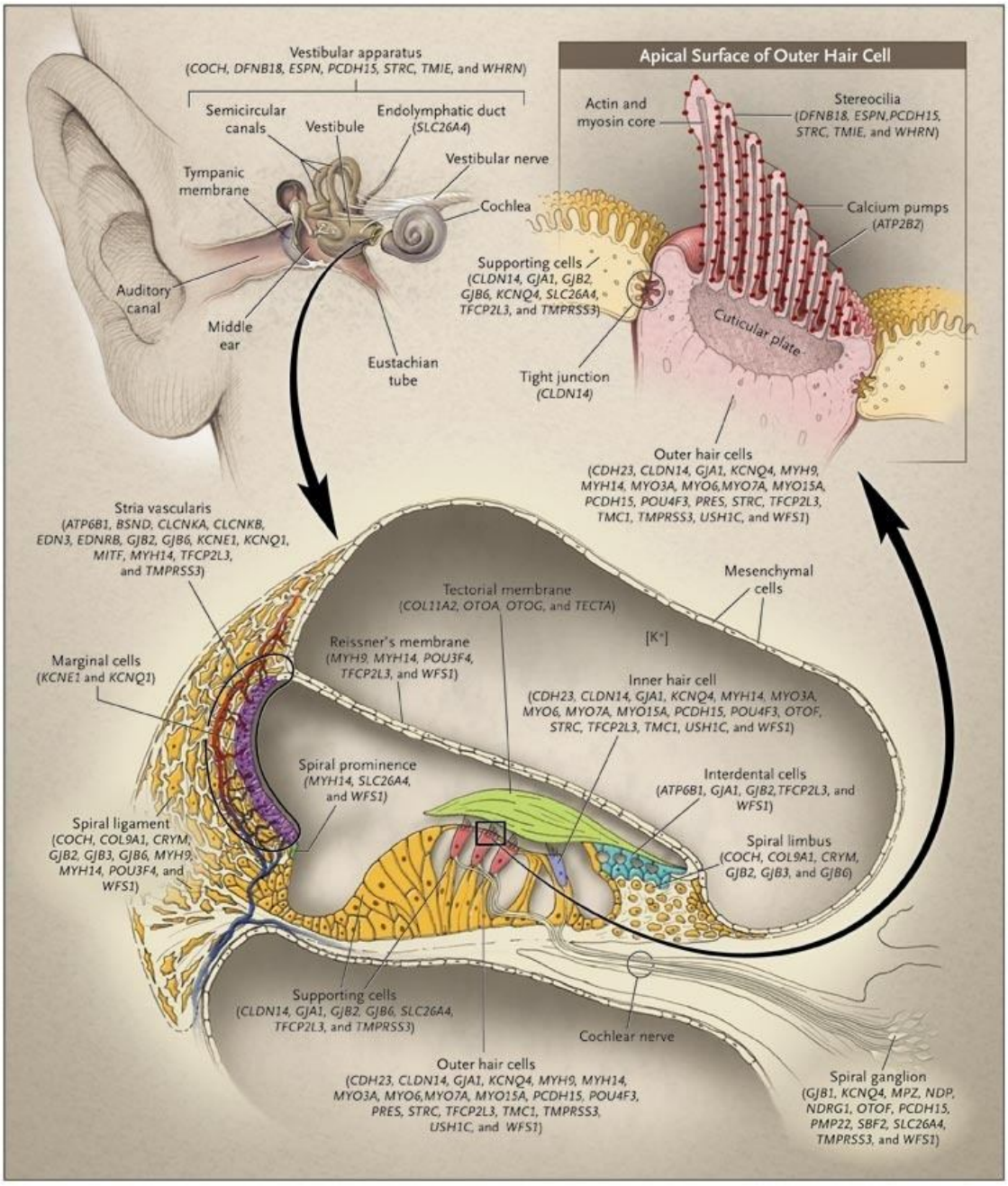


Figure 2.9: Diagram showing the structure and gene expression of the human ear. (Morton & Nance, 2006)

The organ of Corti, when viewed under electron microscopy, reveals three rows of outer hair cells, each with a tuft of stereocilia arranged in a w-pattern and graded according to height, and a single row of inner hair cells are covered by relatively straight rows of stereocilia graded according to height. The stereocilia are anchored in the cuticular plate on the apical end of the hair cell by packed actin and myosin

filaments. For normal auditory function to occur, this intricate arrangement must be maintained at all times (Dallos, 1992, Dallos et al., 2002).

The cochlea inner ear fluids are arranged in a unique manner (figure 2.9). Three extra cellular fluid-filled compartments, each with a unique ionic composition, support the mechanosensory transduction process of hearing (Dallos, 1992; Kumar & Gilula, 1996; Dallos & Fakler, 2002). The scala media contains endolymph, high in K+ and low in Na+ ions. The scala vestibuli and scala tympani contain a fluid low in K+ and high in Na+ ions. The apical surfaces of the hair cells are bathed in the K+ rich endolymph, which is tightly sealed off from the rest of the cell by the reticular lamina. All around the cochlea, these fluids are kept separate by the tight junctions between the cells and the fluid composition is preserved (Dallos & Fakler, 2002; Kikuchi et al., 2000; Kumar & Gilula, 1996). The variety of cell types in the cochlea serves an important purpose, with cells arranged into cell systems to handle the complex functions of the cochlear. Abnormalities in the cell groups could lead to hearing loss.

2.3.3 Gap Junctional Systems of the Human Ear

In the cochlea, all supporting cells are directly connected to adjacent supporting cells via gap junctions. These extend to the root cells and the interdental cells, and form the epithelial cell gap junction system (Kumar & Gilula, 1996). The epithelial gap junction system is separated from the adjacent connective tissue cells by a continuous basement membrane (Kikuchi et al., 2000).

Connective tissue cells in the spiral limbus are also interconnected through gap junctions, forming a large connective tissue cell gap junction system (Kikuchi et al.,

2000). Included in this system is the mesenchymal cell gap junction system lining the scala vestibuli. All in all, the strial intermediate and basal cells, four types of fibrocytes in the cochlear lateral wall, the mesenchymal cells lining the bony otic capsule of the scala vestibuli, as well as the connective tissue cells in the spiral limbus, together form the connective tissue cell gap junction system (Kikuchi et al., 2000). There are no gap junctions between the hair cells and the supporting cells, or between the connective tissue gap junction system and the marginal cells in the cochlear (Goodenough et al., 1996; Kikuchi et al., 2000; Rabionet et al., 2000).

The gap junctional system provides pathways by which K^+ ions are re-circulated from the Organ of Corti to the stria vascularis. K^+ ions which enter the IHC on acoustic stimulation are expelled basolaterally by the hair cells, and are accumulated by the supporting cells (Holt & Corey, 1999; Steel & Bussoli, 1999; Kumar et al., 1996). From the Organ of Corti, ions are moved via the gap junctional system to the spiral ligament, from where they transported to stria vascularis. From the stria vascularis, ions, especially K^+, are actively secreted into the scala media (Kikuchi et al., 2000).

2.3.4 Major Ear Defects in Hereditary Hearing Loss

The major ear defects in hereditary hearing loss are grouped into three main categories (Steel & Brown 1994; Steel, 1995):

i. Morphogenic defects

ii. Cochleo-sacular defects

iii. Neuroepithelial defects

Morphogenic defects occur as a result of interruption of the early events in the development of the labyrinth, leading to a malformed inner ear (Steel & Brown 1994;

Steel, 1995). The neural tube has been shown to have an inductive influence on inner ear morphogenesis. The lateral semicircular canal is the most commonly affected structure in the vestibule. Several genes, which when mutated lead to morphogenic defects, have been identified in animal studies and at least four of these code for transcription factors (Hemond & Morest, 1991; Rubel & Fritzsch, 2002; Alsina et al., 2003). The main effects are failure to develop a normal endolymphatic duct, a tendency to cystic expansion of the inner ear cavities, as well as restricted malformations of the labyrinth such as thin or obliterated semicircular canals. There is often asymmetry in the extent of the malformations.

Cochleosacular defects are characterised by a primary defect of the stria vascularis with a reduction in the endochochlear potential, collapse of Reissner's membrane, and degeneration of hair cells and spiral ganglion cells (Steel & Brown 1994; Steel, 1995). These features have been found to occur commonly in human non-syndromic hearing loss.

Neuroepithelial defects are characterised by a primary defect in the Organ of Corti. Although there is degeneration of the spiral ganglion cells, the stria vascularis, endochochlear potential and Reissner's membrane are all normal (Steel & Brown 1994; Steel, 1995).

2.3.5 Overview of the Physiology of Hearing

Sound pressure is collected by the pinna, modified and directed down the external auditory meatus to the tympanic membrane. This sets the tympanic membrane into vibration and the sound pressure, now in the form of mechanical energy, is conducted

via chain of ossicles to the stapes footplate. Movement of the footplate causes the sound energy to be transmitted to the cochlear fluid, setting up a travelling wave along the basilar membrane, with the area maximal displacement depending on frequency of stimulus**.** There is amplification of the maximum area of displacement, involving OHCs. The outer hair cells have been shown to contain in their lateral walls a protein called prestin which contracts and expands, accounting for the change in length of the hair cell in response to stimulation (Dallos, 1992; Dallos & Fakler, 2002; Zheng et al., 2000). This has been termed the motile response of the OHC, now believed to be part of the mechanism by which the travelling wave is amplified at the basilar membrane (Dallos & Fakler, 2002; Zheng et al., 2000).

Movement of basilar membrane at this point causes a shearing movement between the tectorial membrane and reticular lamina, causing tilting of the rigid stereocilia on the surface of the outer hair cells (OHCs) and opening up the transducer channels near the tip-links. The inner hair cells (IHCs) are stimulated by the viscous drag of the cochlear fluid in the inner sulcus (Dallos et al., 2002; Zheng et al., 2000).

Because of the different ionic composition of the cochlear fluids, high K+ and low Na+ concentration in the endolymph (scala media), compared to the low K+ and high Na+ ions in the scala vestibuli and scala tympani, there is a high endolymph potential of about 100mV in the cochlear. The inrush of K+ ions from fluid of scala media into the hair cells (which contain a low potassium concentration) generates a receptor potential in the hair cells (Dallos, 1992; Dallos et al., 2002; Zheng et al., 2000). With the depolarisation of the hair cells, there is a resultant release of neuro-transmitter at the base of the hair cells, followed by firing of nerve fibres connecting with the basal

end of the hair cells. The K+ ions are then released into the extracellular fluid and from there recirculated back into the endolymph via the gap junctional system of the inner ear (Dallos, 1992; Dallos et al., 2002; Zheng et al., 2000). Thus the mechanical energy is turned into electrical energy through this mechanosensory transduction process. The resultant nerve impulses are transmitted down the auditory nerve fibres to the central connections of the brain where they are interpreted.

The IHCs are the true sensory receptors while the OHCs are responsible for amplifying the travelling wave at the basilar membrane (Dallos, 1992; Dallos et al., 2002; Zheng et al., 2000). As such the IHCs are transducers of vibration, and the source of all the auditory signals passing to the brain (Dallos et al., 2002). The electromotility of the OHCs on the other hand increases the sensitivity and frequency selectivity of the cochlea (Dallos et al., 2002). When the OHCs are damaged, these key features of sensitivity and frequency selectivity are lost, leading to elevated hearing thresholds and poor speech discrimination (Dallos et al., 2002).

Normal hearing thus depends on the integrity of the organ of Corti, the integrity of the hair cells, a correct ionic homeostasis and composition of the inner ear fluids, notably the high K^+ content of the scala media, as well as a normally functioning stria vascularis (Holt et al., 1999; Steel et al., 1999; Kumar et al., 1996; Rabionet et al., 2000; Dallos et al., 2002).

CHAPTER 3: LITERATURE REVIEW AND BACKGROUND INFORMATION – PART II

Summary of the chapter

This chapter details the history of research into deafness genes up to the Human Genome Project and beyond; the advances made in characterizing deafness genes with special attention to the candidate genes chosen for this study, GJB2, PAX3, MITF and the four common mitochondrial genes analyzed for in this study. It also covers the clinical perspectives of childhood hearing loss especially as it relates to the management of the hearing impaired child (suspicion, identification, assessment, investigation, aetiological diagnosis and rehabilitation).

3.1 HISTORY OF RESEARCH INTO GENETIC DEAFNESS

3.1.1 History of the Genetics of Hearing Loss

Although hearing loss has been known over the centuries, the importance of heredity of hearing loss was first documented in the 16th century (Keats & Berlin 1999). Johannes Schenek (1531-1598) is reported to have been the first author who noted a family in which many siblings were congenitally deaf (Goldstein, 1933). By 1621, with growing evidence that heredity was an important co-factor in deafness, Paolus Zacchias (1584-1659), the pope's physician, recommended that deaf persons should not marry and have children to prevent deafness in their offspring (Cranefield & Federn, 1970). More recently, Sir William Wilde is reported to have recognized the different patterns of inheritance and that consanguinity was an important co-factor in deafness (Reardon, 1990). He is also believed to have been the first to recognize and document that there were many more males than females among the congenitally

deaf. Extensive studies in German schools of the deaf (Hartmann, 1881) were to confirm these observations later. Syndrome delineation through observation followed. Development of new techniques in histopathological studies of the temporal bone made identification and classification of hearing firmer (Michaels et al., 1983). When the genetic code was discovered and techniques in mutation detection and tracking were developed, research moved to the molecular basis of disease. Comparative genomics has thus made great advancement in the identification of gene identification in many genetic disorders including hearing loss (Meisler, 1996; Brown and Steel, 1994; Keats and Berlin, 1999). Many genes and loci for hearing loss have now been discovered and documented, changing the scene of auditory research forever. These details are outlined in the sections that follow.

3.1.2 Clinical Phenotypes of Genetic Deafness

In the 1950s and early 1960s, research in genetic deafness centered on syndromic forms of deafness, following the general trend of progress in the study human genetic disease. Many people, including Fraser, Reuben, Yntema, Deol, Bosher, Fisch, Hallpike, Konigsmark, Gorlin, McKusick and others, contributed valuable work in the study of genetic hearing loss in the last century. There are 400 syndromes known, many of which are rare. They were catalogued and described by amongst others, Fraser (1976), Konigsmark and Gorlin (1976), and McKusick (1986). The most important and common genetic syndromes include Waardenburg, Usher, Pendred and Jarvell and Lange-Nielsen.

Syndrome delineation depended on the use of clinical features to group and classify a genetic syndrome (phenotypic classification). Starting with a few patients, clinical

findings were documented, and as more patients were added, the phenotype was gradually established and refined (Cohen, 1989). Initially, because only cases with identical or nearly identical clinical features to the original description were included, a false impression that a syndrome was clinically homogeneous was created. This phenotypic classification was later found to be imprecise. There was a noted phenotypic overlap between different syndromes and many genetic diseases were found to exhibit variable expression and penetrance. An example is Waardenburg syndrome, an autosomal dominant form of genetic hearing loss, in which members of the same family may show different clinical features as well as different degrees and types of hearing loss (Arias 1971; Hageman & Delleman, 1977, Liu et al., 1995).

The finding of phenotypic overlap between different syndromes raised the question of what constituted the core syndrome phenotype and its variants. Pinsky et al. (1977) recognised that overlapping syndrome phenotypes may reflect a biological relationship. The terms ‘phenotype communities’ and ‘syndrome families’ were introduced to describe groups of syndromes sharing a large number of their key clinical features. Today, syndrome families are found as an arbitrary grouping of syndromes in specialized databases. These include OMIM, the London DYSMORPHOLOGY Database (LDDB), or the Pictures of Standardized Syndromes and Undiagnosed Malformations (POSSUM) database (Evans D 1995; Hammosh et al 2002). In spite of these challenges, the concept of syndrome families continues to successfully predict allelic mutations, as demonstrated in several skeletal dysplasias (Pinsky, 1977; Evans D 1995; Hammosh et al 2002).

3.1.3 Histopathologic Phenotypes of Genetic Deafness

Central to the study of medicine is the knowledge of the pathological basis of disease. By the start of the nineteenth century, this had been applied to most of the body, except for the ear. The inaccessibility of the inner ear in life as well as the difficulties of post-mortem preservation of the inner ear structures posed a major challenge. Conventional methods of biopsy and histology in life were not possible. Ultimately post-mortem studies of the temporal bone were the only resort. But it was not until advanced techniques of isolation of the inner ear structures were developed that accurate pathological analysis could be performed (Michaels et al., 1983). The development of early (immediate post-mortem) formalin infusion of the inner ear through the round window, and advances in bone dissolving techniques enabled accurate demonstration of pathological changes in the inner ear.

The landmarks in major anatomical advances that were made during the nineteenth century include:

- 1837 - Lineke published cross-section drawing of the cochlea.
- 1851 - Alfonse Corti discovered the organ of Corti. The drawing was of a rather flattened Organ of Corti due to poor fixation methods.
- 1865 - Harvard University developed better fixation techniques and published a more accurate drawing of the organ of Corti. This was later improved on by Politzer
- 1889 - Renaut made an accurate drawing of hair cells in relation to the reticular lamina
- 1927 - Krause drew organ of Corti with tectorial membrane approximating the form we know today

The study of the developing embryo in the early part of the last century also added to the sea of knowledge. Through experimental studies on the amblystoma (Yntema 1950) and in mice (Deol, 1964), the developing central nervous system was identified as the primary inductor of the bony labyrinth. Ruben and Van der Water (1983) noted the contribution of the surrounding mesenchyme to the developing ear. The end-state nature of the cells of the organ of Corti was demonstrated in cell-kinetic studies (Ruben, 1967). These studies showed that the cells of the organ of Corti are formed by the end of the second month of intrauterine life, indicating that cell death after this period would result in hearing loss. Studies on human foetuses which had been infected with rubella, showed normal sensory epithelia (Bordley, et al., 1968), yet one of the key pathological findings of rubella sensorineural deafness has been shown to be, among other things, a lack of sensory cells (Schuknecht, 1974). This confirmed that some forms of congenital deafness were a result of early cell death or apoptosis.

That apoptosis also affected genetic forms of hearing loss was to be confirmed late in the twentieth century through animal studies (Steel et al., 1996). Later studies on the effects of sound deprivation on the central auditory pathways, either by removal of the developing otocyst from a chick embryo (Levi-Montalcini, 1949) or through destruction of the inner ear (Webster et al., 1977) or through actual sound deprivation (Gottlieb, 1975; Riesen and Zilbert, 1975) all showed reduction in the numbers of structures in the auditory pathways.

It is estimated that there are over 350 different conditions causing deafness, but temporal bones from only about 50 of these have been studied (Marchant, 2004). Histopathologic studies of the temporal bones in genetic deafness are important for

two main reasons. First, by providing insight into the pathological basis of deafness, they verify the validity of animal models used in the study of human deafness. Secondly, the information obtained helps generate hypotheses on mechanisms of hearing loss (Marchant et al., 2004; Smith et al., 1992; Steel & Bock, 1983). This information can then be applied to suitable animal models for confirmation. By 2004, only in 12 of the conditions in which the gene for deafness had been cloned had the otopathology been determined and reported worldwide (Marchant et al., 2004).

Clinically, attempts to classify the various hearing impairments had mainly focussed on audiological features (Smith et al., 1992). It was, however, more difficult to understand and classify the disorders associated with sensorineural hearing losses. Until 1992 the most widely accepted histopathological classification recognized five degrees of malformation (Ormerod, 1960; Schuknecht, 1967). This system posed major limitations, including the fact that it could not provide reliable prognostic information or be used to predict hearing acuity and stability (Jackler et al., 1987). No alternative method of studying the temporal bones had yet emerged, inspite of the fact that in over 80% of congenitally hearing impaired neonates no morphogenetic defect could be identified because the auditory lesions were confined to the membranous labyrinth (Smith et al., 1992).

However, in 1983, based on anatomic and electrophysiologic data from animals, a classification system had been proposed (Steel & Bock, 1983). Based on this, a study was undertaken in 1992 to establish the histopathologic findings in temporal bones stored in temporal bone libraries in England. From these, 42 suitable temporal bones were selected for study covering the following disorders: Jervel and Lange-Nielsen

syndrome, Deaf-mutism, Scheibe malformation, Refsum disease, Hereditary nephritis and hyperprolinemia, Hunter's syndrome, Pendred's syndrome, Alport's syndrome, Treacher Collins syndrome, Ataxia (Friedreich-like), unspecified congenital deafness, and Klippel-Feil syndrome. For these, histopathologic features have been reported (Marchant et al., 2004, Smith et al., 1992). Histopathology has also been ascertained in Usher's syndrome, Waardenburg's syndrome, MELAS, Mohr-Tranebjaerg syndrome and DFNA9 (Marchant et al., 2004, Smith et al., 1992).

3.1.4 Molecular Phenotypes in Syndromic Genetic Disease

With advances in molecular genetics, molecular definition of a syndrome became possible. Molecular findings have clarified the picture of a syndrome, and what actually constitutes the core phenotype and its variants. As more information has become available, a merger and splitting of syndromes has occurred over time. In some cases, syndromes have even disappeared altogether (Lindeman-Kusse et al., 1996). The problem of understanding phenotypic variability has not been completely resolved by molecular classification alone. Many allelic mutations have been associated with phenotypic diversity, modifier genes have been identified and mutations in different genes have been found to cause similar phenotypes (Romeo et al., 1994; Biesecker, 1998; Resendes et al., 2001; Smith & van Camp, 2005). Examples of such genes include *PAX3*, *SOX10* and *MITF* associated with WS (Tassabehji et al., 1993; Farrer et al., 1994; Liu et al., 1995), *COL4A5*, *COL4A3* and *COL4A4* which are involved in Alport syndrome and *COL11A2* and *COL2A1* which are involved in Stickler syndrome.

3.1.5 The Human Genome Project

In the year 2000, it was announced that most of the human Genome had been sequenced. This marked a new direction in the field of human genetics. With this came a great amount of genetic information about the human body. This information carries with it profound consequences on the future of human medicine as a discipline, as well great potential for abuse.

It had taken many years to reach this point. Although Mendel's laws had been around since 1865 (Mendel, 1865), it was not until 1900 that Garrod recognised their importance in the diseases of inborn errors of metabolism in man (Garrod, 1900). The next major breakthrough came with Crick and Watson's discovery of the structure of DNA in 1953, linking it to the genetic code and its role in heredity (Watson & Crick, 1953). It took another 15 years before the role messenger RNA in translating the genetic code to protein was unraveled.

Although sequencing of DNA was not fully perfected until Sanger (1977), and Maxam and Gilbert (1977) established the techniques of DNA sequencing that are still in use today, important steps had already been taken with the revolution of recombinant DNA technology in the 1970s. From 1980, various DNA markers were used for linkage analysis of human disorders (Botstein, White, Skolnick, & Davis, 1980). The first major success came in 1983 with the mapping of the Huntington's disease gene to chromosome 4 (Gusella et al., 1983). During this time, scientists in the US Department of Energy and others proposed an organized effort towards the sequencing of the whole human genome as a way forward from linkage of markers to actual identification of gene loci. This seemingly impossible task met with resistance

even among many researchers in the scientific field. The combined support of some members of the National Academy of Sciences and the US congress led to the establishment of the Human Genome Project (HGP) by the National Institutes of Health and the Energy Department in 1990 (US Dept of Health, 1990).

It was an international project from the outset, with the involvement of countries like Britain, China, Canada, France, Germany, and Japan. The HGP was not only to map and sequence all of the human genome, but also undertook to handle the simpler model organisms such as yeast, bacteria, the fruit fly and the round worm. The ethical, legal and social aspects related to this work and the information generated were also explored and dealt with from the beginning. The HGP was also mandated to avail all map and sequence data into the public domain (US Dept of Health, 1990; Collins & Galas, 1993; Collins et al., 1998).

Massive funding enabled the development of new technology and instrumentation such as capillary sequencing machines. Although the plan sought to complete all sequencing by 2005, the complete sequencing of many yeast and bacterial organisms was done by 1996. In June of 2000, the sequencing consortium announced that they had completed the working drafts of the human genome. Great advances were made in the identification of genes involved in single gene disorders, from 10 in 1990 to over 100 by 1997, because of the availability of genetic and physical maps (Collins, 1995).

The development of the field of genomics thus resulted in the development of high-resolution genetic and physical maps, and in the construction of both genomic and

cDNA libraries, availing the sequence databases of many species (Keats & Berlin, 1999), and so providing tools for finding genes for hearing loss.

3.1.6 Research using the mouse as a model for human deafness

The mouse as a model for human deafness in the early 1990's was a major breakthrough in the study of genetic deafness (Steel & Brown 1994; Steel, 1995). Its major advantage as a deafness model was its capacity to breed very large families in a short period of time, unlike man who has a longer lifespan. This enabled researchers to study many generations of mice at a time. The mouse was also found to share a large number of genes with man, as well as exhibit hearing loss with phenotypes similar to man. Studies on mice identified similarities between deafened mice and human deaf subjects (Avraham et al., 1995). The pathology of both human and mutant mice genetic deafness was found to be similar in many aspects. For example, mutant mice exhibited neuroepithelial defects, the commonest deficit in human genetic deafness (Avraham et al., 1995). The *Whirler* mouse had pigmentary defects and malformations of digits, while the *Shaker-1* mouse had associated vertigo or imbalance, features exhibited by patients with Waardenburg's syndrome and Usher's syndrome respectively.

For all of the above reasons, the mouse was adopted as a model for the study of human genetic deafness, both the syndromic and non-syndromic forms. By identifying the human analogue of mouse deafness for the gene or loci to be identified, human loci for deafness could be searched for. The *Shaker-1* mutant mice were studied using a positional cloning approach. Researchers identified an area of mutation that was mapped to chromosome 7 (Gibson et al., 1995). Three mutated

alleles were found located in this region. This gene was subsequently shown to code for an unconventional myosin, Myosin VII. By linking the findings to humans, research teams were able to identify human homologues. The screening of DNA from a large consanguineous family from Tunisia with profound non-syndromic profound hearing loss led to the mapping of the DFNB2 locus on chromosome 11q13.5 (Guilford et al., 1994). Humans with Usher syndrome type 1B have similar neural epithelial defects as these mice. DNA from persons with this syndrome was screened and mutations were identified in the chromosomal region 11q13, the site of one of the genes for Usher type 1B, *USH1B*. This confirmed that both human and mice deafness were caused by mutations in the same gene.

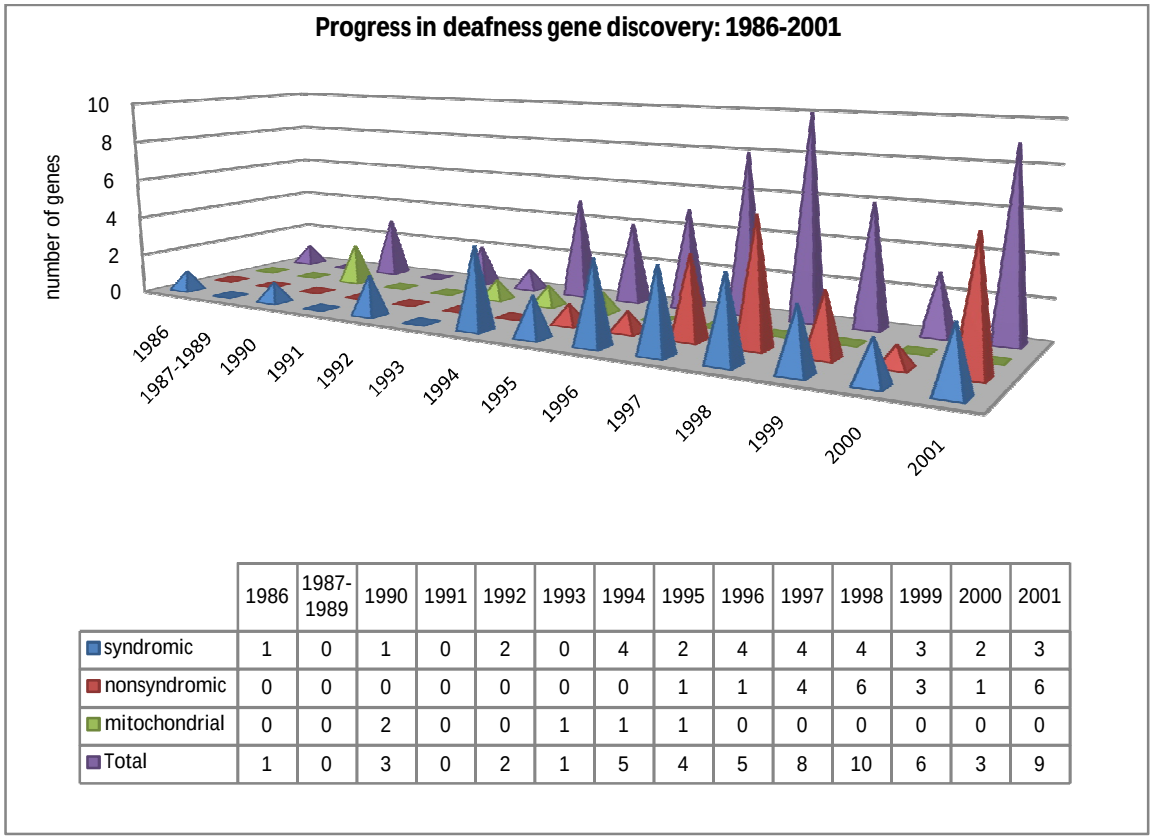
Weil et al. (1995) proposed that a defective Myosin VIIA might also be responsible for DFNB2. This was based on mapping data as well as on similarities between the phenotypes of DFNB2-affected individuals and *Shaker-1* mouse mutants. Sequence analysis of the coding axons of myosin VIIA gene (*MYO7A*) was undertaken in the DFNB2-affected family. In the last nucleotide of exon 15, an A to G transition was detected, a type of mutation known to decrease the efficiency of splicing. These results showed that the different mutations in *MYO7A* result in either an isolated (non-syndromic) or a syndromic form of deafness (Weil et al., 1995).

It was not until 1994 that the first successful linkage study of autosomal recessive non-syndromic hearing loss was reported (Gibson et al., 1995; Weil et al., 1995). Three years later, in 1997, the first recessive deafness genes, *MYO7A* and *GJB2*, were identified (Liu et al., 1997; Kelsell et al., 1997). The identification of genes for

deafness progressed very rapidly between 1994 and 2001, as can be clearly seen in figure 3.1.

Up until 1994, only one autosomal non-syndromic deafness locus was known, DFNA1. It had been identified in a single large Costa-Rican family expressing dominantly inherited deafness (Leon et al., 1992). On the human genome, only three gene loci implicated in nonsyndromic hearing loss had been mapped (Willems, 2000). By 1996, twenty two autosomal loci, eleven recessive and eleven dominant, had been discovered (Steel et al., 1996). Most of these had been found by using very large deaf families in relatively isolated communities. These types of families were found to be ideal for single gene mutations. Their large size also made a linkage analysis approach feasible. Again, by 1996, three mitochondrial mutations, A1555G, T7445C, and 7445insC, as well as three X-linked loci associated with non-syndromic hearing loss had been identified (Steel et al., 1996; de Kok et al., 1995; Tassabehji et al., 1995; Lalwani et al., 1994; Prezant et al., 1993; Reid et al., 1994a; Tiranti et al., 1995).

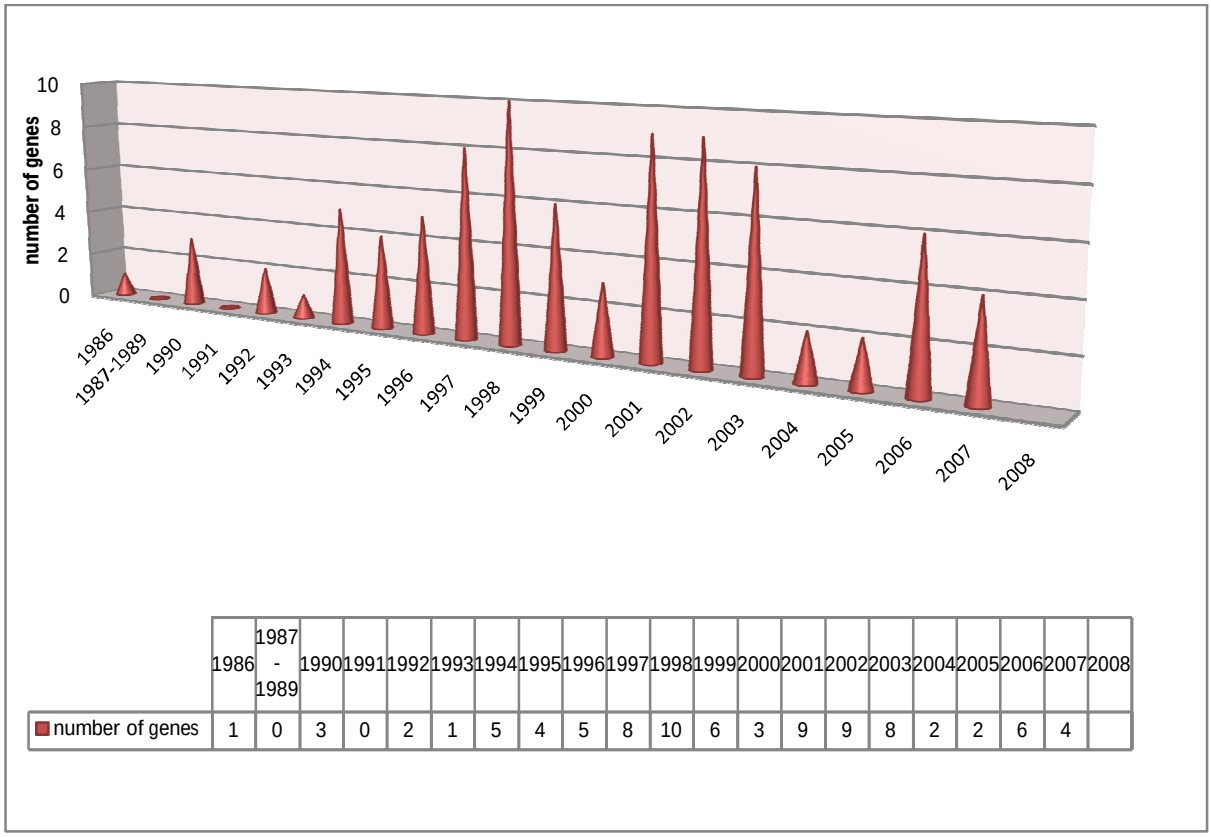
By 2001, more than 80 loci for non-syndromic hearing loss had been localized, while genes causing more than 23 non-syndromic and 30 syndromic forms of hearing loss had been cloned (Petit et al., 2001). With regard to non-syndromic hearing loss, 30 autosomal recessive, 29 autosomal dominant, and 8 X-linked loci had been identified. There were also two mitochondrial mutations, A1555G and A7445G, which had been implicated in non-syndromic recessive hearing loss. The two other mitochondrial mutations, A3243G, and A7511C, had been associated with syndromic types of hearing loss.



	1986	1987-1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
syndromic	1	0	1	0	2	0	4	2	4	4	4	3	2	3
nonsyndromic	0	0	0	0	0	0	0	1	1	4	6	3	1	6
mitochondrial	0	0	2	0	0	1	1	1	0	0	0	0	0	0
Total	1	0	3	0	2	1	5	4	5	8	10	6	3	9

Figure 3.1a: The progress of deafness gene discovery from 1986 to 2001

By 2003, the corresponding mutated gene of at least 35 of the loci mapped for non-syndromic genetic hearing loss had been identified (van Camp 2003 http://dnalab-www.uia.ac.be/dnalab/hhh). In September 2004, the Hereditary Hearing Loss Homepage posted 96 loci for nonsyndromic hearing loss, 51 dominant, 39 recessive and 6 X-linked, as well as 42 deafness genes, 20 for dominant, 21 for recessive and 1 for X-linked nonsyndromic hearing loss respectively. By the end of 2007, a total of 88 deafness genes had been identified (Smith and van Camp, 2009) (Fig. 3.1b).



	1986	1987 - 1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
number of genes	1	0	3	0	2	1	5	4	5	8	10	6	3	9	9	8	2	2	6	4	

Figure 3.1b: Total number of deafness genes identified annually from 1986-2007

3.2 GENE LOCALIZATION AND AUDITORY RESEARCH

3.2.1 Genes implicated in Hearing Loss

As noted, genetic hearing loss exhibits great heterogeneity (Steel & Bock, 1983; Gorlin, 1995). Considering the complexity of the hearing organ, this is to be expected. The ear has over one million moving parts and numerous cell types (Kenneson et al., 2002). It also has many specialized compartments which link intricately to function as one unit. All these units must function in a coordinated manner if normal sound perception and interpretation is to be achieved. This also involves events at cellular, chemical, electrical and molecular level (Morton and Nance, 2006; Kenneson et al., 2002). Failure of function in any one of these areas will lead to hearing loss of

varying degrees. As has been shown in linkage studies, mutations in any one of a number of genes may lead to hearing loss (Van Camp et al., 1997; Kenneson et al., 2002).

There are over fifty genes expressed in the ear (Morton and Nance, 2006). These genes encode a wide variety of proteins necessary for the normal development and function of the inner ear (Morton and Nance, 2006; Resendes et al., 2001). Some act as adhesion molecules (cadhedrin 23), others form gap junction proteins (connexin 26, connexin 30, connexin 31, connexin 43). Many form components of the cytoskeleton (diaphanous 1, espin) and extracellular matrix (cochlin, ∝2(XI) collagen, otoanchorin, ∝-tectorin), and ion channels and transporters (*KCNQ4*, *Pendrin*). The enzymes include TMPRSS3, the integral membrane proteins TM1, TMIE, and macromolecular organizers harmonin. Other proteins are the molecular motors including myosin IIA, myosin VI, myosin VIIA, myosin XVA. Some of the proteins play a role in synaptic function (otoferlin), tight junctions (claudin 14), and as transcription regulators (*EYA4*, *POU3F4*, *POU4F3*, *TFCP2L3*). Still others are crucial in translation, including 12SrRNA, and tRNA-Ser (UNC). The function of many other proteins, such as μ-crystalline, DFNA5, stereocilin and Wolframin, is still to be determined (http://dnalab-www.uia.ac.be/dnalab/hhh).

3.2.2 General functional classification of deafness genes

The various deafness genes and their proteins can be functionally grouped into five clear categories according to their target areas, namely, hair cell structure, extracellular matrix, ion homeostasis, transcription and miscellaneous (Resendes et al., 2001).

3.2.2.1 Genes controlling Hair cell structure

Many conditions characterized by disarray and/or fusion of the stereocilia also cause sensorineural hearing loss. These range from congenital malformations to environmental insults such as acoustic trauma. Maintenance of the intricate nature of the auditory sensory epithelium is a fundamental role of a number of genes for deafness.

The unconventional myosin genes, *MYO7A* (Liu et al., 1997) and *MYO15* (Wang et al., 1998) are known to play a critical role in the structural integrity of the stereocilia (Friedman et al., 1998). Mutations in these genes are implicated in Usher syndrome type 1B (Weil et al., 1997), DFNB2 (Liu et al., 1997b; Weil et al., 1997), DFNA11 (Liu et al., 1997c) and DFNB3 (Wang et al., 1998). Likewise, mutations have been demonstrated in *CDH23*, implicated in Usher syndrome type 1D (Boltz et al., 2001; Bork et al., 2001), in DFNB12 (Bork et al., 2001), and in *PCDH15*, implicated in Usher syndrome type 1F (Kumar et al., 2001). MYO6 is the other myosin gene in which mutations lead to nonsyndromic autosomal dominant hearing loss characterized by disarray and fusion of stereocilia (Melchionda et al., 2001). It accounts for DFNA22 (Melchionda et al., 2001) in humans, and, in mice, Snell's waltzer mouse (Melchionda et al., 2001; Self et al., 1999).

3.2.2.2 Extracellular matrix genes

These genes code for proteins such as collagens, usherin, prestin and α-tectorin. Examples of defective proteins linked to genetic hearing loss abound. For example, mutations in *COL1A2* lead to the production of defective collagen, resulting in osteogenesis imperfect and syndromic hearing loss (Sykes et al., 1986). Mutations in

USH2A lead to defective usherin and Usher syndrome type 2A (Eudy et al., 1998). Mutations in *TECTA* lead to defective α-tectorin, the tectorial membrane protein, causing both a dominant, DFNA8/12 (Verhoeven et al., 1998), and recessive, DFNB21 (Mustapha et al., 1999) nonsyndromic hearing loss. Prestin, the cochlear motor protein (Dallos, 1992; Zheng et al., 2000; Dallos & Fakler, 2002) was shown to be defective in mutations involving the *Prestin* gene (Liu et al., 2003).

3.2.2.3 Genes controlling ion homeostasis

The critical nature in composition of the cochlear fluids and their strict compartmentalization within the cochlear is controlled by numerous gap-junctions and ion channels. These are regulated by different genes which when mutated lead to hearing loss. Among the key genes are *GJB2*, encoding Connexin 26, a gap junction protein responsible for up to 50% nonsyndromic hearing loss in some populations (Rabionet et al., 2000), *GJB6* (Grifa et al., 1999), encoding Connexin 30, which combines with Connexin 26 to form form heteromeric gap junctions in the cell membrane that function as an integral component of the potassium regulation in the inner ear, potassium channels KCNQ4 (Kubisch et al., 1999) and KCNE1 (Tyson et al., 1997), as well as *CLDN14*, which codes for a tight junction protein believed to regulate compartmentalization of endolymph (Wilcox et al., 2001).

3.2.2.4 Genes controlling transcription factors

Transcription factors regulate critical biological pathways in the body, including the auditory system. For example, a pathway that has been identified as essential for embryonic development is that controlled by the *EYA* genes. Mutations in *EYA1* lead to the bronchio-oto-renal (BOR) and branchio-otic (BO) syndromes, while mutations

in *EYA4* lead to DFNA10. *EYA4* is expressed and functions within the mature organ of Corti (Wayne et al., 2001).

Another pathway of interest, especially to the current study, is the interaction between *PAX3*, *MITF* and *SOX10*, the genes involved in the different types of Waardenburg syndrome. *MITF*, the primary regulator of melanocyte development, when interrupted, leads to disrupted pigmentation and hearing loss (Tachibana et al., 1996). *SOX10* and *PAX3* have been shown to synergistically transactivate *MITF*. In pathological mutations of these two genes (*SOX10* and *PAX3*), their binding to and induction of the *MITF* promoter are interfered with (Bondurand et al., 2000; Potterf et al., 2000).

3.2.2.5 Miscellaneous genes

This group contains the pool of genes which do not fit in any of the above groups, or whose function has not been fully elucidated. Genes from this group would be reclassified once their function is clarified.

The expression of genes implicated in hearing loss varies (Morton and Nance, 2006; http://dnalab-www.uia.ac.be/dnalab/hhh). Gene expression is related to the protein produced by the gene, and its function in the hearing mechanism (Cohen-Salmon et al., 2002). Different genes are preferentially expressed, upregulated and down regulated in the ear at every stage of development as well as in response to stressor factors in the ear. Defects in the genes may cause loss of function effect or early apoptosis (Cohen-Salmon et al., 2002). The expression and proposed functions of some of the known genes in the ear is summarized in figure 2.10 and table 3.1.

Major and exciting advances are continuing on this front. One such advance has been the development of transgenic and knockout mice. By breeding knockout mice, researchers were able to observe the effect of the defective gene on the mouse. Initially, an attempt was made to breed Cx26 knockout mice in the hope that in vivo studies would clarify the role of Cx26 in hearing function. These studies were however hampered because these knockout mice died early in utero. Researchers (Cohen-Salmon et al., 2002) were finally able to carry out targeted ablation of Cx26 in two specific cellular networks of the inner ear, producing the homozygous mutant mice, $Cx26^{OtogCre}$. Their results showed that these mice were born with hearing loss but normal vestibular function. Although the inner ears developed normally, cell death appeared from the 14^{th} postnatal day starting with the supporting cells of the inner hair cells (IHCs), and then extended through the entire cochlear epithelial network and sensory hair cells.

3.2.3 Overview of Connexins (Cx) and the Gap Junctional Systems of the Ear

Connexins are gap junction proteins. A group of six connexins forms a hexamer, called a connexon, and defined as "a specialized intercellular structure surrounding a pore" (Pazmekas et al., 2003). Two connexons of adjacent cells come together to form an intercellular gap junction. These gap junctions, when open, provide a low-resistant pathway for the exchange of small ions and signaling molecules that control physiologic and developmental processes of the ear (Cohen-Salmon et al., 2002; Kikuchi et al., 2000; Holt et al., 1999; Steel et al., 1999; Kumar et al., 1996).

To date, mutations in three of the connexin genes, *GJB2* (Cx26), *GJB6* (Cx30) and *GJB3* (Cx 31) have been confirmed as resulting in sensorineural hearing loss (Kelly et

al., 1998; Xia et al., 1998; Rabionet et al., 2000; Goodenough et al., 1996, Grifa et al., 1999). Connexin 26 (Cx26) and Connexin 30 (Cx30) are present in both epithelial and connective tissue gap junctional systems in the ear (Holt et al., 1999; Kikuchi et al., 2000) while Cx31 is found in type II fibroblasts. Cx 26 and Cx 30, combine to form heteromeric gap junctions in the cell membrane, which function as an integral component of the potassium regulation in the inner ear (Kumar and Gilula 1996; Holt and Corey 1999; Steel and Bussoli 1999; Kikuchi, Adams et al. 2000).The major effect of mutations in their genes is believed to be due to failure of correct ionic homeostasis of the inner ear fluids (Cohen-Salmon et al., 2002; Holt et al., 1999; Steel et al., 1999; Kumar et al., 1996; Goodenough et al., 1996; Kikuchi et al., 2000; Rabionet et al., 2000).

The most common mutation in *GJB6* is a 342-kb deletion, *GJB6*-D13S1830, which causes NSHL when homozygous, or when present on the opposite allele of a *GJB2* mutation. One study investigated allele specific *GJB2* expression using RNA extracted from buccal epithelium in three unrelated compound heterozygotes, each with the *GJB6*-D13S1830 deletion and a different variation in *GJB2* (Rodriguez-Paris J, Schrijver I. 2009). The results revealed a lack of *GJB2* expression where the *GJB2* mutation was carried on the same allele as the *GJB6*-D13S1830 deletion. On the other hand, *GJB2* was expressed when the *GJB6*-D13S1830 deletion was found on the opposite allele to that carrying the *GJB2* mutation. This clearly demonstrated a cis-regulatory section within the deleted region of *GJB6*. The study confirmed that a digenic mechanism of inheritance was at play in individuals homozygous for the *GJB6*-D13S1830 mutation while a cis-regulatory mechanism, leading to a lack of *GJB2* mRNA expression, operated where the *GJB6*-D13S1830 deletion occurred in cis with a *GJB2* mutation.

Table 3.1: Gene expression in the human ear

Gene Name	Chromosomal Location	Type of product	Expression
HDIA 1	5q31	Cytoskeletal protein	Inner ear
GJB3	1p34	Gap junction component	Supporting cells, spiral ligament
GJB2 (cx26)	13q12	Gap junction component	Supporting cells, spiral ligament
GJA1 (cx43)	6q21-q23.2	Gap junction component	Non-sensory epithelial cells, Type I fibrocytes in spiral ligament and spiral limbus
GJB6	13q12	Gap junction component	Supporting cells, spiral ligament
KCNQ4	1p34	Channel component	Outer hair cells
DfN5	7p15	Organ of Corti	Stria vascularis
TECTA	11q22-q24	Extracellular	Inner sulcus and Hensen's cells during development; tectorial membrane
COCH	14q12-q13	Extracellular	Chick equivalent of spiral ligament, limbus, stroma below maculae and cristae
EYA4	6q22-q23	A member of vertebrate	EYA family of transcriptional activators, Neuroepithelia of developing inner ear
MYO7A	11q12.3-q21	Motor molecule	Hair cells
COL112A	6p21	Collagen protein	Tectorial membrane
MYO15	17p11.2	Motor molecule	Inner ear
POU3F4	Xq21.1	Transcriptional factor	Mesoderm around the otic vesicle, lateral wall
PUO4F3	5q31	Transcriptional factor	Hair cells
MYH9	22q12.2-q13.3	Non-muscle myosin	Organ of Corti, subcentral region spiral ligament, Reissner's membrane
PDS	7q31.1	Ion transporter	External sulcus region, cells adjacent to maculae, endolymphatic duct and sac
TMPRSS3	21q22.3	A new transmembrane serine protease	Inner ear
OTOF	2p23.1	Vestibular type I hair cells	Inner hair cells, Outer hair cells during development
CDH23	10q21-q22	Cadhelin-like protein	Cochlea
CLDN14	21q22.3	Express protein components of tight junctions	Sensory epithelium of the Organ of Corti
DDP	X22.1	Deafness/Dystonia peptide (involved in neurological development)	Widespread (but mainly in brain)
12SrRNA	**	Mitochondrial protein	Widespread
TRNASer (UCN)	**	Mitochondrial protein	Sensory epithelium of the Organ of Corti
HDIA 1	5q31	Cytoskeletal protein	Inner ear
GJB3	1p34	Gap junction component	Supporting cells, spiral ligament
GJB2 (cx26)	13q12	Gap junction component	Supporting cells, spiral ligament
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KCNQ4	1p34	Channel component	Outer hair cells
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EYA4	6q22-q23	A member of vertebrate	Eya family of transcriptional activators, Neuroepithelia developing inner ear
MYO7A	11q12.3-q21	Motor molecule	Hair cells
COL112A	6p21	Collagen protein	Tectorial membrane
MYO15	17p11.2	Motor molecule	Inner ear
POU3F4	Xq21.1	Transcriptional factor	Mesoderm otic vesicle, lateral wall
PUO4F3	5q31	Transcriptional factor	Hair cells

Gene Name	Chromosomal Location	Type of product	Expression
MYH9	22q12.2-q13.3	Non-muscle myosin	Organ of Corti, subcentral region spiral ligament, Reissner's membrane
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TMPRSS3	21q22.3	A new transmembrane serine protease	Inner ear
OTOF	2p23.1	Vestibular type I hair cells	Inner hair cells, Outer hair cells during development
CDH23	10q21-q22	Cadhelin-like protein	Cochlea
CLDN14	21q22.3	Express protein components of tight junctions	Sensory epithelium of the Organ of Corti
DDP	X22.1	Deafness/Dystonia peptide (involved in neurological development)	Widespread (but mainly in brain)
12SrRNA	**	Mitochondrial protein	Widespread
TRNASer (UCN)	**	Mitochondrial protein	Sensory epithelium of the Organ of Corti

Source: http://dnalab-www.uia.ac.be/dnalab/hhh 2005

3.2.4 Gap junction Gene Variants and Hearing Loss

As early as 1999 (Denoyelle et al., 1999), it was noted that *GJB2* mutations or variants led to hearing impairment of great variability, ranging in severity from mild to profound. From published studies carried out (Liu & Xu, 1994; Denoyelle et al., 1999), it would seem that the hearing impairment due to *GJB2* variations is not only highly variable but is also independent of type of mutation or variation. Researchers have proposed that this may be due to modifying genes and environmental factors. Functional studies of gap junction molecules have shown consistently that the connexin hexons form homomeric, and hexamerix hemichannels, and that these have different molecule transfer capabilities depending on the type of mutation or gene variation (Kikuchi et al., 2000).

3.2.5 GJB2 Mutations and Hearing Loss: Phenotype-Genotype Relationship

A survey of published studies on *GJB2* associated hearing impairment confirms the heterogeneity of this disorder. Not only does it vary among individuals but also among family members. Kenneson (2002) analyzied 22 studies that reported on

sequence variations in the *GJB2* locus and found that the observed variations occured in different proportions among the different hearing impaired population groups, 43% in Israel, 20% in Japan, 20% among Caucasians of northern European descent, 17% in Tunisia, 14% in Australia, and 5% in Korea.

The phenotype due to *GJB2* mutations seems to be variable and independent of mutation, with variation in type, degree and severity of hearing loss (Cohn 1999) but no consistent audiologic phenotype. The degree of hearing loss may range from mild to moderate in a few of cases, but is mainly severe to profound (Liu et al., 1994; Denoyelle et al., 1999; Mueller et al., 1999). Generally stable hearing losses, with fewer cases of progressive hearing loss, have been reported (Mueller et al., 1999). A case with sudden recurrent sensorineural hearing loss was reported in Austria (Janecke et al., 2002). Dominant *GJB2* mutations can be associated with a mild to profound, often progressive hearing loss associated with skin disorders (Heathcote et al., 2000; Richard et al., 1998; Maestrini et al., 1999; van Geel et al., 2002). Patients with severe to profound prelingual onset hearing loss may also demonstrate marked variability in degree of hearing loss (Denoyelle et al., 1999).

3.2.6 GJB2 Mutations and Type of Hearing Loss

It has been shown that up to 50% of recessive nonsyndromic hearing loss in Caucasian and European populations may be due to mutations in *GJB2* gene. However, *GJB2* mutations have also been shown to cause both syndromic and non-syndromic deafness (Denoyelle et al., 1999; Kelsell et al., 1997). Some of the clinical features associated with syndromic *GJB2* mutations include palmoplanter keratoderma (Richard et al., 1998; Heathcote et al., 2000), Vohwinkel syndrome

(Maestrini et al., 1999), and keratitis-ichthyosis/hystrix-like ichthyosis-deafness (KID-HID) (Richard et al., 1998). Mutations in other connexin genes may also cause deafness such as *GJB1* (Cx32) which is also responsible for the X-linked Charcot-Marie-Tooth disease, *GJB3* (cx31) which has been shown to be involved in both deafness and a skin disease, *GJA1* (Cx43) shown to cause a dominant conductive hearing loss, and *GJB6* (Cx30) causing a dominant type of deafness.

A number of *GJB2* mutations have been implicated in dominant deafness (Denoyelle et al., 1999; Morle et al., 2000; Tekin et al., 2001). The *GJB2* mutation M34T has been described as a cause of dominant hearing loss, autosomal recessive hearing loss and a polymorphism (Cucci et al., 2000; Houseman et al., 2001), highlighting the difficulty presented by some of the gene variations. It is currently believed that M34T is likely to have low penetrance but is also affected by other factors in the body in its effect on hearing function (Smith and van Camp, 2005).

3.2.7 Waardenburg syndrome

Waardenburg syndrome (WS) is believed to be the commonest type of autosomal dominant syndromic hearing loss, accounting for approx. 1-2% of all cases of congenital deafness (Smith and van Camp, 2005; Fraser, 1976). It has a worldwide distribution, is known to affect all racial groups, and has been shown to have variable expression and penetrance (Smith and van Camp, 2005; Tassabehji et al, 1995; Liu et al 1995a, & b; Farrer et al, 1994; Tassabehji et al, 1994; Fraser, 1976). The degree of penetrance for profound sensorineural hearing loss is estimated at 0.20, with a mutation rate estimated at 0.5 per 100,000 gametes (Fraser 1976).

WS forms part of the auditory-pigmentary syndromes characterized by hearing loss and abnormal pigmentation of the skin, hair, and eye (Tassabehji et al, 1995). Early reports did not recognize any distinction between types (Fisch 1959, Partington 1964, Goldberg 1966, Reed et al. 1967). First described in 1951 by the geneticist Waardenburg (Waardenburg, 1951), the disorder was later phenotypically classified into types I and II based on the presence or absence of dystopia canthorum (Arias, 1971). As more information and associations with the disorder were made, WS was phenotypically re-classified as Types I, II, III and IV, based on the recommendations of the Waardenburg Consortium and others (Farrer et al., 1994; Liu et al., 1995; Attie et al, 1995; Edery et al, 1996; Pingault et al, 1998; Sanchez-Martin et al, 2002; Selicorni et al, 2002). This classification depends on the presence of a number of clinical features and signs, including dystopia canthorum (lateral displacement of the inner canthi), sensorineural hearing loss and heterochromia irides.

3.2.7.1 Clinical features of Waardenburg Syndrome

The classical clinical features of Waardenburg Syndrome include:

- Lateral development of the inner canthi
- Dystropia of lacrimal punctum
- Horizontal shortening of the palpebral fissures
- Prominent broad nasal root
- Hypertrichosis of the eyebrows
- White forelock
- Heterochromia irides
- Sensorineural hearing loss (any degree)
- Patchy depigmentation of skin (best seen under UV light)

- Synophyris
- Hypoplastic irises

Other reported associated clinical features of Waardenburg Syndrome include:

- Cleft lip
- Cleft palate
- Both cleft lip and palate
- High arched palate
- Changes in iris pigmentation during 1st year of life
- Hirschsprung disease
- Premature graying of hair (before 30yrs age)
- Absent vestibular response
- Pigmentary heterochromia of the fundus
- Disappearance of white forelock after 1st year life
- Dacrocystitis
- Isochromic pale irides
- Mild facial dysmorphism
- Hypoplastic alar nasi

Embryologically, the developing neurocrest cells migrate to different tissues including:

a) the Cochlea: stria vascularis
b) Eye: corneal endothelium and iris
c) GIT: colon
d) Adrenals: the medulla

The underlying pathology of the auditory-pigmentary syndromes is failure of melanocytes to either a) differentiate in the embryonic neurocrest or b) migrate from the neurocrest to the correct final location or c) survive after migration (Steel & Barkway, 1989; Tassabehji et al., 1995; Tachibana et al., 1996; Bondurand et al., 2000; Potterf et al., 2000) The number of pigmentary syndromes and conditions associated with hearing loss include piebaldism with deafness and ataxia, piebaldism with deafness, vitiligo with deafness and achalasia, vitiligo with deafness, albinism with deafness, Hirschsprung's disease with deafness and heterochromia irides, Waardenburg syndrome and many others. They are classified under the London Dysmorphology Database and OMIM (Tassabehji et al., 1995).

Pigmentary disorders are functionally classified into three groups (Steel & Barkway, 1989; Steel et al., 1996 Tassabehji et al., 1995):

1. Melanocytes are present but unpigmented e.g. Albinism
2. Localized absence of melanocytes e.g Waardenburg syndrome Type II, Piebaldism
3. Generalized neurocrest dysfunction e.g. Waardenburg Syndrome Type I, Hirschsprung's disease with hearing loss and pigmentary disturbance.

Studies on humans and mice with auditory-pigmentary defects have demonstrated that pigment cells, located on the lateral wall of the cochlear duct, play a crucial role in the functioning of the stria vascularis (Steel & Barkway, 1989, Steel et al., 1996). The stria vascularis is the power source or battery for the sensory hair cells in the organ of Corti (Steel et al., 1996). All cases of hearing loss associated with pigmentary defects

have demonstrated variable expression indicating that there may be modifying genes involved in these disorders (Tassabehji et al., 1995).

The number of genes identified as mutated in conditions where deafness is associated with pigmentary defects include *PAX3* for Waardenburg syndrome Type I and Type III (Tassabehji et al., 1995; Baldwin et al., 1992), *MITF* in Waardenburg's syndrome Type II (Tassabehji et al., 1995), *EDNRB*, an endothelin-B receptor gene (Puffenberger et al., 1994; Attie et al., 1995) and *EDN3*, an endothelin-3 gene (Edery et al., 1996; Hofstra et al., 1996), both of which are implicated in aganglionic megacolon associated with hearing loss and Shah-Waardenburg syndrome (table 3.2). However, studies have shown the *MITF* gene to be mutated in only 20% of the patients with Waardenburg syndrome Type II, indicating that there is another unidentified gene involved (Tassabehji et al., 1995).

MITF is now known to be the primary regulator of melanocyte development in the embryo and when mutated, leads to disrupted pigmentation and hearing loss (Tachibana et al., 1996). *SOX10* and *PAX3* have been shown to synergistically transactivate *MITF* (Bondurand et al., 2000; Potterf et al., 2000). In pathological mutations of these two genes (*SOX10* and *PAX3*), there is resultant interference with their ability to bind to *MITF*, thus affecting induction of the *MITF* promoter (Bondurand et al., 2000; Potterf et al., 2000).

3.2.7.2 The clinical classification of Waardenburg Syndrome

Only one type of WS was known until 1971 when types 1 and 2 were defined (Arias 1971). In 1990 genetic linkage mapped the gene for WS to chromosome 2q (Foy et al

1990) but it was not until 1992 that identification of WS Type 1 mutations in the *PAX3* gene was achieved (Tassabehji et al 1992). In 1994 WS type 2 was mapped to a gene on chromosome 3p (Hughes et al 1994). In the same year, the *MITF* gene was cloned and mapped to chromosome 3p14.1-p12.3 (Tachibana et al 1994). In 1995 *EDNRB*, identified as causing WS type IV, was mapped to chromosome 13q22 (Attie et al., 1995). In 1996, *EDN3* was identified as one of the genes causing type IV and mapped to chromosome 20q13.2-q13.3 (Edery et al., 1996), while *SOX10*, another gene implicated in WS type IV was mapped on chromosome 22q13 (Pingault et al, 1998) (table 3.2). In 2002, *SNAI2* was mapped to chromosome 8q11 and implicated in WS type 2D (Sanchez-Martin et al, 2002).

Table 3.2 Classes and genes identified for Waardenburg Syndrome.

WS type	WS name	Locus	Gene	Reference	OMIM entry
Type I	WS1	2q35	*PAX3*	Tassabehji et al, 1992	193500
Type IIA	WS2A	3p14.1-p12.3	*MITF*	Tassabehji et al, 1994	193510
Type IIB	WS2B	1p21-p13.3	unknown	Am J Hum Genet 55 (suppl): A14, 1994	600193
Type IIC	WS2C	8p23	unknown	Selicorni et al, 2002	606662
Type IID	WS2D	8q11	*SNAI2*	Sanchez-Martin et al, 2002	608890
Type III	WS3	2q35	*PAX3*	Hoth et al, 1993	148820
Type IV	WS4	13q22	*EDNRB*	Attie et al, 1995	131244
Type IV	WS4	20q13.2-q13.3	*EDN3*	Edery et al, 1996	131242
Type IV	WS4	22q13	*SOX10*	Pingault et al, 1998	602229

Source: Hereditary Hearing loss homepage, downloaded November 2009, van Camp & Smith 2009.

The clinical features of the different classifications can be summarized as follows:

WS type1 (WSI)

- MIM 193500

- Autosomal Dominant Sensorineural hearing loss
- Heterochromia irides
- White forelock/early greying
- Dystopia canthorum (W> 1.95)
- Mild facial dysmorphism
- Maps to 2q35 *PAX3* gene

WS Type 2 (WSII)

- MIM 193510
- Audosomal dominant sensorineural hearing loss
- Heterochromia irides
- White forelock/early greying
- No dystopia canthorum (W<1.95)
- No dysmorphic features
- *MITF* gene mutations in 20% cases

WS Type 3 (WSIII)

- MIM 148820
- Upper limb abnormalities e.g. Flexion contractures hands
- Dystopia canthorum
- Sensorineural hearing loss
- Heterochromia irides
- Premature greying
- *PAX3* gene mutations

Type 4 (WSIV)

- Hirschsprung disease present
- Sensorineural hearing loss

- Early greying
- Lack of retinal pigment
- +/- hypoplastic irises
- +/- Subclinical sensorineural hearing loss
- +/- patchy leukoderma
- +/- white forelock
- *EDNRB* (MIM 131244), *EDN3* (MIM 131242), *SOX* (MIM 602229) mutations

3.2.7.3 Variable penetrance of Waardenburg syndrome

The variable penetrance of Waardenburg syndrome among affected individuals, even among family members with identical mutations, makes it difficult to diagnose WS types clinically (Liu et al., 1994; Tassabehji et al., 1995).

In an extensive study reviewing 435 cases (Hageman & Delleman, 1977), deafness was observed to occur in 36% of WS type 1 and 57% type 2 cases. A more recent study and view of the literature indicated higher figures, 57% and 58% for type 1 and 77% and 77% for type 2 WS syndrome individuals (Liu et al, 1995a; Liu et al 1995b). The latter study used dystopia canthorum as a guide to the diagnosis and differentiation of WS types 1 and 2, and also considered mild and moderate hearing losses (tables 3.4 and 3.5), which were possibly overlooked by Hageman and Delleman (1977). This would have led to a bias in ascertainment, with under-diagnosis of WS type 2 in persons without hearing loss. Liu et al (1995) found that out of 81 affected WS type 2 individuals, 62 demonstrated a congenital sensorineural hearing loss, with bilateral losses in 52, unilateral in 10, but noted that profound hearing loss was found in only 25/62 hearing impaired individuals (Liu et al, 1995).

Both intra-familial and inter-familial variation in hearing loss has been demonstrated (Newton, 1990), further compounding the difficulty in diagnosing WS type2 in normally hearing individuals. Liu et al., (1995a, 1995b) summarized their findings in tables 3.3 and 3.4.

Table 3.3: Phenotypic penetrance of selected Waardenburg syndrome traits (percentages).

	SN	HetI	HypE	WF	EG	Skin	HNR	Eyb
Type I								
Liu et al. (n=60)	58 (52)	15 (14)	15 (10)	48 (46)	38 (40)	37 (37)	100	63
Literature (n=210)	57	31	18	43	23	30	52	70
Type II								
Liu et al. (n=81)	77 (78)	44 (42)	2 (3)	28 (23)	27 (30)	4 (5)	0	5 (7)
Literature (n=43)	77	54	23	16	14	12	14	7

Key: SN = sensorineural hearing loss; HetI= heterochromia irides; HypE= hypoplastic blue eyes; WF= white forelock; EG= early graying; Eyb= bushy confluent eyebrows

Source: Liu et al, 1995

Table 3.4: Penetrance of pigmentary abnormalities in WS patients with and without hearing loss in relation to syndrome type (percentages)

	Type I		Type II	
	Normal hearing (n = 25)	Hearing loss (n = 35)	Normal hearing (n = 18)	Hearing loss (n = 67)
HetI	12	43	39	49
WF	28	60	44	21
EG	16	54	22	28
Skin pigmentation abnormalities:	36	37	6	3
At least 1	84	91	100	76
Two only	16	34	17	19
Three or more	0	31	6	2

Key: SN = sensorineural hearing loss; HetI= heterochromia irides; HypE= hypoplastic blue eyes; WF= white forelock; EG= early graying; Eyb= bushy confluent eyebrows

Source: Liu et al, 1995

Table 3.5: The degree of hearing loss and the frequency of pigmentary abnormalities in relation to syndrome type

dB HL	Frequency of pigmentary abnormalities (%)	
	Type I	Type II
30	0	67
31-60	100	69
61-100	100	64
100+	93	89

Source: Liu et al, 1995

While some studies inferred that pigmentary disturbances occurred more frequently in WS type II compared to WS type II (Saxe et al., 1984; Hildesheimer, 1989), Liu et al. (1995) were able to demonstrate that there was no significant difference, other than heterochromia irides which was found more commonly in WS type II (table 3.3 and 3.4).

An aetiological survey of 3,064 deaf children in schools for the deaf and hard of hearing in Southern Africa during the period 1975-1982 grouped the aetiologies of childhood deafness into five major categories: Genetic syndromes (n=550), Undifferentiated (n=1,749), Acquired (n=765), Cryptogenic deafness without additional anomalies (n=1,418), and Cryptogenic deafness with additional anomalies (n=331). This is summarized in figure 3.2 below. The study relied on history, clinical examination and medical records for aetiological diagnosis. No mutational screening was done at the time.

Among the identified genetic causes, syndromic hearing loss was identified in 203 individuals (7% of the cohort) while nonsyndromic hearing loss was identified in 347

individuals (11% of the cohort). Of the syndromic hearing loss group, 44% had clinically evident Waardenburg syndrome, 16% with Treacher Collins syndrome, 11% with Branchial arch syndrome, 8% with Pendred, 4.4% with Usher's syndrome, and the rest spread over rarer syndromes, making WS by far the largest aetiological factor in syndromic hearing loss among students in South African schools for the deaf during the period of the study. The current status in South Africa is unknown. The establishment of the mutational spectrum of WS in South African populations will therefore be a very important step towards both the diagnosis and genetic counselling for this disorder.

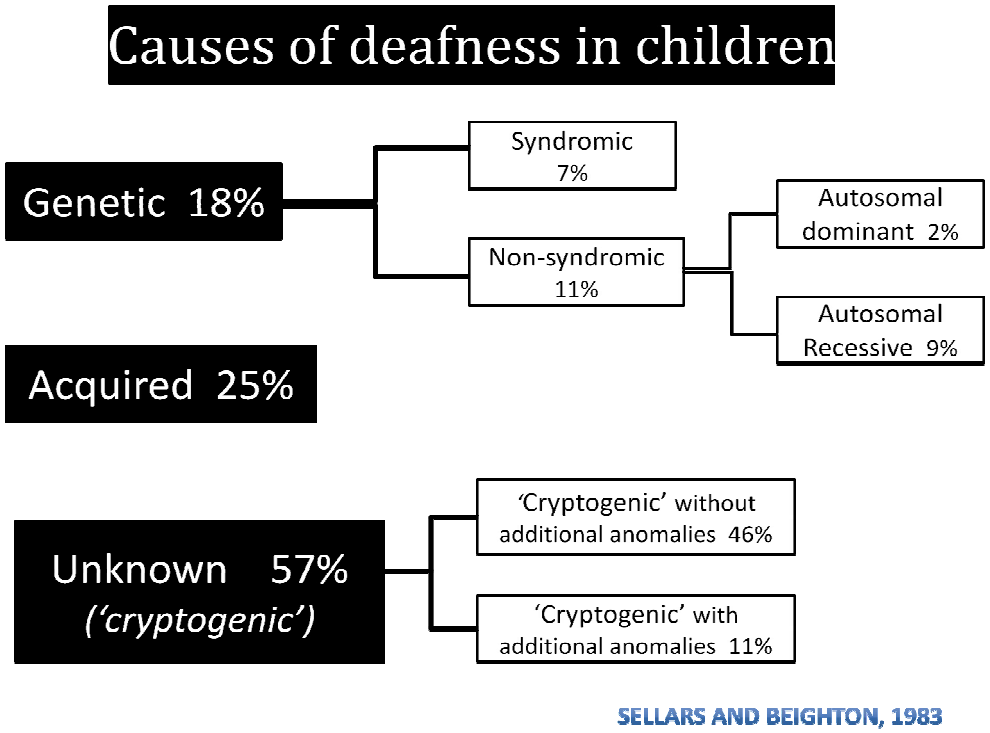


Figure 3.2 Aetiological surveys among 3,064 children in Southern Africa (Sellars et al., 1983b)

3.2.8 Mitochondrial genes

The mitochondrial genome is a 16,569 bp sized circular molecule (Reardon & Harding, 1995). It codes for 22 types of transfer RNA (tRNA), 13 mitochondrial

proteins, and 2 types of ribosomal RNA (rRNA) (Reardon & Harding, 1995; Reid et al., 1994; Fischel-Ghodasian et al., 1995; Sevior et al., 1998; Guan et al., 1998; Verhoeven et al., 199; Sue et al., 1999; Prezant et al., 1993; Fischel-Ghodasian et al., 1993; Casano et al., 1999). Through their control of apoptosis and the production of cellular energy, mitochondria are believed to cause hearing loss through different mechanisms (Reardon & Harding, 1995; Sue et al., 1999).

Mitochondrial mutations are implicated in many conditions affecting different parts of the body, leading to myopathies including cardiomyopathy, neuropathies, diabetes mellitus, retinal degeneration and hearing loss (Reardon & Harding, 1995). The hearing loss may be the only symptom, or may be progressive and occur with the classic mitochondrial disorders such as the MERRF syndrome, the MELAS syndrome, and the Kearns-Sayre syndrome (Reardon & Harding, 1995).

Nonsyndromic hearing loss due to mitochondrial mutations is uncommon, affecting two genes, the *tRNA^{Ser}* gene and the *12S rRNA* gene. These two genes carry the common mitochondrial mutations A7445G (Reid et al., 1994, Fischel-Ghodsain et al., 1995; Sevior et al., 1998; Guan et al., 1998), 7472insC (Verhoeven et al., 1999), T7511C (Sue et al., 1999), found in the *tRNA^{Ser}* gene, and A1555G (Prezant et al., 1993; Fischel-Ghodsain et al., 1993), found in the *12S rRNA* gene.

The A1555G mutation has been demonstrated in patients with aminoglycoside induced nonsyndromic hearing loss (Prezant et al., 1993; Fischel-Ghodsain et al., 1993) while another less common mutation in the *12S rRNA* gene, 961delT, has been shown to predispose the affected individuals to aminoglycoside toxicity and hearing

loss (Casano et al., 1999). Other than hearing loss alone, two of these mutations have also been demonstrated to occur with other clinical features, palmoplanter keratoderma in most patients with the A7445G mutation (Reid et al., 1994, Fischel-Ghodsain et al., 1995; Sevior et al., 1998; Guan et al., 1998), and ataxia and myoclonus in a minority of patients with the T7511C mutation (Sue et al., 1999).

3.2.9 Audiological findings in nonsyndromic genetic hearing loss

Although the types and degree of hearing loss in hereditary hearing loss varies, there are some conclusions that can be made from published literature. As a general rule, autosomal recessive hearing impairment causes prelingual and profound deafness, while autosomal dominant hearing loss causes progressive and postlingual hearing loss. The explanation for this is believed to be due the fact that in recessive disorders, there is complete absence of functional protein production, while in dominant mutations, there is initial function but due to the accumulation of pathology, hearing loss occurs subsequently (Resendes et al., 2001).

An audioprofile is a recording of several audiograms on a single graph (Smith & van Camp, 2005). Audioprofiles have been drawn for the different classes of hereditary hearing loss and some of these have been found to be useful for predicting candidate genes. For example *WFS1* has beeh shown to affect the low frequencies and spare the high frequencies in over three quarters of the patients. If a patient were to present with this type of audioprofile, the candidate genes to be considered for genetic screening would therefore include *WFS1*. These conclusions are summarized in tables 3.6 a-d below.

Table 3.6a: Audiological manifestation of the autosomal dominant nonsyndromic hearing impairment genes

Locus name	Chromosomal locus	Gene symbol	Hearing loss Onset/Decade	Audioprofile
DFNA1	5q31	*DIAPH1*	Postlingual/1st	Low frequency progressive
DFNA2	1p35.1	*GJB3*	Postlingual/2nd	High frequency progressive
	1p34	*KCNQ4*		
DFNA3	13q11-q12	*GJB2*	Prelingual	
	13q12	*GJB6*		
DFNA4	19q13	*MYH14*	Postlingual	Flat/gently downsloping
DFNA5	7p15	*DFNA5*	Postlingual/1st	High frequency progressive
DFNA6/14/38	4p16.1	*WFS1*	Prelingual	Low frequency progressive
DFNA8/12	11q22-q24	*TECTA*		Mid-frequency loss
DFNA9	14q12-q13	*COCH*	Postlingual/2nd	High frequency progressive
DFNA10	6q23	*EYA4*	Postlingual/3rd	Flat/gently downsloping
DFNA11	11q13.5	*MYO7A*	Postlingual/1st	
DFNA13	6p21.3	*COL11A2*	Postlingual/2nd	Mid-frequency loss
DFNA15	5q31	*POU4F3*	Postlingual	High frequency progressive
DFNA17	22q11.2	*MYH9*		
DFNA20/26	17q25	*ACTG1*		
DFNA22	6q13	*MYO6*		
DFNA28	8q22	*TFCP2L3*		
DFNA36	9q13-q21	*TMC1*		Flat/gently downsloping
DFNA39	4q21.3	*DSPP*		High frequency progressive
DFNA48	12q13-q14	*MYO1A*		Progressive

Adopted from van Camp & Smith, 2003; Smith and van Camp, 2006

Table 3.6b: Audiological manifestation of the autosomal recessive nonsyndromic hearing impairment genes

Locus name	Chromosomal locus	Gene symbol	Hearing loss Onset	Type
DFNB1	13q11-112	*GJB2*	Prelingual[1]	Usually stable
	13q12	*GJB6*		
DFNB2	11q13.5	*MYO7A*	Prelingual/ postlingual	Unspecified
DFNB3	17p11.2	*MYO15*	Prelingual	Stable
DFNB4	7q31	*SLC26A4*	Prelingual, postlingual	Progressive, stable
DFNB6	3p21	*TMIE*	Prelingual	Stable
DFNB7/11	9q13-q21	*TMC1*		
DFNB8/10	21q22.3	*TMPRSS3*	Postlingual[2]/Prelingual	Progressive, stable
DFNB9	1p22-p23	*OTOF*	Prelingual	Stable
DFNB12	10q21-q22	*CDH23*		
DFNB16	15q15	*STRC*		
DFNB18	11p15.1	*USH1C*		
DFNB21	11q22-q24	*TECTA*		
DFNB22	16p12.2	*OTOA*		
DFNB29	21q22.3	*CLDN14*		
DFNB30	10p11.1	*MYO3A*		
DFNB31	9q32-q34	*DFN31*		--
DFNB36	1p36.31	*ESPN*		--
DFNB37	6q13	*MYO6*		--

Adopted from van Camp & Smith, 2003; Smith and van Camp, 2006

Table 3.6c: Audiological manifestation of the X-linked nonsyndromic hearing impairment genes

Locus name	Chromosomal locus	Gene symbol	Hearing loss Onset/Decade	Type and degree of HL	Frequencies affected
DFN2	Xq22	--	Prelingual	Stable sensorineural; profound	All
DFN3	Xq21.1	*POU3F4*		Progressive, mixed; variable but progresses to profound	
DFN4	Xp21	--		Stable sensorineural; profound	
DFN5	Withdrawn	--			
DFN6	Xp22	--	Postlingual/1st	Progressive sensorineural; severe to profound	High frequencies evolving to include all frequencies by adulthood
DFN7	Withdrawn	--	--	--	--
DFN8	Reserved	--	--	--	--

Adopted from van Camp & Smith, 2003; Smith and van Camp, 2006

Table 3.6d: Audiological manifestation of the mitochondrial nonsyndromic hearing impairment genes

Gene Symbol	Mutation	Severity	Penetrance
MTRNR1	961(different mutations)	Variable	Highly variable, aminoglycoside induced
	1494 C>T		
	1555 A>G		
MTTS1	7445 A>G		Highly variable
	7472 Ins C		
	7510 T>C		
	7511 T		

Adopted from van Camp & Smith, 2003; Smith and van Camp, 2006

3.2.10 Future Application of Proteomics and Genomics

Following on success of the Human Genome Project, the vast information on the sequence, variation and expression of genes has ushered in a new era in clinical medicine (Collins & McKusick, 2001). Ethical, social and legal issues demand

increased awareness for intervention in matters relating to privacy, genetic discrimination, education and future research. Studies are underway to identify genes that play a significant role in disease causation, including hereditary effects on common diseases. A clear understanding of the normal homeostatic pathways of the human body is being developed, so that gene variants influencing disease pathways can be identified. Sophisticated technology such as gene CHIPS are required and must be developed to achieve this goal (Collins & McKusick, 2001).

The current understanding of the impact of molecular biology in clinical medicine is very low in the developing world and in many countries it is limited. According to Collins and McKusick (Collins et al., 2001) it is projected that by the year 2010, as many as a dozen common conditions will have predictive genetic tests on the market. They stress that this is especially so for conditions which have a strong family history such as breast, colon and ovarian cancer, and believe that this will, however, require a broader and deeper understanding of genetics and molecular medicine by all clinicians, starting at the level of medical school training.

It is predicted that by 2020, clinical medicine will have moved into the pharmacogenomics era in which the standard approach to treatment of many disorders will be to predict drug responsiveness based on the genetic makeup of the individual (Collins & McKusick, 2001). It is believed that new drugs based on gene structure will ultimately be produced to give a more tailored treatment protocol for diseases such as hypertension, and diabetes mellitus, to name but a few (Collins et al., 2001). The diagnosis and treatment of cancer is progressing rapidly. Research teams worldwide are already collecting information revealing the genetic and molecular

basis of malignant change. It is predicted that by 2020 every tumour will have precise molecular fingerprints on the database, indicating which genes have gone wrong and the kind of drug needed to sort it out (Collins & McKusick, 2001).

Many concern groups, while recognizing the advantages of the advances in genetics, have also expressed grave concerns about the potential for harm (Verma, 2000; Collins & McKusick, 2001). The drug STI-571 was designed to block the activity of bcr-abl kinase, a protein product of a translocation mutation between chromosomes 9 and 22 that is characteristic of chronic myelogenous leukaemia. There were dramatic positive results shown when used in patients with advanced chronic myelogenous leukaemia (Verma, 2000). On the downside, the advances in the field of gene therapy have been disappointing so far, with teams still struggling with the challenge of finding optimal gene delivery methods. This was not helped by the death of a volunteer in a gene therapy trial 1999 (Verma, 2000).

In the area of genetic hearing loss, scientists are continuing to explore the interaction of the inner ear proteins. The information gathered has helped to refine our understanding of the molecular basis of hearing and the mechanisms of hearing loss. As expected the picture is changing rapidly and consistently as more information surfaces.

The translation of this information from a laboratory setting into the clinical field has brought hope not only to the patients and their families but also to clinicians. Whereas previously the diagnosis of non-syndromic hearing loss was made by exclusion, it is now possible to give a definitive diagnosis based on molecular genetic analysis. For

many syndromic and non-syndromic types of deafness, the gene involved and type of mutation can now be identified, allowing for more accurate genetic counselling.

It is expected that the understanding of how mutations impact on protein function (proteomics) to cause hearing loss, together with the application of molecular biologic assays (genomics and proteomics) to the study of temporal bones, will truly transform our understanding of the pathophysiology of genetic hearing loss, and lead to the provision of potential therapeutic targets for pharmacological and gene therapy (Verma, 2000; Collins et al., 2001; Collins & McKusick, 2001). The prevention of deafness as well as the progression of deafness becomes a realistic goal for the near future.

3.3 CLINICAL PERSPECTIVES

When considering the various causes of hearing loss, whether hereditary versus environmental, single gene versus multiple gene defects, phenotypic versus genotypic manifestations of hearing loss, it is important to remember the interaction between genetic and environmental factors in the causation of hearing loss (figure 2.6). Therefore, the management of hearing loss should ideally follow a holistic approach, beginning with detection, through diagnosis and finally rehabilitation (HPCSA, 2007; JCIH, 2000).

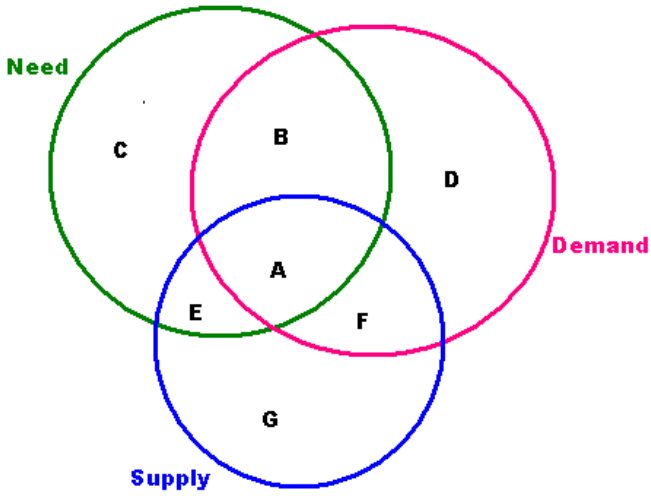
Detection may occur at any age, from neonates to school children and may be by means of high risk registers, caregiver concern, hearing screening programmes or audiological evaluation for any other reason. Details of these follow below.

Interventions include family education and counseling, ongoing training and guidance, amplification and assistive devices, medical treatment, otologic and other surgery, cochlear implantation, speech therapy, occupational therapy and physiotherapy. Genetic counseling and risk assessment are dependent on accurate genetic diagnosis, in the absence of which empiric recurrence risk figures can be used, with caution (Smith & van Camp, 2005).

With the above in mind, resource management, including service planning and resource delivery would have to be carefully handled because the need and demand always outstrip the supply in health services. Ideally, a population based needs assessment should be carried out initially to investigate the epidemiology of the conditions under assessment, and out of this would come population data, including that relating to the incidence and prevalence of the health problems under scrutiny, and covering all levels of disease burden and healthcare provision (HPCSA, 2007; Moodley et al 2000; Olusanya, 2000; Olusanya et al, 2006c).

Needs assessment on the other hand may also be through a measure of the ability to benefit, such as the effectiveness and appropriateness of health services. In Africa, this has been ably demonstrated by Olusanya, working among Nigerian populations, and Swanepoeol and others, working among South African populations, investigating hearing screening and detection among children (HPCSA, 2007; Olusanya, 2001, Olusanya et al., 2004a & 2004b, Olusanya, 2005, Olusanya et al., 2005, Olusanya et al., 2006a; Olusanya & Okolo, 2006). Knowledge of the natural history of these conditions would also help to determine whether intervention, for example, is appropriate and useful.

It is to be remembered that **need** does not equate **want** or **demand**. This applies to hearing loss as well as to other areas of healthcare. Need is not fixed, and is subject to a variety of interpretations and influences, such as the cultural and ethnic determinants of the times, a current research angle, changes in treatment modalities such as in cancer or heart disease. Neither is need always expressed (Olusanya & Okolo, 2006). Demand on the other hand is what people ask for. It is very changeable, even more so than need, and is influenced by social background, the media, educational background and even the medical profession. Supply can be defined as what is provided. It is often influenced by various pressure groups such as the public, politicians, drug companies as well as medical power politics. The difference between the realistic versus the ideal is summarized in figures 3.3 and 3.4. The ideal health service would reflect minimal unexpressed need, minimal unmet need and no inappropriate supply.



Key:
A Demand and need met
B Expressed need (waiting lists)
C Unexpressed need
D Unexpressed demand
E Supplied unexpressed need
F Demand met but not needed
G Unused supply

Figure 3.3: An example of the realistic relationship of need, demand and supply in current health care services.

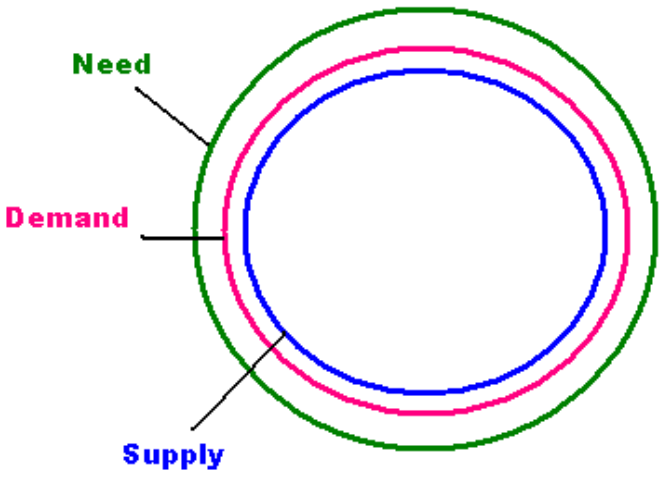


Figure3.4: An example of the relationship of need, demand and supply in the ideal health care services.

3.3.1 Detection of Childhood Hearing Loss

Initial suspicion of hearing impairment in children is often by the parent or caregiver. In Africa and other communities where the extended family system still exists, this may be a close relative such as the grandmother. Sometimes, the teacher may suspect a student to have hearing loss through observed poor academic performance, inattentiveness or disruptive behaviour in class and repeated requests by the student for the teacher to repeat the question. Where medical services are well established, a primary health care worker such as the school nurse or a community speech and hearing therapist may identify the hearing impaired child at a failed hearing-screening test. A hearing impaired child may also be identified when referred for poor language development, or on suspicion for dysmorphology or as part of assessment for a syndrome. Assessment may also be requested from a neuro-developmental paediatric clinic in healthcare facilities that have these services, such as secondary and tertiary level hospitals (HPCSA, 2007; JCIH, 2000).

However, this is not ideal, as most of the mild-to moderate forms of hearing loss may be missed. Children with the mild-to moderate forms of hearing loss have been shown to be the most likely to respond maximally to early intervention (Yoshinaga-Itano, 2004), that is, benefitting the most from amplification and succeeding in mainstream school. They also show the most promise in reaching their full potential since they are more likely to get and keep their jobs (Moeller, 2000; Yoshinaga-Itano, 2004). All initial concern should be taken seriously and the child sent for formal hearing evaluation.

As far back as 1964, the audiologist Marion Downs demonstrated that using behavioural hearing screening techniques could reliably detect severe-to-profound hearing loss among neonates (Downs, 1964). The discovery of otoacoustic emissions by David Kemp in 1978 (Kemp, 1978) and the ABR (Jewett & Williston, 1971) radically changed the scene. Today significant neonatal hearing loss can be identified within 24 hours after birth using objective tests of hearing based on evoked otoacoustic emissions and automated ABR (AABR) (HPCSA, 2007; JCIH, 2000).

This new technology, based on objective physiologic measures, has been shown to be superior to the previous tests based on subjective measures of behavioural testing (BOA, VRA, audiometry) which were highly dependent on tester/observer skill and therefore subject to error, and in the case of VRA, subject to the child's level of maturation. The subjective screening techniques all had poor sensitivity and specificity, and with low pick up rates, are now considered unsuitable and unreliable, except for the profoundly hearing impaired (Northern & Downs, 2002).

Improved technology has made ABR and OAE based hearing screening the ideal because they are fast, cost-effective and accurate (Chu et al., 2003; Hall, 2000; Vohr et al., 1998; Roizen, 1998; Watkin et al., 1991). OAE and AABR based technology has demonstrated specificity of above 95% (Lutman, 2000) and sensitivities of close up to 100% (Vohr et al., 2001a). Their low false positive rates, 2-3% or below (Iwasaki et al., 2004; Lutman, 2000; Vohr et al., 1998) and low false negative rates, 6-15% (Watkin, 1996; Vohr et al., 1998; Kennedy et al., 1998), means they are ideal for hearing screening programmes.

Hearing loss in children between birth and five years of age is detected in three main ways:

1. Systematic surveillance
2. Targeted screening
3. Mass screening or universal hearing screening

3.3.1.1 Targeted Screening

Targeted screening was initially proposed by The Joint Committee on Infant Hearing (JCIH) in 1990 with the drawing up of a list of 10 risk factors for development of significant hearing loss among children (JCIH 1990). The top three risk factors were felt to be admission to neonatal intensive care unit, a family history of hearing loss and craniofacial abnormalities. The National Deaf Children's society in the UK added 'any suspicion of intra-uterine infection during pregnancy and parental consanguinity' to this list in 1994 (National Deaf Children's Society, 1994).

Hearing screening was initially performed using a variety of methods including distraction testing, Crib-O-gram, OAE's and ABR. It was aimed at containing the costs of detecting hearing impairment. Many studies however subsequently showed that up to 50% of children with significant hearing loss did not have any of the listed risk factors (Chu et al., 2003; Davies & Wood, 1992; Watkin, Baldwin & McEnery, 1991). One study in the UK found the yield from targeted screening to be only 43% (Watkin, Baldwin and McEnery 1991). The findings of this and other similar studies led to the push for universal neonatal hearing screening.

3.3.1.2 Universal Neonatal Hearing Screening

According to WHO guidelines for screening programmes, it is a pre-requisite that the method used should be non-invasive, cost-effective, have a high yield while showing acceptable sensitivity and specificity. The disorder screened for should also have amelioration (WHO, 2005).

In 1993, the National Institutes of Health (NIH) in the United States of America recommended that all infants should be screened within the first three months of life for hearing impairment (Joint Committee 1993). The JCIH in 1994 also supported the NIH stance on the basis that it was unacceptable that only 50% of the hearing impaired children could be detected by targeted screening and so benefit from early intervention (Joint Committee 1994).

The Rhode Island Neonatal Hearing Screening Project (White et al., 1994), and other similar studies in Europe and the United States of America confirmed the efficacy, sensitivity of automated ABR and transient otoacoustic emission in neonatal hearing

screening. The findings of the Rhode Island and similar projects raised two further issues:

1) A need to establish early intervention programmes for mild to moderate hearing loss in children. The current programmes then were detecting hearing loss of over 50dB thresholds.

2) The need for extensive in-service training for both the specialized and genetic early intervention staff, especially regarding increased sensitivity to both language and listening needs of the hearing impaired children.

The finding that early detection and management of hearing impairment affected the educational and social outcome of the child (Yoshinaga-Itano, Sedey, Coulter, & Mehl, 1998), and that it could be performed by non-professional personnel (Heyes, 2003) have further paved the way for universal hearing screening.

It is believed that acquired hearing loss may account for up to 7% of significant hearing impairment among children by 5 years of age, with 90% of these as sequelae of meningitis (Davis et al., 1992). Meningitis was therefore added to the list of risk factors for hearing impairment at any age as it was now considered to be the most important cause of acquired SNHL in children (Fortnum & Davis, 1993). The current aim of hearing screening programmes is the early identification and early intervention of significant hearing loss among infants and children (HPCSA, 2007; JCIH, 2000). By 2003, the average age at which hearing loss was confirmed had come down to 2 to 3 months, from 24 to 30 months ten years before (Harrison, Roush, & Wallace, 2003).

The Professional Board for Speech, Language and Hearing Professions of the Health Professions Council of South Africa, in the Position Statement of 2007 (HPCSA

2007) states that it: “advocates early detection of and intervention for, infants with hearing loss (EHDI) programmes through integrated Provincial and District service delivery mechanisms which include all relevant government, private and non-governmental organisation (NGO) role players. This must be attained by inter-sectoral collaboration with governmental departments at all levels of care, including health, social development and education, and the private sector.” (South African Department of Social Development, 2006)

The goal of EHDI is to ensure that all hearing impaired children are given the opportunities and support they need to develop to their full potential. Further on, the position statement states:

> “Universal newborn and infant hearing screening is recommended using objective physiologic measures to identify congenital and early onset bilateral hearing loss.” (pg.3) and elsewhere
>
> “Diagnostic audiological, and if necessary, medical evaluations should be in progress before 3 months of age and diagnosis confirmed by no later than 4 months of age. Those infants with confirmed hearing loss should receive intervention before 6 months of age and no later than 8 months of age from health care professionals and interventionists with experience in infant hearing loss.” (pg3).

The South African government has advocated for the primary health care led health service model in the public sector (Dept of Health, South Africa, April 1997; ANC, 1994; Dept of Health, South Africa, July 2000; Dept of Health, South Africa, 2004). In this model, decisions regarding access to health care, the quality of health care, the

effectiveness and cost of healthcare as well public health priorities are made by the National Department of Health, with policies, guidelines and protocols sent out to all the provinces. The National Department of Health also undertakes to strengthen relationships with the patients, secondary care professionals, with the various health authorities including private health care providers, with social services and with other agencies. A more detailed summary of 'The White Paper for The Transformation of the Health System for South Africa' (1997) is found in appendix 10.

Focus on health and not on services, improved geographical access to services, improved coordination of the services, and closer long-term relationships with the patients are the ideals of this model. If properly implemented, it promises many positive outcomes, with clinicians leading the whole process of health care management, having increased accountability for decision-making, and on the whole resulting in increased value for money. For the patient this means greater satisfaction, with less feeling of being pushed around the system, feeling of involvement in the decisions involving the type of care received, as well as feeling that they are 'known' since the hospital services are near to home.

The School Health Services policy as laid out in the Primary Health Care Package for South Africa (Dept of Health, South Africa, 2000) states that "The introduction of the philosophy of inclusive education means that children with barriers to learning will be included in ordinary schools and communities will have to provide acceptable services for these children." The School Health Teams are an integral part of the primary health team whose service is built on the sub-district approach to service delivery. Norms laid out in the School Health Services policy include:

- A minimum of one School Health Promoting Team per sub-district
- Access to a trained school health nurse on per clinic per district
- Provision of Screening programmes, not limited to certain age groups, that will identify all children at risk of barriers to learning
- Creation of a positive learning environment, by identifying barriers to learning, and developing ways to remove these barriers in a community inclusive way
- Promotion of acceptance and celebration of diversity among individuals through a learner-centred approach

Concrete health data is generally not available to policy makers or the managers implementing policy because appropriate research addressing the priority issues has not been conducted. This is especially true of rural communities in the underdeveloped provinces such as the Limpopo. The South African National Treasury provides funding to the provinces but leaves the province the right to allocate the funds according to local need. This is however within the framework of the principles, guidelines and objectives of the national Department of Health (White Paper on Health 1997).

An aim of the White Paper on Health (1997) with regard to improving health sector planning and the monitoring of health status and services speaks of the development of *"a national health information system that will: facilitate the measurement and monitoring of the health status of the South African population; enable the evaluation of the delivery of health services; and support effective management at all levels of the health service."* A second stated objective is *"building capacity at the provincial,*

district, local and community levels to develop plans based on priority issues and ensure appropriate and cost-effective interventions".

To quote the Policy on Quality in Health Care for South Africa (Dept of Health, South Africa, 2007. pgs 10-11) where health care providers and workers are jointly cautioned: ***"A successful national effort to improve health care quality will need to build on existing resources, experience and expertise. All efforts should promote and strengthen existing innovative work that is being done. Competing with, stifling or slowing down these actions will not advance the agenda for quality improvement."***

In line with the White Paper for transformation of Health (Dept of Health, South Africa, April 1997) objective of ensuring a functional referral system throughout the different levels of healthcare, the Department of Health in Limpopo has provided for transport to patients referred for more specialized care in the public health sector. This system has greatly benefited the rural population of low-income earners who would not have otherwise afforded the transport costs. The Limpopo Department of Health has also provided for the continued education of healthcare workers through funding the attendance of seminars, workshops, conferences and short courses. It has also provided for granting of study leave with full pay for those officers needing to attend classes at tertiary institutions. Hospital facilities have been upgraded from the level of clinics right up to the level specialized hospitals including the Pieterburg/Mankweng hospital, the only tertiary hospital in the province, both in physical facilities and in equipment. Even within the budgetary constraints, hearing aids are being provided in public hospitals.

Notwithstanding, unmet needs and disparities of service provision are still issues to be dealt with all over the world, but especially in the developing world. South Africa, because of its parallel public and private health care systems, shows up these disparities clearly. In the rural areas, services still do not reach all who need them, often because they are not identified but also because of the insufficient resources to meet the guidelines laid down by the Departments of Health. On the other hand, patients with adequate funding from their medical aids are able to have services such as cochlear implants that need expensive intensive post-implant management, and because they can access the specialized medical centers staffed with well-trained personnel, they are also able to benefit fully from these implants.

The challenge to South African health care providers and personnel, in view of all these excellent health and education policies laid down by government, remains in their implementation (HPCSA, 2007). Health care workers and providers, it would seem, balk at the required and much needed paradigm shift, leading to slow change in the status quo.

3.3.2 Principles of Assessment

Many disorders, including hearing loss, vertigo, tinnitus, are often difficult to diagnose pathophysiologically. Medical management is often not an option as it is rarely curative. The outcome measures therefore focus on disability and handicap, with the aim of intervention being to relieve the effects on the patient, their families and caregivers (WHO, 2005; HPCSA, 2007). Since the patients rarely require hospital admission, the impact of their disabilities mainly falls on their communities. These patients will therefore benefit from a comprehensive explanation of the problem,

informed reassurance regarding management and the prognosis, supportive counseling for the associated psychological problems, as well as specific rehabilitative measures such as the fitting of hearing aids.

From a medical perspective, apart from the initial full examination carried out by the audiological physician or paediatrician, there are scheduled regular checkups. The child's GP should be informed especially of the consequences of persistent otitis media with effusion (OME). Communication assessment requires assessment in all four domains of communication, phonology, syntax, semantics and conversational. It is also necessary to determine whether the child has auditory, speech, perception or auditory attention deficits. In this the caregiver/child interaction is also assessed.

In the developed world, the management team includes an otolaryngologist, an audiologist, a clinical geneticist, a paediatrician, an educator for the deaf where available, a neurologist, and an ophthalmologist (Smith & van Camp, 2005; JCIH, 2000). The South African Professional Board for Speech, Language and Hearing professions recommends that for success, EDHI programmes must depend on multidisciplinary teams and facilitate collaboration in their approach (HPCSA, 2007). The essential team members are identified as the 'families, audiologists, paediatricians, primary care physicians, otolaryngologists, speech and language therapists, educators, nurses, community workers, other early intervention professionals and interpreters where needed' (HPCSA, 2007).

The planned assessment and management of the hearing impaired child should be based on a patient-centered approach, always taking into account the needs of the

hearing impaired child. The assessment can be approached as summarized in figure 3.5 below. Management can therefore also reflect a need-based protocol as depicted in figure 3.6. From these a management model can be constructed for the hearing impaired child, taking into consideration the prevailing circumstances in the child's life, as well as the logistical and financial constraints of the available healthcare system and of the child's community.

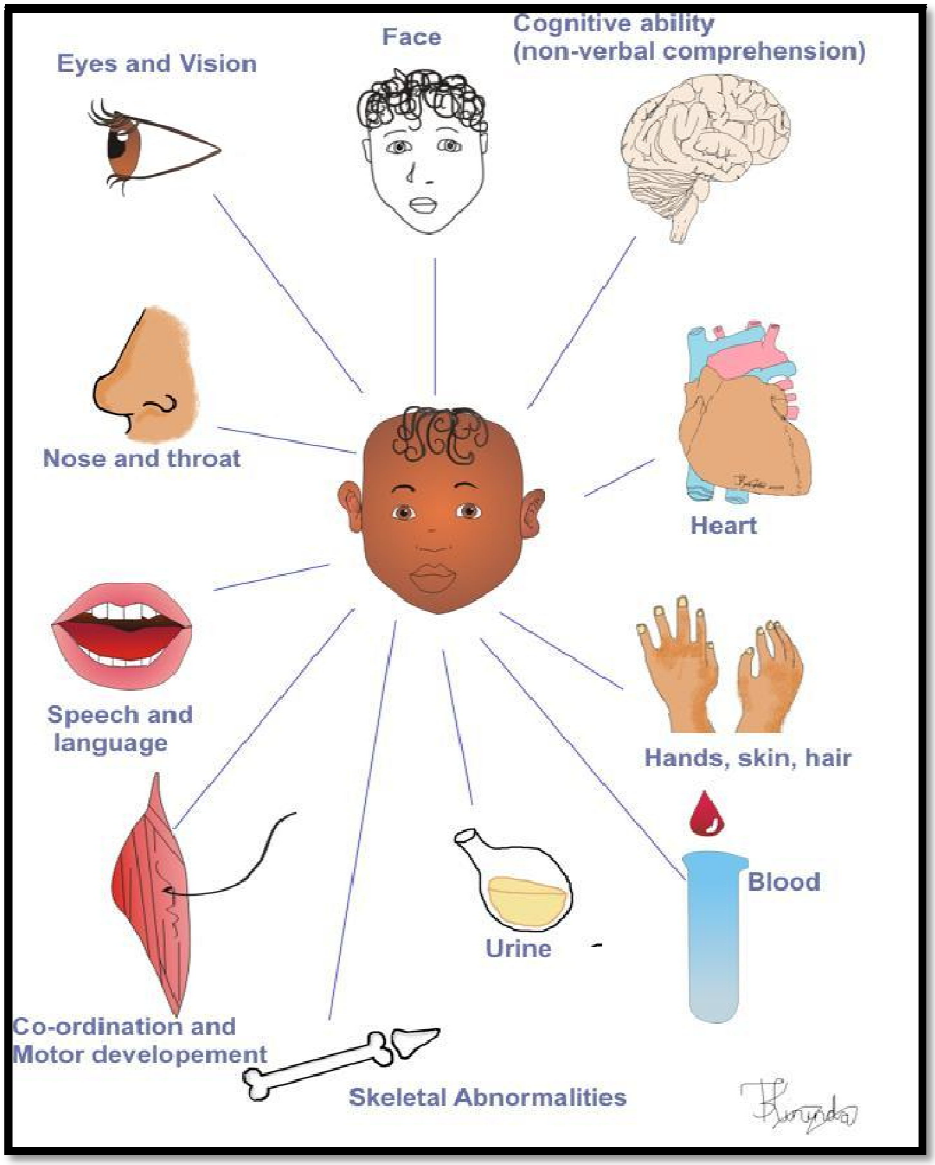


Figure 3.5: Medical assessment of the hearing impaired child

3.3.3 Audiological assessment

This requires specifically modified assessment and management techniques, taking into account the available healthcare facilities and the level of development of the child. The test battery should be structured to assess the whole of the auditory pathway, from the pinna and the rest of the external ear right up to the central auditory pathways in the cortex (Smith & van Camp, 2005; JCIH, 2000; Bamiou et al., 2000).

3.3.3.1 Immitance testing

Immitance testing, which includes tympanometry, acoustic reflex thresholds, and acoustic decay, assesses the peripheral auditory system. Middle ear pressure, tympanic membrane mobility, eustachian tube function, and the mobility of the ossicles (malleus, incus and stapes) in the middle ear, are all assessed.

3.3.3.2 Evoked otoacoustic emissions

Evoked otoacoustic emissions (EOAEs) are sounds measured in the external auditory meatus using a probe and transducer. EOAEs are believed to reflect the electromotile activity of the outer hair cells in the cochlea, and are measurable over a wider frequency range than the ABR (500-4000 Hz). EOAEs therefore have an advantage over ABR in that they can be used to assess low frequency hearing sensitivity (< 1500Hz). They are present in ears with hearing sensitivity better than 40-50 dB hearing level.

3.3.3.3 Auditory brainstem response testing

Auditory brainstem response testing (ABR) uses click stimuli to evoke electrophysiologic responses recorded by surface electrodes (Davis, 1976; Davis,

1981). The responses originate in the eighth cranial nerve and the brainstem, phase locking to changes in a continuous signal. The ABR 'V-wave' detection threshold has been shown to correlate best with the 1500-4000 Hz hearing sensitivity in neurologically normal persons (Davis, 1976; Davis, 1981). The main drawback to ABR is its inability to assess low-frequency, below 1500 Hz, sensitivity.

3.3.3.4 Auditory steady-state response testing

Auditory steady-state response testing (ASSR) is another measure of hearing acuity that can provide an estimate of hearing sensitivity in children who have no response to ABR testing. This is because the ASSR stimulus, being a continuous signal, delivers a higher sound pressure level than the click signal of the ABR.

3.3.3.5 Audiometry

Audiometry is a subjective measure of an individual's ability to process auditory information, in short, hears. This package includes behavioural testing and pure tone audiometry. Behavioural testing includes behavioural observation audiometery (BOA), and visual reinforcement audiometry (VRA). Both BOA and VRA have significant limitations and are subject to error: although BOA can be performed on infant from birth up to six months of age, it is highly dependent on tester skill, while VRA, used in children from six months of age to two and a half years and can provide a reliable complete audiogram, is dependent on both tester skill and the child's maturational age.

Pure tone audiometry on the other hand is a measure of the individual's ability to detect a pure tone as a function of frequency (pitch). Using earphones, frequencies

from 250 Hz to 8000Hz are tested, and the results recorded, a measure of intensity or loudness, in decibels (dB). Decibels can be defined as a ratio between two sound pressures. Air conduction, which depends on the condition of the external ear canal, middle ear and ossicles, as well as bone conduction, which reflects the condition of the inner ear, are tested.

On average, a normal hearing adult has a hearing threshold of 0 dB, the level at which a normal hearing young adult perceives a tone burst 50% of the time, and sound is perceived to be painful at 120 dB. Speech reception thresholds and speech discrimination tests both measure understandability of speech and so represent a more accurate assessment of an individual's ability to hear. Conventional audiometry is used to test individuals above 5 years of age, with the individual indicating when the sound is heard, while conditioned play audiometry is used to test children from 30 months to five years of age. A complete frequency specific audiogram can be obtained for each ear in a cooperative child.

3.3.3.6 Audioprofiles

An audioprofile is a recording of several audiograms on a single graph. The audioprofile may be for one individual at different times, or for a group of individuals, such as a family segregating familial deafness of the autosomal dominant type (Smith and van Camp, 2006). These plots, when recorded according to age over time, can bring out age related progression of the hearing loss. An example of this is the characteristic audioprofile of the hearing loss caused by mutations in the WFS1 gene, the gene for DFNA6/14/38.

3.3.3.7 Description of hearing loss

Hearing loss may be described according to type, onset, degree, progression, or shape of the audiogram. The terms hearing loss and hearing impairment are often used interchangeably to refer to hearing thresholds below those of normal hearing. In terms of **onset,** prelingual hearing loss is present before speech develops. Postlingual hearing loss on the other hand sets in after the development of language, often evident after 2 years of age. For this reason all congenital hearing loss is prelingual, although not all prelingual hearing loss will be congenital.

Regarding **type** of hearing loss, conductive hearing loss is due to abnormalities in sound conduction from the external environment to the oval window, reflecting abnormalities of the external, middle ear and the ossicles. Sensorineural hearing loss reflects a malfunctioning inner ear (cochlea) and mixed hearing loss a combination of conductive and sensorineural hearing loss. Central auditory dysfunction results from malfunction anywhere from the eighth cranial nerve through brain stem to the cortex. Pure tone audiograms (PTAs) when analyzed to determine the degree, type and severity of hearing loss according to recommendations of the European Concerted Action Project on Genetics of Hearing Loss (1996) use the following criteria:

Severity of hearing loss

This was defined by the average of the best ear at 500, 1000, 2000 and 4000 Hz.

Mild hearing loss	> 20 dB and < 40 dB
Moderate hearing loss	> 40 dB and < 70 dB
Severe hearing loss	> 70 dB and < 95 dB
Profound hearing loss	> 95 dB

Asymmetry of hearing loss

- A difference of >10 dB between the ears in at least two frequencies
- A difference of >20 dB in the PTA between two ears

Shape of hearing loss

A **Sloping** audiogram was defined as having a >15 dB difference between the mean hearing thresholds at 500 and 1000 Hz and at 4000 and 8000 Hz. A **Flat** audiogram was defined as having < 15 dB difference in hearing thresholds between 250 and 8000 Hz. The **Mid-Frequency U-Shaped** audiogram was defined as having > 15 dB difference between the poorest thresholds in the mid-frequencies compared to the lower and higher frequency thresholds. A **Low Frequency Ascending** audiogram was defined as having > 15 dB threshold difference from the low frequencies to the higher frequencies.

The description of the scale of hearing impairment does not always describe the full extent of hearing dysfunction experienced by the listener. The term Average Threshold Level (**re-ANSI-1989**) refers to hearing thresholds in the 0.5, 1 and 2 kHz frequencies and says nothing of the higher frequencies. The same limitation is found in the use of the Pure Tone Average (PTA). Therefore, every classification scale used should be supplemented with information regarding the communicative behaviour.

Audiological findings can help to direct further investigation. Progressive hearing loss should be considered among the genetic forms of hearing lossas well as other aetiologies such as neoplasms (NF2), trauma, infections (syphilis), metabolic, immunologic, circulatory and neurological disorders (Smith and van Camp, 2006).

3.3.4 Assessment and Investigations

The aims of the assessment of and investigations on the hearing impaired child include:

1. Identification of the cause of hearing loss,
2. Provision of information relevant to hearing loss management,
3. Identification of coexisting medical problems and prognosis for the child and family,
4. The clarification of phenotypes and epidemiology of the hearing loss (Smith and van Camp, 2006; Bamiou et al., 2000). This is also essential for the appropriate planning of both hearing loss prevention and surveillance programmes (Bamiou et al., 2000).

In some countries, such as the USA and parts of Europe, the diagnosis of genetic forms of hearing loss is by history (especially with regard to family history of hearing loss), physical examination, otologic examination, audiological assessment, ancillary testing (for example CT scans of the temporal bone), as well as molecular genetic testing (Smith and van Camp, 2005; JCIH, 2000; Bamiou et al., 2000).

A battery of tests, depending on the age of the child, age of onset of hearing loss and the suspected aetiology of the hearing loss, is used (Smith and van Camp, 2005; JCIH, 2000; Bamiou et al., 2000) (table 3.6). The choice of appropriate investigations takes into consideration the costs involved versus the extra information obtained from the test. A modified Newton's recommended protocol for investigating the hearing impaired child (Bamiou et al., 2000) is outlined in table 3.6 below. Key points in the protocol are outlined.

Table 3.7: Evaluation strategy of hearing loss in children.

History
Clinical examination
Ophthalmology
Serology
Haematology
Biochemistry
Thyroid tests
Immunology (as required)
Metabolic screen: blood and urine
Urinalysis
Electrocardiography
Radiography
Audiology: affected child and first and second degree relatives
Vestibular investigations
Clinical photographs
Genetic studies
Referral to a geneticist

Adopted from Newton, 1988; Bamiou et al., 2000

3.3.4.1 History

Viral illnesses during pregnancy, drugs, rhesus and ABO incompatibility as well as maternal metabolic disorders, such as diabetes mellitus and hypothyroidsm, are elucidated in the prenatal history. Key features in the perinatal history include prematurity, asphyxia, hypoxia, stay in neonatal ICU, low birth weight (Roizen, 1999); hyperbilirubinaemia (Boo et al., 1994), respiratory distress syndrome (Konkle & Knightly, 1993), neonatal sepsis and ototoxic drug medication (Unhanand et al., 1993), extracorporeal membrane oxygenation (Kawashiro et al., 1996), neonatal

meningitis (Unhanand et al., 1993) and persistent pulmonary hypertension (Kawashiro et al., 1996), all of which have been associated with high frequency hearing loss (Razi & Das, 1994).

The postnatal history seeks information on neuro-developmental milestones to determine whether vestibular hypofunction as well as speech and language delay could be due to a global neurological deficit. Causes of acquired forms of hearing loss are also sought including meningitis (Davis & Wood, 1992), noise exposure including incubator noise (American Academy of Paediatrics, 1997; Luxon, 1998), ototoxic medication including chemoradiotherapy (Freilich et al., 1996; Scott & Griffiths, 1994; Freeman et al., 1996; Bellman, 1996), accidents of all forms (car, bicycle, falls from heights etc), other viral illnesses (such as mumps) or whether the hearing loss followed vaccination with MMR (Nielsen & Walter, 1988), or vaccination for mumps (Kaga, Ichimura & Ihara, 1998), tetanus (Mair & Everland, 1977), or hepatitis B (Orlando et al., 1997).

The family history should cover at least three generations, taking special effort to obtain history pertaining to other hearing-impaired relatives. This list should include metabolic disorders, craniofacial anomalies, pigmentary disorders, visual defects and developmental disorders. The history of consanguinity or origin from ethnically isolated populations is important and should be specifically sought. Proper documentation and later confirmation, wherever possible, through direct medical examination or perusal of their medical records would follow. The salient findings would include any audiograms and clinical photographs previously taken, otologic

examinations, as well as DNA-based genetic testing if available (Smith and van Camp, 2006; Bamiou et al., 2000).

3.3.4.2 Clinical examination

A general clinical examination must be undertaken in all children with hearing loss. This includes physical measurements, such as height and weight, and assessment of the cranial nerves, head circumference and skull shape, as well as the nose and mid-facial region. In the mouth area, the teeth, jaw and palate are assessed. The chest and abdomen should also be examined.

It is recommended that all hearing impaired individuals be examined for associated features of syndromic hearing loss (Smith and van Camp, 2006; Bamiou et al., 2000; Calzolari & Sensi, 1996). Concerning the eye, these include dystropia canthorum, telecanthus, epicanthal folds, hypertrichosis of eyebrows, heterochromia irides, hypoplastic blue eyes, high myopia, and pigmentary retinopathy. Pigmentary anomalies of the skin and hair include patchy depigmentation, vitiligo, premature graying of the hair or parts of the eyebrows and white forelock. Patchy depigmentation may only be visible when the skin is examined under ultraviolet light.

Around the ears, key features include pre-auricular pits, as well as branchial cleft pits, cysts or fistulae. A goiter and craniofacial anomalies should also be sought. A careful physical examination on the proband and available family members will yield valuable information in, especially, autosomal dominant forms of deafness which exhibit variable expressivity and penetrance.

3.3.4.3 Ophthalmology

A baseline examination of the eyes including visual acuity, indirect fundoscopy, as well as both papillary and extraocular muscle assessment should be done on all hearing impaired children (Bamiou et al., 2000). Ophthalmologic findings may expose the aetiology of the hearing loss. Classic features include the chorioretinitis of CMV, and of congenital toxoplasmosis, while the 'salt and pepper' retinopathy, cataract and glaucoma are pathognomonic of congenital rubella syndrome. ERG is essential in a child with bilateral sensorineural hearing loss and delayed milestones as it may unmask early retinitis pigmentosa (Bamiou et al., 2000). Others however feel that it should be mandatory in all children with bilateral SNHL (Young et al., 1996).

3.3.4.4 Serology

The timing of serological investigations is very important as it affects the results of the tests. This is especially valuable in the immediate post-natal period as elevated IgM antibody titres and positive urinary cultures, which are confirmatory of in-utero exposure to certain infections, are cleared in the postnatal period (Smith and van Camp, 2006; Bamiou et al., 2000). These conditions include the TORCH organisms. For example, congenital rubella syndrome is confirmed if rubella specific IgM antibodies are detected in prenatal (third trimester) foetal blood or at birth. These antibodies persist in half the children with congenital rubella at 6 months of age but cannot be detected at 1 year of age (Sutherland, 1993). Postnatal acquisition of the TORCH viral infections may also confound the picture. Postnatal acquisition of CMV, for example, is common yet it does not cause hearing loss. The presence of high CMV antibody titers in a child's blood, therefore, may not necessarily be linked to hearing loss.

3.3.4.5 Haematology and Biochemistry

These are mainly useful for the exposure of underlying illnesses that may contribute to hearing loss. The tests include FBC, urea and electrolytes as well as liver function tests. Epstein-Barr infection, leukemia, thalassemia, sickle cell disease and other blood dyscrasias can be detected through FBC findings. Renal failure that is a feature in some syndromes such as Alport syndrome will show up in elevation of urea and creatinine.

3.3.4.6 Thyroid tests

Hearing loss may be a feature of a number of disorders such as Pendred syndrome, and congenital hypothyroidism. The tests include TSH, T3, T4, thyroid autoantibodies, and the perchlorate test.

3.3.4.7 Immunology

From the history, children with findings suggestive of autoimmune hearing loss, that is, sudden hearing loss, other clinical manifestations or family history, are selected for further analysis. The tests include ESR, complement tests, autoantibodies, immunoglobulins and anticardiolipin antibodies. These children may benefit from treatment (Luetje & Berliner, 1997).

3.3.4.8 Metabolic screen

A metabolic screen on blood and urine includes blood glucose, urine reducing substances, aminoacids, very long chain fatty acids, mucopolysaccharides and others (Blau, Duran & Blascovics, 1996). Because many of these tests are expensive, the choice of test should depend on careful history and clinical findings. However all

cochlear implant candidates receive a metabolic screen. The disorders commonly associated with abnormal findings on metabolic screen include Refsum's disease (elevated phytanic acid levels and very long chain fatty acids in serum), Alstrom syndrome (diabetes mellitus), and Hurler (elevated glycosaminoglycans in urine).

3.3.4.9 Urinalysis

Urinalysis is essential and aids in ruling out hearing loss associated with chronic renal failure due to other causes (Bamiou et al., 2000). This is picked up by the presence of haematuria and proteinuria, as well as renal tubular necrosis (alkaline pH and increased calcium in urine). One important syndrome is Alport syndrome which is characterized by haematuria and signs of chronic renal failure in the late stages.

3.3.4.10 Electrocardiography

All hearing impaired children should have at least one ECG in their assessment, and if it is abnormal or suspicious, the child should be referred to a cardiologist. ECG has been used in the diagnosis of Jervell and Lange-Nielsen syndrome, which is characterized by syncope and sudden death in the first year of life (although this may occur later) and a prolonged QT interval on ECG (Cussimanno, Martines & Rizzo, 1991; Ocal et al., 1997). This test is important both for the affected patient as well as for relatives who may be carriers and have a risk of giving birth to affected offspring.

3.3.4.11 Radiology

Computed tomography (CT) scanning in the first few months of life is performed under no sedation. CT scans of the temporal bones are recommended in the light of the finding that malformations of the inner ear occur with many forms of genetic

hearing loss (Smith and van Camp, 2006; Bamiou et al., 2000), especially the progressive forms of hearing loss. The pick-up rates of aetiologies of hearing loss have been reported to range from 6.8% to 28.4% (Zalzal et al., 1986; Phelps, 1998; Bamiou, Phelps, & Sirimanna, 1999). CT scans exhibit good resolution for bony defects, including the Mondini deformity, Michel aplasia, a dilated vestibular aqueduct, and a dilated internal acoustic meatus. There may be absent semicircular canals in the CHARGE association, which will be demonstrable on CT scan. CT scanning of the temporal bones is also essential for planning intervention, such as cochlear implantation, skull base surgery as well as other otologic surgery (Bamiou, Phelps, & Sirimanna, 1999). Thin section high resolution magnetic resonance imaging (MRI), which shows good resolution of soft tissues, is performed on all cochlear implant candidates to demonstrate the cochlear nerve.

Renal and other abdominal ultrasound, non-invasive radiological tests, are useful for demonstrating renal anomalies which may be associated with hearing loss. Plain radiographs are used to demonstrate skeletal abnormalities in syndromic hearing loss, such as Klippel-Feil syndrome in which there are abnormal cervical spines.

3.3.4.12 Audiology

This is covered in section 3.3.3 above.

3.3.4.13 Vestibular investigations

Vestibular failure is found in many forms of hearing loss, and many are subclinical. These include Usher syndrome and Pendred syndrome. It has been recommended that

all children with SNHL undergo vestibular testing as part of their assessment (European Workgroup on Genetics of Hearing Impairment, 1996).

3.3.4.14 Clinical photographs

Photographs are useful for a number of reasons. They are especially valuable in the delineation of a syndrome phenotype, as well as for future reference.

3.3.4.15 Genetic testing

Although clinical diagnostic testing is available for some of the known auditory genes, there are tests available for research alone, while some identified gene base variations are still under scrutiny for their significance in the causation of hearing loss (Kazazian, Boehm and Seltzer, 2000; Kenneson et al., 2002; Smith and van Camp, 2006).

Genetic testing needs special mention because of the multiple and complex ethical issues it raises. Some groups of deaf individuals argue that it devalues them (Arnos, 1992; Middleton et al., 1998; Arnos 2003; Kenneson et al., 2002) while a group of normally hearing parents with deaf children regarded genetic testing positively (Kenneson et al., 2002). However, due to the ACMG recommendation to establish the aetiology of hearing loss as soon as possible (Kazazian, Boehm and Seltzer, 2000), it is felt that the issues raised by the Deaf community must be taken into consideration by both scientists and society, with the aim to providing both culturally sensitive and acceptable methods for genetic testing as well as research (Middleton et al., 1998; Arnos 2003; Kenneson et al., 2002; Smith and van Camp, 2006).

The challenges for laboratories remain in the interpretation of variants found, the sizes of the auditory genes and low frequency of some of the mutations assessed for (Smith and van Camp, 2006). GJB2 mutations, on the one hand, have been found to account for up to 50% nonsyndromic hearing impairment in some populations (del Castillo et al., 2003; Estivill et al., 1998; Doneyelle et al., 1997), with 35delG as the commonest mutation. In these populations, laboratories offer a simple enzyme-digest based test for the mutation 35delG as a first screening tool (Kenneson et al., 2002; Smith and van Camp, 2006). This screening tool is suitable for some population groups, such as in Caucasians, but are not appropriate for South Africa where this mutation has not been identified and where in fact GJB2 mutations are not prevalent (current study). Therefore, laboratories offering genetic testing need to rely on population based studies to determine the common genes and mutations for the population groups they serve.

The laboratories also need to determine the clinical relevance of all mutations or variations before offering them as tests (Smith and van Camp, 2006). The American Council for Medical Genetics recommendations caution laboratories 'to develop any interpretation made on what is known not only about the sequence variant but also the individual's chance of having the condition, family history, other test results, and the sensitivity and specificity of the test being performed' (Kazazian, Boehm and Seltzer, 2000, Kenneson et al., 2002).

Both prenatal testing and preimplantation genetic diagnosis are a cause of major ethical dilemmas, such as the question of 'designer' babies. What should be done to the hearing impaired fetus? This is of greater concern if used for termination of

pregnancy based on the results of the prenatal tests (Smith and van Camp, 2006). What about the role of variable expression and penetrance so often encountered? Although prenatal testing may be technically feasible, the deafness causing allele must first be identified in a family member before testing can take place. This is performed by extracting DNA from fetal cells following chorionic sampling or amniocentesis at 10-12 weeks' and 15-18 weeks' gestation respectively.

Molecular genetic testing is now offered as part of the test protocol for many genetic forms of hearing loss, including GJB2 and GJB6 (Kenneson et al., 2002; Smith and van Camp, 2006). Genetic testing is recommended for individuals with congenital non-syndromic forms of hearing loss (Smith and van Camp, 2006). It is also considered for individuals demonstrating 'pseudo-dominant inheritance where an autosomal recesive disorder manifests in two or more generations (Smith and van Camp, 2006).

Regarding other genes implicated in syndromic forms of hearing loss such as Pendred syndrome, which on CT scan demonstrates a widened vestibular aqueduct, testing for mutations in the *SLC26A4* gene is recommended (Smith and van Camp, 2006). Another example of CT scan findings directing molecular genetic testing is in the *POU3F4* gene (Vore et al., 2005), which exhibits inner-ear defects on CT. Tests for Alport syndrome, Stickler syndrome, Pendred syndrome, BOR syndrome and NF2 can also be tested for. Some auditory genes on the other hand are of such a large size (*MYO7A, MYO15*) or mutations in them rather rare (*DFNB9, TECTA, POU4F3, HDIA1,* and *COCH*) that routine genetic testing becomes impractical for many laboratories (Smith & van Camp, 2006).

3.3.4.17 Referral to geneticist

The main reason for referring the hearing impaired child and family to the geneticist is for appropriate genetic counseling. A number of key areas are covered which areas are essential for the fulfillment of moral, legal and ethical obligations to the hearing impaired individual and to the health profession. These will be outlined shortly. Genetic counseling has been defined as 'the process of providing individuals and families with information on the nature, inheritance, and implications of genetic disorders to help them make informed medical and personal decisions" (Smith & van Camp, 2006). The areas covered by the geneticist include among others risk assessment, DNA banking, prenatal testing, preimplantation genetic diagnosis, as well as other related genetic counseling issues. Although the field of genetic counseling is large and cannot be delved into in detail, a number of areas key to this PHD are mentioned below.

Risk assessment

This varies according to the mode of inheritance (Smith and van Camp, 2006). All individuals with an **autosomal dominant hereditary hearing loss** are likely to have a deaf parent, and therefore the family history will usually yield positive findings. Before confirming apparently de-novo mutations, careful exploration for alternative paternity should be undertaken. All parents of a de-novo mutation should undergo audiometry and molecular genetic testing. It is believed that the proportion of de-novo mutations is very low (Smith and van Camp, 2006). The risk to a sibling of a proband whose parent has a mutatnt allele is 50%. The proband has a 50% chance of passing on the diseased allele to his/her offspring. In general, for autosomal dominant

hereditary hearing losses, the clinical picture and disease progression will vary according to syndrome type and may not necessarily be predictable.

With **autosomal recessive hereditary hearing loss**, the parents of a proband carry one diseased allele, and are asymptomatic obligate carriers (Smith & van Camp, 2006). They are considered obligate heterozygotes. The siblings of the proband carry, at conception, a 50% chance of having normal hearing and being a carrier and a 25% chance each of being deaf and of having normal hearing yet not carriers (Smith & van Camp, 2006). The at-risk normally hearing sibling carries a 2/3 risk of being a carrier. All heterozygotes are asymptomatic. The offspring of a proband are all obligate carriers, while the siblings of obligate heterozygotes carry a 50% risk of being heterozygotes. The disease phenotype and severity may differ among individuals with the same type of mutation and among syndromes, therefore factors such as age of onset, as well as disease progression, may not always be predictable (Smith & van Camp, 2006).

In the case of mitochondrial mutations, while the mother of a proband carries the mitochondrial mutation and may or may not have symptoms, the father is not at risk of having the disease causing mutation. Secondly, the proband could have acquired the disease causing allele through a de-novo mutation. The genetic status of the mother will dictate the risk to the proband's siblings. Generally, all the siblings of a proband are at risk of inheriting the mitochondrial mutation if the mother has got it. The risk to the offspring of a proband depends on their sex, with the females all standing a chance to inherit it, while none of the males are at risk.

Where a specific diagnosis or the mode of transmission cannot be established, empirical risks are used for genetic counseling (Smith & van Camp, 2006). A **normal hearing couple** with one deaf child and a negative family history carry an 18% chance of deafness in future children. If the couple comes from an inbred community or is consanguineous, there is a 25% chance of deafness occurring in subsequent offspring due to the likelihood of autosomal recessive inheritance. The union of a **deaf individual with a normally hearing individual** carries a 10% empirical risk of deafness in the offspring. On the other hand, a non-sanguineous deaf couple without evidence of autosomal dominant hearing loss carries a 15% empirical risk of deafness to the offspring. If both have Connexin 26 related deafness however, the risk jumps to 100%. The **offspring of a hearing sibling of a deaf proband**, who has been diagnosed as having autosomal recessive nonsyndromic hearing loss, **and a deaf individual** carries a 0.5% empirical risk of deafness, five times the general population risk. If there is related GJB2 or GJB6 related deafness or carrier status however, the empirical risk jumps to 50%.

Related genetic counseling issues

It is important that the following be observed for a successful genetic counseling outcome:

1. A culturally sensitive manner of communication for deaf people, which may be different for normally hearing individuals, is preferred. As such, terms like **probability or chance, deaf, hard of hearing**, are preferred over **risk, hearing impaired, affected, abnormal,** and **disease-causing** and these should be avoided.

2. The counselor should identify and both acknowledge and respect a deaf individual's concerns, queries and fears, since deaf people are also interested in gaining insight into their deafness (Middleton et al., 1998; Arnos 2003). Rather than information about family planning, prevention or reproduction, many deaf people are really looking for information on medical and social services, the cause of their deafness, as well as on education.

3. Many deaf individuals have expressed having a deaf child as preferable over a normally hearing child, viewing deafness as an identifying feature and not a disability requiring cure, treatment or prevention (Arnos et al., 1992).

4. As can be seen from the above, communication with the deaf person, especially in a counseling environment, requires the services of a trained interpreter.

3.3.5 Aetiological Diagnosis

Ultimately this is made on the basis of all the above findings. With the advent of molecular diagnostics and the establishment of a database for known genes for deafness, a candidate gene approach is now possible for mutation screening. Using a number of mutation detection techniques, it is now possible to confidently give a genetic diagnosis in a reasonable number of genetically hearing impaired patients, which was not the case 15 years ago. The batch of unknown aetiology has consistently shrunk over the decade and it is hoped that one day every hearing impaired person will be able to have a firm diagnosis.

3.3.6 Intervention for the Hearing Impaired Child

Early intervention principles involve the comprehensive assessment of the hearing impaired children and a family-centred, well co-ordinated, community based rehabilitation programme for the child (Bamiou et al., 2000; HPCSA, 2007). As with all tools, care must be taken to adjust interventions to suit the needs of the patient taking into consideration factors such as age, lifestyle and co-morbidities (figure 3.6).

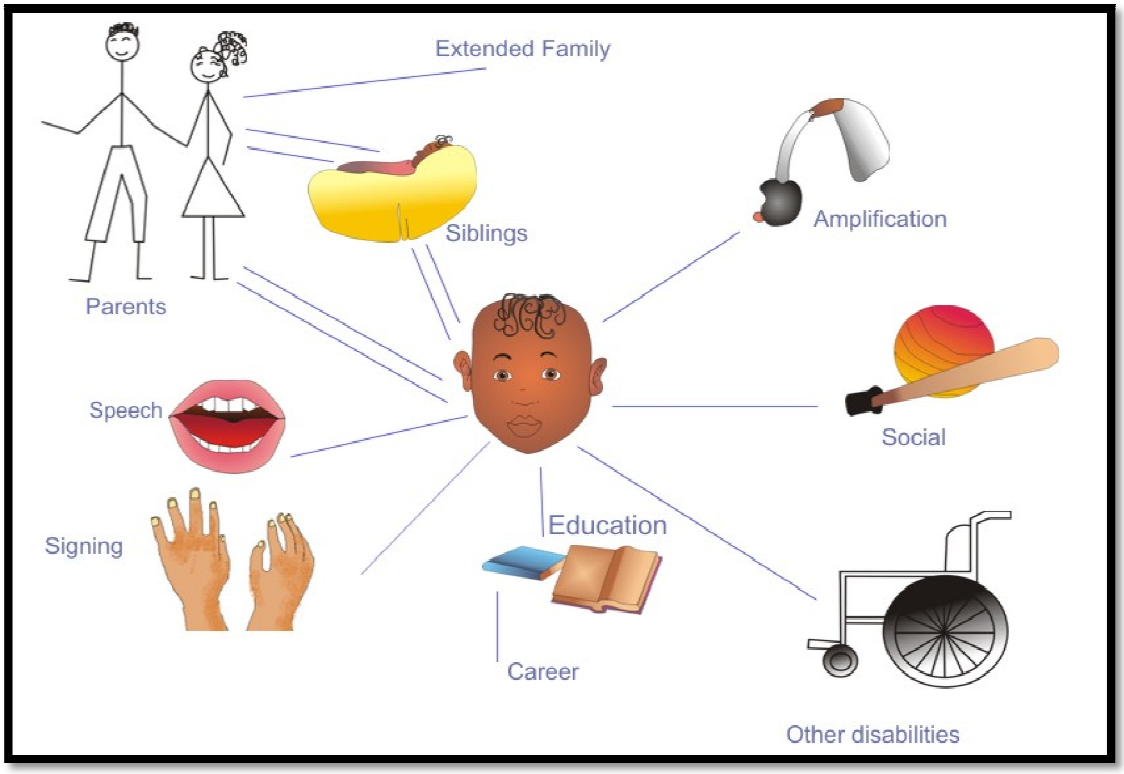


Figure 3.6: The areas to address in a hearing impaired child's management protocol.

It has been demonstrated that, regardless of language of communication (oral, signing or combined), among those infants diagnosed before six months of age, early remediation leads to maintenance of language, social and emotional development appropriate for their physical development, in contrast to diagnosis and intervention after six months of age **(Yoshinaga-Itano, 2003)**. This includes amplification of any

residual hearing and using that as a basis for the development of suitable communication techniques, the management of associated defects in the hearing impaired person, rehabilitation and management (including counselling) of family and important others, and management of other problems as they arise (Bamiou et al., 2000). The progressive blindness of a deaf/blind person (Usher's syndrome), and progressive deafness are such examples. Psychological attributes may occur needing management. These include guilt and blame among the parents, autism, adolescence, the cochlear implanted patient, as well as the introduction of a second deafness gene in the family through the marriage of two deaf individuals (Bamiou et al., 2000). Social factors that may need addressing include education/special schooling, which may necessitate family to move closer to the school of choice.

For most people, the ideal of meeting needs is impossible due to cost constraints. This is more so among the developing countries where incomes are low and government health care is stretched to the limit. Clinicians are called upon to use their discretion in balancing available health resources with the ideals of patient management (South African Department of Health. April 2007). Careful evaluation of the patient will allow the clinician to give the best possible care in the situation. The aim in all this is to maximize the potential of the deaf individual to become a responsible independent member of society (WHO, 2005; HPCSA, 2007).

Figure 3.7 summarizes the various components of an effective paediatric audiological medicine service as laid out by the British Association of Audiological Physicians. It encompasses all the areas relevant to childhood deafness/hearing impairment in a way that links these areas together so that none is left unattended. In the UK the

audiological physician heads the team and links all the various service providers in a patient-centered manner, acting as the hearing impaired child's advocate. Although South African universities do not yet offer this qualification, it was gazetted and registered as a recognized qualification in South Africa in August 1998 (The Interim National Medical and Dental Council of South Africa, 1998).

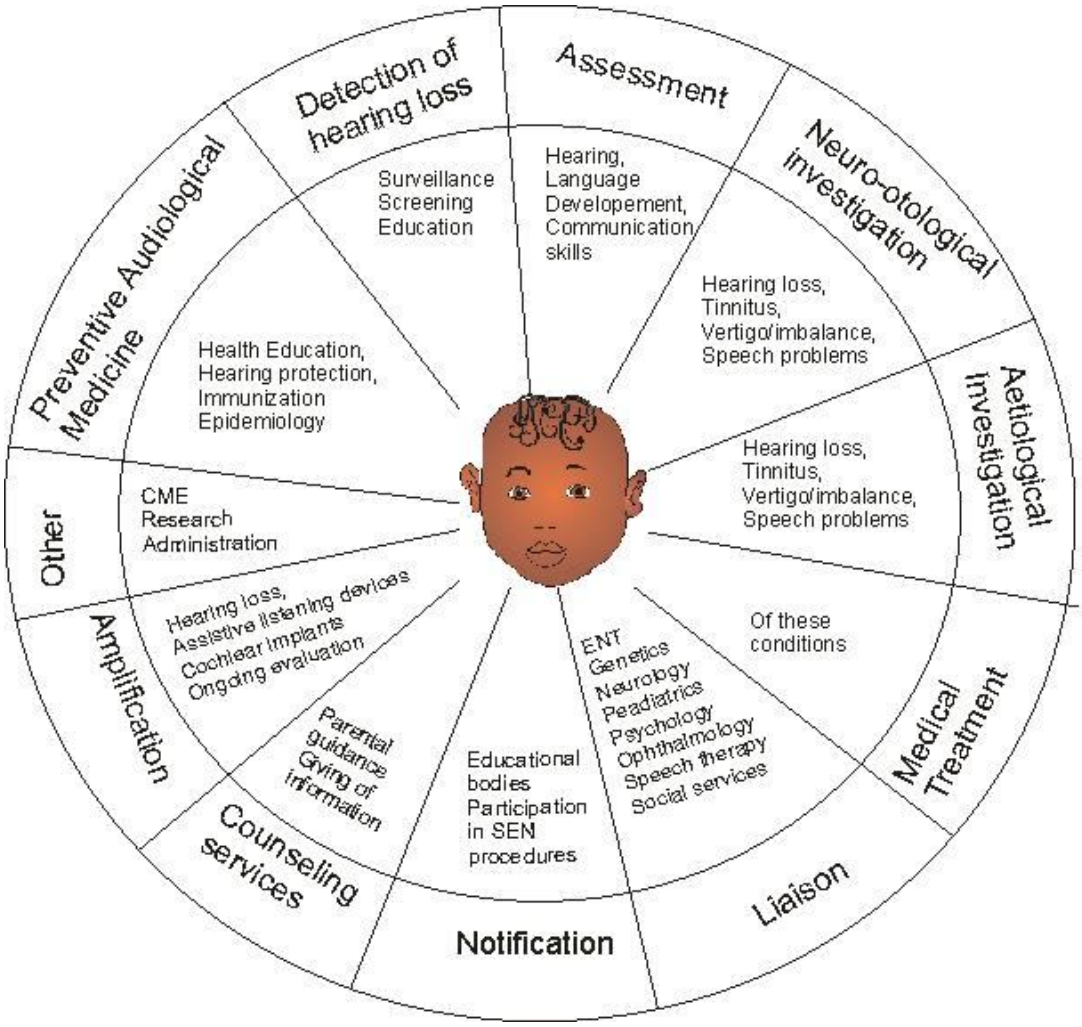


Figure 3.7: Components of a Paediatric Audiological Medicine Service

CHAPTER 4: METHODOLOGY

4.1 PROBLEM STATEMENT, RESEARCH QUESTION, AND PURPOSE OF THE STUDY.

The finding of a high number of deaf children from one geographical area, Nzhelele, in Limpopo province raised the question of whether there are genuine high-risk areas for genetic or environmental deafness in the Limpopo province. If the indigenous people of Nzhelele practiced consanguineous marriage for example, this could predispose them to recessive hereditary disorders, including hearing loss. The perceived high rate of childhood severe to profound hearing loss could therefore be due to the concentration and segregation of a recessive variant in a gene for hearing in this community. On the other hand, what other factors could be contributing to the apparent high incidence of deafness and blindness in the area? Could there be unknown environmental factors involved? Could it perhaps be due to selective admission of students to the Tshilidzini School for the Deaf?

This study intended to investigate the aetiology of genetic hearing loss and explore the effect of consanguineous mating on hearing loss in two Deaf populations in the Limpopo. Scientifically based data is lacking as to the magnitude and effect of hearing impairment in the province, impacting on budget allocation and the structuring of services in both the education and the health sectors. Results of this study may provide data on which decisions could be based for improving and boosting the secondary preventative rehabilitation measures. Through these measures, individuals affected by significant hearing impairment would be assisted to become empowered, self-sufficient, and productive members of their communities.

4.2 AIM AND OBJECTIVES

Aim

To investigate the aetiology of childhood hearing loss found among the people of the Limpopo Province of South Africa, with special reference to the non-syndromic genetic forms.

Objectives

1. To determine the geographical distribution of hearing loss in the Limpopo Province.
2. To determine the type and degree of hearing impairment of students at the Tshilidzini School for the Deaf.
3. To investigate the aetiologies of these hearing disorders, including the influence of consanguinity, on the prevalence of significant childhood hearing loss in these communities.
4. To correlate the clinical findings with other studies on deafness, to determine whether a suitable candidate gene can be identified and examined to determine its potential role in the causation of deafness in this community.
5. To build up a catalogue of clinical signs (including cochlear and vestibular investigations) specific for each of the forms of hearing loss identified, with the view to dividing the collection of sensorial deficiencies into distinct nosological entities.
6. To come up with data that can be used to guide policy and management of hearing loss in the population.

4.3 STUDY DESIGN

Since the current study aimed to ascertain aetiology and evaluate the importance of aetiological factors in the deafness identified in the study population, a combination of sample survey and case-control methods were chosen in the study design. These were the methods felt best suited to answer our research question economically, reliably, within the short time of a PhD study without risking accuracy. The study was therefore designed as a two phase study, in which phase 1 was used for hypothesis formulation and phase 2 was for hypothesis testing. Therefore phase 1 was a descriptive retrospective case study while phase 2 was a combination of sample survey and prospective descriptive case studies.

The risks and potential biases that could result from the chosen methods were noted. In phase 1 for example, data collection was done by questionnaires completed by a parent, caregiver or research assistant who was a teacher, a nurse or a student from the Dept of Speech Pathology and Audiology of the University of the Witwatersrand. The heavy reliance on collating history, language interpretation errors, the indirect approach to aetiology and natural history, the difficulty of disentangling age, time and cohort, the need for sophisticated statistics, the difficulty of ensuring adequate control groups all had to be catered for in methods and procedures and will be discussed in detail in the discussion section of this work.

The following were taken into consideration for mapping and data analysis:

1. The initial plan was to eyeball HL spatial distribution over population within the Limpopo province.

2. Alternative demographic mapping in which area is expanded or reduced proportionate to population density would then be considered.
3. To consider including time (date of birth) with clustering through cross tabulation.
4. To consider interactions of disease frequency vs space, time, person, through cross –tabulation and calculation of odds ratio.
5. To use 1 data set for hypothesis formulation and another data set hypothesis testing.
6. To obtain and utilize some information about the natural history or progress of the disease.
7. To address the areas of bias wherever possible including:
    a. Selection bias
    b. Berksonian bias
    c. Information bias.
8. In managing the issue of controls, to:
    a. Account for differences in known relevant cofactors
    b. Restrict sampling of subjects to certain levels of relevant cofactors
    c. Perform post-hoc adjustment
    d. Check for partial restriction (in matching): where the comparison group parallels the relevant cofactor distribution of the case group

Mapping of geographical disstribution

The smallest possible geographical unit was to be used. In the absence of GPS facilities at the time, x-y coordinates of the participants' home area that are required for mapping could not be generated. The participants' home area was taken as the village or subplacename (StatsSA) in which the home was situated. In searching the

StatsSA database, it soon became apparent that many of the known place names used by the local community were not in the StatsSA database, and where they were, the names were spelt differently, casting doubt on the accuracy of placement. A decision, based on this finding was taken to move to the next biggest unit, the municipality, as the identifying home area. This too is problematic because municipal boundaries artificially divide communities. This is a cause of bias in the current study. Where possible, units of five consecutive wards each were generated in each municipality for the purpose of more detailed analysis (table 5.7).

Spatial distribution of hearing loss

The plan was to initially eyeball the geographical maps displaying the spatial distribution of hearing loss in the Limpopo province, and to follow these on with map-on-map techniques, choropleth mapping. Spot clusters on spatial distribution maps would represent *uneven distribution of population at risk*, that is, of hearing loss in the Limpopo province. Using the municipal ward units mentioned above, possible high risk areas for deafness within the province would be indentified.

Demographic mapping

Ideally, a demographic map, in which area is expanded or reduced in proportion to the population density, would have best suited the current study since spot clusters would have represented a *genuine concentration of risk*, that is hearing loss.

An alternative to the demographic map was chosen however as expertise in this type of mapping was not available, and GPS systems could not be accessed as mentioned above. In the current study, the number of observations of hearing loss was

normalized to African population (the study population and therefore the population at risk), generating frequency distribution figures per 100,000 African population. These were then mapped and clusters observed. By taking population density into account in this way, areas constituting genuine populations at risk for genetic non-syndromic sensorineural hearing loss (NSSNHL) in the Limpopo province of South Africa would be identified.

Hypothesis formulation and hypothesis testing

Phase 1 was used for hypothesis formulation while phase 2 was used for hypothesis testing. By using student records to build up a demographic database of students admitted to Tshilidzini School, and comparing this to the data from Bosele School, the hypothesis was formulated as described in the text and causes of bias during this phase of the study accounted for. During phase 2 of the study, the hypothesis was extensively tested through geographical mapping techniques, frequency tables, tests of association, calculation of the crude odds ratio and risk assessment, as well as through binary logistic regression analysis. The fitted model was further tested for reliability through the Hosmer-Lemeshow goodness-of-fit test and the ROC (receiver operating characteristic) curve.

4.3.1 Reference Population

The reference population for this study was the indigenous Black African population of the Limpopo province of South Africa, comprising mainly of the Venda, Pedi/Northern Sotho and the Shangaan (Tsonga) speaking groups in the province. According to the 2002 census, this comprised 5,107,674 out of 5,273,631, the total population of the Limpopo Province (tables 1-2). The effect of the previous apartheid

policy that encouraged separate development and influenced the location of the special schools caused under-representation of the Shangaan (Tsonga) speaking as well as the other minority language groups in both Tshilidzini and Bosele schools. For instance, the Shangaan or Tsonga speaking population would tend to send their deaf children to the third school for the deaf in the province, Yingisani School for the Deaf, a school that previously catered for Shangaan speaking people, which was not part of this study. Deaf children from the other language groups could be sent to any of these three schools, or to their rural home areas to stay with a relative such as a grandmother and attend their local special school.

4.3.2 Setting: Schools for the Deaf

This study looked to the schools for the deaf for participants. In the Limpopo province, all the three schools for the deaf are boarding schools, and so the children are institutionalised early. Deaf children who cannot cope with mainstreaming attend the special education institutions in the province. They are often identified at a public health service facility such as the clinic by a primary health care giver or the speech and hearing therapist, often in response to parental concern. Healthcare workers may alternatively pick up these children during school screening programmes for hearing impairment. Some children come in as referrals from other health professionals and schoolteachers at normal schools. Hearing loss is confirmed after assessment by the speech and hearing therapist, the audiologist or community speech and hearing therapist. A medical officer and where available, a paediatrician at their local hospital examines the child and fills out the referral forms to the school for the Deaf for placement.

4.3.3 Study Population

The sample size depended on the study phase.

Phase 1: N= 361

This phase included all hearing-impaired students in the two schools for the Deaf in Limpopo Province, namely Tshilidzini and Bosele, registered from 1997 to 2000 for whom complete records could be obtained. From the initial pool, 86 participants from Bosele School and 275 participants from Tshilidzini School were recruited.

Phases 2a: N=184

This phase included currently registered hearing-impaired students at the Tshilidzini (107) and Bosele Schools (77) for the deaf for whom parental consent was obtained. Although the study had been planned to include all deaf participants, only those whose parents could be contacted and who gave consent were included in the study.

Phase 2b: N=184 Controls = 63

This phase included 182 participants with suspected nonsyndromuic SNHL plus one sibling pair with Waardenburg Syndrome type 1. The controls were drawn from the general patient pool attending the ENT department of the Polokwane Provincial Hospital with non-otological conditions, and no history or evidence of hearing loss.

Although the language groups, age and sex were not matched to the study group, these were not significant to the question asked, that is, whether the observed variations in GJB2 in the study group were polymorphisms or not. The condition under investigation is a genetic disorder which is not influenced by age or sex. Since

the variations were found to be common to all the indigenous Africans in the Limpopo province, it was not necessary to allocate equal proportions of language groups among the controls as in the definitive study cohort. The sampling was restricted to known relevant cofactors, that is, indigenous Africans from language groups in the Limpopo province of South Africa. In this, the most important aspect of matching, the comparison group (controls) paralleled the cofactor (indigenous Africans from the Limpopo province language groups) of the case group (the definitive study cohort).

4.3.4. Inclusion Criteria

All participants with hearing impairment attending the two schools for the Deaf, namely, Tshilidzini and Bosele, during the periods under study were eligible.

4.3.5 Exclusion Criteria

In Phase 1 only students with adequate records for the phase were included. In Phase 2, the exclusion criteria were selected to rule out syndromic and acquired hearing loss. Participants without a valid informed consent, defined as consent of a parent or a legal guardian for participantss under 18 years of age, or the consent of a participant over 18 years of age, were also excluded. The criteria used to rule out syndromic and acquired hearing loss included:

- Stigmata of known syndromes
- Craniofacial anomalies
- Signs of neurodegenerative disorders such as neurofibromatosis
- History or signs of Toxoplasmosis, Rubella, Cytomegalovirus, Herpes, Syphilis (TORCH), or HIV infections during pregnancy

- Low birth weight - less than 1500 gms
- Documented low apgar score, less than 4 at 1 minute; less than 6 at 5 minutes
- History of anoxic or hypoxic events or prolonged mechanical ventilation
- History of hyperbilirubinaemia
- History of ototoxic drug use
- History of bacterial meningitis
- History of head trauma

4.3.6 Limitations of the Study

4.3.6.1 Language

The study population did not have a good command of English, and the researcher, who speaks mainly English, did not have a good command of the many local languages spoken in the province. Interpreters made up by a combination of school nurses, teachers, as well as community speech and hearing therapists were therefore used wherever possible. Care was taken to reduce the risk of incomplete data, under-reporting or misunderstanding of terms by training and using of interpreters competent in the local language where possible.

4.3.6.2 Sample Size

Hearing impairment is a complex but common disorder worldwide, its prevalence increasing with age, affecting 10% to 15% of the population (Gorlin et al., 1995, Liu et al., 2001). The sample size of phase 1 of the study was determined using the statistical package nQuery Advior version 4 as shown below:

Setting the required level of significance of the statistical test set at 5%, a two-sided test was assumed, and the Exact test for a single proportion was used. Using the above estimate of the prevalence of hearing loss at 15% of the population, the adjusted sample size of study was calculated at 361. The power of study becomes equal to 34%. Both the sample size of study and power are suitable enough for the study as the design of the study is descriptive and cross-sectional.

In cases where the attribute of study is rare, the power of study has to be made large. It is also not so difficult to find and locate deaf children at the site of study since these are schools for the deaf. In cases such as this where the attribute of study is not so rare in the population being studied however, a power of 34% is robust enough.

4.3.6.3 The Use of Questionnaires

The questionnaires were completed by the parents and/or guardians of the participants, assisted by teachers and nurses at the schools. However, for the parents who did not physically come to the school, forms were sent out to them through the participants. As mentioned in 4.3.6.1 above, care was taken to reduce the risk of incomplete data, under-reporting or misunderstanding of terms by training the interpreters in the completion of the questionnaires before they were handed to the participants. The interpreters and helpers were briefed about and trained about the meaning of the terms used and asked to write down what the parent or guardian said, and not what they themselves understood, to avoid interpreter bias.

4.3.6.4 Attrition

Many of the participants, especially the older ones, moved out of the province after their last year at school and were lost to follow-up. This affected data collection and clarification of issues that arose during analysis.

4.3.6.5 Pedigrees and Family testing

Due to the large size of the study and the limited time and funding available for the PhD programme, it was not possible to draw up pedigrees or test family members' hearing thresholds, except for the Waardenburg family.

4.3.6.6 Unavailability of Investigative Facilities

Vestibular testing, retinography and CT scans of the temporal bones could not be carried out due to unavailability of the tests.

4.3.7 Ethical Considerations

1. Ethical approval was obtained from the University of Witwatersrand ethics committee and the Limpopo Province, then called the Northern Province, department of health research committee.
2. Informed Consent was obtained from the subjects and their guardians.
3. Confidentiality was maintained, within reasonable limits (since interpreters were used).
4. All participants were free to withdraw from study at any time they wished without prejudice.

5. Proper investigation and intervention, including amplification and rehabilitation, were offered to all the participants of the study. This was mainly by referral to the government hospitals.
6. Permission was obtained from the 'Waardenburg' family to use and publish their photographs in this thesis since eye and facial findings were crucial for reporting.

4.3.8 Ethics Approval

Ethical approval was obtained from the University of Witwatersrand Committee for Reasearch on Human Subjects, Ethics committee clearance certificate protocol number M991005 (appendix 11), and approval obtained from the Research Committee of the Department of Health and Welfare of the Northern Province, now Limpopo Province (appendix 12).

4.4 METHODS AND PROCEDURES

4.4.1 Equipment

All the equipment used was either loaned from the Pietersburg Provincial hospital, or used on site in the various laboratories where the molecular work was done. The audiological testing equipment was calibrated by HASS/Phonak group in Pretoria before use.

4.4.2 Audiological Evaluation

Audiological testing was carried out according to standard test protocols (British Society of Audiology, 1981 & 1986). Two fully trained audiologists in private practice with significant experience assisted in the testing. On three occasions, a group of master's students accompanied by their tutors came out to assist with the audiological evaluation, using the department of Audiology, University of the

Witwatersrand, test protocols. The hearing tests were carried out in soundproofed rooms at both schools for the Deaf (figure 4.8). TEOAEs were done in a quiet room at Bosele School and in a soundproofed room at Tshilidzini School.

For the purposes of this study, definition of type of hearing loss was done according to the following criteria (European Concerted Action Project on Genetics of Hearing Loss, 1996):

Autosomal dominant HL

1. Hearing loss in three successive generations without any apparent cause.
2. At least 1 parent had an audiometrically proven non-acquired hearing disorder.
3. Apparent syndromal characteristics of dominantly inherited hearing loss (such as found in Waardenburg Syndrome, Pierre Robin syndrome).

Autosomal recessive hearing loss

1. Sibling with an audiometrically confirmed similar hearing disorder.
2. Consanguinity of parents within four generations.
3. Two relatives of the subject suffering from a non-acquired hearing loss.
4. Observable characteristics of recessively inherited hearing loss (such as Usher syndrome, Pendred syndrome).

Other inherited hearing disorders

1. Hearing loss in combination with Down's syndrome.
2. Syndromes in which a hereditary aetiology is suspected but mode of inheritance is unclear (Wildervanck syndrome).
3. Radiation exposure > 1 Gray during pregnancy.

Acquired hearing disorders

A potential non-genetic factor causing HL identified (pre- peri- & post-natal).

Unexplained hearing disorders

1. There are no obvious causes of acquired hearing loss, and criteria for hereditary hearing loss are not met (undetected viral infections, spontaneous mutations, autosomal recessively inherited hearing loss, are all possible for this group).
2. This group may also contain cases in which 2 or more causes of hearing loss are possible
3. Ear abnormalities

The pure tone audiograms (PTAs) were analyzed to determine the degree, type and severity of hearing loss according to recommendations of the European Concerted Action Project on Genetics of Hearing Loss (1996) as mentioned in section 3.3.3..7 above. Because of the limited testing previously done in audio records used, many of which were not tested at the 8kHz frequency, a modification of the definition of a sloping audiogram was made as having a >15 dB difference between the mean hearing thresholds at 500 and 1000 Hz and at 4000 +/- 8000 Hz where available. A flat audiogram was defined as having < 15 dB difference in hearing thresholds between 250 and 8000 Hz or 4000Hz where 8KHz frequency result was not available.

4.4.3 Procedures

4.4.3.1 Phase1

Research approval.

After obtaining the relevant ethical approval, introductory visits were made to the two schools, Tshilidzini and Bosele. Letters of introduction given to the school principals and the research project was explained to them. They agreed to help where needed.

Collection of demographic data.

A list of all deaf students enrolled in two schools for the hearing impaired, namely Tshilidzini and Bosele, during 1997 - 2000 was obtained. Demographic data was obtained from the admission records and referral letters where these were available. EpiInfo was used to create the database for statistical analysis at a later stage. The key information other than the participants' names, sex and date of birth, included date of onset of hearing loss, past medical history including risk factors for hearing loss, language group, family history of hearing loss, subjects' home/area of origin, parents' home/area of origin, as well as any consanguineous relationship between parents (appendices 2a, 2b, 3 & 4).

Using data from statistics SA, the participants' home areas were identified according to place names and according to municipalities. These were then coded according to the municipal (MDB) code and ward. These codes were used to map the distribution of hearing loss within the province, generating the spatial maps of distribution of hearing loss. This data was not normalized according to population density. The data was also analysed according to language group and school-by-school comparison was made according to the mapping techniques detailed above.

A list of all deaf students enrolled in two schools for the hearing impaired, namely Tshilidzini and Bosele, during 1999 - 2000 was obtained. As is common in this part of the world, the meeting with the parents was preceded by a prayer led by the teacher (figure 4.1). With the permission of the school principals, the parents of the students were addressed at a general meeting, using nurses and teachers as translators (figures 4.2, 4.3) during which the subject information leaflet was read out and translated into

the local language. After this, informed consent was obtained from the parents for inclusion of their children into the study (appendix 1).

Demographic data was collected from the parents of each participant and from admission records (appendix 2a, 2b, 3, and 4). The various class teachers and the principal researcher plus assistants at each school for the deaf helped with the completion of the questionnaires. This information was used to build the demographic database used for epidemiological analysis.

As indicated above, since this phase of the study was mainly used for hypothesis formulation, the main *aim of this phase* was to determine whether there was genuine clustering, and whether indeed the observed high numbers of deaf participants from Nzhelele at the Tshilidzini School represented a high-risk population.

4.4.3.2 Phase 2a

Assessments of the hearing impaired children enrolled at Tshilidzini and Bosele schools for the deaf during 2000, and for whom consent had been obtained, were carried out. A thorough clinical examination, audiological evaluation and urinalysis were done. Mutational screening was carried out in all 184 subjects using genomic DNA. The clinical examination (appendix 5) also included assessment for dysmorphic features (fig 4.5, 4.6). Audiometric assessment (fig 4.7, 4.8) including tympanometry, transient evoked otoacoustic emissions (TEOAEs), as well as pure tone audiometry, were performed on each subject according to standard protocols as mentioned above. These tests were carried out by teams of Master's students from the department of Speech Pathology and Audiology, University of the Witwatersrand, supervised by

their lecturers, as well as two well-qualified audiologists in private practice who were recruited to assist with the testing. Audiometry was performed in testing booths at the two schools for the deaf (fig 4.8).

Although auditory evoked brain-stem responses were initially performed, ABR was abandoned after a few tests due to malfunction of the machine which then had to be sent away for repair. It was later felt that since the participants were old enough to cooperate in the test and the results of the audiograms were adequate to give an accurate enough picture, it was not really necessary to proceed with ABR.

In total, 95 participants had audiograms performed, either by the master's students from the department of Speech Pathology and Audiology, University of the Witwatersrand, or by the two qualified audiologists in private practice who were recruited to test the participants. The rest, 89 participants, could not be tested due to attrition. The reported hearing thresholds used to compile profiles of the hearing loss for these participants were obtained from the school records. As mentioned before in section 3.3 above, the limitations of PTA and Average Hearing Level in accurately describing the hearing thresholds were taken into account and so each audiogram was supplemented with more descriptive information. Pure-tone thresholds were measured from 500 to 4000 Hz and the values used to calculate the PTA as well as the High frequency PTA. These values were then analyzed to determine the severity, the asymmetry and the configuration of the audiogram according to the criteria outlined.

For DNA analysis, from each participant, a 10 millilitre sample of peripheral blood was drawn from a vein in the cubital fossa following the standard procedures of

venepuncture. The blood was immediately stored on ice and later transported to a –20 freezer where it was stored for DNA extraction at a later stage. Urinalysis was done on each of the participants using Combur-9.

Fundoscopy, electronystagmography (ENG) and retinography were not performed due to lack of facilities mainly because of lack of funds for recruiting an ophthalmologist for the study. CT scans were not performed due to the legal and financial challenges of transporting the subjects to PMHC for the CT Scans. Participants believed to have non-syndromic recessive hearing loss (NSRHL) were selected on clinical basis from this group for Phase 2b of the study.

As indicated above, this phase of the study was used for hypothesis testing, and as such, one of the *aims* of this phase of the study was to ascertain whether there was an increased risk for development of NSRHL among the cases with a family history of consanguinity. A total of 184 participants formed the definitive case group.

4.4.3.3 Phase 2b

N=182 (+2); Controls= 63

A total of 184 participants were selected for this final phase of genetic analysis, including 63 controls from the general population. DNA was extracted from the stored peripheral blood samples according to the standard salting out and precipitation procedures at the NHLS laboratories of the University of Witwatersrand (appendix 7).

Collaboration was set up with two teams researching genetic hearing loss, Prof R F Mueller at the St James Hospital, Leeds University, UK; and Dr XZ Liu of the

department of Otorhinolaryngology, University of Miami, Florida, USA. The Leeds team had someone specialized in mitochondrial mutations, as well as with Connexin 26 mutations. Collaboration was later set up with Prof Andrew Read and Dr James O'Sullivan, then based at the St Mary's Hospital Manchester, UK, whose team had specialized in Waardenburg syndrome, a syndromic type of genetic deafness.

Although this study focussed mainly on non-syndromic genetic hearing loss, this last collaboration was important for two reasons. Firstly, three subjects had been identified clinically with features suggestive of Waardenburg syndrome and secondly it was academically challenging to identify the mutations in our African population. Three months were spent at the DNA laboratory, St James's Hospital, Leeds, UK, and two months were spent at the Department of Otorhinolaryngology Research Laboratory, University of Miami, USA, learning the techniques of mutation detection and analysis.

Fig. 4.1 Parents and teachers at prayer in the hall, Bosele School

Fig. 4.2: Translator (Nurse) explains questionnaire to parents

Fig. 4.3: Parents await assistance in completing questionnaire

Fig. 4.4: Subjects waiting for their turn at Bosele School

Fig. 4.5: Doctor completing a subject's medical examination form

Fig. 4.6: Doctor examines subject's ear at Bosele School

Fig. 4.7: TEOAE (transient otoacoustic emission) station – Bosele School

Fig. 4.8: Sound-proofed testing room, Tshilidzini School

4.5 METHODS USED FOR MUTATION DETECTION

With so many genes to choose from, a candidate gene approach was used to analyze genomic DNA extracted from peripheral blood. Candidate genes selected included GJB2 encoding Connexin26, common mitochondrial mutations A1555G, A3243G, A7511C, A7445G and finally PAX and MITF of Waardenburg syndrome. The choice of tests for mutation detection depended on the laboratory protocols and the costs involved.

4.5.1 Specimen Collection

This was done at the schools. Under aseptic conditions, a 10 ml sample of peripheral blood was collected from the cubital fossa of each subject into EDTA tubes. The samples were transported in cooler bags to the Polokwane Hospital laboratory where they were stored in freezers at –20^{o}C. The samples were later transported in cooler bags on ice packs to the NHLS laboratory in Johannesburg where they were stored at –20^{o}C to await DNA extraction.

4.5.2 DNA Extraction

DNA was extracted using the salting out procedure as described by Miller et al (1988) (appendix 7). On the first day, the blood samples, including white blood cells, red blood cells and plasma, were thawed at room temperature. The red blood cells were lysed using cold sucrose-Triton-X lysing buffer. After mixing by inversion, the samples were centrifuged to separate the white blood cells from the lysed red cell debris. The resultant white pellet was then washed with cold sucrose-Triton-X lysing buffer. The white blood cells were then lysed using a mixture of T20E5, 10% SDS and Proteinase-K mix. The detergent SDS acts by breaking up the lipid bilayer of the

cell membrane of the white blood cells while Proteinase-K digests the cell walls. After mixing, the contents were incubated overnight at 42 ^{o}C.

On the second day, saturated NaCl was added to the lysate to precipitate the proteins. The DNA was precipitated (using absolute ethanol) and resuspended in 1xTRIS-EDTA (TE) buffer in an appropriately labeled (patient's name, disease code and date of receipt) new safety-lock Eppendorf tube and stored at 4 ^{o}C. The detailed protocol is found in appendix 7.

4.5.3 Mutation Detection

The polymerase chain reaction (PCR) is a standard first step for mutation detection. The technique involves the synthesis of copies of DNA segments using of primers flanking the area of interest and DNA polymerase, with the primers incorporated into the final reaction product. The amplification process is conducted using a thermocycler. Genomic or cDNA is denatured in the presence of excess (5-50pmol/reaction) primers, dNTPs, buffer and heat stable *Taq* polymerase, usually under hot start conditions. The active reagents are either only combined at high temperature or enzyme formulations such as *Clonetech Advantage, Platinum Taq* or *AmpliTaq Gold* that require heat activation before use are applied to increase the yield and specificity of the reaction. For example, *AmpliTaq Gold* requires a 10-15 minute pre-heating period at 95^{o}C in the initial denaturation step. The mixture is then cooled for primer hybridization then incubated to allow for polymerisation of new strands. The whole process is repeated for up to 25-35 cycles to produce an exponential yield of DNA. The PCR products are analyzed by gel electrophoresis, and visualized by staining with ethidium bromide or silver, by radioactive labelling or by fluorescent

labelling. Since different conditions apply to different DNA products and these are affected by laboratory conditions and equipment, optimization is required for each reaction.

For ***GJB2,*** direct sequencing of the coding exon was then carried out using the ABI377 sequencer. It was sometimes difficult to get clean electropherograms at the extreme ends. Therefore, once the –34 and –15 variants were identified, an enzyme digest assay was designed to conclusively identify the variants in the rest of the samples. Restriction enzyme digestion assay was found to be a sensitive, fast and cost effective way of establishing the variants. Primers were designed based on the catgcttgcttacccagac (forward) and **CACTACTTCCCCATCTCCC** (reverse) sections of the gene, yielding a 350 base-pair (bp) PCR fragment (figure 5.13). To identify the –34 C>T variant, **BsmI (Fermentas Mva1269I)** enzyme, recognition sites **5'..GAATGCN↓…3'** (forward) an **3'…CTTAC↑GN...5'** (reverse), was used. For position –15 variants, **AciI (Fermentas SsiI)**, recognition sites **5'..C↓CGC…3'** (forward) strand and **3'..GGC↑G...5'** (reverse), was used. The digestion products were then analyzed using gel electrophoresis (figures 5.14 and 5.15). In the normal samples, the process yielded two fragments of approximately 275 bp and 75 bp each for position –34, and 256 bp and 84 bp for position –15. In addition to the above, the heterozygous states yielded a third uncut fragment of approximately 350 bp. Only one fragment was produced in the enzyme digest assay of the homozygous samples since they lacked recognition sites.

The four common **mitochondrial** DNA mutations, A1555G, A3243G, A7511C and A7445G were screened for using Restriction Fragment-Length Polymorphism (PCR–

RFLP) analysis. For **Waardenburg syndrome** genomic DNA was first screened by SSCP and hetero-duplex analysis. The first 8 exons of *PAX3* (but not exon 9 and 10) and all of *MITF* genes were analyzed. Samples showing shifts were then sequenced using an ABI 377 sequencer. Standard mutation detection methods were used as according protocols of the department of human genetics at the St Mary's Hospital, Manchester **(Tassabehji et al., 1995).**

A) *GJB2*

Sequencing

Primers

Cx26F 5' –GTT CTG TCC TAG CTA GTG ATT CC– 3'

Cx26R 5' –TGA GCA CGG GTT GCC TCA TCC– 3'

PCR protocol:

94°C x2min

40 cycles of: 94 °C x 30 sec

65 °C x 1 min

72 °C x1 min

Sequencing kit:

BigDye version 3.1 dye terminator cycle sequencing kit, Applied Biosystems.

Sequencing protocol:

94°C x2 min

30 cycles of: 94 °C x 10 sec

50 °C x 10 sec

60 °C x 4 min

Sequence analysis systems used:

ABI377 and ABI 3100 from Applied Biosystems

Restriction Enzyme Digest

PCR Primers

Forward CATGCTTGCTTACCCAGAC

Reverse CACTACTTCCCCATCTCCC

PCR protocol:

94°C x 3 min

35 cycles of: 94 °C x 30 sec

58 °C x 30 sec

72 °C x 30 sec

72°C x 10 min

Enzymes:

Fermentas SsiI **(AciI)**	Position –15	5'..C↓CGC…3'
		3'..GGC↑G...5'
Fermentas Mva1269I **(BsmI)**	Position –34	5'..GAATGCN↓…3'
		3'…CTTAC↑GN...5'

Protocols for digestion:

AciI:

10µl PCR product

17.75µl H_2O

2µl Buffer

0.25µl enzyme

Total reaction volume 30µl

Incubate at 37^0C for 12 hours

BsmI:

10µl PCR product

17.75µl H_2O

2µl Buffer

0.25µl enzyme

Total reaction volume 30µl

Incubate at 37^0C for 3 hours

B) Waardenburg Syndrome: *PAX3* and *MITF*

PCR, Hetero-duplex analysis, SSCP, sequencing as per protocol, department of human genetics, St Mary's Hospital, Manchester UK. (Tassabehji et al., 1995).

C) Multiplex PCR Amplification for *GJB6*-D13S1830: We screened the deletion of *GJB6* (D13S1830) using the method described by Wu, et al. (Wu, Kenna et al. 2003). Polymerase chain reaction (PCR) was used to amplify DNA fragments simultaneously with each of the three sets of primers in a multiplex state.

D) Restriction Fragment-Length Polymorphism (PCR–*RFLP)* analysis for *mtDNA* mutations (A1555G, A3243G, A7445G, and T7511C):

To detect each of the four mtDNA mutations, PCR was used to amplify mtDNA fragment encompassing the mutation site. This was followed by digestion with a

restriction endonuclease that differentially cleaves PCR products containing normal versus mutant sequences. Digestion products were then electrophoresed through 2% agarose gels. We screened for the *12SrRNA* A1555G and tRNA $^{Ser\ (UCN)}$ A7445G mutation using the method described by Pandya A. et al. (Pandya, Xia et al. 1999). For the T7511C mutation, the method described by Sue et al. (Sue, Tanji et al. 1999) was used.

To detect the presence of the A1555G mutation, the PCR fragment was cut with *BsmA*I. The PCR product of 1605 bp is digested in individuals without A1555G to yield three bands of sizes 1106, 293, and 206 bp respectively. Individuals with the A1555G mutation lack the digestion site, yielding two bands of 1399 bp and 206 bp. For the detection of the mtDNA A7445G mutation, the PCR fragment was digested with the restriction enzyme *Xba*I. In unaffected subjects, the digestion normally results in two 400 and 262 bp sized bands. The A7445G mutation leads to the loss of the *Xba*I cutting site, resulting in a single 662 bp size band. To identify the A3243G mtDNA mutation, PCR was performed with the following primers: 5'- GCC TTC CCC CGT AAA TGA TA-3' and 5'- AGG TTG GCC ATG GGT ATG T-3' using standard PCR conditions. Digestion of the PCR product was carried out using the restriction enzyme *Apa*I. The presence of mt DNA A3243G leads to the cleavage of the 161 bp PCR product into two fragments of sizes 87 bp and 74 bp respectively. The A3243G mutation can further be confirmed by bi-directional sequencing.

To screen the T7511C mtDNA mutation, we amplified a 226 bp fragment using primers corresponding to nucleotide positions 7397-7417 "forward" and 7633-7613 "reverse". The mutant mtDNA creates a novel *Mbo*II restriction site, which can be

detected by PCR-RFLP analysis. The wild-type 226 bp PCR product is cleaved into two 196 bp and 30 bp sized fragments respectively, whereas the T7511C mutation leads to cleavage of the PCR product into three fragments of 120 bp, 76 bp and 30 bp size respectively (Sue, Tanji et al. 1999).

4.6 DATA ANALYSIS

4.6.1 Mapping techniques used for epidemiological analysis

Using GIS and SA Explorer the addresses of origin, or ancestral home, of each participant were analysed. Two methods of geographical display were used, choropleth mapping (including the modification of demographic mapping) and map-on-map techniques.

In choropleth mapping, the occurrence of observations, that is the number of deaf participants, was plotted within the municipal boundaries. Because the sparsely populated areas tend to attract attention during interpretation, the modification of demographic mapping was also applied. Since clusters on a choropleth map chiefly represent un even distribution of the populations at risk, spot-clusters on a demographic map, which are computer generated, represent true clustering. Using GIS, cartograms relating population density to the occurrence of deaf participants were generated.

Map-on-map techniques were chosen because they help to bring the disease-observation (deafness) into visual apposition with the various risk factors to significant hearing loss (appendix 6). Maps were superimposed and clusters analysed. Risk factors analysed for included consanguinity of parents, neonatal hypoxia, family

history of hearing loss, childhood illness, neonatal and childhood illness and trauma. A retrospective cohort, comprising all 184 participants from both schools of the Deaf, was assessed for risk factors for hearing loss (appendix 6). The results were then analysed. The null hypothesis for this phase of the study is that significant childhood hearing loss in the Limpopo Province of South Africa has no relationship with the geographical area of origin, or ancestral home area.

4.6.2 Statistical Analysis

The main statistical package was STATA. To a lesser extent GIS and Map Info were also used. The data generated in this study was analysed using statistical tests in order to establish the significance of the results obtained. Below is a summary of the various methods used to analyze this data at each stage of the study.

Phase 1 - GIS system

- STATA
- StatisticsSA packages of census
- Municipal Demarcation Board – SA Explorer Package
- Eye-balling the geographical displays in the first instance
- Correlating using congruent choropleth maps of different variables
- Computer-generated cluster-display to estimated populations-at-risk

Phase 2 - STATA

- Two-by-two tables,
- tests of association,
- binary logistic regression analysis and Chi-square estimation
- Calculation of odds ratios
- Molecular methods of mutation detection and analysis as indicated

4.6.3 The Null Hypothesis (H_0)

The null hypothesis in this study was that there was no high-risk group or population for the development of genetic recessive non-syndromal sensori-neural hearing loss (NSSNHL) in the Limpopo Province of South Africa. Although the null hypothesis is generated to be rejected, it is importanc for testing the research hypothesis (H_1).

CHAPTER 5: RESULTS

5.1 Demographic Information of Subjects

5.1.1 Phase I

A total of 361 records were obtained for analysis (table 5.1), 86 from Bosele and 275 from Tshilidzini. The participants ranged in age from 2 to 24 years, with the majority (90.5%) in the 4 to 17 year age range (age calculated up to 31 December 2000).

Table 5.1: Demographic Information on subjects, Phase 1

	Tshilidzini, n = 275		**Bosele, n = 86**		**Total, n = 361**	
	Frequency	Percent	Frequency	Percent	Frequency	Percent
Age in years						
<5	28	10.2	0	0	17	4.7
5-9	97	35.3	25	29.1	116	32.1
10-14	99	36.0	34	39.5	140	38.8
15-19	40	14.5	20	23.3	67	18.6
20+	1	0.4	5	5.8	9	2.5
Unknown age	10	3.6	2	2.3	12	3.3
Sex						
Male	148	53.8	35	40.7	183	50.7
Female	127	46.2	51	59.3	178	49.3
Language group						
Venda	198	72.0	0	0	198	54.8
Pedi/N. Sotho	39	14.2	80	93.0	119	33.0
Tsonga	38	13.8	0	0	38	10.5
Swati	0	0	6	7.0	6	1.7

The mean age of Phase 1 the participants was 11.06 years, SD 4.27 and variance of estimation 18.22 years. For Bosele School, the mean age was 12.88 years, SD 4.26, variance 18.18 years. For Tshilidzini school, the average age was 10.48 years, SD 4.11, variance 16.90 (figure 5.1a-c). Overall, more than half the subjects were of the Venda language group, 54.8%, while the Sepedi or Northern Sotho language groups comprised 33% of the study population (table 5.1).

School by school analysis revealed 72% of the participants from Tshilidzini to be of the Venda language group while more than 90% of the participants from Bosele were of the Sepedi or Northern Sotho language group (table 5.1)

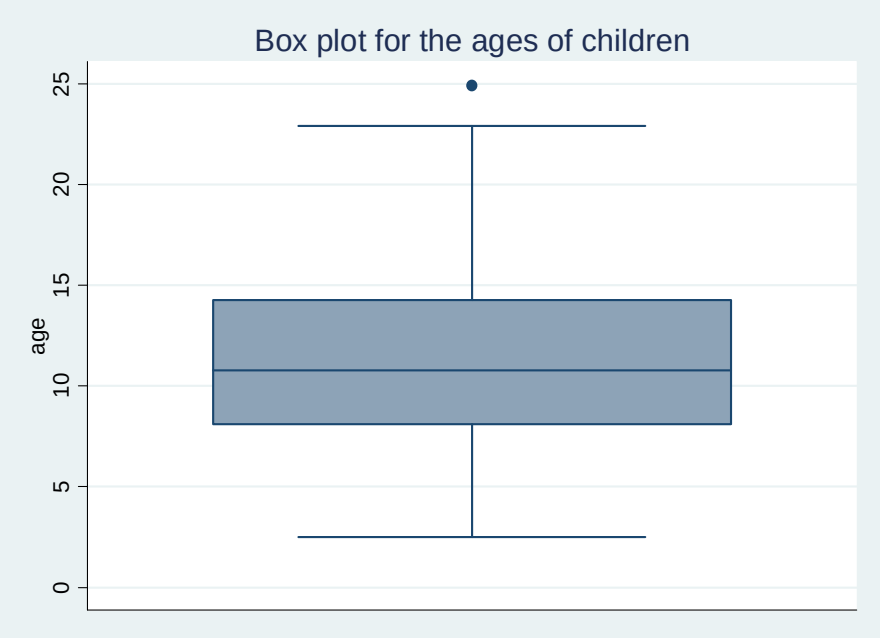


Figure 5.1a: Box and whisker plot showing the ages (in years) of the participants, Phase 1, both schools.

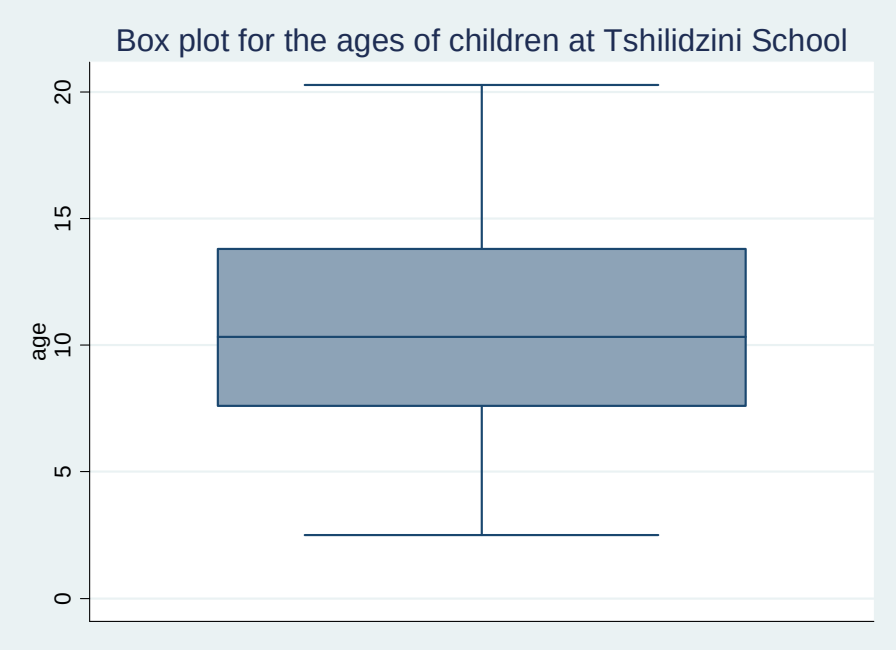


Figure 5.1b: Box and whisker plot showing the ages (in years) of the participants, Phase 1, Tshilidzini School.

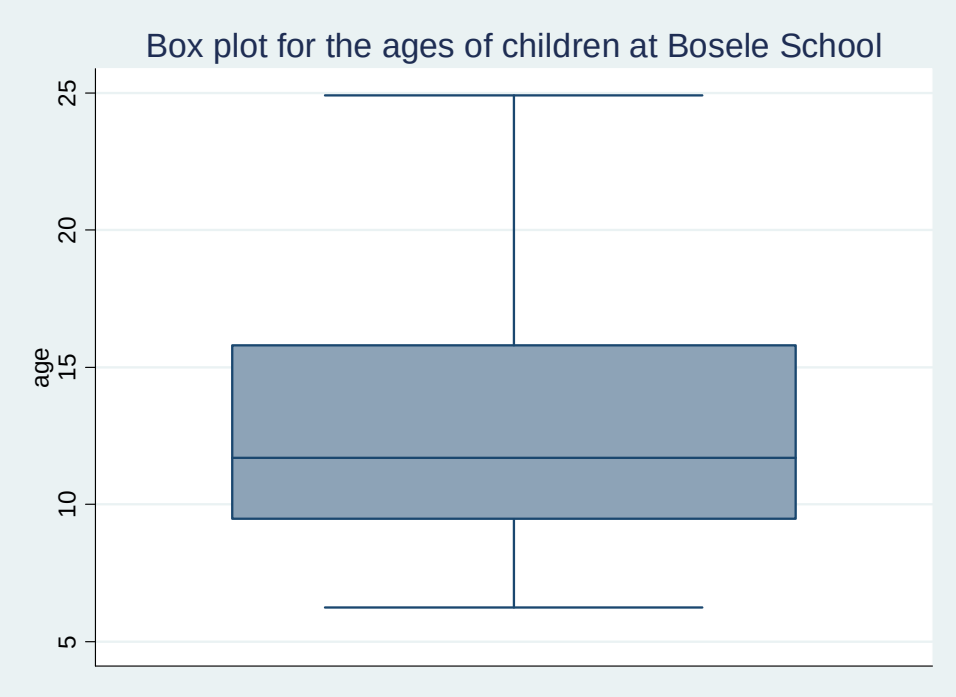


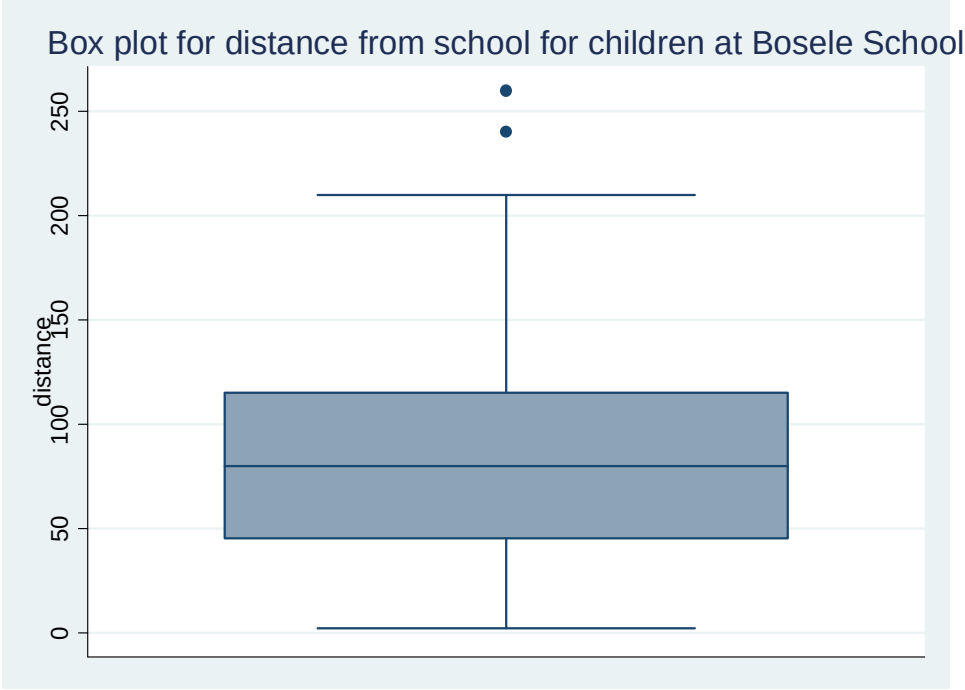
Figure 5.1c: Box and whisker plot showing the ages (in years) of the participants, Phase 1, Bosele School.

There was an almost equal sex distribution (table 5.1) with a male to female ratio of 1:0.97 overall. Analysis by school showed a male preponderance in Tshilidzini with a male to female ratio of 1: 0.68 and a female preponderance in Bosele with a male to female ratio of 1: 1.46.

The overall average distance from school to the children's homes (figures 5.2a-c) was 82.33 kms, with a standard deviation of 66.50 and the variance 4422.89 kms, showing that the homes were highly dispersed. For Bosele School, the mean distance from school was 91.93 kms (SD 56.05, variance 3141.45 kms), while for Tshilidzini School, the average distance was 78.65 kms, with a SD of 69.86, and a variance of 4880.92 kms.

Mean distance 82.33 kms, SD 66.50, Variance 4422.89 kms

Figure 5.2a: Box and whisker plot showing the distance of the participants' homes from school (in kms) , Phase 1, both schools.



Mean distance 91.93 kms, SD 56.05, Variance 3141.45 kms

Figure 5.2b: Box and whisker plot showing the distance of the participants' homes from school (in kms) , Phase 1, Bosele School.

Mean distance 78.65 kms, SD 69.86, Variance 4880.92 kms

Figure 5.2c: Box and whisker plot showing the distance of the participants' homes from the school in kms , Phase 1, Tshilidzini School.

The reported age of detection of hearing loss by the parents or caregivers showed differing patterns between the two schools (table 5.2). Whereas there were no missing data from Bosele School, 23% of the participants from Tshilidzini School had no registered age of detection. On the other hand, 60% of the parents from Tshilidzini School reported that the subjects' onset of deafness was detected before 4 months of age, compared to Bosele where only 9% of the subjects' hearing loss was detected before 4 months of age. Bosele also had a larger proportion of participants detected at a much older age, with 27% between 10-12 months of age and 23% between 19-24 months of age.

Overall, hearing loss was detected in almost two thirds (63.7%) of the subjects by the age of 6 months, and over 10% after 2 years of age (tables 5.3). Both schools had a significant number of participants in whom the hearing loss was detected late, with

8% of Tshilidzini and 9% of Bosele participants detected after 24 months of age. Overall, over 4% participants were detected after 3 years of age.

Table 5.2: Age of detection, by parents, of hearing lossamong subjects, Phase 1

	Tshilidzini		Bosele		Total	
Age of detection of deafness in months	Frequency	Percent	Frequency	Percent	Frequency	Percent
<4	165	60	8	9	173	58
4-6	3	1	14	16	17	5.7
7-9	2	1	8	9	10	3.4
10-12	7	3	23	27	30	10.1
13-18	4	1	5	6	9	3
19-24	8	3	20	23	28	9.4
25+	22	8	8	9	30	10.1
Unknown age	64	23	0	0	64	21.5
Total	275		86		361	100.0

5.1.2 Phase 2

This phase included 182 South African school children with non-syndromic hearing loss and two participants with Waardenburg syndrome. Their age ranged from 5 to 24 years, with 97% of the participants under 19 years of age. The overall mean age was 12.61 years (SD 3.84, variance 14.77 years) for the whole cohort of phase 2. For Bosele, the mean age was 12.91 years, with a SD of 4.13, and variance of 17.04 years. On the other hand, for Tshilidzini the mean age was 12.90627, SD 4.13, and variance 17.04 years (table 5.3, figure 5.3). There was a strong female preponderance with a male to female ratio of 1:1.9 overall. Analysis by school showed a male to female ratio of 1:2.4 in Tshilidzini and a male to female ratio of 1:1.4 for Bosele. This is summarized in table 5.3.

Overall, the Venda and Pedi/Northern Sotho speaking communities were nearly equally represented, 46% Venda and 44% Pedi/N. Sotho, while 7% were Tsonga

speaking. School by school analysis showed however that there was no Venda speaking subject in Bosele School, a school that had 93.5% Pedi/N. Sotho speaking subjects. On the other hand over 78% of the subjects from Tshilidzini were of the Venda language speaking group, while only 9% were Pedi/N. Sotho (table 5.3).

Table 5.3: Demographic Information of participants, Phase 2

	Tshilidzini, n = 107		**Bosele, n = 75 (+2)***		**Total, n = 182 (+2)***	
	Frequency	Percent	Frequency	Percent	Frequency	Percent
Age in years						
<5	0	0	0	0	0	0
5-9	33	30.8	22	29.3	55	30.2
10-14	43	40.2	29 (+1)*	38.7 (39.0)*	72 (+1)*	3.9.6 (39.7)*
15-19	26	24.3	19 (+1)*	25.3 (26.0)*	45 (+1)*	24.7 (25.0)*
20+	1	0.9	5	6.7	6	
Unknown age	4	3.7	0	0	4	
Sex						
Male	31	29.0	31 (+1)*	41.6	62 (+1)*	34 (34.2)*
Female	76	71.0	44 (+1)*	58.4	120 (+1)*	65.9 (65.8)*
Language group						
Venda	84	78.5	0	0	84	46.2
Pedi/N. Sotho	10	9.4	70 (+2)*	93.5	80 (+2)*	44.0 (44.6)*
Tsonga	13	12.1	0	0	13	7.1
Swati	0	0	5	6.5	5	2.7

*(+2) includes the two participants with Waardenburg syndrome Type 1

Table 5.4: Age of detection of hearing loss among the participantss, Phase 2

	Tshilidzini		**Bosele**		**Total**	
Age of detection of deafness in months	Frequency	Percent	Frequency	Percent	Frequency	Percent
<4	58	54.2	8 (+2)*	10.7 (13.0)*	66 (+2)	36.3 (37.0)*
4-6	2	1.9	13	17.3	15	8.2
7-9	1	0.9	8	10.7	9	5.0
10-12	1	0.9	21	28.0	22	12.1
13-18	2	1.9	4	5.3	6	3.3
19-24	0	0	17	22.7	17	9.3
25+	6	5.6	4	5.3	10	5.5
Unknown age	37	34.6	0	0	37	20.3
Total	107	100.0	75 (+2)*	100.0	182 (+2)*	100.0

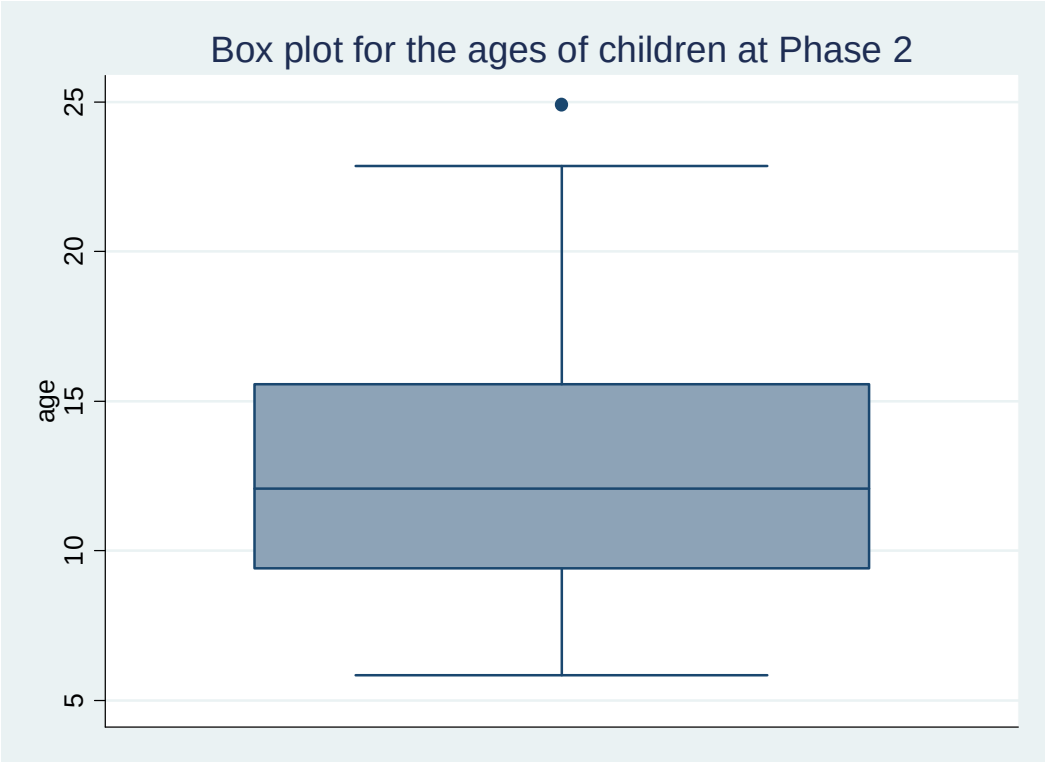


Figure 5.3: Box and whisker plot showing the ages (in years) of all the participants, Phase 2, both schools.

Again the reported age of detection of hearing loss showed differing patterns between the two schools (table 5.4). Over one third of the subjects from Tshilidzini School had no registered age at detection, while none of the Bosele School participants lacked data. On the other hand, 54% of the parents from Tshilidzini School and 13% from Bosele reported that the participants' deafness was detected before 4 months of age. This figure rises to 21% by 6 months of age for Bosele. Of concern are the high numbers of participants from Bosele School detected late, 28% between 10 and 12 months, and 22.7% between 19 and 24 months of age.

5.2 GEOGRAPHICAL DISTRIBUTION OF HEARING LOSS

5.2.1 Phase I

Two districts carried the highest number of subjects, with 67.9% of the Tshilidzini School participants coming from Vhembe district and 62.8% of Bosele School subjects coming from Sekhukhune district (Table 5.5, figure 5.4). Analysis by municipality and school showed the top four municipalities for each school accounted for the majority of participants, with over 74% from Tshilidzini School and just over 58% from Bosele School (table 5.9, figure 5.5).

Table 5.5: Geographical distribution of hearing loss according to district, Limpopo Province, both schools Phase 1

	Tshilidzini, n = 275		**Bosele, n = 86**		**Total, n = 361**	
	Frequency	Percent	Frequency	Percent	Frequency	Percent
Sekhukhune	1	0.4	54	62.8	55	15.2
Mopani	27	9.8	0	0	27	7.5
Vhembe	187	67.9	1	1.2	188	52.1
Capricorn	31	11.3	12	13.9	43	11.9
Waterburg	1	0.4	5	5.8	6	1.7
Unknown	27	9.8	8	9.3	35	9.7
Cross-border	1	0.4	6	7.0	7	1.9
Total	275	100.00	86	100.00	361	100.0

These included Thulamela, Makhado, Mutale and Greater Giyani for Tshilidzini, and Makhudatamaga, Greater Tubatse, Greater Groblersdal and Fetakgomo for Bosele. The combined data from both schools showed Thulamela and Makhado municipalities to have the highest number of subjects, over 44% of the study population (table 5.7). The geographical distribution of hearing loss according to language group and family history of hearing loss is summarized in figures 5.12a and 5.12b below.

5.2.2 Phase 2

Among phase 2 subjects, Vhembe and Sekhukhune districts still carried the highest number of participants, 71% and 61.3% respectively (table 5.6 and figure 5.9). When normalized to the total African population, Vhembe district comes up with the highest frequency with 29.95 deaf participants per 100,000 African population and Sekhukhune second at 14.80 deaf subjects per 100,000 African populations (table 5.9). Capricorn and Mopani districts had the lowest representation with 6.51 and 5.83 deaf participants per 100,000 African populations respectively.

Table 5.6: Geographical distribution of hearing loss according to district, Limpopo Province, both schools Phase 2

	Tshilidzini, n = 107		**Bosele, n = 75**		**Total, n = 182**	
	Frequency	%	Frequency	%	Frequency	%
Sekhukhune	0	0	46	61.3	46	25.3
Mopani	12	11.2	0	0	12	6.6
Vhembe	76	71.0	0	0	76	41.8
Capricorn	5	4.7	11	14.7	16	8.8
Waterburg	0	0	5	6.7	5	2.7
Unknown	13	12.1	8	10.7	21	11.5
Cross-border	1	0.9	5	6.7	6	3.3
Total	107		75		182	

Analysis by municipality came up with interesting results (table 5.10, figure 5.11). Mutale municipality carried the highest normalized frequency for Tshilidzini School, 13.14 per 100,000 African population, while Makhudutamaga carried the highest normalized frequency for Bosele School at 6.71 per 100,000 African population, with Fetakgomo coming in a close second with 6.22 per 100,000 African population (table 5.10, figure 5.11).

A comparison of municipal wards considered high risk areas for hearing loss, which was defined as approximately 5% of the cohort, revealed three common areas for both

phases of the study. These, coded 18C, 18G and 19B, are wards 11-15 of Thulamela (NP343), wards 31-35 of Thulamela (NP343) , and wards 6-10 Makhado (NP344) respectively (table 5.7). There were two areas that were picked up as high risk areas during one phase of the study and are marked in red where they are not considered high risk areas (table 5.7).

Table 5.7: Comparison of municipal wards considered high risk areas for hearing loss

Address (MDB-Wd code)	Phase 1 frequency	Address (MDB-Wd code)	Phase 2 frequency
(10C) NP03A2 wards 11-15	*(12)*	10C	10
18C NP343 wards 11-15	23	18 C	14
18F NP343 wards 26-30	10	*(18F)*	*(6)*
18G NP343 wards 31-35	17	18G	10
19B NP344 wards 6-10	17	19B	9
Total	67		43

High risk= approx. 5% of cohort i.e. equal to or greater than 17 hearing impaired participants (phase1) and 9 hearing impaired participants (phase2) per municipal ward.

Table 5.8: Municipalities with highest geographical distribution of hearing loss, both schools Phase I

Municipal Code	Municipality	Frequency	Percent
CBLC4	Greater Groblersdal	10	2.8
CBLC5	Greater Tubatse	12	3.3
NP03A2	Makhudutamaga	23	6.4
NP03A3	Fetakgomo	6	1.7
NP331	Greater Giyani	22	6.1
NP342	Mutale	25	6.9
NP343	Thulamela	93	25.8
NP344	Makhado	67	18.6
NP351	Blouberg	5	1.4
NP353	Molemole	13	3.6
NP354	Polokwane	18	5.0
NP355	Nkumpi	5	1.4
NP367	Mogalakwena	6	1.7

Table 5.9: Municipalities showing the highest geographical distribution of hearing loss according to school, Phase I

Tshilidzini			Bosele		
Municipality	**Frequency**	**percent**	**Municipality**	**Frequency**	**Percent**
Greater Giyani	22	7.9	Greater Groblersdal	10	11.6
Mutale	25	9.0	Greater Tubatse	12	14.0
Thulamela	93	33.3	Makhudutamaga	22	25.6
Makhado	67	24.0	Fetakgomo	6	7.0
Total	**207**	**74.2**	**Total**	**50**	**58.2**

Table 5.10: The geographical distribution of Hearing Loss for both schools according to Municipality, Normalized to African population, Phase 2

District	**Municipal code**	**Frequency**	**Percent**	**Total African Population**	**Frequency Normallized : per 100,000 African Population**
Sekhukhune	CBLC3	3	1.65	93963	1.06
Sekhukhune	CBLC4	8	4.40	211511	3.78
Sekhukhune	CBLC5	10	5.49	226325	3.75
Bohlabela	CBLC6	1	0.55	536370	0.18
	JHB	2	1.10		
	MP312	1	0.55	176596	0.56
	MP314	1	0.55	32389	3.08
Bohlabela	MP321	2	1.10	54754	3.65
Sekhukhune	NP03A2	18	9.89	268132	6.71
Sekhukhune	NP03A3	6	3.30	96403	6.22
Mopani	NP331	8	4.40	215757	3.7
Mopani	NP332	1	0.55	199429	0.5
Mopani	NP333	2	1.10	328948	0.6
Mopani	NP334	1	0.55	96920	1.03
Vhembe	NP341	1	0.55	28419	3.51
Vhembe	NP342	9	4.95	68454	13.14
Vhembe	NP343	42	23.08	532091	7.89
Vhembe	NP344	24	13.19	443319	5.41
Capricorn	NP351	1	0.55	149961	0.66
Capricorn	NP353	2	1.10	105440	1.89
Capricorn	NP354	9	4.95	393450	2.28
Capricorn	NP355	4	2.20	233409	1.71
Waterberg	NP367	5	2.75	273704	1.82
Unknown		21	11.54		
Total		**182**	**100.00**		

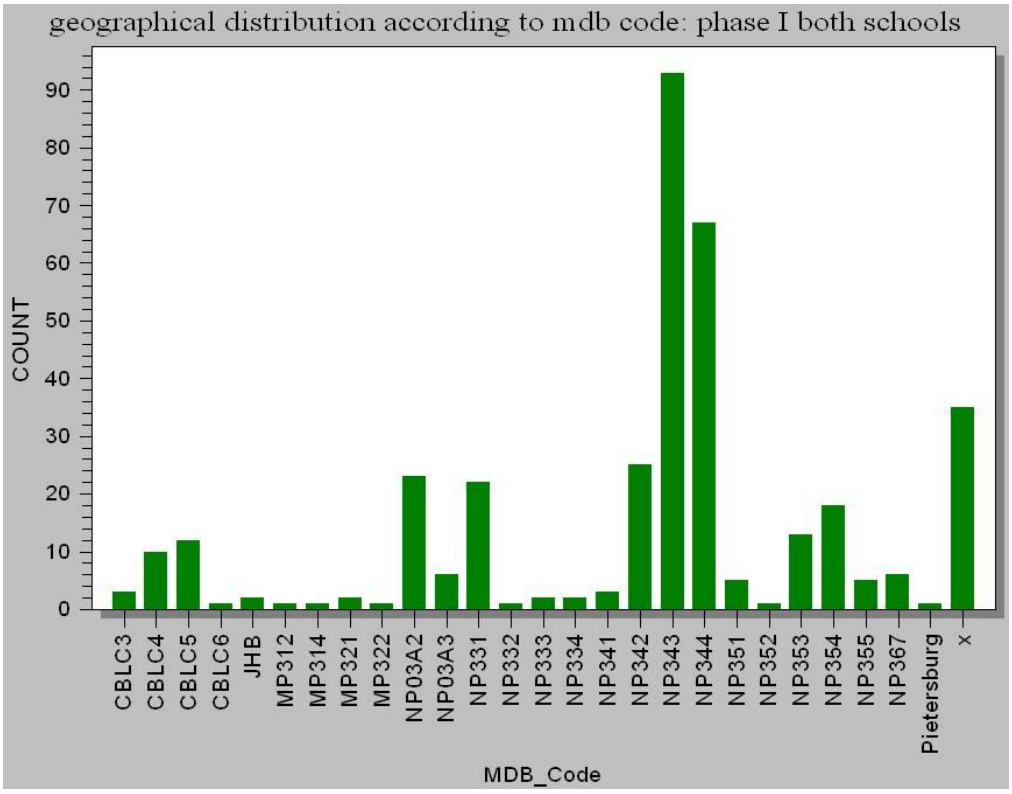


Figure 5.4: Geographical distribution of hearing loss according to municipality, Limpopo Province, both schools Phase 1

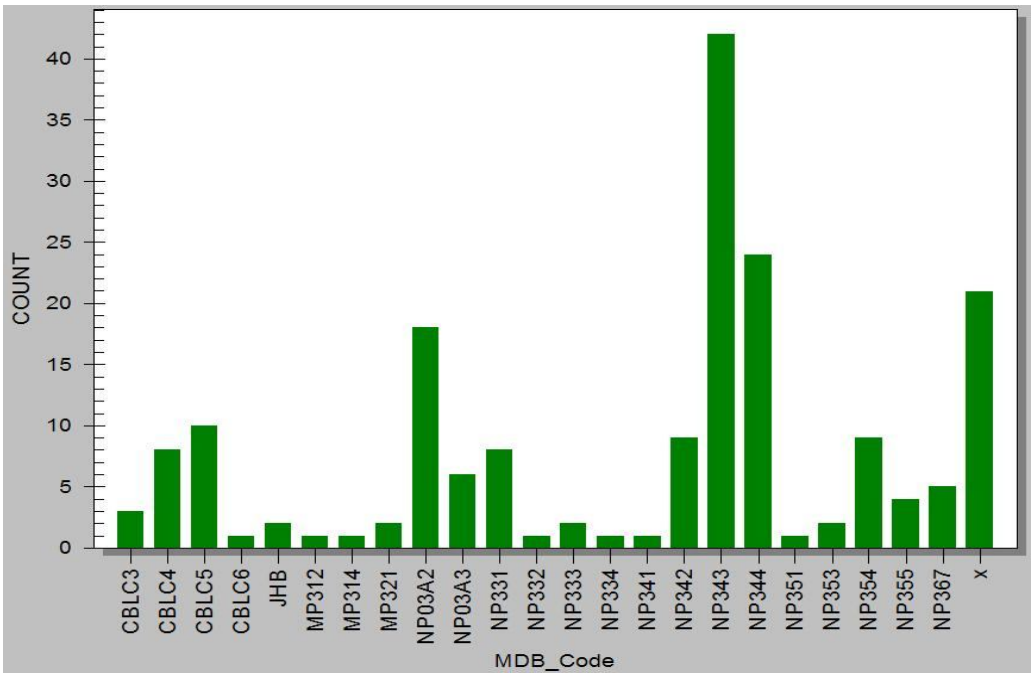


Figure 5.5: Geographical distribution of hearing loss according to Municipality, Limpopo Province, both schools Phase 2

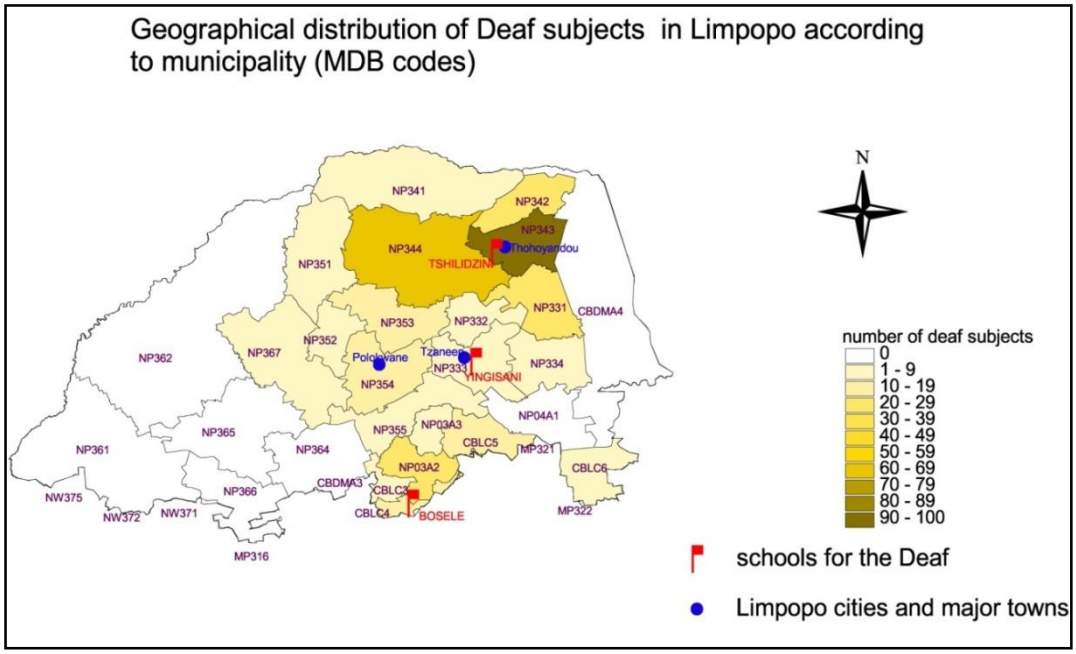


Figure 5.6: Spatial distribution of Hearing Loss according to municipality, Limpopo Province Phase 1

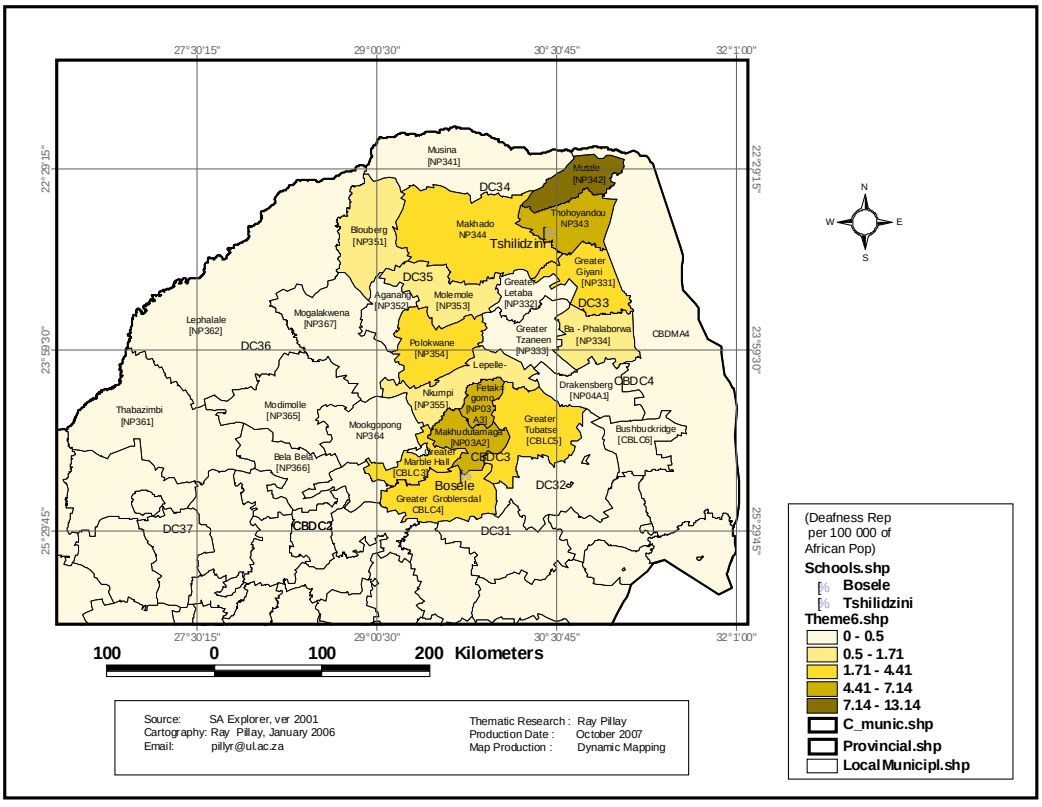


Figure 5.7: Spatial distribution of Hearing Loss according to Municipality, Limpopo Province, Normalized to African Population, Phase 2

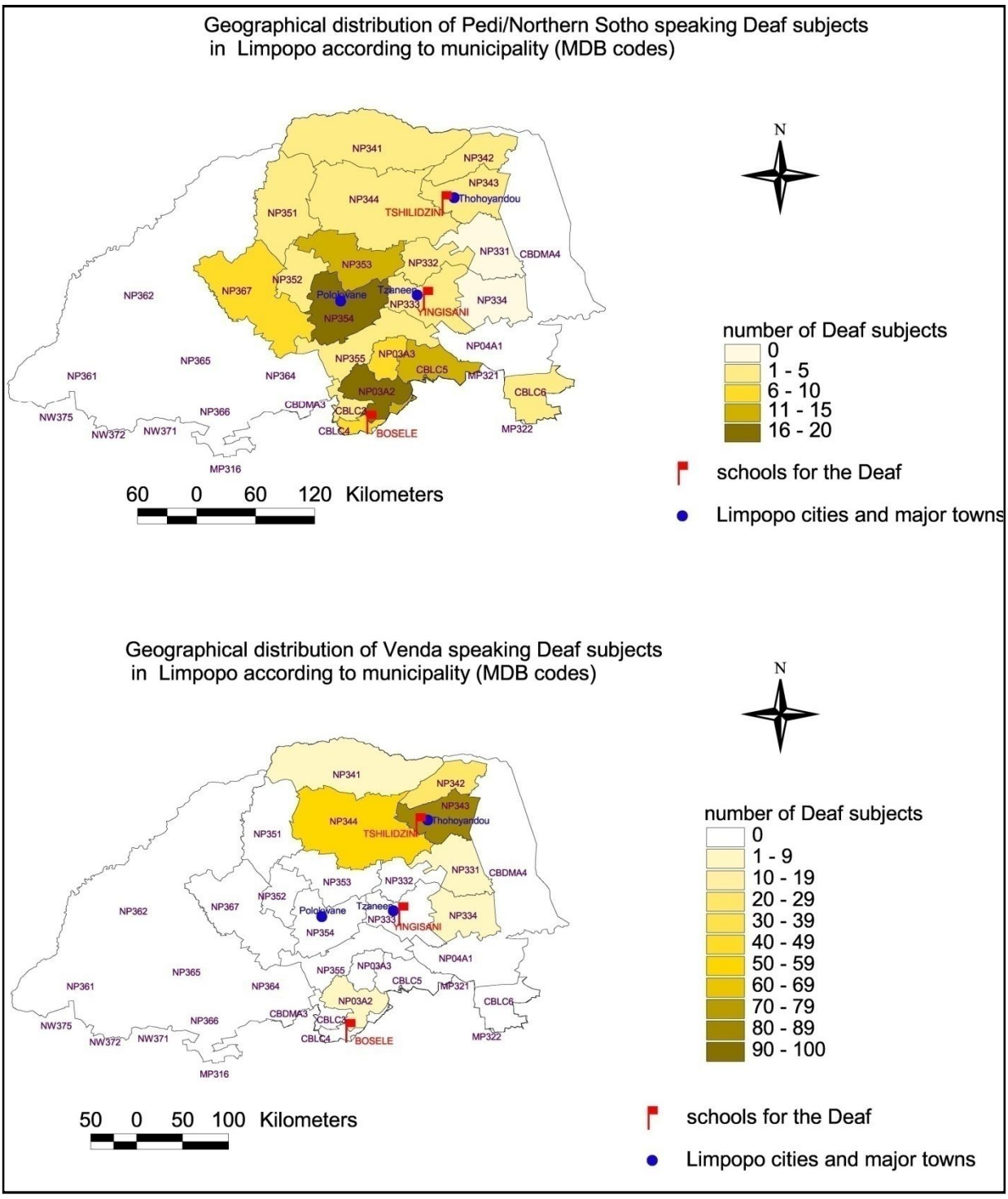
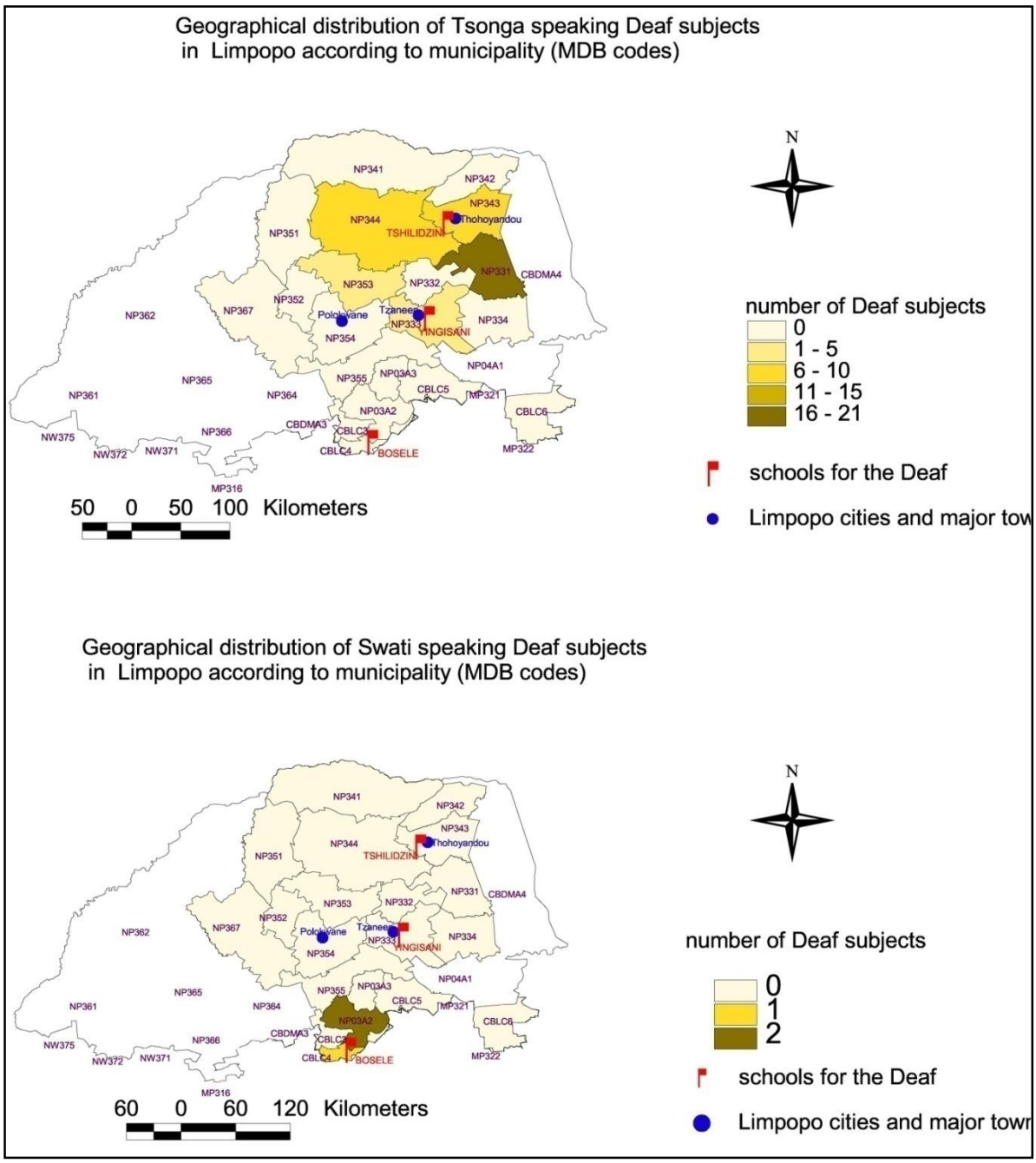


Figure 5.8: Spatial distribution of Hearing Loss in the Limpopo Province according to language group, Phase 1



5.9: Spatial distribution of Hearing Loss in the Limpopo Province according to language group, Phase 1

5.3 TYPE AND DEGREE OF HEARING IMPAIRMENT

The hearing loss was only assessed during the second phase of the study, that is, on the 184 participants who underwent mutation screening. The hearing loss was found to be sporadic in 136 subjects and familial in 48 subjects.

5.3.1 Tympanometry and Transient otoacoustic emissions

Tympanometric results showed type A tympanograms in 71-73%, type B tympanograms in 4-7% of the participants (including one subject with a cleft soft palate). In 22-23% of the participants tympanometry could not be performed for reasons indicated in tables 5.11-5.13.

Transient otoacoustic emissions could not be elicited in all but five participants who were initially tested. These five subjects were all from Tshilidzini School. Three of them were found to have normal hearing in the initial screening process and excluded from the study, while two were later confirmed to suffer from a mild form of cerebral palsy.

Table 5.11: Tympanometric results

Tympanograms	Left ear		Right ear		P-value
	Frequency	Percent	Frequency	Percent	
Type A	133	73	130	71	0.694
Type B	8	4	12	7	
No results	41	23	40	22	

Fisher exact test

Table 5.12: Cross tabulation of Tympanometric results between ears

		Tympanograms Right ear			P-value
		Type A	Type B	No Results	
Tympanograms Left ear	Type A	125	7	1	0.001
	Type B	3	5	0	
	No Result	2	0	39	

Fisher exact test

Table 5.13: Abnormalities for ear with abnormal or no tympanogram results

	Left ear		Right ear	
	Frequency	**Percent**	**Frequency**	**Percent**
Retracted ear drum	2	25	3	25
Impacted Wax	2	25	2	17
Otitis Media with Effusion	4	50	5	42
Foreign Body	-	-	1	8
Eczema	-	-	1	8

5.3.2 Audiometry

The overall results indicate that profound hearing loss was found in almost 75% of the subjects, the majority exhibiting flat 70.1% or sloping 23.4% audiograms, and commonly symmetrical 81.5%. Low frequency ascending audiograms were found in 6% of the participants, while one participant had a mid frequency u-shaped audiogram. Two of the participants with mild hearing loss of a flat configuration were found to have cerebral palsy, while the cleft palate patient had symmetrical flat moderate conductive hearing loss. The two participants with Waardenburg syndrome demonstrated profound hearing loss with flat audiograms. Three out of the five participants with combined visual and balance disorders had flat audiograms while the other two had sloping configurations. On the other hand, two of the four participants with balance problems only had sloping audiograms, with the rest flat. Vestibular tests could not be carried out due to lack of facilities as mentioned above. These findings are summarised in tables 5.14 to 5.21 below.

Table 5.14: Severity of hearing impairment, best ear average 0.5-4kHz, Tshilidzini, Phase 2

Severity	Freq	Percent	Conditions
Mild hearing loss	3	2.8	2 Cerebral Palsy, 1 cleft palate
Moderate hearing loss	2	1.8	
Severe hearing loss	22	20.6	
Profound hearing loss	80	74.8	
Total	**107**	**100.0**	

Table 5.15: Audiogram Configuration among subjects, Tshilidzini, Phase 2

Configuration	Freq	Percent	Conditions
Sloping	17	15.9	
Flat	81	75.7	2 Cerebral Palsy, 1cleft palate
Mid frequency U-shaped	1	0.9	
Low frequency Ascending	8	7.5	
Total	**107**	**100.0**	

Table 5.16: Asymmetry of Hearing Impairment among subjects, Tshilidzini, Phase 2

Asymmetry	Freq	Percent	Conditions
Yes	18	16.9	1cleft palate, 1Cerebral Palsy, 1partially sighted
No	89	83.1	
Total	**107**	**100.0**	

Table 5.17: Severity of hearing impairment, best ear average 0.5-4kHz, Bosele, Phase 2

Severity	Freq	Percent	Conditions
Severe hearing loss	20	26.0	
Profound hearing loss	57	74.0	2 Waardenburg syndrome
Total	**77**	**100.0**	

Table 5.18: Audiogram Configuration among subjects, Bosele, Phase 2

Configuration	Freq	Percent	Conditions
Sloping	26	33.8	2 visual/balance problems 2 balance problems
Flat	48	62.3	2 Waardenburg syndrome, 2 balance disorders, 3 visual/balance disorders
Low frequency Ascending	3	3.9	
Total	**77**	**100**	

Table 5.19: Asymmetry of Hearing Impairment among subjects, Bosele, Phase 2

Asymmetry	Freq	Percent	Conditions
Yes	16	20.8	1 visual/balance problems
No	61	79.2	4 visual/balance problems, 2 Waardenburg syndrome, 4 balance disorder
Total	**77**	**100.0**	

Table 5.20: Severity of hearing impairment, best ear average 0.5-4kHz, both schools, Phase 2

Severity	Freq	Percent	Conditions
Mild hearing loss	3	1.6	2 Cerebral Palsy, 1 cleft palate
Moderate hearing loss	2	1.1	
Severe hearing loss	42	22.8	
Profound hearing loss	137	74.5	2 Waardenburg syndrome
Total	**184**	**100.0**	

Table 5.21: Audiogram Configuration among subjects, both schools, Phase 2

Configuration	Freq	Percent	Conditions
Sloping	43	23.4	2 visual/balance problems 2 balance problems
Flat	129	70.1	2 Cerebral Palsy, 1cleft palate 2 Waardenburg syndrome 3 visual/balance disorder 2 balance disorder
Mid frequency U-shaped	1	0.5	
Low frequency Ascending	11	6.0	
Total	**184**	**100.0**	

Table 5.22: Asymmetry of Hearing Impairment among subjects, both schools, Phase 2

Asymmetry	Freq	Percent	Conditions
Yes	34	18.5	1cleft palate, 1Cerebral Palsy, 1partially sighted, 15 unknown, 1 visual/balance problems
No	150	81.5	4 visual/balance problems, 2 Waardenburg syndrome, 4 balance disorder
Total	**184**	**100.0**	

Table 5.23: Family History of hearing loss among subjects, Phase 2

Family history of hearing loss	**Frequency**	Percent
No	111	60.99
Yes	46	25.27
Unknown	25	13.74
Total	**182**	**100.00**

Table 5.24: Distribution of Family History of Hearing Loss according to Municipality, Limpopo Province, both schools, Normalized to African Population, Phase 2

Municipality	**Family History of hearing loss**				**Total African Popn**	**'Yes' group Normalized to African Popn (Per 100,000)**
	Total	**No**	**Yes**	**Unkown**		
Greater Marble Hall	3	1	2		93963	2.12
Greater Groblersdal	8	5	3		211511	1.41
Greater Tubatse	10	4	6		226325	2.65
Bushbuckridge	1	1	0		536370	0
JHB	1	0	1			
MP312 **	1	1	0		176596	0
MP314 **	1	1	0		32389	0
MP321 **	2	2	0		54754	0
Makhudutamaga	18	13	5		268132	1.86
Fetakgomo	6	4	2		96403	2.07
Greater Giyani	6	6	0		215757	0
Greater Letaba	1	1	0		199429	0
Greater Tzaneen	1	1	0		328948	0
Ba-Phalaborwa	1	0	1		96920	1.03
Musina	1	0	0	1	28419	0
Mutale	9	7	2		68454	2.92
Thulamela	38	31	7		532091	1.31
Makhado	17	9	8		443319	1.8
Blouberg	1	1	0		149961	0
Molemole	1	1	0		105440	0
Polokwane	9	5	4		393450	1.01
Nkumpi	4	2	2		233409	0.85
Mogalakwena	5	3	2		273704	0.73
Total	**144**	**99**	**45**	**1**		

**** cross-border municipalities**

5.4 AETIOLOGICAL INVESTIGATION OF HEARING DISORDERS

Determination of the aetiology of hearing loss in this community was based on school records for Phase 1, while questionnaires (appendices 2a, 2b and 3), clinical findings (appendix 5) and investigations including genetic analysis were used in Phase 2. The known risk factors were assessed for, including pre-natal, intra-partum, and post-partum events, paediatric infections and trauma, consanguinity, language group and a family history of hearing loss (appendix 6).

5.4.1 Family History of Hearing Loss Among the Participants

Phase 1 analysis showed that the highest numbers of participants with a history of family history of hearing loss was Makhado municipality (figure 5.6)

In Phase 2, there was a reported family history of hearing loss of 25.27% % among the participants (table 5.22). These included siblings and other close family relatives (table 5.23, 5.24, 5.25). Twenty-one participants had a deaf sibling while three subjects had a deaf parent. The majority of participants with a positive family history of hearing loss from Tshilidzini School were from two municipalities, Thulamela and Makhado, which had a total of fifteen participants out of eighty-two respondents (table 5.26). On the other hand the two municipalities from Bosele School with the highest numbers of participants with a family history of hearing loss were Greater Tubatse and Makhudutamaga, with eleven participants out of seventy-five participants (table 5.26, figures 5.7 & 5.8). When the results were normalized according to African population, Mutale municipality had the highest incidence among the Tshilidzini participants while Greater Tubatse had the highest among the participants from Bosele (table 5.26, figures 5.7, 5.8 & 5.9).

Forty-six participants reported a positive family history of hearing loss (tables 5.22, 5.23, 5.24). A brother was the most commonly affected sibling, reported among 13 or 28% of the participants, a sister reported among 8 or 17.39% of the subjects, while an uncle was reported among 8 or 17.39% of the participants. The results also show that among those participants with more than one close family member affected, none had two siblings affected (table 5.25). The two participants with Waardenburg Syndrome, while having a positive family history of hearing loss, reported the hearing loss only among the siblings. Out of the four siblings (one male, three female), the first-born girl was reported to have normal hearing but the rest were reported to be hearing impaired. The parents and both sets of grandparents were reported to have normal hearing. The rest of the family members were also reported to have normal hearing.

5.4.2 Consanguinity Among Parents

In Phase 2, fourteen respondents (7.7% of study group) reported consanguinity among the parents. Of these, thirteen were from Bosele School and one was from Tshilidzini School (table 5.29). When analysed by language group, the results showed that all these participants were of the Pedi/N. Sotho language group (table 5.31). Most of the Vendas did not respond to the question. Cross-tabulation with a family history of hearing loss yielded 8 participants, comprising 4.4% of the study population (table 5.30). Two of these participants had a brother with hearing loss, two had a sister with hearing loss, three had an uncle with hearing loss while one participant had a cousin with hearing loss (table 5.33). The three participants with more than one close relative with hearing loss reported a grandfather as the hearing impaired relative (table 5.34). The geographical distribution showed the highest incidence to be in Greater Groblersdal and Molemole municipalities (tables 5.27- 5.28, figures 5.10-5.12).

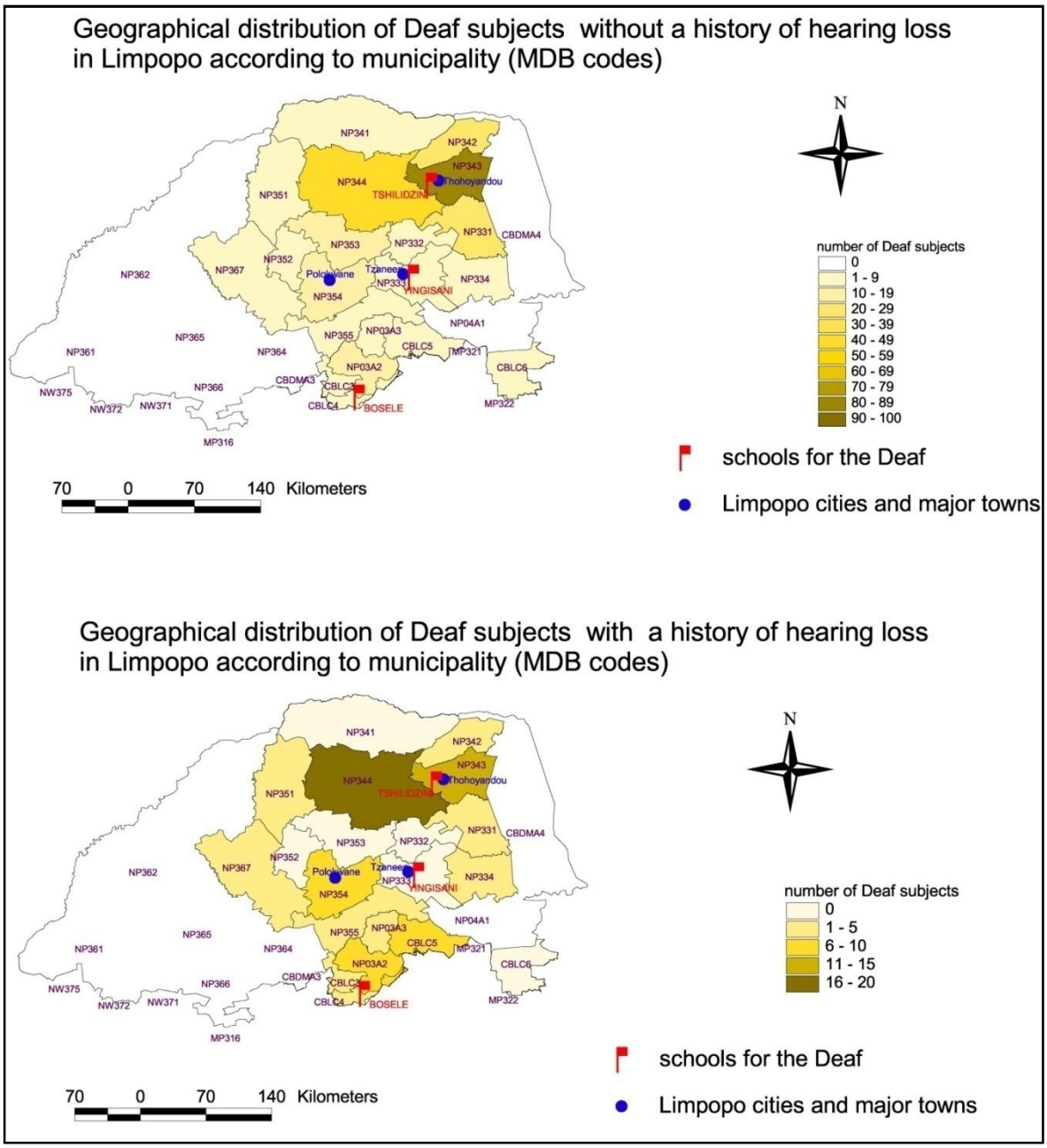


Figure 5.10: Spatial distribution of subjects according to Family History of Hearing Loss per local municipality, Limpopo Province, Phase1

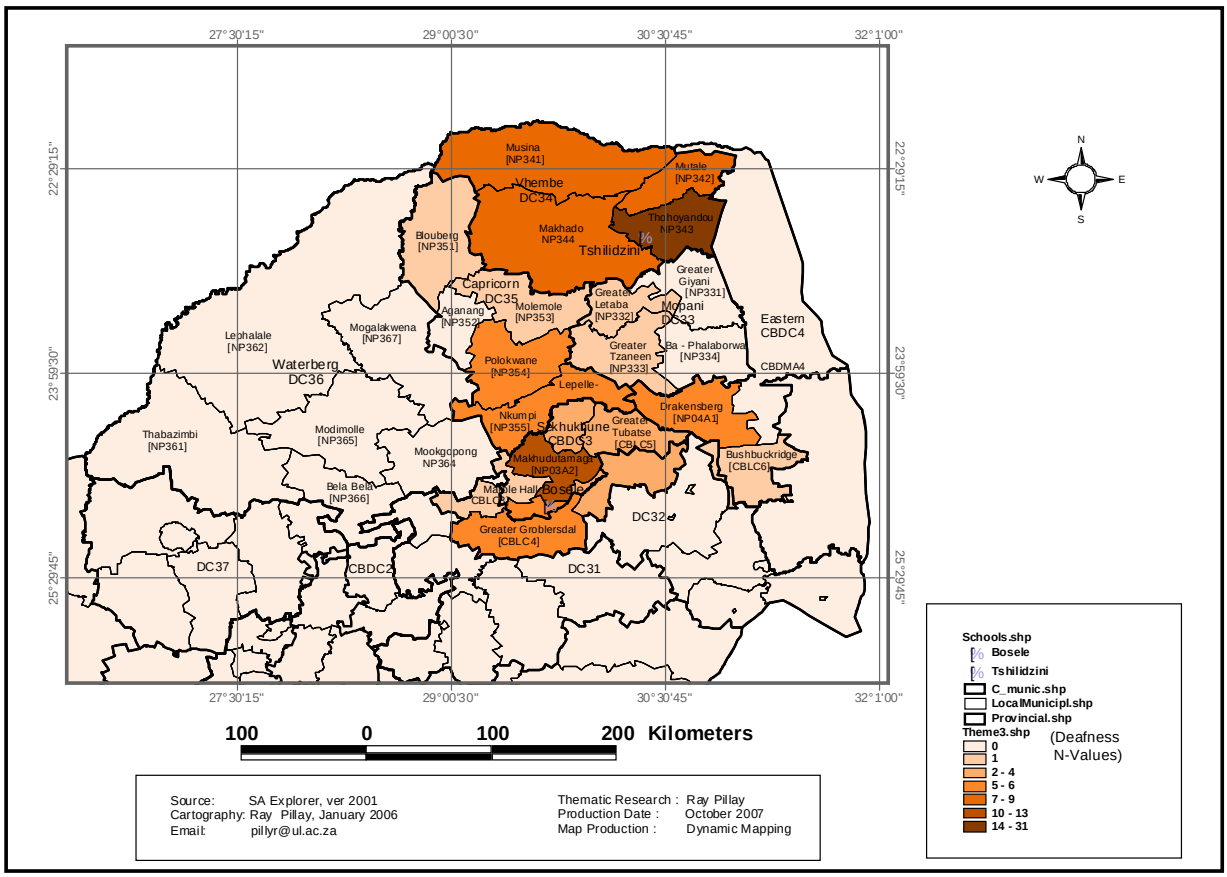


Figure 5.11: Spatial distribution of subjects without a Family History of Hearing Loss per Local Municipality, Limpopo province, Phase 2

Table 5.25: Cross tabulation of consanguinity of parents by municipality, Bosele School, Phase 2

Municipality	**Consangunity of Parents**		
	No	**Yes**	**Total**
Greater Marble Hall	2	1	3
Greater Groblersdal	4	4	8
Greater Tubatse	9	1	10
Bushbuckridge	0	1	1
JHB	1	0	1
MP312 **	1	0	1
MP314 **	1	0	1
MP321 **	2	0	2
Makhudutamaga	17	1	18
Fetakgomo	6	0	6
Blouberg	1	0	1
Polokwane	5	1	6
Molemole	1	3	4
Mogalakwena	4	1	5
Unknown	8	0	8
Total	**62**	**13**	**75**

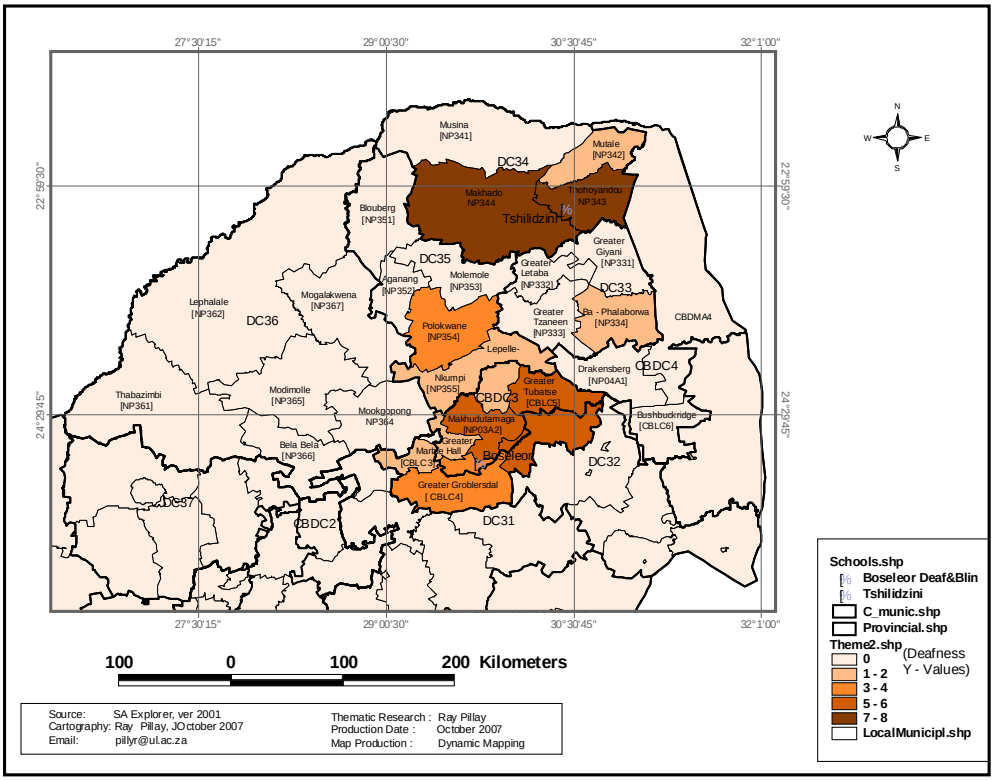


Figure 5.12: A Spatial distribution of subjects with a Family History of Hearing Loss per Local Municipality, Limpopo province, Phase 2

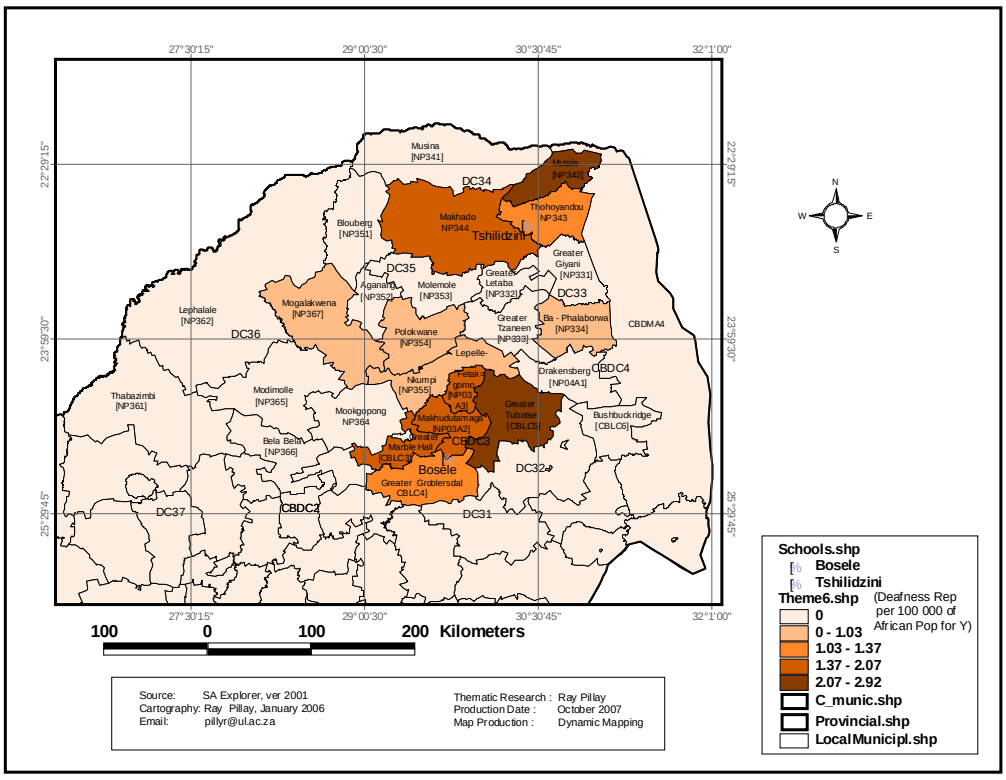


Figure 5.13: A Spatial distribution of subjects with a Family History of Hearing Loss per Local Municipality, Limpopo province, Normalized to the African Population, Phase 2

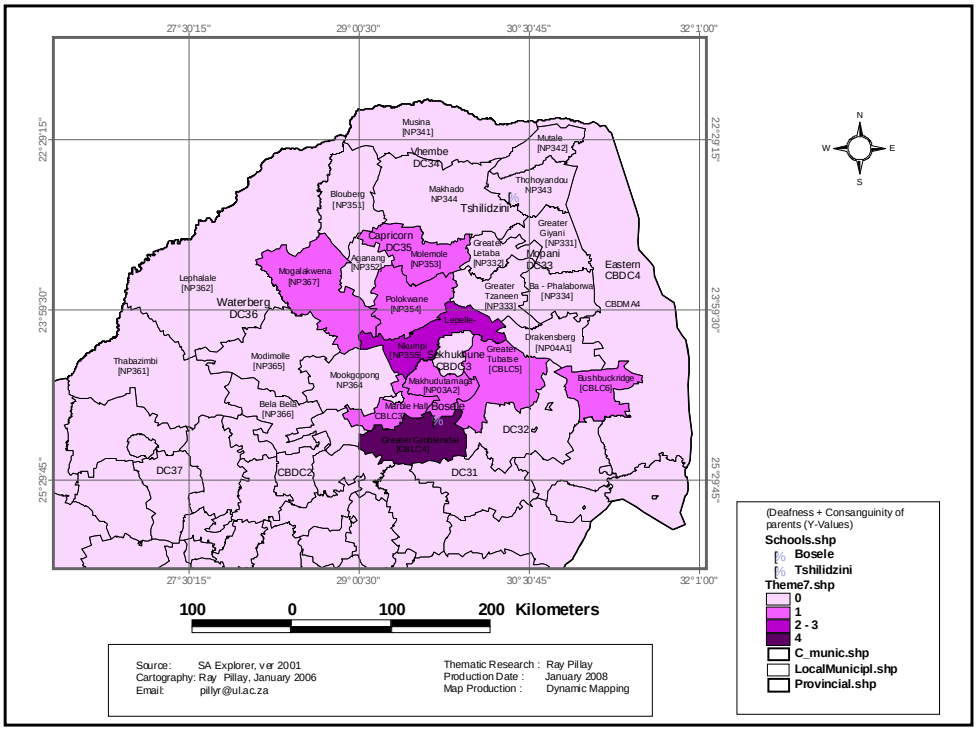


Figure 5.14: A Spatial distribution of subjects with a history of consanguinity among parents per Local Municipality, Limpopo province, Phase 2

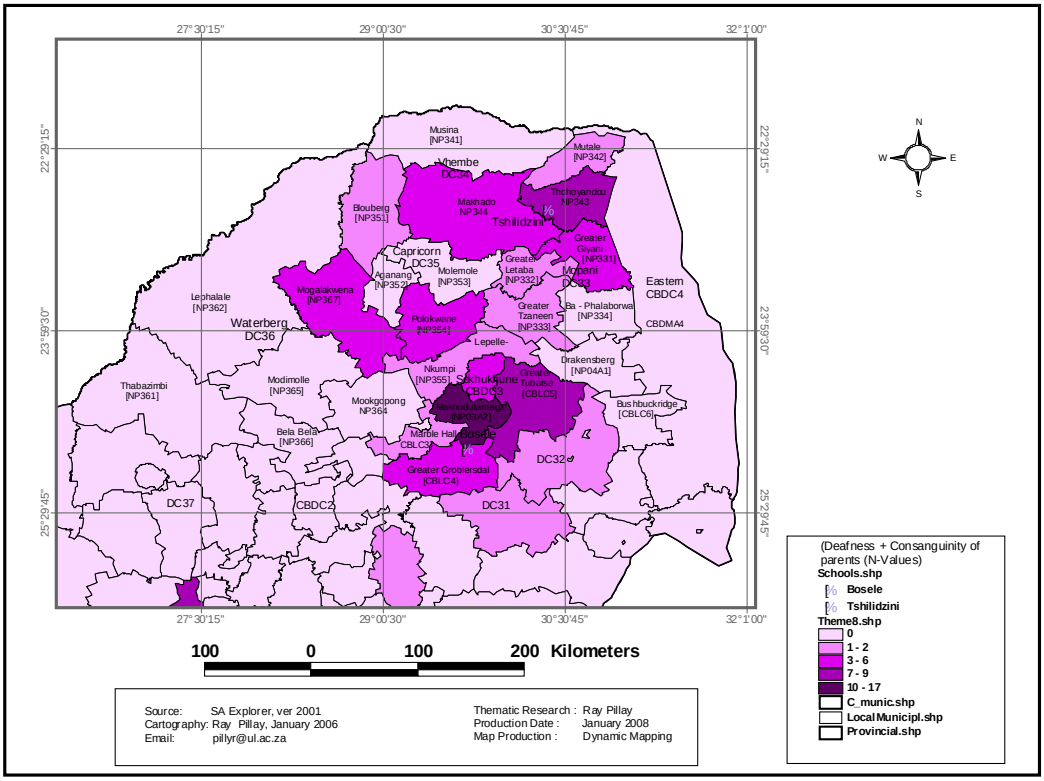


Figure 5.15: Spatial distribution of subjects without a history of consanguinity among parents per Local Municipality, Limpopo province, Phase 2

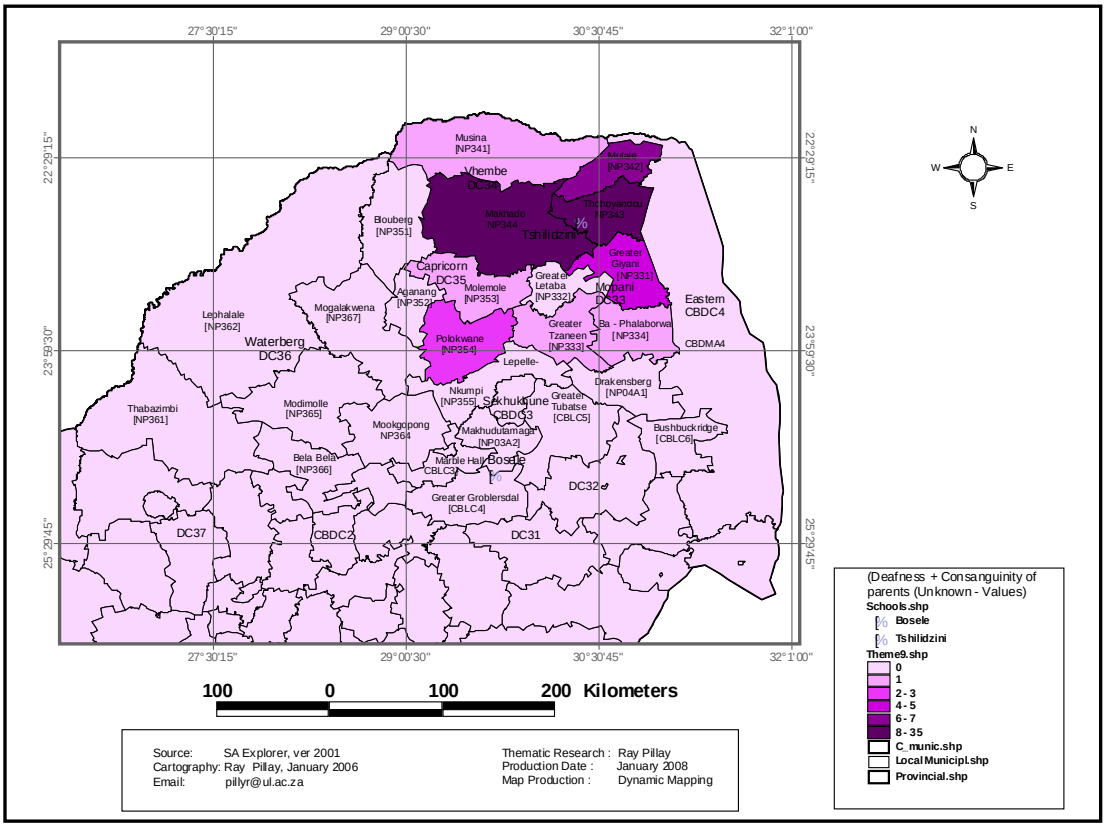


Figure 5.16: Spatial distribution of subjects with unknown history of consanguinity per Local Municipality, Limpopo province, Phase 2

Table 5.26: Cross tabulation of consanguinity of parents by municipality, Tshilidzini School, Phase 2

Municipality	Consanguinity Of Parents			Total
	No	Yes	Unknown	
JHB	0	0	1	1
Greater Giyani	3	0	5	8
Greater Letaba	1	0	0	1
Greater Tzaneen	1	0	1	2
Ba-Phalaborwa	0	0	1	1
Musina	0	0	1	1
Mutale	2	0	7	9
Thulamela	7	0	35	42
Makhado	3	0	21	24
Molemole	0	1	1	2
Polokwane	0	0	3	3
Unknown	1	0	12	13
Total	**18**	**1**	**88**	**107**

5.4.3 Urinalysis Results

The majority of the subjects had normal urine, with only six participants reported terminal haematuria, confirmed on urinalysis (table 5.32). This is pathognomonic of bilharzia in this population. One of the subjects with confirmed Waardenburg Syndrome also reported terminal haematuria, confirmed at urinalysis.

5.4.4 Reported Pregnancy and Perinatal history

Regarding pregnancy history, the respondents to the parental questionnaire (appendix 2a) reported one case of an unspecified illness during pregnancy, and three cases of seizures during pregnancy. On the question of perinatal history, one case of premature rupture of membranes, six cases of ante-partum haemorrhage, one case of labour following a motor vehicle accident, one case of precipitate labour, four cases of prolonged labour and four cases of forceps delivery were reported (table 5.35). From the respondents, most of the labour related complications and their sequelae are reported among the Bosele subjects.

5.4.5 Reported Medical Conditions Among the Subjects

Nine participants reported balance problems, while eight participants reported visual problems (table 5.36). On further analysis, the four participants with both visual and balance disorders were deliverd by forceps. The fifth participant with visual and balance disorders reported delivery after a prolonged labour. The four participants with balance disorders alone did not have any other medical problem. The single participant with a low apgar score had no other medical problem. On the other hand there were two participants with cerebral palsy picked up among the Bosele subjects.

Table 5.27: Cross tabulation of consanguinity of parents by municipality, Bosele School, Phase 2

Municipality	Consangunity of Parents		
	No	Yes	Total
Greater Marble Hall	2	1	3
Greater Grolersdal	4	4	8
Greater Tubatse	9	1	10
Bushbuckridge	0	1	1
JHB	1	0	1
MP312 **	1	0	1
MP314 **	1	0	1
MP321 **	2	0	2
Makhudutamaga	17	1	18
Fetakgomo	6	0	6
Blouberg	1	0	1
Polokwane	5	1	6
Molemole	1	3	4
Mogalakwena	4	1	5
Unknown	8	0	8
Total	**62**	**13**	**75**

Table 5.28: Cross tabulation of consanguinity of parents by municipality, Tshilidzini School, Phase 2

Municipality	Consangunity Of Parents			Total
	No	Yes	Unknown	
JHB	0	0	1	1
Greater Giyani	3	0	5	8
Greater Letaba	1	0	0	1
Greater Tzaneen	1	0	1	2
Ba-Phalaborwa	0	0	1	1
Musina	0	0	1	1
Mutale	2	0	7	9
Thulamela	7	0	35	42
Makhado	3	0	21	24
Molemole	0	1	1	2
Polokwane	0	0	3	3
Unknown	1	0	12	13
Total	**18**	**1**	**88**	**107**

Table 5.29: History of consanguinity of parents by school, Phase 2

School	Consanguinity of Parents			Total
	No	Yes	Unknown	
Bosele	62	13	0	75
Tshilidzini	20	1	86	107
Total	**82**	**14**	**86**	**182**

Table 5.30: Cross tabulation of consanguinity of parents by family history of hearing loss, Phase 2

Consanguinity Of parents	Family History of hearing loss		Total
	No	Yes	
No	59	23	82
Yes	6	8	14
Total	**65**	**31**	**96**

Table 5.31: Cross tabulation of language group by consanguinity of parent, phase 2

Language Group	Consangunity Of Parents			Total
	No	Yes	Unknown	
N/Sotho	58	14	9	81
Venda	12	0	71	83
Tsonga	5	0	8	13
Swati	5	0	0	5
Total	**80**	**14**	**88**	**182**

Table 5.32: Results of urinalysis among participants

School	Urinalysis		Total
	Abnormal/ abnormality	Normal	
Bosele	6 / haematuria	69	75
Tshilidzini	0	107	107
Total	**6**	**176**	**182**

Table 5.33: Cross tabulation of consanguinity of parents by relative with hearing loss

1st affected| Member	Consangunity of Parents			Total
	No	Yes	unknown	
Grandfather	0	0	1	1
Grandmother	1	0	0	1
Maternal Grandmother	1	0	1	2
Paternal Grandfather	2	0	0	2
Aunt	3	0	0	3
Brother	7	2	4	13
Cousin	2	1	1	4
Father	0	0	2	2
Mother	1	0	0	1
Sister	4	2	2	8
Uncle	2	3	3	8
Total	**23**	**8**	**14**	**45**

Table 5.34: Cross tabulation of consanguinity of parents by relative with hearing loss

2nd family Member	Consangunity Of Parents			Total
	No	Yes	Unknown	
Paternal Grandfather	0	3	0	3
Aunt	1	0	0	1
Sister	1	0	1	2
Total	**2**	**3**	**1**	**6**

Table 5.35: History of maternal problems during pregnancy and labour

Maternal condition	School		Total
	Bosele	Tshilidzini	
Bleeding during pregnancy	6	0	6
Forceps delivery	4	0	4
Precipitate labour	1	0	1
Prolonged labour	4	0	4
'Ill' during pregnancy	1	0	1
Premature rupture of membranes	1	0	1
Seizures	3	0	3
Labour followed MVA	0	1	1
No abnormality	55	106	161
Total	**75**	**107**	**182**

Table 5.36: History of other medical conditions among participants

Subject medical Condition	School		Total
	Bosele	Tshilidzini	
Apgar low	1	0	1
Balance disorder	4	0	3
Visual disorder	3	0	2
Visual& balance disorder	5	0	5
Waardenburg Syndrome Type I	2	0	2
None	62	107	167
Total	**77**	**107**	**184**

5.5 MUTATION DETECTION

5.5.1 GJB2

There was a high incidence of the C>T variant at position g.3318-34 in this population, occurring in 84 participants, comprising about 46.2% of the cohort. The C>T variant at position g.3318-15 was found in 39 participants comprising 21.4% of the cohort. The figures for the control group were 42.6% and 35% respectively. In three participants a T>A homozygous variation was detected at –6 in the 5' UTR. None of the mutations reported in the literature were found in the coding region in this study group. Significantly, the 35delG mutation prevalent in the Mediterranean region and among people of Caucasian descent was not found in any of the 182 participants. These findings are summarized in table 5.37 and figures 5.17 to 5.21.

Table 5.37: GJB2 variations observed in a deaf population from the Limpopo Province of South Africa.

Nucleotide exchange	Domain	Description	Cohort Genotype Frequency	Control Group Genotype Frequency	% Of cohort (N=182)	% Of control group (N=63)
G.3318-34C>T (heterozygous)	5"-UTR	Intronic mutation	62	26	34	41
g.3318-34C>T (homozygous)	5"-UTR	Intronic mutation	22	1	12	1.6
g.3318-15C>T (heterozygous)	5"-UTR	Intronic mutation	34	17	18.7	27
g.3318-15C>T (homozygous)	5"-UTR	Intronic mutation	5	5	2.7	8
g.3318-34C>T (homozygous) + g.3318-15C>T (homozygous)	5"-UTR	Intronic mutation	0	0	0	0
g.3318-34C>T (heterozygous) + g.3318-15C>T (heterozygous)	5"-UTR	Intronic mutation	11	6	6	10
g.3318-34C>T (homozygous) + g.3318-15C>T (heterozygous)	5"-UTR	Intronic mutation	1	0	0.5	0
g.3318-34C>T (heterozygous) + g.3318-15C>T (homozygous)	5"-UTR	Intronic mutation	0	1	0	1.6
g.3318-6 T>A (homozygous)	5"-UTR	Intronic mutation	3	0	1.6	0

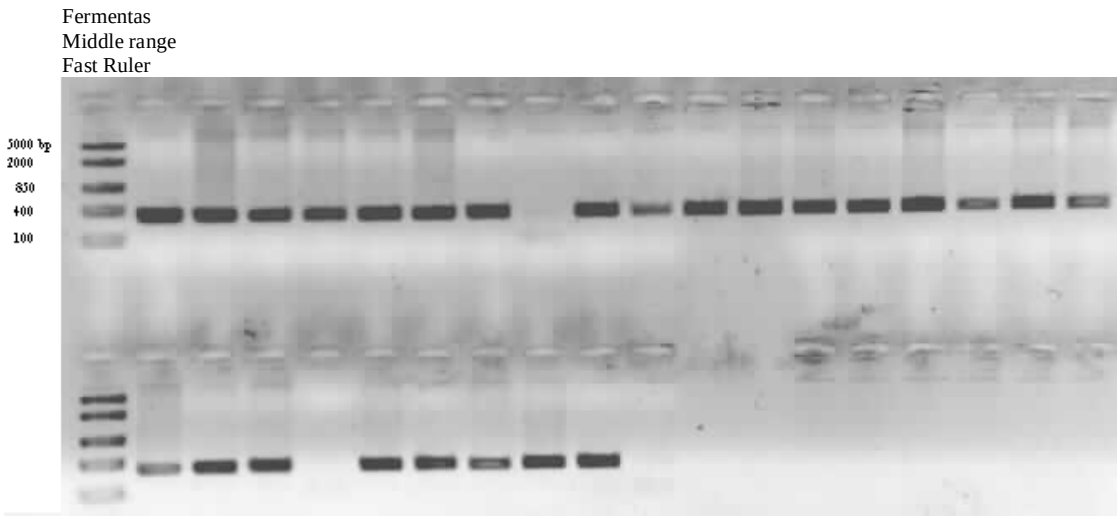


Figure 5.17: Gel electrophoresis showing size of PCR fragment (GJB2)

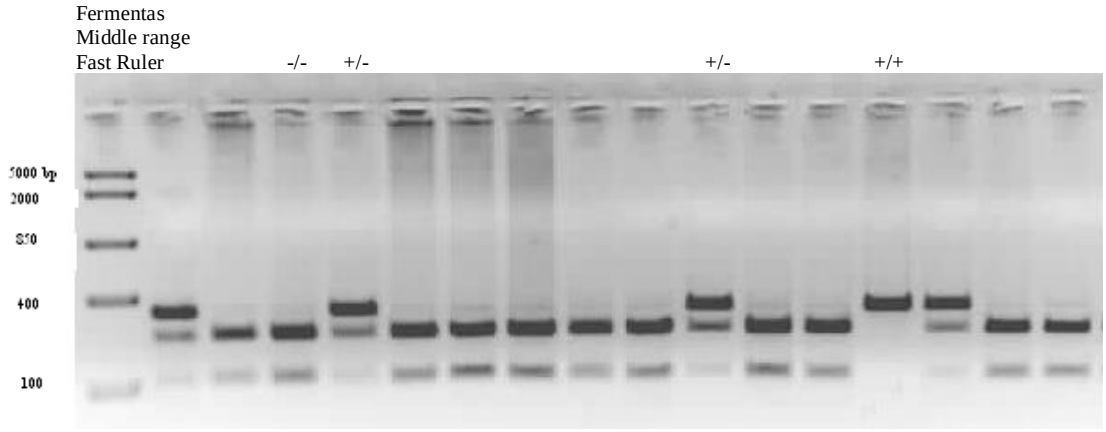


Figure 5.18: Gel electrophoresis (GJB2) following Fermentas SsiI enzyme digest (cutting at position g.3318-15)

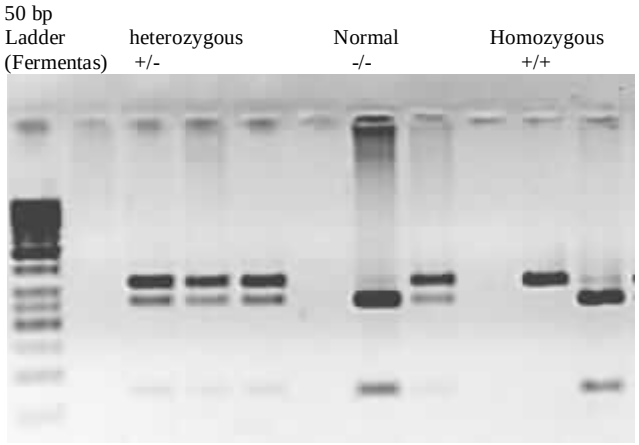


Figure 5.19: Gel electrophoresis (GJB2) following BsmI enzyme digest (cutting at positiong.3318 –34)

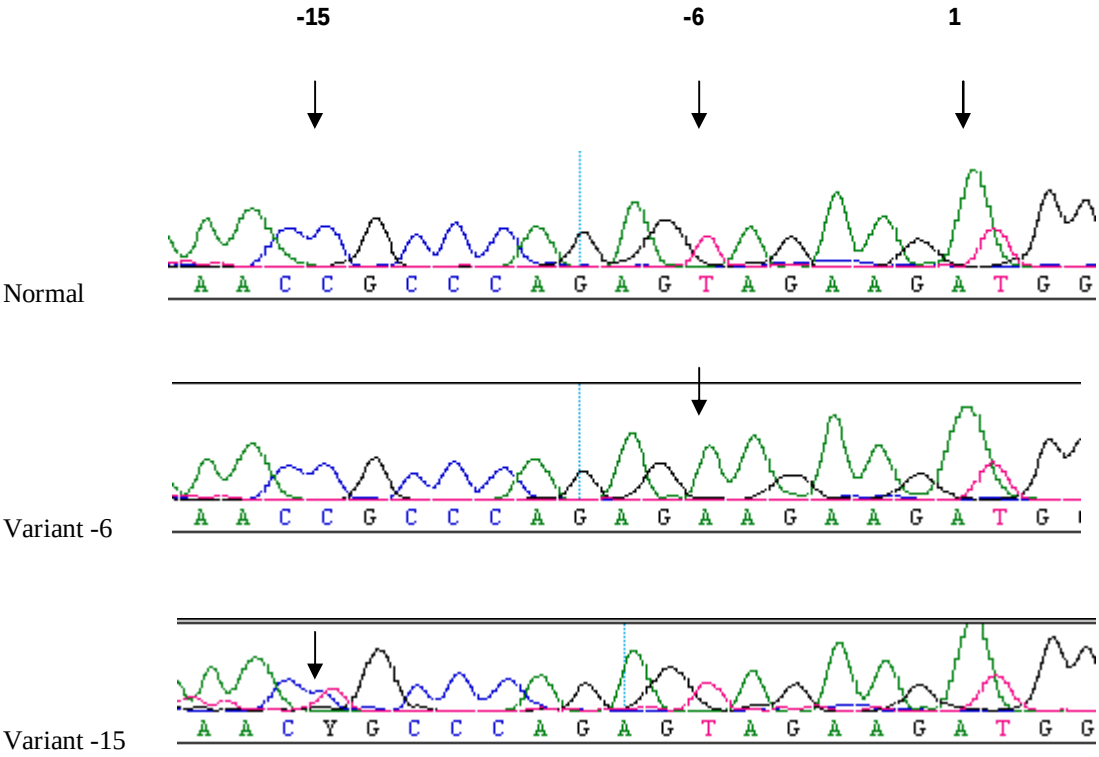


Figure 5.20: Electropherograms showing GJB2 variation T>A at position -6 and C>T variation at position –15

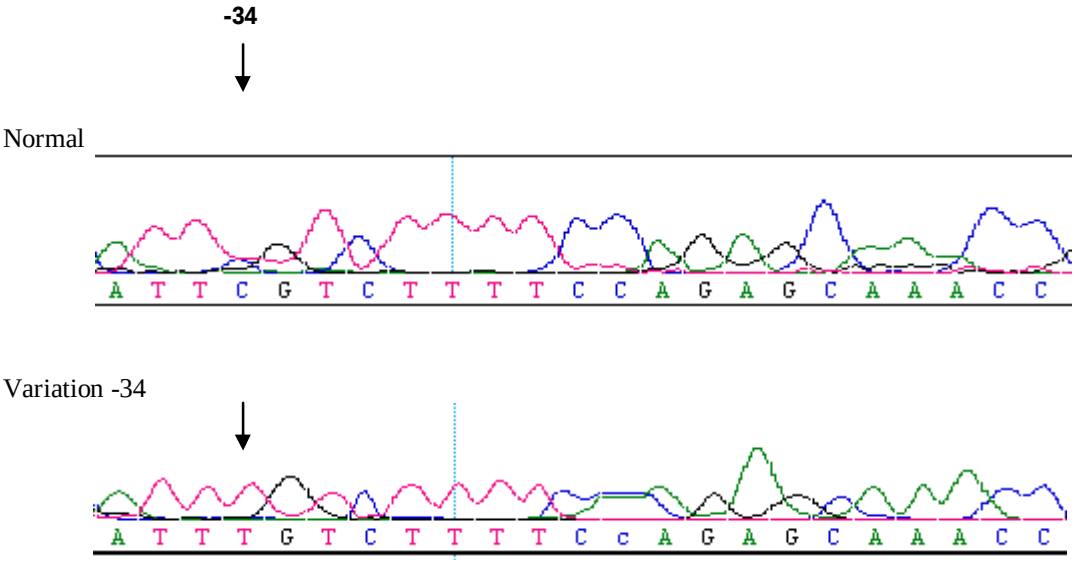


Figure 5.21: Electropherogram showing GJB2 variants at GJB2 position –34

Genotype frequency versus allele frequency of GJB2

There are three genotypes Normal/Normal (designated N/N), Normal/Variant (designated N/Variant), and Variant/Variant at each position, g.3318-34 and g.3318-15. The findings of this study indicate that the occurrence of the C>T variant at position g.3318-34 in this population is not significant in the participant group *(p= 0.261)*, the control group *(p= 0.124)*, or in the combined group *(p= 0.562*), since it is within the Hardy-Weinberg equilibrium. The occurrence of the C>T variant at position g.3318-15 in this population, though not significant in the participant group *(p= 0.187)* or the control group *(p= 0.188)*, is significant in the combined group *(p= 0.038*) where it is not within the Hardy-Weinberg equilibrium (tables 5.40, 5.41).

There was no significant relationship between a history of consanguinity and base variation at position g.3318-34 in the study population (table 5.42). Neither did the family history of hearing loss in the family influence the base variation at position g.3318-34 (table 5.43). The results also indicate that there was no significant relationship between a language group and base variation at position g.3318-34 in the study population (table 5.44), nor in the control group (table 5.38).

5.5.2 Waardenburg Syndrome

The results confirmed WS type I in one sibling pair. These mutations were identical in the sibling pair. Two mutations were all found in the PAX3 gene. A rare mutation, R223X, which is a nonsense mutation at the 223 amino acid: CGA-TGA, R-X, arginine – stop, and a novel silent mutation at the 293 amino acid, GGG>GGT. No mutations were found in the MITF gene.

5.5.3 Mitochondrial Mutations

The four common mitochondrial mutations, A1555G, A3243G, A7511C, and A7445G were not identified in any of the samples.

5.5.4 The *GJB6*-D13S1830 mutation

The 342-kb deletion in *GJB6*, *GJB6*-D13S1830 was not detected in any of the participants of the current study. Since the coding region of *GJB6* was not sequenced, its role in the South African population studied remains uncertain.

Table 5. 38: Cross tabulation of GJB2 variations and Language group in a South African control population (n=74).

Ethnic group	Position g.3318-34				Position g.3318-15			
	C	C/T	T	X	C	C/T	T	X
Venda (N= 8)	2	4	0	2	3	1	1	3
Pedi (N= 45)	24	16	1	4	24	10	3	8
Tsonga (N= 17)	9	4	0	4	7	5	1	4
Tswana (N= 2)	1	1	0	0	1	1	0	0
Swati (N= 1)	0	1	0	0	x	x	x	x
Ndebele (N= 1)	x	x	x	x	x	x	x	x

Table 5. 39: GJB2 (Cx26) variations: Genotype versus allele frequency as observed in a South African population.

Genotype	Position g.3318-34				Position g.3318-15			
	Subjects	BS	TS	Controls	Subjects	BS	TS	Controls
N/N	65	32	33	36	121	53	68	35
N/variant	62	24	38	26	34	14	20	17
Variant/Variant	22	14	8	1	5	2	3	5
Total	149	70	79	63	160	69	91	57

Table 5.40: GJB2 (Cx26) variations tested for Hardy-Weinberg equilibrium: Position g.3318-34

Subjects

	Normal/Normal	Normal/Variant	Variant/Variant	
Observed	65	62	22	149
Expected	61.85235	68.2953	18.85235	149
Chi^2	0.160183	0.580286	0.525542	1.266012
			p =	**0.260516**

Frequs.	
Normal	0.644295
Variant	0.355705
	1

Controls

	Normal/Normal	Normal/Variant	Variant/Variant	
Observed	36	26	1	63
Expeected	38.11111	21.77778	3.111111	
Chi^2	0.116942	0.818594	1.43254	2.368076
			p =	**0.12384**

Frequs.	
Normal	0.777778
Variant	0.222222
	1

Combined

	Normal/Normal	Normal/Variant	Variant/Variant	
Observed	101	88	23	212
Expected	99.17453	91.65094	21.17453	
Chi^2	0.033601	0.145436	0.157375	0.336413
			p =	**0.561907**

Frequs.	
Normal	0.683962
Variant	0.316038
	1

Table 5.41: GJB2 (Cx26) variations tested for Hardy-Weinberg equilibrium: Position g.3318-15

Subjects

	Normal/Normal	Normal/Variant	Variant/Variant	
Observed	35	17	5	57
Expected	33.19737	20.60526	3.197368	
Chi^2	0.097884	0.630806	1.016298	1.744988
			p =	**0.186508**

Frequs.	
Normal	0.763158
Variant	0.236842
	1

Controls

	Normal/Normal	Normal/Normal	Variant/Variant	
Observed	121	34	5	160
Expected	119.025	37.95	3.025	
Chi^2	0.032771	0.411133	1.289463	1.733367
			p =	**0.187982**

Frequs.	
Normal	0.8625
Variant	0.1375
	1

Combined

	Normal/Normal	Normal/Variant	Variant/Variant	
Observed	156	51	10	217
Expected	151.8076	59.38479	5.807604	
Chi^2	0.115779	1.183885	3.026409	4.326074
			p =	0.037533

Frequs.	
Normal	0.836406
Variant	0.163594
	1

Table 5.42: Cross tabulation of consanguinity of parents by base variation: Position g.3318-34

Consanguinity of parents	GJB2 variation C>T at position -34				Total
	N	N C/T	Y	Undetermined	
No	41	22	15	2	80
Unknown	81	0	7	0	88
Yes	6	2	3	2	14
Total	128	25	25	4	

Fisher's exact = 0.000

Table 5.43: Cross tabulation of family history of hearing loss by base variation: Position g.3318-34

Family history of hearing loss	GJB2 variation C>T at position -34				Total
	N	N C/T	Y	Undetermined	
No	75	16	15	3	109
Unknown	22	0	5	0	27
Yes	31	9	5	1	46
Total	128	25	25	4	

Fisher's exact = 0.221

Table 5.44: Cross tabulation of language group by base variation: Position g.3318-34

Language group	GJB2 variation C>T at position -34				Total
	N	N C/T	Y	Undetermined	
Sotho/ N. Pedi	40	24	13	4	81
Venda	73	0	10	0	83
Tsonga	13	0	0	0	13
Swati	2	1	2	0	5
Total	128	25	25	4	182

Fisher's exact = 0.000

5.6 CLINICAL SIGNS IN HEARING LOSS

5.6.1 Eye Findings Among the Subjects

The most striking features were found in the iris. There were two participants with hypoplastic blue eyes, one subject with dark purple irises, one subject with brown speckled irises, and one with radial brown lines in the irises (table 5.49, 5.50). One subject had a dark halo around the irises. The participants with hypoplastic blue eyes had confirmed Waardenburg Syndrome Type I (figure 5.22a). None of these participants had GJB2 mutations or variants.

Six participants had thick bushy eyebrows, hypertrichosis, converging at the root of the nose. Two of these were later confirmed to have Waardenburg syndrome Type 1 (tables 5.52, 5.53, 5.54, figure 5.22a). None of these participants had GJB2 variants.

There were two participants with dystopia canthorum, both confirmed Waardenburg Syndrome Type I (figure 5.22a), and one subject with marked epicanthal folds (table 5.55). None of them had GJB2 variants or mutations.

5.6.2 Skeletal Findings Among the Participants

Special emphasis was paid to the hands and feet (tables 5.45 – 5.54). Nineteen participants had a radially curved small finger bilaterally. This included one participant with confirmed Waardenburg syndrome Type 1. Six participants had all five fingers of the hand curving radially bilaterally. One participant each was found to have the following signs: palmar contracture, wide interdigital spaces in combination with a curved little finger, short curved fingers, polydactyly or extra digits, hyperextensible interphalangeal joints, thin long fingers, and wide interdigital spaces.

The g.3318-34 C>T GJB2 variant was detected in the patient with the wide interdigital spaces, but no GJB2 variants were detected in the rest of this group. The feet were of normal appearance though the subject with thin long fingers also had thin long toes. None of the Waardenburg syndrome participants had other skeletal abnormalities. None of the participants had spinal abnormalities.

5.6.3 Ear, Nose and Throat Findings Among the Subjects

Pinnae shape, size and location were assessed (tables 5.56, 5.57, 5.58). There were two participants with bat ears, two participants with cupped ears and one participant with pixie ears in the group. None of these participants had any other visible abnormality. Three participants had low set ears, while two participants had laid back ears, again all without other visible abnormality. The rest of the participants (179/184) had normally placed pinnae. In terms of size, six participants had microtia with no other abnormality. The rest (178/184 subjects) had normally sized ears.

Miscellaneous ear findings, common to the general ENT population, included otitis media with effusion (OME) in 5 participants, a perforated tympanic membrane in one participant, foreign bodies in 3 participants, eczema in 3 participants, impacted wax in 2 subjects, and an ear tag in 1 participant (tables 5.59, 5.60). One participant had narrow external auditory meati while one participant had bilateral preauricular sinuses. Wax was found in 19 participants, but was impacted in only 2 of these participants.

Nasal findings showed the usual signs of rhinitis and sinusitis in nine participants, two with hypertrophy of the inferior turbinates, three with mucoid nasal discharge, one

with a post-nasal drip, and three with pale congested nasal mucosa (table 5.59). The palate was normal in 176 out of 184 participants. Of the rest (8/184 participants), one had a cleft soft palate, two had high arched palates, one had no uvula and two had uvulae that were laterally fused to the posterior faucial pillars (table 5.60).

5.6.4 Other Systemic Findings Among the Subjects

Central nervous system examination confirmed features of mild cerebral palsy in three participants. The abdominal, respiratory and cardiovascular systems were normal in all the participants. The participants all had normal hair except the Waardenburg syndrome sibling pair which had a white forelock (figure 5.22a). Of special interest, both of the Waardenburg syndrome participants had patchy depigmentation of the skin (figure 5.22b). Except for the three participants with eczema, the participants with a dry scaly dermatitis around the ears and the participant with hypopigmented patches on one pinna, the rest (179/184 subjects) had normal skin.

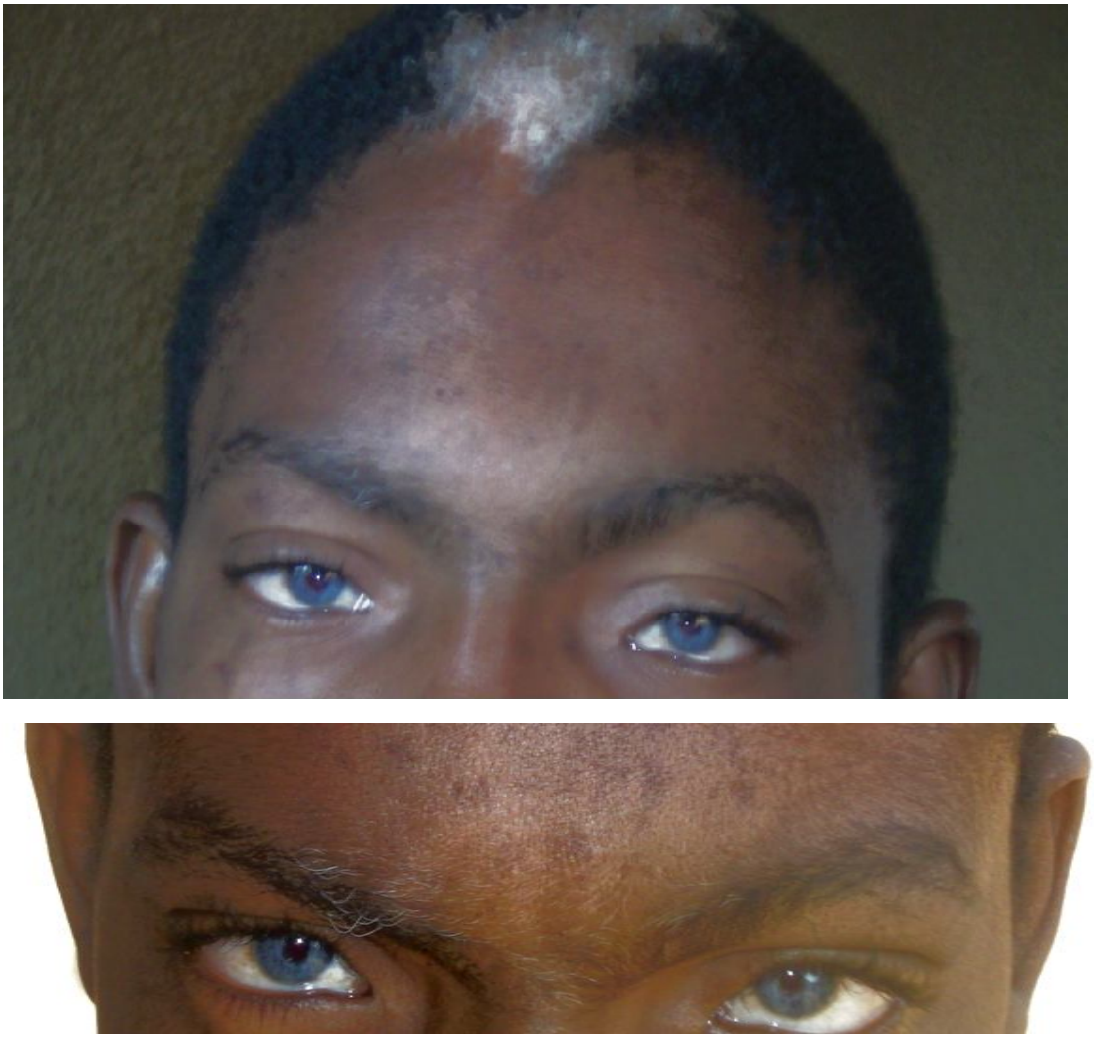


Figure 5.22a: Pale blue hypochromic irises with white forelock in participant with Waardenburg syndrome. Note the hypertrichosis of the eyebrows, as well as patchy graying of the right eyebrow in the close-up. The lateral displacement of the medial canthi is evident.

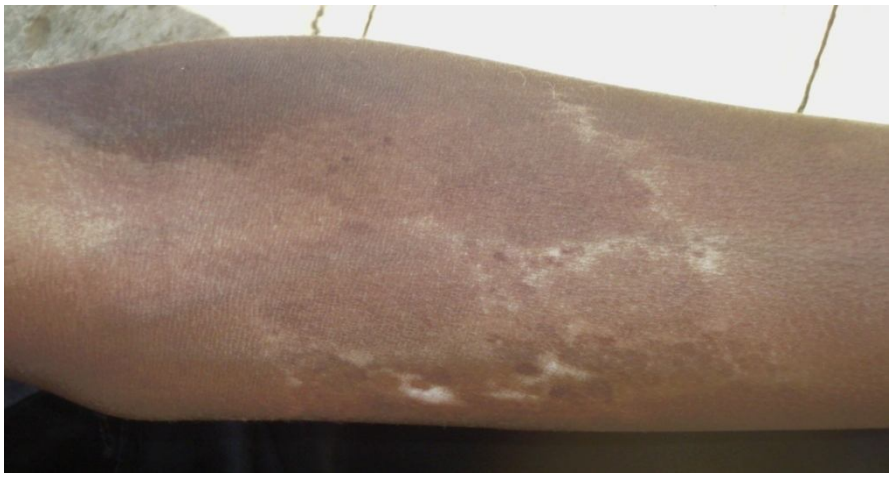


Figure 5.22b: Patchy depigmentation of the skin in participant with Waardenburg syndrome

5.7 TESTS OF ASSOCIATION AND BINARY LOGISTIC REGRESSION ANALYSIS

Fisher's tests of association were performed on the following variables: language group, family history of hearing loss, GJB2 C>T variations at positions -34 and -15, degree of 1st, 2nd and 3rd affected family member, participant's age at detection (of hearing loss), home address, risk factors for hearing loss, maternal medical conditions during pregnancy, participant's medical condition, hand abnormality and consanguinity of parents (tables 5.45-5.54).

Table 5.45 : Levels of significance of results following cross tabulation of participants' age at detection with other variables.

Variables cross tabulated		Fisher's exact chi square test p-value	Comments
Participant's age at detection (AAD)	Language group	0.000	Highly significant association: Venda language group associated with early detection.
AAD	Family history of hearing loss	0.000	Highly significant association: 34/46 with positive FHHL early diagnosis, <12mths age
AAD	GJB2 base variation C>T at position -34	0.000	No real significant association as polymorphisms.
AAD	GJB2 base variation C>T at position -15	0.007	No real significant association as polymorphisms.
AAD	Degree of first affected relative	0.050	Borderline association.
AAD	Degree of second affected relative	0.003	No real association as sample size too small
AAD	Degree of third affected relative	0.171	No association
AAD	Participants' home address	0.008	Significant association: linked to language group.
AAD	Risk factor for hearing loss	0.003	No rel significant association as sample size too small.
AAD	Participants' medical problem	0.065	No significant association
AAD	Maternal medical problem in pregnancy	0.582	No association
AAD	Hand abnormality	0.071	No significant association

Table 5.46 : Levels of significance of results following cross tabulation of risk factors for hearing loss with other variables.

Variables cross tabulated		Fisher's exact chi square test p-value	Comments
Risk factors for hearing loss RFHL)	Language group	0.056	No significant association.
RFHL	Family history of hearing loss	0.085	No significant association.
RFHL	GJB2 base variation C>T at position -34	0.002	No real significant association as polymorphisms.
RFHL	GJB2 base variation C>T at position -15	0.562	No real significant association as polymorphisms.
RFHL	Degree of first affected relative	0.067	No significant association.
RFHL	Degree of second affected relative	0.051	No significant association.
RFHL	Degree of third affected relative	1.000	No association
RFHL	Participants' age at detection in months	0.003	Very highly significant association.
RFHL	Maternal medical problem in pregnancy	1.000	No association
RFHL	Participants' medical problem	0.123	No association
RFHL	Hand abnormality	0.247	No association

Table 5.47 : Levels of significance of results following cross tabulation of consanguinity of parents with other variables.

Variables cross tabulated		Fisher's exact chi square test p-value	Comments
Consanguinity of parents (COP)	Language group	0.000	Highly significant association: especially among Pedi language group.
COP	Family history of hearing loss	0.000	Highly significant association: see section on odds ratio and risk ratio.
COP	GJB2 base variation C>T at position -34	0.000	No real significant association as polymorphisms.
COP	GJB2 base variation C>T at position -15	0.000	No real significant association as polymorphisms.
COP	Degree of first affected relative	0.026	Significant association.
COP	Degree of second affected relative	0.004	Highly significant association.
COP	Participants' home address risk level	0.000	Highly significant association: linked to language group.
COP	Participants' age at detection in months	0.000	Very highly significant association: linked to language group.
COP	Risk factor for hearing loss	0.000	Highly significant association: COP a risk factor for HL.
COP	Participants' medical problem	0.005	Highly significant: linked tolanguage group-COP and participants' medical problems mainly reported in Pedis
COP	Hand abnormality	0.004	Linked to language group: mainly Pedis

Table 5.48:Levels of significance of results following cross tabulation of family history of hearing loss with other variables.

Variables cross tabulated		Fisher's exact chi square test p-value	Comments
Family history of hearing loss (FHHL)	GJB2 C>T variation at position -34	0.221	No association.
FHHL	GJB2 C>T variation at position -15	0.356	No association.
FHHL	Degree of first affected relative	0.000	Highly significant association: 11/33 first degree, 21/33 second degree relative.
FHHL	Degree of second affected relative	0.000	Highly significant association: equal numbers (4 each) first and second degree relatives
FHHL	Participants' address	0.658	No association.
FHHL	Age at detection	0.000	Highly significant association: 34/49 detection <12 mths age, 15/46 detection < 4mths age.
FHHL	Risk factor for hearing loss	0.085	No significant association.
FHHL	Participants' medical condition	0.464	No association.

Table 5.49: Levels of significance of results following cross tabulation of degree of first affected relative with other variables.

Variables cross tabulated		Fisher's exact chi square test p-value	Comments
Degree of first affected relative (DFAR)	Degree of second affected relative	0.000	Highly significant association: linked to family history of hearing loss.
DFAR	Degree of third affected relative	0.065	No significant association.
DFAR	Home address	0.414	No association.
DFAR	Age at detection	0.050	Significant association.
DFAR	Risk factor	0.067	No significant association.
DFAR	Maternal medical problems during pregnancy	1.000	No association.
DFAR	Participants' medical condition	0.260	No association.
DFAR	Participants' hand abnormality	0.047	Weak association.

Table 5.50: Levels of significance of results following cross tabulation of degree of second affected relative with other variables.

Variables cross tabulated		Fisher's exact chi square test p-value	Comments
Degree of second affected relative (DFAR)	Degree of third affected relative	0.001	Highly significant association.
DFAR	Home address	0.819	No association.
DFAR	Age at detection	0.003	Highly significant association.
DFAR	Risk factor	0.051	Significant association: Family history of hearing loss a risk factor.
DFAR	Maternal medical problems during pregnancy	1.000	No association.
DFAR	Participants' medical condition	0.009	Significant association.
DFAR	Participants' hand abnormality	0.429	No association.

Table 5.51 : Levels of significance of results following cross tabulation of language group with other variables.

Variables cross tabulated		Fisher's exact chi square test p-value	Comments
Language group (LG)	Family history of hearing loss.	0.001	Significant association.
LG	GJB2 variation C>T at position -34	0.000	No real significant association as polymorphisms.
LG	GJB2 variation C>T at position -15	0.000	No real significant association as polymorphisms.
LG	Degree of first affected family member	0.627	No association.
LG	Degree of second affected family member	0.461	No association.
LG	Degree of 3rd affected family member	0.385	No association.
LG	Home address	0.000	Highly significant association: linked to language group.
LG	Age at detection	0.000	Highly significant association: linked to language group.
LG	Risk factor	0.056	No significant association.
LG	Maternal medical problems during pregnancy	1.027	Significant association.
LG	Participants' medical condition	0.002	Significant association: mainly Pedis.
LG	Participants' hand abnormality	0.004	Significant association: mainly Pedis.

Table 5.52: Levels of significance of results following cross tabulation of GJB2 variation C>T at position -34 with other variables.

Variables cross tabulated		Fisher's exact chi square test p-value	Comments
GJB2 variation C>T at position -34	GJB2 variation C>T at position -15	0.000	No real significant association as polymorphisms.
GJB2 variation C>T at position -34	Degree of first affected family member	0.449	No association.
GJB2 variation C>T at position -34	Degree of second affected family member	0.074	No significant association.
GJB2 variation C>T at position -34	Degree of third affected family member	0.049	No real significant association as polymorphisms.
GJB2 variation C>T at position -34	Home address	0.329	No association.
GJB2 variation C>T at position -34	Age at detection	0.000	No real significant association as polymorphisms.
GJB2 variation C>T at position -34	Risk factor	0.002	No real significant association as polymorphisms.
GJB2 variation C>T at position -34	Maternal medical problems during pregnancy	1.000	No association.
GJB2 variation C>T at position -34	Participants' medical condition	0.001	No real significant association as polymorphisms.
GJB2 variation C>T at position -34	Participants' hand abnormality	0.084	No significant association.

Table 5.53 : Levels of significance of results following cross tabulation of GJB2 variation C>T at position -15 with other variables.

Variables cross tabulated		Fisher's exact chi square test p-value	Comments
GJB2 variation C>T at position -15	Degree of first affected family member	0.540	No association.
GJB2 variation C>T at position -15	Degree of second affected family member	0.145	No association.
GJB2 variation C>T at position -15	Degree of third affected family member	0.303	No association.
GJB2 variation C>T at position -15	Home address	0.133	No association.
GJB2 variation C>T at position -15	Age at detection	0.007	No real significant association as polymorphisms.
GJB2 variation C>T at position -15	Risk factor	0.562	No association.
GJB2 variation C>T at position -15	Maternal medical problems during pregnancy	1.000	No association.
GJB2 variation C>T at position -15	Participants' medical condition	0.579	No association.
GJB2 variation C>T at position -15	Participants' hand abnormality	0.245	No association.

Table 5.54 : Levels of significance of results following cross tabulation of participants' home addresswith other variables.

Variables cross tabulated		Fisher's exact chi square test p-value	Comments
Participant's home address (PHA)	Language group	0.000	Highly significant association.
PHA	Family history of hearing loss	0.658	No association
PHA	GJB2 base variation C2T at position -34	0.329	No association
PHA	GJB2 base variation C2T at position -15	0.133	No association
PHA	Degree of first affected relative	0.414	No association.
PHA	Degree of second affected relative	0.819	No association
PHA	Degree of third affected relative	1.000	No association
PHA	Participants' age at detection in months	0.008	Significant association: linked to language group.
PHA	Risk factor for hearing loss	0.740	No association.
PHA	Participants' medical problem	0.065	No significant association
PHA	Maternal medical problem in pregnancy	1.000	No association
PHA	Hand abnormality	0.222	No association

Binary logistic regression analysis was then performed (table 5.56) on family history of hearing loss, GJB2 C>T variations at positions -34 and -15, degree of 1st affected family member, participant's age at detection (of hearing loss). The odds ratios were calculated on these variables (table 5.56) to determine which of these outcomes had any predictive value.

Table 5.55 Results of binary logistic regression analysis

```
note: degree3 dropped because of co-linearity

Logistic regression                               Number of obs   =        172
                                                  LR chi2(4)      =      10.98
                                                  Prob > chi2     =     0.0267
Log likelihood = -43.040435                       Pseudo R2       =     0.1132

------------------------------------------------------------------------------
        cop2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
     degree1 | 1.462423   1.393676     0.40   0.690     .2258841    9.468044
     history | 4.783582   2.992357     2.50   0.012     1.403739    16.30122
       GJB2a | 1.926376   1.442086     0.88   0.381     .4441515    8.355088
         aad | .3849309   .3097591    -1.19   0.235     .0795077    1.863615
------------------------------------------------------------------------------
```

Cop= consanguinity of parents aad= participants age (months) at detection
degree1= first affected relative
GJB2= GJB2 variations

5.7.1 Calculation of crude odds ratio

Crude risk ratio (relative risk) and crude odds ratios were calculated as shown in table 5.56. The crude risk ratio was calculated to be 2.57 with a 95% confidence interval of (1.49, 4.36), while the crude odds ratio was found to be 4.64 with a 95% confidence interval of (1.57, 13.61).

5.7.2 Interpretation of crude odds ratio

A child with a family history of hearing loss is 4.64 times as likely to be a product of a consanguineous mating in comparison with a child who does not have a family history of hearing loss.

5.7.3 Interpretation of the odds ratio for family history

From the findings, family history of hearing loss was the only predictor of consanguinity of parents at the 5% level of significance with a p-value of 0.012 (< 0.05). Family history of hearing loss gave an odds ratio of 4.78, SE2.99, p value of 0.012. This means that in this cohort, a child with a family history of hearing loss is 4.78 times as likely to be a product of a consanguineous mating in comparison with a child who does not have a family history of hearing loss.

5.7.4 Assessment of the fitted logistic regression model

The reliability of the fitted logistic regression model was assessed based on the classification table as depicted in table 5.57 below:

Table 5.56 Logistic model for consanguinity of parents

```
              -------- True --------
Classified|     D              ~D  |      Total
-----------+-------------------------+-----------
   +      |      0               0   |          0
   -      |     14             158   |        172
-----------+-------------------------+-----------
  Total   |        14            158   |        172

      Classified + if predicted Pr(D) >= .5
                 True D defined as cop2 != 0
--------------------------------------------------
Sensitivity                     Pr( +| D)    0.00%
Specificity                     Pr( -|~D)  100.00%
Positive predictive value       Pr( D| +)       .%
Negative predictive value       Pr(~D| -)   91.86%
--------------------------------------------------
False + rate for true ~D        Pr( +|~D)    0.00%
False - rate for true D         Pr( -| D)  100.00%
False + rate for classified +   Pr(~D| +)       .%
False - rate for classified -   Pr( D| -)    8.14%
--------------------------------------------------
Correctly classified                        91.86%
--------------------------------------------------
```

From this, it can be seen that percentage specificity is perfect at 100%. This means that the fitted logistic regression model has the capacity to detect participants who are not at risk of hearing loss. The overall percentage of correct classification is very high at 91.86%. This fulfills the required figure of above 75% for a well-fitted regression model. The percentage sensitivity is zero (very poor), meaning that the fitted logistic regression model has no ability to detect participants who are at risk of hearing loss. In conclusion, the fitted model is reliable in spite of the fact that it is poorly sensitive.

5.7.5 The Hosmer-Lemeshow goodness-of-fit test

The Hosmer-Lemeshow goodness-of-fit test was also performed as indicated below.

```
Logistic model for cop2, goodness-of-fit test

       number of observations =       172
 number of covariate patterns =         9
             Pearson chi2(4) =      6.09
              Prob > chi2 =        0.1927
```

With a P-value equal to 0.1927 > 0.05, the fitted model is reliable, and that there is no reason to doubt the adequacy of the fitted logistic regression model.

Table 5.57 Hosmer-Lemeshow goodness-of-fit test

history	cop2 0	cop2 1	Total
0	132	6	138
1	38	8	46
Total	170	14	184

	Exposed	Unexposed	Total
Cases	8	38	46
Noncases	6	132	138
Total	14	170	184
Risk	.5714286	.2235294	.25
	Point estimate		[95% Conf. Interval]
Risk difference	.3478992		.0812167 .6145816
Risk ratio	2.556391		1.499919 4.356992
Attr. frac. ex.	.6088235		.3332973 .7704838
Attr. frac. pop	.1058824		
Odds ratio	**4.631579**		**1.573753 13.61031**

(Cornfield)

chi2(1) = 8.35 Pr>chi2 = 0.0039

5.7.6 Magnitude of area under the ROC (receiver operating characteristic) curve

The magnitude of the area that lies under the ROC curve is a measure of the overall explained variation by the fitted logistic regression model. In this study, the area that lies under the ROC curve is 73.49%, a figure which is fairly close to 75%, the recommended figure for reliable fitted models (figure 5.23).

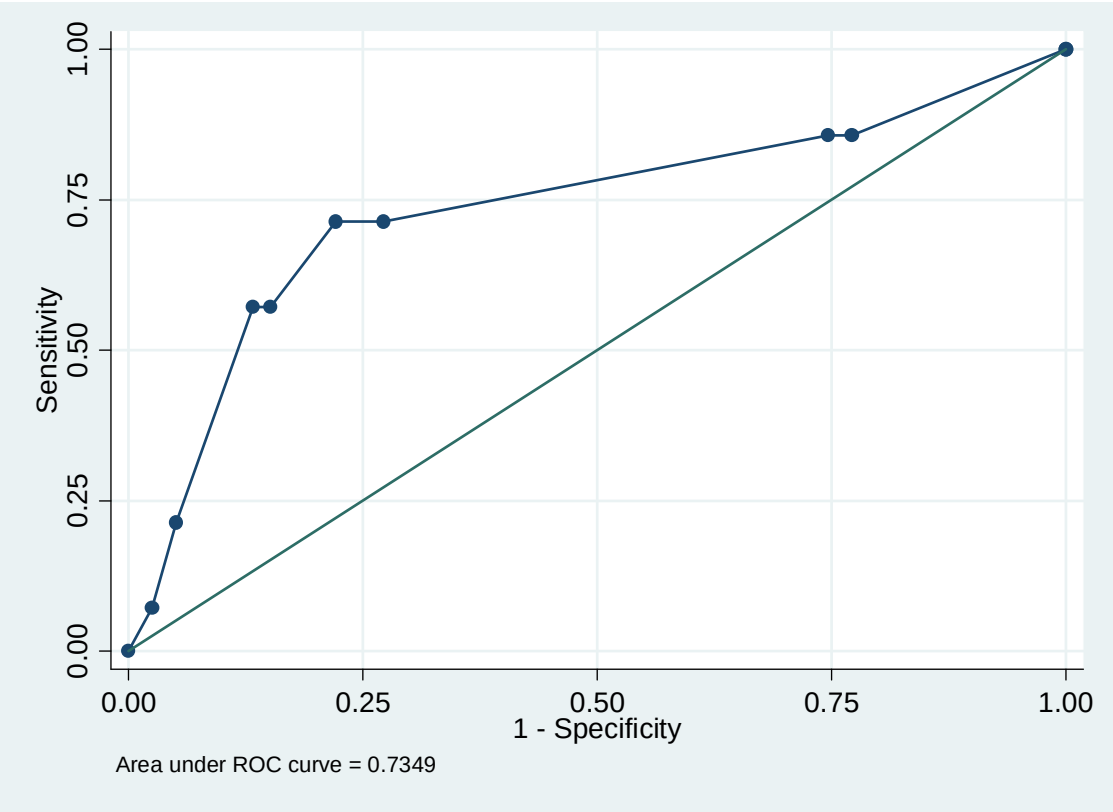


Figure 5.23:The ROC (receiver operating characteristic) curve

5.7.7 Plot of sensitivity/specificity versus probability cut-off point

The plot in figure 5.24 is a standard method of assessing overall sensitivity and specificity. If we drop a perpendicular from the point of intersection of the two curves to the X-axis vertically below, the perpendicular crosses the X-axis fairly close to zero. This shows that the fitted model is reliable.

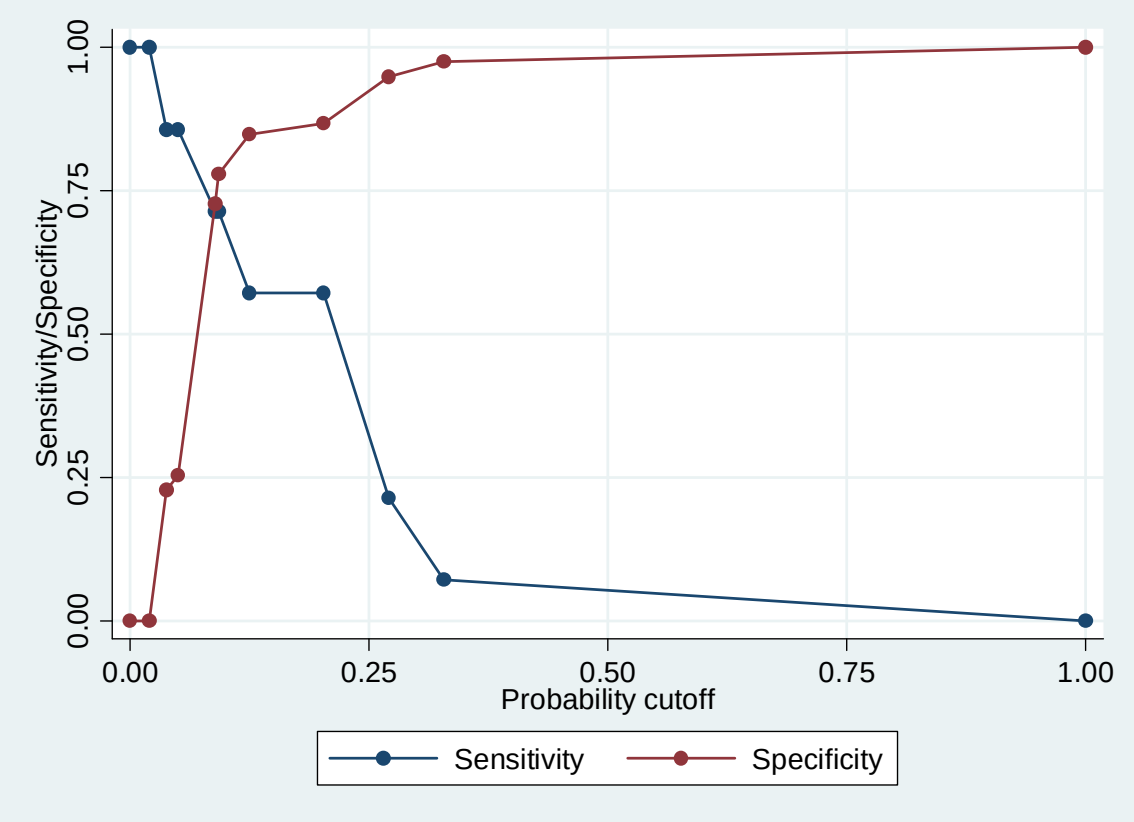


Figure 5.24: Plot of sensitivity/Specificity versus probability cut-off point

CHAPTER 6: DISCUSSION AND RECOMMENDATIONS

6.1 DISCUSSION

6.1.1 Geographical distribution of hearing loss in the Limpopo Province of South Africa.

The hypothesis of this study posed the question: Is there a high-risk area for deafness in the Limpopo province of South Africa? The results of Phase 2 of the study seem to suggest so. To start with, Tshilidzini School was compared against itself in Phases 1 and 2 to see if there would be a similar pattern of distribution of deaf subjects. If the findings showed clustering in the same geographical location in both phases of the study, it would give credibility to the theory that there was a high-risk area for deafness in the province. Secondly, analysis of data according to school was performed to see if a high-risk area would be highlighted at both schools for the deaf. Finally, normalized data of geographical distribution of hearing loss according to 100,000 African population was generated and analyzed to differentiate between apparent and true clustering.

Spot clusters on spatial distribution maps of the Limpopo province, presumed to represent *uneven distribution* of hearing loss, were demonstrated (figures5.4-5.6, tables 5.8-5.10). The alternative to the demographic map chosen in the current study also demonstrated clustering, representing genuine populations at risk for hearing loss within the province. By taking population density into account, Mutale (municipality NP342), was identified as constituting a genuine population at risk for genetic non-syndromic sensorineural hearing loss (NSSNHL) in the Limpopo province of South Africa (table 5.10, figure 5.7).

Secondly, using the municipal ward units mentioned under study design, possible high risk areas for deafness were identified in the region that was previously referred to as Nzhelele by the local population. These are wards 11-15, 26-30 and 31-35 in Thulamela (NP343) municipality and wards 6-10 in Mutale (NP 344) municipality (tables 5.7, 5.8). Together, these units accounted for 67 (18%) of participants in phase 1, and 33 (18%) of the participants in phase 2 of the study. Further study is indicated to tease out the confounding factors of this observed clustering, and this will be undertaken in future research. The clustering observed was not linked to time.

At district level, Vhembe district had the highest number of hearing impaired subjects (tables 5.5 and 5.6), while at municipal level, Thulamela and Makhado municipalities had the highest number of hearing impaired subjects in both phases of the study (tables 5.8-5.10, figures 5.4-5.7). Although further analysis of these municipalities showed wards 31-35 of Thulamela (NP343), and wards 6-10 of Makhado (NP344) municipality to have the highest number of hearing impaired participants, suggesting these areas to be high risk areas for hearing loss in the Limpopo Province, the normalized frequency calculations however give a different picture. With its low African population of just over 68,000, Mutale showed the highest normalized frequency at 13.14 per 100,000 African Population. Thulamela municipality, which had shown impressively high numbers of hearing impaired subjects, had a lower figure at 7.89 per 100,000 African Population. Mutale could well be a high-risk area for deafness in Limpopo. However, both Nzhelele and Mutale are located in the Northern part of Vhembe district with Siloam hospital, the main hospital in Nzhelele sub district, situated only 30 kms to the west of Mutale.

Local municipalities were chosen over villages and place-names as the smallest geographical unit to be used in mapping the spatial distribution of hearing loss. This was mainly because of the availability of municipal data and the fact that many of the local villages' names did not appear on the Subplace database of the Stats SA package used in the current study. This was not ideal as municipalities are too large a geographical unit for use without loss of important data. A much smaller unit such as GPS coordinates would have been ideal in placing the home area of the subject accurately within the province, eliminating the generalized spread that occurred with municipal location. In this way subjects originating 100 meters apart across a municipal boundary would not have been confused when analysing for clustering. However, due to low manpower and the lack of funds needed for the costly drive all over the province physically identifying the home area of the subject, a compromise had to be reached. Municipalities were chosen as the mapping unit to be used.

Since 1994, there have been repeated changes in the demarcation of local municipalities for electoral purposes, with the effect that many of the villages have changed location within municipalities as the municipal boundaries have been enlarged and shrunk. A future study will be needed to fully resolve the question of whether Nzhelele is indeed a high risk area for genetic hearing loss in the Limpopo province of South Africa or not, taking these issues into account in the design of the study.

The hearing impaired population at Schools for the Deaf comprises a selected and therefore biased population which does not necessarily represent the whole non-syndromic genetically hearing-impaired population in the province, as has been

shown in section 5.7.1. While these findings cannot be generalized to reflect the situation in the general population, they call for further research into hearing impairment among the people of Mutale municipality in the Limpopo province.

A school-by-school analysis demonstrated a high normalized frequency for hearing loss at Makhuduthamaga and Fetakgomo among participants from Bosele School, 6.71 and 6.22 respectively (tables 5.9, 5.10). These two areas will need to be revisited in future research to determine if the observed occurrence of deaf participants is higher than expected and the true aetiology of the observed hearing loss.

The frequency of participants originating from a particular municipality alone however cannot be relied upon to make a conclusion about high-risk populations. Other variables could be influencing these results. For example, there could have been selective intake of participants to Tshilidzini School, although this was ruled out in the current study. This could be because a teacher at the school originated from these communities or a community leader influenced the intake at the school. This is not uncommon in these communities in which many people are not highly educated and need direction from the people they look up to. Secondly, Tshilidzini School is situated in Thulamela municipality. It is also possible that the communities in the areas surrounding the school would send their children to this school. However, this is refuted by the results of the comparison with Bosele school did not yield a similar picture, and the analysis of distance from school showed wide variance reflecting widely spread homes (figures 5.2a – 5.2c). Thirdly, there is great variation in the population density in the various municipalities, often influenced by availability of work level of development of the area, and issues of land ownership.

Many of the people from the previous Venda homeland own their land, although the rural areas are often underdeveloped and there are few job opportunities. Some of the people are employed on farms as labourers, or in small and end medium enterprises (SMMEs). A few are employed in mines. However, the government, through the public service, is the largest employer in the province (Labour Force Survey of March 2004, Stats SA). Since most of the government and municipal offices are located in the towns and cities, there is higher population density clustered around the cities and towns. This is not a new phenomenon, affecting many rural communities worldwide. Migrant labour practices are also still rife in this province (and in South Africa in general). The bread winners move as far away as Johannesburg (over 500km away) to get employment in the industries and mines, and only come home at the end of the month. Due to the high cost of living in these large cities, the wife and children stay behind in the rural homestead.

These causes of bias had to be dealt with if the results of this study were to be meaningful. First of all, the 2-phase study design was essential for hypothesis formulation (phase 1) and hypothesis testing (phase 2). This has achieved the desired outcome of answering the research question, that is, whether there were high risk areas for hearing loss in the Limpopo province of South Africa. As has been noted under section 2.2.3 (pg 55), in epidemiological research, it is not even possible to design a single step study that covers all aspects of prevalence estimation, only a multi staged study is suitable. Secondly, Fisher's tests of association were done on the major outcomes to determine the influence of co-factors on the observed hearing loss.

From from the calculated odds ratio for family history, at the 5% level of significance, in this cohort, family history of hearing loss was the only predictor of consanguinity of parents with a p-value of 0.012 (< 0.05). Family history of hearing loss gave an odds ratio of 4.78, SE2.99, p value of 0.012. This means that in this cohort, a child with a family history of hearing loss is 4.78 times as likely to be a product of a consanguineous mating in comparison with a child who does not have a family history of hearing loss.

6.1.2 Accounting for bias in this study

6.1.2.1 Bias due to migratory labour practice

In the first phase, demographic data from a larger number of participants who had attended Tshilidzini School over the previous three years (1996-1999) was collected and a distribution pattern of their **homes of origin** was analyzed. The term **home,** when used alone, could be misleading and confusing to the participant and invalidate the research findings because it could also easily describe the place the parents or family stayed because they had had to move to an area where the parents/breadwinner got employment. By using the term 'home of origin' to describe the rural or ancestral home, it was hoped that the bias from migratory labour was to be overcome. This was however only partially successful as many records did not have a physical address and had registered the students at the school using their postal address (a post office). As such 34 participants in Phase 1 and 22 subjects in phase 2 were registered as '**unknown home of origin**'. These findings are summarized in figures 5.1 and 5.2.

6.1.2.2 Bias due to non-random admission into schools

Discrete inquiries were made at Tshilidzini School as to whether there was any known person on the staff coming from these communities or with close links with community leaders from these communities who could influence the admission into the school. This was denied. Anecdotal information seems to indicate that the schools for the deaf in Limpopo province have an open admission system whereby all qualifying students referred to the school by relevant medical personnel, including doctors, speech and hearing therapists, audiologists and social workers, are accepted into the schools without bias or discrimination. In particular the schools did not turn away deaf participants needing admission to the school during the period under study, as their capacity has not been exhausted.

6.1.2.3 Bias due to proximity to the schools

It was can be argued that if there was bias because subjects living closer to the schools for the deaf had a higher chance of attending the school, there would be similar distribution patterns of participants from both schools. By comparing demographical data from Tshilidzini School, the initial area of interest, to Bosele School in Sehukhuneland, this bias would be picked up. The findings of this study do not support this bias coming into play (tables 5.5-5.9, figures 5.1-5.3).

6.1.2.4 Bias due to varying population density within the province

The geographical distribution of participants could easily be skewed by population distribution patterns. Areas with higher population will naturally have higher numbers of affected participants for any given variable. This alone does not translate the region into a high-risk area for that variable. Normalisation of data had to be done to

distinguish real clustering from apparent clustering. It was not possible to get the provincial population data and to compute a normalized frequency for the three-year period, 1996-1999, necessary for phase 1 of this study. As such the occurrence of subjects, and not normalized frequency, was compared for both schools in this phase. However, for Phase 2, population data was available for the province and a normalized frequency per 100,000 of the African population was used to compare the two schools. The African population was obtained from the South African 2001 census *(StatsSA 2002)*. These findings are summarized in table 5.9 and figure 5.4.

6.1.3 The Type and Degree of Hearing Impairment of the Subjects

All the participants in the definitive case group had significant sensorineural hearing loss of severe (22.8%) to profound severity (75%), the majority exhibiting flat (70.1%) or sloping (23.4%) audiograms that were commonly symmetrical (81.5%). Low frequency ascending audiograms were found in 6% of the participants, while one subject had a mid frequency u-shaped audiogram. This study did not test for progression of the hearing loss, as the contact time with the participants was limited to the scope of the PhD study. Overall, there was no clear pattern in the audiological findings. These results agree with the findings of other studies that have shown that generally, congenital non-syndromic forms of hearing loss have no established audiological pattern (Sellars et al., 1983b; Gasmelseed et al., 2004; Mueller et al., 1999; Denoyelle et al., 1999; Kenneson et al., 2002; Liu et al., 1994).

In the African setting, among students at schools for the deaf, the most recent study on 524 individuals (139 Sudanese and 385 Kenyan) with congenital non-syndromal sensorineural hearing loss revealed a severe or profound hearing loss in 98.2% and

83.5% of the cohorts respectively (Gasmelseed et al., 2004). The rest had a moderate hearing loss. The Ghanaian study on 365 unrelated deaf individuals used audiometric findings of congenital non-syndromal profound sensorineural hearing loss as an inclusion criterion but did not catergorize the audiometric findings of all of the deaf students in the schools for the deaf (Hamelmann et al., 2001). Neither did the study on childhood deafness in Southern Africa (Sellars et al., 1983b), reporting only that the cohort consisted of students at schools for the 'profoundly deaf' and 'hard of hearing'. The findings of both of the above studies cannot therefore be compared to the current study.

A Chinese study (Liu et al., 1994) analyzed the audiometric features of 136 individuals with nonsyndromic genetic hearing loss, 83 with autosomal dominant (AD), 50 with autosomal recessive (AR) and 3 with X-linked hearing loss. Three main audiogram shapes were identified, sloping (50.3%), residual (26.5%), and flat (21.0%). The study also found that although there were both intrafamilial and interfamilial variations among the AD and AR types, there also exhibited significant differences. The main shapes in the AR group were residual and sharply sloping, while the AD group had sharply sloping, flat and gently sloping shapes. The study also found a significant difference in the degree of hearing loss, with milder forms in the AD group compared to the AR group, as well as more marked intrafamilial variation in the AD group. Notwithstanding, the Chinese study concluded that the audiograms of nonsyndromic hearing loss are usually non-specific.

Looking at specific phenotype-genotype relationships, Mueller (Mueller et al., 1999) noted that individuals with severe and profound hearing loss were more likely to have

mutations in the GJB2 gene than those with mild or moderate hearing loss. Denoyelle (Denoyelle et al., 1999) on the other hand reported that individuals with two GJB2 mutations exhibited hearing loss ranging from mild to profound severity. In these studies, the degree of hearing loss in individuals with identifiable GJB2 mutations seemed to vary significantly from those without GJB2 mutations (Mueller et al., 1999; Denoyelle et al., 1999).

In the UK, out of a group of 275 subjects with congenital non-syndromal sensorineural hearing loss, 100 (36.4%) had profound, 64 (23.3%) had severe, 92 (33.4%) moderate and 19 (6.9) mild hearing losses (Mueller et al., 1999). Of those in whom a configuration could be determined, the majority, 159 (59.3%), had sloping, while 72 (26.8%) had flat audiograms, 31 (11.5%) u-shaped and 6 (2.2%) ascending audiograms. In France, out of 118 subjects, 56 (47.4%) had profound, 29 (24.5%) severe, 19 (16.1%) moderate, and 14 (11.8%) mild hearing loss, with predominantly flat or sloping configuration (Denoyelle et al., 1999).

In a Ghanaian deaf population, Hamelmann (Hamelmann et al., 2001) had noted that the *GJB2* R143W mutation phenotype exhibited profound hearing loss. Gasmelseed (Gasmelseed et al., 2004) did not report the genotype-phenotype relationship among the deaf Sudanese and Kenyan individuals. The current study did not identify pathogenic *GJB2* genotypes and so cannot be compared to these studies. Of significance, there were no identifiable pathological *GJB2* mutations found in the current study. After analyzing a number of studies looking at *GJB2* variations, Kenneson et al (Kenneson et al., 2002), in a review paper, concluded that, although data seemed to suggest that *GJB2* variants were associated with moderate to severe or

profound hearing loss, the samples were too small and that population based studies were needed to confirm the findings.

The sibling pair with Waardenburg syndrome demonstrated symmetrical profound sensorineural hearing loss with flat audiograms. This degree of hearing loss has been noted to occur in up to 57% of WS Type I individuals, so it is not unexpected (Liu, Newton & Read 1995). Bilateral symmetrical hearing loss was also reported to be more common than unilateral HL (Liu et al., 1995) occurring in 77% of patients with WS Type I.

6.1.4 The Aetiology of Hearing Loss in the Limpopo

One of the goals of this study was to determine the aetiology of hearing loss among the subjects, who were all presumed to have congenital non-syndromic hearing loss, in phase 2 of the study. The aim was to have a clean definitive study group, comprising only of individuals with a genetic cause of hearing loss. The ascertainment criteria were clearly laid out and out of the total hearing-impaired population at the schools, 182 participants were selected to take part in phase 2 of the study.

The observed hearing loss in this cohort is a genetic, non-syndromic form, which is mainly severe and severe to profound, although without any clear defining configuration or shape. It is a stable, non-progressive and prelingual form of hearing loss, implying that this may be a recessive form of deafness. No identifiable environmental confounding factors or associations were identified. The deafness is not linked the common known auditory gene mutations in *GJB2* (coding for

Connexin26), the *GJB6*-D13S1830 mutation or the common mitochondrial mutations A1555G, A3243G, A7511C and A7445G. There was a high prevalence of *GJB2* variations, believed to be polymorphisms, demonstrated in the study population and among the normal hearing controls. Therefore, in conclusion, *GJB2*, the main deafness gene for non-syndromic hearing loss among Caucasians and Orientals, is not a significant deafness gene in the indigenous African population of the Limpopo province of South Africa.

Neither is the *GJB6*-D13S1830 mutation, a finding similar to studies among other African populations (Pandya 2003; Joy Samanich, et al. 2007). The *GJB6*-D13S1830 mutation is most frequent in Spain, France, the United Kingdom, Israel, and Brazil (5.9–9.7% of all DFNB1 alleles), but less frequent in the USA, Belgium, and Australia (1.3–4.5% of all DFNB1 alleles), and very rare in Southern Italy (del Castillo et al., 2003). In Northern Italy, it was found at frequencies similar to those of other European countries (Gualandi, F et al., 2004). The deletion was also detected in Germany (Bolz, H. et al., 2004), but not in Austria (Gunther, B et al., 2003), Turkey (Tekin, M et al., 2003; Uyguner, O. et al., 2003), China (Liu, X. Z et al., 2002), nor among African American populations (Pandya 2003; Joy Samanich, et al. 2007). Since the coding region of *GJB6* was not sequenced, its role in the South African population studied remains uncertain.The significant gene for this form of hearing loss is as yet to be identified and will be searched for in future studies.

Waardenburg Syndrome Type I has been genotypically confirmed in one sibling pair of indigenous African descent in this study. For the first time in Africa, a rare mutation, R223X, previously identified only once out of a cohort of 470 WS patients

(St Mary's Hospital, Manchester, UK 2003), has been identified in the *PAX3* gene in this sibling pair. A novel silent change GGG>GGT at amino acid 293, was also identified. These identical findings document, for the first time, a molecular defect in WS type I in indegenous Africans, and confirm WS Type I in this family. Whether these mutations are common to other WS type I Africans in the Limpopo province of South Africa remains to be seen.

Controls were used in phase 2 of the study to investigate the significance of the high prevalence of *GJB2* variations g.3318-34C>T and g.3318-15C>T in the study population. The finding of a high prevalence of these two variants, 42.6% and 35% respectively, among the 63 normally hearing controls indicate that these variations are polymorphisms and do not contribute to the aetiology of the observed non-syndromic SNHL in this population.

Confounding factors were assessed for by cross-tabulating the important outcome variables and testing them for association (tables5.45-5.50, 5.60, appendix 14). By including time (date of birth) with clustering, this study demonstrated that time was not a confounding factor to the observed deafness. The interactions of disease frequency with home address (space), age of participant (time), and language group (person), this study has demonstrated that space, time and person are not confounding factors to the observed deafness in the province (tables 5.45 – 5.50). Neither were maternal factors during pregnancy, participant's medical condition, or risk factors for hearing loss in childhood significantly associated with the observed hearing loss (tables 5.45-5.60).

Overall, 61.5% of the cohort reported a definite pre-lingual onset of hearing loss. This makes up 112 participants for whom data on the age of onset of deafness was available. The rest were not so sure about the age of onset but rather reported age at diagnosis. Twenty-three participants (12.6% of the total cohort) reported age at diagnosis of between 13 and 24 months of age, ten participants (5.5% of the cohort) after 25 months and thirty-seven partcipants (20.3% of the cohort) were unsure. These candidates could fall into either a pre-lingual or post-lingual onset of hearing loss group. It was not possible to get collateral information supporting either forms of hearing loss because of inability to get hold of informed parents or caregivers. Reliance therefore had to be made on information gleaned from the questionnaires.

The questionnaires used in the study served the purpose of collecting information that would help rule out syndromic and acquired forms of hearing loss. This information included maternal factors during the pregnancy, peripartum and post partum periods, as well as childhood history of the hearing impaired subjects (appendices 2a, 2b, 3, 6). The questionnaires were completed by the parent/care giver or by a research assistant as mentioned in methods. This is an indirect approach to the aetiology and natural history of hearing loss, and is bound to introduce bias due to the heavy reliance on collating history. In this particular study the use of questionnaires proved to be a limitation of the study when a large percentage of the respondents from Tshilidzini School failed to report on the question of consanguinity among the parents. Many of them relied on interpreter since they are of low education standard. It is possible that interviewer error led to this. In a future study this question will have to be tackled differently to ensure that there is no missing data.

Cognisance should be made of the fact that school records were resorted to when searching for missing data, but they too were often found to be inadequate. Secondly, it was very difficult to get the parents or caregivers to come to the school to directly answer some of the queries raised in the completion of the questionnaires. For example, biased or wrong information given by the respondent or errors in transcribing by the translator / research assistant cannot be ruled out.

In short, although it was difficult to completely rule out acquired or environmental causes of hearing loss in some of these subjects, and excepting the eight individuals (4.4%) mentioned below, the rest of the definitive study group (95.6%) are presumed to have a genetic non-syndromal type of hearing loss. Of these 61.5% gave a definite pre-lingual onset of hearing loss, while 20.3% were unsure. This means that 38.5% of the cohort could not with certainty be identified as pre- or post-lingually hearing impaired. Eight participants who were originally thought to have a non-syndromic recessive type of hearing loss were re-classified as having acquired hearing loss on further analysis, based on information obtained on clinical examination during a subsequent visit to the school, or information that came in after further inquiry from the caregivers. The group of eight includes one subject with a cleft soft palate, the two subjects with a mild form of cerebral palsy, and the five subjects with a history of forceps delivery. Again it is difficult to conclusively rule out a genetic form of hearing loss in these eight individuals, and more evaluation would have been desirable.

The finding of three participants with a mild hearing loss, two with mild cerebral palsy, and the participant with a cleft soft palate with moderate conductive hearing

loss due to otitis media with effusion (OME) raises a question about the medical and audiological assessment of children prior to admission to the schools for the deaf. This is a special school only supposed to admit students with significant hearing impairment who cannot be mainstreamed. Clearly, these four participants did not fall into that category. The three children could all have been mainstreamed with assisted support. CP patients are also prone to OME and should have had regular follow-up and management by the Otolaryngologist. The CP children needed normal language stimulation by a speech and hearing therapist and remedial teaching at school if there was an element of learning disorder. The cleft palate child should have been referred to a plastic surgeon and an Otolaryngologist for repair and the management of the associated OME at an early age, and regular follow-up thereafter. The reason they were picked up in this study was because each child was given a clinical examination by a qualified otolaryngologist.

As mentioned before, in the Limpopo province, children with suspected hearing impairment are initially identified by the parent or care giver, schoolteacher, primary health care personnel on school screening programmes, or health personnel at their local government clinic. They may also present through a general practitioner, a paediatrician or other specialist where they had gone for treatment of another condition. The hearing impaired child is then sent to the local hospital for evaluation by the doctors and speech and hearing therapists who then refer the child to a school for the deaf with the evaluation results and recommendations. Given the fact that there were only six speech and hearing therapists (table 1.4), twelve community speech and hearing therapists and four otolaryngologists in the province in 2004, it is clear that this assessment may not have been as thorough as was necessary. If there were three

participants picked up from an apparently otherwise healthy group of children, there could be many others with treatable or aidable hearing impairment that are currently found in the group with associated disabilities at special schools.

On the flip side is the inclusion of a sibling pair with Waardenburg Syndrome type 2. The opportunity to screen for the mutation came through a link with the Manchester team that has specialized in Waardenburg Syndrome. It was felt that the genetic analysis would provide valuable information on a possible mutation spectrum of Waardenburg syndrome in this population, data that could, in a future study, be used for mutation detection in other hearing impaired individuals among the South African populations.

Hearing-impaired children in the South Africa have free access to education in the schools for the deaf, and it is up to the parents to make use of this opportunity. Most of these schools have boarding facilities that cater for children who live far from the school and cannot commute daily. Thus many of these children are institutionalized early, although they are able to go home during the school holidays. One could assume that these participants represent all the hearing impaired children in these communities. It is believed, however, that some children with any form of disability, including hearing loss, are kept hidden away by the family because of fear of stigma. The findings of this study cannot therefore be considered representative of the childhood hearing impaired community in this province, and only a community-based study could confirm or disprove the findings of this study.

6.1.5 The Influence of Consanguinity on the Prevalence of Significant Childhood Hearing Loss in the Limpopo

Family history of hearing loss was found to be a significant predictor of consanguinity of the parents, with a p-value of 0.012 at the 5% level of significance. This is means that a child with a family history of hearing loss is 4.78 times as likely to be a product of a consanguineous union in comparison with a child who does not have a family history of hearing loss

Consanguinity refers to the marriage of persons closely related to each other, for example cousins. In some societies however, marriage between cousins is both encouraged and acceptable. Some of the reasons put forward include the preservation of wealth within the family, and 'keeping the blood line pure' especially among the so-called royal families. So what is the cut-off point for consanguinity in a given community, considering that some societies even encourage marriage between first cousins? There is no clear cut line for it depends on how one views the situation, whether from a sociological point of view, or from a genetic point of view. In scientific and genetic studies like this one, it is important to ignore the social definition and look rather at the genetic material to determine the cut off point. This is because the closer the genetic material, the more likely it is for autosomal recessive characteristics to emerge. The finding of many genetic recessive diseases among inbred communities, such as among the Pakistani, Indian, Bedouin, and Jewish communities, has confirmed this.

This study defined consanguinity as marriage between cousins up to second generation. It was difficult to obtain the information regarding the relationship

between parents. Attempts to get this information using self-reported questionnaires were not always successful as some of the respondents did not respond to the question. The reasons for this are not clear but could be linked to the limitations mentioned below.

Overall, the results were disappointing as only nineteen out of one hundred and seven subjects (17.8%) from Tshilidzini School responded to this question, with the majority (82.2%) of the respondents leaving it blank. However, all seventy-five subjects (100%) from Bosele School volunteered the information regarding consanguinity in the family. Because of the excellent response from Bosele, binary logistic regression analysis was used to answer this question and reliable results obtained from the model applied as demonstrated by the Hosmer-Lemeshow goodness-of-fit test and ROC curve.

It could be concluded that the respondents who indicated that there was no history of consanguinity among the parents and the one respondent who gave a positive history of consanguinity from Tshilidzini School were reliable. Similarly it may be that the non-responders did not wish to divulge this information for fear of stigmatization, because of undisclosed cultural beliefs, or due to poor interviewer/interpretation technique among the individuals who assisted the respondents. The answer however may lie with the more deeply rooted reluctance of the people of this region to speak about personal family matters such as this, just like many people are reluctant to speak about their incomes.

Consanguinity and inbreeding among communities are known to lead to segregation and concentration of recessive traits in a community, often leading to the high incidence of recessive disorders in that community. This is also true for hearing loss. As mentioned before in the section on literature review, it was through the screening of inbred communities that the first major breakthrough came in the mapping of a recessive gene. For example, screening of DNA from a large consanguineous family from Tunisia with profound non-syndromic profound hearing loss led to the mapping of the DFNB2 locus onto chromosome 11q13.5 (Guilford et al., 1994). Bengkala, an isolated old (over 700 yrs) village on the northern shore of Bali with a population of 2185 was identified to have a high incidence (2.2%) of hearing loss. The hearing loss was of a profound, fully penetrant nonsyndromal, congenital, sensorineural type. Using a combination of strategies including genome-wide disequilibrium search and homozygosity mapping, an autosomal recessive mutation was mapped at the DFNB3 locus on chromosome 17 (Friedman et al., 1995).

A study among 162 unrelated Sudanese deaf subjects showed a high rate of consanguinity, with over half of the cohort reporting marriage between cousins, while consanguinity was absent among the 406 unrelated Kenyan deaf subjects (Gasmelseed et al., 2004). All the subjects had non-syndromic deafness. A family history of hearing loss was reported among 74 Sudanese (45.7%) and 45 Kenyan individuals (11%) respectively. This confirms the effect of consanguinity on segregating and concentrating recessive mutations within a given population. A study on unrelated deaf children in Ghana did not analyze or report on the prevalence of consanguinity in the study population (Hamelmann et al., 2001). Information on consanguinity in the

current study was important in determining whether an inbred community existed in the northern part of the Limpopo province.

The findings of the current study show no clear correlation between consanguinity of parents and the prevalence of hearing loss. Almost as many respondents reported a family history of hearing loss as did not, 6 and 8 participants respectively (table 5.30). All these subjects were of the Pedi/N. Sotho language group (table 5.31), with most of the Vendas not responding to the question. It is therefore not surprising that the highest incidence was found in Greater Groblersdal and Molemole municipalities (tables 5.27-5.28, figures 5.10-5.12), areas traditionally inhabited by Pedis/N. Sothos. The N Sotho and Pedi people are known to practice consanguineous mating where cousin marriages are encouraged (Venter, Christianson, Hutamo, Makhura, & Gericke, 1995). However, as mentioned above, the lack of data on consanguinity by the respondents of the participants from Tshilidzini School makes the findings of this study biased. As such no firm conclusion can be derived from these results.

Since the results of the current study did not conclusively rule out consanguinity among the parents of the Tshilidzini School subjects, a future study will have to be conducted to answer this question. The size of this study and the limited time frame and resources determined how far one could go in getting the required information. On a positive note, this study highlights the complex family social setup facing many South African households and families, and this information can be used in structuring a broader population based study that would specifically answer these questions.

6.1.6 Mode of Inheritance of Hearing Loss in the Study Population

The results indicated that overall the observed hearing loss seemed to be recessively inherited among the definitive study group. However, no firm conclusion can be made as to the mode of inheritance of the observed hearing loss since pedigrees were not drawn. Because of the size of this study, this part of the study was shelved for a wider study in the future that will look deeper into the genetics of hearing loss among this population.

The sibling pair with WS reported normal hearing parents and grandparents, suggesting that this could be a sporadic mutation that first occurred in these siblings or in one of their parents since it is an autosomal dominant disorder, or else reflects the variable penetrance of WS. A family study with genetic testing of the pedigree would help determine this.

6.1.7 The significance of the Candidate Genes for Deafness in Limpopo

As mentioned above, one of the objectives of the current study was correlation of the mutation detection results with other studies on deafness, using a candidate gene approach, to determine whether a suitable candidate gene may have already been found. The results are significant. The observed deafness is not linked the common known auditory gene mutations in *GJB2* (coding for Connexin26), or the common mitochondrial mutations A1555G, A3243G, A7511C and A7445G. Waardenburg Syndrome Type I has been genotypically confirmed in one sibling pair of indigenous African descent in this study. For the first time in Africa, a rare mutation, R223X, previously identified only once out of a cohort of 470 WS patients (St Mary's Hospital, Manchester, UK 2003), has been identified in the *PAX3* gene in this sibling

pair. A novel silent change GGG>GGT at amino acid 293, was also identified. The controls were used in phase 2 of the study to investigate the significance of the high prevalence of *GJB2* variations g.3318-34C>T and g.3318-15C>T in the study population demonstrated a high prevalence of these two variants, 42.6% and 35% respectively, among the 63 normally hearing controls, indicating that these variations are polymorphisms and do not contribute to the aetiology of the observed non-syndromic SNHL in this population.

6.1.7.1 *GJB2* (Connexin26)

It was not surprising that the reported *GJB2* mutations common to other population groups worldwide were not present in the Venda, N. Sotho/Pedi, and Tsonga speaking African populations of the Limpopo Province of South Africa. These findings tie in well with the findings of Kenneson (2002) that *GJB2* variations are found in different proportions among different hearing impaired population groups. He noted prevalences of 43% in Israel, 20% in Japan, 20% among Caucasians of northern European descent, 17% in Tunisia, 14% in Australia, and 5% in Korea.

Until relatively recently, there was very little data on *GJB2* variations among African population groups. The study among Sudanese and Kenyan deaf populations (Gasmelseed et al., 2004) found a low incidence of NSRHL *GJB2* variations, with only the 35delG mutation found among 5 Sudanese individuals, and absent among the Kenyans (Gasmelseed et al., 2004). The study however identified 14 other variants between 95 Kenyan and 21 Sudanese individuals, all believed to be polymorphisms. In contrast, a 1998 study detected a high prevalence of R143W *GJB2* mutation among

the Ghanaian deaf population (Brobby, Muller-Myhsok, & Horstmann, 1998; Hamelmann et al., 2001).

GJB2 variations have been reported in many parts of the world, with variation in the reported distribution patterns for different ethnic groups (Kelsell et al., 1997; Estivill et al., 1998). While they seem to occur almost preferentially in some population groups, they also seem to be absent in others (Zelante et al., 1997; Brobby et al., 1998) et al 1998; (Gasmelseed et al., 2004; Denoyelle et al., 1999; Hamelmann et al., 2001).

Founder effects have been demonstrated for mutations in *GJB2*, with 35delG, 167delT and 235delC found predominantly among Caucasoid, Jewish Ashkenazi and Oriental populations respectively (Del Castillo et al., 2003). None of these mutations were observed in the current study. The current study was conducted in a population with a long history of apartheid or "separate development" where inter-racial marriages were previously strongly discouraged, and at one time punishable by law. As such it may be more representative of the genetic pool of indigenous Africans in this region.

Of interest is the significantly high prevalence of two of the variants reported among the Sudanese and Kenyan deaf children, namely g.3318-34C>T and g.3318-15C>T. Whereas the prevalence of these variations among Kenyan and Sudanese deaf subjects found was 12.7% (g.3318-34C>T) and 6.45% (g.3318-15C>T) respectively, the current study documents a prevalence of 46.2% and 21.4% respectively among the definitive study populations of the Limpopo province of South Africa. The T>A

variant at –6 was only found in the homozygous state. Its siginificance remains to be determined. None of the other variants identified in the Sudanese and Kenyan populations were identified in the South African population.

The further finding of a high prevalence of these two variants, g.3318-34C>T (42.6%) and g.3318-15C>T (35%), among the 63 normally hearing control participants is significant. The results suggest that these variations are polymorphisms and do not contribute to the aetiology of the observed non-syndromic SNHL in this population. These results are significant as they indicate that *GJB2* does not play a significant role in non-syndromic genetic hearing loss among the Venda, Tsonga and Pedi/N. Sotho speaking people of the Limpopo Province of South Africa.

Although the language groups, age and sex were not matched to the study group, they were not significant to the question asked, that is, whether the observed variations in *GJB2* in the study group were polymorphisms or not. The condition under investigation is a genetic disorder which is not influenced by age, sex or home address. The variations were found in high levels in all the language groups under investigation and so it was not essential to allocate equal proportions of language groups as in the definitive study cohort. Therefore, sampling was restricted to known the relevant cofactors, that is, indigenous Africans from language groups in the Limpopo province of South Africa. In this, the most important aspect of the matching, the comparison group (controls) paralleled the cofactors (indigenous Africans from language groups normally found in the Limpopo province of South Africa) of the case group (the definitive study cohort). This therefore fulfilled the requirement for partial restriction in the matching of the control and definitive study groups.

It would be interesting to explore the ethnic origins of the participants found with mutations in the *GJB2* coding region among the Ghanaian, and the 5 Sudanese deaf subjects (Hamelmann et al 2001, Gasmelseed et al 2004). It is possible that the observed mutations were found in participants with mixed ancestry due to intermarriage. This could be Middle Eastern or Mediterranean people among the Sudanese, and Caucasians among the Ghanaians. Population based studies are needed to answer these questions and would yield valuable information required for the development of molecular diagnostic protocols for hearing loss that are appropriate for African populations.

6.1.7.2 Common Mitochondrial Mutations A1555G, A3243G, A7511C and A7445G

None of these four mutations were identified in any of the individuals involved in this study. This was not surprising since these mutations are not supported by the medical history or clinical findings on examination of these subjects. For example, none of the subjects had reported hearing loss following injection with streptomycin.

The decision to screen for these mutations was made because one of the collaborators (T. Hutchin) was doing extensive work on mitochondrial mutations, making these mutations an easy selection for the candidate gene list. The results provide an important negative finding. They clear the way for the search of a gene for deafness in this population group through a genome screen, an expensive exercise but justifiable, having ruled out specific mitochondrial mutations as part of the aetiology.

6.1.7.3 Waardenburg syndrome

This study revealed identical mutations in the *PAX3* gene, with none in the *MITF* gene, of a sibling pair. One, R223X, was a rare nonsense mutation at the 223 amino acid (CGA-TGA, R-X, arginine – stop), which is believed to be a loss of function mutation. This mutation had been encountered only once before in a cohort of 470 Waardenburg patients (0.2%) analyzed as of February 2003 at the mentioned laboratory (James O'Sullivan, personal communication). The second mutation was a novel silent mutation at 293, GGG>GGT. A study of the mutational spectrum of Waardenburg syndrome (Tassabehji et al., 1995) screening for mutations in *PAX3* and *MITF*, identified *PAX* mutations in WS type I and WS type III individuals, and *MITF* mutations were in WS type II individuals. The results of the current study therefore confirmed the clinical diagnosis of WS Type I in this Pedi speaking sibling pair**.** These results reveal, for the first time, the mutations of WS in an indigenous population from South Africa.

Although the aim of this study was to investigate the molecular basis of non-syndromic recessive hearing loss among the study population, the finding of a sibling pair with classical clinical features of WS type I was attractive for mutation screening. Collaboration with a laboratory specializing in WS yielded rewarding results.

6.1.8 Nosological entities of Hearing Loss in the Limpopo Province

One of the objectives of the current study was to analyze the clinical findings on the subjects, and to see if that information could be built into a catalogue of clinical signs, specific for each of the forms of hearing loss identified. In this way, the collection of

sensorial deficiencies would be divided into distinct nosological entities, providing a phenotypic-genotypic correlation.

The clinical features of the participants with non-syndromal hearing loss were analysed and cross-tabulated to identify correlations. The results showed that there were no significant correlations. This was not surprising as it has been the general finding among studies on NSSNHL worldwide, and what makes an aetiological diagnosis of NSSNHL without mutation screening both difficult and challenging. Most of the identified clinical features in the current study were insignificant as they occurred in numbers too small to be linked to the observed hearing loss.

Hyper extensible fingers are common to collagen disorders, such as Marfan syndrome, Ehlers-Danlos syndrome and Stickler's syndrome. There was one participant with hyper extensibility of the fingers without other features that could link him to any of the above syndromes. The participant had normal vision and no evidence of sublaxation of the lens. He had a sensorineural type of hearing loss and no evidence of skin hyper elasticity. He also had a normal cardiovascular system. He was referred to a geneticist for further assessment.

Flexion contractures of the hands as have also been associated with WS type III. The subject in the current study found with palmer contractures however did not have any of the clinical features of WS type III (dystopia canthorum, heterochromia, premature greying) associated with his hearing loss. The presence of extra digits or polydactyly is also insignificant to the hearing loss. It is a common dominantly inherited condition, reportedly occurring in 10.40 cases per 1000 livebirths in the 'Black' South

African population (Kromberg & Jenkins, 1982), while the Mankweng Hospital survey, Limpopo province, reported 15.49 cases per 1000 livebirths in the 'Black' South African population (Venter et al., 1995).

There were 6 out of 184 participants with thick bushy eyebrows congruent in the midline at the root of the nose. This feature has been reported in Waardenburg syndrome. This subject did not have any of the other clinical features of Waardenburg syndrome. Further classification is not possible at the moment. Further studies need to be done to clarify the aetiology of this hearing loss.

The iris findings were interesting. Two participants, one with speckled irises and the other with brown speckled irises, could have iris freckles (common benign lesions composed of small spindle and dendritic cells in the superficial layers of the iris) and the Lisch nodules (collections of melanocytes and glial cells lying on the anterior layer of the iris) respectively. The iris freckles are not related to hearing loss. Lisch nodules on the other hand are as they are often found in Neurofibromatosis Type I, an autosomal dominant condition with 80% penetrance, half the cases occurring as spontaneous mutations. Lisch nodules are rare under 5 years of age, appearing after 6 years. There was no discernible skin lesion linked to Neurofibromatosis in this subject. They were referred for ophthalmic and geneticist review.

The ear findings are also inconclusive. Pre-auricular sinuses occur commonly in the general population and are not linked to any form of hearing loss. Low set ears, found in 3/182 participants, can be found in normal hearing individuals as well as in syndromes such as Down's syndrome. None of the participants showed any dysmorphic features or signs that could be linked to a syndrome. It is therefore

unclear how to interpret these findings. Together with the 2 participants with the laid back ears, these cases could not be grouped into any particular class.

One subject had small hypopigmented areas around the external meatal opening, which are possible pigmentary defects. The question arising from this is whether this could be a condition of hearing loss associated with pigmentary defects or if it is an incidental finding of say vitiligo. This subject could therefore fall under any of the Waardenburg syndrome disorders. Further genetic analysis would be required to rule this out since these conditions have been shown to have variable penetrance of any of the features (Steel et al., 1996). The eczema and dermatitis are non-specific findings, commonly found in the normal hearing population.

The palatal findings were for most part insignificant. As discussed above, the cleft soft palate represents a misdiagnosis and therefore a mismanagement of a child who could have had early surgical repair and been mainstreamed as she does not belong in a School for the Deaf.

Haematuria was found in six participants and was confirmed to be Bilharzia, a concomitant finding unrelated to the hearing loss. Bilharzia is endemic in this population and should continue to be routinely screened for and treated at primary care level, just as it is in the normal hearing population. Bladder bilharzia is a treatable condition and may occur sporadically without any relationship to the hearing loss. On the other hand, the haematuria of Alport syndrome, a condition in which nephritis and late onset hearing loss is an autosomal dominant condition and would have ruled these participants out of the current study.

The combination of dystopia canthorum, heterochromia irides, bushy eyebrows converging at the root of the nose are pathognomonic of the Waardenburg Syndrome type I, as laid out by the Waardenburg consortium and others (Tassabehji et al., 1995; Liu et al., 1994; Liu, Newton, & Read, 1995). The detection of PAX3 mutations in a sibling pair in the current study confirmed the genetic diagnosis of WS Type I, in line with the clinical findings. In this population group, it may be assumed that hearing impaired individuals with these clinical features are likely to have mutations in PAX3 as concluded by the Waardenburg Consortium (Farrer et al., 1994). However, this needs to be confirmed in a wider study among WS hearing impaired individuals from this region. Remembering that WS has variable penetrance, with clinical subtypes often indistinct, participants with WS Type I may be missed unless mutational screening is performed. Tassabehji et al (Tassabehji et al., 1995) concluded that PAX 3 mutations are not a common cause of auditory -pigmentary syndromes other than WSI.

6.2. Conclusions

6.2.1 High risk areas for hearing loss in the Limpopo province of South Africa

The null hypothesis has been rejected by the finding that there seem to be areas of genuine populations at risk for hearing loss in the Limpopo province of South Africa, namely Mutale and parts of Makhado and Thulamela municipalities. Using the municipal ward units mentioned above, possible high risk areas for deafness were identified, in Thulamela (NP343) wards 11-15, 26-30 and 31-35, and in Mutale (NP 344) wards 6-10, within the province. Together, these units accounted for 67 (18%) of participants in phase 1, and 33 (18%) of the participants in phase 2 of the study. This will require further study, to be undertaken in future research.

The results of this study also demonstrated a high, normalized frequency for hearing loss at Makhuduthamaga and Fetakgomo municipalities in the Limpopo province of South Africa. These areas could also turn out to be high-risk areas for nonsyndromal genetic hearing loss and follow-up study will be required to conclusively establish this.

6.2.2 Clinical Perspectives

The results of this study demonstrated no significant clinical features associated with the non-syndromal recessive hearing loss in this population. Most of the identified clinical features are insignificant as they occurred in numbers too small to be linked to the observed hearing loss. Audiological assessment indicated that whereas the majority of subjects exhibited significant sensorineural hearing loss of severe (22.8%) to profound severity (75%), with the majority exhibiting flat (70.1%) or sloping (23.4%) audiograms that were commonly symmetrical (81.5%), there was no clear pattern overall in the audiological findings.

The presence of balance disorders among 5/184 participants indicates that future studies should be designed to include vestibular testing as well as CT scans to identify features such as a widened vestibular aqueduct.

6.2.3 Genetic Perspectives

This study has established firmly that GJB2 is not a significant gene for deafness in the Venda, N. Sotho/Pedi or Tsonga speaking African population groups in the Limpopo Province of South Africa. The high prevalence of GJB2 variants, g.3318-

34C>T and g.3318-15C>T, among both the hearing impaired and the normal hearing Venda, N. Sotho/Pedi or Tsonga speaking African population groups in the Limpopo Province of South Africa, suggests that these are common polymorphisms in this South African population. Due to its close proximity to the start codon, the effect of the T>A homozygous variation will need to be explored. The large number of subjects in this study without a confirmed aetiology of deafness poses the possibility of another unidentified significant deafness gene in this population. When compared to the study on Kenyan deaf children with suspected non-syndromic hearing loss (Gasmelseed et al., 2004) in whom no significant mutations in the GJB2 gene could be detected, the results of the current study suggest that different genes vary in significance among different populations.

Although it is known that consanguineous mating is practiced widely especially among the African populations of the current study (Venter et al., 1995), no conclusive relationship could be established between the observed hearing loss and the prevalence of consanguinity. But because of the high level of non-responders, segregation of a recessive deafness gene due to consanguinity in this population group could not be ruled out. The combination of geographical clustering of hearing loss as well as the historical and cultural evidence of consanguinity in this South African population, could contribute to a founder effect. The full implication of these findings will only become clear in light of the results from wider population based studies among non-Caucasian hearing impaired populations.

Similar to studies among other African populations, the 342-kb deletion, *GJB6*-D13S1830, was not detected in the current study. Since the coding region of *GJB6* was not sequenced, its role in the South African population studied remains uncertain. Identification of the common genes for deafness in the African population of the Limpopo will require either a genome screen, or else the screening of deaf pedigrees. The finding of novel TMC1 variants in a Sudanese pedigree (Meyer CG et al., 2005) indicates that latter approach may be the preferred first step. Deafness genes associated with balance disorders include the Myosin genes (USH1B, Myosin VI, and Myosin VII) as well as other recessive genes such as Pendred (which has been found to localize close to the USH1B gene) are possible candidates for this search.

The results of the current study confirmed the clinical diagnosis of WS Type I in a Pedi speaking sibling pair. Two mutations were found in the PAX3 gene, a rare mutation, R223X, which is a nonsense mutation at the 223 amino acid: CGA-TGA, R-X, arginine – stop, and a novel silent mutation at the 293 amino acid, GGG>GGT. No mutations were found in the MITF gene. These identical findings document, for the first time, the molecular defect in WS type II in sub-Saharan Africans, and suggest a possible mutational spectrum of WS among Africans in the Limpopo Province of South Africa. Further studies among a larger sample are needed to confirm these findings.

6.2.4 Policy Issues

The current study demonstrated seemingly genuine populations at risk for genetic nonsyndromic hearing loss within the Limpopo province of South Africa, and demonstrated that parents of genetically hearing impaired children in these areas are

able to detect hearing loss at an early age, with over 60% suspecting their children's hearing loss below 6 months of age.

Although apparently at odds, policy and epidemiology are complimentary in the provision of services. Both are especially essential to the delivery of appropriate medical services. From a policy stand point, services are delivered where needed. In the health sector for example, if there are 50 individuals suffering from disease xx in an area, if enough noise is made from the ground, it may constitute a political crisis for the policy makers and move them to take urgent intervention measures, for fear of losing their electorate. This may either be the provision of a health service and/or medication to the affected individuals, regardless of the population density in the area. Government often responds to a 'wants' instead of 'needs', and therefore need epidemiologists to guide the switching and allocation of funds within the chronically limited budgetary framework.

To the epidemiologist on the other hand, the occurrence of 50 affected individuals in village may or may not constitute a crisis, depending on other factors such as population density, the disease type and the natural history of the disease. If the condition constitutes a risk to the community at large, as occurs with communicable diseases such as meningococcal meningitis, or demonstrates high prevalence or incidence, or is life threatening, it may well constitute an emergency, requiring the readjustment of medical provision. The aetiological investigation will therefore govern further action. This, then, is how data from medical records and audits, and the aetiological diagnosis of disease, often leading to community based research, guides policy in the provision of appropriate healthcare.

The results of the current study reveal that over 70% of the parents detected hearing loss in their children before the age of 6 months, implying that these children could have been picked up soon after birth through a Neonatal Hearing Screen, and rehabilitated at an early age. This would have fulfilled the goal laid out in the position statement of the Professional Board For Speech, Language and Hearing Professions at the Health Professions Council of South Africa on universal hearing screening (HPCSA 2007) that intervention for infants with confirmed hearing loss to be instituted by 6 months of age and no later than 8 months of age.

The planned assessment and management of the hearing impaired child should be based on a patient-centered approach, always taking into account the needs of the hearing impaired child, and reflecting a need-based protocol as depicted in figure 3.6. From these a management model can be constructed for the hearing impaired child, taking into consideration the prevailing circumstances in the child's life, as well as the logistical and financial constraints of the available healthcare system and of the child's community. The various components of an effective paediatric audiological medicine service, summarized in figure 3.7, is feasible in the South African environment It encompasses all the areas relevant to childhood deafness/hearing impairment in a way that links these areas together so that none is left unattended. In the UK the audiological physician heads the team and links all the various service providers in a patient-centred manner, acting as the hearing impaired child's advocate.

Although South African universities do not yet offer this qualification, it was gazette and registered as a recognized qualification in South Africa in August 1998. There is currently one registered audiological physician in South Africa. Although the

Professional Board for Speech, Language and Hearing Professions HPCSA 2007 joint statement clearly notes the current gaps in the management of the hearing impaired child, especially with regards to early detection and intervention of hearing loss, this proposed model goes further by articulating the management areas and the team needed, and clearly shows how they are linked together in a patient centered manner..

The Health and the Education Policies that cover these issues and the required infrastructure are in place (South African Department of Health. July 2000; South African Department of Health. July 2004; South African Department of Health. March 2000; South African Department of Education. 2001). National Treasury already funds the personnel posts indicated in this model (South African Department of Health. April 2007; South African Department of Social development. 2006). The three areas under education are covered policy wise and financially. Assessment is also covered. Likewise neuro-otological investigations, aetiological investigation, and medical treatment are covered under the health services. All the disciplines under liaison already exist within the South African health services, both public and private. Counseling services, amplification (including cochlear implants) are also established and provided for in the health service system. Research and continued medical education (CME) policies have been implemented for all health professions. Preventative audiological medicine is already on the ground, but, more importantly, the structures for its improvement are well established under the PHC programme.

Notification structures are already in place, and work extremely well for diseases under the PHC and immunization programmes, for example the communicable diseases such as TB, measles, polio etc. The same structures and models can be used

to develop and implement effective Special Education Needs (SEN) assessment tools, and, together with the department of Education and employers, improve access to effective education for the hearing impaired, all the way into university and other tertiary institutions and beyond. At a recent meeting with representatives of government, the head of Worcester (a center for the Deaf in South Africa) announced that according to their records, the hearing impaired matriculants' pass rate was a dismal 26%.

As mentioned above, what is required for implementation of this model is a paradigm shift from the current fragmented model of service delivery to a cohesive patient-centered approach, based on concrete data from appropriate community based research, in which all the relevant parties communicate and share resources.

The failure of adequate management of the hearing impaired individuals in South Africa, especially the children, clearly lies in implementation of policy at the provider level and not at policy maker level. What has been lacking is the cohesive approach to the management of the hearing impaired individual, as demonstrated in the fragmented manner in which the various healthcare workers operate around the patient. Improved communication between and co-operation among the key healthcare workers managing the hearing impaired person need to be addressed as a priority. This includes not only the healthcare professionals but also the policy makers, the various departments linked with the deaf child including education and social welfare, as well as the principals and teachers at both the schools for the Deaf and regular schools that receive mainstreamed hearing impaired students.

As previously noted, concrete health data is generally not available to policy makers or the managers implementing policy, because appropriate research addressing the priority issues has not been conducted. Therefore, appropriate research and better planning among the health care professionals, combined with a paradigm shift away from a provider-centered to a patient-centered approach to patient management, is mandatory if the status quo is to be broken. Both policy and personnel are in place for the formation of a team approach to the management of the hearing impaired child. This approach would eliminate the gaps in management without necessarily increasing the costs since all the personnel and facilities are already funded. For this reason, the model used in the UK (figure 3.3), modified to suit the South African environment, is proposed. This should start with pilots in the areas where the infrastructure and personnel are already available and later spread out to areas where the need is identified.

The aim should be to move our health care from an inadequate system (figure 3.1) towards the ideal system (figure 3.2). To quote the Policy on Quality in Health Care for South Africa (Dept of Health 2007, pages 10-11) where health care providers and workers are jointly cautioned: *"A successful national effort to improve health care quality will need to build on existing resources, experience and expertise. All efforts should promote and strengthen existing innovative work that is being done. Competing with, stifling or slowing down these actions will not advance the agenda for quality improvement."*

6.3 RECOMMENDATIONS

There were three broad goals of the current study as indicated below.

First, this study intended to establish more specifically the various aetiologies of genetic hearing loss among the people of the Limpopo province of South Africa, through scientifically acceptable methods.

Secondly, this research sought to determine the level of consanguineous mating and its possible impact on the aetiology of genetic hearing loss in this population, with the purpose of educating families and the communities about the consequences of consanguineous mating.

Thirdly, this study intended to provide basic data on hearing loss in the province that could be used for improving and boosting the secondary preventative rehabilitation measures. For example, the results of this study could lay a foundation for ***early identification*** *and* ***early appropriate rehabilitation*** *of significant childhood hearing loss to be instituted in the province. Through these measures, individuals affected by significant hearing impairment would be assisted to become empowered, self-sufficient, and productive members of their communities.*

These goals have been met to a large extent, although non-disclosure limited the amount of data obtained about consanguinity in the province. Nevertheless, significant deductions have been drawn. As a result of the findings of the current study, it is recommended that:

1. A continuation of this study into a wider search for genes for deafness in this population be carried out, starting with pedigrees and families with nonsyndromal genetic hearing loss.

2. An expanded study on the mutational spectrum of Waardenburg Syndrome should be carried out among the deaf population with clinical signs of WS, first in the Limpopo province and then nationally, to establish the mutational spectrum of Waardenburg syndrome in South Africa.

3. It would be beneficial to the hearing impaired patient if the healthcare professionals managing hearing impaired individuals established a team approach, with more stringent diagnostic and medical evaluation procedures for all children with hearing loss prior to referral to schools for the deaf. This would ensure correct placement and maximize the rehabilitation of all hearing impaired children. There is also a need for regular and streamlined follow-up of hearing impaired subjects as a whole, but especially in the public sector. The model of a team approach as practiced in the UK and in the United States of America, modified to suit South African environment, would go a long way to meet these identified needs. If developed for South Africa, it would yield great results in the management of the hearing impaired individuals in this country.

4. There is a need to move away from the tendency of institutionalizing disabled people in South Africa, recognizing that they too need to enjoy a normal family life if they are to become fully integrated, responsible and productive members of their communities. Early diagnosis and management, including catering for their special needs in both the educational and public institutions

in this country through adequately funded programmes, will facilitate the achievement of this vision.

5. The establishment of Universal Neonatal Hearing Screening Programmes, especially in public health facilities, is urgently needed for the early detection of genetic hearing loss. As mentioned before in the section on epidemiological studies of hearing loss, Davidson et al. **(Davidson et al., 1989)** postulated that if the exact distribution of hearing level at birth and how this level changed over time were known, any data could be compared with ease. Universal neonatal hearing screening would establish the distribution of hearing level at birth, while later hearing screening combined with data from health facilities would serve as a basis for health planning with a resultant improvement in service delivery, providing a more efficient, appropriate cost-effective health care service. The move towards the ideal relationship between needs, wants and supply would then be possible (figure 3.2).

6. With the current trend moving towards molecular diagnostics, aggressive research into genetic hearing loss in the South African setting is mandatory if proper diagnosis and management of hearing impaired patients is to be achieved. Increased funding and collaboration between institutions would help to rapidly acquire the necessary molecular diagnostic skills required for the development of an effective healthcare system where highly qualified/trained healthcare professionals serve a satisfied clientele. This need to foster evidence-based practice and innovation has been summed up in the recommendations found in the Policy on Quality in Health Care for South Africa (2007) as quoted below (pg 13):

'Both public and private sector funding needs to focus on:

- **Basic, clinical, prevention and health services research specific to the needs of South Africa;**
- **Strengthening the scientific evidence base for health care practices through collaborating in technology assessment and research targeted at gaps in existing knowledge, in the South African context; and**
- **Encouraging widespread adoption of innovations that have been demonstrated to be effective, through awareness raising, information, and technical and other support for implementation programmes.'**

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APPENDICES

Appendix 1: Participant information and informed consent

PARTICIPANT INFORMATION

We are carrying out research on childhood hearing loss in the Northern Province in order to better manage the hearing impaired people of the province.

Many causes of hearing loss are known to exist, including infections, injury to the hearing organ, chemical effects, as well as hereditary causes. Because of this, the study will include a full clinical examination of all the subjects as well as full audiological evaluation. This will include hearing tests such as pure tone audiogram, tympanometry, otoacoustic emission testing and auditory evoked response audiometry. These tests are painless and not harmful to your health. A sample of urine will also be collected for analysis. Blood tests will involve collection of not more than 20ml (four teaspoonfuls) of blood from the inner aspects of the elbow using a sterile disposable needle under aseptic conditions. The minimal discomfort involved in blood collection will not pose a threat to your health. The blood samples will be analysed in the hospital laboratories.

We will also collect information about your birth and childhood history as well as family social history. The close family members of all participants considered to have genetic types of hearing loss will also be given a full clinical and audiological assessment. A selection of about 100 per subjects and their families will undergo genetic studies to see if their hearing loss could be caused by a gene defect. For this 10mls (two teaspoonfuls) of blood will be collected as above. This blood will be assessed at the laboratory for the cause.

Strict confidentiality will be followed throughout this study. Code numbers will be used to represent the persons taking part in the study, and their identity will not be exposed. All the participants found with hearing loss will be rehabilitated as necessary, including hearing aid fitting where possible.

Participation in the study is voluntary. Anyone can withdraw at any stage of the study without prejudice to future treatment or loss of benefits. For further information contact:

DR R I Kabahuma: Pietersburg Provincial Hospital **tel 015-2973163**

Appendix 1 cont…

Informed Consent

I Mr./Prof./Dr./Miss/Mrs. ……

1. Hereby acknowledge that I understand the nature of the research project.
2. Understand that all information given by me will be treated as strictly confidential.
3. Understand that my partiipation is voluntary and that I am free to withdraw at any stage of the research without penalty or loss of benefits.
4. Have been informed that I may be required to undergo a full medical examination, investigations such as blood tests, urine tests, x-rays, CT-scans, as well as vestibular testing.
5. Photographs of body parts (such as head and neck, extremities) may be taken.
6. Aseptic techniques will be used to draw not more than 20ml of blood.
7. Understand the relative risks involved in venepuncture and x-ray exposure.
8. Consent *to take part / for my child to take part* in this research.

Signed Witness

Signature…….. *Signature*……

Names (print)……. *Names (print)*……

(Participant/guardian/parent)

Position…….

Appendix 2a: **Demographic data for each hearing impaired student in a school for the deaf in the Northern Province**

Student's names …….

Date of Birth ……

Place of birth ……...

Present home area (village, headman/chief) …….

Home area at birth (village, headman/chief) ……

Type and degree of hearing loss ……..

Medical record available? Yes/No ……..

Cause of hearing loss ……..

Hearing aid usage ……

Balance disorders (dizziness or clumsiness) ……..

Other medical disorders ……..

Parent's names ……

Mother's home area (origin) ……

Father's home area (origin) …….

Relationship between parents (if any) …….

Hearing status of mother …….

Hearing status of father ……...

Siblings (sex, date of birth, hearing status of each child, name of biological father and biological mother of each child-indicate only if any of the parents is different from the deaf student's parents)

1 ……...

2 ……...

3……

4 ……...

5 ……...

6 ……...

7 ……...

8 ……...

9 ……...

10 …….

Appendix 2b: Audiological Case History Questionnaire for Parents of Hearing Impaired children
(After Northern and Downs, 1993)

Chief complaint

When was problem first noted? ……...
Extent of problem ……
Previous examinations and evaluations ……...
…….

Prenatal history

Exposure to disease during pregnancy? ……..
Which disorder?……...
During which pregnancy month? ……
Drugs during pregnancy? ……
Trauma during pregnancy? ……..

Birth history

Gestation age at birth ……...
Birth weight …….Bilirubin level high?……
Asphyxia? ……Meningitis? ……

Family history

Childhood deafness in family ……..
Relationship to patient …….
Birth defect or abnormalities ……...
In any other relatives?……..

Development history

Age of first smile response? ……
Age when sat up alone? ……...
Age when first crawled? ……..
Age of "stranger anxiety"? ……..
Age of walking? ……..

Physical history

Cleft lip or palate ……Submucous cleft …….
Low-set ears ……Poorly formed ears……..
High fever with illness ……Seizures ……..
Ear Infections ……..How many?…….
Previous treatment for ear conditions? ……
……..

What do you (parents) really think caused this hearing problem?

…….
…….
Name of child's paediatrician …….
Names of other physicians who have seen this child……...
……
……
……

Appendix 3: Sensorineural Hearing-Impaired Child Assessment form
(After Northern and Downs, 1993)

Name(s) ……..Surname ……..
Age ……..
Date of Birth …….
Hospital number …….
Age child identified by GP/MO (months) …….
Age suspected of hearing loss by mother (months)……

FAMILY HISTORY

Were parents relatives before marriage Yes/No
Family history of kidney disease Yes/No

Family history of thyroid problems

Yes/No

Family history of progressive blindness Yes/No
Family history of previous stillbirths and miscarriages Yes/No
Family history of hearing loss Yes/No
Another affected child in family Yes/No

MARTENAL FACTORS

Drugs (including antibiotics) Yes/No
Specify…….
Exposure to chemicals Yes/No
Specify…….
Exposure to radiation Yes/No
Specify…….
Amniocentesis Yes/No
Rh immunoglobulin given Rh or ABO incompatible Yes/No
Maternal illness during pregnancy Yes/No
Specify……
Bleeding Yes/No
Anaemia Yes/No
Diabetes Yes/No
Toxemia Yes/No
Paternal illness during pregnancy Yes/No
Specify……
Mother worked outside home Yes/No
Specify…….
Father worked during pregnancy Yes/No
Specify…….
Durign pregnancy mother exposed to
Measles Yes/No
Mumps Yes/No
Chiken Pox Yes/No
German measles Yes/No
Syphilis Yes/No
Herpes virus Yes/No
Influenza Yes/No
Cytomegalovirus Yes/No
Toxoplasmosis
Other (specify) ……...

DELIVERY/LABOUR

Full-term pregnancy Yes/No
Labour induced Yes/No
Labour less than 3hr Yes/No
Labour longer than 24hr Yes/No
Premature membrane rupture Yes/No
Bleeding Yes/No
Forceps/assisted delivery Yes/No
Caeserian section Yes/No
Other Yes/No
Specify……..…
……
……

INFANT/NEWBORN FACTORS

Small birthweight (<2kg/5lb) Yes/No
Birthweight ……...
Apgar low at birth Yes/No
In an intensive care unit Yes/No
How long? …….
Breathing problems Yes/No
Oxygen given Yes/No
How long? ……
Bilirubin>15mg/100ml Yes/No
Congenital rubella Yes/No
Defect of ear, nose, throat Yes/No
Specify ……
Congenital heart disease Yes/No
Drugs including antibiotics Yes/No
Specify ……
Exposure to chemicals Yes/No
Specify ……
Exposure to radiation Yes/No
Specify ……
Paralysis
Seizures
Septicaemia

INFANT/CHILHOOD HISTORY

Eye problems Yes/No
Specify ……..
Balance/gait/incoordination/dizziness Yes/No
Cerebral palsy Yes/No
Seizures Yes/No
Head trauma/injury Yes/No

INFANT/CHILDHOOD HISTORY(continued)

Ever hospitalized for:

Meningitis	Yes/No
Encephalitis	Yes/No
Measles	Yes/No
Influenza	Yes/No
Rubella	Yes/No
CMV	Yes/No
Chicken pox	Yes/No
Septicaemia	Yes/No
Diabetes	Yes/No
Sickle cell disease	Yes/No
Other (including conductive hearing loss)	Yes/No

Specify …….
……..
……..
……..

Appendix 4: Family Tree for Hearing Impaired Child

Subject's Surname				**Date of birth**	**Sex**
Subject's First name(s)				**Place of birth**	
Mother's names			DOB		Place of birth
Father's names			DOB		Place of birth
Siblings	**1**	DOB	Birth place	Sex M/F	Hearing status N/I
	2	DOB	Birth place	Sex M/F	Hearing status N/I
	3	DOB	Birth place	Sex M/F	Hearing status N/I
	4	DOB	Birth place	Sex M/F	Hearing status N/I
	5	DOB	Birth place	Sex M/F	Hearing status N/I
	6	DOB	Birth place	Sex M/F	Hearing status N/I
	7	DOB	Birth place	Sex M/F	Hearing status N/I
	8	DOB	Birth place	Sex M/F	Hearing status N/I

Maternal grandmother	Place of birth	Hearing status N/I
Maternal grandfather	Place of birth	Hearing status N/I
Paternal grandmother	Place of birth	Hearing status N/I
Paternal grandfather	Place of birth	Hearing status N/I
Maternal greatgrandma	Place of birth	Hearing status N/I
Maternal greatgrandpa	Place of birth	Hearing status N/
Paternal greatgrandma	Place of birth	Hearing status N/I
Paternal greatgrandpa	Place of birth	Hearing status N/I

Relationships noted:

Appendix 5

MEDICAL EXAMINATION

Name: Address:	Contact	DOB: Sex: **M F**	School:
		Code **T000 /**	

General examination: *Temp* ºC *Pulse* /min *Other* *Height* cm *Weight* kg	**Pigmentation:** *Skin* *Hair* *Iris*
Skeleton: *Hands* ***R*** ***L*** *Feet* ***R*** ***L*** *Spine*	**Eyes:** size shape position R L med.canthal distance other R L
Ears: **Pinna** size shape position other R L **EAM** size shape position other R L **R** **L** **Tympanic membrane**	**Throat:** Gums Dentition Palate Tongue

Nose	**CVS** *BP* mm/Hg Heart sounds *Murmers* *Other*

Respiratory System	**Abdomen**
CNS	

Investigations

Urinalysis

FBC

U&E

TEOAEs

Tymps ARTs

Audiogram

Appendix 6
IDENTIFYING RISK FACTORS FOR SENSORINEURAL HEARING LOSS IN

NEONATES AND INFANTS

NEONATES (BIRTH TO 28 DAYS)		**INFANTS** (29 DAYS TO 2 YEARS)	
CODE	RISK FACTOR	CODE	RISK FACTOR
A	FAMILY HISTORY OF SENSORINEURAL HEARING LOSS	1	PARENTAL/CARE GIVER CONCERN REGARDING HEARING, SPEECH, LANGUAGE, AND/OR DEVELOPMENTAL DELAY
B	KNOWN OR SUSPECTED MATERNAL INFECTION	2	BACTERIAL MENINGITIS
C	CRANIOFACIAL ANOMALIES	3	NEONATAL RISK FACTORS AS LISTED ABOVE
D	BIRTH WEIGHT LESS THAN 1500gm	4	HEAD TRAUMA
E	HYPERBILIRUBINAEMIA	5	STIGMA OF KNOWN SYNDROME
F	OTOTOXIC DRUG USE	6	OTOTOXIC DRUG USE
G	BACTERIAL MENINGITIS	7	NEURODEGENERATIVE DISORDERS (E.G NEUROFIBROMATOSIS)
H	ANOXIC OR HYPOXIC EVENTS	8	CHILDHOOD INFECTION DISEASES (E.G. MUMPS, MEASLES)
I	PROLONGED MECHANICAL VENTILATION		
J	STIGMATA OF KNOWN SYNDROMES		

Adopted from JCIH 1990, The National Deaf Children's society,UK (1994)

Appendix 7

STANDARD OPERATING PROCEDURE

MODIFIED FROM: Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* 16 (3): 1215.

NOTE: All samples in this extraction process are centrifuged in the Beckman Benchtop GS-6R centrifuge at 2400 r.p.m. at 4°C.

RESPONSIBILITY
The Medical Scientist who has been assigned the task for the week. If on leave the second medical scientist or the Head of the Molecular Genetics Diagnostic Laboratory.

PROCEDURE:
DAY 1:

1. Collect blood into purple-top EDTA.
2. Decant no more than 10ml of whole blood into a 50 ml polypropylene tube (a NUNC), i.e. 2 or sometimes 3 ACDs/EDTAs per NUNC tube. If only 3-5 ml of blood is available, halve the volumes of solutions in the protocol. Mark these tubes with an asterisk on the lid. Freeze the blood at -40^{o} C until required (all blood samples are preferably frozen before DNA extraction). The extraction procedure is usually done in a batch of 16 samples.
3. Centrifuge additional blood tubes for 10 minutes to separate the whole blood into its three phases, viz. plasma layer on top, a middle buffy layer (consisting of leukocytes or white blood cells) and the red blood cell layer at the bottom. Discard the plasma and aspirate the buffy coat, together with the top layer of the red blood cell layer (containing young nucleated red blood cells) into a new 10ml tube. Freeze at -40°C as a back-up sample in a box that is labelled appropriately.
4. Carry out salting-out extraction procedure.
5. Thaw the whole blood including white blood cells, red blood cells and plasma at room temperature when ready to start the extraction of the DNA. Usually 1 hour is sufficient to thaw the blood.
6. Fill each 'full' 50ml NUNC tube containing the whole blood to the 40ml mark and each 'half' sample to 20ml with cold sucrose-Triton-X lysing buffer (which should be kept cold during the procedure). This detergent lyses the red blood cells.
7. Invert the NUNC tubes several times to mix.
8. Centrifuge the NUNC tube for 10 minutes.
9. A reddish-white pellet is visible.
10. Pour off the supernatant fluid (SNF), containing lysed red blood cells into a beaker clearly labelled as "Blood Waste". Ensure that the pellet does not dislodge when pouring off the SNF.

11. For all samples (full and half), wash the pellet well with 20 - 25ml of the cold sucrose-Triton-X lysing buffer.
12. Put the NUNC tube on ice or in -40°C freezer for 5 minutes.
13. Centrifuge the NUNC tube for 5 minutes. These washes are carried out several times (although 2 washes are sufficient) to ensure that most of the red cell debris is removed in the supernatant.
14. Pour off the SNF into the Blood Waste beaker.
15. Add 3ml T20E5, 0.2ml (200µl) 10% SDS and 0.5 ml (500µl) Proteinase-K mix to each full sample. This step lyses the white blood cells and degrades the protein. The detergent SDS acts by breaking up the lipid bilayer of the cell membrane.
16. Mix the components of the NUNC tube well by inversion.
17. Incubate overnight in the 42°C incubator (No need to agitate).

DAY 2:

18. Agitate saturated NaCl solution.
19. Add 1ml-saturated NaCl (clear liquid) to the lysate.
20. Agitate vigorously for 15 seconds by inversion.
21. Place the tube with the salt at -40°C or on ice for 10 minutes.
22. Centrifuge for 30 minutes.
23. A white pellet should be visible, containing proteins precipitated by salt. If no pellet is visible spin again.
24. Transfer the SNF containing the DNA to a new NUNC tube, labelled with the patient's name and disease code on the tube and with just the disease code on the lid.
25. Add 2 volumes of absolute ethanol kept at room temperature (the DNA is precipitated out of solution with the addition of absolute ethanol. In the presence of high salt concentration, DNA is not soluble in ethanol).
26. Agitate gently and spool, fish or precipitate the DNA (refer to next section).
27. Wash DNA in 70% ice-cold ethanol (the DNA is fished out and washed in 70% ethanol to remove excess salt that could interfere with enzymatic reactions e.g. PCR).
28. Place DNA o the side of a new safety-lock Eppendorf tube labelled with patient's name, disease code and date of receipt. Remove all traces of liquid from the Eppendorf tube. Air dries the DNA and resuspended in an appropriate amount of 1xTRIS-EDTA (TE) buffer (usually 200µl to 1000µl).
29. The resuspended DNA is stored at 4°C until required.

PROCEDURE FOR PRECIPITATION OF DNA

1. If the DNA is not visible or is fragmented, precipitate the DNA either at -20°C overnight or at -70°C for 30 minutes.
2. Centrifuge the NUNC tube for 20 minutes to pellet the DNA.
3. To avoid salts and proteins precipitating out as well, do not spin for too long.
4. Pour off the SNF.
5. Wash the DNA pellet with 70% ethanol (~10ml) and centrifuge for 10 min.
6. Pour off the SNF and invert the NUNC tube on a paper towel to dry the pellet.
7. Resuspended the DNA pellet in an appropriate amount of 1x TE buffer (usually 20-100µl).

8. The resuspended DNA is stored at 4°C until required in a tube labelled with the patient's name, disease code, date received and "ppt".
9. Dialyse half the sample before use in a test.

SOLUTIONS USED IN EXTRACTION PROCEDURE

SUCROSE-TRITON-X-LYSING BUFFER:
10 ml 1M Tris-HCl pH8
5ml 1M $MgCl_2$
10ml Triton-X 100
Make up to 1L with dH_2O
Autoclave
Keep solution chilled at 4°C
Add 109.5g sucrose just before use.
(Do not keep longer than 1 day)

20mM Tris 5mM EDTA (T20E5):
20ml 1M Tris-HCl (pH 8.0)
10ml 0.5M EDTA (pH8.0)
Make up to 1L with dH_2O
(Autoclave)

1x TRIS EDTA (TE) BUFFER:
10ml 1M Tris-HCl (pH8)
2ml 0.5M EDTA
Add dH_2O to volume (1L)
Autoclave.

PROTEINASE-K MIX:
For 16 extractions make up:
400µl 10% SDS
16µl 0.5M EDTA
2.8ml autoclaves dH_2O
Add 800µl Proteinase-K (10mg/ml stock)

SATURATED NaCl:
Autoclave 100 ml of dH_2O
Slowly add 40g NaCl until absolutely saturated i.e. some NaCl will precipitate out.
Before use, agitate and let NaCl settle, use clear supernatant.

0.5M EDTA:
93.06g EDTA
Make up to 500ml with dH_2O
pH to 8.0 with NaOH
(EDTA will only dissolve once correct pH is reached)
Autoclave.

1M $MgCl_2$:
Add 101.66g $MgCl_2$
Make up to 500ml with dH_2O
Autoclave

1M TRIS-HCl pH 8.0:
121.1g Tris
make up to 1L with dH_2O.
Autoclave

10% SDS
Add 10g to 100ml autoclaved dH_2O
Just before use.

SAFETY MEASURES

Refer to section B, C, D and E of the NHLS safety manual.

REAGENT STORAGE

Proteinase K is stored in the 20°C diagnostic freezer.
All other reagents are stored at room temperature on the diagnostic shelf.

REAGENT PREPARATION

1. The sucrose component for the Sucrose-Triton X solution must be added on the day of the extraction. This solution must be chilled at 4°C prior to use.

2. All other reagents can be prepared in advance, autoclaved and stored until ready to use. It is important to label the solution appropriately with the solution name and concentration, date of preparation and the initial of the person that prepared the solution.
3. If a reagent has been depleted at the time of an extraction procedure, it can be prepared concurrently and used without autoclaving. The remainder of the solution must be autoclaved and can be stored for future use.

WASTE DISPOSAL

1. All blood-contaminated items must be disposed of in the biohazard bin.
2. For blood waste, a 1/10 volume of bleach solution must be added to disinfect it and then disposed of in a waste bottle that has been labelled appropriately.

Appendix 8: Demographic data of the people of the Limpopo Province: Mid-year estimates 2004

Source: Department of Health and Social Services, Limpopo Province, Population and Development Unit report, 2005

Table 1: Population of Limpopo showing citizenship by district

Citizenship	Mopani	Vhembe	Capricorn	Waterberg	Sekhukhune	Bohlabela	Limpopo
South Africa	951,320	1,186,212	1,151,469	608,114	744,599	590,964	**5,232,678**
SADC Countries	12,385	12,679	2,287	5,566	704	4,037	**37,658**
Rest of Africa	109	215	246	97	75	27	**769**
Europe	196	134	268	238	15	98	**949**
Asia	102	586	324	87	61	34	**1,194**
North America	80	27	69	39	3	27	**245**
Central & South America	12	27	16	12	9	12	**88**
Australia & New Zealand	27	3	6	3	-	3	**42**
Total	**964,231**	**1,199,882**	**1,154,685**	**614,156**	**745,467**	**595,202**	**5,273,623**

Table 2: Age distribution in five – year intervals by population group, males, Limpopo Province

Age group	Black African	Coloured	Indian/Asian	White	Total
0 - 4	294583	526	433	4109	**299651**
5 - 9	353174	589	395	4605	**358763**
10 - 14	368168	561	353	5575	**374657**
15 - 19	338368	541	321	5647	**344877**
20 - 24	214916	457	494	3754	**219621**
25 - 29	147639	402	622	4332	**152995**
30 - 34	116261	385	518	4857	**122021**
35 - 39	104273	312	369	4717	**109671**
40 - 44	85916	277	296	4861	**91350**
45 - 49	71468	203	221	4263	**76155**
50 - 54	58999	168	253	3926	**63346**
55 - 59	42695	117	175	3331	**46318**
60 - 64	38910	82	117	2744	**41853**
65 - 69	27737	67	83	2117	**30004**
70 - 74	25697	53	48	1703	**27501**
75 - 79	14764	28	31	955	**15778**
80 - 84	12207	8	8	576	**12799**
85+	7186	7	12	217	**7422**
	2322961	**4783**	**4749**	**62289**	**2394782**

Table 3: Age distribution in five – year intervals by population group, females, Limpopo province

Female	Black African	Coloured	Indian/Asian	White	Total
0 - 4	297903	543	428	3996	**302870**
5 - 9	356023	529	340	4326	**361218**
10 - 14	373407	578	352	5249	**379586**
15 - 19	344230	598	324	5531	**350683**
20 - 24	252840	513	316	3621	**257290**
25 - 29	208873	484	413	4678	**214448**
30 - 34	169009	392	300	5048	**174749**
35 - 39	159830	427	259	4892	**165408**
40 - 44	128790	297	236	4849	**134172**
45 - 49	108885	260	226	4379	**113750**
50 - 54	83088	204	213	3945	**87450**
55 - 59	60553	153	137	3383	**64226**
60 - 64	67297	113	112	2910	**70432**
65 - 69	60403	116	74	2358	**62951**
70 - 74	58994	68	52	1966	**61080**
75 - 79	27788	41	20	1422	**29271**
80 - 84	28709	33	22	849	**29613**
85+	19031	30	12	585	**19658**
Total	**2805653**	**5379**	**3836**	**63987**	**2878855**

Table 4: Citizenship by population group in the Limpopo province

	Black African	Coloured	Indian/Asian	White	Total
South Africa	5090368	10046	7403	124727	**5232544**
SADC countries	37281	67	31	371	**37750**
Rest of Africa	726	3	18	20	**767**
Europe	39	23	15	865	**942**
Asia	144	13	1107	23	**1287**
North America	28	12	12	174	**226**
Central and South America	28	0	3	63	**94**
Australia and New Zealand	0	0	0	32	**32**
Total	**5128614**	**10164**	**8589**	**126275**	**5 273 642**

Table 5: Limpopo Province disabled population according to racial group and sex

Disability and sex	Black African	Coloured	Indian/Asian	White	Total
Sight:					
Male	29368	77	43	595	**30083**
Female	42670	68	13	576	**43327**
Total	**72038**	**145**	**56**	**1171**	**73410**
Hearing:					
Male	19843	47	10	654	**20554**
Female	25914	44	6	543	**26507**
Total	**45757**	**91**	**16**	**1197**	**47061**
Communication:					
Male	5176	12	8	113	**5309**
Female	4945	17	4	100	**5066**
Total	**10121**	**29**	**12**	**213**	**10375**
Physical:					
Male	25893	74	22	880	**26869**
Female	28639	112	26	869	**29646**
Total	**54532**	**186**	**48**	**1749**	**56515**
Intellectual:					
Male	13606	36	19	410	**14071**
Female	12859	25	7	350	**13241**
Total	**26465**	**61**	**26**	**760**	**27312**
Emotional:					
Male	19277	31	9	220	**19537**
Female	16880	32	4	266	**17182**
Total	**36157**	**63**	**13**	**486**	**36719**
Total Male	113163	277	111	2872	**116423**
Total Female	131907	298	60	2704	**134969**
Grand Total	**245070**	**575**	**171**	**5576**	**251392**

Appendix 9

Chi square test –34 locus

For -34 locus						
	N/N	N/V	V/V			
Participants, Observed	65	62	22	149		
Participants Expected	70.98585	61.84906	16.16509			Chi
Chisqr	0.504754	0.000368	2.106151		Total Chisq	8.787141
Controls, Observed	36	26	1	63	P =	0.012357
Controls Expected	30.01415	26.15094	6.834906			
Chisqr	1.193783	0.000871	4.981213			
Totals	101	88	23	212		

Chi square test –15 locus

For -15 locus						
	N/N	N/V	V/V			
Observed (Participants)	35	17	5	57		
Participants Expected	40.97696	13.39631	2.626728			
	0.871808	0.969413	2.144272			Chi
Observed (Controls)	121	34	5	160	Total Chisq	5.405324
Controls Expected	115.023	37.60369	7.373272		P =	0.067027
	0.310582	0.345353	0.763897			
Totals	156	51	10	217		

Appendix 10

EXTRACT FROM 'A SYNOPSIS OF THE WHITE PAPER FOR THE TRANSFORMATION OF THE HEALTH SYSTEM FOR SOUTH AFRICA' (1997)

3.1 Introduction

The White Paper for the Transformation of the Health System in South Africa was published as Notice 667 of 1997 in the Government Gazette no. 17910. It was preceded by a document called Towards A National Health System (NHS) and was widely consulted on before publication. Its basis was the RDP and the ANC's National Health Plan.

3.2 Aims and Objectives

The objective of the White Paper is to "present to the people of South Africa a set of policy objectives and principles upon which the Unified National Health System of South African will be based" (p. 1). In addition the document contains a series of implementation strategies designed to meet the needs of South Africans within the constraints of available resources.

3.3 Contents

The White Paper contains 21 chapters. These are:

- mission, goals and objectives of the health sector;
- reorganising the health service;
- financial and physical resources;
- developing human resources for health;
- essential national health research;
- health information;
- nutrition;
- maternal, child and women's health;
- HIV/AIDS and STDs;
- communicable diseases;
- environmental health;
- mental health and substance abuse;
- oral health;
- occupational health;
- academic health service complexes;
- national health laboratory services;
- the role of hospitals;
- health promotion and communication;
- the role of donor agencies and non-government organisations (NGOs);
- international health; and
- Year 2000 health goals, objectives and indicators for South Africa.

Five key strategies are outlined in the White Paper based on the principles of the RDP. These are: "(a) the health sector must play its part in promoting equity by developing a single, unified health system; (b) the health system will focus on districts as the major locus of

implementation, and emphasise the PHC approach; (c) the three spheres of government, NGOs and the private sector will unite in the promotion of common goals; (d) the national, provincial and district levels will play distinct and complementary roles; and (e) an integrated package of essential PHC services will be available to the entire population at the first point of contact" (p. 12).

The mission of the health sector is to "provide leadership and guidance to the National Health System in its efforts to promote and monitor the health of all people in South Africa, and to provide caring and effective services through a primary health care approach" (p. 13).

The White Paper spells out seven key goals (and a range of related objectives). The goals are:

- To unify fragmented health services at all levels into a comprehensive integrated NHS;
- To promote equity, accessibility and utilisation of health services;
- To extend the availability and ensure the appropriateness of health services;
- To develop health promotion activities;
- To develop the human resources available to the health sector;
- To foster community participation across the health sector; and
- To improve health sector planning and the monitoring of health status and services.

Chapter two sets out the roles and functions of the national, provincial and district levels of system and spells out how the National Department of Health will be restructured (note: the National Department has undergone additional change since 1997). The chapter also points to ways in which the public and private health sectors can work together and how communities can become involved in the health system.

The next chapter deals with financial and physical resources. It lists the principles by which financial and physical resources will be planned. These are: "health care financing and resource allocation policies should promote equity of access to health services among all South Africans, between urban and rural areas, between rich and poor people, and between the public and private sectors. Policies should also promote the optimal utilisation of resources. Financial resources should be allocated equitably. Physical resources should be distributed equitably" (p. 40).

In terms of increasing access to PHC services the goal set is 2,8 and 3,5 consultations per person per year by 2000/01 and 2005/06 respectively. Another important section of this chapter is that on 'revised procedures for budgeting' (pp. 46-48). This section includes strategies for budget controls and criteria for budget reprioritisation.

Chapter four sets out the principles and strategies for the development of human resources for health. Three principles are listed including: (a) a national framework for the training and development of health personnel will be established; (b) the skills, experiences and expertise of all health personnel should be used optimally to ensure maximum coverage and cost-effectiveness; and (c) health personnel should be distributed throughout the country in an equitable manner. The chapter emphasises the need to train health personnel in the PHC approach and also the need to create a caring ethos. Various principles and strategies to change the nature of management from authoritarian to one that is participative and democratic are listed.

With regard to clinical skills development the White Paper lists both principles and strategies to be used. The need for affirmative policies and practices are also mentioned in this chapter.

The next chapter focuses on essential national health research (ENHR) and lists three principles to be used in developing an ENHR programme. These are: (a) "the research agenda should be developed to address the country's major health problems and initiate a process involving scientist decision-makers and population representatives as equal, inclusive partners; (b) health problems should be addressed by means of a full range of methodologies including epidemiology, social and behavioural, clinical and biomedical, health system and policy analysis. Priorities should be set by the stakeholders involved; (c) research should be relevant to health needs and aimed at informing health planning, effective delivery, management and policy development" (p. 74).

Chapter six lists three principles of the national health information system that should be established. These include: (a) "the National Health Information System (NHISSA) should be nationally co-ordinated in order to support the effective delivery of services at all levels of the health system; (b) the NHISSA should be used to monitor the implementation and success of the health priority programmes, both of the Department of Health and the Reconstruction and Development Programme (RDP); and (c) Reporting of NHISSA data at all levels should be timeous, accurate and complete" (p. 79).

Three principles are listed in the White Paper with regard to nutrition (chapter 7). These are: "(a) Nutrition for all South Africans should be promoted as a basic human right and an integral component and outcome measure of the country's social and economic development; (b) nutrition programmes should be integrated, sustainable, environmentally sound, people and community driven and should target the most vulnerable groups, especially children and women; (c) nutritional well-being should be promoted and monitored within nationally-defined goals.

A three pronged nutrition strategy is proposed: health facility-based nutrition programme; community-based programme; and nutrition promotion, including communication, advocacy and legislation.

Chapter eight covers maternal, child and women's health and contains six key principles: (a) "maternal, child and women's health (MCWH) services should be accessible to mothers, children, adolescents and women of all ages, the focus being on the rural and urban poor and farm workers; (b) MCWH services should be comprehensive and integrated; (c) clear objectives and targets should be set at the national, provincial, district and community levels in accordance with the goals of the RDP, the health sector and the United Nations Convention on the Rights of the Child; (d) individuals, households and communities should have adequate knowledge and skills to promote positive behaviour related to maternal, child and reproductive health; (e) MCWH services should be efficient, cost-effective and of a good quality; and (f) women and men will be provided with services which will enable them to achieve optimal reproductive and sexual health.

HIV/AIDs and sexually transmitted diseases are major problems which are tackled in chapter eight of the White Paper. The National AIDS Control Programme focuses on five key objectives: (a) prevention of the spread of the disease through the promotion of safer sex behaviour, adequate provision of condoms and control of STDs; (b) protection and promotion of the rights of people living with HIV/AIDS by ensuring that discrimination against them is

outlawed; (c) reduction of the personal and social impact of HIV/AIDS through the provision of counselling, care and support, including social welfare services for persons with HIV/AIDS, their families and communities; (d) use of the mass media to popularise key prevention concepts and the development of life skills education for youth in and out of school; and (e) mobilisation and unification of local, provincial, national and international resources to prevent and reduce the impact of HIV/AIDS.

Decreasing morbidity and mortality rates through strategic interventions

- Improving quality of care
- Speeding up delivery of an essential package of primary health care services through the district health system
- Revitalisation of hospital services
- Improving resource mobilisation and the management of resources without neglecting the attainment of equity in resource allocation
- Improving human resource development and management
- Reorganisation of certain support services

The key strategies listed to achieve the above include:

- life-skills programmes targeted at the youth;
- use of mass communication media to popularise key prevention concepts in AIDS;
- appropriate treatment and management of patients seeking treatment for STDs;
- improved access to barrier methods; and
- promotion of appropriate care and support.

Besides HIV/AIDS the White Paper also includes a series of principles and implementation strategies with respect to other infectious and communicable diseases. The six principles contained in the White Paper include: (a) communicable disease control services (CDCS) should be assessable and integrated into comprehensive PHC services; (b) CDCS should be efficient, cost-effective and of good quality; (c) health care staff should be adequately trained in clinical management and on strategies of communicable disease control; (d) communities and individuals should be adequately informed about communicable diseases and should be involved in communicable disease control (CDC) activities; (e) the CDC programmes should ensure accountability through the use of recording and reporting systems, by establishing a financial management system and through regular evaluation and review; and (f) CDCS should ensure effective infection control, including control and management of epidemics.

Chapter ten advocates the Directly Observed Treatment Strategy (DOTS) as the major strategy to be used to achieve the target of 85% cure rate of new smear positive cases. It also advocates community involvement in DOTS as treatment supporters and in malaria control. A range of other implementation strategies, including training of health workers, health promotion and involvement of employers in CDCS is also proposed.

Chapters 11 and 14 deal with environmental and occupational health respectively. Some of the strategies advocated to improve environmental health include: (a) development of more appropriate human resources; (b) equitable distribution of environmental health officers; (c) intersectoral collaboration with other government departments; (d) making environmental health a shared responsibility with each individual taking some responsibility; (e)

strengthening the enforcement of health legislation; (f) doing health impact assessments as part of environmental impact assessments; and (g) community empowerment and advocacy.

With respect to occupational health five principles are proposed. These are: (a) effective interdepartmental coordination and organisation of the various components of occupational health and safety; (b) the development of occupational health services at national, provincial, regional and district levels; (c) the development of norms and standards for a healthy and safe environmental in collaboration with stakeholders; (d) the extension of benefit examinations for the identification of compensable diseases in former miners; and (e) the development of occupational health and safety across Southern Africa.

Mental health is dealt with in chapter 12. Policies and implementation strategies are to be guided by three principles viz., (a) a comprehensive and community-based mental health and related service should be planned and co-ordinated at the national, provincial, district and community levels and integrated with other health services; (b) essential national health research should include an analysis of mental health and substance abuse; and (c) human resource development for mental health services should ensure that personnel at various levels are adequately trained to provide comprehensive and integrated mental health services based on the PHC approach.

Chapter 13 deals with oral health services. Various implementation strategies are listed. These are: (1) prioritisation of service delivery (focusing on prevention and equitable distribution of services); (2) prevention of oral diseases; (3) integration of oral health care with other health services based on a basic package of oral health services; (4) review of training of oral health personnel; (5) water fluoridation; and (6) reduction of the consumption of refined sugar.

Chapter 15 lists four principles that should underpin implementation strategies with respect to Academic Health Service Complexes (AHSC). These are listed as: “The activities of different AHSCs will be co-ordinated with those of other stakeholders. Services in provincial and district facilities that are part of an AHSC will be linked with similar facilities, for the benefit of all communities. AHSCs should be accountable to both the national department and provincial health authorities. AHSCs should maximise the benefits from available resources and adopt cost-effective approaches. The curricula of AHSCs will be revised to place greater emphasis on the needs of the communities, in accordance with primary health care principles” (p. 153).

Seven principles related to the provision of hospital services are listed in chapter seventeen. These include: (a) the role of hospitals will be redefined to be consistent with the PHC approach; (b) plans will be developed to rationalise hospital services, facilities, staffing and capital investment; (c) decentralised hospital management will be introduced to promote efficiency and cost-effectiveness; (d) hospital boards will be established to increase local accountability and power; (e) a targeted, efficient and equitable user fee system will be introduced and facilities will retain part of the revenue generated to encourage efficient collection and improved services; (f) policy and regulations pertaining to private hospitals will be implemented to encourage cost containment in the private sector, and ensure that private hospitals contribute optimally to the National Health System; and (g) hospitals providing unique or highly specialised services will be treated as a national resource. A list of implementation strategies are contained in the White Paper linked to each of the principles listed above.

Chapter eighteen lists principles and implementation strategies on health promotion and communication. With respect to health promotion the five areas outlined by the Ottawa Charter are promoting health public policy; creating supportive environments; supporting community action; developing personal skills especially in the education sector; and reorienting health services. Priority groups identified include: children, women, youth, the aged, the disabled and the poor. Priority health problems to be targeted include violence, substance abuse, HIV/AIDS and problems related to lifestyle.

Chapter sixteen proposes the creation of a National Health Laboratory Service (NHLS) which will be nationally controlled or co-ordinated. It would include the following services: (a) pathology; (b) environmental health services like water, food and water; (c) occupational health; forensic services; and (d) other laboratory-based services.

The White Paper (chapter 19) contains a set of policy guidelines for donor funding and assistance. This chapter also emphasises the importance of a healthy relationship between the Department of Health and NGOs and includes guidelines for funding of NGOS by the Department.

Chapter twenty deals with international relations and lists five principles: (a) an effective mechanism for international health liaison will be established and awareness of international issues and opportunities created; (b) international health relations should serve the interests of South Africans, and contribute to the advancement of global health goals; (c) development co-operation and donor assistance should support health reform; (d) international liaison activities should support regional health sector co-operation in Southern Africa; and (e) South African participation in international health science development should be encouraged.

The final chapter includes a list of Year 2000 health goals, objectives and indicators to be used in monitoring the implementation of the principles and strategies contained in the White Paper.

3.4 Additional Information

Additional information on the contents of the White Paper can be obtained by the reading the original document which is available from the Government Printer as Government Gazette No. 17910 (16 April 1997). The document may also be downloaded from the internet at www.doh.gov.za.

Appendix 11

Ethics Clearance certificate, University of the Witwaatersrand

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Kabahuma

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M991005

PROJECT Genetic Aspects of Hearing Loss In Nzhelele Northern Province South Africa

INVESTIGATORS Dr RI Kabahuma

DEPARTMENT Speech Pathology & Audio, Wits University

DATE CONSIDERED 991029

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 00/01/31 **CHAIRMAN** .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Prof C Penn
Dept of Speech Pathology & Audio, Wits University

Works2\lain0015\HumEth97.wdb\M 991005

==========

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE .....SIGNATURE .....

PROTOCOL NO.: M 991005

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix 12

Research clearance, Department of Health, Limpopo Province

NORTHERN PROVINCE

DEPARTMENT OF HEALTH AND WELFARE

TEL: (015) 290 9000
(015) 290 9001
FAX: (015) 291 5961
(015) 291 5146

PRIVATE BAG X9302
PIETERSBURG
0700

Enquiries: Sinah Mahlangu.

Reference: Research and quality Improvement

20 March 2000

DR R I KABAHUMA
PIETERSBURG/MANKWENG HOSPITAL COMPLEX
PRIVATE BAG X9316
PIETERSBURG
0700

GENETIC ASPECTS OF HEARING LOSS IN NDZELELE, NORTHERN PROVINCE OF SOUTH AFRICA.

1. **Permission is hereby granted to conduct a study on the above topic in namely, Nzhelele of the Northern Province.**
2. **The Department of Health & Welfare needs a copy of the research findings for its own resource centre.**
3. **The researcher should be prepared to assist in interpretation and implementation of the recommendations where possible.**
4. **Implications: Permission should be requested from institutional management to do research.**

Sincerely,

SUPERINTENDENT _ GENERAL
DEPARTMENT OF HEALTH & WELFARE
NORTHERN PROVINCE

DR JAN MOOLMAN BUILDING
34 HANS VAN RENSBURG STREET
PIETERSBURG 0700

Appendix 14 Ststistical Analysis Data

Fisher’s exact chi-square tests of associations at Phase 2

Fisher’s tests of associations

```
. tab2   lang_group fam_hhl cx26_34t cx26_15t degree1 degree2 degree3
address2
aad__mths2 rf_22 mmpdp2 smp2 shand_rt_abn2 shand_lt_abn2 cop,  cell   exact
```

-> tabulation of lang_group by fam_hhl

lang_group	N	fam_hhl Unknown	Y	Total
1	51	3	27	81
	28.02	1.65	14.84	44.51
2	45	22	16	83
	24.73	12.09	8.79	45.60
3	9	2	2	13
	4.95	1.10	1.10	7.14
4	4	0	1	5
	2.20	0.00	0.55	2.75
Total	109	27	46	182
	59.89	14.84	25.27	100.00

Fisher's exact = **0.001**

-> tabulation of lang_group by cx26_34t

lang_group	N	cx26_34t N C/T	Y	x	Total
1	40	24	13	4	81
	21.98	13.19	7.14	2.20	44.51
2	73	0	10	0	83
	40.11	0.00	5.49	0.00	45.60
3	13	0	0	0	13
	7.14	0.00	0.00	0.00	7.14
4	2	1	2	0	5
	1.10	0.55	1.10	0.00	2.75
Total	128	25	25	4	182
	70.33	13.74	13.74	2.20	100.00

Fisher's exact = 0.000

-> tabulation of lang_group by cx26_15t

lang_group	cx26_15t N	N C/T	Y	x	Total
1	60	14	1	6	81
	32.97	7.69	0.55	3.30	44.51
2	75	0	8	0	83
	41.21	0.00	4.40	0.00	45.60
3	13	0	0	0	13
	7.14	0.00	0.00	0.00	7.14
4	4	1	0	0	5
	2.20	0.55	0.00	0.00	2.75
Total	152	15	9	6	182
	83.52	8.24	4.95	3.30	100.00

Fisher's exact = 0.000

-> tabulation of lang_group by degree1

lang_group	degree1 0	1	2	Total
1	54	6	12	72
	31.95	3.55	7.10	42.60
2	68	5	7	80
	40.24	2.96	4.14	47.34
3	11	0	1	12
	6.51	0.00	0.59	7.10
4	4	0	1	5
	2.37	0.00	0.59	2.96
Total	137	11	21	169
	81.07	6.51	12.43	100.00

Fisher's exact = 0.627

```
-> tabulation of lang_group by degree2
```

lang_group	degree2 0	1	2	Total
1	75	2	4	81
	41.21	1.10	2.20	44.51
2	81	2	0	83
	44.51	1.10	0.00	45.60
3	13	0	0	13
	7.14	0.00	0.00	7.14
4	5	0	0	5
	2.75	0.00	0.00	2.75
Total	174	4	4	182
	95.60	2.20	2.20	100.00

Fisher's exact = 0.461

```
-> tabulation of lang_group by degree3
```

lang_group	degree3 0	1	Total
1	79	2	81
	43.41	1.10	44.51
2	83	0	83
	45.60	0.00	45.60
3	13	0	13
	7.14	0.00	7.14
4	5	0	5
	2.75	0.00	2.75
Total	180	2	182
	98.90	1.10	100.00

Fisher's exact = 0.385

```
-> tabulation of lang_group by address2


           |              address2
lang_group | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         1 |        10         62          9 |        81
           |      5.49      34.07       4.95 |     44.51
-----------+---------------------------------+----------
         2 |        33         25         25 |        83
           |     18.13      13.74      13.74 |     45.60
-----------+---------------------------------+----------
         3 |         0         12          1 |        13
           |      0.00       6.59       0.55 |      7.14
-----------+---------------------------------+----------
         4 |         0          5          0 |         5
           |      0.00       2.75       0.00 |      2.75
-----------+---------------------------------+----------
     Total |        43        104         35 |       182
           |     23.63      57.14      19.23 |    100.00

           Fisher's exact =                 0.000

-> tabulation of lang_group by aad__mths2


           |             aad__mths2
lang_group | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         1 |        12         28         41 |        81
           |      6.59      15.38      22.53 |     44.51
-----------+---------------------------------+----------
         2 |        41         39          3 |        83
           |     22.53      21.43       1.65 |     45.60
-----------+---------------------------------+----------
         3 |         8          5          0 |        13
           |      4.40       2.75       0.00 |      7.14
-----------+---------------------------------+----------
         4 |         1          3          1 |         5
           |      0.55       1.65       0.55 |      2.75
-----------+---------------------------------+----------
     Total |        62         75         45 |       182
           |     34.07      41.21      24.73 |    100.00

           Fisher's exact =                 0.000
```

```
-> tabulation of lang_group by rf_22

           |               rf_22
lang_group | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         1 |         2         72          7 |        81
           |      1.10      39.56       3.85 |     44.51
-----------+---------------------------------+----------
         2 |         0         82          1 |        83
           |      0.00      45.05       0.55 |     45.60
-----------+---------------------------------+----------
         3 |         0         13          0 |        13
           |      0.00       7.14       0.00 |      7.14
-----------+---------------------------------+----------
         4 |         0          4          1 |         5
           |      0.00       2.20       0.55 |      2.75
-----------+---------------------------------+----------
     Total |         2        171          9 |       182
           |      1.10      93.96       4.95 |    100.00

           Fisher's exact =                 0.056

-> tabulation of lang_group by mmpdp2

           |        mmpdp2
lang_group | High risk   Low risk |     Total
-----------+----------------------+----------
         1 |         0         81 |        81
           |      0.00      44.51 |     44.51
-----------+----------------------+----------
         2 |         0         83 |        83
           |      0.00      45.60 |     45.60
-----------+----------------------+----------
         3 |         0         13 |        13
           |      0.00       7.14 |      7.14
-----------+----------------------+----------
         4 |         1          4 |         5
           |      0.55       2.20 |      2.75
-----------+----------------------+----------
     Total |         1        181 |       182
           |      0.55      99.45 |    100.00

           Fisher's exact =                 0.027

-> tabulation of lang_group by smp2

           |               smp2
lang_group | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         1 |         7         68          6 |        81
           |      3.85      37.36       3.30 |     44.51
-----------+---------------------------------+----------
         2 |         0         83          0 |        83
           |      0.00      45.60       0.00 |     45.60
-----------+---------------------------------+----------
         3 |         0         13          0 |        13
           |      0.00       7.14       0.00 |      7.14
-----------+---------------------------------+----------
```

```
         4 |         0          4          1 |         5
           |      0.00       2.20       0.55 |      2.75
-----------+---------------------------------+----------
     Total |         7        168          7 |       182
           |      3.85      92.31       3.85 |    100.00

           Fisher's exact =                 0.002
```

-> tabulation of lang_group by shand_rt_abn2

```
           |          shand_rt_abn2
lang_group | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         1 |        21         59          1 |        81
           |     11.54      32.42       0.55 |     44.51
-----------+---------------------------------+----------
         2 |         4         77          2 |        83
           |      2.20      42.31       1.10 |     45.60
-----------+---------------------------------+----------
         3 |         1         12          0 |        13
           |      0.55       6.59       0.00 |      7.14
-----------+---------------------------------+----------
         4 |         1          4          0 |         5
           |      0.55       2.20       0.00 |      2.75
-----------+---------------------------------+----------
     Total |        27        152          3 |       182
           |     14.84      83.52       1.65 |    100.00

           Fisher's exact =                 0.004
```

-> tabulation of lang_group by shand_lt_abn2

```
           |          shand_lt_abn2
lang_group | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         1 |        21         59          1 |        81
           |     11.54      32.42       0.55 |     44.51
-----------+---------------------------------+----------
         2 |         4         77          2 |        83
           |      2.20      42.31       1.10 |     45.60
-----------+---------------------------------+----------
         3 |         1         12          0 |        13
           |      0.55       6.59       0.00 |      7.14
-----------+---------------------------------+----------
         4 |         1          4          0 |         5
           |      0.55       2.20       0.00 |      2.75
-----------+---------------------------------+----------
     Total |        27        152          3 |       182
           |     14.84      83.52       1.65 |    100.00

           Fisher's exact =                 0.004
```

-> tabulation of fam_hhl by cx26_34t

fam_hhl	cx26_34t				Total
	N	N C/T	Y	x	
N	75	16	15	3	109
	41.21	8.79	8.24	1.65	59.89
Unknown	22	0	5	0	27
	12.09	0.00	2.75	0.00	14.84
Y	31	9	5	1	46
	17.03	4.95	2.75	0.55	25.27
Total	128	25	25	4	182
	70.33	13.74	13.74	2.20	100.00

Fisher's exact = 0.221

-> tabulation of fam_hhl by cx26_15t

fam_hhl	cx26_15t				Total
	N	N C/T	Y	x	
N	91	9	4	5	109
	50.00	4.95	2.20	2.75	59.89
Unknown	25	0	2	0	27
	13.74	0.00	1.10	0.00	14.84
Y	36	6	3	1	46
	19.78	3.30	1.65	0.55	25.27
Total	152	15	9	6	182
	83.52	8.24	4.95	3.30	100.00

Fisher's exact = 0.356

-> tabulation of fam_hhl by degree1

fam_hhl	degree1			Total
	0	1	2	
N	109	0	0	109
	64.50	0.00	0.00	64.50
Unknown	27	0	0	27
	15.98	0.00	0.00	15.98
Y	1	11	21	33
	0.59	6.51	12.43	19.53
Total	137	11	21	169
	81.07	6.51	12.43	100.00

Fisher's exact = 0.000

```
-> tabulation of fam_hhl by degree2


           |             degree2
   fam_hhl |         0          1          2 |     Total
-----------+---------------------------------+----------
         N |       109          0          0 |       109
           |     59.89       0.00       0.00 |     59.89
-----------+---------------------------------+----------
   Unknown |        27          0          0 |        27
           |     14.84       0.00       0.00 |     14.84
-----------+---------------------------------+----------
         Y |        38          4          4 |        46
           |     20.88       2.20       2.20 |     25.27
-----------+---------------------------------+----------
     Total |       174          4          4 |       182
           |     95.60       2.20       2.20 |    100.00

           Fisher's exact =                 0.000

-> tabulation of fam_hhl by degree3


           |        degree3
   fam_hhl |         0          1 |     Total
-----------+----------------------+----------
         N |       109          0 |       109
           |     59.89       0.00 |     59.89
-----------+----------------------+----------
   Unknown |        27          0 |        27
           |     14.84       0.00 |     14.84
-----------+----------------------+----------
         Y |        44          2 |        46
           |     24.18       1.10 |     25.27
-----------+----------------------+----------
     Total |       180          2 |       182
           |     98.90       1.10 |    100.00

           Fisher's exact =                 0.084

-> tabulation of fam_hhl by address2


           |             address2
   fam_hhl | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         N |        25         59         25 |       109
           |     13.74      32.42      13.74 |     59.89
-----------+---------------------------------+----------
   Unknown |         7         17          3 |        27
           |      3.85       9.34       1.65 |     14.84
-----------+---------------------------------+----------
         Y |        11         28          7 |        46
           |      6.04      15.38       3.85 |     25.27
-----------+---------------------------------+----------
     Total |        43        104         35 |       182
           |     23.63      57.14      19.23 |    100.00

           Fisher's exact =                 0.658
```

```
-> tabulation of fam_hhl by aad__mths2


           |            aad__mths2
   fam_hhl | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         N |        43         40         26 |       109
           |     23.63      21.98      14.29 |     59.89
-----------+---------------------------------+----------
   Unknown |         4         23          0 |        27
           |      2.20      12.64       0.00 |     14.84
-----------+---------------------------------+----------
         Y |        15         12         19 |        46
           |      8.24       6.59      10.44 |     25.27
-----------+---------------------------------+----------
     Total |        62         75         45 |       182
           |     34.07      41.21      24.73 |    100.00

           Fisher's exact =                 0.000

-> tabulation of fam_hhl by rf_22


           |              rf_22
   fam_hhl | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         N |         0        104          5 |       109
           |      0.00      57.14       2.75 |     59.89
-----------+---------------------------------+----------
   Unknown |         0         27          0 |        27
           |      0.00      14.84       0.00 |     14.84
-----------+---------------------------------+----------
         Y |         2         40          4 |        46
           |      1.10      21.98       2.20 |     25.27
-----------+---------------------------------+----------
     Total |         2        171          9 |       182
           |      1.10      93.96       4.95 |    100.00

           Fisher's exact =                 0.085

-> tabulation of fam_hhl by mmpdp2


           |        mmpdp2
   fam_hhl | High risk   Low risk |     Total
-----------+----------------------+----------
         N |         1        108 |       109
           |      0.55      59.34 |     59.89
-----------+----------------------+----------
   Unknown |         0         27 |        27
           |      0.00      14.84 |     14.84
-----------+----------------------+----------
         Y |         0         46 |        46
           |      0.00      25.27 |     25.27
-----------+----------------------+----------
     Total |         1        181 |       182
           |      0.55      99.45 |    100.00

           Fisher's exact =                 1.000
```

```
-> tabulation of fam_hhl by smp2

           |                smp2
   fam_hhl | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         N |         4        101          4 |       109
           |      2.20      55.49       2.20 |     59.89
-----------+---------------------------------+----------
   Unknown |         0         27          0 |        27
           |      0.00      14.84       0.00 |     14.84
-----------+---------------------------------+----------
         Y |         3         40          3 |        46
           |      1.65      21.98       1.65 |     25.27
-----------+---------------------------------+----------
     Total |         7        168          7 |       182
           |      3.85      92.31       3.85 |    100.00

           Fisher's exact =                 0.464

-> tabulation of fam_hhl by shand_rt_abn2

           |           shand_rt_abn2
   fam_hhl | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         N |        14         94          1 |       109
           |      7.69      51.65       0.55 |     59.89
-----------+---------------------------------+----------
   Unknown |         3         23          1 |        27
           |      1.65      12.64       0.55 |     14.84
-----------+---------------------------------+----------
         Y |        10         35          1 |        46
           |      5.49      19.23       0.55 |     25.27
-----------+---------------------------------+----------
     Total |        27        152          3 |       182
           |     14.84      83.52       1.65 |    100.00

           Fisher's exact =                 0.315

-> tabulation of fam_hhl by shand_lt_abn2

           |           shand_lt_abn2
   fam_hhl | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         N |        14         94          1 |       109
           |      7.69      51.65       0.55 |     59.89
-----------+---------------------------------+----------
   Unknown |         3         23          1 |        27
           |      1.65      12.64       0.55 |     14.84
-----------+---------------------------------+----------
         Y |        10         35          1 |        46
           |      5.49      19.23       0.55 |     25.27
-----------+---------------------------------+----------
     Total |        27        152          3 |       182
           |     14.84      83.52       1.65 |    100.00

           Fisher's exact =                 0.315
```

-> tabulation of cx26_34t by cx26_15t

cx26_34t	cx26_15t N	N C/T	Y	x	Total
N	112	10	6	0	128
	61.54	5.49	3.30	0.00	70.33
N C/T	20	3	0	2	25
	10.99	1.65	0.00	1.10	13.74
Y	19	2	3	1	25
	10.44	1.10	1.65	0.55	13.74
x	1	0	0	3	4
	0.55	0.00	0.00	1.65	2.20
Total	152	15	9	6	182
	83.52	8.24	4.95	3.30	100.00

Fisher's exact = 0.000

-> tabulation of cx26_34t by degree1

cx26_34t	degree1 0	1	2	Total
N	98	8	14	120
	57.99	4.73	8.28	71.01
N C/T	16	2	4	22
	9.47	1.18	2.37	13.02
Y	20	0	3	23
	11.83	0.00	1.78	13.61
x	3	1	0	4
	1.78	0.59	0.00	2.37
Total	137	11	21	169
	81.07	6.51	12.43	100.00

Fisher's exact = 0.449

-> tabulation of cx26_34t by degree2

cx26_34t	degree2 0	1	2	Total
N	125	2	1	128
	68.68	1.10	0.55	70.33
N C/T	22	2	1	25
	12.09	1.10	0.55	13.74
Y	23	0	2	25
	12.64	0.00	1.10	13.74
x	4	0	0	4
	2.20	0.00	0.00	2.20
Total	174	4	4	182
	95.60	2.20	2.20	100.00

Fisher's exact = 0.074

-> tabulation of cx26_34t by degree3

cx26_34t	degree3 0	1	Total
N	128	0	128
	70.33	0.00	70.33
N C/T	23	2	25
	12.64	1.10	13.74
Y	25	0	25
	13.74	0.00	13.74
x	4	0	4
	2.20	0.00	2.20
Total	180	2	182
	98.90	1.10	100.00

Fisher's exact = 0.049

```
-> tabulation of cx26_34t by address2


           |              address2
  cx26_34t | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         N |        33         67         28 |       128
           |     18.13      36.81      15.38 |     70.33
-----------+---------------------------------+----------
     N C/T |         4         19          2 |        25
           |      2.20      10.44       1.10 |     13.74
-----------+---------------------------------+----------
         Y |         6         14          5 |        25
           |      3.30       7.69       2.75 |     13.74
-----------+---------------------------------+----------
         x |         0          4          0 |         4
           |      0.00       2.20       0.00 |      2.20
-----------+---------------------------------+----------
     Total |        43        104         35 |       182
           |     23.63      57.14      19.23 |    100.00

           Fisher's exact =                 0.329

-> tabulation of cx26_34t by aad__mths2


           |            aad__mths2
  cx26_34t | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         N |        52         55         21 |       128
           |     28.57      30.22      11.54 |     70.33
-----------+---------------------------------+----------
     N C/T |         2          8         15 |        25
           |      1.10       4.40       8.24 |     13.74
-----------+---------------------------------+----------
         Y |         6         11          8 |        25
           |      3.30       6.04       4.40 |     13.74
-----------+---------------------------------+----------
         x |         2          1          1 |         4
           |      1.10       0.55       0.55 |      2.20
-----------+---------------------------------+----------
     Total |        62         75         45 |       182
           |     34.07      41.21      24.73 |    100.00

           Fisher's exact =                 0.000
```

-> tabulation of cx26_34t by rf_22

cx26_34t	rf_22 High risk	Low risk	Moderate	Total
N	1	125	2	128
	0.55	68.68	1.10	70.33
N C/T	0	21	4	25
	0.00	11.54	2.20	13.74
Y	0	22	3	25
	0.00	12.09	1.65	13.74
x	1	3	0	4
	0.55	1.65	0.00	2.20
Total	2	171	9	182
	1.10	93.96	4.95	100.00

Fisher's exact = 0.002

-> tabulation of cx26_34t by mmpdp2

cx26_34t	mmpdp2 High risk	Low risk	Total
N	1	127	128
	0.55	69.78	70.33
N C/T	0	25	25
	0.00	13.74	13.74
Y	0	25	25
	0.00	13.74	13.74
x	0	4	4
	0.00	2.20	2.20
Total	1	181	182
	0.55	99.45	100.00

Fisher's exact = 1.000

-> tabulation of cx26_34t by smp2

cx26_34t	smp2 High risk	Low risk	Moderate	Total
N	3	124	1	128
	1.65	68.13	0.55	70.33
N C/T	3	17	5	25
	1.65	9.34	2.75	13.74
Y	1	23	1	25
	0.55	12.64	0.55	13.74
x	0	4	0	4
	0.00	2.20	0.00	2.20
Total	7	168	7	182
	3.85	92.31	3.85	100.00

Fisher's exact = 0.001

-> tabulation of cx26_34t by shand_rt_abn2

cx26_34t	shand_rt_abn2 High risk	Low risk	Moderate	Total
N	14	111	3	128
	7.69	60.99	1.65	70.33
N C/T	9	16	0	25
	4.95	8.79	0.00	13.74
Y	4	21	0	25
	2.20	11.54	0.00	13.74
x	0	4	0	4
	0.00	2.20	0.00	2.20
Total	27	152	3	182
	14.84	83.52	1.65	100.00

Fisher's exact = 0.084

-> tabulation of cx26_34t by shand_lt_abn2

cx26_34t	shand_lt_abn2 High risk	Low risk	Moderate	Total
N	14	111	3	128
	7.69	60.99	1.65	70.33
N C/T	9	16	0	25
	4.95	8.79	0.00	13.74
Y	4	21	0	25
	2.20	11.54	0.00	13.74

```
         x |         0          4          0 |         4
           |      0.00       2.20       0.00 |      2.20
-----------+---------------------------------+----------
     Total |        27        152          3 |       182
           |     14.84      83.52       1.65 |    100.00

           Fisher's exact =                 0.084
```

```
-> tabulation of cx26_15t by degree1
```

```
           |             degree1
  cx26_15t |         0          1          2 |     Total
-----------+---------------------------------+----------
         N |       117          9         17 |       143
           |     69.23       5.33      10.06 |     84.62
-----------+---------------------------------+----------
     N C/T |         9          2          2 |        13
           |      5.33       1.18       1.18 |      7.69
-----------+---------------------------------+----------
         Y |         6          0          2 |         8
           |      3.55       0.00       1.18 |      4.73
-----------+---------------------------------+----------
         x |         5          0          0 |         5
           |      2.96       0.00       0.00 |      2.96
-----------+---------------------------------+----------
     Total |       137         11         21 |       169
           |     81.07       6.51      12.43 |    100.00

           Fisher's exact =                 0.540
```

```
-> tabulation of cx26_15t by degree2

stage 1:  enumerations = 0
```

```
           |             degree2
  cx26_15t |         0          1          2 |     Total
-----------+---------------------------------+----------
         N |       147          2          3 |       152
           |     80.77       1.10       1.65 |     83.52
-----------+---------------------------------+----------
     N C/T |        13          1          1 |        15
           |      7.14       0.55       0.55 |      8.24
-----------+---------------------------------+----------
         Y |         8          1          0 |         9
           |      4.40       0.55       0.00 |      4.95
-----------+---------------------------------+----------
         x |         6          0          0 |         6
           |      3.30       0.00       0.00 |      3.30
-----------+---------------------------------+----------
     Total |       174          4          4 |       182
           |     95.60       2.20       2.20 |    100.00

           Fisher's exact =                 0.145
```

-> tabulation of cx26_15t by degree3

cx26_15t	degree3 0	1	Total
N	151	1	152
	82.97	0.55	83.52
N C/T	14	1	15
	7.69	0.55	8.24
Y	9	0	9
	4.95	0.00	4.95
x	6	0	6
	3.30	0.00	3.30
Total	180	2	182
	98.90	1.10	100.00

Fisher's exact = 0.303

-> tabulation of cx26_15t by address2

cx26_15t	address2 High risk	Low risk	Moderate	Total
N	36	83	33	152
	19.78	45.60	18.13	83.52
N C/T	4	11	0	15
	2.20	6.04	0.00	8.24
Y	3	4	2	9
	1.65	2.20	1.10	4.95
x	0	6	0	6
	0.00	3.30	0.00	3.30
Total	43	104	35	182
	23.63	57.14	19.23	100.00

Fisher's exact = 0.133

-> tabulation of cx26_15t by aad__mths2

cx26_15t	aad__mths2 High risk	Low risk	Moderate	Total
N	52	68	32	152
	28.57	37.36	17.58	83.52
N C/T	2	4	9	15
	1.10	2.20	4.95	8.24
Y	6	2	1	9
	3.30	1.10	0.55	4.95

```
         x |         2          1          3 |         6
           |      1.10       0.55       1.65 |      3.30
-----------+---------------------------------+----------
     Total |        62         75         45 |       182
           |     34.07      41.21      24.73 |    100.00

           Fisher's exact =                 0.007
```

```
-> tabulation of cx26_15t by rf_22
```

```
           |               rf_22
  cx26_15t | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         N |         2        143          7 |       152
           |      1.10      78.57       3.85 |     83.52
-----------+---------------------------------+----------
     N C/T |         0         13          2 |        15
           |      0.00       7.14       1.10 |      8.24
-----------+---------------------------------+----------
         Y |         0          9          0 |         9
           |      0.00       4.95       0.00 |      4.95
-----------+---------------------------------+----------
         x |         0          6          0 |         6
           |      0.00       3.30       0.00 |      3.30
-----------+---------------------------------+----------
     Total |         2        171          9 |       182
           |      1.10      93.96       4.95 |    100.00

           Fisher's exact =                 0.562
```

```
-> tabulation of cx26_15t by mmpdp2
```

```
           |        mmpdp2
  cx26_15t | High risk   Low risk |     Total
-----------+----------------------+----------
         N |         1        151 |       152
           |      0.55      82.97 |     83.52
-----------+----------------------+----------
     N C/T |         0         15 |        15
           |      0.00       8.24 |      8.24
-----------+----------------------+----------
         Y |         0          9 |         9
           |      0.00       4.95 |      4.95
-----------+----------------------+----------
         x |         0          6 |         6
           |      0.00       3.30 |      3.30
-----------+----------------------+----------
     Total |         1        181 |       182
           |      0.55      99.45 |    100.00

           Fisher's exact =                 1.000
```

-> tabulation of cx26_15t by smp2

cx26_15t	smp2 High risk	Low risk	Moderate	Total
N	5	140	7	152
	2.75	76.92	3.85	83.52
N C/T	2	13	0	15
	1.10	7.14	0.00	8.24
Y	0	9	0	9
	0.00	4.95	0.00	4.95
x	0	6	0	6
	0.00	3.30	0.00	3.30
Total	7	168	7	182
	3.85	92.31	3.85	100.00

Fisher's exact = 0.579

-> tabulation of cx26_15t by shand_rt_abn2

cx26_15t	shand_rt_abn2 High risk	Low risk	Moderate	Total
N	22	128	2	152
	12.09	70.33	1.10	83.52
N C/T	4	10	1	15
	2.20	5.49	0.55	8.24
Y	0	9	0	9
	0.00	4.95	0.00	4.95
x	1	5	0	6
	0.55	2.75	0.00	3.30
Total	27	152	3	182
	14.84	83.52	1.65	100.00

Fisher's exact = 0.245

-> tabulation of cx26_15t by shand_lt_abn2

cx26_15t	shand_lt_abn2 High risk	Low risk	Moderate	Total
N	22	128	2	152
	12.09	70.33	1.10	83.52
N C/T	4	10	1	15
	2.20	5.49	0.55	8.24
Y	0	9	0	9
	0.00	4.95	0.00	4.95

```
         x |         1          5          0 |         6
           |      0.55       2.75       0.00 |      3.30
-----------+---------------------------------+----------
     Total |        27        152          3 |       182
           |     14.84      83.52       1.65 |    100.00

           Fisher's exact =                 0.245

-> tabulation of degree1 by degree2


           |             degree2
   degree1 |         0          1          2 |     Total
-----------+---------------------------------+----------
         0 |       137          0          0 |       137
           |     81.07       0.00       0.00 |     81.07
-----------+---------------------------------+----------
         1 |         8          2          1 |        11
           |      4.73       1.18       0.59 |      6.51
-----------+---------------------------------+----------
         2 |        18          1          2 |        21
           |     10.65       0.59       1.18 |     12.43
-----------+---------------------------------+----------
     Total |       163          3          3 |       169
           |     96.45       1.78       1.78 |    100.00

           Fisher's exact =                 0.000

-> tabulation of degree1 by degree3


           |        degree3
   degree1 |         0          1 |     Total
-----------+----------------------+----------
         0 |       137          0 |       137
           |     81.07       0.00 |     81.07
-----------+----------------------+----------
         1 |        10          1 |        11
           |      5.92       0.59 |      6.51
-----------+----------------------+----------
         2 |        21          0 |        21
           |     12.43       0.00 |     12.43
-----------+----------------------+----------
     Total |       168          1 |       169
           |     99.41       0.59 |    100.00

           Fisher's exact =                 0.065

-> tabulation of degree1 by address2


           |             address2
   degree1 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |        32         77         28 |       137
           |     18.93      45.56      16.57 |     81.07
-----------+---------------------------------+----------
         1 |         4          6          1 |        11
           |      2.37       3.55       0.59 |      6.51
-----------+---------------------------------+----------
         2 |         2         13          6 |        21
```

```
           |      1.18       7.69       3.55 |     12.43
-----------+---------------------------------+----------
     Total |        38         96         35 |       169
           |     22.49      56.80      20.71 |    100.00

           Fisher's exact =                 0.414
```

-> tabulation of degree1 by aad__mths2

```
           |            aad__mths2
   degree1 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |        48         63         26 |       137
           |     28.40      37.28      15.38 |     81.07
-----------+---------------------------------+----------
         1 |         5          2          4 |        11
           |      2.96       1.18       2.37 |      6.51
-----------+---------------------------------+----------
         2 |         4          8          9 |        21
           |      2.37       4.73       5.33 |     12.43
-----------+---------------------------------+----------
     Total |        57         73         39 |       169
           |     33.73      43.20      23.08 |    100.00

           Fisher's exact =                 0.050
```

-> tabulation of degree1 by rf_22

```
           |              rf_22
   degree1 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |         0        132          5 |       137
           |      0.00      78.11       2.96 |     81.07
-----------+---------------------------------+----------
         1 |         1          9          1 |        11
           |      0.59       5.33       0.59 |      6.51
-----------+---------------------------------+----------
         2 |         0         20          1 |        21
           |      0.00      11.83       0.59 |     12.43
-----------+---------------------------------+----------
     Total |         1        161          7 |       169
           |      0.59      95.27       4.14 |    100.00

           Fisher's exact =                 0.067
```

-> tabulation of degree1 by mmpdp2

```
           |        mmpdp2
   degree1 | High risk   Low risk |     Total
-----------+----------------------+----------
         0 |         1        136 |       137
           |      0.59      80.47 |     81.07
-----------+----------------------+----------
         1 |         0         11 |        11
           |      0.00       6.51 |      6.51
-----------+----------------------+----------
         2 |         0         21 |        21
           |      0.00      12.43 |     12.43
```

```
-----------+----------------------+----------
     Total |         1        168 |       169
           |      0.59      99.41 |    100.00

           Fisher's exact =                 1.000

-> tabulation of degree1 by smp2


           |                smp2
   degree1 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |         4        129          4 |       137
           |      2.37      76.33       2.37 |     81.07
-----------+---------------------------------+----------
         1 |         1          9          1 |        11
           |      0.59       5.33       0.59 |      6.51
-----------+---------------------------------+----------
         2 |         0         20          1 |        21
           |      0.00      11.83       0.59 |     12.43
-----------+---------------------------------+----------
     Total |         5        158          6 |       169
           |      2.96      93.49       3.55 |    100.00

           Fisher's exact =                 0.260

-> tabulation of degree1 by shand_rt_abn2


           |          shand_rt_abn2
   degree1 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |        17        118          2 |       137
           |     10.06      69.82       1.18 |     81.07
-----------+---------------------------------+----------
         1 |         1          9          1 |        11
           |      0.59       5.33       0.59 |      6.51
-----------+---------------------------------+----------
         2 |         7         14          0 |        21
           |      4.14       8.28       0.00 |     12.43
-----------+---------------------------------+----------
     Total |        25        141          3 |       169
           |     14.79      83.43       1.78 |    100.00

           Fisher's exact =                 0.047

-> tabulation of degree1 by shand_lt_abn2


           |          shand_lt_abn2
   degree1 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |        17        118          2 |       137
           |     10.06      69.82       1.18 |     81.07
-----------+---------------------------------+----------
         1 |         1          9          1 |        11
           |      0.59       5.33       0.59 |      6.51
-----------+---------------------------------+----------
         2 |         7         14          0 |        21
           |      4.14       8.28       0.00 |     12.43
-----------+---------------------------------+----------
```

```
     Total |        25        141          3 |       169
           |     14.79      83.43       1.78 |    100.00

           Fisher's exact =                 0.047
```

```
-> tabulation of degree2 by degree3
```

```
           |        degree3
   degree2 |         0          1 |     Total
-----------+----------------------+----------
         0 |       174          0 |       174
           |     95.60       0.00 |     95.60
-----------+----------------------+----------
         1 |         2          2 |         4
           |      1.10       1.10 |      2.20
-----------+----------------------+----------
         2 |         4          0 |         4
           |      2.20       0.00 |      2.20
-----------+----------------------+----------
     Total |       180          2 |       182
           |     98.90       1.10 |    100.00

           Fisher's exact =                 0.001
```

```
-> tabulation of degree2 by address2
```

```
           |             address2
   degree2 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |        42         98         34 |       174
           |     23.08      53.85      18.68 |     95.60
-----------+---------------------------------+----------
         1 |         1          3          0 |         4
           |      0.55       1.65       0.00 |      2.20
-----------+---------------------------------+----------
         2 |         0          3          1 |         4
           |      0.00       1.65       0.55 |      2.20
-----------+---------------------------------+----------
     Total |        43        104         35 |       182
           |     23.63      57.14      19.23 |    100.00

           Fisher's exact =                 0.819
```

```
-> tabulation of degree2 by aad__mths2
```

```
           |            aad__mths2
   degree2 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |        59         74         41 |       174
           |     32.42      40.66      22.53 |     95.60
-----------+---------------------------------+----------
         1 |         3          1          0 |         4
           |      1.65       0.55       0.00 |      2.20
-----------+---------------------------------+----------
         2 |         0          0          4 |         4
           |      0.00       0.00       2.20 |      2.20
-----------+---------------------------------+----------
     Total |        62         75         45 |       182
```

```
           |     34.07      41.21      24.73 |    100.00

           Fisher's exact =                 0.003

-> tabulation of degree2 by rf_22


           |              rf_22
   degree2 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |         2        165          7 |       174
           |      1.10      90.66       3.85 |     95.60
-----------+---------------------------------+----------
         1 |         0          4          0 |         4
           |      0.00       2.20       0.00 |      2.20
-----------+---------------------------------+----------
         2 |         0          2          2 |         4
           |      0.00       1.10       1.10 |      2.20
-----------+---------------------------------+----------
     Total |         2        171          9 |       182
           |      1.10      93.96       4.95 |    100.00

           Fisher's exact =                 0.051

-> tabulation of degree2 by mmpdp2


           |        mmpdp2
   degree2 | High risk   Low risk |     Total
-----------+----------------------+----------
         0 |         1        173 |       174
           |      0.55      95.05 |     95.60
-----------+----------------------+----------
         1 |         0          4 |         4
           |      0.00       2.20 |      2.20
-----------+----------------------+----------
         2 |         0          4 |         4
           |      0.00       2.20 |      2.20
-----------+----------------------+----------
     Total |         1        181 |       182
           |      0.55      99.45 |    100.00

           Fisher's exact =                 1.000

-> tabulation of degree2 by smp2


           |               smp2
   degree2 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |         5        163          6 |       174
           |      2.75      89.56       3.30 |     95.60
-----------+---------------------------------+----------
         1 |         2          2          0 |         4
           |      1.10       1.10       0.00 |      2.20
-----------+---------------------------------+----------
         2 |         0          3          1 |         4
           |      0.00       1.65       0.55 |      2.20
-----------+---------------------------------+----------
     Total |         7        168          7 |       182
           |      3.85      92.31       3.85 |    100.00
```

```
           Fisher's exact =                 0.009

-> tabulation of degree2 by shand_rt_abn2


           |          shand_rt_abn2
   degree2 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |        25        146          3 |       174
           |     13.74      80.22       1.65 |     95.60
-----------+---------------------------------+----------
         1 |         1          3          0 |         4
           |      0.55       1.65       0.00 |      2.20
-----------+---------------------------------+----------
         2 |         1          3          0 |         4
           |      0.55       1.65       0.00 |      2.20
-----------+---------------------------------+----------
     Total |        27        152          3 |       182
           |     14.84      83.52       1.65 |    100.00

           Fisher's exact =                 0.429

-> tabulation of degree2 by shand_lt_abn2


           |          shand_lt_abn2
   degree2 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |        25        146          3 |       174
           |     13.74      80.22       1.65 |     95.60
-----------+---------------------------------+----------
         1 |         1          3          0 |         4
           |      0.55       1.65       0.00 |      2.20
-----------+---------------------------------+----------
         2 |         1          3          0 |         4
           |      0.55       1.65       0.00 |      2.20
-----------+---------------------------------+----------
     Total |        27        152          3 |       182
           |     14.84      83.52       1.65 |    100.00

           Fisher's exact =                 0.429

-> tabulation of degree3 by address2


           |             address2
   degree3 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |        43        104         35 |       182
           |     23.37      56.52      19.02 |     98.91
-----------+---------------------------------+----------
         1 |         0          2          0 |         2
           |      0.00       1.09       0.00 |      1.09
-----------+---------------------------------+----------
     Total |        43        106         35 |       184
           |     23.37      57.61      19.02 |    100.00

           Fisher's exact =                 1.000

-> tabulation of degree3 by aad__mths2
```

```
           |            aad__mths2
   degree3 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |        60         77         45 |       182
           |     32.61      41.85      24.46 |     98.91
-----------+---------------------------------+----------
         1 |         2          0          0 |         2
           |      1.09       0.00       0.00 |      1.09
-----------+---------------------------------+----------
     Total |        62         77         45 |       184
           |     33.70      41.85      24.46 |    100.00

           Fisher's exact =                 0.171
```

```
-> tabulation of degree3 by rf_22
```

```
           |              rf_22
   degree3 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |         2        171          9 |       182
           |      1.09      92.93       4.89 |     98.91
-----------+---------------------------------+----------
         1 |         0          2          0 |         2
           |      0.00       1.09       0.00 |      1.09
-----------+---------------------------------+----------
     Total |         2        173          9 |       184
           |      1.09      94.02       4.89 |    100.00

           Fisher's exact =                 1.000
```

```
-> tabulation of degree3 by mmpdp2
```

```
           |        mmpdp2
   degree3 | High risk   Low risk |     Total
-----------+----------------------+----------
         0 |         1        181 |       182
           |      0.54      98.37 |     98.91
-----------+----------------------+----------
         1 |         0          2 |         2
           |      0.00       1.09 |      1.09
-----------+----------------------+----------
     Total |         1        183 |       184
           |      0.54      99.46 |    100.00

           Fisher's exact =                 1.000
   1-sided Fisher's exact =                 0.989
```

```
-> tabulation of degree3 by smp2
```

```
           |              smp2
   degree3 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
         0 |         5        168          9 |       182
           |      2.72      91.30       4.89 |     98.91
-----------+---------------------------------+----------
```

```
        1 |         2          0          0 |         2
          |      1.09       0.00       0.00 |      1.09
----------+---------------------------------+----------
    Total |         7        168          9 |       184
          |      3.80      91.30       4.89 |    100.00

          Fisher's exact =                 0.001
```

```
-> tabulation of degree3 by shand_rt_abn2
```

```
          |          shand_rt_abn2
   degree3 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
        0 |        28        151          3 |       182
          |     15.22      82.07       1.63 |     98.91
-----------+---------------------------------+----------
        1 |         1          1          0 |         2
          |      0.54       0.54       0.00 |      1.09
-----------+---------------------------------+----------
    Total |        29        152          3 |       184
          |     15.76      82.61       1.63 |    100.00

          Fisher's exact =                 0.318
```

```
-> tabulation of degree3 by shand_lt_abn2
```

```
          |          shand_lt_abn2
   degree3 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
        0 |        28        151          3 |       182
          |     15.22      82.07       1.63 |     98.91
-----------+---------------------------------+----------
        1 |         1          1          0 |         2
          |      0.54       0.54       0.00 |      1.09
-----------+---------------------------------+----------
    Total |        29        152          3 |       184
          |     15.76      82.61       1.63 |    100.00

          Fisher's exact =                 0.318
```

```
-> tabulation of address2 by aad__mths2
```

```
             |           aad__mths2
    address2 | High risk   Low risk  Moderate  |     Total
-------------+---------------------------------+----------
   High risk |        21         13          9 |        43
             |     11.41       7.07       4.89 |     23.37
-------------+---------------------------------+----------
    Low risk |        24         53         29 |       106
             |     13.04      28.80      15.76 |     57.61
-------------+---------------------------------+----------
Moderate risk |        17         11          7 |        35
             |      9.24       5.98       3.80 |     19.02
-------------+---------------------------------+----------
       Total |        62         77         45 |       184
             |     33.70      41.85      24.46 |    100.00

          Fisher's exact =                 0.008
```

```
-> tabulation of address2 by rf_22

           |               rf_22
  address2 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
 High risk |         0         42          1 |        43
           |      0.00      22.83       0.54 |     23.37
-----------+---------------------------------+----------
  Low risk |         2         97          7 |       106
           |      1.09      52.72       3.80 |     57.61
-----------+---------------------------------+----------
Moderate r |         0         34          1 |        35
           |      0.00      18.48       0.54 |     19.02
-----------+---------------------------------+----------
     Total |         2        173          9 |       184
           |      1.09      94.02       4.89 |    100.00

           Fisher's exact =                 0.740

-> tabulation of address2 by mmpdp2

           |        mmpdp2
  address2 | High risk   Low risk |     Total
-----------+----------------------+----------
 High risk |         0         43 |        43
           |      0.00      23.37 |     23.37
-----------+----------------------+----------
  Low risk |         1        105 |       106
           |      0.54      57.07 |     57.61
-----------+----------------------+----------
Moderate r |         0         35 |        35
           |      0.00      19.02 |     19.02
-----------+----------------------+----------
     Total |         1        183 |       184
           |      0.54      99.46 |    100.00

           Fisher's exact =                 1.000

-> tabulation of address2 by smp2

           |               smp2
  address2 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
 High risk |         2         41          0 |        43
           |      1.09      22.28       0.00 |     23.37
-----------+---------------------------------+----------
  Low risk |         5         92          9 |       106
           |      2.72      50.00       4.89 |     57.61
-----------+---------------------------------+----------
Moderate r |         0         35          0 |        35
           |      0.00      19.02       0.00 |     19.02
-----------+---------------------------------+----------
     Total |         7        168          9 |       184
           |      3.80      91.30       4.89 |    100.00

           Fisher's exact =                 0.065
```

```
-> tabulation of address2 by shand_rt_abn2

           |          shand_rt_abn2
  address2 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
  High risk |         6         37          0 |        43
           |      3.26      20.11       0.00 |     23.37
-----------+---------------------------------+----------
   Low risk |        21         83          2 |       106
           |     11.41      45.11       1.09 |     57.61
-----------+---------------------------------+----------
Moderate risk |         2         32          1 |        35
           |      1.09      17.39       0.54 |     19.02
-----------+---------------------------------+----------
      Total |        29        152          3 |       184
           |     15.76      82.61       1.63 |    100.00

          Fisher's exact =                 0.222

-> tabulation of address2 by shand_lt_abn2


           |          shand_lt_abn2
  address2 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
  High risk |         6         37          0 |        43
           |      3.26      20.11       0.00 |     23.37
-----------+---------------------------------+----------
   Low risk |        21         83          2 |       106
           |     11.41      45.11       1.09 |     57.61
-----------+---------------------------------+----------
Moderate risk |         2         32          1 |        35
           |      1.09      17.39       0.54 |     19.02
-----------+---------------------------------+----------
      Total |        29        152          3 |       184
           |     15.76      82.61       1.63 |    100.00

          Fisher's exact =                 0.222

-> tabulation of aad__mths2 by rf_22


           |              rf_22
 aad__mths2 | High risk   Low risk  Moderate  |     Total
-----------+---------------------------------+----------
  High risk |         0         61          1 |        62
           |      0.00      33.15       0.54 |     33.70
-----------+---------------------------------+----------
   Low risk |         0         75          2 |        77
           |      0.00      40.76       1.09 |     41.85
-----------+---------------------------------+----------
Moderate risk |         2         37          6 |        45
           |      1.09      20.11       3.26 |     24.46
-----------+---------------------------------+----------
      Total |         2        173          9 |       184
           |      1.09      94.02       4.89 |    100.00

          Fisher's exact =                 0.003

-> tabulation of aad__mths2 by mmpdp2
```

```
               |        mmpdp2
   aad__mths2 | High risk   Low risk |     Total
--------------+----------------------+----------
    High risk |         1         61 |        62
              |      0.54      33.15 |     33.70
--------------+----------------------+----------
     Low risk |         0         77 |        77
              |      0.00      41.85 |     41.85
--------------+----------------------+----------
Moderate risk |         0         45 |        45
              |      0.00      24.46 |     24.46
--------------+----------------------+----------
        Total |         1        183 |       184
              |      0.54      99.46 |    100.00

           Fisher's exact =                 0.582
```

-> tabulation of aad__mths2 by smp2

```
               |               smp2
   aad__mths2 | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
    High risk |         3         59          0 |        62
              |      1.63      32.07       0.00 |     33.70
--------------+---------------------------------+----------
     Low risk |         4         68          5 |        77
              |      2.17      36.96       2.72 |     41.85
--------------+---------------------------------+----------
Moderate risk |         0         41          4 |        45
              |      0.00      22.28       2.17 |     24.46
--------------+---------------------------------+----------
        Total |         7        168          9 |       184
              |      3.80      91.30       4.89 |    100.00

           Fisher's exact =                 0.065
```

-> tabulation of aad__mths2 by shand_rt_abn2

```
               |          shand_rt_abn2
   aad__mths2 | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
    High risk |         5         56          1 |        62
              |      2.72      30.43       0.54 |     33.70
--------------+---------------------------------+----------
     Low risk |        12         63          2 |        77
              |      6.52      34.24       1.09 |     41.85
--------------+---------------------------------+----------
Moderate risk |        12         33          0 |        45
              |      6.52      17.93       0.00 |     24.46
--------------+---------------------------------+----------
        Total |        29        152          3 |       184
              |     15.76      82.61       1.63 |    100.00

           Fisher's exact =                 0.071
```

-> tabulation of aad__mths2 by shand_lt_abn2

```
              |          shand_lt_abn2
   aad__mths2 | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
    High risk |         5         56          1 |        62
              |      2.72      30.43       0.54 |     33.70
--------------+---------------------------------+----------
     Low risk |        12         63          2 |        77
              |      6.52      34.24       1.09 |     41.85
--------------+---------------------------------+----------
Moderate risk |        12         33          0 |        45
              |      6.52      17.93       0.00 |     24.46
--------------+---------------------------------+----------
        Total |        29        152          3 |       184
              |     15.76      82.61       1.63 |    100.00

           Fisher's exact =                 0.071
```

```
-> tabulation of rf_22 by mmpdp2
```

```
              |        mmpdp2
        rf_22 | High risk   Low risk |     Total
--------------+----------------------+----------
    High risk |         0          2 |         2
              |      0.00       1.09 |      1.09
--------------+----------------------+----------
     Low risk |         1        172 |       173
              |      0.54      93.48 |     94.02
--------------+----------------------+----------
Moderate risk |         0          9 |         9
              |      0.00       4.89 |      4.89
--------------+----------------------+----------
        Total |         1        183 |       184
              |      0.54      99.46 |    100.00

           Fisher's exact =                 1.000
```

```
-> tabulation of rf_22 by smp2
```

```
              |               smp2
        rf_22 | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
    High risk |         0          1          1 |         2
              |      0.00       0.54       0.54 |      1.09
--------------+---------------------------------+----------
     Low risk |         7        159          7 |       173
              |      3.80      86.41       3.80 |     94.02
--------------+---------------------------------+----------
Moderate risk |         0          8          1 |         9
              |      0.00       4.35       0.54 |      4.89
--------------+---------------------------------+----------
        Total |         7        168          9 |       184
              |      3.80      91.30       4.89 |    100.00

           Fisher's exact =                 0.123
```

```
-> tabulation of rf_22 by shand_rt_abn2
```

```
           |          shand_rt_abn2
    rf_22 | High risk   Low risk  Moderate  |     Total
----------+---------------------------------+----------
 High risk |         0          2          0 |         2
           |      0.00       1.09       0.00 |      1.09
----------+---------------------------------+----------
  Low risk |        28        143          2 |       173
           |     15.22      77.72       1.09 |     94.02
----------+---------------------------------+----------
Moderate r |         1          7          1 |         9
           |      0.54       3.80       0.54 |      4.89
----------+---------------------------------+----------
    Total |        29        152          3 |       184
           |     15.76      82.61       1.63 |    100.00

      Fisher's exact =                 0.247
```

-> tabulation of rf_22 by shand_lt_abn2

```
           |          shand_lt_abn2
    rf_22 | High risk   Low risk  Moderate  |     Total
----------+---------------------------------+----------
 High risk |         0          2          0 |         2
           |      0.00       1.09       0.00 |      1.09
----------+---------------------------------+----------
  Low risk |        28        143          2 |       173
           |     15.22      77.72       1.09 |     94.02
----------+---------------------------------+----------
Moderate r |         1          7          1 |         9
           |      0.54       3.80       0.54 |      4.89
----------+---------------------------------+----------
    Total |        29        152          3 |       184
           |     15.76      82.61       1.63 |    100.00

      Fisher's exact =                 0.247
```

-> tabulation of mmpdp2 by smp2

```
           |               smp2
   mmpdp2 | High risk   Low risk  Moderate  |     Total
----------+---------------------------------+----------
 High risk |         0          1          0 |         1
           |      0.00       0.54       0.00 |      0.54
----------+---------------------------------+----------
  Low risk |         7        167          9 |       183
           |      3.80      90.76       4.89 |     99.46
----------+---------------------------------+----------
    Total |         7        168          9 |       184
           |      3.80      91.30       4.89 |    100.00

      Fisher's exact =                 1.000
```

-> tabulation of mmpdp2 by shand_rt_abn2

```
           |          shand_rt_abn2
   mmpdp2 | High risk   Low risk  Moderate  |     Total
----------+---------------------------------+----------
```

```
    High risk |         0          1          0 |         1
              |      0.00       0.54       0.00 |      0.54
--------------+---------------------------------+----------
     Low risk |        29        151          3 |       183
              |     15.76      82.07       1.63 |     99.46
--------------+---------------------------------+----------
        Total |        29        152          3 |       184
              |     15.76      82.61       1.63 |    100.00

           Fisher's exact =                 1.000

-> tabulation of mmpdp2 by shand_lt_abn2


              |          shand_lt_abn2
       mmpdp2 | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
    High risk |         0          1          0 |         1
              |      0.00       0.54       0.00 |      0.54
--------------+---------------------------------+----------
     Low risk |        29        151          3 |       183
              |     15.76      82.07       1.63 |     99.46
--------------+---------------------------------+----------
        Total |        29        152          3 |       184
              |     15.76      82.61       1.63 |    100.00

           Fisher's exact =                 1.000

-> tabulation of smp2 by shand_rt_abn2


              |          shand_rt_abn2
         smp2 | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
    High risk |         3          4          0 |         7
              |      1.63       2.17       0.00 |      3.80
--------------+---------------------------------+----------
     Low risk |        22        143          3 |       168
              |     11.96      77.72       1.63 |     91.30
--------------+---------------------------------+----------
Moderate risk |         4          5          0 |         9
              |      2.17       2.72       0.00 |      4.89
--------------+---------------------------------+----------
        Total |        29        152          3 |       184
              |     15.76      82.61       1.63 |    100.00

           Fisher's exact =                 0.034

-> tabulation of smp2 by shand_lt_abn2


              |          shand_lt_abn2
         smp2 | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
    High risk |         3          4          0 |         7
              |      1.63       2.17       0.00 |      3.80
--------------+---------------------------------+----------
     Low risk |        22        143          3 |       168
              |     11.96      77.72       1.63 |     91.30
--------------+---------------------------------+----------
Moderate risk |         4          5          0 |         9
```

```
             |      2.17       2.72       0.00 |      4.89
-------------+---------------------------------+----------
       Total |        29        152          3 |       184
             |     15.76      82.61       1.63 |    100.00

          Fisher's exact =                 0.034
```

```
-> tabulation of shand_rt_abn2 by shand_lt_abn2
```

```
             |          shand_lt_abn2
shand_rt_abn2 | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
    High risk |        29          0          0 |        29
              |     15.76       0.00       0.00 |     15.76
--------------+---------------------------------+----------
     Low risk |         0        152          0 |       152
              |      0.00      82.61       0.00 |     82.61
--------------+---------------------------------+----------
Moderate risk |         0          0          3 |         3
              |      0.00       0.00       1.63 |      1.63
--------------+---------------------------------+----------
       Total |        29        152          3 |       184
              |     15.76      82.61       1.63 |    100.00

          Fisher's exact =                 0.000
```

```
->  tab2 cop lang_group, exact

-> tabulation of cop by lang_group
```

```
             |                lang_group
         cop |         1          2          3          4 |     Total
-------------+--------------------------------------------+----------
          No |        58         12          5          5 |        80
     Unknown |         9         71          8          0 |        88
         Yes |        14          0          0          0 |        14
-------------+--------------------------------------------+----------
       Total |        81         83         13          5 |       182

          Fisher's exact =                 0.000
```

```
->  tab2 cop fam_hhl, exact

-> tabulation of cop by fam_hhl
```

```
             |            fam_hhl
         cop |         N    Unknown          Y |     Total
-------------+---------------------------------+----------
          No |        58          0         22 |        80
     Unknown |        45         27         16 |        88
         Yes |         6          0          8 |        14
-------------+---------------------------------+----------
       Total |       109         27         46 |       182

          Fisher's exact =                 0.000
```

```
->  tab2 cop cx26_34t, exact

-> tabulation of cop by cx26_34t


           |                  cx26_34t
       cop |         N      N C/T          Y          x |     Total
-----------+-------------------------------------------+----------
        No |        41         22         15          2 |        80
   Unknown |        81          0          7          0 |        88
       Yes |         6          3          3          2 |        14
-----------+-------------------------------------------+----------
     Total |       128         25         25          4 |       182

          Fisher's exact =                 0.000

->  tab2 cop cx26_15t, exact

-> tabulation of cop by cx26_15t


           |                  cx26_15t
       cop |         N      N C/T          Y          x |     Total
-----------+-------------------------------------------+----------
        No |        61         12          3          4 |        80
   Unknown |        82          0          6          0 |        88
       Yes |         9          3          0          2 |        14
-----------+-------------------------------------------+----------
     Total |       152         15          9          6 |       182

          Fisher's exact =                 0.000

->  tab2 cop degree1, exact

-> tabulation of cop by degree1


           |             degree1
       cop |         0          1          2 |     Total
-----------+---------------------------------+----------
        No |        58          4         11 |        73
   Unknown |        73          5          6 |        84
       Yes |         6          2          4 |        12
-----------+---------------------------------+----------
     Total |       137         11         21 |       169

          Fisher's exact =                 0.026

->  tab2 cop degree2, exact

-> tabulation of cop by degree2


           |             degree2
       cop |         0          1          2 |     Total
-----------+---------------------------------+----------
        No |        77          2          1 |        80
   Unknown |        86          2          0 |        88
       Yes |        11          0          3 |        14
-----------+---------------------------------+----------
```

```
        Total |       174          4          4 |       182

           Fisher's exact =                 0.004

->  tab2 cop degree3, exact

-> tabulation of cop by degree3

              |        degree3
          cop |         0          1 |     Total
--------------+----------------------+----------
           No |        78          2 |        80
      Unknown |        90          0 |        90
          Yes |        14          0 |        14
--------------+----------------------+----------
        Total |       182          2 |       184

           Fisher's exact =                 0.334

->  tab2 cop address2, exact

-> tabulation of cop by address2

              |             address2
          cop | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
           No |        13         55         12 |        80
      Unknown |        29         38         23 |        90
          Yes |         1         13          0 |        14
--------------+---------------------------------+----------
        Total |        43        106         35 |       184

           Fisher's exact =                 0.000

->  tab2 cop aad__mths2, exact

-> tabulation of cop by aad__mths2

              |            aad__mths2
          cop | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
           No |        21         29         30 |        80
      Unknown |        39         47          4 |        90
          Yes |         2          1         11 |        14
--------------+---------------------------------+----------
        Total |        62         77         45 |       184

           Fisher's exact =                 0.000

->  tab2 cop rf_22, exact

-> tabulation of cop by rf_22

              |              rf_22
          cop | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
           No |         0         76          4 |        80
```

```
      Unknown |         0         89          1 |        90
          Yes |         2          8          4 |        14
--------------+---------------------------------+----------
        Total |         2        173          9 |       184

           Fisher's exact =                 0.000
```

```
->  tab2 cop mmpdp2, exact

-> tabulation of cop by mmpdp2
```

```
              |        mmpdp2
          cop | High risk   Low risk |     Total
--------------+----------------------+----------
           No |         1         79 |        80
      Unknown |         0         90 |        90
          Yes |         0         14 |        14
--------------+----------------------+----------
        Total |         1        183 |       184

           Fisher's exact =                 0.511
```

```
->  tab2 cop smp2, exact

-> tabulation of cop by smp2
```

```
              |               smp2
          cop | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
           No |         7         68          5 |        80
      Unknown |         0         88          2 |        90
          Yes |         0         12          2 |        14
--------------+---------------------------------+----------
        Total |         7        168          9 |       184

           Fisher's exact =                 0.005
```

```
->  tab2 cop shand_rt_abn2, exact

-> tabulation of cop by shand_rt_abn2
```

```
              |          shand_rt_abn2
          cop | High risk   Low risk  Moderate  |     Total
--------------+---------------------------------+----------
           No |        18         62          0 |        80
      Unknown |         7         81          2 |        90
          Yes |         4          9          1 |        14
--------------+---------------------------------+----------
        Total |        29        152          3 |       184

           Fisher's exact =                 0.004
```

```
->  tab2 cop shand_lt_abn2, exact

-> tabulation of cop by shand_lt_abn2
```

cop	shand_lt_abn2 High risk	Low risk	Moderate	Total
No	18	62	0	80
Unknown	7	81	2	90
Yes	4	9	1	14
Total	29	152	3	184

Fisher's exact = 0.004

Cross-tabulation of language group by family history of hearing loss

Lang. group	N	Fam. hhl Unknown	Y	Total
1	51 28.02	3 1.65	27 14.84	81 44.51
2	45 24.73	22 12.09	16 8.79	83 45.60
3	9 4.95	2 1.10	2 1.10	13 7.14
4	4 2.20	0 0.00	1 0.55	5 2.75
Total	109 59.89	27 14.84	46 25.27	182 100.00

Fisher's exact = **0.001**

Since P=0.001 < 0.05, there is a significant association between language group and family history of hearing loss, at the 5% level of significance.

Cross-tabulation of language group by GJB2 variation C.T at position -34

Lang. group	N	Cx26_34t N C/T	Y	x	Total
1	40 21.98	24 13.19	13 7.14	4 2.20	81 44.51
2	73 40.11	0 0.00	10 5.49	0 0.00	83 45.60
3	13 7.14	0 0.00	0 0.00	0 0.00	5 2.75
4	2 1.10	1 0.55	2 1.10	0 0.00	5 2.75
Total	128 70.33	25 13.74	25 13.74	4 2.20	182 100.00

Fisher's exact = 0.000

Cross-tabulation of language group by GJB2 variation C.T at position -15

Lang. group	N	cx26_15t N C/T	Y	x	Total
1	60 32.97	14 7.69	1 0.55	6 3.30	81 3.30
2	75 41.21	0 0.00	8 4.40	0 0.00	83 45.60
3	13 7.14	0 0.00	0 0.00	0 0.00	13 7.14
4	4 2.20	1 0.55	0 0.00	0 0.00	5 2.75
Total	152 83.52	15 8.24	9 4.95	6 3.30	182 100.00

Fisher's exact = 0.000

Cross-tabulation of language group by degree of first affected relative in familial hearing loss

	Degree of 1st affected relative			
Lang. group	0	1st	2nd	Total
1	54 31.95	6 3.55	12 1. 7.10	72 42.60
2	68 40.24	5 2.96	7 4.14	80 47.34
3	11 6.51	0 0.00	1 0.59	12 7.10
4	4 2.37	0 0.00	1 0.59	5 2.96
Total	137 81.07	11 6.51	21 12.43	169 100.00

Fisher's exact = 0.627

Cross-tabulation of language group by degree of second affected relative in familial hearing loss

	Degree of 2nd affected relative			
Lang. group	0	1	2	Total
1	75 41.21	2 1.10	4 2.20	81 44.51
2	81 44.51	2 1.10	0 0.00	83 45.60
3	13 7.14	0 0.00	0 0.00	13 7.14
4	5 2.75	0 0.00	0 0.00	5 2.75
Total	174 95.60	4 2.20	4 2.20	182 100.00

Fisher's exact = 0.461

Cross-tabulation of language group by degree of third affected relative infamilial hearing loss

Degree of 2nd affected relative			
Lang. group	0	1	Total
1	79 43.41	2 1.10	81 44.51
2	83 45.60	0 0.00	83 45.60
3	13 7.14	0 0.00	13 7.14
4	5 2.75	0 0.00	5 2.75
Total	180 98.90	2 1.10	182 100.00

Fisher's exact = 0.385

Cross-tabulation of language group by home address according to risk category

Home address according to risk				
Lang. group	High risk	Low risk	Moderate	Total
1	10 5.49	62 34.07	9 4.95	81 44.51
2	33 18.13	25 13.74	25 13.74	83 45.60
3	0 0.00	12 6.59	1 0.55	13 7.14
4	0 0.00	5 2.75	0 0.00	5 2.75
Total	43 23.63	104 57.14	35 19.23	182 100.00

Fisher's exact = 0.000

Cross-tabulation of language group by participant's age at detection of hearing loss

Age at detection in months				
Lang. group	Under 5	5-12	Over 12	Total
1	12 6.59	41 22.53	28 15.38	81 44.51
2	41 22.53	3 1.65	39 21.43	83 45.60
3	8 4.40	0 0.00	5 2.75	13 7.14
4	1 0.55	1 0.55	3 1.65	5 2.75

Total	62 34.07	45 24.73	75 41.21	182 100.00

Fisher's exact = 0.000

Results from binary logistic regression analysis

```
. use c:\rosemary\logistic regression.dta, clear

. logistic cop2 degree1 degree2 degree3 history GJB2a GJB2b
aad


note: degree2 != 0 predicts failure perfectly
      degree2 dropped and 4 obs not used

note: GJB2b != 0 predicts failure perfectly
      GJB2b dropped and 8 obs not used

note: degree3 dropped because of collinearity

Logistic regression                               Number of obs   =        172
                                                  LR chi2(4)      =      10.98
                                                  Prob > chi2     =     0.0267
Log likelihood = -43.040435                       Pseudo R2       =     0.1132

------------------------------------------------------------------------------
        cop2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
     degree1 |   1.462423   1.393676     0.40   0.690     .2258841    9.468044
     history |   4.783582   2.992357     2.50   0.012     1.403739    16.30122
       GJB2a |   1.926376   1.442086     0.88   0.381     .4441515    8.355088
         aad |   .3849309   .3097591    -1.19   0.235     .0795077    1.863615
------------------------------------------------------------------------------
```

Only family history of hearing loss is a significant predictor of cop2 at the 5% level of significance. This is because its P-value is 0.012 < 0.05.

Interpretation of the odds ratio for family history

A child with a family history of hearing loss is 4.78 times as likely to be a product of a consanguineous mating in comparison with a child who does not have a family history of hearing loss.

Assessment of the fitted logistic regression model

The reliability of the fitted logistic regression model is assessed based on the classification table and the Hosmer-Lemeshow goodness-of-fit test as shown below:

```
. lstat

Logistic model for cop2

              -------- True --------
Classified |         D            ~D  |      Total
-----------+--------------------------+-----------
     +     |         0             0  |          0
     -     |        14           158  |        172
-----------+--------------------------+-----------
   Total   |        14           158  |        172

Classified + if predicted Pr(D) >= .5
True D defined as cop2 != 0
--------------------------------------------------
Sensitivity                     Pr( +| D)    0.00%
Specificity                     Pr( -|~D)  100.00%
Positive predictive value       Pr( D| +)       .%
Negative predictive value       Pr(~D| -)   91.86%
--------------------------------------------------
False + rate for true ~D        Pr( +|~D)    0.00%
False - rate for true D         Pr( -| D)  100.00%
False + rate for classified +   Pr(~D| +)       .%
False - rate for classified -   Pr( D| -)    8.14%
--------------------------------------------------
Correctly classified                        91.86%
--------------------------------------------------
```

Percentage sensitivity is zero (very poor). The fitted logistic regression model has no ability to detect participants who are at risk of hearing loss. The model is too blind to detect them. As a result, intervention cannot be done with a view to help them. The poor sensitivity of the fitted model constitutes a minor limitation of study. The problem is a result of the fact that the sample drawn for the study is not a good representative of the population of study.

Percentage specificity is perfect at 100%. The fitted logistic regression model has the capacity to detect participants who are not at risk of hearing loss.

The overall percentage of correct classification is very high at 91.86%. This figure is above 75%. As such, it shows that the fitted model is reliable in spite of the fact that it is poorly sensitive.

The Hosmer-Lemeshow goodness-of-fit test

```
. lfit

Logistic model for cop2, goodness-of-fit test

       number of observations =       172
 number of covariate patterns =         9
             Pearson chi2(4) =      6.09
                 Prob > chi2 =    0.1927
```

The P-value from the Hosmer-Lemeshow goodness-of-fit test is equal to 0.1927 > 0.05. This shows that the fitted model is reliable. That is, we have no reason to doubt the adequacy of the fitted logistic regression model.

Calculation of crude odds ratio

To obtain a crude risk ratio (relative risk) and crude odds ratio,

```
. use c:\rosemary\logistic regression.dta, clear

. tab2  history   cop2


-> tabulation of history by cop2

           |        cop2
   history |         0          1 |     Total
-----------+----------------------+----------
         0 |       132          6 |       138
         1 |        38          8 |        46
-----------+----------------------+----------
     Total |       170         14 |       184

. csi   8   38   6   132,  or

                |   Exposed   Unexposed  |      Total
----------------+------------------------+------------
          Cases |         8          38  |         46
       Noncases |         6         132  |        138
----------------+------------------------+------------
          Total |        14         170  |        184
                |                        |
           Risk |  .5714286    .2235294  |        .25
                |                        |
```

```
                |      Point estimate    |    [95% Conf. Interval]
                |------------------------+------------------------
 Risk difference |         .3478992       |    .0812167    .6145816
      Risk ratio |         2.556391       |    1.499919    4.356992
 Attr. frac. ex. |         .6088235       |    .3332973    .7704838
 Attr. frac. pop |         .1058824       |
      Odds ratio |         4.631579       |    1.573753    13.61031 (Cornfield)
                +-------------------------------------------------
                              chi2(1) =     8.35  Pr>chi2 = 0.0039
```

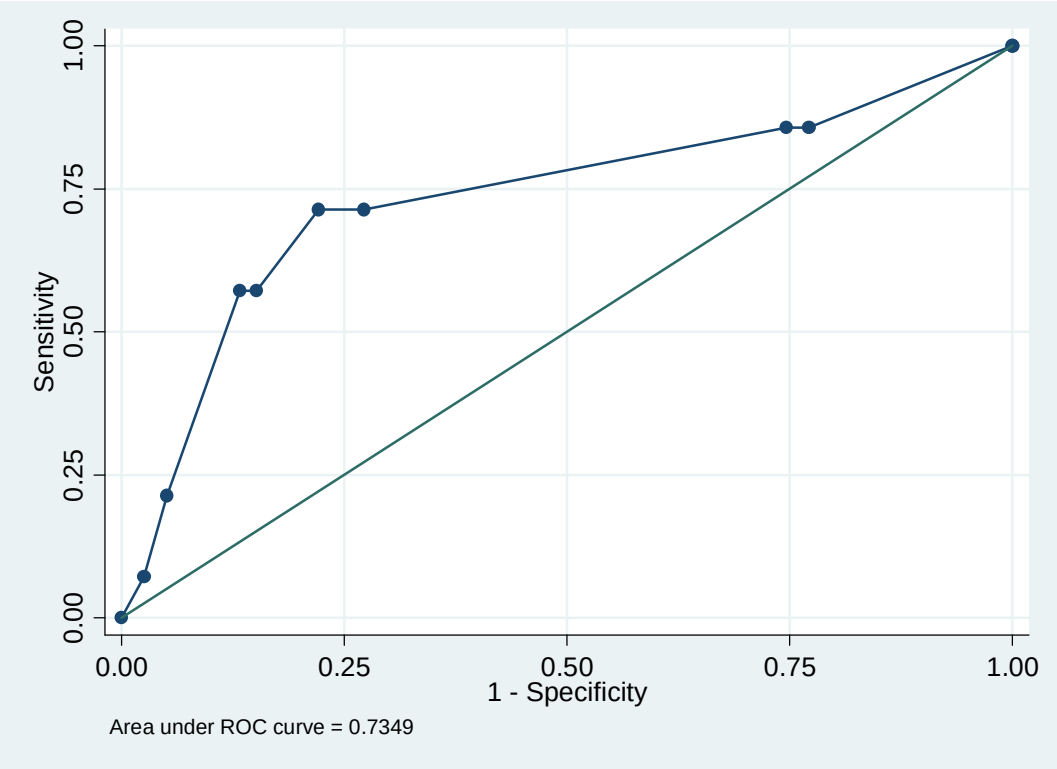
The crude risk ratio is 2.57 with a 95% confidence interval of (1.49, 4.36).

The crude odds ratio is 4.64 with a 95% confidence interval of (1.57, 13.61).

Interpretation of crude odds ratio

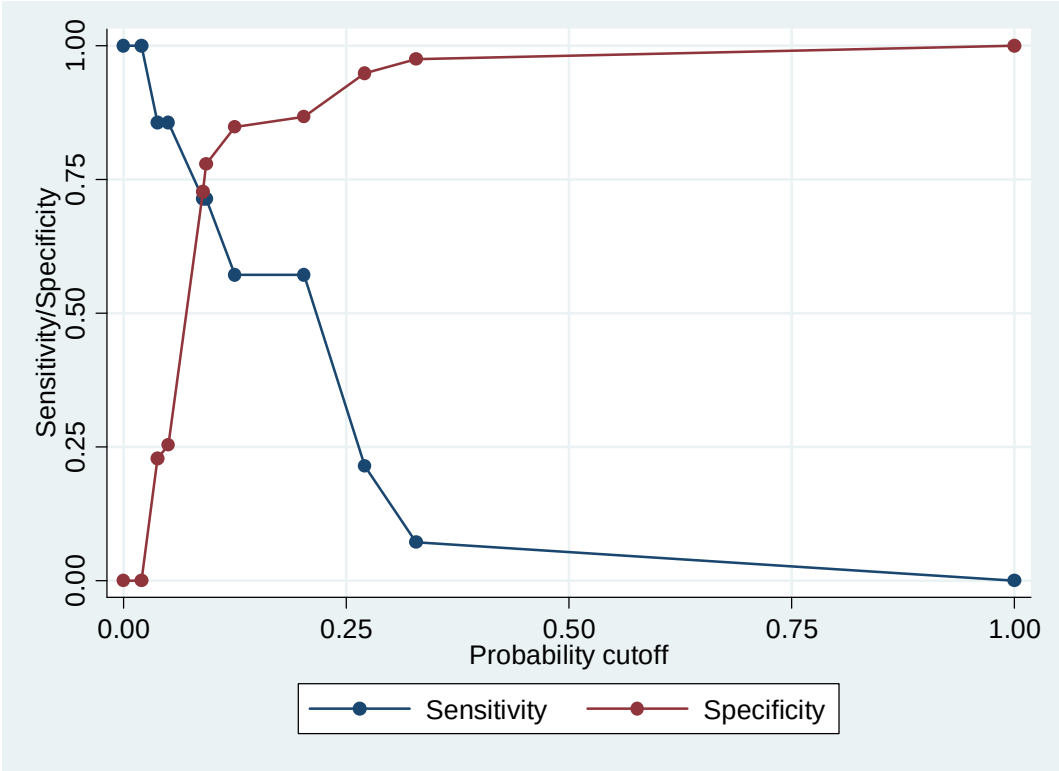
A child with a family history of hearing loss is 4.64 times as likely to be a product of a consanguineous mating in comparison with a child who does not have a family history of hearing loss.

Magnitude of area under the ROC (receiver operating characteristic) curve



The magnitude of area that lies under the ROC curve is a measure of overall explained variation by the fitted logistic regression model. In this study, the area that lies under the ROC curve is 73.49%, a figure which is fairly close to 75% (the recommended figure for reliable fitted models).

Plot of sensitivity/Specificity versus probability cut-off point



The above plot is a standard method of assessing overall sensitivity and specificity. If we drop a perpendicular from the point of intersection of the two curves to the X-axis vertically below, the perpendicular crosses the X-axis fairly close to zero. This shows that the fitted model is reliable.