Significance Of Connexin Genes In Non-Syndromic Deafness In Africans

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I- Plagiarism Declaration

I, **Jason Bosch**, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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II- Acknowledgements

I owe a huge debt of gratitude to the many people who helped me during the experiments and writing necessary to prepare this thesis.

My supervisors, Ambroise Wonkam, Collet Dandara and Karen Fieggen who provided a lot of excellent advice, feedback and support during the entire duration of my MSc. Nomlindo Makubalo, who travelled to the Eastern Cape to retrieve the blood and clinical data of the South African patients and for translating the relevant documentation from English to Xhosa. Zeinonisaa Latief for assisting me with and teaching me how to extract DNA from blood and saliva and for instructions regarding health and safety. Alvera Vorster for showing me around the lab and generally providing advice. Marelize Swart for support, help with DNA sequencing and being a great person to discuss results with. Yolandi Roodt for help and advice regarding sequencing. Our collaborators from SANBI, Galen Wright, Jean-Baka Domolevo Entfellner and Nicki Tiffin, who did the original bioinformatics analysis which I was later able to replicate with a little further help. The rest of the Wonkam team in the lab, Maryam Rumaney and Valentina Ngo Bitoungi, and all the other members of the Division of Human Genetics who provided friendship and support throughout my time there.

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Last, but certainly not least, I want to thank my parents and family for their support. I could not have done it without them.

III- Publications

This is a list of all articles published, in press or in preparation that have stemmed from my MSc.

A) Wonkam, A., Noubiap, N., **Bosch, J.**, Dandara, C. & Toure, G. B. Heterozygous p.Asp50Asn mutation in the *GJB2* gene in two Cameroonian patients with keratitis-ichthyosis-deafness (KID) syndrome. *BMC Medical Genetics* **14,** 81 (2013).

B) **Jason Bosch**, Jean Jacques Noubiap Nziale, Collet Dandara, Nomlindo Makubalo, Galen Wright, Jean-Baka Domolevo Entfellner, Nicki Tiffin, Ambroise Wonkam; Mutations in *GJB2* do not play a significant role in recessive non- syndromic sensorineural deafness in patients from sub-Saharan Africa. *Genetics in Medicine* (Submitted, 2013)

C) **Jason Bosch**, Kamogelo Lebeko, Jean Jacques Noubiap Nziale, Collet Dandara, Nomlindo Makubalo, Ambroise Wonkam; Hearing loss in Africans is not linked to variations in the *GJB6* or *GJA1* genes. (In preparation, 2013)

IV- Abstract

Introduction: Deafness is the most common sensory disability, occurring in approximately 2 per 1000 births in the developed world and up to 6 per 1000 births in South Africa. It has long-term detrimental impacts on a child's well-being and social life. Early detection is associated with better outcomes for the child. Although deafness is a highly heterogeneous condition, it has been found that mutations in the *GJB2* gene encoding connexin 26 (CX26) are responsible for a large proportion of deafness cases in Europeans and Asians. To date, there have been few studies examining the genetic causes of deafness among Africans. We investigated the significance of mutations in two connexon (CX) coding genes on deafness among Africans.

Methods: Patients were recruited from both Cameroon and South Africa. The cohort consisted of patients with recessive, non-syndromic, sensorineural deafness of unknown or putative genetic origin (N = 217), two patients with keratitis-icthyosis-deafness (KID) syndrome, known to be due to mutations in *GJB2* (CX26), and healthy, population-matched controls (N = 81). DNA, extracted from either blood or saliva, was used for genotyping the coding regions of the *GJB2* (CX26) and *GJA1* (CX43) genes using PCR and sequencing. The data was compared to that generated by the 1000 Genomes Project enabling the construction of a phylogenetic tree and the use of Principal Components Analysis (PCA) to detect any differences between these groups.

Results: Our results confirm the role for the p.(D50N) mutation in *GJB2* (CX26) in KID syndrome. However, among non-syndromic deafness cases, only two mutations in *GJB2* (CX26) were found, both in the heterozygous state, in two unrelated patients. These mutations alone could not explain the hearing loss in these patients. Subsequent sequencing of the *GJA1* (CX43) gene did not yield any mutations. No statistically significant differences were observed between cases and controls overall. However, among the South Africans, they were significant differences in *GJB2* (CX26) haplotypes (P = 0,03). Using PCA and the phylogeny, samples generally grouped together according to their geographic origin, with *GJB2* being more discriminatory compared to *GJA1*.

Discussion: The observation of the p.(D50N) mutation in *GJB2* (CX26) in the KID syndrome patients points to it not being population-specific, as this mutation is the most frequent in all populations. Contrary to what is reported in Europeans and Asians, our data confirm that mutations in *GJB2* (CX26) do not explain deafness in African patients with non-syndromic deafness. Our investigation into variations of *GJA1* (CX43) similarly did not reveal any mutations. Our findings support the hypothesis that there are likely to be other genes of importance in deafness among Africans, which have yet to be described.

Conclusion And Perspective: Our results show that neither *GJB2* (CX26) nor *GJA1* (CX43) is significantly associated with non-syndromic deafness in Africans. Future studies may benefit from massively parallel sequencing, such as whole exome sequencing, as opposed to single gene approaches, in order to unravel the genetic aetiology of non-syndromic hearing loss in Africans.

1- Introduction

Deafness is a global problem but is most serious in the developing world. The United States and Europe have prevalence rates of childhood hearing loss of less than 2 per 1000 live births.[1,2] In the developing world, the numbers are greater with 7 per 1000 children in Nigeria[3] and 5,5 per 1000 live births in South Africa[4] suffering from deafness. Deafness is a highly complex condition caused by both genetic and environmental factors as well as a combination of the two.[5]

About 25% of permanent childhood deafness in the US and Europe is hereditary, with another 25% acquired and around half of unknown aetiology[1,6,7]. Aetiological studies conducted in Africa paint a very different picture. Recent work in Cameroon shows the aetiology of hearing loss to be 15% genetic, 52% acquired and 33% unknown, with meningitis accounting for 34% of all cases.[8]

Globally, it has been found that genetic hearing loss is caused primarily by mutations in gap junction beta 2 (*GJB2*) which encodes connexin 26 (CX26). The major mutations in *GJB2* have been shown to be population-specific, due to founder effects, and include c.35delG affecting Caucasians,[9] c.167delT affecting those of Ashkenazi Jewish ancestry[10] and c.235delC affecting East Asians.[11] In contrast, studies of *GJB2* in Africans or African Americans have not shown a large contribution to deafness and that the common mutations are only present at low frequencies[12,13,14]. Mutations in other connexins are also known to cause deafness, with *GJA1* occasionally being included in diagnostic testing after an early paper suggested its involvement in deafness in patients of African ancestry.[15]

This study aimed to ascertain the significance of connexin mutations in *GJB2* and *GJA1* in a sample of African patients from both Cameroon and South Africa with recessive, non-syndromic deafness. In addition, we included two patients with a rare syndromic condition for genotyping as the disease was known to be caused by *GJB2* mutations and had not been molecularly diagnosed in a patient of sub-Saharan African origin.

2- Literature Review

2.1- Normal Hearing Function[16,17]

The ear is divided into three sections, the outer, middle and inner ear, all of which play an important role in hearing. The structure of the ear is presented in Figure 1. In normal hearing, sound is collected by the outer ear, amplified by the middle ear and converted to a nerve impulse in by the inner ear.

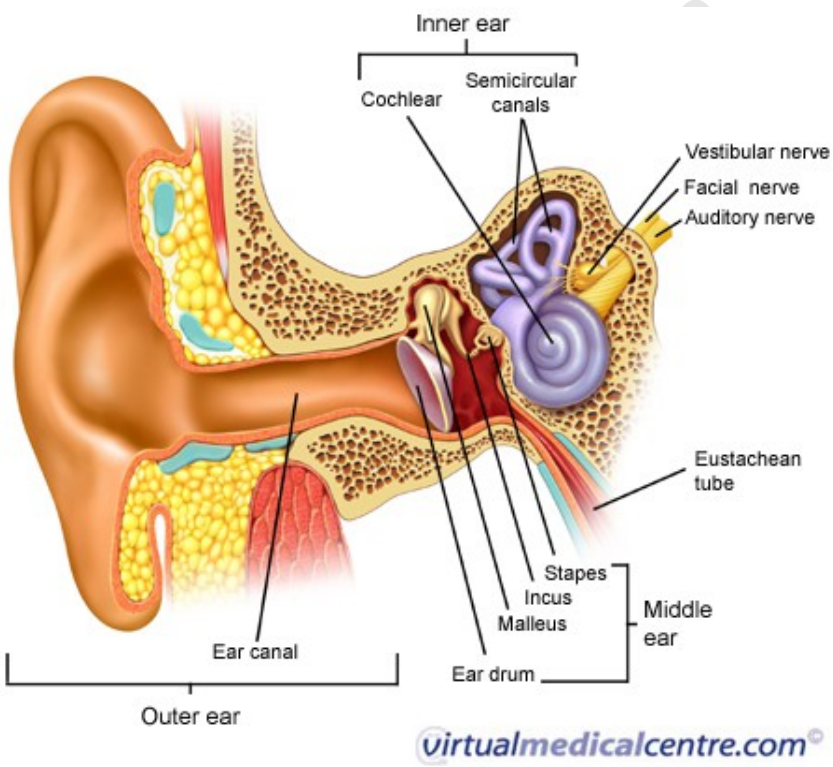

Figure 1: Anatomy of the ear. (Source: Vitualmedicalcentre.com[126])

The pinna collects sound and directs it down the ear canal to the tympanic membrane. Sound is then conducted through the tympanic membrane and the three ossicles to the cochlea. During this period the sound is amplified due to the relative differences in size between the pinna, tympanic membrane and the ossicles. The ossicles act as a series of levers which further amplifies the sound.

As the stapes, the third ossicle, vibrates, it transfers that movement to the perilymph of the cochlear. The cochlear (Figure 2) is divided into three tunnels, the scala vestibuli and scala tympani, which are connected and both filled with perilymph, and the scala media which is closed and filled with endolymph. The hair cells, which trigger the nerve pulses, are mounted on the basilar membrane in the scala media. As the basilar membrane moves, because of the movement of the perilymph, it causes the hair cells to stimulate the neurons. This stimulus is caused through the change in action potential due to an influx of potassium ions, through mechanically-gated ion channels, into the hair cell. The nerve impulses then travel away from the cochlea, via the auditory nerve, to the brain where they are interpreted as sound.

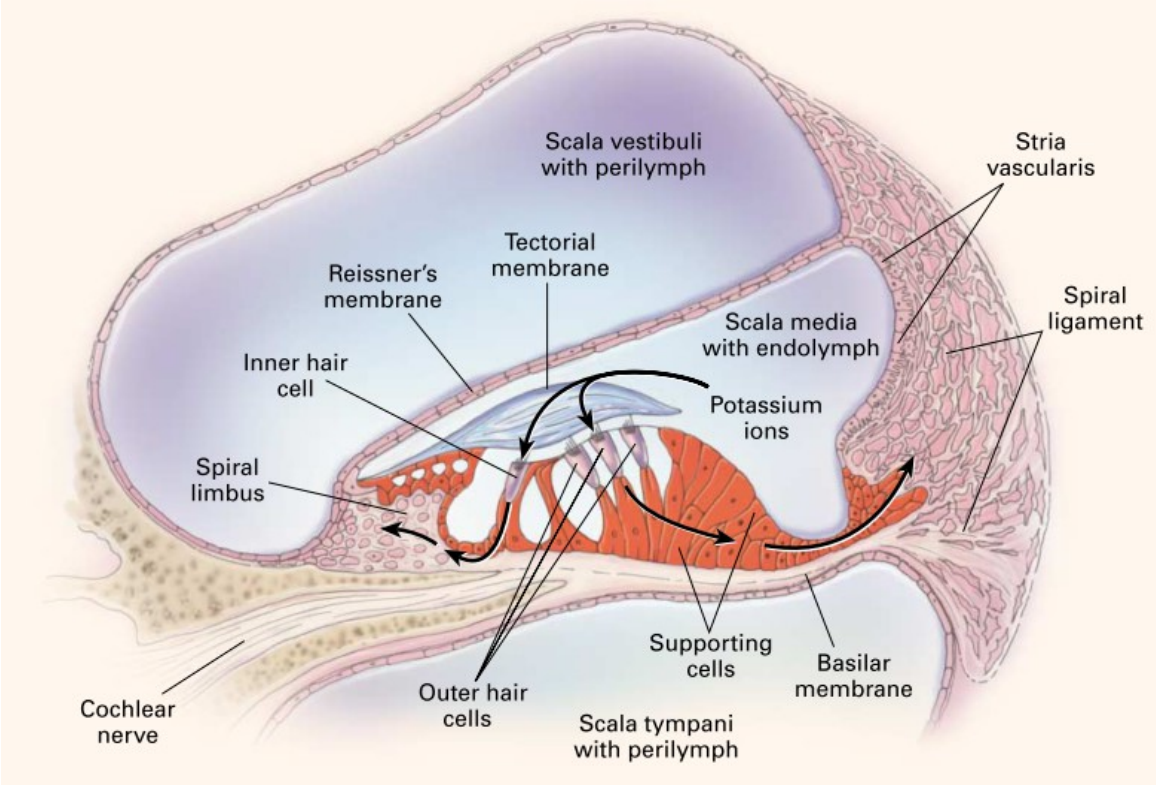

Figure 2: Cross-section through the cochlear. Red cells express Connexin 26. (Source: Willems 2000.[17])

2.2- Hearing Loss

Hearing loss is a condition where an individual's ability to hear is reduced for any reason. It has far-reaching effects on both individuals and their families, leading to stress, increased risk of abuse, reduced educational performance and lowered earning potential.[18] The earlier it is detected, the sooner treatment can begin and the less of an effect it will have. Hearing loss is often grouped according to the severity of the condition, but the criteria for each category differs slightly from study to study which can make comparisons difficult. Table 1 gives the categories of hearing loss according to the World Health Organisation (WHO) but the American Speech-Language-Hearing Association (ASHA), for example, uses slightly different criteria.[19]

Table 1: Hearing loss categories. (Source: WHO 2012[20])

Grade of Impairment	Corresponding audiometric ISO value	Performance	Recommendations
0 – No impairment	25 dB or better (better ear)	No or very slight hearing problems. Able to hear whispers.	
1 – Slight impairment	26 – 40 dB (better ear)	Able to hear and repeat words spoken in normal voice at one metre.	Counselling. Hearing aids may be needed.
2 – Moderate impairment	41-60 dB (better ear)	Able to hear and repeat words spoken in raised voice at one metre.	Hearing aids usually recommended.
3 – Severe impairment	61-80 dB (better ear)	Able to hear some words when shouted into better ear.	Hearing aids needed. If no hearing aids available, lip-reading and signing should be taught.
4 – Profound impairment, including deafness	81 dB or greater (better ear)	Unable to hear and understand even a shouted voice.	Hearing aids may help understanding words. Additional rehabilitation needed. Lip-reading and sometimes signing essential.

2.2.1- Classification Of Hearing Loss

The Palo Alto Medical Foundation describes three types of hearing loss, conductive, sensorineural and mixed.[21] Conductive hearing loss concerns the outer and/or middle ear and occurs when sound is prevented from easily passing through to the inner ear. Sensorineural hearing loss occurs when there is a problem with the inner ear, so that the nerves or sensory cells do not transmit the sound to the brain. Mixed hearing loss is when there is a combination of both conductive and sensorineural hearing loss. If the hearing loss occurs with no other symptoms or signs it is referred to as non-syndromic. However, if there are other features present, it is known as syndromic hearing loss.

Depending on the time when hearing loss presents, it can be described as either acquired or congenital. Congenital hearing loss is that which is present when a baby is born, though it is not necessarily genetic. Acquired hearing loss is that which occurs after birth, either from delayed genetic effects, the environment or natural ageing. Also referring to the timing of hearing loss are the categories prelingual and postlingual. These apply depending on when hearing loss occurred with respect to language acquisition. All congenital deafness is prelingual.

There are also many other descriptors of hearing loss that are useful to understanding how a particular individual is affected.[22] It might be that the hearing loss only affects certain frequencies, such as high-frequency hearing loss or low-frequency hearing loss. This can be seen on an audiogram such as in Figure 3. The graph shows severe to profound impairment of hearing at all frequencies but particularly at the higher frequencies. In a healthy person, the graph will be a flat line, running along the very top, which would indicate no reduction in hearing at any frequency.

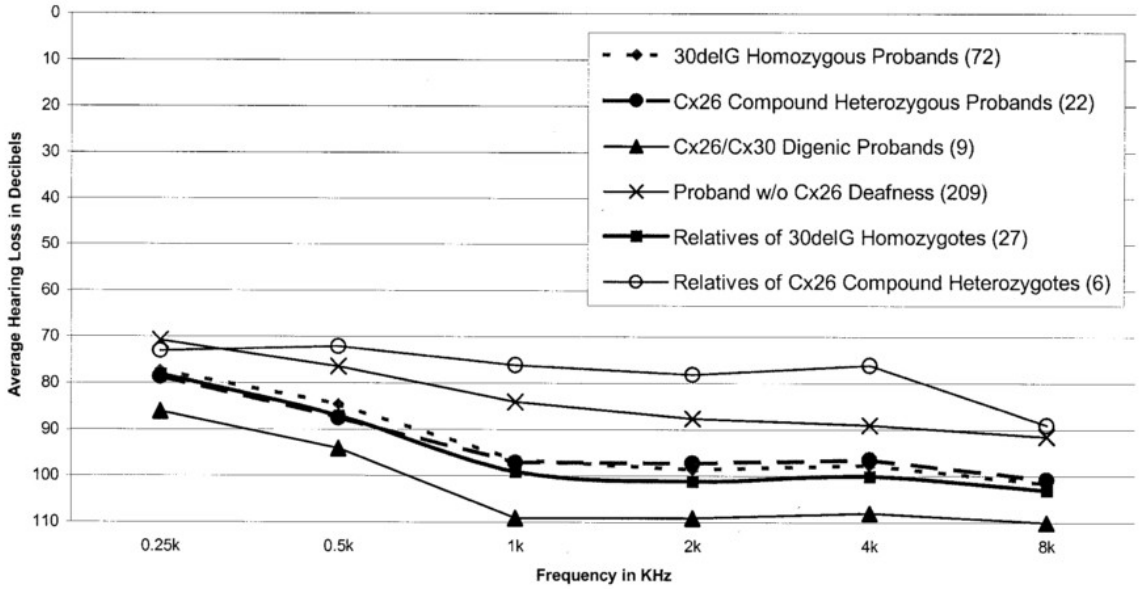

Figure 3: Example of an audiogram. In this case, the audiogram tracks average hearing loss for patients of multiple genotypes. (Source: Pandya et al. 2003[50])

Hearing loss may occur in only one or both ears or there could be a different amount of hearing loss in each ear. If the ears have the same magnitude of hearing loss and the same cause then the hearing loss is symmetrical. If the hearing loss differs in severity or cause then it is asymmetrical. Some tests may not detect asymmetrical hearing loss and the degree of hearing loss is measured according to the ability of the better ear.

Hearing loss may occur suddenly or it may be progressive and increase in severity with time, for example hearing loss caused by noise or old age. Hearing loss may also be stable over time or fluctuate with the degree of hearing loss increasing or decreasing over a period of time. The epidemiology of hearing loss is similarly variable.

2.2.2- Epidemiology Of Congenital Hearing Loss

The prevalence rate of congenital hearing loss in Europe is approximately 1,47 per 1000 births.[2] A review of children screened for hearing loss in the United States in 2004 found that 1,1 per 1000 infants had permanent hearing loss.[1] In China, a 2010 study showed the prevalence of congenital deafness in the Hubei province to be 0,5 per 1000 births[23] and a trial in Shanghai found a rate 1,5 per 1000 births.[24] Japan, although a developed country, has a higher rate of hearing loss than Europe and the US with about 3 per 1000 births suffering from congenital hearing loss.[25] The rates of congenital hearing loss are much higher in the developing world, with an estimated average of 6 per 1000 live births in Sub-Saharan Africa developing hearing loss in the first few weeks of life.[18] A study of infant screening programmes in Lagos, Nigeria, found that the incidence of hearing loss was 7 per 1000 individuals.[3]

The rate of congenital and early-onset hearing loss for South Africa has been estimated at 5,5 per 1000 individuals, much higher than in Europe and America and more in line with other Sub-Saharan countries.[4] This is most likely due to poor health care as the prevalence rate reported in the private health sector is 3 per 1000, compared to 6 per 1000 in the public health sector. A community screening programme in the Western Cape found 4,5 per 1000 infants screened during vaccination.[26]

2.2.3- Detection Of Hearing Loss

Screening for hearing loss in newborns is standard practice in some countries and is the most effective way to detect hearing problems and reduce the negative effects of hearing loss. Testing may also be conducted at schools. Outside of those situations, hearing tests are recommended for people who find they have trouble hearing during daily life or for a child if a parent notices the child often does not hear them or turns the volume up high on the television or radio.

South Africa does not have a national, neonatal screening programme and, at best, only 7,5% of public hospitals provide screening for hearing loss with only 1% providing universal screening.[4] The situation is even worse for neighbouring countries and it has been found that many doctors have not had contact with deaf children and do not respond to parental concerns appropriately.[18] In the South African private health sector 53% of hospitals with obstetric units provide hearing screening on request but only 14% offer universal screening.[27]

There are a number of different tests for hearing loss that operate on a variety of principles and may be suitable for different situations. These tests include pure tone audiometry[5], speech testing,[28] otoacoustic emissions,[5,29] auditory brain-stem response[5,29] and immittance testing.[5,30]

2.2.4- Aetiology Of Childhood Hearing Loss

There are many causes of hearing loss such as loud noises, physical trauma, infections, drugs and genetic causes. The most recent review of the aetiology of childhood hearing loss was performed by Korver et al., primarily based on data from Western countries, and showed 48,3% of cases of hearing loss were of unknown cause, 30,4% were genetic and 19,2% were acquired.[7] It also found 9,8% of cases were non-syndromic and 11,8% were syndromic. This is in agreement with Mehra et al.'s review of the aetiology of hearing loss in America.[1]

Krover and Mehra et al.'s reviews show a different result from Morzaria et al., who examined studies published in English and found the biggest causes of hearing loss could be classed as unknown (37,70%) followed by non-syndromic genetic (29,21%), prenatal (12,04%), perinatal (9,62%), postnatal (8,21%) and syndromic genetic (3,15%).[6] This could be due to different criteria used for the inclusion of studies as they all used papers published around the same time.

A 1997 review of childhood hearing loss in sub-Saharan Africa found that the main cause of hearing loss was infection, particularly meningitis and measles, with very few cases attributed to a genetic aetiology.[31] There were more cases of genetic hearing loss, mostly syndromic, in Zimbabwe and South Africa. It is worth noting that very little genetic analysis was done in the African countries.

This illustrates that the frequencies of these causes are not absolute and will vary by place and time. As we learn more about the causes, particularly genetic causes, of hearing loss and develop better tests we will see a reduction in cases of unknown aetiology. With improved health care there will also be a reduction in the number of cases of hearing loss caused by disease and an increase in the proportion attributable to genetics.

2.2.4.1- Non-Genetic Causes

As medicine has improved it has led to a reduction in the non-genetic causes of hearing loss. When Morzaria et al compared their 1966-1989 cohort with those from 1990-2002 they found changes in the aetiology of hearing loss, such as Rubella dropping from 5,77% to 1,28% while asphyxia and prematurity rose from 1,98% and 1,38% to 2,57% and 2,84% respectively.[6] Roizen observed an increase in the proportion of deafness in neonatal intensive care units due to low birth weight and perinatal factors from 2% in the 1960s and '70s to 17% in 1983-1992.[32]

It must be remembered though that those figures are for the developed world. While there are reductions in disease due to vaccinations, these are not always available in Africa. The 1997 review mentioned above paints a much grimmer picture for the developing world, with meningitis and measles accounting for up to 31% and 19% of acquired hearing loss respectively.[31] In many African countries, malnutrition of children is a real problem that is also a significant risk factor for developing hearing loss.[33] As vaccines and more effective

treatments for disease have been developed, researchers' interest in deafness has been focussed on the genetic causes of deafness.

2.2.4.2- Genetic Causes

Genetic causes are thought to contribute up to 30% of cases of hearing loss worldwide, vary by population group and can be divided both by how hearing loss presents and by the mode of inheritance. There are differing figures for the relative proportions of syndromic and non-syndromic hearing loss but both have multiple aetiologies. Syndromic hearing loss is made up of more than 400 different syndromes[34] and, by July 2013, the Hereditary Hearing Loss website[35] listed a total of 65 genes involved in non-syndromic hearing loss. When considering mode of inheritance (Figure 4), Hone & Smith claimed that 75-80% of hearing loss was recessive, 20% dominant, 2-5% X-linked and a very small number mitochondrially inherited.[34] Bayazit and Yılmaz described approximately the same proportions in their 2006 review.[36]

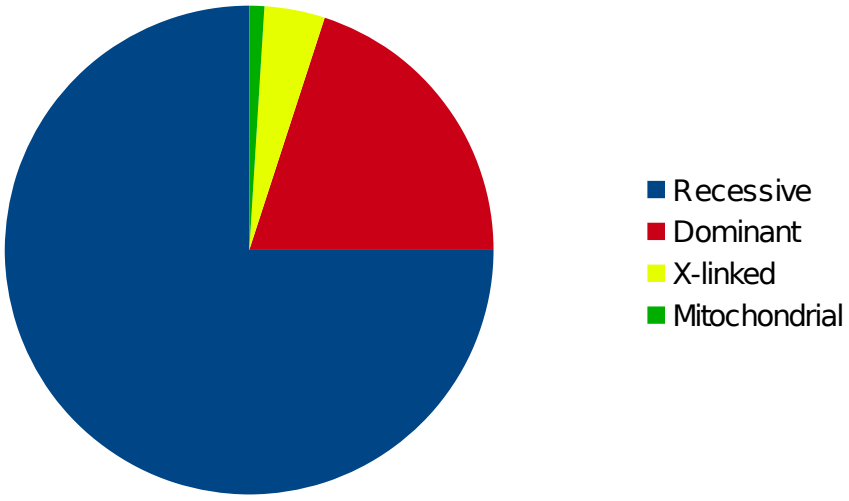

Figure 4: Contribution to genetic hearing loss.

Mitochondrial mutations are the rarest cause of hearing loss. However, they are particularly important in a South African context as they contribute to aminoglycoside-induced hearing loss. Aminoglycosides are used in the treatment of tuberculosis and so individuals carrying mitochondrial mutations have an increased risk of permanent hearing loss if treated inappropriately.

On the other end of the spectrum, recessive genetic conditions are the largest contributor to genetic hearing loss and, of the genes involved in recessive hearing loss, the most important is *GJB2*, encoding connexin 26. It is one of the leading causes of hearing loss in many populations and, depending on the specific mutation, can be inherited in either a recessive or a dominant pattern. Aside from connexin 26, other connexins have also been linked to hearing loss.

2.3- Connexins

2.3.1- Structure And Function

There are 21 human connexins,[37] which are expressed in almost every cell.[38] The connexin proteins are named according to their weight.[37] Thus, connexin 26 is a protein with a molecular weight of 26 kDa. If there are multiple proteins with similar mass then they are distinguished by the use of a decimal point, such as with CX30 and CX30.3.

The names of the genes encoding connexins are determined according to how they are grouped and the order of their discovery and all begin with "GJ" which refers to "gap junction."[37] Gap junctions are intercellular channels that allow ions, secondary messengers and small metabolites to be exchanged by adjacent cells. The proteins that make up gap junctions fall into one of three groups; connexins in chordates, innexins in non-chordates and pannexins in vertebrates.[38] The two major groups of connexins are the alpha and beta connexins based on sequence similarity of the cytoplasmic loop. So, *GJB2* was the second beta connexin gene to be identified. The actual value of the grouping is disputed, however, and genes are being renamed to fit a phylogenetic classification.[37]

Connexins all share a basic structure, shown in Figure *5*, with four transmembrane domains and two extracellular loops which play a role in protein docking and recognition.[38] Connexins use the extracellular domains to form gap junctions with connexins in neighbouring cells. Most of the connexin structure is conserved, although the c-terminus and cytoplasmic loop are free to vary.

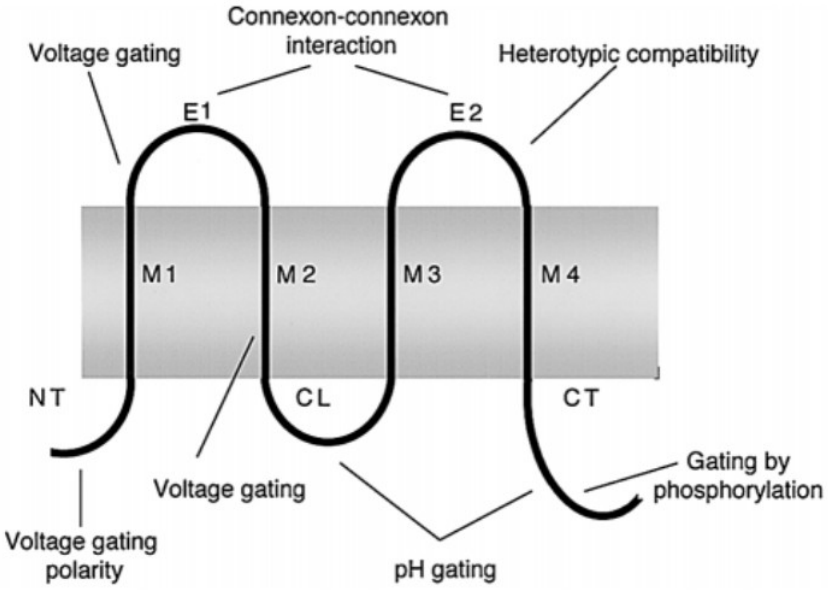

Figure 5: Generalised structure of a connexin and the putative function of each domain. M1–M4: transmembrane domains 1–4; E1/E2: extracellular domains 1 and 2; CL: cytoplasmic loop; NT: amino-terminus; CT: C-terminus. (Source: Richards, 2000[64])

Connexins form intercellular channels by combining in groups of six to form a structure called a connexon.[17] Connexons from adjacent cells join together to form gap junctions. These gap junctions have variable properties as connexons can consist of just a single type of connexin, described as homomeric, or a combination thereof, heteromeric, and connexons in adjacent cells may be composed by the same, homotypic, or different proteins, heterotypic.[38] These variations in structure and assembly are illustrated in Figure 6.

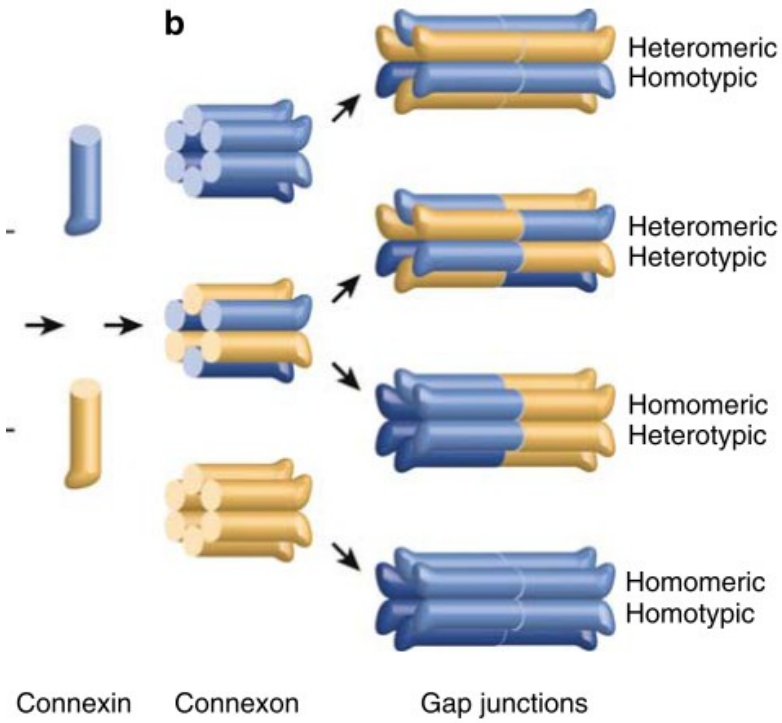

Figure 6: Illustration of how connexins join to form connexons, how connexons join to form gap junctions and the various combinations that can occur. (Source: Meşe et al. 2007[38])

2.3.2- GJB2/Connexin 26

GJB2 is located on chromosome 13 and codes for the gap-junction protein connexin 26. Connexin 26 is involved in the transport of potassium ions and other small molecules and is expressed in the cochlea, shown in Figure 2, skin, liver and placenta.[38,39,40] The structure of connexin 26 is shown in Figure 7 where one can see that there are few mutations in the intercellular loop or C-terminus. It is also worth noting that syndromic deafness mutations are all dominant.

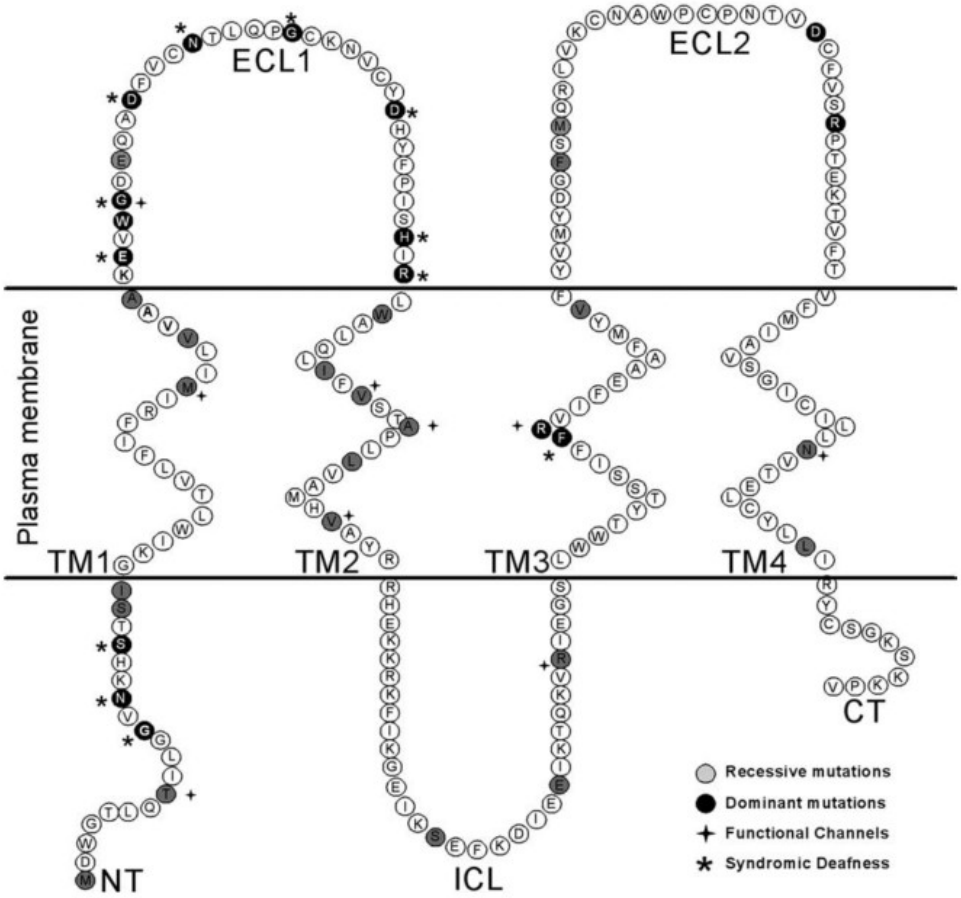

Figure 7: Structure of connexin 26 with positions of deafness-associated mutations shown. (Source: Martínez et al., 2009[78])

GJB2 has been identified as a major cause of autosomal recessive hearing loss in several population groups, although the specific mutations contributing to hearing loss are generally population-specific. *GJB2* has also been linked to other diseases such as autosomal dominant deafness and syndromic diseases with a combination of hearing loss and skin disorders such as Vohwinkel syndrome and keratitis-icthyosis-deafness syndrome.[41]

It is believed that mutations in Connexin 26 affect its ability to transport potassium ions and therefore regulate endocochlear potential, which is required for nerve impulses involved in hearing, but the mode of action is not definitive. It is possible that it is involved in transport of other metabolites which may also have an effect on hearing.[39] This is supported by some studies that have found certain mutations to have no effect on the ability to transport ions but a reduced ability to transport other molecules such as glucose.[42]

2.3.2.1- Molecular Epidemiology Of *GJB2*

2.3.2.1.1- Global Mutation Spectrum

A lot of focus is given to studies of *GJB2* performed on Caucasian populations and its contribution to hearing loss in that context. However, more broadly, it has been shown that the contribution of *GJB2* to hearing loss differs from population to population and, even more importantly, the actual mutations contributing to hearing loss differ between populations.

GJB2 was first associated with deafness in patients with dominant, syndromic, genetic deafness, however, in 1997, it was implicated in recessive, non-syndromic deafness in a consanguineous Pakistani family.[40] The mutation detected was p.(W77X) and, after screening another two families, a second mutation, p.(W24X), was discovered. A later study on Pakistani patients found *GJB2* mutations in 16,6% of alleles, although only 6,1% were pathogenic, and also found p.(W24X) and p.(W77X) to be the most common mutations.[43] Similar results have been found in the British Pakistani community with a study finding that 7,69% of hearing impaired individuals had pathogenic *GJB2* mutations with all of them being homozygous for p.(W24X).[44] p.(W24X) is also the most prevalent *GJB2* mutation in India, with some evidence suggesting this may be due to a founder effect.[45,46]

When research began to focus on European populations and their descendants in America the same gene was found but with different mutations. Zelante et al. found 90% of chromosomes from Mediterranean patients with non-syndromic, sensorineural deafness had *GJB2* mutations and 60% of chromosomes carried the c.35delG mutation.[47] Rabionet found fewer *GJB2* mutations, in only 37% of hearing loss chromosomes, but a similarly high prevalence of the c.35delG mutation, 82% of the mutant chromosomes.[48] In America, Green et al. found 58% of individuals with hearing loss had *GJB2* mutations and the carrier rate of the c.35delG mutation was 2,5%.[49] This was extrapolated to give a total *GJB2* mutation carrier rate of 3% for the mid-Western US. Other studies have given different figures for the prevalence of *GJB2* variations. Pandya et al. described *GJB2* alterations in approximately 33% of alleles, with 31% of the total alleles being pathogenic,[50] and Wu et al. found nearly 43% of alleles have a variation.[51] There is a consensus that the most common mutations are c.35delG, p.(M34T), and c.167delT with prevalence rates of approximately 24%, 2,2% and 2,7% of alleles respectively. As non-syndromic hearing loss can be caused by many genes, and even more mutations, a single gene with a few highly prevalent mutations being responsible for the majority of deafness was an encouraging find.

When one moves away from the Caucasian populations the mutation spectrum changes, just like how a different array of mutations was observed in Pakistanis. In East Greenland, it was found that only around 8% of alleles in individuals with hearing loss had a *GJB2* variant and that dropped to about 3% when considering mutations, including c.35delG, suggesting *GJB2* did not play a major role in hearing loss in that population.[52] Similarly in the Ashkenazi Jews the c.35delG mutation is present but its effect is far less than that of the c.167delT mutation, which is the most common mutation in that group. This mutation has a carrier rate of 4% yet was not detected in any other population.[10]

Studies in the Chinese population have found that although *GJB2* is an important cause of hearing loss, with variants in 39% of patients with hearing loss and mutations in 16%, the c.35delG mutation plays only a tiny role.[53] While c.35delG accounted for 0,85% of alleles, c.235delC and c.299-300delAT accounted for 20% and 4% of alleles respectively. Similar results were reported by Chen et al. in 2011, with 18% of hearing loss patients having a *GJB2* mutation.[54] Chen et al.'s study found c.235delC mutation accounted for 9,7% of the alleles, the c.299-300delAT mutation accounted for 1,5% and the c.35delG mutation for 0,25%.

2.3.2.1.2- Reported *GJB2* Mutations/Variations In Africans

The importance of c.35delG, and even that of *GJB2*, for deafness in African populations is questionable. Very little research has been done on non-syndromic sensorineural hearing loss in African populations, despite the continent containing a large and rapidly growing proportion of the world's population. Most research on genetic, recessive hearing loss has been focussed on Caucasian populations, yet research on other population groups has shown the mutations leading to hearing loss are population-specific due to founder mutations.[9,10,11] This means that current genetic tests for deafness, which focus on *GJB2*, may not be appropriate for African populations.

A recent study in South Africa reported no common *GJB2* mutations, the absence of the *GJB6:*D13S1830 deletion and none of four mitochondrial mutations (c.A1555G, c.A3243G, c.A7445G, and c.T7511C).[14] Two *GJB2* variations were found at high frequencies, g.3318-34C>T and g.3318-15C>T at 46% and 21% respectively. They were also present in 43% and 35% of the control group respectively, indicating that they are most likely not pathogenic.

A study of the Kenyan and Sudanese population found 23% of cases had variations in *GJB2,* but less than 4% were located in the coding region of the gene and there were no controls against which to assess pathogenicity.[12] The c.35delG mutation was detected in 3% of Sudanese individuals, a much lower figure than in Europeans, and not at all in Kenyans. The g.3318-34C>T and g.3318-15C>T variations detected in South Africa were both found in the combined Kenyan and Sudanese cohort at frequencies of 13% and 6,5% respectively.

Studies on African Americans have also found low prevalence of common mutations. One study reported 9% of African American alleles contained *GJB2* variations, the majority being c.35delG, compared to 33% of Caucasian alleles.[50] A second study found that about 8,7% of hearing-impaired individuals had a polymorphism in *GJB2* as well as only polymorphisms in the controls.[55]

There are two African countries where *GJB2* mutations have been shown to play a substantial role. They are Tunisia and Ghana. In Tunisia, approximately 17% of families affected by hearing loss have mutations in the *GJB2* gene with c.35delG as the predominant mutation followed by p.(E47X).[56] These results were confirmed by a subsequent study that found 27% of deaf patients had a *GJB2* mutation[57] and the most recent, which found *GJB2* mutations, 85% of which are c.35delG, in 39% of families.[58] This can be explained by the fact that the Tunisian population is primarily Arab.

In Ghana, around 17% of hearing impaired individuals carry a *GJB2* mutation.[59] The most common being the p.(R143W) mutation, which has previously been found in all deaf individuals in one Ghanaian village.[60] This mutation is seldom found elsewhere and appears to represent an African founder effect in Ghana, much like c.35delG in Caucasians[9] and c.235delC in Asians.[11]

2.3.3- *GJA1*/Connexin 43

2.3.3.1- Expression

Connexin 43 (CX43) is widely expressed in the human body. This is well-illustrated by the list of symptoms of oculodentodigital dysplasia (ODDD), a rare, autosomal dominant disease caused by mutations in *GJA1*, which includes skeletal and tooth malformations, syndactyly, deafness, mental retardation, dry and scaly skin and other abnormalities.[61,62,63] It should be noted that ODDD is highly variable and many of the features occur infrequently.

CX43 is a major component of gap junctions in the skin,[64] where it plays a role in wound healing.[65] It is abundantly expressed in bone cells,[66] and is known to be expressed in the heart. Some studies suggest a relationship between *GJA1* and pulse rate[67] or that mutations in *GJA1* may contribute to sudden infant death[68] while others show *GJA1* is not likely to be important in congenital heart disease.[69]

There have been few studies of CX43 expression in the cochlea, the most important site if it is involved in hearing loss, but there is good evidence of its expression. Liu et al. gave preliminary results of CX43 expression in the satellite cells of the spiral ganglion.[70] This was followed up by a more sensitive method of observing CX43 expression and revealed that the protein was produced in many of the cells making up the organ of corti as shown in Figure 8.[71] The evidence provided by CX43 expression in the cochlea makes it biologically plausible that mutations in *GJA1* might affect hearing loss.

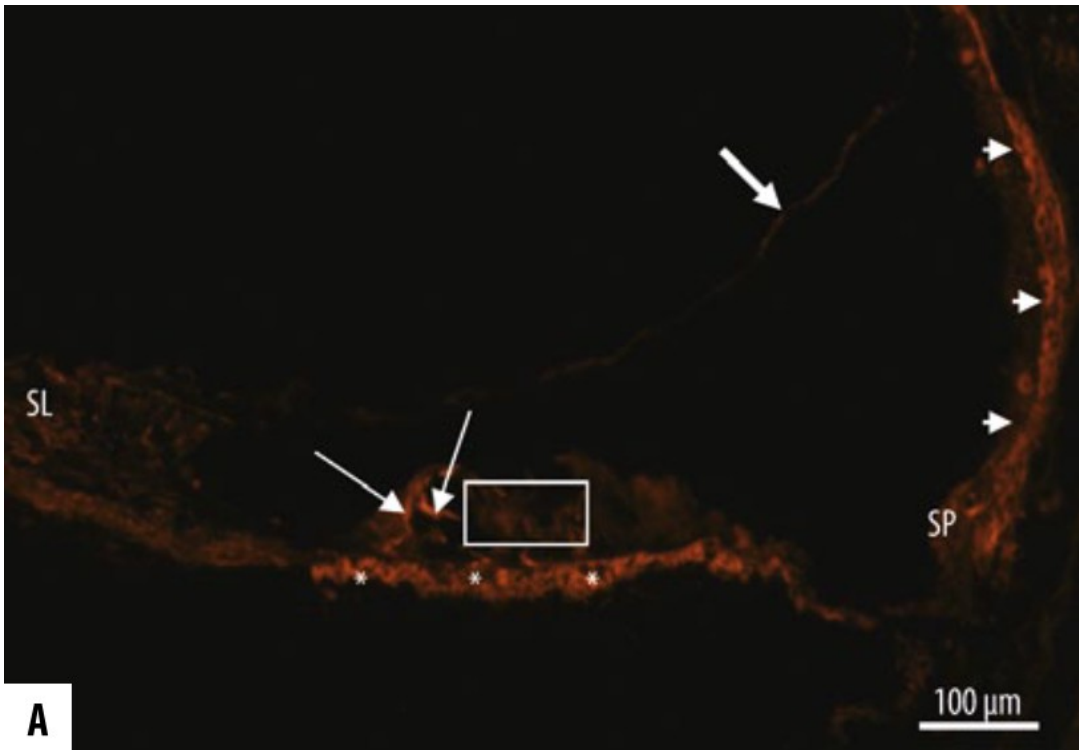

Figure 8: Connexin 43 immunohistochemistry of the cochlea. Arrowheads = basal cell layer of stria vascularis; thin arrows = pillar cells; asterisks = covering cell layer beneath the basilar membrane; SL = spiral limbus; SP = spiral prominence; square = Deiters cells; thick arrow = vestibular membrane. (Source: Liu et al. 2011[71])

2.3.3.2- *GJA1* And Hearing Loss

After successfully linking a number of other gap junction mutations to deafness, most notably with *GJB2* and *GJB6*, researchers turned their attention to *GJA1*. In 2001, Liu et al. screened 26 deaf African Americans, 100 controls (split among 40 African Americans and 60 from other races) and 510 deaf probands from other races in a DNA repository. In the African American cohort, Liu et al. found three individuals homozygous for one change and one homozygous for a second change, concluding that *GJA1* could be a common cause of recessive deafness in people of African ancestry.[15]

Two years later, in a paper on oculodentodigital dysplasia, Paznekas et al. revealed personal communication that showed that the results of Liu et al. were due to changes to the *GJA1* pseudogene.[61] A study conducted in Turkey of approximately 60 deaf patients and 210 controls found no variations in *GJA1*.[72] At this point there was no evidence of a link between *GJA1* and hearing loss.

Work done in 2007 on Taiwanese patients provided fresh evidence of a link between *GJA1* and hearing loss. Yang et al. examined multiple connexins, including connexin 43 and its pseudogene, in a cohort of deaf patients and found several variants.[73] Three polymorphisms (defined as occurring in both patients and controls) were discovered in the pseudogene, two of which occurred at a statistically significantly different rate between patients and controls and only in the homozygous form. Yang et al. also detected two pseudogene mutations, 205T>C (p.(S69P)) and 932delC and one *GJA1* mutation, 977C>T (T326I). These were not highly prevalent, with the exception of the pseudogene mutation 932delC which occurred, heterozygously, in 6,15% of patients. In addition, they found three synonymous mutations, one in the pseudogene and two in *GJA1*.

The three non-synonymous Taiwanese mutations were further tested with a dye transfer assay. All three mutant proteins showed correct trafficking in the cell but impaired dye transfer with two showing no dye transfer at all.[74] This further supports the link between the mutations and deafness, however the contribution from *GJA1* is likely small as a second screening of 253 Taiwanese patients with hearing loss found only one *GJA1* heterozygote and no variant in any of the 120 controls.[75]

In 2012, a study in Iran found another possible, though also unlikely, link between *GJA1* and deafness.[76] Three variants, c.758C>T (p.(A253V)), synonymous c.717G>A and c.3*dupA, were discovered in 4/34 (11,8%) patients and 7/200 (3,5%) controls. All the patients carried a *GJA1* variant as well as being heterozygous for c.35delG in *GJB2*.

While not linked to any specific mutation, it is worth noting that when Paznekas et al. examined 177 ODDD individuals with known *GJA1* mutations and available phenotypic data, 26% had conductive hearing loss.[77]

2.3.4- Syndromic Conditions

2.3.4.1- Overview Of Syndromic Connexin Diseases

While the majority of this thesis is concerned with non-syndromic deafness, it was already mentioned that two Cameroonian samples that formed an off-shoot of the connexin study had a syndromic condition, both suffer from KID syndrome. Syndromic deafness is the term used when deafness presents with a second clinical feature, in the case of connexin mutations this is usually a skin condition. *GJB2* can cause a number of syndromes that present with deafness, including keratitis-icthyosis-deafness (KID) syndrome, hystrix-like icthyosis deafness syndrome, Bart-Pumphrey syndrome and Vohwinkel syndrome.[39] *GJA1* is involved in the syndromic condition, ODDD, described briefly in 2.3.3.1.

Although non-syndromic hearing loss can be found in both dominant and recessive forms it has been found that all syndromic hearing loss by connexin 26 is dominant. As the the common c.35delG mutation in Caucasians leads to a truncated protein and recessive, non-syndromic hearing loss we can assume that the loss of connexin 26 does not cause syndromic effects. It is hypothesised that syndromic deafness is caused by mutations which also affect other proteins.[78] There is experimental evidence to support this hypothesis.

It is well-known that different connexins can form hemichannels with one another (heteromeric connexons). Liu et al. showed that not only did connexins 26 and 31 stain at the same position in the cochlea but that immunoprecipitation with anti-CX31 antibodies showed reaction with CX31, CX26 and CX30 in Western blotting.[79] It has also been shown that in *X. laevis* oocytes that dominant CX26 mutations can affect the junctional

conductance of other connexins expressed in the same oocyte.[80]

2.3.4.2- KID Syndrome

Keratits-icthyosis-deafness Syndrome (OMIM: 148210) is an extremely rare disease that has only been reported in approximately 100 patients worldwide. It has three major symptoms; keratitis, inflammation of the cornea, icthyosis, dry, thickened or scaly skin and severe sensorineural deafness. Aside from the diagnostic manifestations, the phenotype can include many other conditions such as increased risk of infection and increased occurrence of squamous cell carcinoma.[81]

It is usually caused by mutations in *GJB2*[82,83] which generally occur *de novo* with the most common being p.(D50N).[84] Interestingly a p.(V37E) mutation in *GJB6* (connexin 30) has also been linked to KID syndrome, suggesting there may be more than one genetic cause.[85] That same mutation in *GJB6* has also been linked to Clouston syndrome (OMIM: 129500) in a Scottish patient whose skin phenotype overlaps that of KID syndrome.[86] The reason why the same mutation could lead to two different syndromes may be due to the KID syndrome patient having the *GJB6* mutation as well as being homozygous for the p.(V27I) mutation in *GJB2*. While the p.(V27I) change in *GJB2* is considered non-pathogenic on its own it has been speculated that perhaps interactions between p.(V37E)-*GJB6* and p.(V27I)-*GJB2* lead to the different observed phenotypes. While discussing the overlap between these connexin diseases it should also be noted that p.(D50N)-*GJB6,* the same change that causes KID syndrome in *GJB2*, can be responsible for Clouston syndrome on its own.[87]

An autosomal recessive form of KID syndrome (OMIM: 242150) has been proposed but this has not been confirmed with molecular work and, as most cases of KID syndrome arise sporadically, this must be treated with caution.

2.4- Rationale

Currently *GJB2* mutations are considered to explain the majority of cases of non-syndromic, sensorineural hearing loss but there are few studies of *GJB2* on Africans. We examined *GJB2*, using Cameroon as a proxy for African diversity as the country has been shown to contain high levels of genetic diversity.[88] We also chose to sample from the previously-unstudied Xhosa population of South Africa. In addition, despite very limited evidence of *GJA1* being involved in deafness it is sometimes included in clinical testing for Black Africans in South Africa. We present here the results of the first study of *GJA1* conducted on Africans. This adds to our knowledge of the genetics of hearing loss in Africa and can be used to guide clinical practices and direct future studies.

3- Aim And Objectives

3.1- Aim

The aim was to ascertain the significance of connexin mutations in *GJB2* and *GJA1* in a sample of African patients from both Cameroon and South Africa with recessive, non-syndromic deafness. A secondary aim was to genotype two Cameroonian patients with KID syndrome, a disease known to be caused by mutations in *GJB2*.

3.2- Objectives

The aims were achieved through the following objectives:

1. Recruitment of Cameroonian and Black South African patients with congenital, non-syndromic hearing loss of unknown or putative genetic origin.
2. Extraction of DNA, from either peripheral blood or saliva, and sequencing the coding regions of *GJB2* (CX26) and *GJA1* (CX43) in patients and population-matched controls.
3. Ascertaining of the role of genetic variation in both *GJB2* and *GJA1* in deafness among African populations.
4. Comparing sequence variations both between patients and controls and between published data, including data from the 1000 Genomes Project.

4- Methodology

4.1- Ethical Considerations

Recruitment of patients from Cameroon was approved by Cameroon's National Ethics Committee, authorisation number N°123/CNE/SE/2010. Ethics approval for this study was granted by the University of Cape Town's Human Research Ethics Committee, HREC REF: 080/2011. Written and signed informed consent was obtained from all participants, if they were 18 years or older, or from the parents/guardians with verbal assent from the children.

4.2- Patients And Settings

4.2.1- Procedure

Patients were recruited from seven of the ten regions of Cameroon, mainly from schools for the deaf, and those procedures have been reported previously.[8] Xhosa patients were recruited from a school for the deaf in the Eastern Cape Province of South Africa.

During recruitment, information on participants' medical and family history was obtained from the participants themselves, their parents and medical records, depending on which sources were available. In the majority of cases, general systemic and otological examination was performed as well as an audiological evaluation using either pure tone audiometry or auditory brain stem response test. Audiological test results that were obtained before admission to schools for the deaf were also reviewed for some subjects. When syndromic deafness was suspected, additional tests, when possible, were later performed to confirm or exclude the diagnosis.

4.2.2- Inclusion And Exclusion Criteria

For the present study, the following inclusion criteria were used for patients:

- Patients of Black African descent with non-syndromic hearing loss as confirmed by a clinical and audiological report.
- Deafness of putative genetic origin, as revealed by one or more affected member in the families or consanguinity, or deafness of unknown origin.
- Two patients with KID syndrome, a condition known to be caused by mutations in the *GJB2* gene, were also included for genotyping.

Patients were excluded from the study if they failed to meet inclusion criteria or declined to participate in the study. Reasons for exclusion were primarily that patients had syndromic deafness, with the exception of KID syndrome patients, or their hearing loss had obvious environmental causes, mostly meningitis, trauma, prematurity or exposure to ototoxic drugs.

Following the above criteria, 194 non-syndromic and two KID syndrome Cameroonian patients with available DNA samples were recruited from the previously reported cohort. Forty-four South African patients were prospectively recruited from Efata School for the Blind and Deaf in the Eastern Cape Province. Upon review of the South African patients' information sheets, 19 patients were excluded from the study due to suspected syndromic or acquired deafness, resulting in a final cohort of 25. Although all patients were included in the *GJB2* portion of the study, only a selected subset was included for *GJA1*. The patients included are detailed at the beginning of the respective chapters.

4.2.3- Controls

Healthy and normal hearing controls were recruited from the same population background. In total, there were 17 South African and 64 Cameroonian controls.

4.3- Molecular Methods

4.3.1- DNA Extraction, Quantification And Integrity Check

DNA was extracted either from blood, using a modified version of the salting out method,[89] or purified from saliva according to the manufacturer's instructions with minor changes (Oragene® kit; DNA Genotek®, USA). The protocols are available in appendices I.VI and I.VII.

All DNA samples were subjected to spectrophotometry to determine concentration and purity and gel electrophoresis to assess DNA integrity before use. Spectrophotometric measurements were carried out with a Nanodrop ND-1000 (Thermo Fischer Scientific, Waltham, USA) spectrophotometer that was blanked with either distilled water or DNA rehydration solution, depending on what the DNA was dissolved in. The spectrophotometer readings gave both a measure of DNA quality through the 260/280 and 260/230 ratios and concentration.

Cameroonian samples showed a mean concentration of 170 ng/µl (1,7 - 528 ng/µl), mean 260/280 ratio of 1,8 (0,97 – 3,8) and mean 260/230 ratio of 0,86 (0,03 - 1,94). One Cameroonian sample did not have a valid 260/230 reading. South African samples showed a mean concentration of 642 ng/µl (54 – 1681 ng/µl), mean 260/280 of 1,86 (1,79 – 1,91) and mean 260/230 of 1,93 (1,06 – 2,2).

Once the concentration of the stock DNA was known, each sample was diluted with distilled water to give a working concentration of 100 ng/µl. If the stock concentration was below 100 ng/µl then the DNA was kept at the original concentration and a portion of the stock aliquoted into a working tube. The DNA dilutions were stored at 4 °C and used in all further experiments while the stock DNA was stored at -20 °C until it was needed for further use.

Following dilution, 35-40 ng was run on a 1% agarose gel (LE agarose and 1X TBE) and visualised with ethidium bromide under UV light. This was done to make sure that the DNA was intact and there was no obvious contamination. Good quality DNA was observed in most of the samples. Figure 9, below, is representative agarose gel electrophoresis of working stock DNA. Samples were considered to pass the integrity check so long as they displayed a single band at the top of the gel. None of the samples showed degraded DNA. DNA was subsequently used for genotyping for variations in *GJB2* and *GJA1*, using primers that were synthesised at the Synthetic DNA Laboratory of the Department of Molecular and Cell Biology, University of Cape Town.

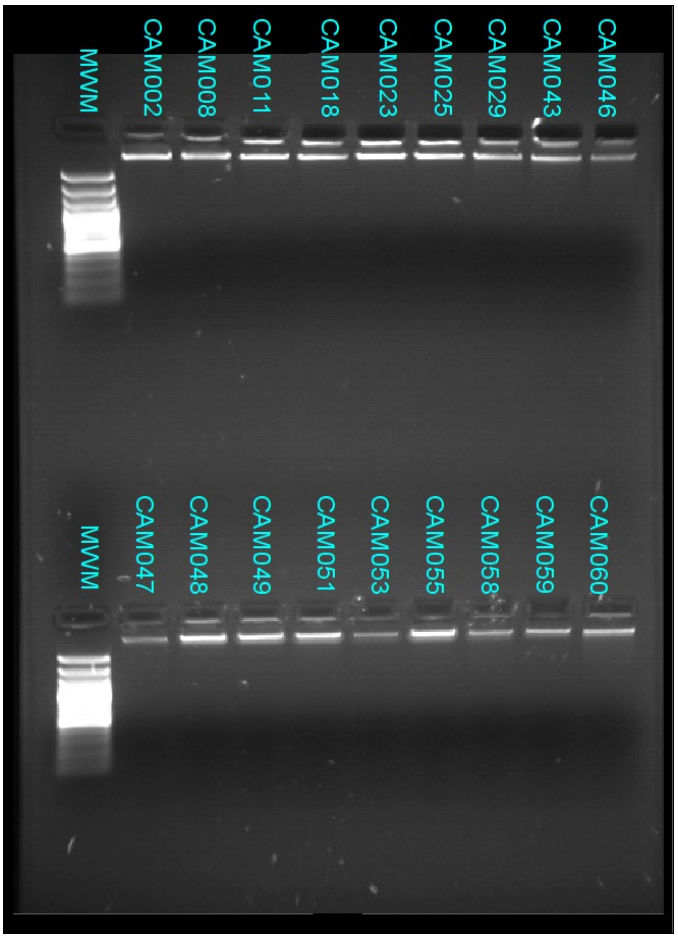

Figure 9: An example of an integrity gel. All samples, including those not shown, have undamaged DNA that migrates slowly.

4.3.2- Amplification Of *GJB2*

Amplification of the *GJB2* gene was performed according to the methods of Liu et al.[53] Two primers were used to amplify a 900 bp region of the *GJB2* gene which contained the complete coding region of exon 2. The structure of *GJB2* can be seen in Figure 10.

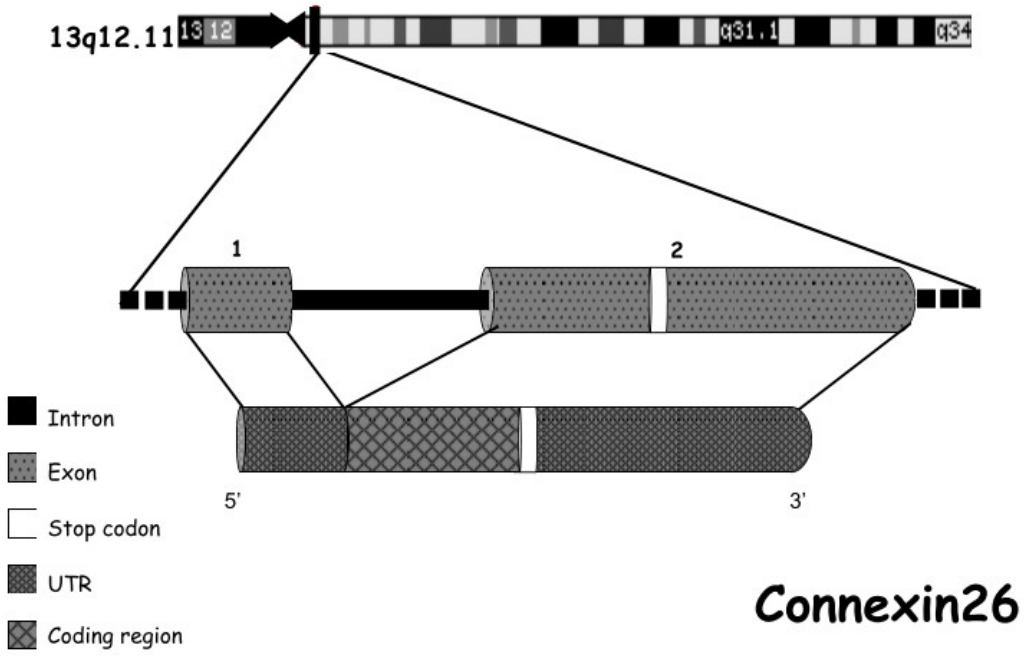

Figure 10: Structure of *GJB2*/CX26. From top to bottom the figure shows the position of *GJB2* on chromosome 13, the position of the two exons in *GJB2* (labelled 1 and 2) and the structure of the mRNA. The coding region falls completely within exon 2. (Source: Iossa et al. 2011[39])

The PCR mixture consisted of 1X GoTaq colourless buffer (Promega, Madison, USA), 200 µM nucleotide mix, 0,4 µM of each primer, 0,5 U of GoTaq Polymerase (Promega, Madison, USA), 100 ng of DNA and was made up to 25 µl with distilled water. Amplification consisted of an initial denaturation step at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 1 minute, annealing at 60 °C for 1 minute and extension at 72 °C for 1 minute; followed by a final extension at 72 °C for 5 minutes.

4.3.3- Amplification Of *GJA1*

Structurally, *GJA1* is very similar to *GJB2* with the entire coding region residing in the second exon. The most noticeable difference between the two genes is their size, while the coding region of *GJB2* comprises 680 bp, that of *GJA1* is 1148 bp, at least 400 bp longer. There is an even greater difference in the size of their introns, 3180 bp versus 11 000 bp respectively. A 1348 bp fragment of *GJA1*, containing the complete coding region, was amplified using primers and a method adapted from that of Huang et al.[69]

As only minimal details were provided in the paper it was necessary to perform a temperature gradient PCR to determine the optimum annealing temperature, Figure 11. This was performed at the following cycling conditions and with the same reagent concentrations as described in section 4.3.2. Amplification consisted of an initial denaturation step at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, an annealing temperature gradient of 56-62 °C for 30 seconds and an extension at 72 °C for 1,5 minutes; followed by a final extension step at 72 °C for 5 minutes. Based on the results of the temperature gradient, the optimal annealing temperature was chosen to be 57°C.

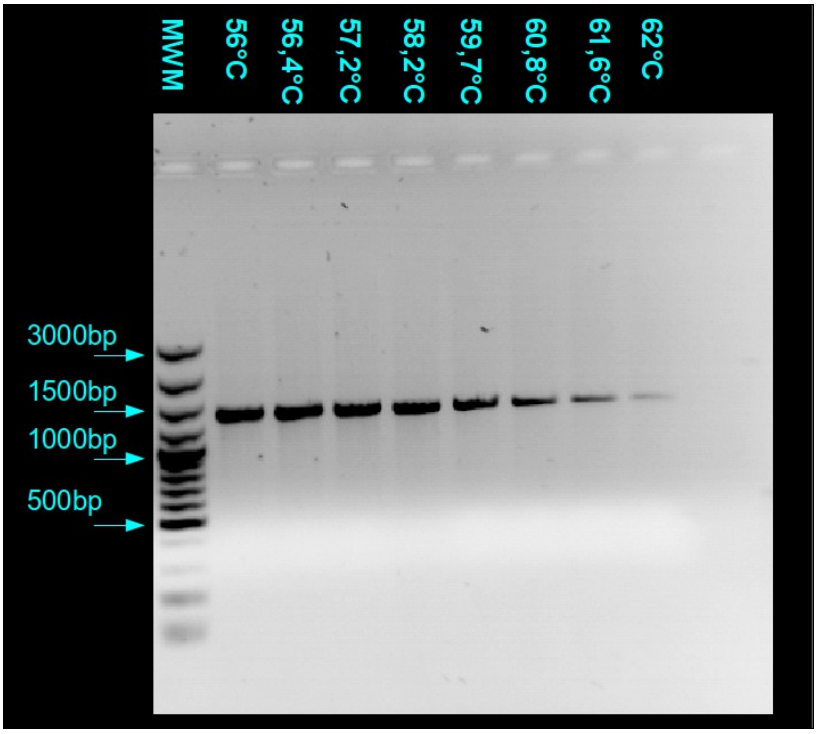

Figure 11: *GJA1* temperature gradient. An annealing temperature of 57 °C was chosen to minimise non-specific binding and maximise yield.

4.3.4- Sequencing Of *GJB2* And *GJA1*

After amplification of the desired region, PCR products were genotyped by sequencing, following a sequencing protocol that included PCR clean-up, sequencing reaction, sequencing clean-up and electrophoresis. PCR products were cleaned by mixing 5 µl of the product with 1 µl of FastAP Thermosensitive Alkaline Phosphatase (Thermo Fischer Scientific, Waltham, USA), 0,1 µl Exonuclease I (Thermo Fischer Scientific (Fermentas), Waltham, USA) and made to 20 µl final volume with distilled water. The mixture was incubated at 37 °C for one hour followed by 75 °C for 15 minutes.

After PCR clean-up, 3 µl of the cleaned product was combined with 2 µl 5X sequencing buffer (Applied Biosystems, Foster City, USA), 1 µl BigDye V3.1 Terminator (Applied Biosystems, Foster City, USA), 1 µl either forward or reverse primer and made up to 10 µl in distilled water. All samples were sequenced in both the forward and reverse direction

with the same primers used for amplification. Samples were run in a thermal cycler for 1 cycle of 5 minutes at 98 °C and either 30 (*GJB2*) or 35 (*GJA1*) cycles of denaturation for 30 seconds at 96 °C, annealing for 15 seconds at 50 °C and extension for 4 minutes at 60 °C. After the sequencing reaction, samples were cleaned by ethanol precipitation as described in Appendix I.VIII.

After the sequencing reaction, electrophoresis was performed in 96-well plates on an ABI3130XL Genetic Analyser (Applied Biosystems, Foster City, USA). Wells contained a mixture of 8 µl Hi-Dye formamide and 5 µl cleaned sequencing reaction. *GJB2* was sequenced in both directions and overlapped over almost the complete length of the product but, due to its length, we did not have forward and reverse strand overlaps for the majority of the *GJA1* coding region. However, we decided this was a minor limitation as any ambiguous results could be resequenced.

4.4- Bioinformatics And Statistical Analysis

4.4.1- Sequencing Quality-Check

Chromatogram files were manually checked using FinchTV 1.3.1 (GeoSpiza, Seattle, USA) then aligned in BioEdit 7.0.5.3, running through WINE 1.4. Sequences were aligned to the *GJB2* reference sequence (Ensembl transcript ENST00000382848 (*GJB2*-001) retrieved 31 August 2012) or the *GJA1* reference sequence (ENSG00000152661, retrieved 13 March 2013).

4.4.2- Molecular Analysis

Detected variations were checked against dbSNP[90] and the effects of non-synonymous variants were predicted using Polyphen-2.[91] SHEsis[92,93] (http://analysis2.bio-x.cn/) was used

to check for statistical differences between the cases and controls. SHEsis analysed allele frequency, genotype frequency, haplotype frequency and linkage disequilibrium. Significance of SHEsis results is reported using Fischer's p-values.

4.4.3- Phylogeny Construction And Principal Components Analysis (PCA)

SNP frequencies for the sequenced region of *GJB2* and *GJA1* was downloaded from the 1000 Genomes Browser (http://browser.1000genomes.org) for all populations.[94] A phylogeny was then constructed with PopTree software using the Neighbour Joining algorithm, Nei's DA genetic distance[95] and 1000 bootstraps. PCA was performed in R[96] using the FactoMineR package with the same 1000 Genomes data. As the 1000 Genomes data is from apparently healthy individuals, only control data was used for comparison.

5- Results

5.1- GJB2

5.1.1- Patients

One South African control, four Cameroonian controls and 12 Cameroonian patients were excluded from the study due to low DNA concentrations, failure to amplify the *GJB2* coding region or failure when sequencing. This left our total number of participants for *GJB2* analysis at 25 South African patients, 16 South African controls, 180 Cameroonian patients and 60 Cameroonian controls. A summary of the participants' sociodemographic information can be seen in Table 2.

Table 2: Sociodemographics of *GJB2* study participants.

Demographic		South Africa		Cameroon	
		Case (Freq.)	Control (Freq.)	Case (Freq.)	Control (Freq.)
Gender	Male	20 (0,80)	0 (0,00)	94 (0,52)	36 (0,60)
	Female	4 (0,16)	6 (0,38)	84 (0,47)	24 (0,40)
	Unknown	1 (0,04)	10 (0,63)	2 (0,01)	0 (0,00)
Age	Average	13,95	47,75	11,81	13,53
	Unknown	4 (0,16)	8 (0,50)	3 (0,02)	0 (0,00)
Age of onset	Prelingual (<2 years)	3 (0,12)	NA	157 (0,87)	NA
	Perilingual (2-4 years)	6 (0,24)	NA	0 (0,00)	NA
	Postlingual (>4 years)	3 (0,12)	NA	14 (0,08)	NA
	Unknown	13 (0,52)	NA	9 (0,05)	NA
Transmission	Familial	5 (0,20)	NA	47 (0,26)	NA
	Sporadic/unknown	20 (0,80)	NA	133 (0,74)	NA
		N=25	N=16	N=180	N=60

Of the 47 familial cases (26,11%) in Cameroon, our analysis includes two cases of two affected siblings, two cases of three affected siblings and one case of four affected siblings. Consanguinity was not reported in the South African cohort but 10 (5,56%) Cameroonian patients were from known consanguineous unions.

The majority of Cameroonian patients (70%) suffered from sensorineural hearing loss, a small number (4,4%) suffered from mixed hearing loss and the type of hearing loss was not determined for the remainder. Hearing loss was not symmetrical for all patients but was always severe. Audiological information was not available for patients recruited from South Africa. The breakdown by level of hearing loss per ear can be seen in Table 3.

Table 3: Breakdown of severity of hearing loss in Cameroonian patients genotyped for *GJB2*. Level of deafness given according to BIAP classification.[97]

Severity of Deafness	Left Ear (Frequency)	Right Ear (Frequency)
Severe 1 (71-80)	6 (0,03)	7 (0,04)
Severe 2 (81-90)	19 (0,11)	20 (0,11)
Profound 1 (91-100)	53 (0,29)	54 (0,30)
Profound 2 (101-110)	49 (0,27)	46 (0,26)
Profound 3 (111-119)	17 (0,09)	22 (0,12)
Total (120)	7 (0,04)	2 (0,01)
Unknown	29 (0,16)	29 (0,16)
	N=180	N=180

5.1.2- Molecular Analysis Of *GJB2*

Sequence variations were confirmed on both the forward and reverse strand. Once spurious readings were eliminated, we were left with a total of 10 variations, both in the 5'UTR and the coding region. The chromatogram results for all the variations are available in Figure 12 and the details of the variations are in Table 4.

Table 4: *GJB2* variations detected in Cameroon and South Africa. Domains: IC = Intracellular domain, EC = extracellular domain, TM = transmembrane domain.

Genomic	Transcript	Protein	Domain	RS number	Pathogenicity
g.3318-41G>A	c.-41G>A	NA	Intron	Novel	Polymorphism
g.3318-34C>T	c.-34C>T	NA	Intron	rs9578260	Polymorphism
g.3318-15C>T	c.-15C>T	NA	5'UTR	rs72561725	Polymorphism
g.3318-6T>A	c.-6T>A	NA	5'UTR	rs148136545	Polymorphism
g.3332G>A	c.15G>A	p.=	IC1	Novel	Polymorphism
g.3503C>T	c.186C>T	p.=	EC1	Reported	Polymorphism
g.3542G>T	c.225G>T	p.=	EC1	rs149137695	Polymorphism
g.3741_3743delTTC	c.424_426delTTC	p.(F142del)	TM3	Reported	**Pathogenic**
g.3774G>A	c.457G>A	p.(V153I)	TM3	rs111033186	Polymorphism
g.3816G>A	c.499G>A	p.(V167M)	EC2	rs111033360	**Possibly pathogenic**

Sequencing revealed two pathogenic or probably pathogenic mutations in Cameroon, g.3741_3743delTTC (p.(F142del)) and g.3816G>A (p.(V167M)). Each was detected in only a single individual and both in the heterozygous state. There were no pathogenic mutations detected in South African patients.

A number of variations in the *GJB2* sequence in both Cameroonian and South African patients were also uncovered. We detected two novel variations, g.3318-41G>A and g.3332G>A, both in the heterozygous form. g.3318-41G>A occurs in the first intron of *GJB2* and g.3332G>A is a synonymous variation. The most common variants in both South African and Cameroonian patients were the intronic change g.3318-34C>T and two changes in the 5'UTR, g.3318-15C>T and g.3318-6T>A.

There were no statistically significant differences between genotype (Table 5) or allele (Table 6) frequencies in cases and controls among both South Africans and Cameroonians.

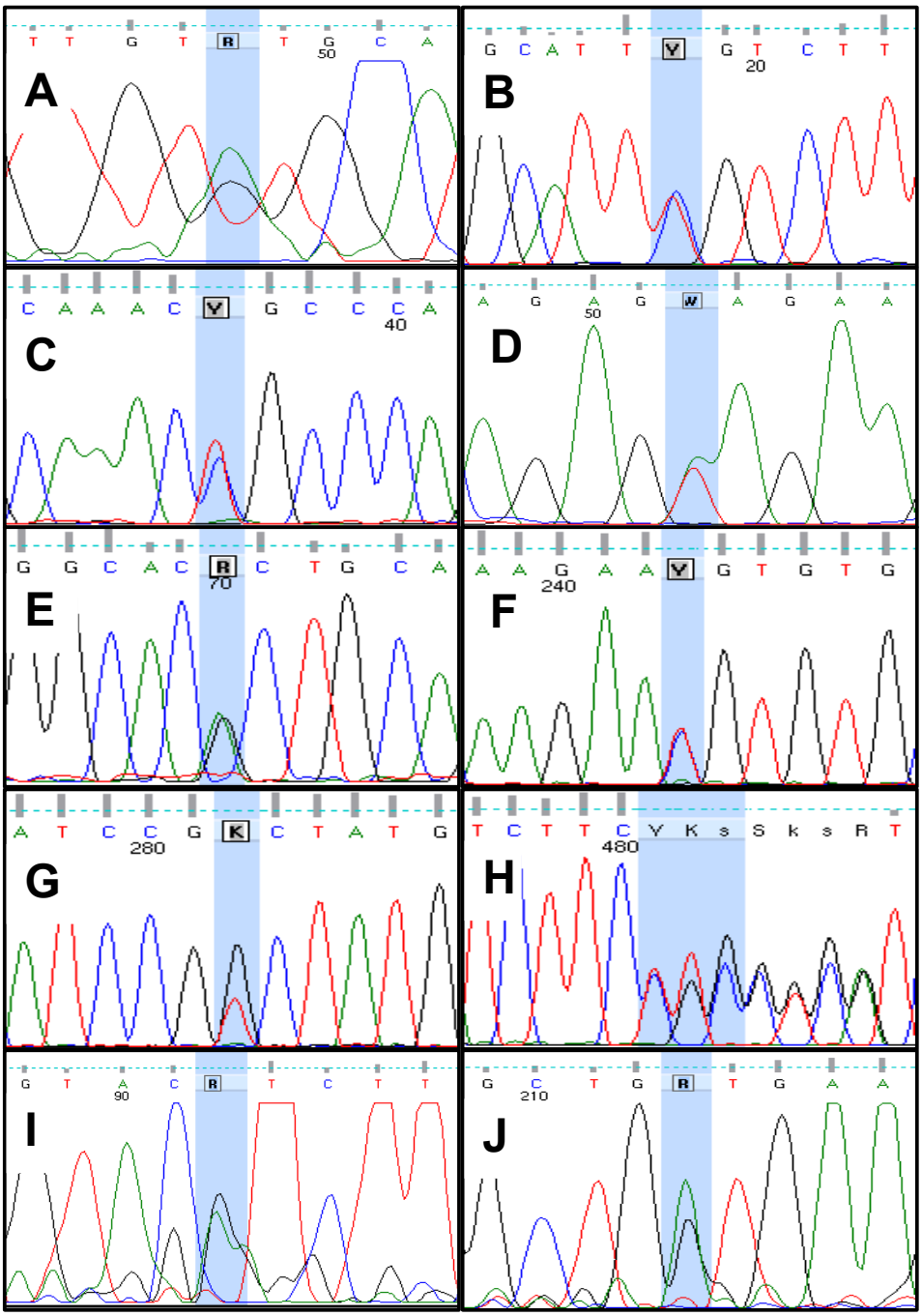

Figure 12: *GJB2* variations detected in Cameroon and South Africa. A = g.3318-41G>A (Novel), B= g.3318-34C>T, C= g.3318-15C>T, D= g.3318-6T>A, E = g.3332G>A (Novel), F = g.3503C>T, G = g.3542G>T, H = g.3741_3743delTTC (Pathogenic), I = g.3774G>A, J = g.3816G>A (Possibly pathogenic).

Table 5: *GJB2* genotype frequencies in the South African and Cameroonian cohorts. Freq. = frequency.

Variant	South Africa							Cameroon						
	Cases			Controls				Cases			Controls			
	Hom. MT (Freq.)	Het. (Freq.)	Hom. WT (Freq.)	Hom. MT (Freq.)	Het. (Freq.)	Hom. WT (Freq.)	P-Value	Hom. MT (Freq.)	Het. (Freq.)	Hom. WT (Freq.)	Hom. MT (Freq.)	Het. (Freq.)	Hom. WT (Freq.)	P-Value
g.3318-41G>A	0	0	25 (1,000)	0	0	16 (1,000)	NA	0	1 (0,006)	179 (0,994)	0	0	60 (1,000)	0,563
g.3318-34C>T	4 (0,160)	11 (0,440)	10 (0,400)	1 (0,062)	5 (0,312)	10 (0,625)	0,336	15 (0,083)	70 (0,389)	95 (0,528)	6 (0,100)	28 (0,467)	26 (0,433)	0,448
g.3318-15C>T	0	7 (0,280)	18 (0,720)	0	1 (0,062)	15 (0,938)	0,087	0	15 (0,083)	165 (0,917)	1 (0,017)	5 (0,083)	54 (0,900)	0,222
g.3318-6T>A	0	1 (0,040)	24 (0,960)	0	0	16 (1,000)	0,418	0	4 (0,022)	176 (0,978)	0	1 (0,017)	59 (0,983)	0,794
g.3332G>A	0	0	25 (1,000)	0	0	16 (1,000)	NA	0	2 (0,011)	178 (0,989)	0	0	60 (1,000)	0,412
g.3503C>T	0	0	25 (1,000)	0	0	16 (1,000)	NA	0	1 (0,006)	179 (0,994)	0	0	60 (1,000)	0,563
g.3542G>T	0	0	25 (1,000)	0	0	16 (1,000)	NA	0	0 (0,000)	180 (1,000)	0	1 (0,017)	59 (0,983)	0,083
g.3741_3743 delTTC	0	0	25 (1,000)	0	0	16 (1,000)	NA	0	1 (0,006)	179 (0,994)	0	0	60 (1,000)	0,563
g.3774G>A	0	0	25 (1,000)	0	1 (0,062)	15 (0,938)	0,206	0	0	180 (1,000)	0	0	60 (1,000)	NA
g.3816G>A	0	0	25 (1,000)	0	0	16 (1,000)	NA	0	1 (0,006)	179 (0,994)	0	0	60 (1,000)	0,563

Table 6: *GJB2* minor allele frequencies in South African and Cameroonian cohorts. Freq. = frequency.

Variant	Minor Allele	South Africa			Cameroon		
		Cases (Freq.)	Controls (Freq.)	P-value	Cases (Freq.)	Control (Freq.)	P-value
g.3318-41G>A	A	0	0	NA	1 (0,003)	0	0,563
g.3318-34C>T	T	32 (0,364)	7 (0,219)	0,134	100 (0,278)	40 (0,333)	0,246
g.3318-15C>T	T	13 (0,148)	1 (0,031)	0,079	15 (0,042)	7 (0,058)	0,450
g.3318-6T>A	A	1 (0,011)	0	0,545	4 (0,011)	1 (0,008)	0,795
g.3332G>A	A	0	0	NA	2 (0,006)	0	0,413
g.3503C>T	T	0	0	NA	1 (0,003)	0	0,563
g.3542G>T	T	0	0	NA	0	1 (0,008)	0,083
g.3741_3743delTTC	delTTC	0	0	NA	1 (0,003)	0	0,563
g.3774G>A	A	0	1 (0,031)	0,096	0	0	NA
g.3816G>A	A	0	0	NA	1 (0,003)	0	0,563

There was no statistically significant difference between haplotypes in the Cameroonian cohort (Table 7) but there was a statistically significant difference in the South African cohort (P = 0,03) (Table 8).

Table 7: Haplotype frequencies between cases and controls in the Cameroonian Cohort. Freq. = frequency.

Haplotype	Case (Freq.)	Control (Freq.)	P-Value
G C C A G C G 1 G	1,79 (0,005)	1,00 (0,008)	0,515
G C C T G C G 1 G	242,67 (0,674)	71,02 (0,592)	0,101
G C C T G C T 1 G	0	1,00 (0,008)	0,083
G C T T G C G 1 G	12,63 (0,035)	6,98 (0,058)	0,268
G T C T G C G 1 G	93,24 (0,259)	39,98 (0,333)	0,116
G T T T G C G 1 G	1,46 (0,004)	0,02 (0,000)	0,877
A C C T G C G 1 G	1,00 (0,003)	0	0,563
G C C T G T G 1 G	1,00 (0,003)	0	0,563
G C T A G C G 1 G	0,91 (0,003)	0	0,582
G T C A G C G 1 G	1,30 (0,004)	0	0,510
G T C T A C G 1 G	2,00 (0,006)	0	0,413
G T C T G C G 1 A	1,00 (0,003)	0	0,563
G T C T G C G 2 G	1,00 (0,003)	0	0,563
Global			0,594

Haplotypes were constructed from the following SNPs: g.3318-41G>A, g.3318-34C>T, g.3318-15C>T, g.3318-6T>A, g.3332G>A, g.3503C>T, g.3542G>T, g.3741_3743delTTC (1 = WT, 2 = deletion) and g.3816G>A.

Table 8: Haplotype frequencies between cases and controls in the South African cohort.

Haplotype	Case (Frequency)	Control (Frequency)	P-Value
C C T A	0	1,00 (0,031)	0,209
C C T G	24,00 (0,480)	24,00 (0,750)	**0,016**
T C T G	19,00 (0,380)	6,00 (0,188)	0,065
T T T G	0	1,00 (0,031)	0,297
C T A G	1,00 (0,020)	0	0,421
C T T G	6,00 (0,120)	0	**0,042**
Global			**0,030**

Haplotypes were constructed from the following SNPs: g.3318-34C>T, g.3318-15C>T, g.3318-6T>A and g.3774G>A. Values that are statistically significant (P < 0,05) are in bold.

Linkage analysis results showed a low rate of recombination between markers but also that they were uninformative of one another. This is can be explained by the low frequency of most variants and the fact that they all appear within 600 bp of each other.

5.1.3- Phylogeny Construction And Principal Components Analysis (PCA) Using Variation In *GJB2*

Phylogenetic comparison of our South African and Cameroonian data and the 1000 Genomes populations showed samples clustering into three clades, representing Asians, Africans and Europeans with the admixed populations positioned closer to the base of the tree.

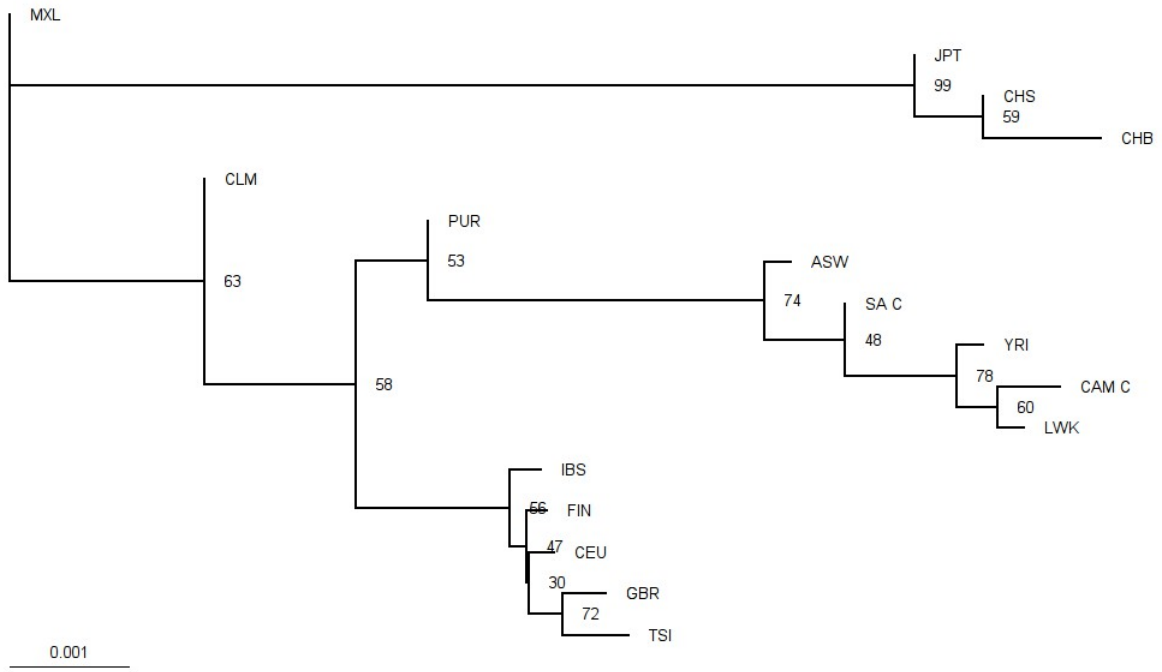

Figure 13: Phylogeny constructed from *GJB2* SNP data. The three clades (from top to bottom: Asian, African European) are clearly visible. Numbers indicate bootstrap values over 1000 iterations. Population abbreviations are: ASW, Americans of African Ancestry in SW USA; CEU, Utah Residents (CEPH); CHB, Han Chinese in Beijing; CHS, Southern Han Chinese; CLM, Colombians from Medellin Colombia; FIN, Finnish in Finland; GBR, British in England and Scotland; IBS, Iberian population in Spain; JPT, Japanese in Tokyo Japan; LWK, Luhya in Webuye Kenya; MXL, Mexican Ancestry from Los Angeles USA; PUR, Puerto Ricans from Puerto Rico; TSI, Toscani in Italia; YRI, Yoruba in Ibadan Nigeria; SA_C, South African control; CAM_C, Cameroonian control.

The first plane of the *GJB2* PCA (Figure 14) contained only 40% of the variation between population groups but does show that different populations are characterised by different SNPs. African groups clustered in the bottom left section of the plot while Asians grouped towards the right. There was overlap between the European populations and admixed populations which occurred at the centre and to the top left of the plot. A variable factor map (Figure 15) was also generated, showing all the SNPs that were considered in the PCA and the effect they had on the position in the PCA of the populations that contained them.

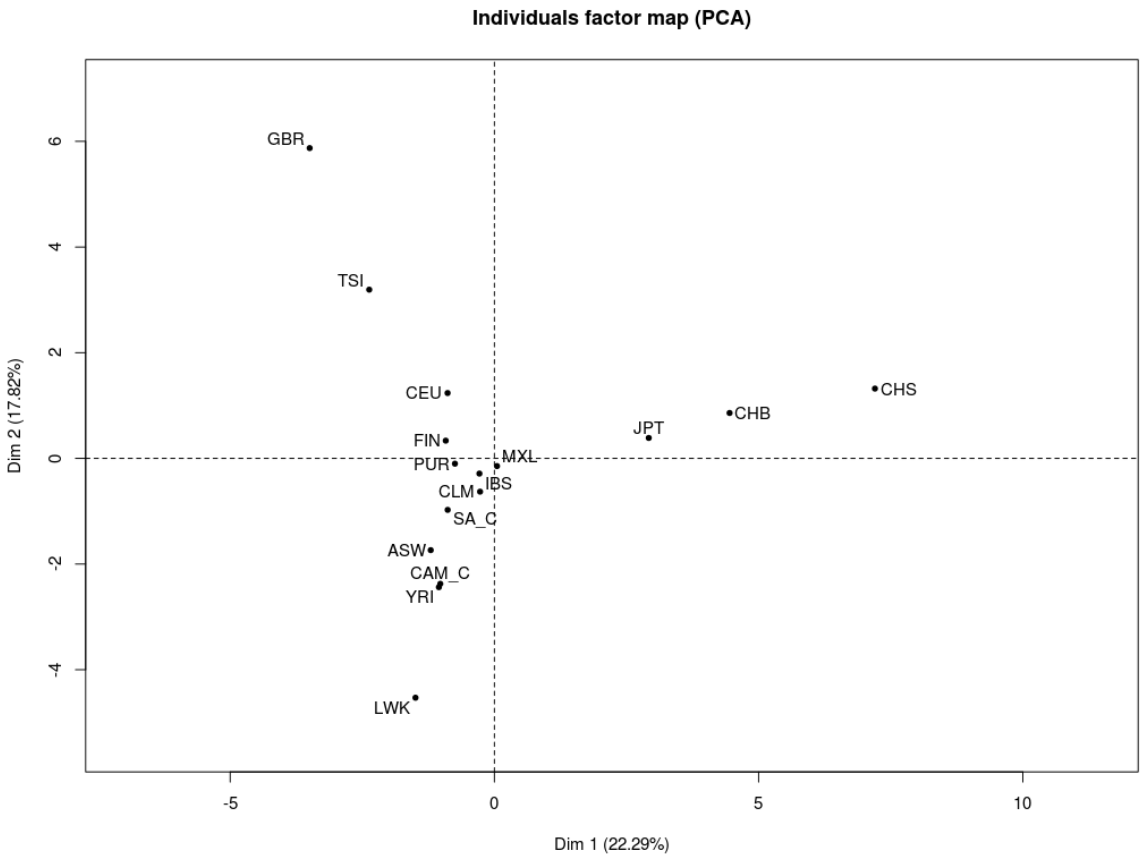

Figure 14: PCA of study and 1000 Genomes Project populations with respect to variation in *GJB2*. Population abbreviations are: ASW, Americans of African Ancestry in SW USA; CEU, Utah Residents (CEPH); CHB, Han Chinese in Beijing; CHS, Southern Han Chinese; CLM, Colombians from Medellin Colombia; FIN, Finnish in Finland; GBR, British in England and Scotland; IBS, Iberian population in Spain; JPT, Japanese in Tokyo Japan; LWK, Luhya in Webuye Kenya; MXL, Mexican Ancestry from Los Angeles USA; PUR, Puerto Ricans from Puerto Rico; TSI, Toscani in Italia; YRI, Yoruba in Ibadan Nigeria; SA_C, South African control; CAM_C, Cameroonian control.

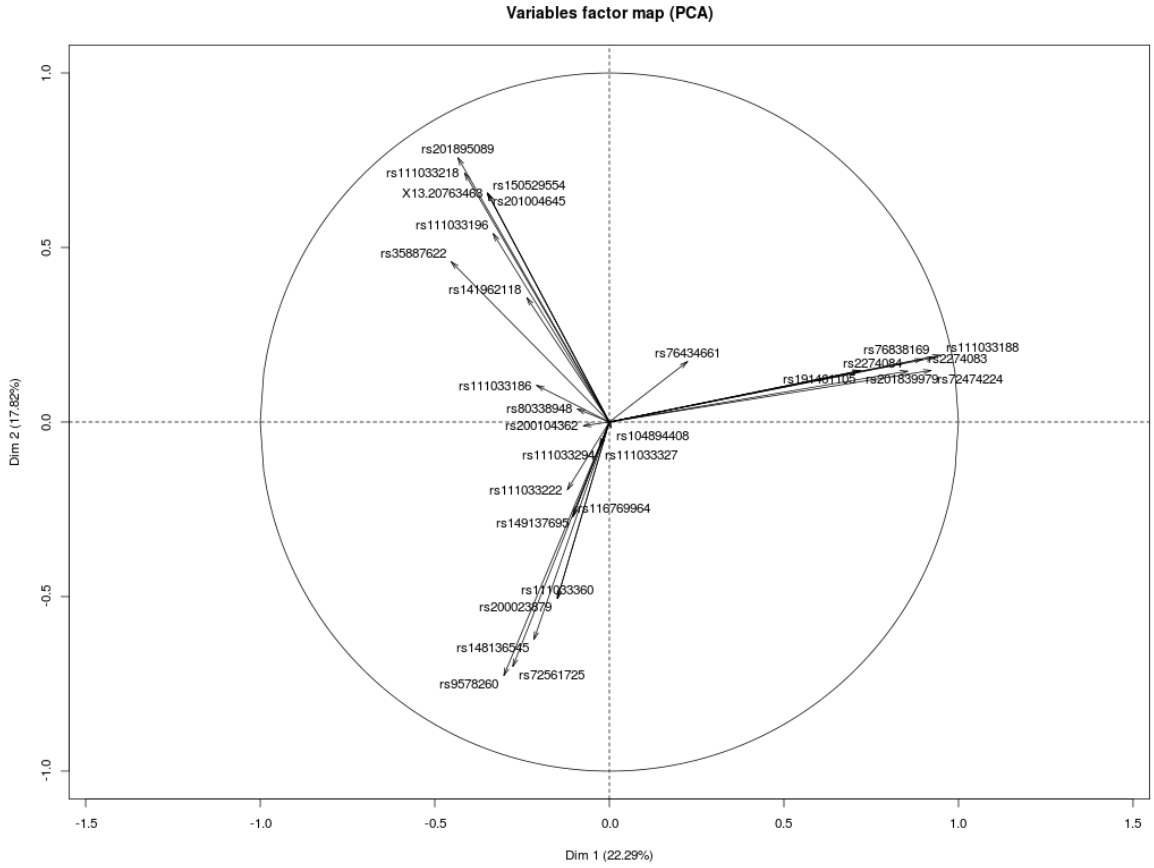

Figure 15: Contribution of SNPs to the *GJB2* PCA. This plot shows how possession of a specific SNP, shown here by its rs number, influenced the position of a population on the main PCA plot.

5.2- KID Syndrome In Two Cameroonian Patients

5.2.1- Clinical Presentation

Patient 1:

A five-year-old girl that presented with a profound, bilateral, sensorineural deafness diagnosed at two years of age. She was born at term to unrelated, healthy parents after an uneventful pregnancy and normal vaginal delivery. She presented at birth with generalized erythema and had a history of chronic otitis externa and hypohidrosis. Her psychomotor development was normal, however physical examination revealed a generalized, thickened skin and xeroderma, palmoplantar keratoderma and rippled hyperkeratotic plaques on the knees and elbows . She had an aged facial appearance, hypotrichosis (sparse eyelashes and eyebrows), and hyperkeratotic lesions in the external auditory canal. Ophthalmologic examination revealed a mild vascularizing keratitis which explained her photophobia and reduced visual acuity. Oral examination showed dental dysplasia and histopathological examination of the skin revealed an acanthotic dyskeratosis. There was no family history of a similar condition.

Patient 2:

A two-year-old girl that presented with a prelingual, bilateral, profound, sensorineural deafness. She was born at term after an uncomplicated pregnancy and delivery. Since two months of age, she presented with thick, reddened patches of the skin that were dry and scaly. The thickness of the skin gradually increased as she grew older. At the time of presentation at the health facility, physical examination revealed generalized ichthyosiform erythrokeratoderma, palmoplantar keratoderma, alopecia universalis and atrichosis (absence of eyelashes and eyebrows). Joint mobility of the elbows, knees and ankles was significantly reduced by keratoderma. She had photophobia and ocular irritation and an ophthalmologic examination revealed a vascularizing keratitis. The intraoral examination was normal. She had no major neurological abnormalities and her non-consanguineous

parents and young sister were all healthy and there was no family history of similar clinical presentation.

The two patients were unrelated with their parents originating from, and living in, two geographically distinct of area of Cameroon (Western and Centre provinces, about 400 km apart). Furthermore, the parents belonged to two different ethno-linguistic groups, the Bantu and Nilo-Sahelian respectively.

5.2.2- Molecular Characterisation Of KID Patients

Sequencing of the entire coding region of *GJB2* in the two KID patients revealed, in both patients, the presence of the c.148G>A (p.(D50N)) change (Figure 16). The same mutation was not present in Cameroonian, non-syndromic deafness individuals or in 60 healthy persons of Cameroonian origin. While patient 1 presented only with this mutation, patient 2 was also heterozygous for the most-common variant, g.3318-34C>T. Even though there is no molecular data on the parents of these two KID patients, the lack of clinical manifestations on their part and the absence of proven familial cases with reduced penetrance suggests the mutations in these two patients are most likely *de novo*.

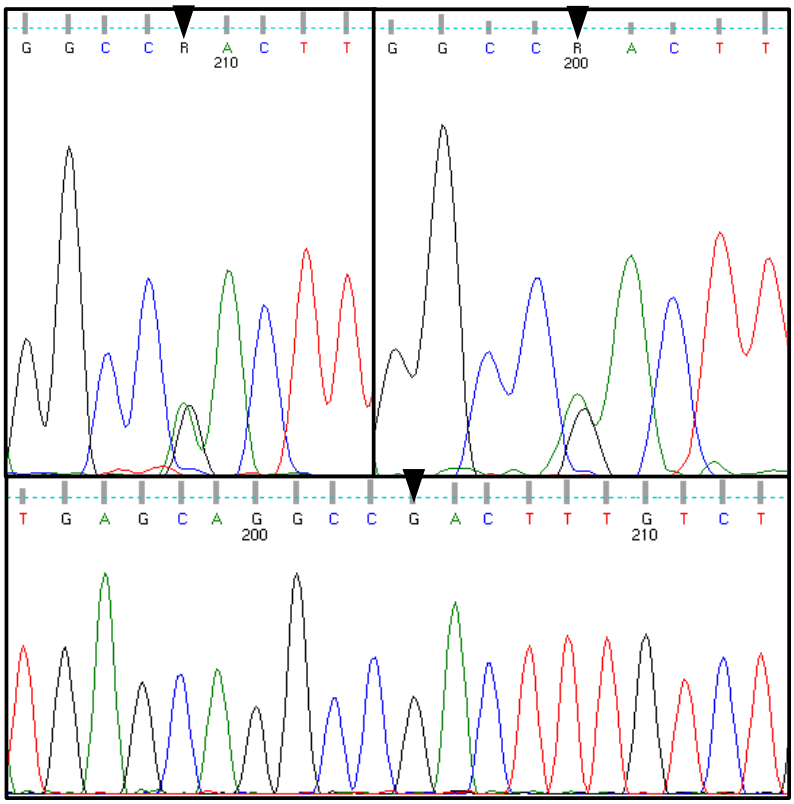

Figure 16: Molecular diagnosis of KID syndrome. Top two panels are from Cameroonian patients with KID syndrome. Bottom panel is the chromatogram from a control.

5.3- GJA1

5.3.1- Patients

For analysis of *GJA1*, where the evidence for a link between the gene and hearing loss is weak, we chose a subset of the patients that we thought would give us the greatest chance of detecting a link if one existed. We chose 100 patients which included six consanguineous Cameroonian patients, 52 familial Cameroonian cases, five familial South African cases, two Cameroonian patients with heterozygous *GJB2* mutations, 15 prelingual Cameroonian cases and the 20 remaining South African cases. All but four Cameroonian patients had previously been genotyped for *GJB2*. The same control samples used for *GJB2* were used for *GJA1*.

Samples were excluded if they failed amplify or sequence, leaving us with a final figure of 67 Cameroonian, 23 South African patients, 16 Cameroonian controls and 17 South African controls. The sociodemographic breakdown of the cohort can be seen in Table 9.

Table 9: Sociodemographics of *GJA1* study participants.

Demographic		South Africa		Cameroon	
		Case (Frequency)	Control (Frequency)	Case (Frequency)	Control (Frequency)
Gender	Male	19 (0,83)	0 (0,00)	35 (0,52)	8 (0,50)
	Female	3 (0,13)	6 (0,38)	31 (0,46)	8 (0,50)
	Unknown	1 (0,04)	10 (0,63)	1 (0,01)	0 (0,00)
Age	Average	14,4	47,75	12,12	14,31
	Unknown	3 (0,13)	8 (0,50)	2 (0,03)	0 (0,00)
Age of Onset	Prelingual (<2 Years)	2 (0,09)	NA	63 (0,94)	NA
	Perilingual (2-4 Years)	5 (0,22)	NA	0 (0,00)	NA
	Postlingual (>4 years)	3 (0,13)	NA	3 (0,04)	NA
	Unknown	13 (0,57)	NA	1 (0,01)	NA
Transmission	Familial	5 (0,22)	NA	47 (0,70)	NA
	Unknown	18 (0,78)	NA	20 (0,30)	NA
		N=23	N=17	N=67	N=16

While there were no reported cases of consanguinity in the South African cohort, nine (13,43%) patients in the Cameroonian cohort were the result of consanguineous unions. There were 57 (85,07%) patients with sensorineural, one (1,49%) with mixed and nine (13,43%) with undetermined hearing loss. The degree of hearing loss is shown in in Table 10.

Table 10: Breakdown of severity of hearing loss in Cameroonian patients examined for *GJA1*. Level of deafness given according to BIAP classification.

Severity of deafness	Left Ear (Frequency)	Right Ear (Frequency)
Severe 1 (71-80)	1 (0,01)	2 (0,03)
Severe 2 (81-90)	0 (0,00)	3 (0,04)
Profound 1 (91-100)	17 (0,25)	14 (0,21)
Profound 2 (101-110)	16 (0,24)	16 (0,24)
Profound 3 (111-119)	6 (0,09)	7 (0,10)
Total (120)	3 (0,04)	1 (0,01)
Unknown	19 (0,28)	18 (0,27)
	N=67	**N=67**

5.3.2- Molecular Analysis Of *GJA1*

Genotyping for *GJA1* by sequencing resulted in the detection of five sequence variants (Described in Table 11 and examples visible in Figure 17), none of which were pathogenic. Of the five, four are known SNPs and one, g.11522T>C, is novel. Only g.11873G>A was detected in more than one individual. The majority of the variations were detected in the South African cohort with Cameroonians only presenting with g.11873G>A and g.11090A>G.

Table 11: *GJA1* variations detected in Cameroon and South Africa. Domains: EC = extracellular domain, IC = intracellular domain.

Genomic	Transcript	Protein	Domain	RS number	Pathogenicity
g.11090A>G	c.-67A>G	NA	Intron	rs189167598	Polymorphism
g.11345T>C	c.189T>C	p.=	EC1	rs139688042	Polymorphism
g.11522T>C	c.366T>C	p.=	IC2	Novel	Polymorphism
g.11873G>A	c.717G>A	p.=	IC3	rs57946868	Polymorphism
g.11914C>T	c.758C>T	p.(A253V)	IC3	rs17653265	Polymorphism

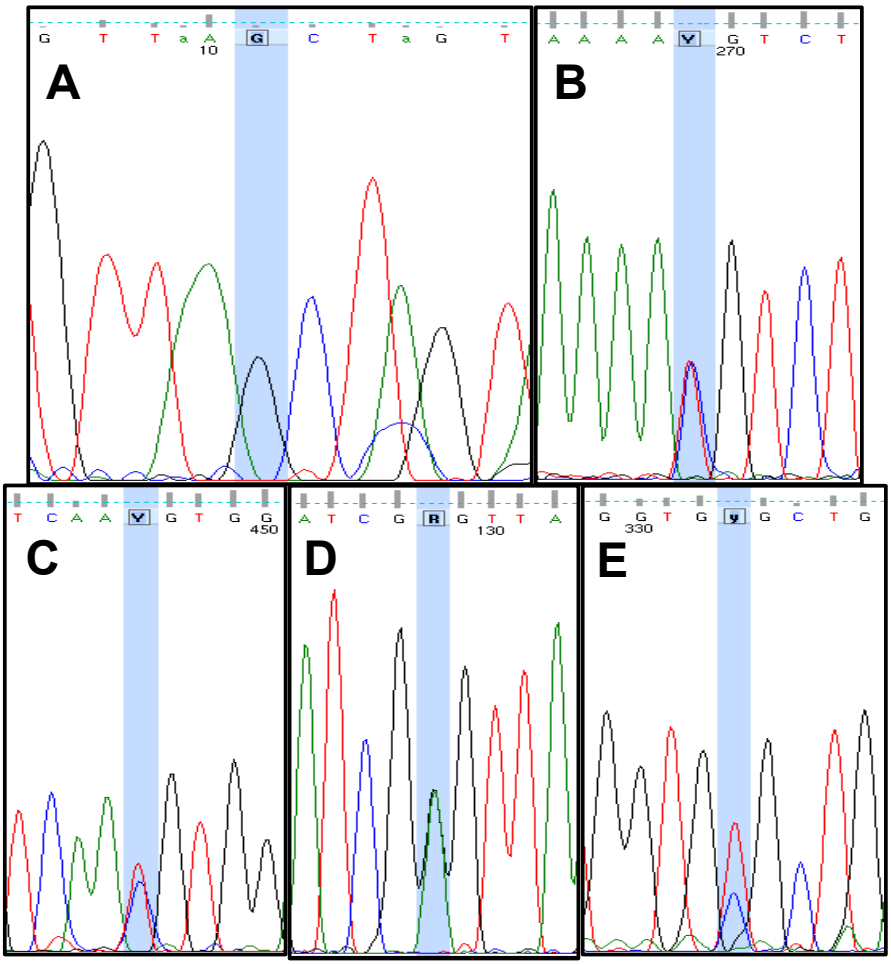

Figure 17: Chromatogram of *GJA1* variations. A = g.11090A>G, B = g.11345T>C, C = g.11522T>C, D = g.11873G>A, E = g.11914C>T

Comparison of allele, genotype and haplotype frequencies showed no statistically significant differences in either the Cameroonian or South African cohort (Tables 12, 13, 14 and 15). Just as with *GJB2,* there was very little information for linkage disequilibrium. In both populations the D` values were essentially 1 but the r^2 values were almost 0.

Table 12: *GJA1* genotype frequencies in South African and Cameroonian cohorts. Freq. = frequency.

	South Africa							Cameroon						
	Case			Control				Case			Control			
Variant	**Hom. MT (Freq.)**	**Het. (Freq.)**	**Hom. WT (Freq.)**	**Hom. MT (Freq.)**	**Het. (Freq.)**	**Hom. WT (Freq.)**	**P-Value**	**Hom. MT (Freq.)**	**Het. (Freq.)**	**Hom. WT (Freq.)**	**Hom. MT (Freq.)**	**Het. (Freq.)**	**Hom. WT (Freq.)**	**P-Value**
g.11090A>G	0	0	23 (1,000)	0	0	17 (1,000)	NA	1 (0,015)	0	66 (0,985)	0	0	16 (1,000)	0,623
g.11345T>C	0	1 (0,043)	22 (0,957)	0	0	17 (1,000)	0,384	0	0	67 (1,000)	0	0	16 (1,000)	NA
g.11522T>C	0	1 (0,043)	22 (0,957)	0	0	17 (1,000)	0,384	0	0	67 (1,000)	0	0	16 (1,000)	NA
g.11873G>A	0	2 (0,087)	21 (0,913)	0	3 (0,176)	14 (0,824)	0,397	0	11 (0,164)	56 (0,836)	0	1 (0,062)	15 (0,938)	0,299
g.11914C>T	0	1 (0,043)	22 (0,957)	0	0	17 (1,000)	0,384	0	0	67 (1,000)	0	0	16 (1,000)	NA

Table 13: *GJA1* minor allele frequencies in South African and Cameroonian cohorts. Freq. = frequency.

Variant	Minor allele	South Africa			Cameroon		
		Case (Freq.)	Control (Freq.)	P-value	Case (Freq.)	Control (Freq.)	P-value
g.11090A>G	G	0	0	NA	2 (0,015)	0	0,487
g.11345T>C	C	1 (0,022)	0	0,387	0	0	NA
g.11522T>C	C	1 (0,022)	0	0,387	0	0	NA
g.11873G>A	A	2 (0,043)	3 (0,088)	0,414	11 (0,082)	1 (0,031)	0,318
g.11914C>T	T	1 (0,022)	0	0,387	0	0	NA

Table 14: Haplotype frequencies in patients and controls from Cameroon.

Haplotype	Case (Frequency)	Control (Frequency)	P-Value
A A	11,00 (0,082)	1,00 (0,031)	0,318
G A	121,00 (0,903)	31,00 (0,969)	0,229
G G	2,00 (0,015)	0	0,487
Global			0,467

Haplotypes were constructed from the following SNPs: g.11090A>G and g.11873G>A.

Table 15: Haplotype frequencies in patients and controls from South Africa.

Haplotype	Case (Frequency)	Control (Frequency)	P-Value
A T T C	2,00 (0,043)	3,00 (0,088)	0,414
G T T C	41,00 (0,891)	31,00 (0,912)	0,763
G C T C	1,00 (0,022)	0	0,387
G T C C	1,00 (0,022)	0	0,387
G T T T	1,00 (0,022)	0	0,387
Global			0,583

Haplotypes were constructed from the following SNPs: g.11345T>C, g.11522T>C, g.11873G>A and g.11914C>T.

5.3.3- Phylogeny Construction And Principal Components Analysis (PCA) Using Variation In *GJA1*

The phylogeny created from *GJA1* (Figure 18) does not show the same structure or grouping as that of *GJB2*. Only the African populations form one of the expected clusters, although with much lower bootstrap values than in the *GJB2* phylogeny. There is a predominantly Asian cluster but it also includes the admixed Puerto Rican population as well as the Finnish population.

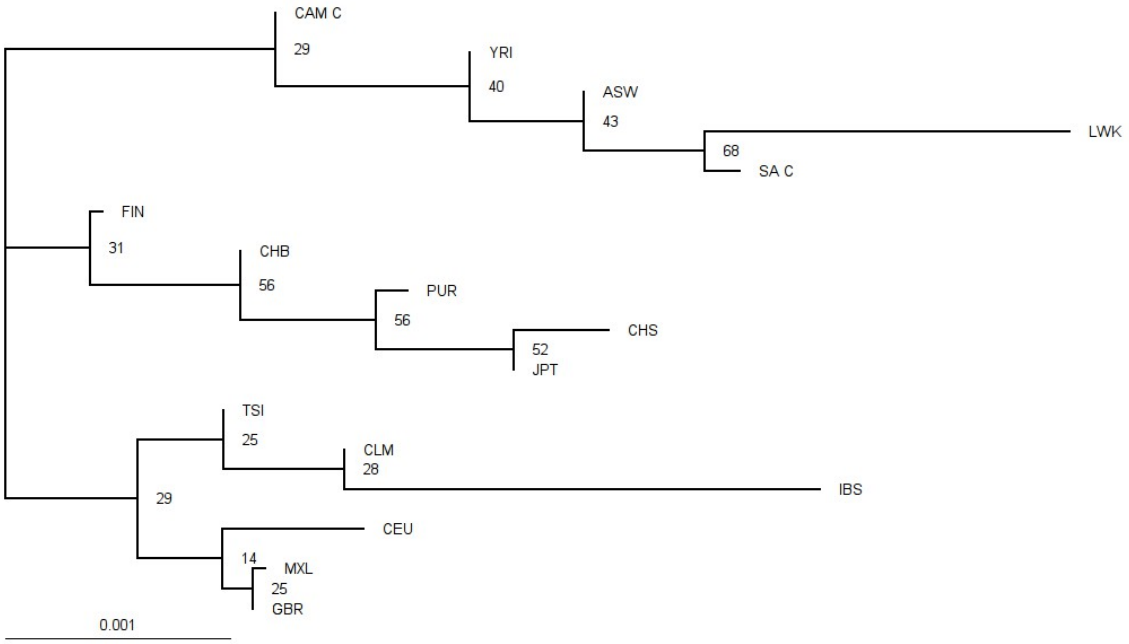

Figure 18: Phylogeny constructed with *GJA1* SNP data. Numbers indicate bootstrap values over 1000 iterations. Population abbreviations are:ASW, Americans of African Ancestry in SW USA; CEU, Utah Residents (CEPH); CHB, Han Chinese in Beijing; CHS, Southern Han Chinese; CLM, Colombians from Medellin Colombia; FIN, Finnish in Finland; GBR, British in England and Scotland; IBS, Iberian population in Spain; JPT, Japanese in Tokyo Japan; LWK, Luhya in Webuye Kenya; MXL, Mexican Ancestry from Los Angeles USA; PUR, Puerto Ricans from Puerto Rico; TSI, Toscani in Italia; YRI, Yoruba in Ibadan Nigeria; SA_C, South African control; CAM_C, Cameroonian control.

Principal Components Analysis (PCA), Figure 19, divided the samples in a manner that explained 56% of the variation between populations. It was also more informative than the phylogeny in distinguishing groups. The African populations form an almost straight line of increasing prevalence of the g.11873G>A variant. The Asian populations form a single group at the bottom of the PCA with no other populations in their region. The European and admixed populations are not easily distinguishable, similar to what was observed in the *GJB2* PCA.

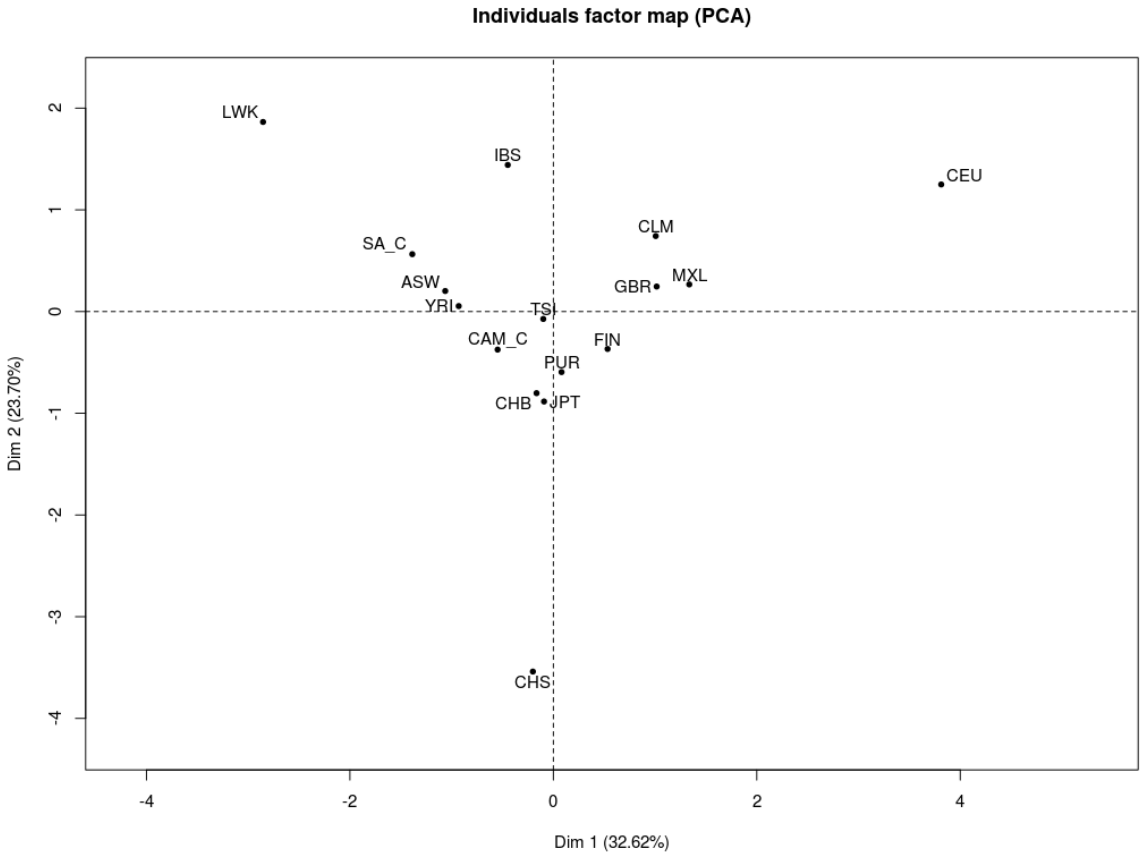

Figure 19: PCA of study and 1000 Genomes Project populations with respect to variation in *GJA1*. Population abbreviations are:ASW, Americans of African Ancestry in SW USA; CEU, Utah Residents (CEPH); CHB, Han Chinese in Beijing; CHS, Southern Han Chinese; CLM, Colombians from Medellin Colombia; FIN, Finnish in Finland; GBR, British in England and Scotland; IBS, Iberian population in Spain; JPT, Japanese in Tokyo Japan; LWK, Luhya in Webuye Kenya; MXL, Mexican Ancestry from Los Angeles USA; PUR, Puerto Ricans from Puerto Rico; TSI, Toscani in Italia; YRI, Yoruba in Ibadan Nigeria; SA_C, South African control; CAM_C, Cameroonian control.

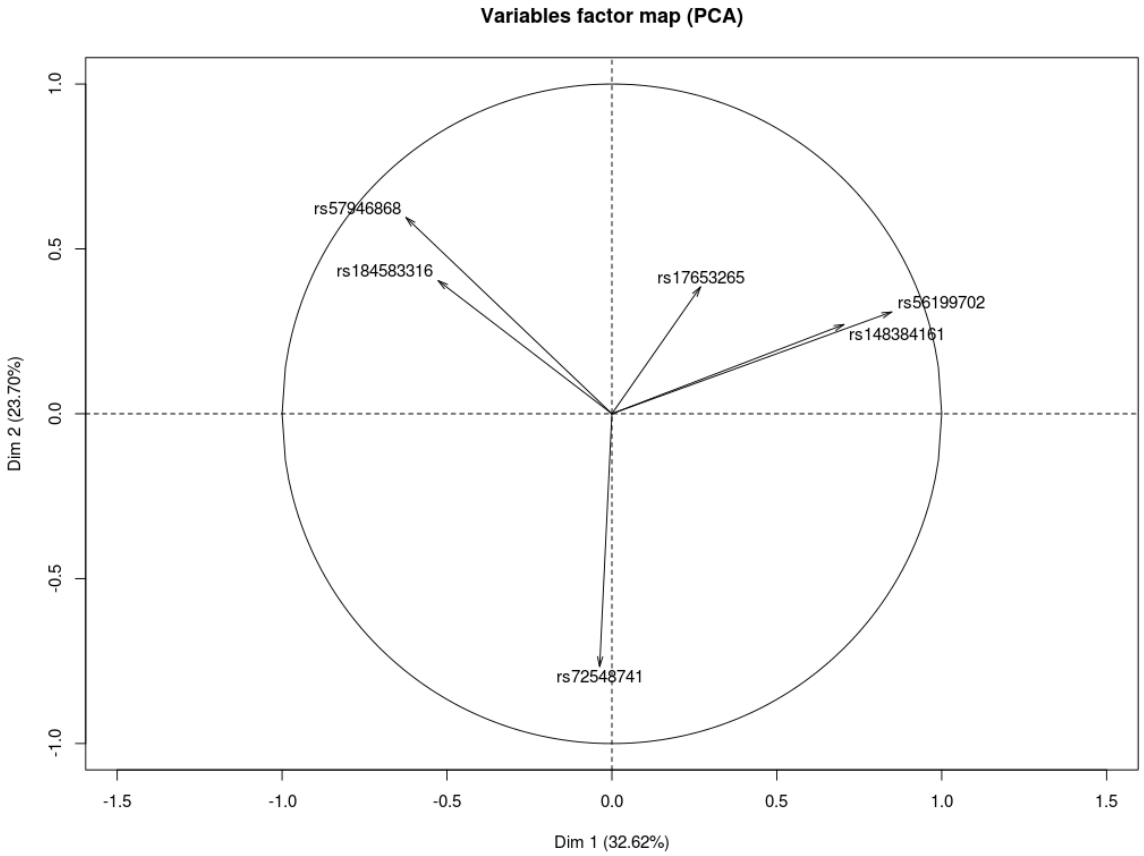

Figure 20: Contribution of SNPs to the *GJA1* PCA. This plot shows how possession of a specific SNP, shown here by its rs number, influenced the position of a population on the main PCA plot.

6- Discussion

6.1- Strengths Of The Study

This comprehensive report on the significance of connexin mutations in deafness in Africans offers a substantial contribution to the literature on the topic. Our inclusion of Cameroonian patients is of major importance as Cameroon is frequently referred to as "Africa in miniature," because of its central location on the continent, its many geographical and cultural attributes and the diversity of it's population (There are more than 200 distinct local languages in the country). The country spans two main geographical zones of almost equal size: the equatorial rain forest in the south and the tropical savannah and the Sahel region in the north. At the genetic level, Cameroonian population diversity mimics that of various ethno-linguistic groups in African populations[88] and it is anticipated that results from a carefully selected sample in this population could represent a snapshot of many African populations. Furthermore, we include patients from the Xhosa population of South Africa, a formerly unstudied population but an offshoot of the Bantu population that migrated from areas around Cameroon.

This study reports the first molecular diagnosis of KID Syndrome due to p.(D50N) in *GJB2* in sub-Saharan Africa and conduct the first analysis of *GJA1* in an African population. Data from the 1000 Genomes Project is used to derive meaning from our findings. The information presented here builds on previously published studies on connexins and their effect on deafness in African patients and contributing novel findings that further enrich the literature and influence clinical practice on the continent in terms of suitable genetic testing.

6.2- GJB2

GJB2 mutations have been shown to explain a large proportion of deafness in many different population groups.[45,47,54] With only two mutations detected, both in heterozygotes, mutations in *GJB2* were unable to explain deafness for any of the African patients. This is

similar to other results from African studies that have also reported low frequencies of deafness-associated mutations in *GJB2* (Table 16).

Both mutations detected here have been described previously. The p.(F142del) (g.3741_3743delTTC) mutation has been detected and associated with deafness twice before, in the heterozygous state in an Egyptian patient suffering from moderate hearing loss[98] and in four Chinese patients with non-syndromic hearing loss.[99] p.(V167M) (g.3816G>A) has been reported in three studies and is possibly exclusive to patients of African ancestry. It has been detected in a heterozygous state in 4/406 (~1%) Kenyans with prelingual, non-syndromic hearing loss,[12] 1/94 (~1%) African American controls[55] and in 1/19 (~5%) patients from a predominantly African American cohort of patients with congenital CMV infection and hearing loss, although the exact ethnicity of the individual with the variant is not mentioned.[100]

Gasmelseed et al.[12] proposed a possible dominant mode of inheritance for the p.(V167M) mutation but that does not fit with the results that have since become available, particularly its detection in unaffected controls, which supports a recessive mode of inheritance. While it may be pathogenic, it has not been reported in a homozygous form and predictive tools give ambiguous results. Polyphen-2[91] predicts it as "possibly damaging with a score of 0,618 (sensitivity: 0,87; specificity: 0,91)" with HumDiv and "possibly damaging with a score of 0,537 (sensitivity: 0,82; specificity: 0,82)" with HumVar. The Polyphen-2 score ranges from 0 to 1 and represents the probability that the change is damaging.

Three common variants were detected in our cohort, all of which have previously been reported at high frequencies in African populations. These are g.3318-34C>T, g.3318-15C>T and g.3318-6T>A, which occured in 60%, 28% and 4% of our South African patients respectively and 47,22%, 8,3% and 2,22% of Cameroonian patients respectively. Our results are similar to those of Kabahuma et al. where g.3318-34C>T and g.3318-15C>T were the only variants detected in 182 case and 63 control individuals from the Limpopo province, South Africa.[14] They found the g.3318-34C>T change in 46.2% and

42.6% of patients and controls respectively and the g.3318-15C>T change in 21,4% and 35% of patients and controls respectively. Although also from South Africa, the study did not include members of the Xhosa population. The same variants, including the g.3318-6T>A change, were also among those detected in deaf individuals from both Kenya and Sudan.[12] They found the g.3318-34C>T and g.3318-15C>T changes in 12,73%, 6,45% of patients respectively. The g.3318-6T>A change was only found in 0,49% of Kenyan patients.

These variants appear to be almost exclusive to those of African ancestry, perhaps best illustrated by Tang et al.[101] Looking at Tang et al.'s control data, which is stratified by ethnicity, we can see the g.3318-34C>T, g.3318-15C>T and g.3318-6T>A variants occurring in 27,8%, 2,78% and 2,08% respectively of African Americans but just 3,29%, 1,97% and 0% of Hispanics and none of the three variants occurring in either Asians or Caucasians. Tang et al. also found a lack of polymorphisms in the coding region of *GJB2* among African Americans but a high prevalence of variations in the 3'UTR, an area we did not genotype in this study.

There has been one previous study of *GJB2* and hearing loss in Cameroon whose results are partially supported by this study.[102] Out of 67 deaf and 66 normal hearing controls, two *GJB2* variants, the c.186C>T change (in two patients and one control) and c.296insT change (in one control) were detected. There was no mention of the variants we report here at high frequencies but this discrepancy could be due to differences between the primer sequences used for genotyping. An additional weakness of Trotta et al.'s study could be the sampling of all participants from a single school in the North of the country, whereas participants in our study were recruited from across Cameroon.

PCA offers some SNPs that appear to differentiate our cohorts, and other African populations included in the 1000 Genomes Project, from non-African populations (Figure 15). However, for some of these SNPs, the frequencies are too low, and are limited to just a single population, to provide a reliable way of separating out the populations. The same situation arises when the patient cohorts are included in the analysis as they contain many low frequency, unique SNPs which then appear to distinguish African populations. The three SNPs that we found at a high prevalence (g.3318-34C>T, g.3318-15C>T and g.3318-6T>A) appear to be the most important and reliable way of distinguishing African populations.

Although we see a statistically significant difference in haplotype frequencies between cases and controls in South Africa, this result is likely due to the small sample size of our South African cohort with no other results suggesting a difference between the two groups. None of the variants seen in the South African cohort are known to be pathogenic, making their connection to deafness unlikely. We did not find a difference between cases and controls in South Africa and Cameroon, probably pointing to the lesser role that *GJB2* variations play in deafness in Africans when compared to other populations.

A detailed comparison of our findings and those from other African and African American studies can be seen in Table 16. This table only includes specific data from deaf individuals, although variants detected in controls are also referred to. The table shows that most variations occur at very low frequencies and the major variants are common to South Africa, Cameroon, Kenya and Sudan. The three common variants were not reported in Ghana, possibly because they fell outside of the sequenced region. The p.(R143W) (c.427C>T) change, common in Ghana, has not been detected in other African populations but has been seen in African Americans who could have originated from Ghana.

Our results are in agreement with other African studies, showing a unique African mutation spectrum. Studies have found that *GJB2* mutations are detected in 18% of alleles in Iran,[103] 14,2% in Japan,[104] 37% in Italy and Spain[48] and 22% in the US.[50] In contrast, we

found *GJB2* mutations in less than 1% of Cameroonian and 0% of South African alleles. Similarly to Africans, Caribbean Hispanics do not show a large contribution to deafness from *GJB2* variations.[13] Neither the recessive c.35delG nor c.235delC variants were detected in the 1000 Genomes Project data, despite the c.35delG carrier frequency having been estimated at 2% in European populations and the c.235delC carrier frequency estimated at 1% in East Asian populations.[105] This may be due to the fact that accurate genotyping of small insertions and deletions with next-generation sequencing remains challenging.[106]

The difference between African and non-African populations is not merely limited to the frequency of *GJB2* variations but the identity of the changes themselves. Our results here do not include the mutations that are common in other populations, in agreement with the literature that states that they do not occur in Africa. An exception is Tunisia, on the North coast of Africa, which has a high frequency of the c.35delG change.[57,58,107] This is due to the Tunisian population being predominantly Arab. The three common African SNPs described above and p.(R143W) (c.427C>T) in Ghana are seldom detected outside of Africa.

Table 16: Comparison of results between selected studies of *GJB2* and hearing loss in African populations.

Variation			Country (Observed/Total alleles (%))					
Genomic	**Coding**	**Pathogenicity**	**Cameroon**	**Ghana[59]**	**Kenya/Sudan[12]**	**South Africa**	**Tunisia**	**African American**
g.3318-3201G>A	c.-3201G>A (IVS1+1G>A)	Pathogenic					1/262 (0,4%)[%]	
g.3318-41G>A	c.-41G>A	Polymorphism	1/360 (0,3%)[*]					
g.3318-35T>G	c.-35T>G	Polymorphism			1/1178 (0,1%)			
g.3318-34C>T	c.-34C>T	Polymorphism	100/360 (27,8%)[*]		85/1178 (7,2%)	19/50 (38,0%)[*], 119/364 (32,7%)[@]		
g.3318-15C>T	c.-15C>T	Polymorphism	15/360 (5,8%)[*]		38/1178 (3,2%)	7/50 (14,0%)[*], 56/364 (15,4%)[@]		1/46 (2,2%)[+], NA[+]
g.3318-6T>A	c.-6T>A	Polymorphism	4/360 (1,1%)[*]		2/1178 (0,2%)	1/50 (2,0%)[*]		
g.3332G>A	c.15G>A	Polymorphism	1/360 (0,3%)[*]					
g.3352_3353insG	c.35dupG	**Pathogenic**		1/730 (0,1%)				
g.3352delG	c.35delG	**Pathogenic**			10/1178 (0,8%)		44/204 (21,6%)[$] 45/190 (23,7%)[&] 82/262 (31,3%)[%]	7/100 (7,0%)[~]
g.3395C>T	c.78C>T	Polymorphism			1/1178 (0,1%)			
g.3396C>T	c.79G>A	**Pathogenic**						2/46 (4,3%)[+], NA[+]
g.3419T>C	c.101T>C	**Pathogenic**						NA[+]
g.3426G>A	c.109G>A	**Pathogenic**			1/1178 (0,1%)		1/190 (0,5%)[&] 2/262 (0,8%)[%]	
g.3455_3460del	c.138_143del	**Pathogenic**			1/1178 (0,1%)			
g.3456G>T	c.139G>T	**Pathogenic**					3/190 (1,6%)[&] 8/262 (3,1%)[%]	
g.3503C>T	c.186C>T	Polymorphism	NA[*], 2/122 (1,6%)[#]		3/1178 (0,3%)			
g.3512C>A	c.195C>A	**Pathogenic**			1/1178 (0,1%)			
g.3542G>T	c.225G>T	Polymorphism	1/360 (0,3%)[*]					

Genomic	Coding	Pathogenicity	Cameroon	Ghana	Kenya/Sudan	South Africa	Tunisia	African American
c.3552delC	c.235delC	**Pathogenic**					2/262 (0,8%)[%]	
g.3553T>C	c.236T>C	**Pathogenic**		1/730 (0,1%)				
g.3566C>G	c.249C>G	**Pathogenic**						1/100 (1,0%)[~]
g.3586_3587insT	c.269_270insT	**Pathogenic**	NA[#]					
g.3627A>C	c.310A>C	Polymorphism			1/1178 (0,1%)			
g.3650_3651delAA	c.333_334delAA	**Pathogenic**					2/190 (1,1%)[&]	
g.3658A>G	c.341A>G	**Pathogenic**						NA[+]
g.3697G>A	c.380G>A	**Pathogenic**			1/1178 (0,1%)			
g.3706G>C	c.389G>C	**Pathogenic**					1/262 (0,4%)[%]	
g.3741_3743delTTC	c.424_426delTTC	**Pathogenic**	1/360 (0,3%)[*]					
g.3744C>T	c.427C>T	**Pathogenic**		110/730 (15,1%)				1/100 (1,0%)[~]
g.3774G>A	c.457G>A	Polymorphism			2/1178 (0,2%)	NA[*]		
g.3795G>A	c.478G>A	**Pathogenic**			1/1178 (0,1%)			NA[+]
g.3816G>A	c.499G>A	**Pathogenic**	1/360 (0,3%)[*]		4/1178 (0,3%)			NA[+]
g.3850T>C	c.533T>C	**Pathogenic**		4/730 (0,5%)				
g.3868G>C	c.551G>C	**Pathogenic**					1/190 (0,5%)[&]	
g.3868G>A	c.551G>A	**Pathogenic**		1/730 (0,1%)				
g.3906G>T	c.589G>T	**Pathogenic**		1/730 (0,1%)				
g.3925-3926delinsAA	c.608_610delinsAA	**Pathogenic**		2/730 (0,3%)				
g.3958C>T	c.641T>C	**Pathogenic**		1/730 (0,1%)				

Variant information was obtained through the relevant paper's own results and a combination of Deafness Variation Database[108] and The Connexin-Deafness Homepage.[109] NA refers to variations that were found during the study but only in the control group. *This study, [#]Trotta et al.,[102] [@]Kabahuma et al.,[14] [$]Belguith et al.,[107] [&]Trabelsi et al.,[57] [%]Riahi et al.[58], [+]Sammanich et al.,[55] [~]Pandya et al.[50]

6.3- KID Syndrome

While KID syndrome has been diagnosed in African patients before,[110] this is the first observation of the p.(D50N) mutation in sub-Saharan Africa. Both patients are unrelated and have no family history of skin conditions. The parents did not provide their own DNA for comparison so we can not say for certain whether this is a *de novo* mutation or not, although it seems likely.

Our confirmation of the p.(D50N) mutation in Cameroonians, having previously been observed in patients of European ancestry,[82,83] from Egypt/Middle East,[111] South Korea[112] and Algeria,[84] confirms that p.(D50N) is not linked to ethnicity. In contrast, *GJB2* mutations causing non-syndromic hearing loss have been reported in many parts of the world and have been observed to have marked variations in their frequencies among different ethnicities. This is due to the major mutations in various groups being due to population-specific founder effects, as shown for c.35delG in Caucasians,[9] c.167delT in Ashkenazi Jews[10] and c.235delC in East Asians.[11]

6.4- *GJA1*

Of the five variants detected in *GJA1,* only one is a non-synonymous change and none are known to be pathogenic. This means that these results do not allow us to assign a cause of deafness to any of the patients. This result is not entirely unexpected and confirms what other studies have shown while expanding the range of populations examined. A comparison of the variants discovered in all published studies on *GJA1* and deafness is available in Table 17.

The p.(A253V) variant has been detected before in a Caucasian control allele,[61] a Brazilian patient with ichthyosiform erythrokeratodermia[113] and both cases and controls in a study on sudden infant deaths.[68] It was also the only non-synonymous change found in the Iranian study of *GJA1* and hearing loss.[76] It is generally regarded as having little effect on the protein and Polyphen2 predicts the variant to be "benign" with a score of 0,008 (sensitivity: 0,96; specificity: 0,76) using HumDiv and a score of 0,002 (sensitivity: 0,99; specificity: 0,18) using HumVar. While p.(A253V) does not appear to be linked to any disease, there is evidence that it may act as a modifier of disease severity in Axenfeld-Rieger syndrome[114] and has been shown *in vitro* to result in a loss of tumour-suppressor activity.[115]

No mention of the other variants was found in a literature search, using Google Scholar (http://scholar.google.co.za/) with "*GJA1*" and either either the RS number, coding change description or protein change description as keywords. In addition no links to published literature were available on their respective dbSNP pages. Both g.11873G>A and g.11914C>T were present in our data as well as multiple populations in the 1000 Genomes dataset.

The poor differentiation between population groups in both the phylogeny and, to a lesser extent, the PCA is the result of a relative lack of variation in the *GJA1* gene. It's worth noting that included patients in the *GJA1* PCA only affected the South African cohort

because of the relatively large number of unique variants, albeit at such low frequencies that they can be discounted. Not only did we find more variations in *GJB2*, 10 in *GJB2* versus five in *GJA1*, but the number of patients with each *GJB2* variant was higher than the number per *GJA1* variant.. This is also despite a greater region being sequenced and compared, 760 bp in *GJB2* versus 1242 bp in *GJA1*. There were 29 different SNPs in the 1000 Genomes data for *GJB2* but only six in the *GJA1* data, with the Japanese population reporting no changes. This lack of sequence diversity may be due to the wide expression pattern of *GJA1*, demonstrated by the variable phenotype of ODDD, which can include skeletal abnormalities, dry skin, mental retardation and hearing loss,[61,62,63] suggesting that the gene is under a high level of selection.

Table 17: Comparison of *GJA1* variations in studies of *GJA1* and deafness.

Variation				Country (Observed/Total Alleles (%))				
Genomic	**Transcript**	**Protein**	**Pathogenicity**	**Cameroon***	**Iran#**	**South Africa***	**Thailand**	**Turkey@**
g.11090A>G	c.-67A>G	NA	Polymorphism	2/134 (1,5%)				
g.11345T>C	c.189T>C	p.=	Polymorphism			1/46 (2,2%)		
g.11409G>C	c.253G>C	p.(L181F)	**Pathogenic**				1/506 (0,2%)$	
g.11522T>C	c.366T>C	p.=	Polymorphism			1/46 (2,2%)		
g.11780C>T	c.624C>T	p.=	Polymorphism				1/520 (0,2%)%	
g.11873G>A	c.717G>A	p.=	Polymorphism	11/134 (8,2%)	1/68 (1,5%)	2/46 (4,3%)	1/520 (0,2%)%	
g.11914C>T	c.758C>T	p.(A253V)	Polymorphism		1/68 (1,5%)	1/46 (2,2%)		
g.12132C>T	c.976C>T	p.(T326S)	**Unknown**				2/520 (0,4%)%	
g.12308_12309dupA	c.3*_4*dupA	NA	Unknown		2/68 (3%)			

Pathogenicity obtained from the study's description or prediction with Polyphen2. The p.(T326S) variant received conflicting predictions so is classed as unknown. No variations were detected in Turkey in 120 chromosomes. * = this study, # = Kooshavar et al.,[76] $ = Yang et al.,[75] % = Yang et al.,[73] @ = Uyguner et al.[72]

6.5- Limitations Of Our Study

There are a number of limitations to the study that need to be acknowledged. Our methods only amplified the coding region of *GJB2* and can not detect non-coding mutations elsewhere in the gene which may affect transcription or mRNA stability.[116] In addition, we have classified newly identified SNPs as benign based on a combination of their location, frequencies in patients and controls, whether the variant alters the amino acid and according to predictive tools. It is possible that such a classification may incorrectly identify a variant as benign either due to the low prevalence of the variant or as the variant may have a more subtle effect, such as splicing or codon usage bias.

The South African cohort had a small sample size and this could have resulted in missing variants that do not occur at high frequencies. Taking into account previous work in South Africa and our results from Cameroon, we do not feel that this is particularly likely. Another limitation is the poor clinical description of the South African cohort included in this study. Patients were not fully phenotyped and it is possible that some were included inappropriately. Despite these limitations, the careful phenotyping and the diversity of the Cameroonian samples, strongly validates our findings, particularly in the samples that were used to investigate *GJA1* mutations, where 70% of case were familial and likely genetic in origin.

6.6- Practice Implications

Our results support the currently published literature and the hypothesis that mutations in *GJB2* are not a common cause of recessive, non-syndromic deafness in Africa. We add to the literature by showing that mutations in *GJA1* do not contribute to the genetic causes of non-syndromic hearing loss in Africans. Thus, genetic testing for these two genes in Africans patients with non-syndromic hearing loss in clinical practice is not recommended.

7- Perspectives And Research Recommendations

Our future work is focussed on investigating the genetic variation in *GJB6* (connexin 30) because the large deletion, del(*GJB6*-D13S1830), in compound heterozygosity with *GJB2* variants, has been shown to be the second most frequent cause of genetic deafness in some European populations.[117,118] Work is currently ongoing but provisional results do not show del(*GJB6*-D13S1830) or sequence variations in the same 100 patient cohort as selected for *GJA1*. If no mutations are found in *GJB6,* the most efficient way to identify deafness-causing mutations will by examining a large number of genes simultaneously through either whole genome or whole exome sequencing (WES). Indeed, the identification of the causative mutation in families with hearing loss, using classical single gene screening approaches, is difficult and unaffordable as 65 different genes are known to be involved in non-syndromic hearing loss.[35]

WES provides unprecedented opportunities to identify causative DNA variants in many families with hereditary diseases and has proven successful at elucidating the causes of deafness in a variety of genes and populations, even in small families.[119,120,121,122,123,124] We are fortunate to have enough samples and families to be able to combine homozygosity mapping and WES in Cameroonian and South African families with autosomal recessive non-syndromic hearing loss. To achieve these goals, we have began setting up collaborations with laboratories that are able to perform such high-throughput sequencing using platforms like OtoSCOPE, which combines targeted genomic enrichment and next generation sequencing to examine 66 deafness genes at once.[120,125] We have also initiated a pilot study to perform whole exome sequencing in a selected group of patients. Hopefully, both approaches could help to unravel the genetic aetiology of hearing loss in some African families.

Even with WES, the results will heavily depend on our cohort. While our Cameroonian cohort is well-described, our South African cohort lacks audiological and

sociodemographic data and suffers from a small sample size. In future experiments it will be necessary to recruit a larger cohort as well as collaborating with audiologists to generate the required data. Collaborations with other researchers will also allow recruitment of larger cohorts, more resources and the ability to recruit patients from countries where there has been, to date, no investigation into the genetics of deafness.

8- Conclusions

Our results show that neither *GJB2* (CX26) nor *GJA1* (CX43) is significantly associated with non syndromic deafness in Africans. We have expanded the range of the KID-causing p.(D50N) mutation in two Cameroonian patients. This confirms that, contrary to non-syndromic hearing loss, syndromic deafness due to *GJB2* is not population-specific. These finding have implications in clinical practice as the data suggests that investigation of *GJB2* (CX26) and *GJA1* (CX43) are inappropriate in most African patients with non-syndromic deafness.

Our results support the currently-published literature and the hypothesis that mutations in *GJB2* are not a common cause of recessive, non-syndromic deafness in Africa. This leaves the genetic basis of deafness in Africans still unexplained. It is possible that deafness in Africa is not caused primarily by a single gene but by small contributions from each of the full spectrum of deafness genes. Future studies may benefit more from whole genome approaches, such as whole exome sequencing, as opposed to a single gene approach, to unravel the genetic aetiology of non-syndromic hearing loss in Africans.

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I- Appendix

I.I- Recruitment Sheet

Date of inclusion …../…../….. Place of Recruitment……Form N-° ……..

I- IDENTIFICATION

Full name : ……

Year of birth: ……. ……..Sex : Male ☐[1] Female ☐[2]

Ethnicity: Xhosa ☐[1] Other……

Grade at school: Efata school ☐[1] Other……..

Residence: …….. Contact number: …….

II- CHARACTERISTICS OF DEAFNESS

Age of diagnosis of deafness: ……..

Threshold of the tonal audiometry test: ……..

Bilateral: Yes ☐[1] No ☐[2] Symmetrical ☐[1] Asymmetrical ☐[2]

Mechanism: Sensorineural ☐[1] Conductive ☐[2] Mixed ☐[3]

Audiometric curve shape: …….

Has the cause of deafness been established? Yes ☐[1] No ☐[2]

If yes, please specify……

Treatment…….....

III- CLINICAL EXAMINATION

A- Medical History

a) Prenatal

Documented maternal infection :

CMV ☐[1] Syphilis ☐[2] Rubella ☐[3] Toxoplasme ☐[4] Herpes ☐[5]

Potential terogens in during pregnancy:

Aminoglycosides ☐ [1] Alcohol ☐ [2]

b) Perinatal

Kernicterus ☐ [1] Severe IUGR ☐ [2] Neonatal anoxia ☐ [3] Obstetrical trauma ☐ [3] Teatment with aminoglycosides ☐ [4]

GA at birth……WEEKS

c) Postnatal

Recurrent Otitis media ☐ [1] Meningitis ☐ [2] Febrile convulsions ☐ [3]

Mumps ☐ [2] Measles ☐ [3] Mental retardation ☐ [4]

Delay in walking ☐ [5] Blindness ☐ [6] Night-blindness ☐ [5]

Severe myopia ☐ [6] Discomfort/fainting ☐ [7] Head trauma ☐ [8]

Diabetes millitus ☐ [9] Diabetes insipidus ☐ [10] Hypothyroidy ☐ [12]

Excessive urination/excessive thirst ☐ [13] Hematuria ☐ [14]

Hirschprung's disease ☐ [15] Kidney disease ☐ [16] specify……..

2-Family History

Deafness ☐ [1] Kidney disease ☐ [2] specify ……..

Diabetes melitus ☐ [3] White Forelock or white hair ☐ [4] Goiter ☐ [5]

B- PHYSICAL EXAMANINATION

Facial dysmorphism ☐ [1] Malformation of the external ear ☐ [2]

Microcephaly ☐ [3] Leukocoria ☐ [4]

Dystopia Canthaorum ☐ [5] Microphthalmia ☐ [6]

Residual cervical branchial ☐ [7] Vestibular ataxia ☐ [8]

White hair and white eyebrows ☐ [9] Goiter ☐ [10]

Patchy skin ☐ [11] Heterochromia (eyes) ☐ [12]

IV- FAMILY TREE

V- COMPLEMENTARY INVESTIGATIONS

Investigations	Results
Renal ultrasound Yes ☐ [1] No ☐ [2]	
Cervical ultrason Yes ☐ [1] No ☐ [2]	
Electrocardiogramme Yes ☐ [1] No ☐ [2]	
Opthalmological examination Yes ☐ [1] No ☐ [2]	
CT Brain Yes ☐ [1] No ☐ [2]	
Urine dipstick Yes ☐ [1] No ☐ [2]	

VI- MOLECULAR ANALYSIS

Blood for DNA Yes ☐ [1] No ☐ [2]

I.II- Consent Form (English)

1. I, _____ , am consenting to provide my / my child's (DELETE WHERE NOT APPLICABLE) genetic material for a study on hearing loss in Black Africans.

   Child's name (IF APPLICABLE):_____

2. I understand that the genetic material for analysis is to be obtained from my / my child's (DELETE WHERE NOT APPLICABLE) blood cells.

3. I request that **no** portion of the sample be stored for later use. ☐ (MARK IF APPLICABLE)

   Or

   I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE):

   (a) possible re-analysis

   (b) analysis for the benefit of members of my immediate family

   (c) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.

4. I authorise EFATA to provide relevant clinical details to the Division of Human Genetics, UCT.

5. I understand that:

   (a) there are benefits associated with genetic analysis and storage of biological material and these have been explained to me.

(b) the analysis procedure is specific to the genetic condition mentioned above and cannot determine the complete genetic make-up of an individual.

(c) the genetics laboratory is under an obligation to respect medical confidentiality .

(d) genetic analysis may not be informative for some families or family members.

(e) even under the best conditions, current technology of this type is not perfect and could lead to incorrect results.

(f) where biological material is used for research purposes, there may be no direct benefit to me.

6. I understand that I may withdraw my consent for any aspect of the above at any time without this affecting my future medical care.

7. When blood is drawn there will be someone available who can answer any questions I have in my own language.

Patient's signature or parent/guardian's signature if patient is under 18:

_____

Witness of Consent: _____

Contact details of patient or parent/guardian of patient:

Telephone: _____

Email: _____

I.III- Consent Form (Xhosa)

1. Mna**,** _____, ndinika imvume yokuba kungathathwa imbewu yemfuzo efumaneka egazini kumntwana wam ukuze kufundwe ngesifo sokungeva kubantu abamnyama bese Afrika

   Igama lomntwana:_____

2. Ndiyaqonda ukuba imbewu yemfuzo ezokuxilongwa izakuthathwa egazini lomntwana wam. (CIMA APHO KUNGEKHO MFUNEKO)

3. Ndiyacela ukuba kungabikho ntsalela yesampule yegazi eyakuthi igcinwe umphelo ☐ (YENZA UPHAWU UKUBA UFUNA OKU)

   Okanye

   Ndicela ukuba intsalela yesampule yegazi ingagcinwa umphelo ukuze

   (a) Iphinde ixilongwe

   (b) Ixilongwe ukwenzela inzuzo kumalungu osapho lwam ngqo.

   (c) Injongo zophando, ngokunikwa imvume yi Komiti Yezophando nNgomntu yakwi Yunivesithi yase Kapa phantsi komqathango wokuba incukacha zabantu ezikoluphando zizohlala ziyimfihlo.

4. Ndinika imvume e EFATA ukuba inikise ngenkcukacha yesigulo kwi Ncandelo Lwezenzululwazi Ngemfuzo Ebantwini, e UCT.

5. Ndiyaqonda ukuba:

   (a) Kukho inzuzo enxulumelene nokuxilongwa kwe mfuzo kunye nokugcinwa kwegazi futhi oku kucacisiwe kum.

(b) Indlela yokuxilonga yeyesigulo sikhankanywe ngasentla qha futhi asikwazi ukubona yonke indlela imuzo yomntu eyenziwe ngayo.

(c) Indlu yemfuzo exilongayo inyanzelekile ukuba ihloniphe ukugcina inkcukacha zengulo yomntu ziyimfihlo.

(d) Uxilongo lwemfuzo kunga nganikiki lwazi lungaphaya kwamanye amakhaya okanye kumalungu asekhaya.

(e) Nangona kusetyenzwa phantsi kwemeko ezingcono, izixhobo zalemihla azigqibelelanga, sise okunika ingxelo ezingezizo.

(f) Apho kusetyenziswe khona igazi ngejongo zophando, kungangabikho nzuzo iqondene nam.

6. Ndiyaqonda ukuba ndingarhoxa kwisivumelwano nakowuphi umba ongasentla nangeliphi ixesha kwaye oko akusayi kuchaphazela isihoyo sempilo yam kwixa elizayo.

7. Xa kutsalwa igazi kuzakubakho umntu ozokubakhona ukuphendula nawuphi umbuzo endinawo ngolwimi lwam.

Ukutyikitya komguli/ komzali/ ummeli womguli xa eneminyaka engaphantsi kwe 18

_____

Ukutyikitya kwengqina lesivumelwano_____

I.IV- Patient Information Sheet (English)

This study will be conducted by Jason Bosch for fulfilment of the requirements for an MSc degree. The research will be supervised by Dr Ambroise Wonkam, Dr Collet Dandara and Dr Karen Fieggen and performed at University of Cape Town in Department of Clinical and Laboratory Services, Division of Human Genetics.

This study intends to increase our knowledge of the genetics of non-syndromic sensorineural hearing loss in Black South Africans by examining a selection of genes known to be involved in hearing loss. We will compare the information obtained to known mutations and use family trees to determine what mutations are responsible for hearing loss among Black South Africans.

We are performing this study as there is currently very little information on the genetics of hearing loss in Black South Africans, or Africa as a whole. This is important because hearing loss has many different causes which are often unique to specific populations. Without the information that this study will provide we cannot know whether genetic tests for hearing loss are appropriate for African populations. This uncertainty can delay assistance for those with hearing loss and could result in people spending money on tests that are not suitable.

The knowledge of which genes and mutations cause hearing loss will also be valuable in providing counselling to parents both before they have children and about the probability of having a second child with hearing loss.

There is no risk to anyone participating in the study as all that is needed is a blood sample and information about the participants family and condition.

More information can obtained from Dr Nomlindo Makubalo who is assisting in patient recruitment and the translation of all documentation into Xhosa.

- Dr Nomlindo Makubalo

Telephone: 021 406 6698 Email: np.makubalo@uct.ac.za

Other persons involved in the research can be contacted through the UCT Division of Human Genetics (Telephone: 021 406 6297) or by email.

- Jason Bosch

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- Dr Ambroise Wonkam

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- Dr Collet Dandara

Email: collet.dandara@uct.ac.za

- Dr Karen Fieggen

Email: karen.fieggen@uct.ac.za

I.V- Patient Information Sheet (Xhosa)

Olufundo luzakwenziwa ngu Jason Bosch ukuze abenako ukufezekisa izidingo zesidanga se MSc. Oluphando luzakube luphantsi koqwalaselo luka Dr Ambroise Wonkam, no Dr Collect Dandara kunye no Dr Karen Fieggen, futhi luzakuqhutyelwa kwiYunivesithi yase Kapa kwi Sebe le Clinical and Laboratory Service kwiCandelo Lwezululwazi ngemfuzo ebantwini.

Olufundo lunenjongo lokunyusa ulwazi ngemfuzo eyenza ukungeva ngeendlebe kubantu abamnyama base Mzantsi Afrika ngokuthi kuxilongwe isixa sembewu yemfuzo eyaziwayo ukuba ngunobangela wokungeva. Siyakuthi sifanise ulwazi olufunyenweyo kutshintsho lwembewu kunye neminombo yamakhaya ukuzama ukufumana eyona mbewu ingunobangela yo kungeva ngendlebe kubantu abamnyama base Mzantsi Afrika.

Senza olufundo ngokuba luncinci ulwazi lwembewu yemfuzo eyenza ukungeva ngendlebe ebantwini abamnyama base Mzantsi Afrika, ne Afrika jikelele. Oku kubalulekile ngokuba zininzi izinto ezingunobangela wokubasisithulu kwaye zahlukile ngokwengingqi yokuhlala. Ngaphandle kokuthi senze olufundo angeke siyazi ukuba uxilongo lwembewu yemfuzo olusetyenziswayo lufanele na ukusetyenziswa Kubantu base Afrika. Oku kuthandabuza kungatsalelisa emva uncedo lwabo bazizithulu futhi lwenze uchitho mali kwindlela zokuvavanya indlebe ezingezizo. Ulwazi lwemfuzo notshintsho olungunobangela lokungeva ngendlebe lungaluncedo kakhulu ekunikeni ingcebiso kubazali phambi kokuba baphinde bazale, futhi bacebiswe nangoqikelelo lokuphinda babenomntwana osisithulu.

Akukho bungozi nakubani othi athathe inxaxheba koluphando , into efunekayo yisampuli yegazi kunye nenkcukacha zekhaya nemeko yomthathi -nxaxheba.

Ingcaciso egcweleyo ingafumaneka ku Dr Nomlindo Makubalo oncedisa ngokufumana izithulu ezinokusetyenziswa koluphando nenguqulelo yamaphepha onke esiXhoseni.

- Dr Nomlindo Makubalo

Umnxeba : 021 4066698 Email: np.makubalo@uct.ac.za

Abanye abantu aba yinxalenye yoluphando ungaqhagamshelana nabo ngokusebenzisa Isebe lase UCT Lwezifundo Ngemfuzo Eebantwini (umnxeba : 021 4066297) okanye em

- Jason Bosch

Email : Jason.bosch@uct.ac.za

- Dr Ambroise Wonkam

Email: ambroise.wonkam@uct.ac.za

- Dr Collect Dandara

Email: collet.dandara@uct.ac.za

- Dr Karen Fieggen

Email: Karen.fieggen@uct.ac.za

I.VI- Dna Extraction From Blood

1. When fresh blood arrives and the volume is 3 ml or less, transfer into a 15 ml conical tube. If fresh blood is more than 3 ml, make a buffy and take 3 ml into the 15 ml conical tube.
2. Add 9 ml of filter-sterilised Red Blood Cell (RBC) lysis buffer, Mix well and leave at room temperature for 30 minutes. Vortex briefly every 5 minutes or leave tubes on a roller.
3. Vortex then centrifuge for 10 minutes at 2000 rpm.
4. Decant supernatant, add 1 ml of fresh RBC lysis buffer and mix by vortexing to break up and mix the pellet so that it is smooth. (Repeat steps 3 and 4 for cleaner pellet.)
5. Add 3 ml of filter-sterilised cell lysis buffer and mix well with vortex.
6. After mixing all of the samples, add 12,5 µl of 20 mg/ml Proteinase K and 100 µl of 20% SDS to each tube. Mix well with vortex.
7. Place tubes in 37 °C water bath for 1-3 days.
8. After incubation the liquid should be relatively clear with no pellet before moving to the next step.
9. Mix with 1 ml of 6 M (saturated) NaCl and vortex vigorously.
10. Centrifuge at 2000 rpm for 20 minutes. (Repeat if supernatant is still turbid.)
11. Transfer the 5 ml supernatant to a clean 15 ml tube. Avoid touching the salt-protein deposit at the bottom of the tube.
12. Add 2X the volume (10 ml) of 100% ethanol and mix gently, either by inverting 50X or using a roller for 5-10 minutes. Centrifuge at 2000 rpm for 10 minutes.
13. Decant supernatant and add 2 ml ice-cold 70% ethanol and centrifuge at 2000 rpm for 10 minutes. Carefully remove ethanol without disturbing the DNA pellet.

14. Air dry the pellet for at least 2 hours at room temperature.
15. Reconstitute DNA pellet in 150 µl DNA Rehydration solution. Leave at room temperature for one week to ensure complete reconstitution. (If not completely reconstituted place in 37 °C water bath and vortex occasionally.)

- Red Blood Cell Lysis Buffer (RBC Lysis Buffer)
    - NH_4Cl (Ammonium chloride): 8,82 g
    - NH_4HCO_3 (Ammounium bicarbonate powder): 0,79 g
    - EDTA (0,5 M, pH 7,4): 0,2 ml
    - Make up volume to 1 l with ddH_2O
    - Filter sterilise with 0,2 µM micropore filter

- DNA Lysis Buffer/Cell Lysis Buffer
    - Tris-HCl (1 M, pH 7,5): 25 ml
    - NaCl (3 M): 16,7 ml
    - EDTA (0,5 M): 1 ml
    - Make up volume to 500 ml with ddH_20 (Sterile water)

Excess blood was stored in the form of a buffy coat:

1. Centrifuge blood sample for 10 minutes at 2000 rpm.
2. Transfer 1 ml of buffy coat (all leucocytes and a little of the erythrocytes and plasma) into a 1,5 ml microcentrifuge tube.
3. Centrifuge for 10 minutes at 3000 rpm then store at -4 °C until storage box is full.
4. Once box is full samples are moved -20 °C for long-term storage.

I.VII- DNA Extraction From Saliva

1. Mix the Oragene DNA/Saliva sample in the Oragene vial by inversion and gentle shaking for a few seconds.
2. Incubate the sample at 50 °C in a water bath for a minimum of 1 hour or in an air incubator for a minimum of 2 hours.
3. Transfer 1 ml of the mixed Oragene DNA/saliva sample in a 1,5 ml microcentrifuge tube.
4. For 1 ml of Oragene DNA/saliva, add 40 µl (1/25th volume) of Oragene DNA Purifier (OG-L2) to the microcentrifuge tube and mix by vortexing for a few seconds.
5. Incubate on ice for 10 minutes.
6. Centrifuge at room temperature for 5 minutes at 13 000 rpm (15 000X g).
7. Carefully transfer the clear supernatant with a pipette tip into a fresh microcentrifuge tube. Discard the pellet containing the impurities.
8. To 750 µl of supernatant, add 500 µl of room temperature 95-100% ethanol. Mix gently by inversion 10 times.
9. Allow the sample to stand at room temperature for 10 minutes to allow the DNA to fully precipitate.
10. Place the tube in the microcentrifuge in a known orientation. Centrifuge at room temperature for 2 minutes at 13 000 rpm (15 000X g).
11. Carefully remove supernatant with a pipette tip ad discard it. Take care to avoid disturbing the DNA pellet.
12. Ethanol wash step. Carefully add 500 µl of chilled 70% ethanol. Centrifuge for 1 minute at 13 000 rpm. Carefully remove the ethanol without disturbing the pellet.
13. Air-dry the pellet overnight.
14. Add 100 µl of DNA Rehydration Solution (DNA Buffer) to dissolve the DNA pellet.

I.VIII- Ethanol Precipitation

1. Add the following to a 1,5 ml microcentrifuge tube; 22 µl of ice-cold absolute ethanol, 1 µl 5M NaOAc pH 5 and 10 µl of sequencing reaction.
2. Store the tubes at -20 °C for at least one hour or overnight.
3. Centrifuge at 10 000 rpm for 9 minutes.
4. Decant the supernatant and blot the tube on a paper towel.
5. Add 50 µl ethanol to the tubes, vortex and centrifuge at 10 000 rpm for 9 minutes.
6. Decant supernatant, blot dry and leave tubes to air dry for at least 30 minutes but longer is better.
7. Resuspend in 10 µl of distilled water and vortex.

I.IX- Minor allele frequency of *GJB2* SNPs available from the 1000 Genomes Project data

SNP	ASW	CEU	CHB	CHS	CLM	FIN	GBR	IBS	JPT	LWK	MXL	PUR	TSI	YRI
rs111033327	0	0	0	0	0,008	0	0	0	0	0	0	0	0	0
rs111033294	0	0	0	0	0,008	0	0	0	0	0	0	0	0	0
rs76838169	0	0	0,052	0,065	0	0	0	0	0,051	0	0	0	0	0
rs201004645	0	0	0	0	0	0	0,011	0	0	0	0	0	0	0
rs200104362	0	0	0	0	0	0	0	0	0	0	0	0,018	0	0
rs111033360	0	0	0	0	0	0	0	0	0	0,005	0	0	0	0
rs111033186	0	0	0	0	0	0	0	0	0	0	0	0	0,020	0
rs80338948	0	0	0	0	0	0,005	0	0	0	0	0	0	0	0
rs116769964	0	0	0	0	0	0	0	0	0	0	0	0	0	0,006
rs76434661	0	0,006	0,005	0	0	0	0	0	0	0	0	0	0	0
rs111033196	0	0	0	0	0	0	0,006	0	0	0	0	0	0,020	0
rs111033188	0	0	0,010	0,015	0	0	0	0	0,006	0	0	0	0	0
rs150529554	0	0	0	0	0	0	0,006	0	0	0	0	0	0	0
rs2274083	0	0	0,253	0,195	0	0	0	0	0,152	0	0	0	0	0
rs201839979	0	0	0	0,005	0	0	0	0	0	0	0	0	0	0
13:20763463	0	0	0	0	0	0	0,006	0	0	0	0	0	0	0
rs111033218	0	0,006	0	0	0	0	0,006	0	0	0	0	0	0,005	0
rs200023879	0	0	0	0	0	0	0	0	0	0,005	0	0	0	0

SNP	ASW	CEU	CHB	CHS	CLM	FIN	GBR	IBS	JPT	LWK	MXL	PUR	TSI	YRI
rs72474224	0	0	0,041	0,065	0,008	0	0	0	0,011	0,005	0,008	0	0	0
rs35887622	0,016	0,024	0	0	0,008	0,027	0,022	0	0	0	0,008	0,018	0,010	0
rs2274084	0,025	0	0,351	0,28	0,075	0	0	0	0,393	0	0,136	0,018	0	0
rs104894408	0	0	0	0	0	0	0	0	0	0	0,015	0	0	0
rs111033222	0,008	0	0	0	0	0	0	0	0	0	0	0	0	0
rs148136545	0,016	0	0	0	0,008	0	0	0	0	0,036	0	0	0	0
rs72561725	0,049	0,006	0	0	0	0	0	0	0	0,088	0,008	0,009	0	0,125
rs201895089	0	0	0	0	0	0	0,006	0	0	0	0	0	0,005	0
rs141962118	0	0	0	0	0	0	0	0	0	0	0	0	0,005	0
rs9578260	0,213	0	0	0	0,033	0	0	0	0	0,253	0,008	0,036	0	0,233
rs191461105	0	0	0	0,01	0	0	0	0	0	0	0	0	0	0

I.X- Minor allele frequency of *GJA1* SNPs available from the 1000 Genomes Project data

Name	ASW	CEU	CHB	CHS	CLM	FIN	GBR	IBS	JPT	LWK	MXL	PUR	TSI	YRI
rs56199702	0	0,012	0	0	0,008	0,005	0,006	0	0	0	0,008	0	0	0
rs72548741	0	0	0	0,005	0	0	0	0	0	0	0	0	0	0
rs148384161	0	0,006	0	0	0	0	0	0	0	0	0	0	0	0
rs57946868	0,066	0,012	0,005	0	0,033	0,011	0,011	0,071	0	0,082	0,008	0	0,020	0,057
rs17653265	0	0,006	0	0	0,017	0	0,017	0,036	0	0	0,015	0,009	0,015	0
rs184583316	0	0	0	0	0	0	0	0	0	0,021	0	0	0	0

I.XI- Heterozygous p.Asp50Asn Mutation In The *GJB2* Gene In Two Cameroonian Patients With Keratitis-Ichthyosis-Deafness (KID) Syndrome

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Abstract

Background

Keratitis-Ichthyosis-Deafness (KID) syndrome (OMIM 148210) is a congenital ectodermal defect that consists of an atypical ichthyosiform erythroderma associated with congenital sensorineural deafness. KID appears to be genetically heterogeneous and most cases are caused by *GJB2* mutations. Mutations in African patients have been rarely described.

Case presentation

We report on two unrelated Cameroonian individuals affected with sporadic KID, presenting with the classic phenotypic triad. The two patients were heterozygous for the most frequent p.Asp50Asn mutation. This first report in patients from sub-Saharan African origin supports the hypothesis that the occurrence of KID due to p.Asp50Asn mutation in *GJB2* seems not to be population specific.

Conclusions

Our finding has implication in medical genetic practice, specifically in the molecular diagnosis of KID in Africans. These cases also reveal and emphasize the urgent need to develop appropriate policies to care for patients with rare/orphan diseases in Sub-Saharan Africa, as many of these cases become more and more recognizable.

Keywords:

KID syndrome; *GJB2* gene; p.Asp50Asn mutation; Africa; Cameroon

Background

The Keratitis-ichthyosis-deafness (KID) syndrome (OMIM 148210 and 242150) is a rare congenital ectodermal disorder of unknown prevalence. Approximately 100 cases have been reported in the world to date [1]. KID appears to be genetically heterogeneous and most cases are caused by mutations in the connexin 26 gene, *GJB2*. Connexins are membrane proteins with a common structure consisting of four transmembrane domains linked by one cytoplasmic and two extracellular loops (namely, EC1, and EC2, respectively), with both cytoplasmic N-terminal and C-terminal [2]. Different mutations in the genes encoding connexins can disturb the gap junction system of one or several ectodermal epithelia, which in the case in KID syndrome, where the epidermis, the inner ear and the corneal epithelium are affected [3].

Skinner et al. reviewed 18 affected patients and proposed the acronym 'KID syndrome' to describe three main symptoms, ichthyosis, vascularizing keratitis, and often profound sensorineural hearing loss [4]. KID is genetically heterogeneous and is caused by missense mutations in the connexin (CX) genes *GJB2* and *GJB6*, which cluster at chromosome 13q11-q12 and encode the closely related gap junction β-2 protein (coded by CX26) and β-6 protein (coded by CX30) [5,6]. Inheritance of KID syndrome is usually sporadic but autosomal recessive and dominant cases have been reported [5].

Mutations in cases of KID syndrome have been rarely reported in patients of African descent [7], but p.Asp50Asn mutation in the *GJB2* gene have been previously only reported in a black patient from the Emirates [8]. In this article, we report on two unrelated Cameroonian patients with KID syndrome presenting with heterozygous p.Asp50Asn mutation in the *GJB2* gene.

Case presentation

Patients and methods

We previously published on aetiological factors of congenital hearing loss on 582 Cameroonians [9]. Amongst them, two young patients presented with clinical features of KID syndrome.

DNA amplification and mutation analysis

Genomic DNA samples were extracted from peripheral blood of the two patients, using Puregene blood Kit® (Qiagen, USA), following the manufacturer's protocol and this was carried out in the Molecular Diagnosis Laboratory of the Gyneco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon. The *GJB2* gene was amplified following the method of Liu et al. [10]. Exon 2 was amplified and then sequenced using an ABI 3130XL Genetic Analyze® automated sequencer (Applied Biosystems, USA), in the Molecular Research laboratory in the Division of Human Genetics, University of Cape Town, South Africa.

Clinical data

Patient 1

A five-year-old girl that presented with a profound bilateral sensorineural deafness diagnosed at 2 years old. She was born at term to unrelated healthy parents after an uneventful pregnancy and normal vaginal delivery, and presented at birth with generalized erythema. She had a history of chronic otitis externa and hypohidrosis. Her psychomotor development was normal; however, physical examination revealed a generalized thickened skin and xeroderma, palmoplantar keratoderma and rippled hyperkeratotic plaques on the knees and elbows (Figure 1A-D). She had aged facial appearance, hypotrichosis (sparse of eyelashes and eyebrows), and hyperkeratosis lesions in the external auditory canal. Ophthalmologic examination revealed a mild vascularizing keratitis which explained her photophobia and reduced visual acuity. Oral examination showed dental dysplasia and histopathological examination of the skin revealed an acanthotic dyskeratosis. The parents were non-consanguineous and there was no family history of similar condition.

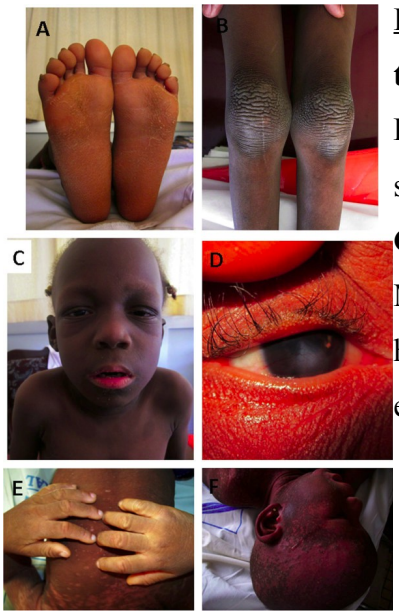

Figure 1. Illustrations of some clinical features of the two Cameroonian KID cases (Case 1; panels A-D; Case 2 panels E and F). A) Keratoderma of the soles **B)** Rippled hyperkeratotic plaques on the knees; **C)** Hypotrichosis of the eyelashes and eyebrows; **D)** Mild vascularizing keratitis; **E)** Hyperkeratosis of the hands; **F)** Alopecia, hypotrichosis, ichthyosiform erythrokeratoderma.

Patient 2

A two-year-old girl that presented with a prelingual bilateral profound sensorineural deafness. She was born at term after an uncomplicated pregnancy and delivery. Since two months of age, she presented with thick, reddened patches of the skin that were dry and scaly. The thickness of the skin gradually increased as she grew older. At the time of presentation at the health facility physical examination revealed generalized ichthyosiform erythrokeratoderma, palmoplantar keratoderma, alopecia universalis and atrichosis (absence of eyelashes and eyebrows) (Figure 1E-F). Joint mobility of the elbows, knees and ankles was seriously reduced by keratoderma. She had photophobia and ocular irritation, and an ophthalmologic examination revealed a vascularizing keratitis. The intraoral examination was normal. She had no major neurological abnormalities and her non-consanguineous parents and young sister were all healthy and there was no family history of similar clinical presentation.

On familial history, the two patients were unrelated: their parents originated from, and live in, two geographically distinct of area of Cameroon (Western and Centre provinces, about 400 km apart). The parents belonged to two different ethno-linguistic groups: Bantu and Nilo-Sahelian, respectively.

Genetic analysis

Analysis of *GJB2* exon 2 in the genomic DNA of the two unrelated patients revealed a heterozygous missense mutation c.148G > A, resulting in a putative amino acid change from aspartic acid (GAC) to asparagine (AAC) in codon 50 p.Asp50Asn (Figure 2). p.Asp50Asn were not present in more than 180 unrelated individuals who were screened for recessive deafness mutations or in 60 healthy control persons of Cameroonian origin [*unpublished results*].

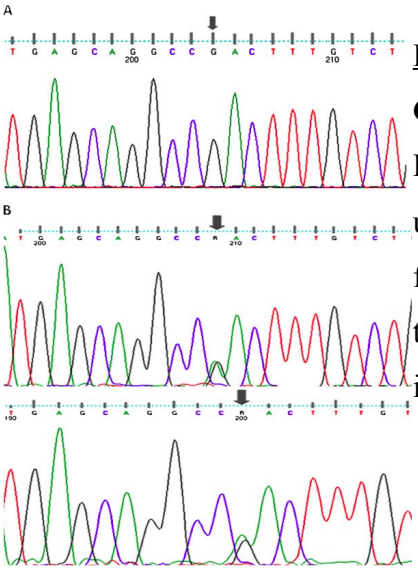

Figure 2. Mutation analysis of *GJB2* in the two Cameroonian individuals affected with sporadic KID. Panel **A**: Sequence chromatograms of *GJB2* from unaffected individual; Panel **B**: Sequence chromatograms from affected patients depicting the heterozygous transition 148G → A at codon 50 encoding asparagine instead of aspartic acid (p.Asp50Asn) (Panel **B**).

Moreover, sequences data from both patients did not show any other common changes, but the p.Asp50Asn variant. The DNA sequence in patient 1 did not have any other changes than the p.Asp50Asn variant, while patient 2 was also heterozygote for the most common non pathogenic polymorphism, g.3318-34C > T. Thus, there was no further need to compare the haplotypes from the two patients to establish unrelatedness.

Discussion

To the best of our knowledge, this is the first report of sub-Saharan African patients with KID syndrome due to the p.Asp50Asn mutation in *GJB2*.

GJB2 mutations responsible for non-syndromic hearing loss have been reported in many parts of the world with marked variations in distribution patterns among different ethnic groups, with a propensity to occur frequently in some population groups (in Europe, North America and Asia) [10,11], while seemingly absent in African populations [12]. Contrary to

GJB2 mutations causing autosomal recessive non-syndromic hearing loss (HL) which are ethnically specific because of founder effect in some specific populations, *GJB2* mutations responsible for syndromic hearing loss seem not to be population specific [5].

The two patients reported here presented with the classic phenotypic triad of KID syndrome including diffuse hyperkeratotic erythroderma, vascularizing keratitis, and profound bilateral sensorineural hearing loss. Several other features were associated including alopecia, hypotrichosis and hypohydrosis, porokeratotic eccrine ostial and dermal duct nevus, follicular occlusion triad and dental anomalies previously [13,14]. Moreover, the p.Asp50Asn mutation has also been reported in KID patients with Dandy-Walker malformation [15]; although brain imaging was not performed, the two cases described here did not have neurologic features that would suggest the occurrence of Dandy-Walker malformation. The p.Asp50Asn mutation has been reported mostly in sporadic cases, but also in a case of autosomal dominant inheritance [5]. In a cohort of 14 patients affected with KID syndrome originating from 11 families, where parent to child transmission in families was verified by molecular analysis, twelve patients (86%) were heterozygous for the p.Asp50Asn mutation. The disease was sporadic in 64%, whereas 36% was familial, suggestive of autosomal dominant inheritance with one parent clinically affected in all the families. A family with p.Asp50Asn mutation was suggestive of germinal mosaicism, as the parent was clinically normal [16]. Similar report of germinal mosaicism was also reported in family with dizygotic twins suffering from a lethal form of KID; the two patients were heterozygous for the p.G45E mutation of *GJB2*, whereas the mutation was not detected in the two parents [7]. The two pairs of Cameroonian parents did not consent for their molecular analysis, claiming in both cases, that this will not have any implication in improving the care of their KID-affected children. However, since none of the parents were clinically affected, and in absence of reported proven familial case with reduce penetrance, mutations in these two Cameroonian cases are most likely *de novo*.

p.Asp50Asn appears to be the most prevalent mutation in unrelated KID patients of Caucasian ancestry [5,16], and the two unrelated Cameroonian cases provide additional information that this mutation may also be important in African populations. The amino

acid replacement, in p.Asp50Asn mutation, occurs in the highly conserved first extracellular loop of CX26, which is crucial for voltage gating and connexon-connexon interactions [2,17]. The majority of dominantly-acting connexin mutations, associated with autosomal dominant syndromic HL are situated in this domain and are missense mutations, while the majority that cause recessive HL are nonsense mutations or small deletions [17]. Moreover, it has been observed that alteration of calcium ion fluxes due to the effects of mutations such as p.Asp50Asn, result in cell death by necrosis [18]. In addition, other functional analyses showed that p.Asp50Asn have consequences for protein localization and gap junction permeability [19]. However, more evidences are needed to associate the variable phenotypes observed in KID with effects on protein trafficking or gap junction permeability.

The management of KID syndrome in Cameroon as in other low-income countries is a critical issue. The most beneficial treatment for the profound hearing impairment in our patients could be cochlear implantation [20], a procedure that is unavailable in Cameroon. There is no universal medical insurance in Cameroon and even hearing aids are not affordable for most patients. Keratitis (also observed in the two cases) can result in progressive decline of visual acuity and may eventually lead to blindness which combined with a profound hearing loss constitute a disastrous disability. In addition, a life-long follow-up of these patients is necessary because the KID syndrome is associated with malignant tumours, particularly squamous cell carcinoma [1]. Although the provision of service for medical care for KID patients is limited in Cameroon, better awareness of the disease within the region would help patients if upon diagnosis, better prevention of the worst effects of disease can be instituted, which besides follow-up for cancerous lesions, would include simple preventative measures against fatal septicemia from recurrent skin infections; specifically for the keratitis; there is a need to develop an appropriate specialist services in the country that could help to prevent blindness.

Conclusion

In conclusion, our report supports the occurrence of KID due to p.Asp50Asn mutations in *GJB2* in Africans and seems to indicate that this mutation is not ethically specific. These cases reveal and emphasize the urgent need to develop appropriate policies to care for patients with rare/orphan diseases in Sub-Saharan Africa, as many of these cases become more and more recognizable.

Consent

The study was performed in accordance with the guidelines of the Helsinki Declaration and was approved by the National Ethics Committee of Cameroon (ethics approval N°123/CNE/SE/2010), and the Faculty of Health Sciences Research Ethics Committee, University of Cape Town, HREC REF: 080/2011. Written informed consents were obtained from patient parents for publication and the accompanying images. Copies of the written consents are available for review.

Abbreviations

KID: Keratitis-ichthyosis-deafness; EC1 and EC2: Extracellular loops 1 and 2; CX: Connexion.

Competing interests

The authors declaim no competing interests.

Authors' contributions

AW and GBT designed the study, raised fund, assured general supervision of the research group, drafted the manuscript and compelled the revisions; JJNN acquired clinical data and performed DNA extraction and draft the manuscript, JB and CD performed and supervised the molecular analysis of *GJB2* gene. All the authors revised and approved the final version the manuscript.

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I.XII- Mutations in *GJB2* do not play a significant role in non-syndromic sensorineural deafness in Africans

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Running title: MUTATIONS IN *GJB2* IN DEAFNESS IN AFRICANS

ABSTRACT

Background: Mutations in the *GJB2* gene, encoding connexin 26, could account for 50% of congenital, non-syndromic, recessive deafness cases in some Caucasian/Asian populations. There is a scarcity of published data in sub-Saharan Africans.

Methods: We Sanger sequenced the coding region of the *GJB2* gene, in 205 Cameroonian and Black South African patients with congenital, non-syndromic deafness; and performed bioinformatic analysis of variations in the *GJB2* gene, incorporating data from the 1000 Genomes Project.

Results: Amongst Cameroonian patients, 26.1% were familial and 5.6% were consanguineous. The majority of patients (70%) suffered from sensorineural hearing loss. Ten *GJB2* genetic variants were detected by sequencing. A previously reported pathogenic mutation, g.3741_3743delTTC (p.F142del), and a putative pathogenic mutation, g.3816G>A (p.V167M), were identified in single heterozygous samples. Amongst eight the remaining variants, two novel variants, g.3318-41G>A and g.3332G>A, were reported. There were no statistically significant differences in allele frequencies between cases and controls.

On a phylogenetic tree, Cameroonian and South African controls cluster with other African populations. Principal Components Analyses differentiated between Africans, Asians and Europeans but only explained 40% of the variation.

Conclusion: Mutations in *GJB2* do not play a major role in congenital non-syndromic genetic deafness in Africans.

Keywords: non-syndromic deafness; Africans; Cameroon; South Africa; *GJB2;* Connexin 26

INTRODUCTION

Deafness is a global problem that is most serious in the developing world, with 7 per 1000 children in Nigeria born with deafness and 5.5 per 1000 live births in South Africa [1, 2]. These incidences are about five times higher than observed in the United States and Europe [3, 4].

Deafness is a highly complex condition caused by both genetic and environmental factors as well as a combination of the two [5].Estimates for the aetiology of permanent childhood deafness in the US and Europe are approximately 20-30% genetic, another 20-30% acquired and 40-55% unknown [3, 6].These proportions have changed over time with improved healthcare reducing the number from infectious diseases, increasing the number due causes such as prematurity and improving the detection of genetic causes. There is a scarcity of data on the aetiology of hearing loss in Africa but recent work in Cameroon shows the causes to be 15% genetic, 52% acquired and 33% unknown [7].

Deafness is non-syndromic, when hearing loss is the only feature, or syndromic, where hearing loss is accompanied by other clinical features. Syndromic hearing loss is made up of more than 400 different syndromes and the Hereditary Hearing Loss Homepage (http://hereditaryhearingloss.org/) lists 65 genes reported to be involved in non-syndromic hearing loss to date, with 75-80% of these exhibiting recessive inheritance [8].

Although deafness is a highly heterogeneous condition, it has been found that mutations in the Gap Junction Beta 2 (*GJB2*) gene, encoding connexin 26, are responsible for up to 50% of cases deafness in populations of Europeans descent [9]. The major mutations in *GJB2* have been seen to be population specific,, resulting from founder effects. These include c.35delG affecting Caucasians[10], c.167delT affecting those of Ashkenazi Jewish ancestry[11] and c.235delC affecting East Asians[12]. In contrast, the few studies available suggest that *GJB2*

in Africans or African Americans is not a major contributor to deafness in these populations. Studies have reported pathogenic *GJB2* mutations in up to 37% of alleles[9] while in the Kenyan and Sudanese populations less than 4% of variants were found to occur in the coding region of *GJB2*[13] and none at all were found in South Africans[14].

The aim of this study was to ascertain the significance of mutations in *GJB2* in a selected group of Cameroonian and South African patients with non-syndromic deafness.

MATERIALS AND METHODS Ethical Considerations

The study was approved by the Cameroon National Ethics Committee (ethics approval N°123/CNE/SE/2010) and the Human Research Ethics Committee of the University of Cape Town (ethics approval HREC REF: 080/2011). Written and signed informed consent was obtained from all participants aged 18 years old or more, or from the parents/guardians with verbal assent from the children.

Settings, Patients and Controls

Cameroonian Patients were recruited from seven of the ten regions of Cameroon, mainly from schools for the deaf, and those procedures have been reported previously[7]. South African patients, all from the Xhosa ethnic group, were recruited from Efata School for the Blind and Deaf in the Eastern Cape Province, South Africa.

During recruitment, information on participants' medical and family history was obtained from the participants themselves, their parents and medical records, depending on which sources were available. In the majority of cases, general systemic and otological examination was performed as well as an audiological evaluation using either pure tone

audiometry or auditory brain stem response test. Audiological test results that were obtained before admission to schools for the deaf were also reviewed for some subjects. When syndromic deafness was suspected, additional tests, when possible, were later performed to confirm or exclude the diagnosis.

We included (1) Patients of Black African descent with non-syndromic hearing loss, as confirmed by a clinical or audiological report, with deafness of either (2) putative genetic origin, as revealed by one or more affected family members or consanguinity, or (3) unknown origin, and who consented to participate in the study. We excluded patients with syndromic hearing loss and those with obvious environmental causes such as meningitis, rubella, mumps, measles, severe prematurity and/or birth weight less than 1500g, neonatal hyperbilirubinemia, neonatal asphyxia, ototoxicity, severe head trauma.

Following the above selection criteria, a total of 180 Cameroonian patients from a large cohort previously reported[7] and 25 South African patients.

Normal hearing individuals from the same population background as patients were recruited: 17 South African and 64 Cameroonian controls.

DNA Amplification and Mutation Analysis

At the Molecular Diagnosis Laboratory of the Gyneco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon, genomic DNA samples were extracted from peripheral blood of the patients, following instructions on the available commercial kit (Puregene blood Kit® (Qiagen, USA)). At the Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, a modified version of the salting out method was used to extract DNA from peripheral blood specimen of South African patients[15], or DNA was purified from saliva (Oragene® kit; DNA Genotek®, USA) according to the manufacturer's

instructions..

The *GJB2* gene was amplified following the method of Liu et al. [16]. Primers were validated using BLAST® and primer analysis software. The complete coding region of exon 2 was amplified with the aforementioned primers and then sequenced using an ABI 3130XL Genetic Analyzer® (Applied Biosystems, Foster City, USA), in the Molecular Research Laboratory in the Division of Human Genetics, University of Cape Town, South Africa.

Bioinformatic and Statistical Analyses

Chromatogram files were manually checked using FinchTV 1.3.1 (GeoSpiza) and aligned in BioEdit 7.0.5.3 to the *GJB2* reference sequence (Ensembl transcript ENST00000382848 (*GJB2*-001) retrieved 31 August 2012). Detected variations were checked against dbSNP[17], and the effects of non-synonymous mutations were predicted using Polyphen-2[18]. SHEsis (http://analysis2.bio-x.cn/) was used to analyse for statistical differences allele, genotype and haplotype frequencies between the cases and controls, as well as the analysis of linkage disequilibrium[9,20]. The Chi-square test and the Fisher's exact test were used to compared SHEsis results, and a p-value less than 0.05 was considered statistically significant.

Data for the sequenced region of *GJB2* was downloaded from the 1000 Genomes browser (http://browser.1000genomes.org) for all available populations [21]. A phylogeny was constructed with PopTree software using the Neighbour Joining algorithm, Nei's DA genetic distance[22] and 1000 bootstraps. Principle Components Analysis (PCA) was performed in R[23] using the FactorMineR Package and the same 1000 Genomes Project data. As the 1000 Genomes Project data is from apparently healthy individuals, only control data was used for comparisons.

RESULTS

Socio-demographics

Table 1 summarizes the socio-demographic data of the study participants. Of the 47 familial cases (26.1%) amongst the Cameroonian participants, our analysis includes two cases of two affected siblings, two cases of three affected siblings and one case of four affected siblings(supplementary data 1). Consanguinity was not reported in the South African cohort but 10 (5.6%) Cameroonian patients were from known consanguineous unions. Admixture with other non-African population groups was highly unlikely in all participants.

Clinical Information

Audiological information was only available for patients from Cameroon. Supplementary data 2 shows the severity of deafness. The majority of patients (70%) suffered from sensorineural hearing loss and a small number (4.4%) suffered from mixed hearing loss. The type of hearing loss for the remainder of the patients was not determined.

Variations/Pathogenic Mutation Analysis

Two pathogenic or probably pathogenic mutations were detected in two unrelated Cameroonian participants, g.3741_3743delTTC (p.F142del) and g.3816G>A (p.V167M). Both were detected in only a single individual in the heterozygous state. There were no pathogenic mutations detected in South African patients.

Sequencing revealed a number of likely non-pathogenic variants in the *GJB2* sequence (Supplementary data 3) in both Cameroonian and South African patients. We also detected two novel variants, g.3318-41G>A and g.3332G>A, both in the heterozygous form. The

g.3318-41G>A change occurs in the first intron of *GJB2* and the g.3332G>A change leads to a synonymous mutation. The most common variants in both the South African and Cameroonian cohorts were the intronic change g.3318-34C>T and two changes in the 5'UTR, g.3318-15C>T and g.3318-6T>A.

Analysis with SHEsis showed that there were no statistically significant differences between allele, genotype or haplotype frequencies between cases and controls in Cameroon. In South African patients, there was a statistically significant difference between haplotypes ($p = 0.023$) (Supplementary data 4). Linkage analysis showed a low rate of recombination between our markers but also that they were uninformative of one another.

Phylogenetics and Principal Components Analysis (PCA)

The phylogeny (Figure 1) shows the populations from the 1000 Genomes Project clustering into Asians, Africans and Europeans with the admixed populations positioned closer to the base of the tree. As expected, the South African and Cameroonian controls grouped with the other African populations. The PCA explains 40% of the variation between population groups (Figure 2) and that different populations are characterised by different SNPs (Supplementary data 5).

DISCUSSION

This comprehensive report on the significance of *GJB2* mutations in deafness in two African populations offers a substantial contribution to the literature on the topic. The inclusion of a carefully selected group of Cameroonian patients is of major importance.

Indeed, Cameroon is frequently referred to as "Africa in miniature," because of its central location on the continent, its many geographical and cultural attributes and the diversity of its population (There are more than 200 distinct local languages in the country). The country spans two main geographical zones of almost equal size: the equatorial rain forest in the south and the tropical savannah and the Sahel region in the north. At the genetic level, Cameroonian population diversity mimics that of various ethno-linguistic groups in African populations[24] and it is anticipated that, results from a carefully selected sample in this population could represent a snapshot of that of many African populations. Furthermore, we included patients from the Xhosa population of South Africa, a formerly unstudied offshoot of the Bantu population that migrated from areas around Cameroon[24].

To our knowledge, the present study is the first to have conducted a comparison of African *GJB2* sequences with the data from the 1000 Genomes Project and revealed the variation between population groups. This unique finding has emphasised the hypothesis that the prevalence of mutations in *GJB2* in non syndromic deafness amongst European and Asian populations is due to founder effects arising after these individuals migrated out of Africa[10,11,12], and not to a putative "protective" variant in the genomic structure of *GJB2* in Africans. Moreover, the exception to this low prevalence of *GJB2* mutations in African patients with deafness, is a specific mutation, p.R143W (c.427C>T), occurring at a high rate in the Ghanaian population from Adamarobe village[25]. This could also be attributed to a founder effect as this mutation has not been reported in other African populations but has been reported only in few African Americans (Table 2), who were probably brought from Ghana during the slave trade. In addition, the description of de *novo* p.Asp50Asn mutation, in two Cameroonian patients diagnosed with KID syndrome also confirms that mutations in *GJB2* do occur in Africans[26]. These mutations probably occur at a frequency that is

comparable to that of other world populations, since *de novo* p.Asp50Asn mutation is the most prevalent in KID syndrome globally[27].

Our results both build on the previously published studies of *GJB2* deafness in Africa while distinguishing itself with comprehensive bioinformatic analyses and a comprehensive review. This study will both add to the literature and could influence molecular diagnosis of hearing loss on the continent.

Globally, studies have found that pathogenic *GJB2* mutations account for 14.2% of alleles in Japan[28] , 18% in Iran[29] , 22% in the USA[30] and 37% in Italy and Spain[9]. In contrast, we found *GJB2* mutations in less than 1% of Cameroonian and 0% of South African alleles. Similar to Africans, Caribbean Hispanics, probably due to their African ancestry, do not show a large contribution to deafness from *GJB2* variations[31]. Mutations in *GJB2* were unable to explain deafness for any of the African patients, with non-syndromic hearing loss included in this study. This is similar to other African studies, which have also reported low frequencies of deafness-associated mutations (Table 2). The pathogenic p.F142del mutation has been detected and associated with deafness twice before, in the heterozygous state in an Egyptian patient suffering from moderate hearing loss[32] and in four Chinese patients with non-syndromic hearing loss[33]. p.V167M has been reported in three studies, only in patients of African ancestry. It has been detected in a heterozygous state in 4/406 (~1%) Kenyans with prelingual, non-syndromic hearing loss[13], 1/94 (~1%) African American controls[34], and in 1/19 (~5%) patients from a predominantly African American cohort of patients with congenital CMV infection and hearing loss[35]. While it may be pathogenic, it has not been reported in a homozygous form and predictive tools give ambiguous results. Polyphen-2[18] predicts it as "possibly damaging with a score of 0.618 (sensitivity: 0.87; specificity: 0.91)" with HumDiv and "possibly damaging with a score of 0.537 (sensitivity: 0.82; specificity:

0.82)" with HumVar. The Polyphen-2 score shows the probability that a specific change will be pathogenic.

Three common variants were detected in our cohort, all of which have previously been reported at high frequencies in African populations. These are the g.3318-34C>T, g.3318-15C>T and g.3318-6T>A changes, which occured in 60%, 28% and 4% of our South African patients respectively and 47%, 8% and 2% of our Cameroonian patients respectively. Our results are similar to those of Kabahuma et al. where g.3318-34C>T and g.3318-15C>T were the only mutations detected in 182 cases and 63 controls from the Limpopo province, South Africa. The authors described the g.3318-34C>T change in 46% and 42% of patients and controls respectively and the g.3318-15C>T change in 21% and 35% of patients and controls respectively[14]. The same variants, including the g.3318-6T>A change, were also present in deaf individuals from both Kenya and Sudan[13]; the g.3318-34C>T and g.3318-15C>T changes in 12.73%, 6.45% of patients respectively. The g.3318-6T>A change was only found in 0.49% of Kenyan patients.

These variants appear to be almost exclusive to those of African ancestry, perhaps best demonstrated by the control data of Tang et al., which is stratified by ethnic background[36]. The results show g.3318-34C>T, g.3318-15C>T and g.3318-6T>A occurring in 28%, 3% and 2% respectively of African Americans but just 3%, 2% and 0% of Hispanics and none of the variations occurring in either Asians or Caucasians. Tang et al. also found a lack of polymorphisms in the coding region of African Americans but a high prevalence of variations in the 3'UTR, an area we did not examine.

There has been one previous study of *GJB2* and hearing loss in Cameroon whose results are partially supported by this study[37]. Out of 67 deaf and 66 normal hearing controls two *GJB2* variants were detected, the c.186C>T variant in two patients and one control and the

c.296insT variant in one control. However, the Trotta et al. study was limited to a single school in the North of the country.

PCA offers a number of SNPs that appear to differentiate African populations from others included in the 1000 Genomes Project data (Supplementary data 5). Many SNPs occur at either very low frequencies or just single populations. The three SNPs that we found at a high prevalence appear to be a reliable way to distinguish African from other populations. Although we see a difference in haplotype frequencies between cases and controls in South Africa, that result could easily be due to the low sample size. None of the variants seen in the South African cohort are known to be pathogenic, making their connection to deafness unlikely. All the other tests for significance fail to find a difference between cases and controls in South Africa and Cameroon, supporting the hypothesis that *GJB2* does not play a significant role in deafness in Africans.

Study Limitations

The molecular methods described here only amplified the coding region of *GJB2* and cannot detect non-coding mutations elsewhere in the gene. A further limitation is the poor clinical description of the South African participants which may have resulted in inappropriate inclusion of some of them. However, despite these limitations, the careful phenotyping and the diversity of the Cameroonian samples, adding to previous data and the bioinformatics analysis performed in this study strongly validates our findings.

Practice Implications

Our results support the currently published literature and the hypothesis that mutations in *GJB2* are not a common cause of recessive, non-syndromic deafness in Africans. Thus,

genetic testing of *GJB2* in African patients with non-syndromic hearing loss is not recommendable in clinical practice.

Research Recommendations

The identification of the causal mutations in families with hearing loss, using classical single genes screening approaches, is difficult and expensive due to the heterogeneous nature of non-syndromic hearing loss. It could be appropriate to move to explore the involvement of *GJB6,* the second biggest cause of genetic deafness in some European populations[38] or *GJA1*, which is sometimes used for clinical screening in South Africa.

Another option is to explore our cohort with high-throughput sequencing using platforms like OtoSCOPE®, which combines targeted genomic enrichment and next generation sequencing to examine 66 deafness genes at once[39]. Probably the most efficient perspective for this study is to use whole exome sequencing, which has proven successful at elucidating the causes of deafness in a variety of genes and populations, even in small families[40],

In conclusion, our results confirm that *GJB2* is not significantly associated with non-syndromic deafness in Africans and support that investigation of *GJB2* is unnecessary in most African patients with non-syndromic deafness.

Conflict Of Interest

The authors declare no conflict of interest.

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Legends

Figure 1. Phylogeny constructed from 1000 Genomes and study control data.

The phylogeny tree shows the 1000 Genomes various populations' clusters. As expected, the South African and Cameroonian controls grouped with the other African populations. Numbers indicate bootstrap values over 1000 iterations. Population abbreviations are: ASW, Americans of African Ancestry in SW USA; CEU, Utah Residents (CEPH); CHB, Han Chinese in Beijing; CHS, Southern Han Chinese; CLM, Colombians from Medellin Colombia; FIN, Finnish in Finland; GBR, British in England and Scotland; IBS, Iberian population in Spain; JPT, Japanese in Tokyo Japan; LWK, Luhya in Webuye Kenya; MXL, Mexican Ancestry from Los Angeles USA; PUR, Puerto Ricans from Puerto Rico; TSI, Toscani in Italia; YRI, Yoruba in Ibadan Nigeria; SA_C, South African control; CAM_C, Cameroonian control.

Figure 2. Principal component analysis (PCA) study and 1000 Genomes populations with respect to variation in *GJB2*.

The PCA explains 40% of the variation between population groups and different populations are characterised by different SNPs.(supplementary data 5).

Population abbreviations are: ASW, Americans of African Ancestry in SW USA; CEU, Utah Residents (CEPH); CHB, Han Chinese in Beijing; CHS, Southern Han Chinese; CLM, Colombians from Medellin Colombia; FIN, Finnish in Finland; GBR, British in England and Scotland; IBS, Iberian population in Spain; JPT, Japanese in Tokyo Japan; LWK, Luhya in Webuye Kenya; MXL, Mexican Ancestry from Los Angeles USA; PUR, Puerto Ricans from Puerto Rico; TSI, Toscani in Italia; YRI, Yoruba in Ibadan Nigeria; SA_C, South African control; CAM_C, Cameroonian control.

Table 1. Socio-demographic data of study participants.

Demographic		South Africa		Cameroon	
		Case (Freq.)	Control (Freq.)	Case (Freq.)	Control (Freq.)
Gender	Male	20 (0.80)	0 (0.00)	94 (0.52)	36 (0.60)
	Female	4 (0.16)	6 (0.38)	84 (0.47)	24 (0.40)
	Unknown	1 (0.04)	10 (0.63)	2 (0.01)	0 (0.00)
Age	Average	13.95	47.75	11.81	13.53
	Unknown	4 (0.16)	8 (0.50)	3 (0.02)	0 (0.00)
Age of onset	Prelingual (<2 years)	3 (0.12)	N/A	157 (0.87)	N/A
	Perilingual (2-4 years)	6 (0.24)	N/A	0 (0.00)	N/A
	Postlingual (>4 years)	3 (0.12)	N/A	14 (0.08)	N/A
	unknown	13 (0.52)	N/A	9 (0.05)	N/A
Transmission	familial	5 (0.20)	N/A	47 (0.26)	N/A
	sporadic/unknown	20 (0.80)	N/A	133 (0.74)	N/A
		N=25	N=16	N=180	N=60

NA = Not applicable; Freq. = Frequency

Table 2. Comparison of results between selected studies of *GJB2* in African populations.

Variation			Country (Observed/Total alleles)				
Genomic	**Coding**	**Pathogenicity**	**Cameroon**	**Ghana[&]**	**Kenya/Sudan[$]**	**South Africa**	**African American**
g.3318-41G>A	c.-41G>A	Polymorphism	1/360[*]				
g.3318-35T>G	c.-35T>G	Polymorphism			1/1178		
g.3318-34C>T	c.-34C>T	Polymorphism	100/360[*]		85/1178	19/50[*], 119/364[@]	
g.3318-15C>T	c.-15C>T	Polymorphism	15/360[*]		38/1178	7/50[*], 56/364[@]	1/46[+], NA[+]
g.3318-6T>A	c.-6T>A	Polymorphism	4/360[*]		2/1178	1/50[*]	
g.3332G>A	c.15G>A	Polymorphism	1/360[*]				
g.3352_3353insG	c.35dupG	**Pathogenic**		1/730			
g.3352delG	c.35delG	**Pathogenic**			10/1178		7/100[~]
g.3395C>T	c.78C>T	Polymorphism			1/1178		
g.3396C>T	c.79G>A	**Pathogenic**					2/46[+], NA[+]
g.3419T>C	c.101T>C	**Pathogenic**					NA[+]
g.3426G>A	c.109G>A	**Pathogenic**			1/1178		
g.3455_3460del	c.138_143del	**Pathogenic**			1/1178		
g.3503C>T	c.186C>T	Polymorphism	NA[*], 2/122[#]		3/1178		
g.3512C>A	c.195C>A	**Pathogenic**			1/1178		
g.3542G>T	c.225G>T	Polymorphism	1/360[*]				
g.3553T>C	c.236T>C	**Pathogenic**		1/730			
g.3566C>G	c.249C>G	**Pathogenic**					1/100[~]
g.3586_3587insT	c.269_270insT	**Pathogenic**	NA[#]				
g.3627A>C	c.310A>C	Polymorphism			1/1178		
g.3658A>G	c.341A>G	**Pathogenic**					NA[+]
g.3697G>A	c.380G>A	**Pathogenic**			1/1178		
g.3741_3743delTTC	c.424_426delTTC	**Pathogenic**	1/360[*]				
g.3744C>T	c.427C>T	**Pathogenic**		110/730			1/100[~]

g.3774G>A	c.457G>A	Polymorphism			2/1178	NA*	
g.3795G>A	c.478G>A	**Pathogenic**			1/1178		NA+
g.3816G>A	c.499G>A	**Pathogenic**	1/360*		4/1178		NA+
g.3850T>C	c.533T>C	**Pathogenic**		4/730			
g.3868G>A	c.551G>A	**Pathogenic**		1/730			
g.3906G>T	c.589G>T	**Pathogenic**		1/730			
g.3925-3926delinsAA	c.608_610delinsAA	**Pathogenic**		2/730			
g.3958C>T	c.641T>C	**Pathogenic**		1/730			

NA refers to variations that were found during the study but only in the control group. Variant information was obtained through the relevant paper's own results and a combination of the Deafness Variation Database (http://deafnessvariationdatabase.org/) and The Connexin-Deafness Homepage (http://davinci.crg.es/deafness/index.php). Study references: *This study, #Trotta et al.,[37] @Kabahuma et al.,[14] $Gasmelseed et al.,[13] &Hamelmaan et al.,[25] +Sammanich et al.,[34] ~Pandya et al.[30]

Figure 1

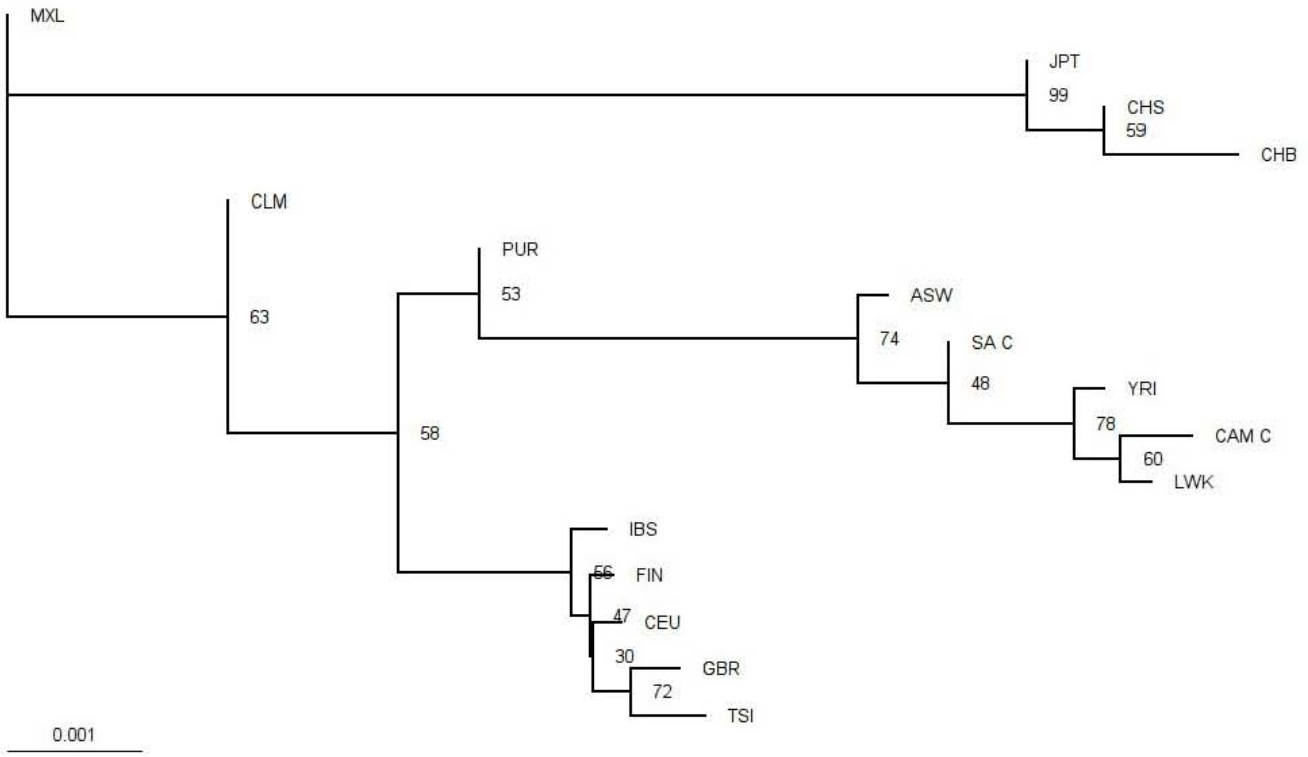
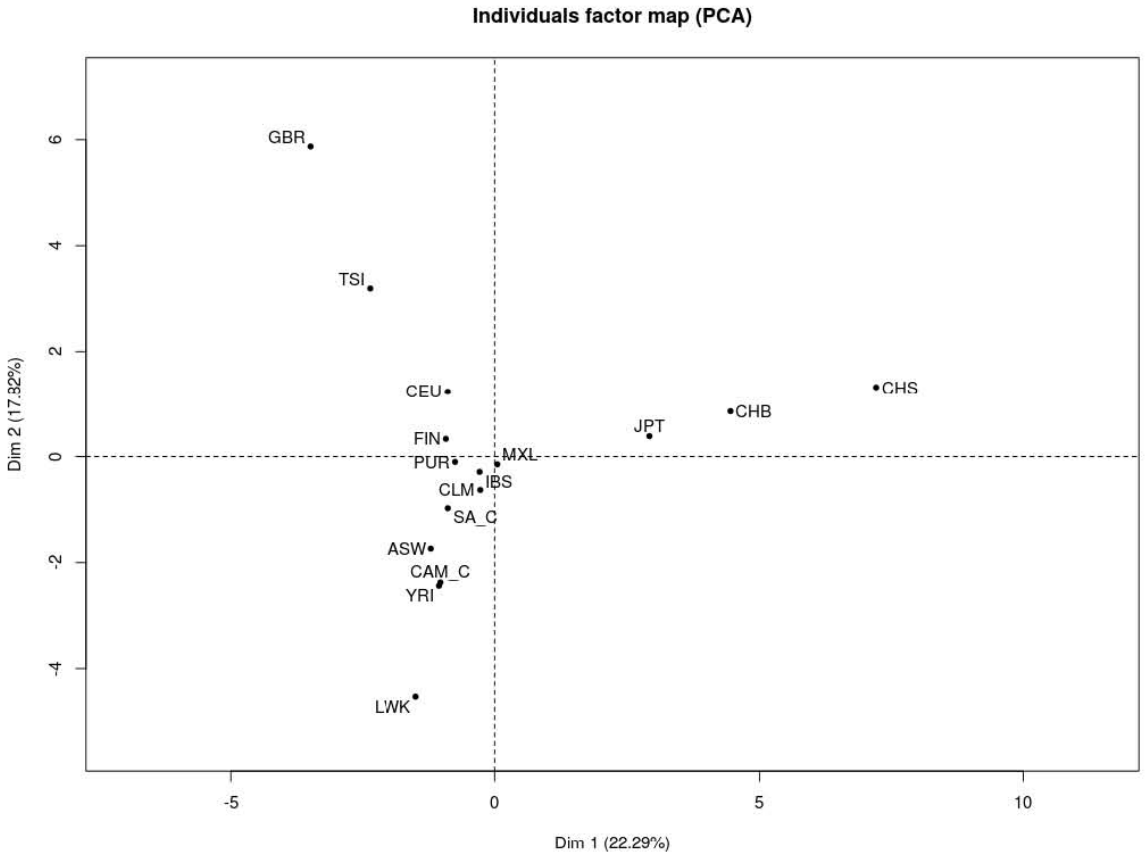
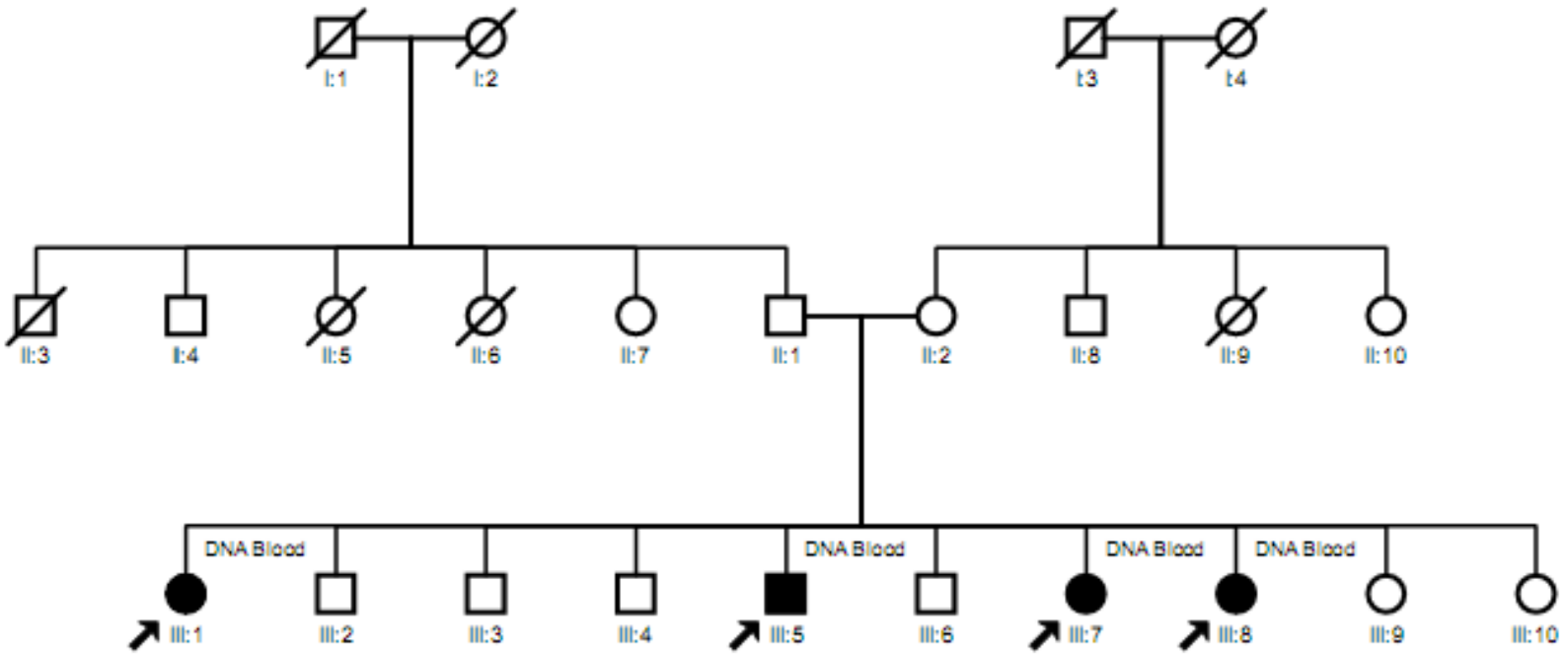

MXL
JPT
99
CHS
59
CHB
CLM
PUR
53
63
ASW
74
SA C
48
YRI
78
CAM C
60
LWK
58
IBS
56
FIN
47
CEU
30
GBR
72
TSI
0.001

Figure 2


Individuals factor map (PCA)
GBR
TSI
CEU
FIN
PUR
CLM
SA_C
ASW
CAM_C
YRI
LWK
MXL
IBS
JPT
CHB
CHS
Dim 2 (17.32%)
Dim 1 (22.29%)
6
4
2
0
-2
-4
-5
0
5
10

Supplementary Data 1



Supplementary data 2. Severity of hearing loss in the Cameroonian cohort.

Level of Deafness[α]	Left Ear (Freq.)	Right Ear (Freq.)
Severe 1 (71-80)	6 (0.03)	7 (0.04)
Severe 2 (81-90)	19 (0.11)	20 (0.11)
Profound 1 (91-100)	53 (0.29)	54 (0.30)
Profound 2 (101-110)	49 (0.27)	46 (0.26)
Profound 3 (111-119)	17 (0.09)	22 (0.12)
Total (120)	7 (0.04)	2 (0.01)
Unknown	29 (0.16)	29 (0.16)
	N=180	N=180

[α]The level of deafness is given according to BIAP classification; http://www.biap.org/en/recommendations/65-ct-2-classification-des-surdites/5-recommandation-biap-021-bis.

Supplementary data 3. *GJB2* variations detected and the frequency of the change in South African and Cameroonian cohorts.

Genomic	Coding	Protein	Domain[αα]	RS number	Pathogenicity	South Africa			Cameroon		
						Cases (Freq.)	Controls (Freq.)	P-value	Cases (Freq.)	Control (Freq.)	P-value
g.3318-41G>A	c.-41G>A	NA	Intron	Novel	Polymorphism	0	0	NA	1 (0.003)	0	0.563
g.3318-34C>T	c.-34C>T	NA	Intron	rs9578260	Polymorphism	19 (0.380)	7 (0.219)	0.126	100 (0.278)	40 (0.333)	0.246
g.3318-15C>T	c.-15C>T	NA	5'UTR	rs72561725	Polymorphism	7 (0.140)	1 (0.031)	0.106	15 (0.042)	7 (0.058)	0.450
g.3318-6T>A	c.-6T>A	NA	5'UTR	rs148136545	Polymorphism	1 (0.020)	0	0.421	4 (0.011)	1 (0.008)	0.795
g.3332G>A	c.15G>A	p.T5=	IC1	Novel	Polymorphism	0	0	NA	2 (0.006)	0	0.413
g.3503C>T	c.186C>T	p.N62=	EC1	Reported	Polymorphism	0	0	NA	1 (0.003)	0	0.563
g.3542G>T	c.225G>T	p.R75=	EC1	rs149137695	Polymorphism	0	0	NA	0	1 (0.008)	0.083
g.3741_3743 delTTC	c.424_426 delTTC	p.F142del	TM3	Reported	**Pathogenic**	0	0	NA	1 (0.003)	0	0.563
g.3774G>A	c.457G>A	p.V153I	TM3	rs111033186	Polymorphism	0	1 (0.031)	0.209	0	0	NA
g.3816G>A	c.499G>A	p.V167M	EC2	rs111033360	**Possibly pathogenic**	0	0	NA	1 (0.003)	0	0.563
						N=50	N=32		N=360	N=120	

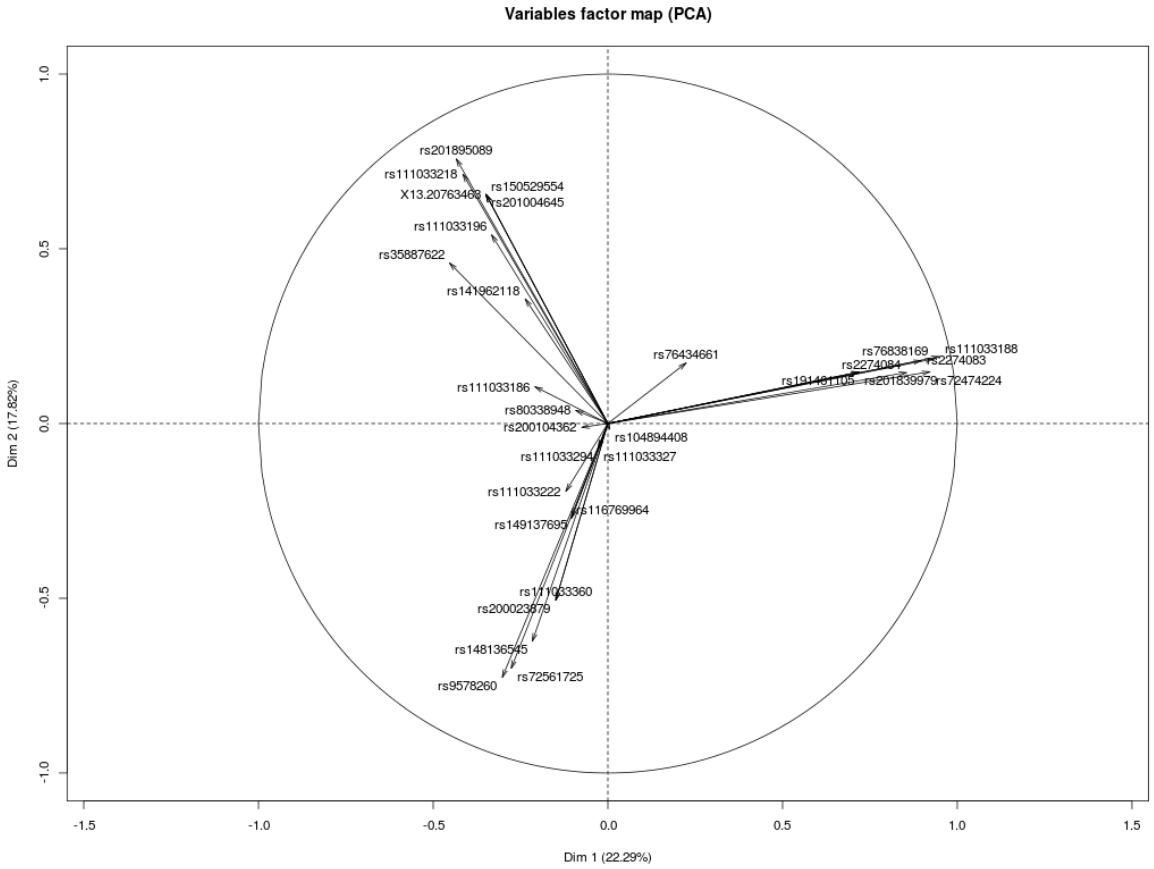
[αα]IC = Intracellular domain, EC = extracellular domain, TM = transmembrane domain.

Supplementary data 4. Haplotype frequencies in the South African cohort.

Haplotype[ααα]	Case (freq.)	Control (freq.)	P-Value
C C T A	0.00 (0.000)	1.00 (0.031)	0.209
C C T G	24.00 (0.480)	24.00 (0.750)	**0.016**
T C T G	19.00 (0.380)	6.00 (0.188)	0.065
T T T G	0.00 (0.000)	1.00 (0.031)	0.297
C T A G	1.00 (0.020)	0.00 (0.000)	0.421
C T T G	6.00 (0.120)	0.00 (0.000)	**0.042**
Global			**0.030**

[ααα]Haplotypes were constructed from the following SNPs: g.3318-34C>T, g.3318-15C>T, g.3318-6T>A and g.3774G>A. Values that are statistically significant ($P < 0.05$) are in bold.

Supplementary data 5.



I.XIII- Hearing loss in Africans is not linked to variations in the *GJB6* or *GJA1* genes

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Abstract

Background: Deafness is the most common sensory disability in the world and has a variety of causes. Globally, mutations in *GJB2* have been shown to play a major role in non-syndromic deafness, but this has not been seen in Africans. Two other connexin genes, *GJB6* and *GJA1*, have been implicated in hearing loss but have been seldom investigated.

Methods: We recruited a subset of 100 patients from a cohort that was previously shown not to have *GJB2* deafness and Sanger sequenced the full coding regions of *GJB6* and *GJA1*. In addition, we checked for the large-scale *GJB6*-D3S1830 deletion.

Results: No pathogenic mutations were detected in either *GJB6* or *GJA1*, nor was the *GJB6*-D3S1830 deletion detected. There were no statistically significant differences between patients and controls.

Conclusion: Mutations in *GJB6* and *GJA1* are not a major cause of deafness in African populations and we advise against using them for clinical screening.

Keywords: non-syndromic deafness, Africans, *GJA1* (CX43), *GJB6* (CX30)

Introduction

Deafness is one of the leading causes of disability in the world and is most severe in the developing world.[1] It is a highly variable and extremely heterogeneous condition that can range from mild to total hearing loss and present either as a single symptom or as one of many clinical features. Deafness can be caused by environmental conditions, genetics, normal aging or a combination of factors.

At current, 65 different genes have been identified that contribute to non-syndromic deafness and there are many more causative mutations.[2] Mutations in *GJB2* (connexin 26) have been shown to be a major contributor to deafness globally but, with the exception of Ghana, not in sub-Saharan Africa.[3] Other potential candidate genes that could lead to non-syndromic deafness in Africans are *GJB6* (connexin 30) and *GJA1* (connexin 43).

The second biggest genetic cause of deafness in the European population is the *GJB6*-D13S1830 deletion identified by del Castillo et al.[4] and present in up to 9.7% of patients in some European countries.[5] Although originally considered to be a case of digenic inheritance, as connexins 26 and 30 are known to interact, other evidence suggests that the deletion includes an unidentified cis-regulatory region for *GJB2*.[6]

GJA1 emerged as a possible candidate for hearing loss in Black Africans when *GJA1* mutations were detected in African Americans.[7] Those results were due to failure to differentiate between *GJA1* and its pseudogene and the changes were only present in the pseudogene.[8] Subsequent studies of *GJA1* and hearing loss have either found no causative variants or variants at very low frequencies.[9–12]

Variations in both *GJB6* and *GJA1* are checked for clinical screening in South Africa and the *GJB6*-D13S1830 deletion is the second largest global cause of non-syndromic hearing loss. We aimed to validate the utility of testing for *GJB6* and *GJA1* in an African context.

Methods

Ethical Considerations:

Recruitment of patients from Cameroon was approved by Cameroon's National Ethics Committee, authorisation number N°123/CNE/SE/2010. Ethics approval for the *GJB6* and *GJA1* research was granted by the University of Cape Town's Human Research Ethics Committee, reference numbers 042/2013 and 080/2011 respectively. Written and signed

informed consent was obtained from all participants, if they were 18 years or older, or from the parents/guardians with verbal assent from the children.

Patient Selection:

A subset of 100 patients from our earlier study (Bosch et al., unpublished data) was chosen in order to maximise the probability of finding a genetic cause of deafness. Our cohort included six consanguineous Cameroonian patients, 52 familial (including four consanguineous) Cameroonian cases, five familial South African cases, two patients with heterozygous *GJB2* mutations, 15 prelingual Cameroonian cases and the 20 remaining South African cases. All but four Cameroonian patients had previously been genotyped for *GJB2*.

Methods:

Detection of del(*GJB6*-D13S1830) was performed using the primers described by del Castillo et al.[4,5] The entire coding region of *GJB6* was amplified using the method described by Chen et al.[13] A 1348 bp fragment consisting of the entire *GJA1* coding region was amplified using the F1 and R3 primers described by Huang et al.[14] Amplified products were Sanger sequenced, in both the forward and reverse direction, on an ABI 3130XL Genetic Analyser (Applied Biosystems, Foster City, USA). The same primers were used for amplification and sequencing. Differences in allele, genotype and haplotype frequencies between cases and controls were assessed using SHEsis (http://analysis2.bio-x.cn/myAnalysis.php).[15,16]

Results

Patients:

The Cameroonian cohort was evenly distributed in terms of gender (46% female) while the South African cohort was 80% male. In the Cameroonian cohort, 94% of patients experience prelingual hearing loss while age of onset was unknown for the majority of the South African cohort. No information on consanguinity was available for the South African cohort but 10 patients in the Cameroonian cohort were from consanguineous marriages. There was no audiological information available for the South African cohort but all the Cameroonian patients presented with severe or greater (≥71 db) bilateral hearing loss. The majority (85%) had sensorineural deafness, one had mixed hearing loss and the rest were

undetermined.

GJB6:

Two Cameroonian patients failed to amplify for both *GJB6* experiments. None of the 98 patients had the *GJB6*-D13S1830 deletion. Only one variant (rs145762940) was detected, in the heterozygous state, in the coding region of *GJB6,* leading to the synonymous c.480G>A change. No variations in *GJB6* were detected in 31 controls (12 South African and 19 Cameroonian).

GJA1:

Two South African and eight Cameroonian patients failed to amplify for *GJA1* and were excluded. Five variants were detected in *GJA1* (Table 1), one of which occurred in the intron, but none of which are known to be pathogenic. Forty-one controls (17 South African, 24 Cameroonian) were also sequenced but only the synonymous c.717G>A change was detected. In addition, there were no statistically significant differences between cases and controls.

Discussion

Like previous studies in Africans,[17] as well as studies in Chinese,[13] Indians, [18] Turkish[19] and both African American and Caribbean Hispanics with *GJB2* mutations,[20] we did not find either the *GJB6*-D13S1830 deletion or coding region changes. This supports the hypothesis that the *GJB6*-D13S1830 deletion is the result of a founder effect.[5] In addition, there remains little evidence for coding region variations in *GJB6* that lead to deafness.

Although variants were detected in *GJA1* there were no significant differences between patients and controls. We have identified the novel c.366T>C (p.=) *GJA1* variant (Figure 1) which has not, to our knowledge, been described before. Only one variant, the c.758C>T (p.(A253V)) change, was non-synonymous. However c.758C>T is a known change that is not considered to be pathogenic. It has been reported both in cases and controls in various studies on *GJA1[10,21–23]* but has been suggested to modify disease severity in certain cases.[24]

As the two major genetic causes of global non-syndromic deafness, *GJB2* and *GJB6*, have not been shown to be associated with non-syndromic deafness in Africans and there are at

least 65 candidates,[2] we recommend that future research should take advantage of the power of massively parallel sequencing to screen multiple genes at once. This approach has previously been shown to be effective for non-syndromic deafness[25] and offers the best chance of uncovering the genetic causes of deafness in a setting with a genetically diverse population.

Conclusion And Perspective

We did not find evidence that mutations in either *GJB6* or *GJA1* are linked to non-syndromic deafness in sub-Saharan African patients. We recommend against using either gene for clinical testing in patients of African ancestry.

Conflict Of Interest

The authors declare no conflict of interest.

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Table 1: Genotypes of patients and controls with respect to variations in *GJA1*. EC = Extracellular domain, IC = intracellular domain.

Transcript	Protein	Domain	RS number	South Africa					Cameroon					
				Case		Control		P-Value	Case			Control		P-Value
				Het. (freq.)	WT (freq.)	Het. (freq.)	WT (freq.)		Hom. (freq.)	Het. (freq.)	WT (freq.)	Het. (freq.)	WT (freq.)	
c.-67A>G	NA	Intron	rs189167598	0	23 (1.000)	0	17 (1.000)	NA	1 (0.015)	0	66 (0.985)	0	24 (1.000)	0.547
c.189T>C	p.=	EC1	rs139688042	1 (0.043)	22 (0.957)	0	17 (1.000)	0.384	0	0	67 (1.000)	0	24 (1.000)	NA
c.366T>C	p.=	IC2	Novel	1 (0.043)	22 (0.957)	0	17 (1.000)	0.384	0	0	67 (1.000)	0	24 (1.000)	NA
c.717G>A	p.=	IC3	rs57946868	2 (0.087)	21 (0.913)	3 (0.176)	14 (0.824)	0.397	0	11 (0.164)	56 (0.836)	2 (0.083)	22 (0.917)	0.332
c.758C>T	p.(A253V)	IC3	rs17653265	1 (0.043)	22 (0.957)	0	17 (1.000)	0.384	0	0	67 (1.000)	0	24 (1.000)	NA

Figure 1: Novel *GJA1* variant, c.366T>C (p.=) detected in one South African patient.