CONTRIBUTION TO THE STUDY OF EPIDEMIOLOGICAL FACTORS ASSOCIATED WITH SENSORINEURAL HEARING LOSS IN THE POPULATION OF SÃO TOMÉ AND PRÍNCIPE

CRISTINA MARIA DE PAIVA CHAVES LOPES CAROÇA TOMÉ DE JESUS

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Cristina Maria de Paiva Chaves Lopes Caroça Tomé de Jesus

Leader: João Paço, Professor Catedrático FCML

Co-leader: Susana Nunes Silva, Investigadora FCML

Helena Caria, Professora Auxiliar FCUL

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"Humanitarian aid is a fundamental expression of the universal value of solidarity among peoples, as well as a moral imperative."[1]

[1] (http://www.instituto-camoes.pt/cooperacao-para-desenvolvimento/ajuda-humanitaria#sthash.mEFTQ3BE.dpuf)

Summary

Otorhinolaryngology, like other medical and non-medical services, can contribute to its humanitarian support.

At the end of 2010, the ORL department of CUF Infante Santo Hospital, led by Professor João Paço, was invited to participate in humanitarian missions in one of the Portuguese-speaking countries, former Portuguese colony - Democratic Republic of São Tomé and Príncipe.

Without any knowledge of the main ENT pathologies we started this adventure.

From research on sub-Saharan African countries, an increase in deafness was expected from chronic otological infections. The mission was organized to have audiological assessment, ENT assessment and surgical interventions.

It was in February 2011 that we started the adventure through the hot lands of São Tomé and Príncipe.

Deafness was one of the main problems found, but contrary to what we expected, deafness was mainly sensorineural, affecting a high percentage of children, some without language acquisition and with bilateral profound deafness.

At the end of the first mission, the results led to the curiosity to understand the reason for the increased prevalence of sensorineural deafness, irreversible and with socio-cultural repercussions important for the development of a country.

The absence of clinical records, laboratory exams, imagiologic or audiologic tests led us to a greater clinical challenge of etiological cause of hearing loss in this country.

The present doctoral project was born at the end of the first mission.

Admission to the doctoral course was accepted in July 2011, and a challenge to research began.

The initial research project has undergone several modifications due to there are several diagnostic and economic constrictions.

In June 2013, in the scope of the doctoral course, during genetics curricular unit, and due to difficulties in carrying out complementary examinations in the island, born the idea was born to study the association between neurossensorial hearing loss and some of some of the most frequent hemoglobinopathies of the region. These hemoglobinopathiesare known to give some protection for the development of one of the major scourges of the country – Malaria. For that, was carried-out the collection of blood samples on Dried Blood Spot or Guthrie paper from São Tomé and Príncipe for analysis in Portugal.

Since São Tomé and Príncipe is an island that has been colonized for many years by Portuguese, we could hypothesize for the existence of an associated genetic factor, and for that reason the evaluation of the main mutations of our people should be investigated.

Since São Tomé and Príncipe is an island that has been colonized for many years by Portuguese, we hypothesize the existence of the same genetic factors, and for that reason the evaluation of the main mutations from Portuguese population was investigated.

In this country, they had a significant improvement in primary health care, since the implementation of humanitarian aid by the IMVF in cooperation with the Camões Institute. São Tomé and Príncipe have good vaccination coverage, but some vaccines that may have an impact on the appearance of deafness, such as rubella, were not implemented until this project.

Rubella is a known etiologic factor for congenital deafness. In São Tomé and Príncipe it is not possible to determine the existence of the disease by laboratory tests, being a pathology often forgotten at diagnosis. For this reason, the research project also contemplated the serological study of rubella in order to confirm the existence of the disease in the community and demonstrate the need to implement adequate measures - vaccination.

Knowing that there is not only an etiological factor for deafness, the birth of this project undoubtedly is stimulating for a clinician and a researcher. The curiosity that awakens and the attempt to stop this scourge that interferes so much with the development of a community, leads to the effort which is always rewarded.

Analyzing the main effects of the risk factors evaluated, it was found that female gender is in high risk to have HL than male gender; rubella disease could lead to HL if it is during gestational period and represents a double risk to HL. In this sample, self-report of malaria infection augments the risk of HL, around three times more; sickle cell disease (HbSS) have almost two and half folder risk to develop HL and sickle cell trait is protecting to HL; regarding G6PD non-B variant in association with gender, even though it was not found to be statistically significant, male gender almost duplicated the risk to develop HL; the mutations in GJB2 and GJB6 of control group, unilateral and bilateral did not reveal significant difference. There was a wide variability in mutations found, which led to conclude that it is not possible to define a standard and can be justified by the existence of multiple people to colonize the island.

Simultaneously with the etiological research project, there is a need to the diagnosis to minimize the effects of deafness. The acquisition of equipment for audiological evaluation (audiogram and auditory evoked potentials) and the acquisition and adaptation of hearing aids to children and young adults with work activity were born with the help of patronage, in order to reduce the effects of social isolation.

This project gave rise to another humanitarian project - "Projecto sem Barreiras" of the Fundação Calouste Gulbenkian with the IMVF and Universidade Católica Portuguesa which included the national implementation of the Sign Language of São Tomé and Príncipe (LGSTP) and the Neonatal Auditory Screening of São Tomé and Príncipe (RANSTP), with the acquisition of acoustic oto-emission equipment.

Undoubtedly a project that was born with Professor João Paço, cherished by all, with the ambition to contribute to the development of a country - Democratic Republic of São Tomé and Príncipe and scientific knowledge.

Dedication

To Ana Cristina and Pedro

Acknowledgment

To Ana Cristina and Pedro for the ability they had to share their mother with the work and numerous trips in mission.

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List of abbreviations

ABR	Auditory Brainstem Response
ACT	Artemisin combination therapy
AG	Aminoglycosides
ANSD	Auditory neuropathy spectrum disorder
ARHI	Age related hearing impairment
BBB	Blood-brain barrier
BIND	Bilirrubin-induced neurologic dysfunction
CDC	Center for disease control
CM	Cerebral malaria
CMV	Cytomegalovirus
CNS	Central Nervous system
CRS	Congenital Rubella syndrome
CS	Congenital Syphilis
Cx 26	Conexin 26
Cx 30	Conexin 30
DBS	Dried blood spot
DFNB1	Nonsyndromic hearing loss and deafness
DNA	Deoxyribonucleic acid
ENT	Ear, nose and throat diseases
EP	Endocochlear potential
FDA	Food and drug administration
G6PD	Glucose-6-Phosphate Dehydrogenase
GJB2	Gap Junction β2
GJB6	Gap Junction β6
GNI	Gross National Income
HA	Hemolytic anemia
HbAS	Sickle cell trait
HBB	Beta globin

HbS	Sickle Hemoglobin
HL	Hearing loss
HSV	Herpes simplex virus
IC	Intermediate cells
ICAM-1	Intercellular Adhesion Molecule 1
IEPs	Individualized education plans
IHC	Inner hair cells
ILD	Interaural level differences
IMVF	Instituto Marquês de Valle Flôr
IPAD	Instituto Português de Apoio ao Desenvolvimento
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
ITD	Interaural time differences
LDC	Least Developed Country
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NICU	Neonatal Intensive Care Unit
OAE	Otoacustic Emissions
OHC	Outer hair cells
OR	Odds Ratio
PCR	Polymerase chain reaction
PLHIV	People living with HIV
PNH	Paroxysmal nocturnal hemoglobinuria
RBC	Red blood cells
SCD	Sickle cell disease
SNHL	Sensorineural hearing loss
STP	São Tomé and Príncipe
UHL	Unilateral hearing loss
VLBW	Very low birth weight
WHO	World Health Organization
WT	Wild Type

1 Introduction

Hearing loss is a hearing impairment responsible for social isolation, professional, low education, and may even lead to depression and suicide (Olusanya et al. 2014).

Underdeveloped countries are those with the highest prevalence of deafness. In some situations, chronic infectious processes would trigger this hearing loss, lack of hygiene and health care as well as medical therapy shortages. Actually we recognize numerous causes of hearing loss and identified some of the most frequent and most of them could be prevented.

São Tomé and Príncipe is an island located in sub-Saharan Africa, discovered and colonized by the Portuguese people (João de Santarém and Pedro Escobar) in 1470. It was a Portuguese colony until 12th July, 1975, when it became independent. It is an underdeveloped country that is the subject of humanitarian aid.

In humanitarian aid during the project - "Health for All - Specialty" promoted by the Marquês de Valle Flor Institute (IMVF) and Camões Institute, a group of otolaryngologists found an increased prevalence of deafness in the STP islands. This diagnosis led to the emergence of a study on factors that could be contributing to the development of sensorineural deafness in São Tomé and Príncipe (Figure 1-1).

Figure 1-1 - First contact in São Tomé during the first mission, with a deaf child, that didn't answered to call. (Personal photo)

Being an island country with an endemic disease in the region - malaria, there was the need to assess the influence of some of the most frequent hemoglobinopathies in the region and the effect that may be triggering the hearing loss. Assuming also, that these are prevalent in these regions and are acting as a protective factor for severe malaria.

Another possible causes of hearing loss, and considering that it is a country previously colonized by diverse populations and cultures including European, is genetic. Consequently the identification of mutations associated with genetic deafness would be important. One of the objectives in the present study was be the identification of mutations in genes related with familial inheritance GJB2 (Cx26) and GJB6 (Cx30).

According to the statement that the majority of causes of hearing loss are preventable, and assessing the main characteristics of public health, such as common diseases and preventive actions in the country, it was found that vaccination for rubella and its diagnosis is not contemplated in São Tomé and Príncipe, have been a factor to be assessed and included in the investigation.

The etiology of hearing loss can't be attributed to one solely factor. This situation usually results from the interaction of numerous factors that influence the emergency of hearing loss.

The deaf people should be integrated in society. For this reason was created the sign language adapted to the country and also adapted hearing aids to promote hearing and stimulate oral language.

This was an enthusiastic work developed during five years of humanitarian missions and, on the end; this study will contribute also for the objective to improve social conditions of this country.

1.1 Socio-cultural context in São Tomé and Príncipe

São Tomé and Príncipe are a group of islands of volcanic origin, located in the African equatorial zone (Figure 1.1-1), with an approximate surface of 1,000 km2, with about 187,000 inhabitants (CENSUS 2012).

Figure 1.1-1 - Map of São Tomé and Príncipe.
(http://www.ezilon.com/maps/africa/são-tomé-and-principe-maps.html)

At 1471, João de Santarém and Pêro Escobar discovered the island of São Tomé in one of the attempts to reach the sea route to India.

They landed in the north of the island on December 21th, 1470, giving the name of the day of the saint to the island - São Tomé [Figure 1.1-2A and 1.1-2B), and later on 17th January of 1471, Santo António Island of Principe, city of Santo António.

Figure 1.1-2A - Pattern of discoveries - standard of Anobóm and Figure 1.1-2B - Ana Chaves Baia where Portuguese navigators arrived at São Tomé Island. (Personal photo)

The islands were uninhabited, and they were delivered to the captains of the granary João de Paiva (São Tomé, 1485-1522) and António Carneiro and his descendants (Santo António / Príncipe, 1500-1753).

Several attempts at settlement were made, having been extremely difficult, serving as a warehouse for slavery from the Guinean coast.

Only after about 20 years (1493) was it settled under the command of Alvaro Caminha by "gentlemen and merchants", degraded and about 2000 Jewish children (in which only 600 survived in 1506).

These Jewish children were from the Jewish movement fleeing from Spain in 1492. They negotiated with King John II to pay an individual tax when they crossed the border and could stay here for 8 months, and king should have ships to they leave Portugal. After finishing this period anyone who could not leave Portugal would be

declared a slave. It was in this context that about 2000 Jewish children under the age of 8 embarked for São Tomé to learn Christian doctrine and become good Christians, helping the settlement of that island through the crossing of the children with the inhabitants of the islands.

It was at this time that, taking advantage of the acquired knowledge in the Atlantic islands that began the sugar cane plantation, beginning the export to the Northern European trade.

Despite the success of sugar economic exploitation, social conflicts between a white, bellicose minority among themselves and sovereign members; and a black majority subject to harsh conditions of slavery with attitudes of revolt.

In this way, the population of the islands suffered marked reductions.

During the period from 1540 to 1710 São Tomé was the target of invasions by other peoples, namely Angolans, Dutch, and French. All this political turmoil led to the change of the capital of the country to the Príncipe in 1753. After about 100 years it returned to the city of São Tomé. Slavery was abolished in 1875. Later the first political parties appeared and on 12th July 1975 is enacted independence the São Tomé and Príncipe (Malheiro & Morais 2013).

Since the independence it was created the Democratic Republic of São Tomé and Príncipe.

The weather is hot and humid, with the rainy season, from October to May and the dry season from June to September.

Socio-economic development is low, being STP included in underdeveloped countries group with poor hygienic and sanitary conditions.

The age-sex pyramid of population presents a broad-based, classically associated with underdeveloped countries, with rapid growth and low expectancy live (Figure 1.1-3).

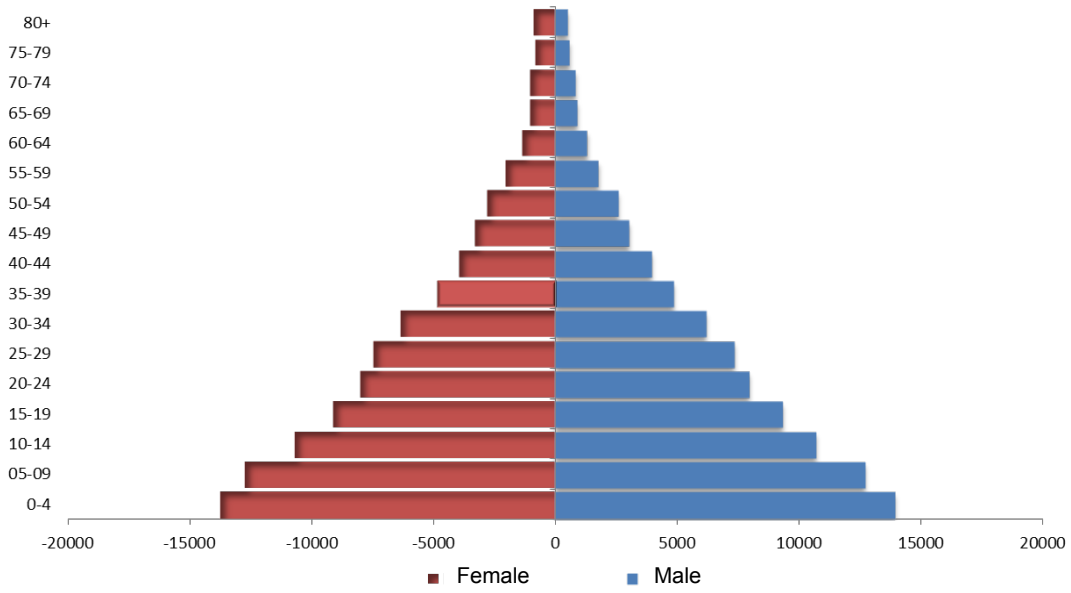

Figure 1.1-3 - Age-sex pyramid of Republic Democratic of São Tomé and Príncipe (Instituto Nacional de Estatística 2012).

The average life expectancy is short when compared with developed countries, usually because the scarcity of medical care, being 65.2 years in overall population. Female gender has a longer life expectancy (68.7 years) than males (62.1 years) as presented in Figure 1.1-4.

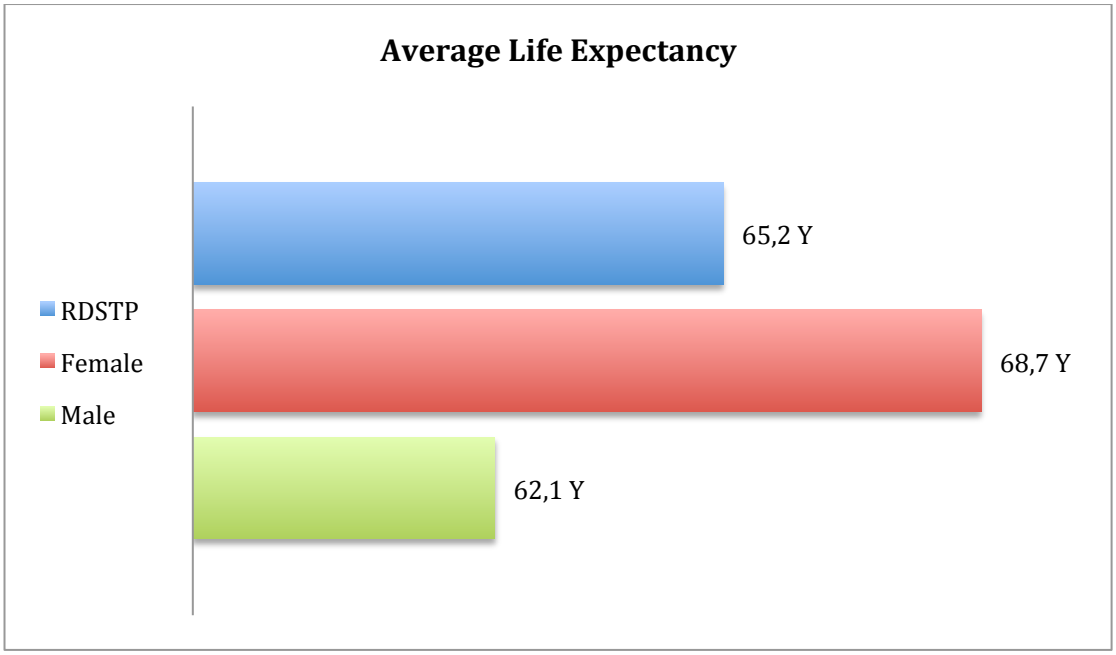

Figure 1.1-4 - Average life expectancy in Population of São Tomé and Príncipe (Instituto Nacional de Estatística 2013).

São Tomé and Príncipe is divided into seven administrative districts: Água Grande, Mé-Zochi, Cantagalo, Caué, Lembá, Lobata and Região Autónoma do Príncipe, with a higher density population in Água Grande district (Figure 1.1-5), each with different number of individuals.

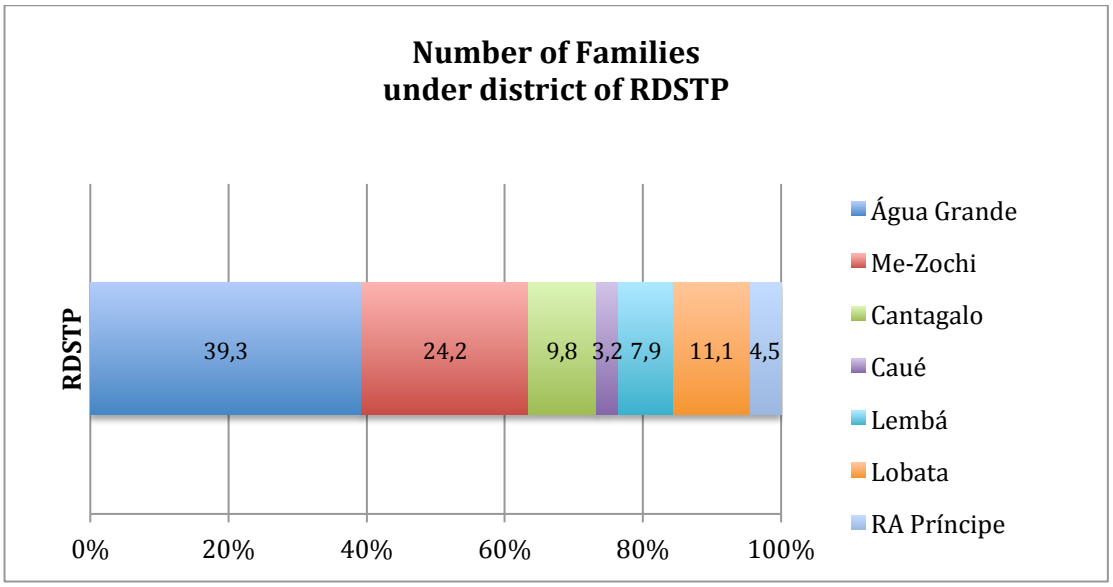

Figure 1.1-5 - Number of Families under district of Republic Democratic of São Tomé and Príncipe (Instituto Nacional de Estatística 2013).

Approximately 67% of the population live in urban areas, while the remaining 33% in rural areas (Instituto Nacional de Estatística 2013).

The male gender is more prevalent in rural areas, while women in urban areas.

The water supply for drinking in families is supplied mostly by public net at about 83.6%, and may be appealed by spring or river / stream at 6.9% or 6.4% respectively (Instituto Nacional de Estatística 2013).

The houses (Figure 1.1-6) in all districts are mostly constructed (80.1%)(Instituto Nacional de Estatística 2013).

Figure 1.1-6 – Example of wood constructions. (Personal photo)

In accommodation there is not always sanitary facility (57.4%), being the most used the latrine installation (24.6%) (Instituto Nacional de Estatística 2013).

The most commonly used fuel for cooking is the wood, followed by the oil and then coal. Electrical power is an energy resource at the time of CENSOS inquiry (2012) was not in all homes of STP. Only in 57.9% of households had electric power in the accommodation, the Água Grande district and Príncipe was more favored by the existence of electricity, than Caué and Cantagalo districts that are the most devoid of electric energy.

Even all districts are dedicated to farming, Mé-Zóchi has a higher activity and Príncipe has a lower activity. The male sex is who dedicate more to agriculture activity.

In São Tomé and Príncipe 14.5% of the female population over 15 years old, has illiteracy, while in male, illiteracy reaches 5.06%. In all districts the female gender has higher illiteracy rate, being always higher than the male (more than double) (Figure 1.1-7) (Instituto Nacional de Estatística 2013).

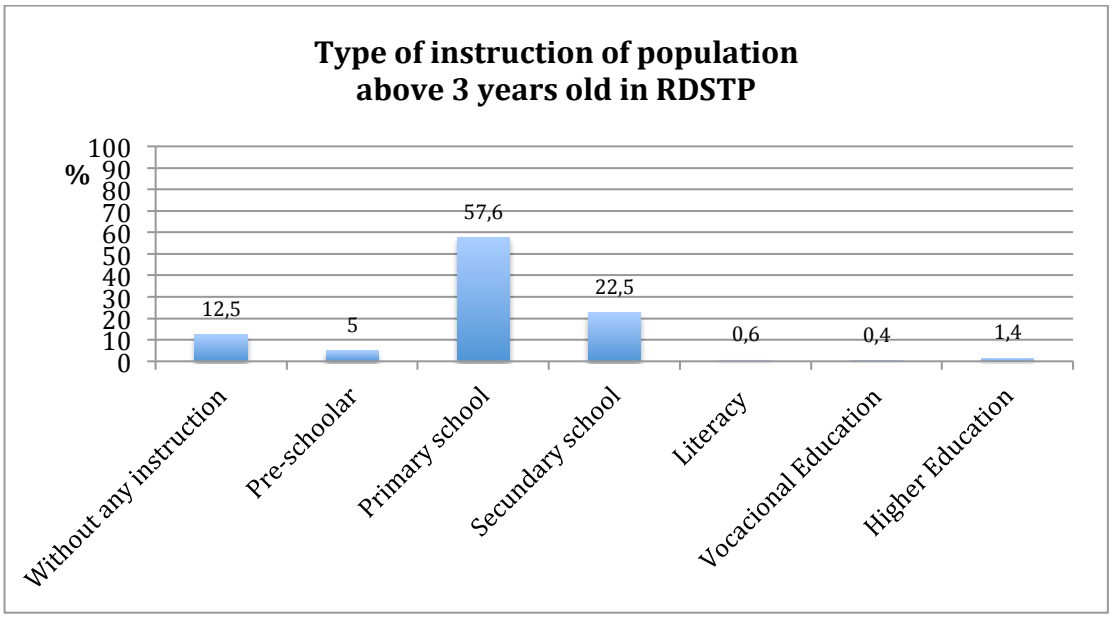

Figure 1.1-7 - Level of instruction of population above 3 years old in RDSTP (Instituto Nacional de Estatística 2013).

From 3 years old, 5% attend preschool, 57.6% attend primary school and 22.5% secondary education. Still consider that 0.6% has only literacy, 1.4% higher education and 0.4% vocational education. Note that even 12.5% showed without any instruction (Instituto Nacional de Estatística 2013).

According disabilities, in the result of CENSUS 2012, about 29.1% report severe hearing impairment.

1.2 Humanitarian Missions in São Tomé and Príncipe

1.2.1 Five years of Humanitarian Missions in São Tomé and Príncipe

The data presented has been published by Caroça C and Paço J (2016) as is possible to see in

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ARTIGO DE REVISÃO

Five Years of Humanitarian Missions in São Tomé and Príncipe

Cinco Anos de Missões Humanitárias em São Tomé e Príncipe

Cristina Caroça, João Paço[1,2]

Autor Correspondente:
Cristina Caroça [cristinacaroca@netcabo.pt]

ABSTRACT

INTRODUCTION: Since February 2011, a group of otolaryngologists from CUF Infante Santo Hospital, a private healthcare unit in Portugal, invited by a non-governmental organization to provide equipment and properly skilled professionals to help and treat otolaryngology diseases in São Tomé and Príncipe. These missions included surgical procedures, consultation and hearing evaluation.

METHODS: This work is a retrospective chart review of all otolaryngology cases performed during these missions since 2011 to 2016, and what we done during mission.

RESULTS: During these missions, we have found some common pathologies. Deafness is the most prevalent after which follows the lymphoid tissue of oropharynx pathology. On these 18 missions a total of 1057 otolaryngology assessments were conducted. The main surgery was oral cavity with adenoidectomy and tonsillectomy. The results of all audiological tests performed during these 18 missions, reveal an increase of sensorineural deafness.

DISCUSSION: These missions' purpose is to allow healthcare access to all, to identify people with hearing and language problems and to adapt prosthetics, if possible, mainly for children and young adults.

We have witnessed a considerable improvement on the children to whom we have adapted prosthetics. Some of them return to school, have friends and became more social.

As the result of this work, we conclude that all Humanitarian Missions must be adapted to each country's needs as we have done over the past five years.

KEYWORDS: Humanitarian Missions; Otolaryngology; Sensorineural Hearing Loss; São Tomé and Príncipe

1. Otolaryngology Department, Nova Medical School/Faculty of Medical Sciences, Universidade Nova de Lisboa, Lisboa, Portugal
2. Otolaryngology Department, CUF Infante Santo Hospital, Lisboa, Portugal

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ARTIGO DE REVISÃO

RESUMO

INTRODUÇÃO: *Desde fevereiro de 2011, um grupo de Otorrinolaringologistas do Hospital CUF Infante Santo, tem sido convidado, por uma organização não-governamental, para integrar missões humanitárias em São Tomé e Príncipe. Esta equipa médica tem sob a sua responsabilidade a organização e disponibilização dos recursos humanos e dos equipamentos necessários para efetuarem procedimentos cirúrgicos, consultas e rastreios auditivos aos que têm patologias em Otorrinolaringologia.*

MÉTODOS: *Este trabalho resulta de uma revisão retrospetiva de todos os casos de Otorrinolaringologia com que esta equipa contactou, entre 2011 e 2016 durante as missões humanitárias supramencionadas. É também o resultado de todas as atividades que foram desencadeadas no decurso das referidas missões.*

RESULTADOS: *No decurso destas missões encontrámos patologias comuns. Entre as mais recorrentes destacamos os casos de surdez e de patologias dos tecidos linfoides no contexto de uma orofaringe. Em 18 missões foram efetuados 1057 rastreios em Otorrinolaringologia e as principais cirurgias foram efetuadas na cavidade oral com recurso a adenoidectomia e a amigdalectomia. Os resultados de todos os testes conduzidos, durante as 18 missões, revelam um aumento da surdez neurossensorial.*

DISCUSSÃO: *Estas missões pretendem promover o acesso a todos, na medida do possível, aos cuidados de saúde, no contexto da Otorrinolaringologia. Para tal, procede-se à identificação dos que têm problemas auditivos e/ou problemas relacionados com a linguagem. Neste sentido, também procurámos adaptar próteses, quando possível, sobretudo em crianças e jovens adultos. Constatámos um bom desenvolvimento das crianças às quais foi efetuada uma adaptação das próteses existentes. Algumas regressaram à escola, fortaleceram amizades, tornando-se assim mais sociáveis.*

Em última análise, concordamos que as missões humanitárias têm que ser adaptadas às especificidades do país onde decorrem, tal como temos feito nestes últimos cinco anos.

PALAVRAS-CHAVE: *Missões Humanitárias; Otorrinolaringologia; Surdez Neurossensorial; São Tomé e Príncipe*

INTRODUCTION

Since February 2011, a group of otolaryngologists from a private hospital in Portugal - hospital CUF Infante Santo was invited by a NGO to provide equipment and properly skilled professionals to help and treat otolaryngology diseases in São Tomé and Príncipe. These missions included surgical procedures, consultation and hearing evaluation.

São Tomé and Príncipe is an archipelago in western equatorial Africa, near Gabon, Equatorial Guinea, Camaroon and Nigeria. Was discovered by Portuguese explorers in 1470 (João de Santarém and Pero Escobar), who decided that these islands were good locations for bases to trade with the mainland. São Tomé and Príncipe have economic and political autonomy since 1975. The official language is Portuguese.[1]

With a resident population of approximately 187.000 inhabitants, this Country shows a low average age distribution (17-18 years) who have a low socioeconomic power and poor sanitary conditions. Many have also suffered from Malaria, a public health infection.[2-4]

The main hospitals in São Tomé and Príncipe are Hospital Ayres de Menezes in São Tomé city, and Hospital Manuel Quaresma Dias da Graça in Príncipe island.

The IMVF (Institute Marquês of Valle Flôr) is an NGO (Non-Governmental Organization) with the objective of implementing in this country the "Health for All – Medical Specialities", supported by Portuguese Institute to Development Support – Camões Institute and Callouste Gulbenkian Foundation in partnership with the Ministry of Health of São Tomé e Príncipe.

Doctors from the University Clinical Center of Hospital CUF Infante Santo embraced this project, since February of 2011. At the very beginning an Humanitarian Mission in this Country consists of observing patients during a regular consultation, surgery and hearing assessments for one week and then three or four times per year.

The aim was to be part of the project Health for all - Medical Specialities and to implement at São Tomé and Príncipe assessments and surgical interventions. These were our top priority. Due to absence of audiological exams for more than 30 years in these islands, our team also brought them. Thus, this project's global aim was not only to consolidate the Health System by bringing specialized medical care in São Tomé and Príncipe but also to complement its preventive and primary healthcare with specialized assistance for secondary and tertiary care.

FIGURE 1. First mission (February 2011) and five years later (February 2016).

FIGURE 2. Briefing when arrive, before work. (Feb 2011, Feb 2016).

The first team of five arrived on February of 2011: two ENT doctors, two nurses and one clinical audiologist with audiometric equipment and one year after we initiated speech therapy and hearing aids (Fig. 1). Each team of doctors tends to include one or two senior surgeons and one other in acadamic training. Nurses are specialized in ENT operating room.

Before arriving in São Tomé and Príncipe for these missions we were already expecting to find problems related to ear infections, chronic otitis media and conductive hearing loss.

Before going, we need to prepare all equipment and material. For the assessment, audiological evaluation and operating room. Surgical material, supplies and equipment arrived in STP by plane and boat.

Before going to São Tomé we had acquired a microscope with possible connection to a monitor to allow showing and explaining the surgical intervention system, to the local professionals.

As we arrive all mission members plan the activities for the week (Fig. 2).

The hospital has 2 operating rooms for the whole country. These are spacious, each has a large window and air conditioning (Fig. 3). Yet, the lack of reliable electricity and water supply compromises the proper delivery of oxygen and suction as well as cleanliness. The sterilization equipment is quite basic and it results on the instruments gradual degradation. Besides, dressings are often washed and reused.

The post-surgical recovery area is located on the hall right outside the operating room and has no nursing staff.

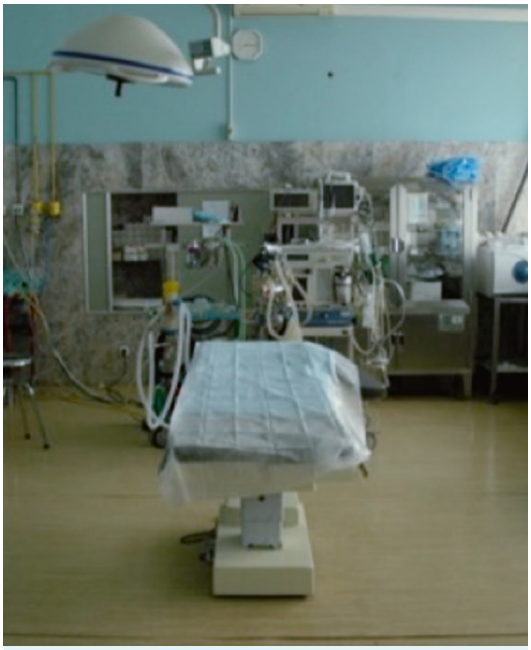

FIGURE 3. Operating room – Hospital Ayres de Menezes.

TABLE 1. Common pathologies found during missions.

ENT Assessment	Audiology	Operating Room
Chronic adenoiditis and tonsillitis	**Sensorineural Hearing Loss (NSHL) severe to profound**	**Adenoidectomy**
Simple chronic middle ear disease and Cholesteatoma	Cofosis	**Tonsillectomy**
Otosclerosis	Children and adults without oral language	Ear surgery
Thyroglossal duct cyst		Head and neck surgery
Branchial cyst and fistula		Biopsies
Tumors		

Otolaryngology assessments are held in a pavilion at Ayres de Menezes Hospital which shares a physical space with ophtalmology. All assessments take place on the arrival day and on the following 3 afternoons.

METHODS

This work is a retrospective chart review of all otolaryngology cases observed during these missions, which occurred from 2011 until 2016. It also includes all activities and actions taken in this context.

RESULTS

During these missions, we have found some common pathologies (Table 1).

Deafness is the most prevalent pathology followed by the lymphoid tissue of oropharynx.

After the first mission, we found that we needed to change our organization, because we found a high prevalence of sensorineural hearing loss. So, we needed to acquire auditory brainstem response to assess what we could not with pure tone audiogram.

The need for fitting hearing aids, also required speech therapy, to improve hearing and oral language. In some cases, it was impossible to acquire oral language and lead the necessity to develop a language for communication - sign language.

The mission grew after 1 year with one more audiologist for hearing aids and a speech therapist. After one year, a teacher of sign language has been associated to help them creating this language.

In these 18 missions 1057 otolaryngology assessments were conducted. Patients were referred for consultation by radio or television (Fig. 4).

Operating room activities are performed on the five working days during the morning and are always prepared one day in advance. Before surgery and early in the morning, all patients were placed in a room next to the operating room for preparation - veins were channeled (Fig. 5). All men and women, children and adults wait all morning for it.

Only sixteen of eighteen missions were surgical and during all missions we made 465 surgical procedures (Fig 6). Surgery of the lymphoid tissue and less complex surgery is done which allows larger number of patients. The main surgery is oral cavity with adenoidectomy and tonsillectomy. Here we have also chronic otitis media surgery and otosclerosis surgery. The rhinologic surgery is mainly chronic rhinosinusitis with polyps, rhinitis and masses. The cervical surgery consists in glands surgery and brachial masses.

After the mission all patients are re-observed by a local otolaryngology doctor who is on a temporary assignment and not a native of São Tomé and Príncipe.

FIGURE 4. Patients are referred for consultation by radio or television.

FIGURE 5. Before surgery early in the morning all patients are placed in a room next to the operating room and are all prepared for surgery - veins are channeled.

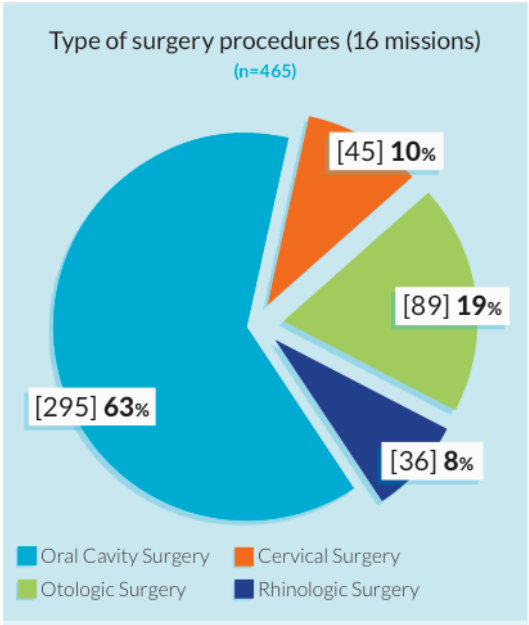

FIGURE 6. Surgical statistic in 18 missions (16 surgical missions).

FIGURE 7. Hearing tests are performed in an isolated room, to minimize noise as much as possible.

We have found a high prevalence of sensorineural hearing loss so we have started audiology assessments on the fourth mission. Since then we are completing a survey to identify risk factors, personal history and record all examinations.

Exactly 1377 audiology assessments were held and included either a tonal audiogram or auditory brainstem response (ABR), in compliance with each individual in a total of 2073 exams (1705 assessments made by tonal audiogram and 368 made by ABR).

Assessments were recorded for each ear. In some cases, only one ear was evaluated, because they have abundant purulent otorrhea.

Hearing tests are performed in an isolated room. This is a closed room with no fan or air conditioning (Fig. 7). This is to minimize noise as much as possible.

The results of all audiological tests performed during these 18 missions, reveal an increase of sensorineural deafness, instead a high prevalence of conductive or mixed hearing loss as we expected (Fig. 8). More than 50% of all ears have from slight to profound deafness, according to the classification adopted by the World Health Organization (Fig. 9). Seeing that normal hearing is 25dB or better.[5]

According to our results we structured the intervention to promote communication and integration of these individuals, adapting earing aids, if possible, and trying to acquire oral language. If we could not have oral language, we send to sign language (Fig. 10).

Since February 2012, seventy-eight hearing aids were adapted (Fig. 11). These prosthetics were offered to children and young adults in STP, especially for those who are working or studying.

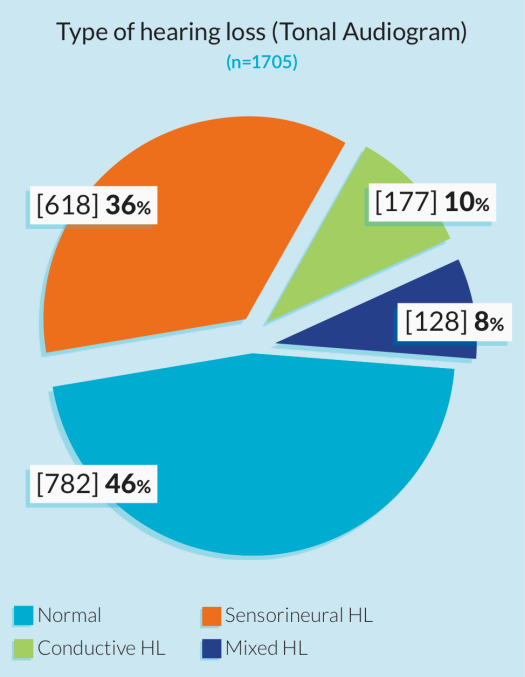

FIGURE 8. Type of hearing loss obtained during 18 missions.

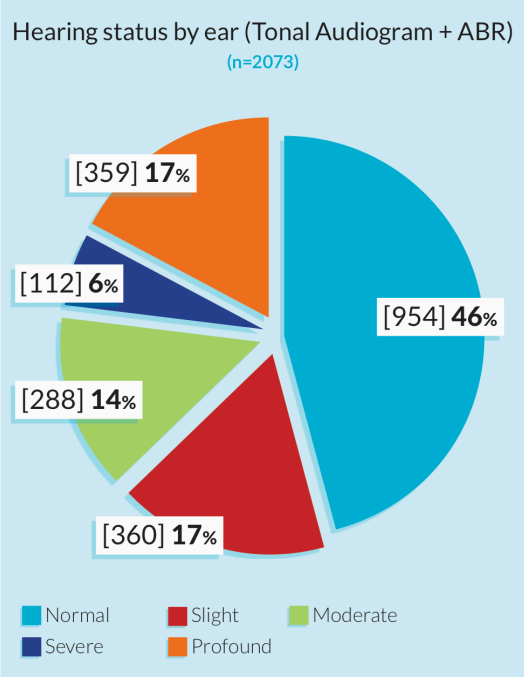

FIGURE 9. Hearing status by ear analyzed during 18 missions.

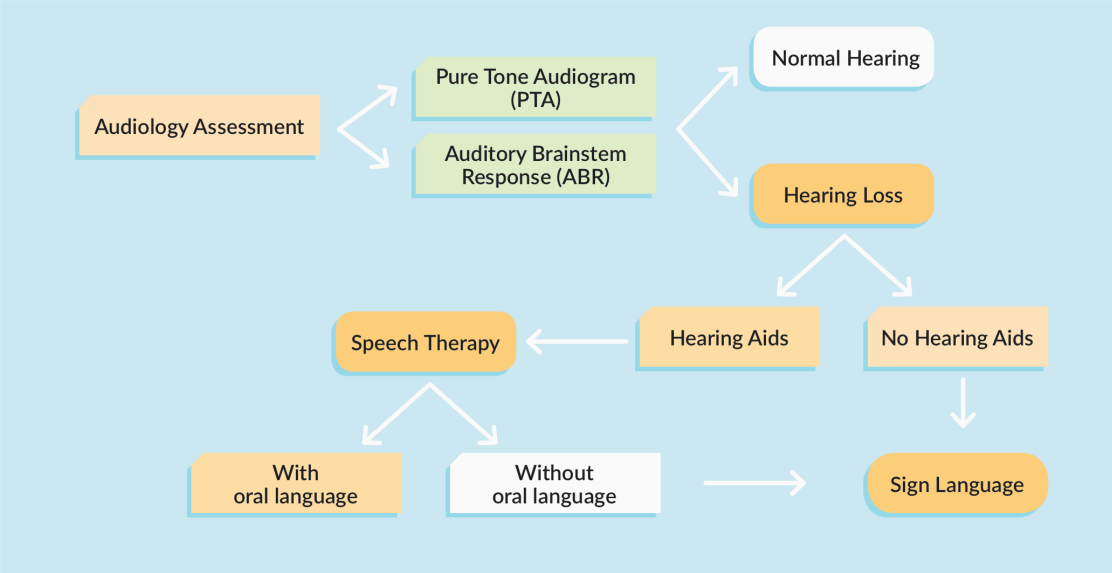

FIGURE 10. Scheme of actuation during the Missions.

In speech therapy we try to have a caretaker present (Fig. 12). These sessions are also important for training the caretaker. For the rest of the year he will be responsible to carry on the work with the children. Some acquired oral language and others did not.

This project leads us to fulfill the need for sign language by creating a sign language alphabet, started in February 2013. The sign language dictionary was created to widely spread the São Tome e Príncipe sign language (Fig. 13).

Since November 2014 we had started neonatal hearing screening in Hospital Ayres de Menezes and in February 2015 in Hospital Manuel Quaresma Dias da Graça (Fig. 14), with an actuation protocol (Fig. 15).

FIGURE 11. Hearing aids adapted in children and young adults that are studying or working. This is a photo of a child in the left on February 2012, when adapted to hearing aids and in the right in last mission (February 2016).

FIGURE 12. In speech therapy we try to have a caretaker present.

FIGURE 13. This project leads us to fulfill the need for sign language by creating a sign language alphabet. The sign language dictionary was created to widely spread the São Tomé e Príncipe sign language.

DISCUSSION

São Tomé and Príncipe is a small country in extreme need. They have limited access to surgical expertise, otolaryngology and audiology evaluation.

These humanitarian missions try to make healthcare accessible to all as well as to identify people with earing and language problems so prosthetics can be adapted, if possible, mainly for children and young adults.

We mainly perform surgical procedures for the lymphoid tissue as we try to treat the vast majority of citizens.

By analyzing Chadwick J *et al* paper, we have the advantage that an otolaryngologist lives in that country. This allows some surgeries that we couldn't do if a colleague wasn't available to take care of patients for the post-surgical time. Yet for any doubt he has our contact to ask for help.[6]

From our experience we agree that we are often unable to make the follow-up of a ear surgery as most patients do not return for the post-surgery follow-up. According to Horlbeck G *et al*, they got good results related with otology surgery but that is based on the follow-up of 64% of cases only. [7]

The most complex cases we must evacuate to Portugal.

We have noticed a positive development in children to whom we have adapted their prosthetics. Some of them returned to school, have friends and became more social.

During the missions, clinical sessions were held, addressing the results of previous missions and clinical topics.

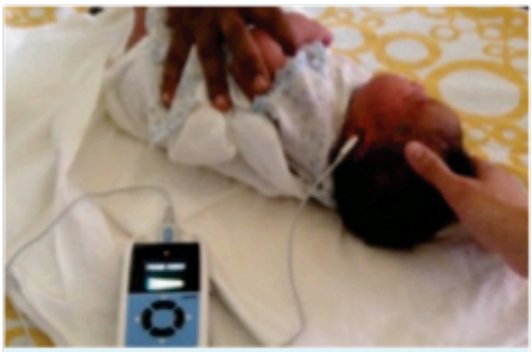

FIGURE 14. Neonatal hearing screening in Hospital Ayres de Menezes.

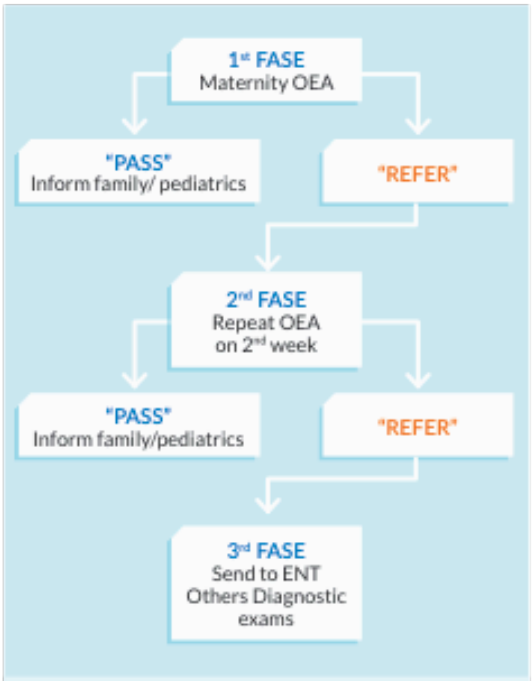

FIGURE 15. Protocol for neonatal hearing screening in São Tomé and Príncipe.

There are otolaryngology conditions that other doctors and health personnel can learn to take care of. That was for that reason, that we started the ENT seminars. First ENT seminar was about ear pathology, second on nasal and oropharynx pathology, third on larynx and cervical pathology and the last on urgent pathology (Fig. 16).

These Missions are projected for otolaryngology and audiology assessment, surgical procedures, speech therapy, ear rehabilitation and finally sign language. We form a multidisciplinary team.

During these years an investigational project is running to determine why they have a high prevalence of sensorineural hearing loss.

CONCLUSIONS

For five years we have carried hope for the people of São Tomé and Príncipe. Yet it is necessary to continue our commitment with these friendly and welcoming people. In order to prevent social problems it is important to secure the hearing screening of newborns so hearing loss is identified as soon as possible and sign language is implemented in time. Furthermore, surgical procedures, during humanitarian missions promote best comfort to patients who have their families near them while providing a reduced economic cost.

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Nurses: Andreia Torrão, Barbara Carvalheda, Carla Ramalho, Cláudia Guiomar, Isabel Aragoa, Isabel Beirão, Elsa Mota, Madalena Januário, Paula Afonso, Patricia Neves, Sandra Paço, Sara Simas;

Audiologists: Adriana Amarante, Cláudia Sobral, Cláudia Sofia Silva, Daniela Batista, Diogo Ribeiro, Nadine Martins, Vera Lourenço, Tania Martins, Sandy Batista, Wendy Lopes;

Speech Therapists: Ana Mafalda Almeida, Tânia Constantino.

CONFLICTS OF INTEREST: The authors have no conflicts of interest to declare.

FINANCING SUPPORT: This work has not received any contribution, grant or scholarship.

CONFIDENTIALITY OF DATA: The authors declare that they have followed the protocols of their work center on the publication of patient data.

PROTECTION OF HUMAN AND ANIMAL SUBJECTS: The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

FIGURE 16. Seminars presented during this five years of missions.

CONFLITOS DE INTERESSE: Os autores declaram a inexistência de conflitos de interesse na realização do trabalho.

FONTES DE FINANCIAMENTO: Não existiram fontes externas de financiamento para a realização deste artigo.

PROTEÇÃO DE PESSOAS E ANIMAIS: Os autores declaram que os procedimentos seguidos estavam de acordo com os regulamentos estabelecidos pelos responsáveis da Comissão de Investigação Clínica e Ética e de acordo com a Declaração de Helsínquia da Associação Médica Mundial.

CONFIDENCIALIDADE DOS DADOS: Os autores declaram ter seguido os protocolos do seu centro de trabalho acerca da publicação dos dados de doentes.

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1.2.2 Hearing Loss in São Tomé and Príncipe: 2 Years of Humanitarian Missions

The data presented has been published by Caroça C, Campelo P, Silva SN and Paço J (2016) as is possible to see in

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Surdez em São Tomé e Príncipe: Análise de 2 anos de missões humanitárias

Hearing loss in Sao Tome and Principe: 2 years of humanitarian missions

Cristina Caroça • Paula Campelo • Susana Nunes Silva • João Paço

RESUMO

Introdução: A audição é um importante sentido para a integração de um indivíduo na comunidade, por esse motivo é importante a identificação dos fatores associados à surdez. Neste trabalho propomos revelar os dados audiológicos verificados durante 2 anos de missões humanitárias de Otorrinolaringologia e a identificação de eventuais fatores de risco para a surdez.

Desenho do Estudo - Material e Métodos: Avaliámos todos os indivíduos que procuraram a consulta de audiologia no decurso das missões humanitárias em São Tomé e Príncipe de 2012 a 2014. Foram observados por um médico de otorrinolaringologia, onde para além de observação foi efetuado um questionário clínico onde foram pesquisados fatores de risco (história familiar de surdez, co-sanguinidade, história de malária clínica, terapêutica antimalárica, história gestacional e peri-parto, história otítica, história de traumatismo craniano) e realizada avaliação audiológica por um audiologista. Os dados foram processados e analisados numa base de dados da IBM SPSS 20.0.

Resultados: Dos 721 indivíduos observados, foram excluídos 77 por não conterem registo de avaliação audiológica, obtendo 644 registos clínicos para o estudo. Verificámos uma prevalência de surdez neurossensorial de 35,7%, surdez de condução de 2,9% e mista de 1,9%. Os restantes indivíduos eram normouvintes (59,5%). Destes indivíduos normouvintes, 26% eram indivíduos apenas com um ouvido ouvinte – surdez unilateral. Dos factores de risco analisados a história de malária clínica foi o único factor de risco que se revelou mais significativo.

Discussão: A prevalência de surdez na amostra avaliada foi elevada, afastando-se dos valores esperados. Numa população de um país subdesenvolvido, e de acordo com os dados da Organização Mundial de Saúde, seria de esperar uma maior prevalência da surdez de condução e mista. No entanto, neste trabalho verificamos uma maior prevalência da surdez neurossensorial. A malária ou mesmo a terapêutica instituída no seu tratamento podem estar a contribuir para os resultados audiológicos obtidos.

Conclusão: A surdez nem sempre tem uma causa única. Existem um conjunto de fatores que ao interagirem podem desencadear o aparecimento de surdez. Neste trabalho a malária revelou ser o fator de risco mais significativo na associação com a surdez neurossensorial em São Tomé e Príncipe. Mais estudos estão a ser realizados na identificação de outros fatores de risco.

Palavras-chave: Malária; São Tomé e Príncipe; Surdez Neurossensorial

ABSTRACT

Introduction: Hearing is an important sense for the integration of an individual in the community; therefore the identification of factors associated with deafness is important. In this work we reveal the audiometric data verified during 2 years of Otorhinolaryngology humanitarian missions and the identification of possible risk factors for deafness.

Study design - Material and Methods: We evaluated all individuals who have sought audiology consultation in the course of humanitarian missions in Sao Tome and Principe from 2012 to 2014. All patients were observed by an ENT doctor, held earing evaluation by an audiologist and answered a clinical questionnaire in which risk factors were accessed (family history of deafness or co-sanguinity, previous clinical malaria, previous malaria treatment, gestational and peri-partum history, previous ear infections and history of head trauma). Data were processed and analyzed in IBM SPSS 20.0 database.

Results: Of the 721 observed individuals, 77 individuals were excluded because they do not contain audiological registration, obtaining 644 medical records for the study. We found a prevalence of sensorineural hearing loss of 35.7%, conductive deafness of 2.9% and 1.9% mixed. The remaining individuals were normal hearing (59.5%). From the normal hearing individuals, 26% had a unilateral deafness. Of the risk factors analyzed, the history of clinical malaria was the only risk factor that showed more association.

Discussion: Away from the expected values, particularly with regard to the type of deafness encountered, the prevalence of hearing loss in the study sample was high with sensorineural

Cristina Caroça
Médica ORL Hospital CUF Infante Santo, Assistente Convidada de ORL da FCML-UNL

Paula Campelo
Médica Interna ORL Hospital CUF Infante Santo

Susana Nunes Silva
Investigadora, Centro de Toxicogenómica e Saúde Humana (ToxOmics), NOVA Medical School/ FCML-UNL

João Paço
Médico ORL, Diretor Clínico Hospital CUF Infante Santo; Regente da Unidade Curricular de ORL da FCML-UNL

Correspondência:
Cristina Caroça
Av. Infante Santo, 34, 6º
1350-179 Lisboa
Tm: 918902967
Mail: cristinacaroca@netcabo.pt

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hearing loss as the most prevalent type. According to WHO in developing countries, conductive and mixed deafness were expected to have a higher prevalence Malaria and antimalarial treatment may be contributing to the development of deafness. Conclusion: Deafness does not always have a single cause. A number of interacting factors may trigger the onset of deafness. In this paper, malaria seemed to be the most significant risk factor in association with sensorineural hearing loss in Sao Tome and Principe. Further studies are being made on the identification of other risk factors.
Keywords: Hearing Loss, Sensorineural; Malaria; Sao Tome and Principe

INTRODUÇÃO

A audição é um dos sentidos importantes para a aprendizagem e integração do indivíduo numa comunidade ouvinte.

Em qualquer idade, a surdez tem um impacto profundo na comunicação interpessoal, psicológica, bem-estar, qualidade de vida e independência económica.[1]

De acordo com a Organização Mundial de Saúde (OMS) a audição na criança é um dos sentidos mais importantes para o desenvolvimento da linguagem. A criança com surdez apresenta um risco aumentado de baixa aprendizagem, abuso físico, social, emocional e sexual, podendo mesmo levar à morte. Por outro lado, no adulto a surdez está associado ao isolamento social, estigmatização, abuso, distúrbios psiquiátricos, depressão, dificuldade no relacionamento com parceiros ou crianças, escolha de carreiras profissionais restritas e stress ocupacional.[1]

Em 2012, existiam no mundo cerca de 141 milhões de surdos e a maioria, cerca de 127 milhões, nos países em desenvolvimento.1 A incidência estimada de surdez congénita ou de início precoce nestes países é de 6:1000 nados vivos, cerca do triplo da que ocorre nos países desenvolvidos.[1]

A surdez tem vindo a aumentar desde 1995 até 2011. O grande peso deste aumento vem dos países subdesenvolvidos, sobretudo Ásia e África subsaariana.[1]

O custo-eficácia da prótese auditiva, implante coclear e outros equipamentos auditivos tem-se demonstrado proibitivo para a maioria dos países subdesenvolvidos ou em desenvolvimento, devido aos custos na aquisição e manutenção destes equipamentos.[1] Por esse motivo é importante atuar na prevenção e detecção precoce da doença.

A República Democrática de São Tomé e Príncipe (STP) é um país localizado na zona equatorial, próximo do Gabão, Guiné Equatorial e Nigéria. Ocupa uma área de aproximadamente 1000 km^2, constituída por duas ilhas (Ilha de São Tomé e Ilha do Príncipe) e ilhéus adjacentes (Rolas, Cabras...), num total de sete distritos administrativos, sendo a capital a cidade de São Tomé.

Atualmente apresenta cerca de 187000 habitantes, com uma base de pirâmide populacional alargada, representando uma população muito jovem de acordo com os resultados do CENSUS 2012.[2]

O desenvolvimento socioeconómico é baixo, incluindo-se a República Democrática de São Tomé e Príncipe no grupo dos países subdesenvolvidos.

Devido à sua situação económica, STP é alvo de ajudas humanitárias, donde se destaca a atuação da organização não-governamental - Instituto Marquês de Valle Flor (IMVF), com o projeto - "Saúde para Todos - Especialidades".

Nunca esteve disponível em STP, avaliação audiológica, pelo que não é conhecido o perfil audiológico desta população.

No que diz respeito à saúde pública desta população, existe uma patologia que os assombra desde há longo tempo. A malária esteve praticamente erradicada há cerca de 40 anos atrás. Posteriormente houve um retrocesso com o aumento da incidência mas nos últimos anos tem-se verificado uma diminuição dos casos de malária clínica.[3,4]

Em fevereiro de 2011, no âmbito do projeto "Saúde para Todos Especialidades" uma equipa constituída por médicos, enfermeiros e audiologista iniciou missões humanitárias de uma semana com periodicidade de três a quatro vezes por ano.

Após a primeira missão verificámos um número aumentado de indivíduos que recorriam à consulta do Hospital de São Tomé por surdez unilateral e bilateral, ligeira a profunda.

A constatação de um número elevado de doentes com surdez, levou-nos à necessidade de insistir nos rastreios e iniciar medidas para tentar minimizar o isolamento, insucesso ou mesmo o abandono escolar.

Perante a situação clínica encontrada, tornou-se importante caracterizar a população avaliada em consulta de audiologia, estudando a prevalência da surdez e identificando possíveis factores de risco associados ao aparecimento da surdez.

MATERIAL E MÉTODOS

Analisámos os casos avaliados durante os anos 2012 a 2014, nas missões humanitárias de otorrinolaringologia (consulta de audiologia) através de um estudo observacional.

Os casos foram recolhidos na consulta de Audiologia, realizada em ambiente hospitalar (Hospital Ayres de Menezes – São Tomé, Hospital Manuel Quaresma Dias da Graça – Ilha do Príncipe) e em rastreios realizados em escolas e num hotel da cidade de São Tomé.

Este trabalho foi autorizado pela Comissão de Ética de São Tomé e Príncipe.

Todos os casos foram observados inicialmente por um médico otorrinolaringologista da equipa e submetidos a questionário clínico, para identificação de possíveis factores de risco para o desenvolvimento de surdez (história familiar, co-sanguinidade, história prévia de malária clínica, história gestacional e perinatal, história de infeções otológicas e história de traumatismo craniano). Este tipo de questionário, leva sem dúvida a um viés de

memória, mas perante a inexistência de registos clínicos, optámos pela colheita de informação, mesmo assumindo o viés.

Foram também registados os dados relativos à presença ou ausência de oralidade, observação na otoscopia, limiares auditivos e resposta à questão "acha que ouve mal?".

A avaliação audiológica foi realizada por um audiologista. Utilizámos como instrumento de medida a audiometria tonal, os potenciais evocados auditivos e a impedanciometria. A aplicação de cada um destes exames dependeu do grau de colaboração do indivíduo e da própria idade. Os exames foram realizados numa sala isolada, minimizando assim o ruído, tornando-o aceitável para a realização de avaliação audiológica fora de uma cabine insonorizada de acordo com o ANSI S3.1-1999 (R2013).

O grau de surdez foi determinado de acordo com a classificação adoptada na OMS, em que o indivíduo é classificado considerando o grau de limiar auditivo obtido no melhor ouvido.

Esta classificação considera normouvinte o indivíduo com um limiar inferior a 25 dB no melhor ouvido, surdez ligeira de 26 a 40 dB, moderada de 41 a 60 dB, severa de 61 a 80 dB e se superior ou igual a 81 dB será profunda.

Registámos todos os dados informaticamente numa base de dados e o tratamento dos resultados foi efetuado através do SPSS Statistics versão 20, utilizando a análise descritiva e de frequências para o estudo descritivo e o teste do $\chi 2$ para a avaliação da significância estatística.

RESULTADOS

Observámos 721 indivíduos na consulta, 77 dos quais foram excluídos por não apresentarem registo de avaliação audiológica (Fig. 1).

Incluímos no estudo 644 indivíduos com uma distribuição etária do 1 aos 83 anos, sendo a média de idades observada de 20,21 anos e a mediana de 15. O sexo feminino na amostra apresentou uma prevalência de 52% e o masculino de 48%.

Todos os distritos administrativos de São Tomé e Príncipe foram abrangidos pela consulta de audiologia, quer através do deslocamento dos indivíduos aos hospitais centrais, quer através da realização de rastreios em escolas e num hotel da cidade, sendo o distrito de Água Grande o que apresentou maior percentagem de indivíduos (52,6%).

Avaliámos por audiograma total 80% dos indivíduos e 20% por potenciais evocados auditivos. Considerámos o limiar auditivo electrofisiológico para a determinação do limiar auditivo do indivíduo quando não se conseguia a colaboração no audiograma tonal.

Destes 644 indivíduos, 47,1% consideravam que ouviam mal do ouvido direito, confirmando-se pelos exames audiológicos, 48,3% de indivíduos com hipoacusia. Apesar do ouvido esquerdo apresentar mais ouvidos normouvintes, apresentou maior prevalência de surdez profunda e severa.

Foi avaliada a queixa subjetiva de surdez através da questão "acha que ouve mal?" tendo-se verificado de

FIGURA 1

Amostra observada durante as Missões Humanitárias e incluídas no estudo

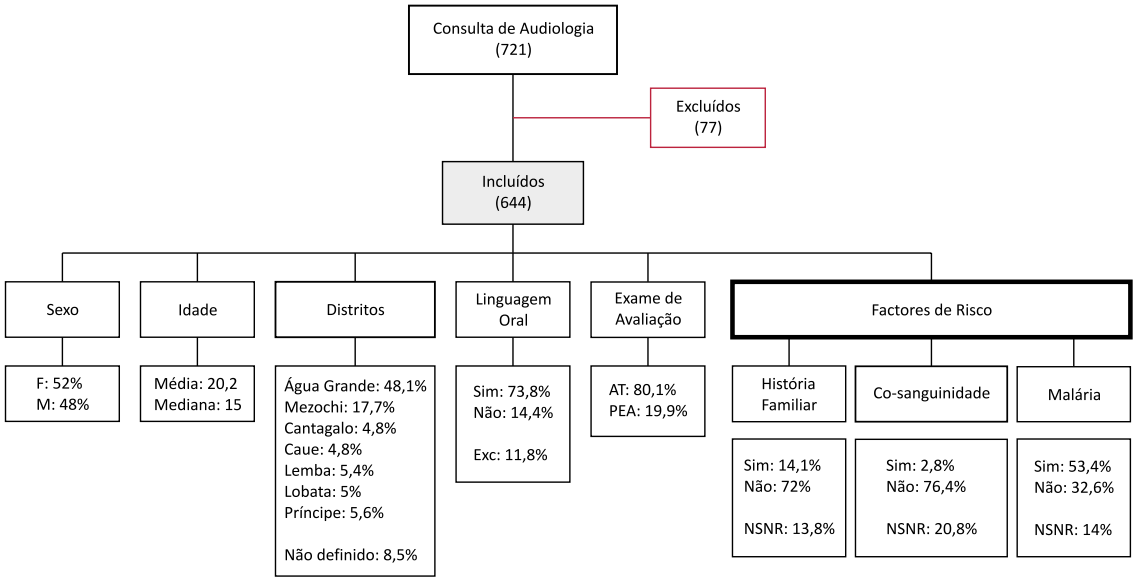
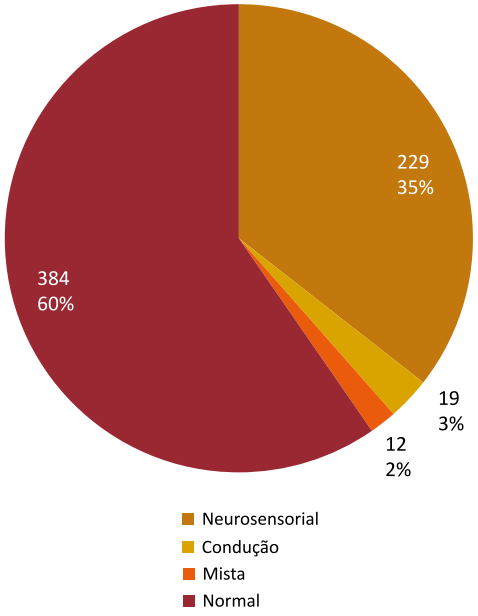


Gráfico: Fluxograma de avaliação

F: Feminino; M: Masculino; Exc.: Excluídos; AT: Audiograma Tonal; PEA: Potenciais Evocados Auditivos; NSNR: Não Sabe/ Não Responde

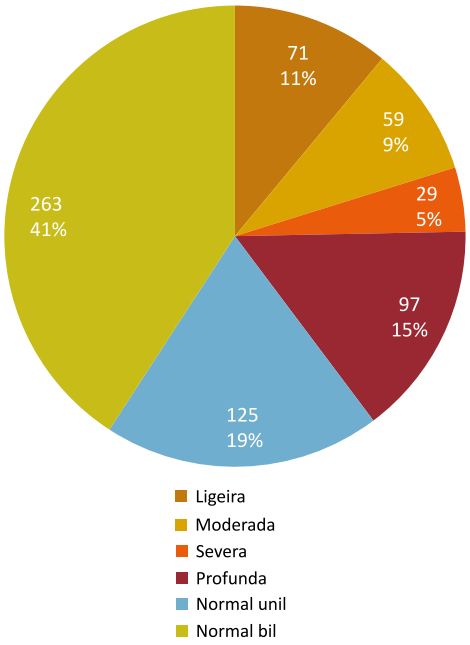
acordo com a classificação no melhor ouvido (quando os dois ouvidos apresentavam hipoacusia) e os exames audiológicos, uma sensibilidade de 65% e 84% de especificidade, um valor preditivo positivo e negativo de 71,2% e 79,7% respectivamente. A prevalência de indivíduos com queixas auditivas foi de 34,5 % e os que efetivamente apresentavam hipoacusia foi de 37,9%.
O tipo de surdez encontrado foi diversificado, verificando-se uma prevalência de surdez neurossensorial de 35,6% enquanto que a surdez de condução e mista apresentaram uma prevalência de 3% e 1,9% respetivamente (Fig. 2).

FIGURA 2
Tipo de surdez (n=644)



Os restantes indivíduos eram normouvintes (59,6%).
Nos indivíduos normouvintes, 74% destes apresentavam os dois ouvidos normouvintes, o que significa que os restantes (26%) tinham uma surdez unilateral, apesar de serem classificados como normouvintes de acordo com a classificação adoptada.
Avaliámos a perda auditiva por graus de acordo com a classificação da OMS, apresentando 60,2% dos indivíduos normouvintes, seguindo-se por ordem decrescente a surdez profunda (15,1%), ligeira (11%), moderada (9,2%) e por fim a severa (4,5%) (Fig. 3).
Calculámos a prevalência de surdez nos distritos. Verificámos que o distrito com maior prevalência de surdez foi Mé-Zóchi com 44%, seguido de Cantagalo e Caué com 42%, Lobata com 41%, Lembá e Água Grande com 35%. O distrito com menor prevalência de surdez foi Pagué (Príncipe) com 25%.
Analisámos o desenvolvimento da linguagem oral na amostra global, havendo 14,4% sem oralidade e 73,8% com oralidade. Os restantes 11,8% não apresentavam

FIGURA 3
Grau de perda auditiva (n=644)



maturidade suficiente para ter oralidade.
Quanto aos factores de risco elegíveis, analisamos a história familiar, co-sanguinidade e história prévia de malária clínica. Outros fatores de risco, tais como a história gestacional e perinatal, foram avaliados, mas devido à baixa taxa de resposta foram excluídos deste trabalho.
Ao serem questionados sobre a existência de surdez na família, apenas 14,1% responderam afirmativamente e outros 13,8% não se recordam ou não sabem. Os restantes 72% responderam não ter história familiar de surdez. Ao aplicar-se o teste do $\chi 2$ verificou-se que nesta amostra, não existe significância estatística entre a história de surdez familiar e a surdez ($\chi 2 = 1,436$; $p = 0,231$).
A relação entre a surdez e a co-sanguinidade nesta amostra revelou que apenas 2,8% responderem afirmativamente sobre a questão, tendo 20,8% optado por responder que não sabiam. Não se verificou existir significância estatística pelo teste do $\chi 2$ ($\chi 2 = 0,716$; $p = 0,398$).
Ao questionar sobre a história de episódio de malária clínica, cerca de 53,4% responde afirmativamente que em determinado momento da vida sofreu infeção por malária, tendo realizado terapêutica antimalárica.
A terapêutica antimalárica variou desde o quinino à terapêutica mais recente com artesunato e amodiaquina, mas nem todos conseguiram responder à questão sobre o tipo de terapêutica realizada, tendo sido excluída da análise o tipo de tratamento antimalárico instituído.
O grupo etário mais jovem foi o que respondeu mais negativamente sobre a história de malária clínica. Cerca de 14% não se recordava de ter tido malária em algum

momento da vida. A associação entre os episódios de malária e a surdez revelaram-se com um $\chi 2 = 3,663$ e $p = 0,056$ que apesar de não apresentar significância estatística, está mais próximo de obter significância.

DISCUSSÃO

A OMS estima que cerca de 5% da população mundial (360 milhões) apresenta incapacidade auditiva. A elevada prevalência é encontrada na Ásia Pacífica, Sul da Ásia e África Subsaariana.[5]

Tal como já foi referido, nos países subdesenvolvidos, a surdez é uma patologia frequente que leva muitas vezes ao abandono escolar, desinteresse, isolamento social e que por conseguinte leva ao agravamento do subdesenvolvimento.

O tipo de surdez mais frequentemente encontrado nestes países é a surdez de condução ou mista. A principal causa de surdez nas idades jovens, particularmente nos países de baixo e médio desenvolvimento é a infecção otológica não tratada, a qual apresenta otorreia crónica.[5,6] Devido à carência de serviços e cuidados médicos é frequente a evolução de uma otite média aguda em crónica. Outras doenças infeciosas como a rubéola, a meningite, a papeira e o sarampo são também causas importantes de surdez. A sua identificação e estudo na população é particularmente importante pelo facto de poderem ser prevenidas através de um programa de vacinação.[5]

Em São Tomé e Príncipe não existe possibilidade de realizar avaliação audiológica a não ser durante as missões humanitárias, por esse motivo não há conhecimento da prevalência da surdez na população. No CENSUS 2012 foi realizada uma questão sobre a audição: "Tem dificuldade permanente de ouvir?" onde 97,09% responderam que não tinham nenhuma dificuldade e os restantes 2,91% que tinham dificuldade. Estes resultados não são compatíveis com os dados da OMS. Por outro lado, neste trabalho foi avaliado, no questionário clínico, a avaliação subjetiva da audição através da questão "acha que ouve mal?", tendo-se verificado uma sensibilidade de 65% (na identificação dos indivíduos surdos) e especificidade de 84% (identificando os indivíduos normouvintes).

Perante o trabalho realizado durante as missões, destacamos uma prevalência aumentada de surdez neurossensorial (35,7%). Ao contrário do esperado a surdez de condução (2,9%) e mista (1,9%), em São Tomé e Príncipe, está pouco representada nos casos que recorreram à consulta de Audiologia.

A oralidade reflete o efeito da surdez na aquisição da linguagem oral, por essa razão é importante identificar os casos que não têm oralidade ou a apresentam de forma deficiente. Como seria expectável perante a prevalência de surdez neurossensorial aumentada, a existência de oralidade também está diminuída, neste segmento da amostra.

A malária é uma patologia que afeta este país desde há vários anos, sendo responsável por um elevado índice de mortalidade e morbilidade. Recentemente a incidência de malária clínica grave tem diminuído, assim como a mortalidade devido a esta patologia. Não existem registos clínicos nos processos hospitalares sobre o diagnóstico desta patologia, pelo que o questionário se baseou apenas na resposta do indivíduo ou responsável pelo indivíduo. A terapêutica antimalárica instituída também chama à atenção como corresponsável pela surdez,[7] mas neste estudo, foram poucos os que sabiam a terapêutica que tinham efectuado.

No país desde há alguns anos está implementada a terapêutica profilática antimalárica durante a gravidez, tendo sido verificado através do questionário que tem tido forte aderência. A malária clínica na ilha do Príncipe está neste momento erradicada, mas na ilha de São Tomé ainda está em fase decrescente de casos.[4]

Muitos têm sido os trabalhos apresentados sobre a surdez em países subdesenvolvidos, com vista à explicação para esse fenómeno.

De entre vários trabalhos, salienta-se um trabalho de Lasisi et al que fez uma revisão dos casos que recorreram à consulta no University College Hospital, Ibadan na Nigéria e onde 14% apresentavam surdez.[8] Este estudo pretendeu identificar as causas de surdez na Nigéria, concluindo que nos países subdesenvolvidos cerca de 85 a 90% das causas de surdez nunca são conhecidas, levando a um atraso na intervenção e efeitos irreversíveis. Verificou-se que o fator genético contribuiu com 25% dos casos, seguindo-se infeções por sarampo (13%) e meningite (8%).[8]

Sendo a Nigéria um país próximo de São Tomé e Príncipe com algumas semelhanças culturais e epidemiológicas, considerámos um trabalho útil para fazer alguma analogia com São Tomé e Príncipe. Neste, à semelhança do que se verificou no nosso trabalho, a inexistência de registos clínicos e de exames complementares de diagnóstico, laboratoriais e imagiológicos, era importante e limitava a investigação etiológica.

Numa meta-análise de artigos ingleses publicados entre 1996 e 2002 tentou-se identificar o factor etiológico da surdez.[9] Da análise constataram que a etiologia da surdez neurossensorial bilateral é desconhecida em 41,5%, seguindo-se a genética não sindrómica (27,2%), a pré-natal (11,5%), a perinatal (9,7%), a pós-natal (6,6%) e a genética sindrómica em 3,5%. A surdez neurossensorial bilateral profunda está mais associada a surdez genética não sindrómica.[9] Por outro lado, a surdez genética não–sindrómica tem vindo a aumentar a sua relevância na causa da surdez, assim como a prematuridade e asfixia neonatal.

Os resultados sugerem que se devem adaptar instrumentos de diagnóstico médico (laboratoriais, neurofisiológicos e oftalmológicos) de modo a ajudar no diagnóstico das causas de surdez que são maioritariamente desconhecidas.[10]

Em São Tomé e Príncipe, os cuidados médicos são limitados, não havendo exames complementares de diagnóstico laboratoriais disponíveis. Por esse motivo as causas infeciosas na população não são completamente conhecidas. A malária é a principal patologia conhecida e

diagnosticada. Não existe possibilidade de realizar testes serológicos que ajudem na identificação de patologias infeciosas que possam estar implicadas no diagnóstico de surdez, nomeadamente as doenças que fazem parte do grupo TORCH (toxoplasmose, rubéola, citomegalovírus e herpes).[11]

A rubéola tem vindo a diminuir o seu papel etiológico devido à implementação da vacina na grande maioria dos países,[9] mas no caso de São Tomé e Príncipe, esta poderá ser uma causa importante, pois não está implementada esta imunização e não se conhece o perfil epidemiológico da doença no país (em estudo, dados não apresentados).

Nesta população existe um programa nacional de vacinação, com uma boa cobertura vacinal em todo o país, mas não contempla a vacina anti-rubéola.[12] Esta poderá ser outra importante causa de surdez congénita, evitável.[11]

Existem nomeadamente casos de patologia cardíaca e oftalmológica na ilha que podem ser enquadrados em casos de rubéola gestacional.

O quadro infecioso de meningite, devido à elevada prevalência de malária, por vezes é diagnosticado como malária cerebral, não havendo por esse motivo história de surdez associado ao diagnóstico de meningite.

É importante salientar, uma vez mais, que neste país não existem exames audiológicos a não ser durante o período em que estão as missões humanitárias. Por esse motivo também não existe possibilidade de recorrer à realização de exames audiológicos a não ser nesse momento, o que limita o diagnóstico.

De acordo com a pesquisa bibliográfica, será de esperar a existência de vários fatores relacionados com o ambiente e o próprio indivíduo que possam estar associados ao desenvolvimento de surdez, dos quais se salientam: a malária[13,14]; a ototoxicidade[7] e a terapêutica antimalárica que, apesar de em alguns artigos não ser considerada como ototóxica[15,16], nas crianças[17] como a dose frequentemente não é adequada ao peso da criança, poderá causar surdez irreversível; as infeções, nomeadamente a rubéola, apesar de desconhecida na população, poderá estar a contribuir para a surdez em São Tomé e Príncipe. Existem outros factores, nomeadamente as hemoglobinopatias que são prevalentes nesta região subsaariana e poderão estar associadas à surdez na forma homozigótica da doença de células falciformes,[18,19] assim como o défice de glucose-6-fosfato-desidrogenase (G6PD) em parte associado ao aumento de casos de icterícia neonatal grave e consequente surdez neurossensorial neonatal.[20, 21]

Não podemos esquecer, que sendo São Tomé e Príncipe, um conjunto de ilhas no seio do Atlântico, com poucos recursos, poderá estar associado a mais casos de co-sanguinidade e maior prevalência de surdez genética (em estudo por Cristina Caroça).

CONCLUSÃO

Dos fatores de risco avaliados, a malária revelou ser o mais significativo. Neste momento estão a decorrer outros estudos tendo como objetivo a identificação de outros fatores de risco para a surdez neurossensorial em São Tomé e Príncipe. As causas genéticas e ambientais poderão estar a ser responsáveis pelo aumento de prevalência de surdez neurossensorial em São Tomé e Príncipe, sendo de salientar que poderá não estar implicada apenas uma causa isolada, mas um conjunto de fatores que em sinergia levam ao aparecimento de surdez.

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Protecção de pessoas e animais

Os autores declaram que os procedimentos seguidos estavam de acordo com os regulamentos estabelecidos pelos responsáveis da Comissão de Investigação Clínica e Ética e de acordo com a Declaração de Helsínquia da Associação Médica Mundial.

Confidencialidade dos dados

Os autores declaram ter seguido os protocolos do seu centro de trabalho acerca da publicação dos dados de doentes.

Conflito de interesses

Os autores declaram não ter nenhum confito de interesses relativamente ao presente artigo.

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1.3 State of Art

1.3.1 Hearing

The interaction between a person and his or her surrounding environment is mediated through sensory experiences. The sense of hearing, in particular, facilitates communication and faster's social interaction. Hearing is the key to learning spoken language and is important for the cognitive development of children (Krug, Cieza, Chadha, Sminkey, Martinez, White, et al. 2016).

The human organ responsible for the ability to the sensory experience of hearing is the ear and auditive pathway to brain.

The function of the ear is to convert physical vibration into an encoded nervous impulse. It can be thought of as a biological microphone. Like a microphone the ear is stimulated by vibration: in the microphone the vibration is transduced into an electrical signal, in the ear into a nervous impulse which in turn is then processed by the central auditory pathways of the brain (Alberti 2001).

1.3.1.1 Sound Conducting Mechanisms

The most apparent function is to bring (conduct) the sound signal from the air to the inner ear (Figure 1.3-1) (Gelfand 2009).

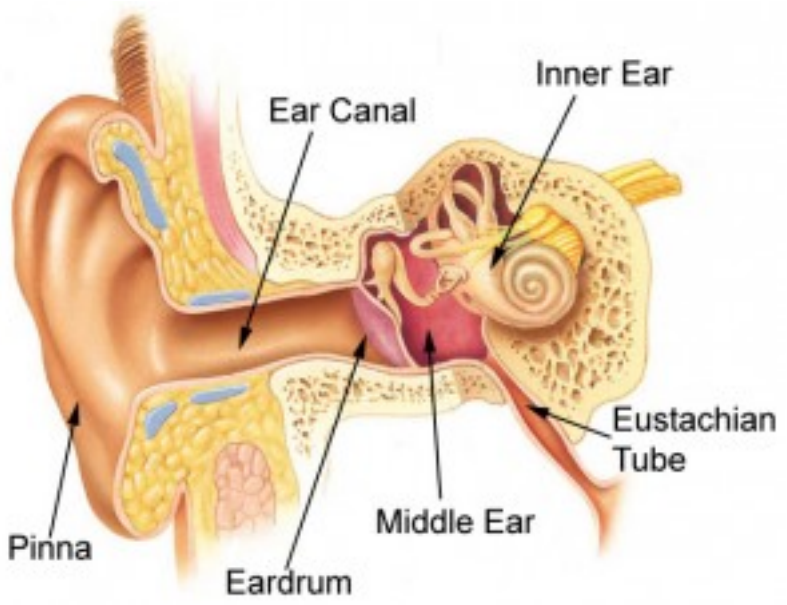

Figure 1.3-1 - Ear's structure and function
(http://www.healthcareinsighter.com/ear-infections-outer-middle-inner/)

1.3.1.1.1 The outer ear

The outer ear transmits sound to the tympanic membrane, it includes the pinna and ear canal (Alberti 2001).

Anatomically the auricle, pinna or outer ear, is a uniquely constructed organ. It is perfectly designed to "catch" incoming sound waves and then funnel them down the external auditory canal (Figure 1.3-2) (Weber 2015).

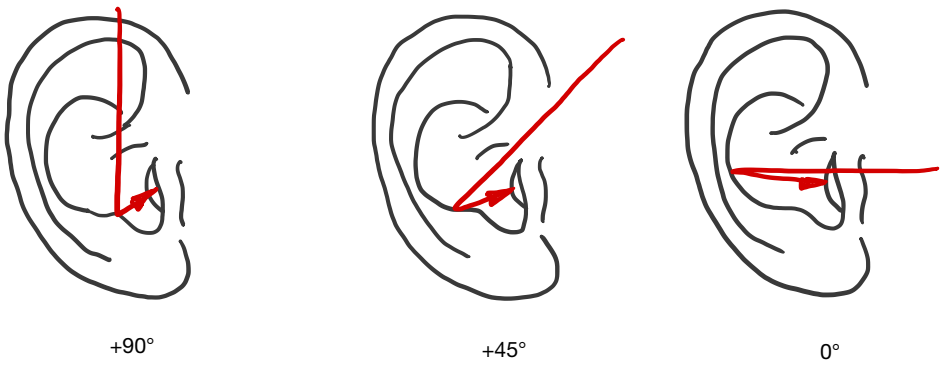

Figure 1.3-2 - Pinna (external ear) reflections of sound for different elevations

1.3.1.1.2 The middle ear

The middle ear is the air-filled cavity behind the eardrum, also known as tympanic cavity. Seen from the ear canal, the membrane is slightly concave and is suspended by a bony ring. Normally it is under some degree of tension. Its surface area is approximately 85 mm^2. The main part of the tympanic membrane, the pars tensa with an area of approximately 55 mm^2 (Moller 2006).

This cavity connects to the pharynx by the eustachian tube. Medial to the middle ear is the inner ear (Figure 1.3-1) (Alberti 2001).

The middle ear space houses three little bones, the malleus, incus and stapes which conduct sound from the tympanic membrane to the inner ear (Figure 1.3-1). This ossicular chain act as a bridge from the eardrum to the oval window, which is the entrance to the inner ear (Gelfand 2009).

1.3.1.2 The Sound transduction Mechanism

The cochlea and eighth cranial nerve compose the sensorial system. It involves the physiological response to the stimulus, activation of the associated nerve cells, and the encoding of the sensory response to a neural sign.

The aspects of the central nervous system that deal with this neurally encoded message are generally called the central auditory nervous system (Gelfand 2009).

1.3.1.2.1 The inner ear

1.3.1.2.1.1 Structure

The inner ear, or cochlea, transduces vibration transmitted to the perilymph via the ossicular chain into a nervous impulse which is then taken to the brain where it is perceived as sound (Alberti 2001).

The bony cochlea is so called because it is shaped like a snail shell, it has two and a half turns and houses the organ of hearing known as the membranous labyrinth surrounded by fluid called the perilymph. The cochlea has a volume of about 0.2 of

a milliliter. Uncoiled the cochlea has a length of 3.1 – 3.3 cm. The height of the cochlea is approximately 0.5 cm in humans (Moller 2006). In this space lie up to 30,000 hair cells which transduce vibration into nervous impulses and about 19,000 nerve fibers which transmit the signals to and from the brain (Alberti 2001).

Vibration of the footplate of the stapes vibrates the perilymph in the bony cochlea. This fluid is essentially incompressible. Therefore, there has to be a counter opening in the labyrinth to allow fluid space to expand when the stapes foot plate moves inwards and in turn to move inwards when the stapes foot plate moves outwards. The counter opening is provided by the round window membrane which lies beneath the oval window in the inner wall of the middle ear. It is covered by a fibrous membrane which moves synchronously but in opposite phase with the foot plate in the oval window (Figure 1.3-3) (Alberti 2001).

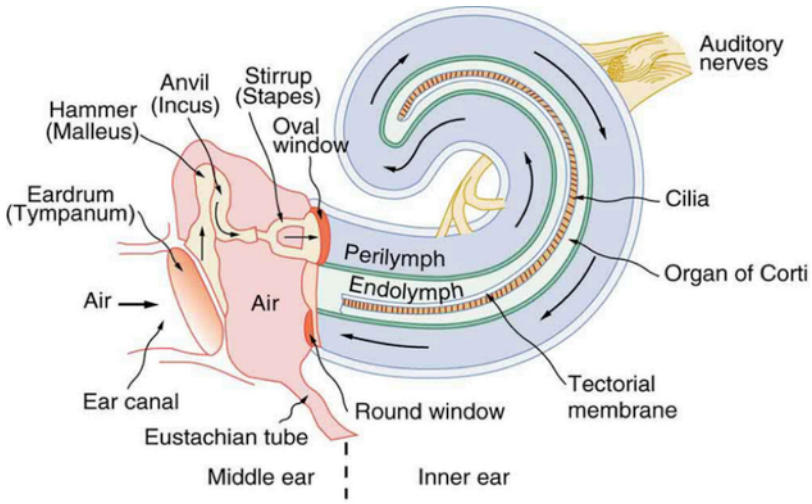

Figure 1.3-3 - The sound vibrations cause the fluid inside the cochlea to ripple, and a traveling wave forms along the membrane. (http://www.deliceslaurentiens.com/tag/cochlea/)

The cochlea has three fluid-filled canals: the scala vestibuli; the scala tympani; and the scala media. The scala media, located in the middle of the cochlea, is separated from the scala vestibuli by Reissner's membrane and from the scala tympani by the basilar membrane (Moller 2006). The scala media narrows towards the apex of the cochlea ending just short of the apical termination of the bony labyrinth. An

opening near the apical termination of the bony labyrinth, called the helicotrema. This allows communication between the scala vestibuli and scala tympani. The basilar membrane separates sounds according to their frequency (spectrum) and the organ of Corti, located along the basilar membrane, contains the sensory cells (hair cells) that transform the vibration of the basilar membrane into a neural code (Figure 1.3-4) (Moller 2006).

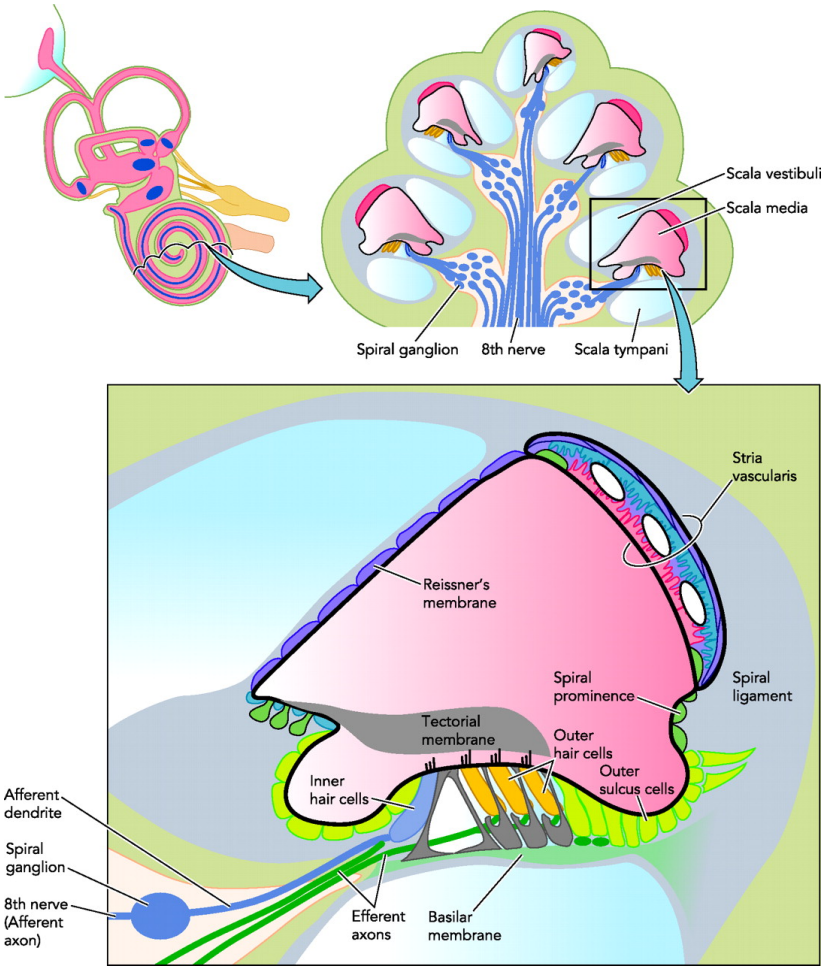

Figure 1.3-4 - Sensory cells on the basilar membrane in the organ of Corti stretch along the entire length of the cochlea (http://physiologyonline.physiology.org/content/24/5/307)

Physical characteristics of the basilar membrane cause different frequencies to reach maximum amplitudes at different positions. Much as on a piano, high frequencies are at one end and low frequencies at the other (Figure 1.3-5). High frequencies are transduced at the base of the cochlea whereas low frequencies are transduced at the apex.

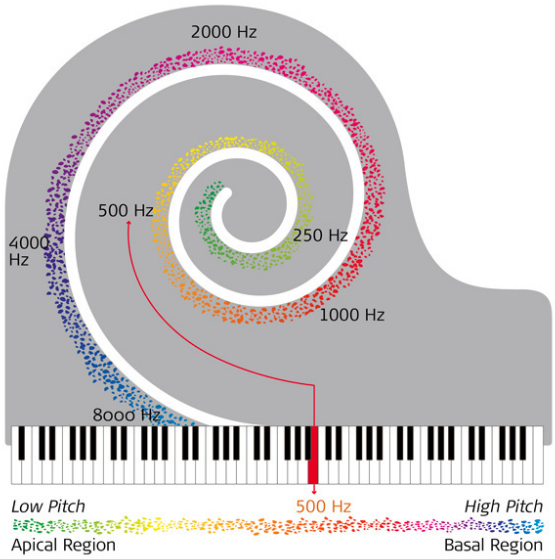

Figure 1.3-5 – Cochlea as a piano, high frequencies are at one end and low frequencies at the other (http://www.medel.com/technology-complete-cochlear-coverage/)

1.3.1.2.1.2 Function

Transduction of vibration in the audible range to a nervous impulse is performed by the inner hair cells; when the basilar membrane is rocked by a travelling wave, the cilia of the inner hair cells are bent in relation to the body of the cell, ion passages are opened or closed in the body of the cell and the afferent nerve ending which is attached to the hair cell base is stimulated (Figure 1.3-6) (Alberti 2001).

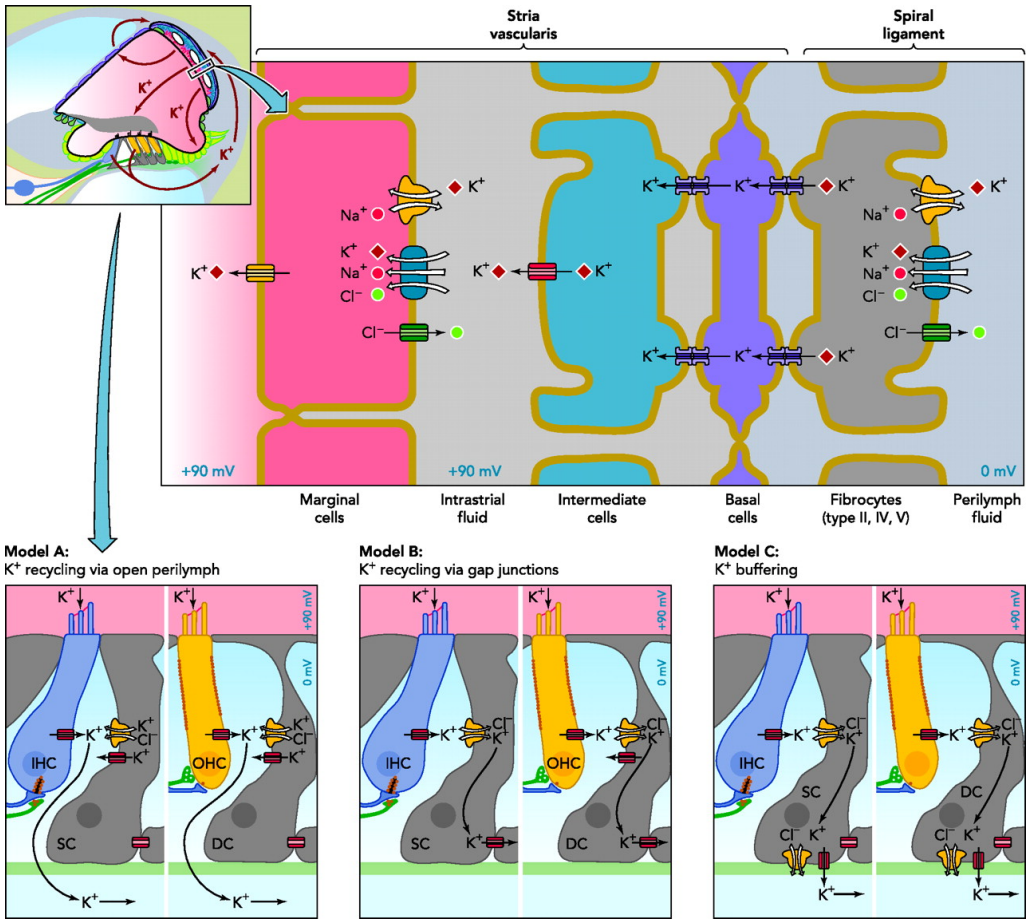

Figure 1.3-6 - Overview over the stria vascularis and its K^+ transport mechanisms and two alternative K^+ pathways removing K^+ from the hair cells.
(http://physiologyonline.physiology.org/content/24/5/307**)**

1.3.1.2.2 Hearing Physiology

1.3.1.2.2.1 The outer and middle ears

The outer and middle ears serve to amplify the sound signal.

Sound entering the outer ear is picked up by the tympanic membrane. The vibrations of the eardrum are transmitted to the ossicular chain, which vibrates essentially in the right-left plane. The vibration is represented as a rocking motion of the stapes footplate in the oval window (Gelfand 2009).

1.3.1.2.2.2 The inner ear

The function of the inner ear is to transduce vibration into nervous impulses.

Stimulation is transmitted to the cochlear fluids by the in-and-out motions of the stapedial footplate at the oval window at the base of the cochlea. This movement is transmitted to the cochlear fluid. The oval window leads into the upper chamber (scala vestibuli) (Gelfand 2009). The vibratory stimulation into the bending of the hair cell stereocilia in the right direction is necessary to activate the sensory process. There is another mechanism for accomplishing this activity and promote the ability to hear different pitches (Gelfand 2009).

1.3.1.3 Central Auditory Processing

The nervous impulses are carried along the 8th from the cochlea to the brain stem.

Auditory messages are conveyed to the brain via two types of pathway: the primary auditory pathway which exclusively carries messages from the cochlea, and the non- primary pathway (also called the reticular sensory pathway) which carries all types of sensory messages (Figure 1.3-7) (Choclea 2013).

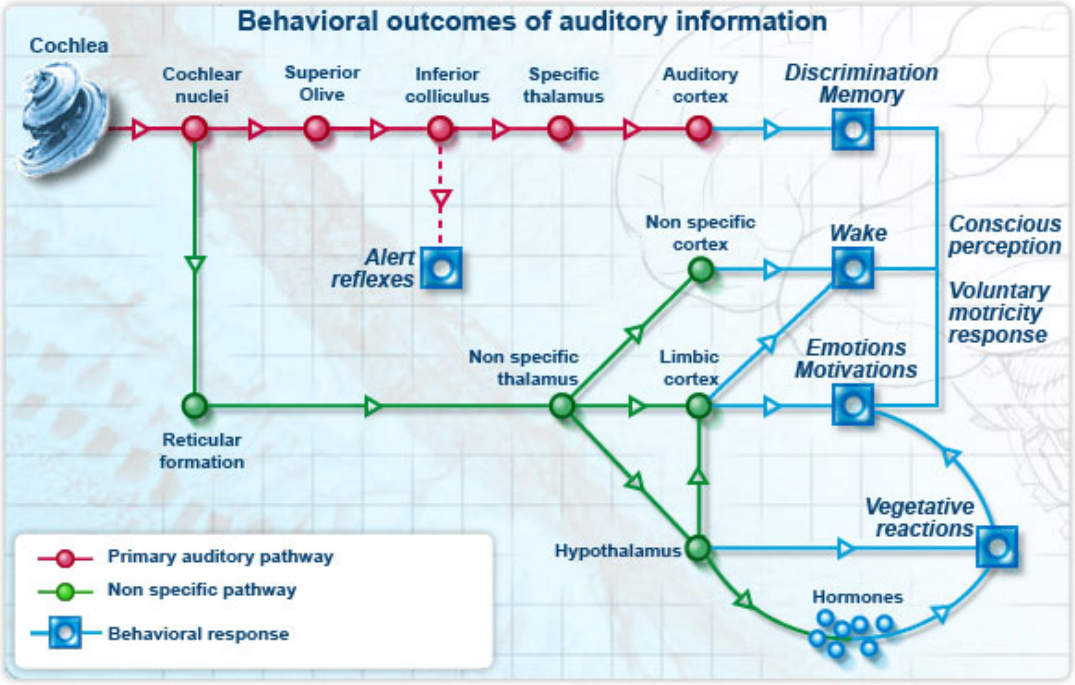

Figure 1.3-7 – Behavioral outcomes of auditory information. (http://www.cochlea.eu/en/auditory-brain)

Schematically, this pathway is short (only 3 to 4 relays), fast (with large myelinated fibers) and ends in the primary auditory cortex (Choclea 2013).

The pathway carries messages from the cochlea, and each relay nucleus does a specific work of decoding and integration (Choclea 2013).

From the cochlear nuclei, small fibers connect with the reticular formation where the auditory message joins all other sensory messages (Choclea 2013).

The main function of these pathways, also connected to wake and motivation centers as well as to vegetative and hormonal systems is to select the type of sensory message to be treated first (Choclea 2013).

Conscious perception requires the integrity of both types of pathways. For instance, during sleep the primary auditory pathway functions normally, but no conscious perception is possible because the link between reticular pathways and the wake and motivation centers is inactive (Choclea 2013).

The Central Auditory Processing is a network that permits some of the characteristics of hearing: sound interpretation, hearing discrimination and discrimination in noisy spaces, ability to block out unwanted sounds, spatial sound localization, on and off sounds.

1.3.1.3.1 The ability to Block Out Unwanted Sounds

In a crowded noisy room a young person with normal hearing can tune in and out conversations at will. This is known technically as the cocktail party effect. The brain quite automatically adjusts time of arrival and intensity differences of sound from different signal sources so that the one which is wanted passes to the cortex and all others which do not meet these criteria are suppressed by feedback loops (Alberti 2001).

1.3.1.3.2 Spatial localization

The ability of localizing sources of sound, also referred to as directional hearing, belongs to auditory abilities of higher level. In everyday life sound localization is mainly used for monitoring of the surroundings (Przewoźny 2015).

Giovanni Battista Venturi (1746–1822), who suggested that localization of sounds was dependent upon inequalities at the two ears, is considered to be the precursor of studies on sound localization in humans (Przewoźny 2015).

The idea is that sound localization is based on interaural time differences (ITD) at low frequencies and interaural level differences (ILD) at high frequencies. If the head remains stationary neither a given ITD nor an ILD can sufficiently define the position of a sound source in space (Figure 1.3-8). On such a theoretical basis cones of confusion which open outward from each ear can be predicted ambiguously projecting any source on the surface of such a cone onto an interaural axis (Paulus 2003). Our restricted ability at localizing sound sources in the vertical median plane is another example of possible ambiguity (Paulus 2003).

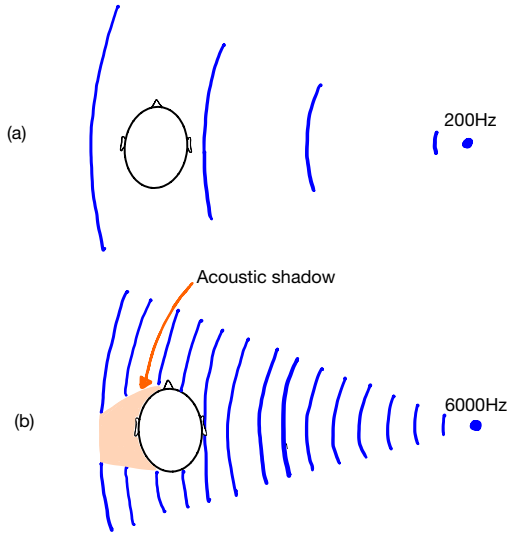

Figure 1.3-8 Sound coming from a loudspeaker off to the right side. a) At lower frequency sounds their wavelengths are small compared with the size of the head, so there isn't an acoustical shadow

(the head shadow effect); b) An acoustical shadow (the head shadow effect) occurs for high frequency sounds because their wavelengths are small compared with the size of the head.

At the end of the 19th century scientists already realized that occlusion of the pinnae cavities decreases localization competence. As a result of later achievements in physics and signal-theory it became more obvious that the pinnae may provide an additional cue for spatial hearing and that the outer ear together with the head and the upper torso form a sophisticated direction-dependent filter (Paulus 2003).

1.3.1.3.3 On and Off Sounds

Hearing has an alerting function especially to warning signals of all kinds. There are brain cells which respond only to the onset of a sound and others which respond only to the switching off of the sound, i.e. a change. These cells allow the ear to respond to acoustic change - one adjusts to constant sound - change is immediately noticeable (Alberti 2001).

1.3.1.3.4 Interaction of Sound Stimuli with other parts of the Brain

Sound stimuli produce interaction with other parts of the brain to provide appropriate responses (Alberti 2001). Certain sounds can evoke anger, others pleasure. The point is that the sensations produced by hearing are blended into the body mechanism in the central nervous system to make them part of the whole milieu in which we live (Alberti 2001).

1.3.2 Type of Hearing Loss

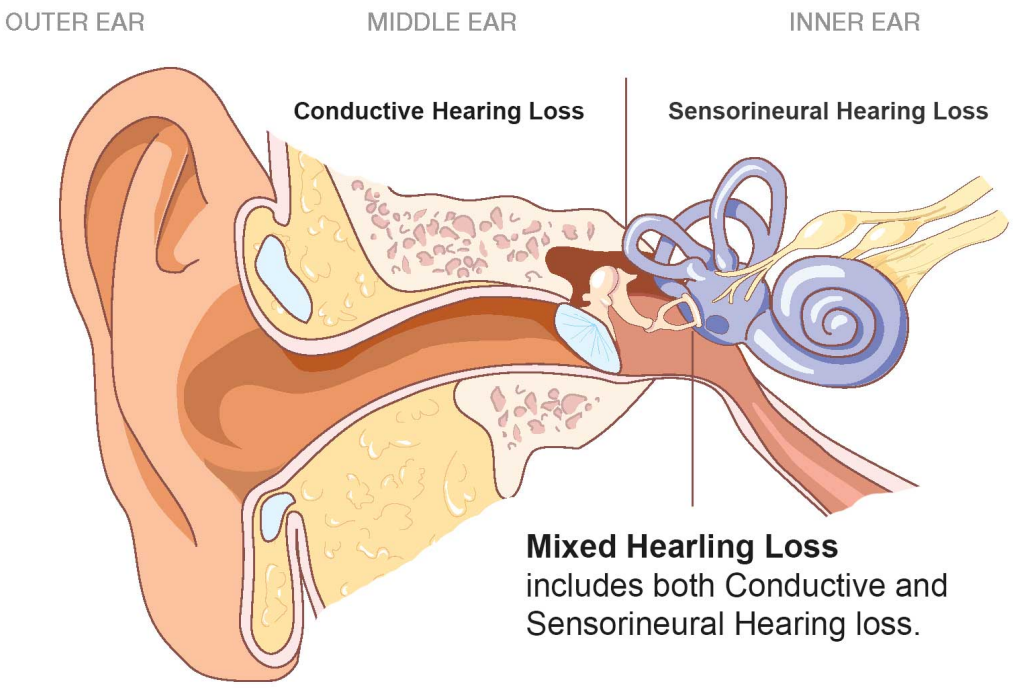

Figure 1.3-9 – Anatomic ear representing the different types and affected regions on hearing loss. (https://audiology.nmsu.edu/hearing-loss-education/hearing-loss-facts/mixed-hearing-loss/)

1.3.2.1 Conductive / Transmission

Conductive lesions impair the transmission of sound from environment to the cochlea, so the signal reaching the sensorineural system is weaker than it should be. A conductive hearing loss is the amount by which the signal is attenuated due to the disorder, and is expressed by the size of air-bone-gap (Figure 1.3-9) (Gelfand 2009).

An increased airway threshold characterizes conductive hearing loss, without altering the bone conduction threshold.

1.3.2.2 Sensorineural / Neurosensorial

Sensorineural lesions involve the cochlea and/or auditory nerve and may affect sensory receptor (hair) cells, auditory neurons, and/or any of the many structures and processes that enable them to be activated and to function properly. The resulting impairment of auditory functioning is called a sensorineural hearing loss (Figure 1.3-9) (Gelfand 2009).

Sensorineural hearing loss appears as an increase of bone conduction threshold following the airway. Usually represents an irreversible hearing loss.

1.3.2.3 Mixed

A mixed hearing loss is the combination of a sensorineural loss and a conductive loss in the same ear. Mixed losses may be caused by the presence of two separate disorders in the same ear (e.g., noise–induced hearing loss plus otitis media) or by a single disorder that affects the conductive and sensorineural systems (e.g., head trauma or advanced otosclerosis) (Figure 1.3-9) (Gelfand 2009).

There is a consequent increase of bone and air conduction threshold with an air-bone gap greater than 10 dB.

1.3.3 Hearing loss Epidemiology

The WHO estimates that the number of individuals with a hearing loss greater than 40 dB in adults and 30 dB in children (disabling hearing loss) has increased in the last 10 years (Olusanya 2008; Olusanya et al. 2014). Of 360 million people, about 5.3% of the world's population, with hearing disability in 2011, about 32 million (9%) are children under 15 years (Figure 1.3-10). Male gender presents proportionally a greater prevalence of disabling hearing loss in relation to the opposite sex.

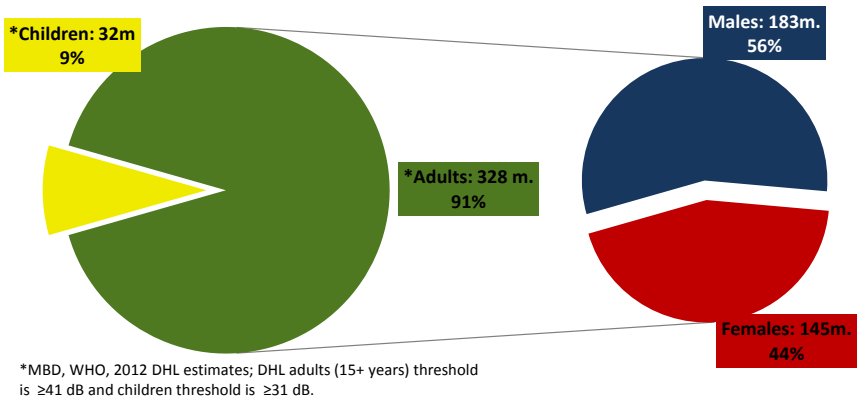

Figure 1.3-10 - Distribution of disabling hearing loss in Adults and Children, Man and Woman (WHO Media centre 2012). Legend: m-million

The countries of Asia Pacific, South Asia and sub-Saharan Africa are those who contribute to this high prevalence (Figure 1.3-11).

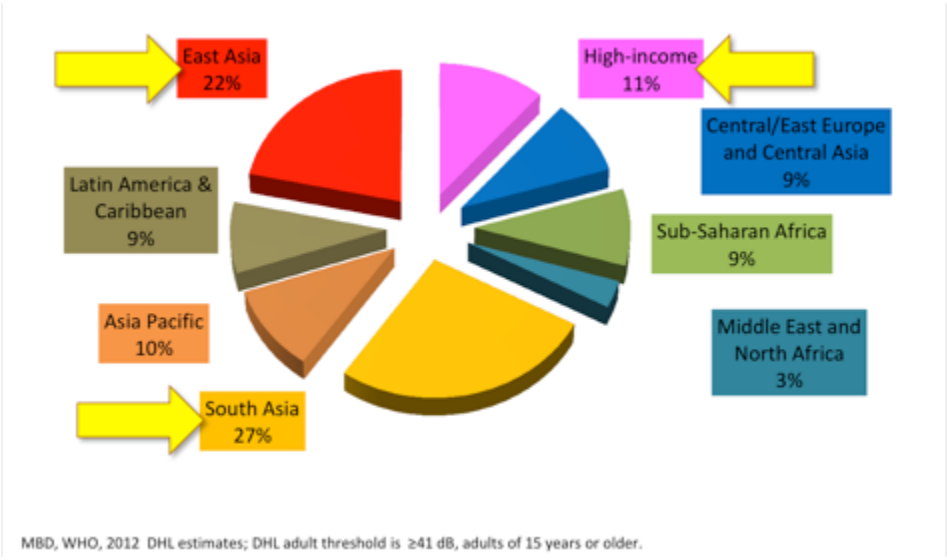

Figure 1.3-11 – Mundial distribution of disabling hearing loss (WHO Media centre 2012).

About 15% of the adult world population presents some degree of hearing loss and only 1/3 of these, feature disabling hearing loss (WHO 2013d).

These epidemiological data, increases more and more, not only by the fact that the average life expectancy is increasing in the general population, as well as

diagnostic tests are becoming more accessible and more early conducted, identifying the cases immediately. Hearing loss arises throughout life, and aging (presbycusis) is the most frequent cause. Despite all the causes that may exist, it is estimated that about half of the cases of hearing loss can be avoided.

The vast majority of cases of congenital or early-onset of hearing loss at 2012, in underdeveloped countries is 6:1000 newborns, while in developed countries is of 2:1000 newborns (Olusanya et al. 2014). The importance of primary prevention should be strengthened especially in those countries where the birth rate is high and where present prevalence of disabling hearing loss increases. According to WHO data, (WHO 2013a) in the case of underdeveloped countries the type of hearing loss that usually arises is conductive and mixed hearing loss, such fact is being observed in countries with weak medical and therapeutic resources, leading to the perpetuation of infectious processes and consequent degradation of the hearing (WHO 2013c).

On the other hand, the cause of hearing loss is not a single, but a number of factors that can interact and trigger this change. Sensorineural hearing loss is a hearing disability classically irreversible by medical or surgical point of view. In this hearing loss type, the only possibility of recovery, in some cases, is the hearing rehabilitation with hearing aids or even implants (osteo-integrated, middle ear or cochlear). These types of rehabilitation, in addition to the high economic weight that involve, entail need for support by specialized professionals and multidisciplinary teams. In countries where the prevalence of hearing loss is common, this possibility is scarce and practically impossible, and should therefore be a priority the prevention of deafness (WHO 2013c; WHO Media centre 2014).

For prevention, knowledge of the various risk factors for hearing loss is required. The prevalence of hearing loss increases with age and is of common knowledge. In children the hearing loss prevalence is 1.7% and in adults (≥ 15 years) is about 7%, increasing in adults over 65 years in the proportion of 1:3 individuals. However, in children the prevalence decreases as the gross national income (GNI)

per capita of the country increases (Figure 1.3-12) decreasing the number of children with hearing disability as the average literacy level of the parent's increases (WHO Media centre 2012).

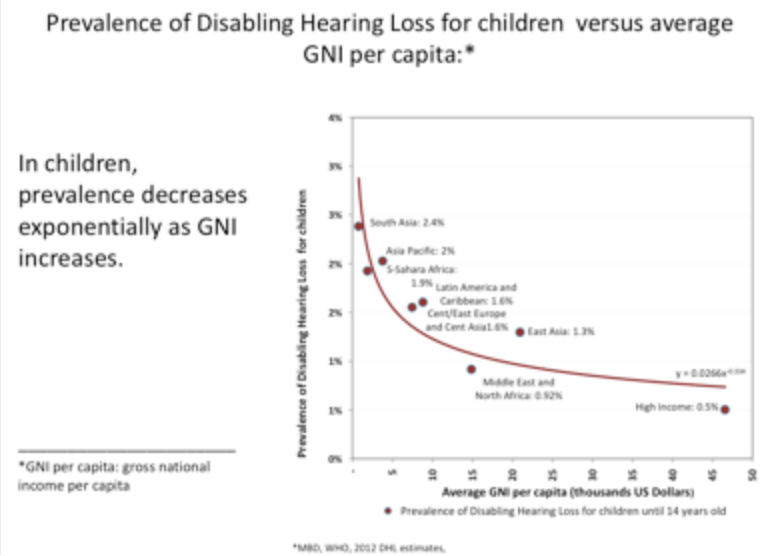

Figure 1.3-12 – Relation between hearing loss prevalence in children and Gross National Income per capita (GNI).(WHO Media centre 2012)

In general we may lead to think that the cause of hearing loss according to ages will change, being at birth more common a genetic cause and while in adult the otological infectious, acoustic trauma and hearing disorders such as Ménière's disease.

1.3.4 Contributing factors to hearing loss

There are many known risk factors for the onset of hearing loss in the individual. These may be inherent to the individual himself or in association with the environment that surrounds it. The interaction between individual and environment could induce to hearing loss. All factors may exercise a triggering role of hearing loss and his intervention may arise at different stages from the prenatal period, perinatal or postnatal period.

According WHO around 60% of HL are preventable (figure 1.3-13) (Krug, Cieza, Chadha, Sminkey, Martinez, White, et al. 2016).

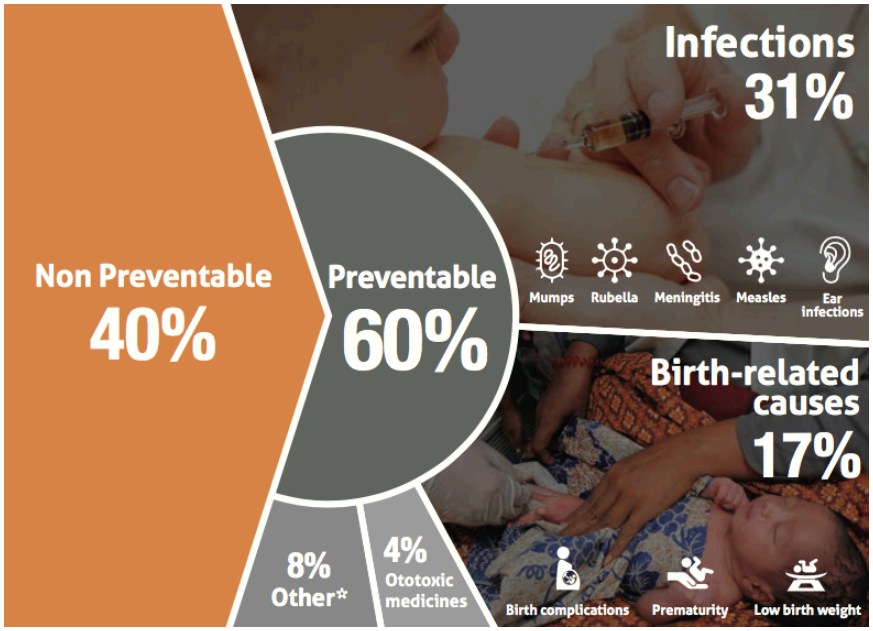

Figure 1.3-13 – Overview of the causes of preventable HL. (Krug, Cieza, Chadha, Sminkey, Martinez, Stevens, et al. 2016)

The proportion of hearing loss due to preventable causes is much higher in middle- and lower-middle-income countries (75%) than in high-income areas (49%) (Krug, Cieza, Chadha, Sminkey, Martinez, White, et al. 2016). This situation is probably explained by the high prevalence of infections in the middle- and lower-middle-income countries and the better maternal and child health care in high-income countries (Krug, Cieza, Chadha, Sminkey, Martinez, White, et al. 2016).

This chapter pretends to evidence principal factors that could contribute for HL.

1.3.4.1 Demographic conditions

1.3.4.1.1 Age

Age Related Hearing Impairment (ARHI) is the most prevalent form of hearing loss in humans. It is characterized by decreased hearing sensitivity, decreased ability to

understand speech in a noisy environment, slowed central processing of acoustic stimuli, and impaired sound localization (Yang et al. 2015; Quaranta et al. 2015).
Age is the most frequent cause of hearing loss, one out of three people aged 65-74 has some level of hearing loss. After age 75, that ratio goes up to one out of every two people (Ratini 2014).
Aging can lead to degenerative audiological process, being individual to each other and where genetic can has a role. Usually between 60 to 69 years, 40% has hearing loss and over 80 years has around 90% of hearing loss (Figure 1.3-14) (Sharashenidze et al. 2007).

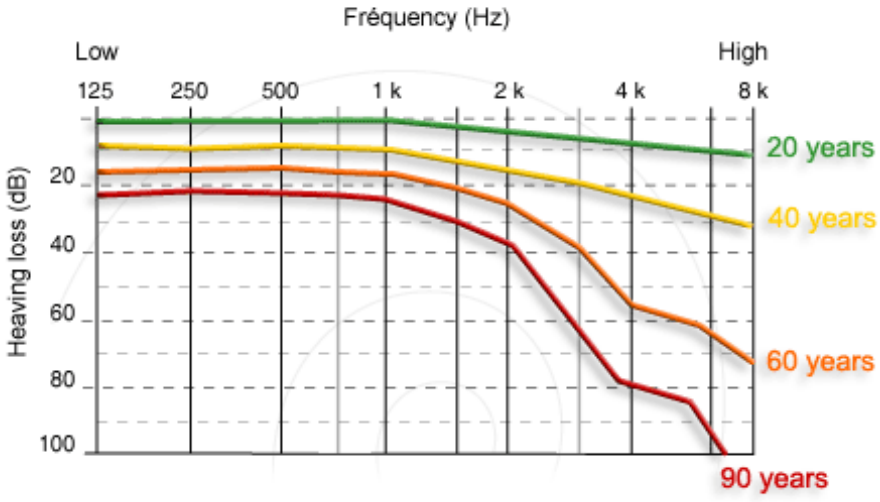

Figure 1.3-14 – Aging hearing loss evolution. (Kiezberlinois 2016)

Epidemiological studies provide insight into the modifiable and non-modifiable risk factors associated with hearing loss and into the mechanistic pathways underlying ARHL. Risk factors to ARHL are classified into four categories: 1 - Cochlear aging; 2 - Environmental factors (noise exposure, ototoxic drugs, socioeconomic factors); 3 - Genetic factors (gender, race, genes or specific loci) and 4 - Comorbidities (hypertension, diabetes, stroke, smoking) (Quaranta et al. 2015).

1.3.4.1.2 Gender

Woman generally presents a better hearing; it is thought that the hormonal factor is contributing in any way to this phenomenon especially among the 30 and 50 years. Usually, women present a better hearing than men particularly in the higher frequencies, whereas hearing degradation in men is two times faster (Figure 1.3-15). The hearing threshold of men begins to decline around the age of 30 years while women this process begins much later (Sax 2010; Pearson et al. 1995; University of Washington Medical Center 1997; Sharashenidze et al. 2007; Jerger et al. 1993).

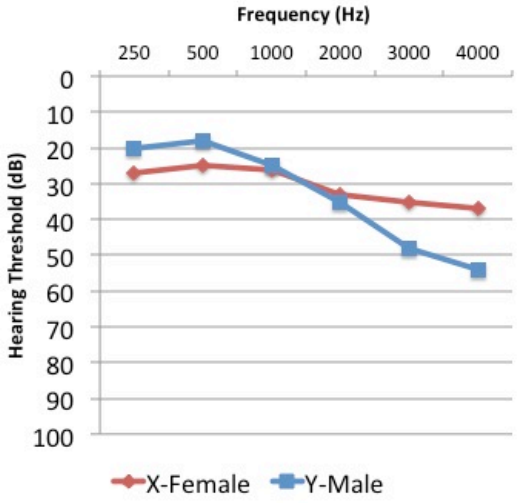

Figure 1.3-15 - The gender reversal phenomenon. Average audiograms of 341 males and 346 females in age range 50 to 89 years. Adapted from J Am Acad Audiol (1993;4:42-49)

First signs of ARHI in males may already be evident at an age of 30–39 years, beginning at the highest frequency range of 10–16 kHz; by 40–49 years hearing impairment has progressed to frequencies of 6–8 kHz. Presbycusis in females generally begins a decade later, and the principal speech band (0.5–4 kHz) is affected in both genders by 60–69 years. Besides gender differences, epidemiological studies in the USA have established a higher prevalence in Caucasians than in African-Americans (Yang et al. 2015).

1.3.4.1.3 Race

In addition to age and sex, race has been identified as the third most important risk factor non-modifiable for hearing loss. Although skin color has been identified as an important risk factor in hearing loss, few human studies have investigated cochlear melanin content and its association with hearing function (Murillo-Cuesta et al. 2010).

The primary skin pigment, melanin, is found in the mammalian inner ear, and data from animal studies suggest a biological role for melanin in hearing preservation. Melanin in derma is proportional to melanin in inner ear (cochlea). There are studies that associate the higher levels of melanin to higher protection to hearing loss (Sun et al. 2014; Murillo-Cuesta et al. 2010).

Melanin in inner ear is produced by melanocytes derived from neural crest progenitors. In *stria vascularis*, melanocytes are called intermediate cells (IC), and play a role in producing endolymph. In mice they had revealed that is also important to establishment of endocochlear potential (EP)(Murillo-Cuesta et al. 2010; Sun et al. 2014).

Murillo-Cuesta and colleagues, revealed that melanin precursors, such as L-DOPA, have a protective role in the mammalian cochlea in age-related and noise-induced hearing loss (Murillo-Cuesta et al. 2010).

In the study of Sun et al. African-American individuals have been shown to have odds of hearing loss 40% to 60% lower than Caucasians, and this difference exists even after adjustment for noise exposure and socioeconomic status (Sun et al. 2014).

Also another study (Mujica-Mota et al. 2015) involving melanin revealed that eye color may be an indicator of inner ear melanin content and has been associated with hearing loss. Evidence suggests that melanin can be protective against radiation-induced sensorineural hearing loss, but may predispose individuals to cisplatin ototoxicity (Mujica-Mota et al. 2015).

1.3.4.2 Family history

One of the possible alerts to the involvement of genetic facts is the familial history of hearing loss. It is sometimes questioned the validity because it usually is applied

on questionnaire leading to a bias. According to Driscolland and colleagues, the degree of parenting influences the prognosis; siblings and mother are the parenting factor with the greatest impact on hearing loss (Driscoll et al. 2015).

Only, the higher degrees of hearing loss are more commonly associated with congenital deafness. For this reason it is important tracking all children with a family history of hearing loss and monitor their development during childhood (Driscoll et al. 2015).

Consanguinity is another risk factor although not always identified nor accepted by some communities, is common practice, instituted as cultural habit that leads to the perpetuation of genetic defects, which encompasses deafness (Figure 1.3-16).

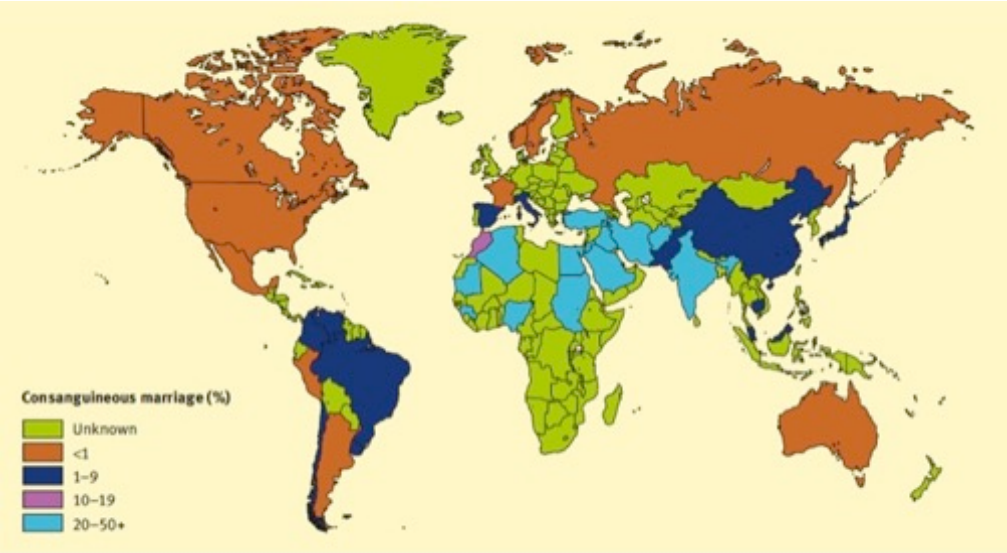

Figure 1.3-16 – Consanguinity in World (Zakzouk 2002)

The highest rates of consanguineous marriage occur in north and sub-Saharan Africa, the Middle East, and west, central, and south Asia. In these regions even couples who regard themselves as unrelated may exhibit high levels of homozygosity, because marriage within clan, tribe, caste, or biraderi boundaries has been a long-established tradition (Bittles & Black 2010).

Consanguinity as common practice is found especially in underdeveloped countries, requiring your discouragement, according to Zakzouk et al. in which the incidence of hereditary hearing loss is higher in these countries compared to developed countries (Zakzouk 2002). On the other hand, the consanguinity as

tradition, with marriages between siblings leads to increased autossomic recessive diseases, among which the hearing loss. The effect of consanguinity in the development of hearing loss in children depends on the close relationship of the parents (Zakzouk et al 2002).

1.3.4.3 Cranio-facial Malformations

Cranio-facial malformations by itself can mean changing the anatomical structures of the ear (external, middle and internal) and therefore lead to hearing loss. These malformations can fit in genetic or syndromic conditions (Bartel-Friedrich & Wulke 2007).

When we talk about changes during the perinatal period can think of changes arising shortly after birth.

1.3.4.4 Low Birth Weight

The low birth weight (under 1.5 Kg), leads to important metabolic alterations with changes in neuromotor development, being the Central Nervous System (CNS) the most affected and associated with an increased sensorineural hearing loss in a proportion of 20-40:1000 newborns.

The risk of hearing loss decreased with increasing birthweights, with adjusted odds ratios of 4.4, 3.8, 1.7 and 1.4 for the birthweights 1500–1999, 2000–2499, 2500–2999 and 3000–3499 g respectively (Engdahl & Eskild 2007). The risk of both mild to moderate and severe/profound hearing losses were influenced by birthweight (Engdahl & Eskild 2007).

The risk of hearing loss associated to low birth weight it is also associated to patients that are commonly exposed to other risk factors for hearing loss such as ototoxic drugs, hypoxia and hyperbilirubinemia, which may lead to early or delayed-onset sensorineural hearing loss as well as progression of a mild pre-existing sensorineural hearing loss years after hospital discharge (Cristobal & Oghalai 2008).

At same time, prevalence of failed hearing screening in neonates with Very Low Birth Weight (VLBW) is significantly higher than in neonates with normal birth weight because they experience higher rates of transient middle ear fluid accumulation and conductive hearing loss (Cristobal & Oghalai 2008).

1.3.4.5 Neonatal Jaundice

Associated to metabolic changes described previously, the neonatal jaundice is undoubtedly one of the situations that often arise. In about 84% of the newborn (pre-term and term) at the end of the first week the jaundice appears. This should be treated with caution because it is associated with important neurological damage and essentially the auditory pathway (Olds & Oghalai 2015; Watchko et al. 2015).

The neurological damage due to acute or chronic exposure of the CNS to bilirubin was named - BIND (Bilirrubin-Induced Neurologic Dysfunction). This includes the different pathological situations associated with neurological damage caused by hyperbilirubinemia, such as kernicterus, encephalopathy and neuronal dysfunction alone (Olds & Oghalai 2015). The prevalence of BIND is not known because it is difficult to know the incidence of CNS dysfunction that can be subtle, transient or located. Auditory system damage can occur even with bilirubin levels that might think they were soft, in the absence of other signs of kernicterus. Auditory effects goes through subtle changes in hearing and speech processing to complete deafness (Olds & Oghalai 2015).

The pathophysiological process associated with this situation is explained by the fact that unconjugated bilirubin go through passive diffusion the blood-brain barrier and barrier cell membrane. Because bilirubin cannot get out by active transport, accumulates in the cells of the CNS and leads for this mode the changes described (Olds & Oghalai 2015). Thus, the exposure of neurons to bilirubin leads to increased oxidative stress and decreased proliferation of glutaminergic central neural synapses causing presynaptic neurodegeneration. At the same time the cell bodies of the spiral ganglia decrease, decreasing also the cell density and consequently the selective loss of myelin fibers (Figure 1.3-17) (Olds & Oghalai 2015).

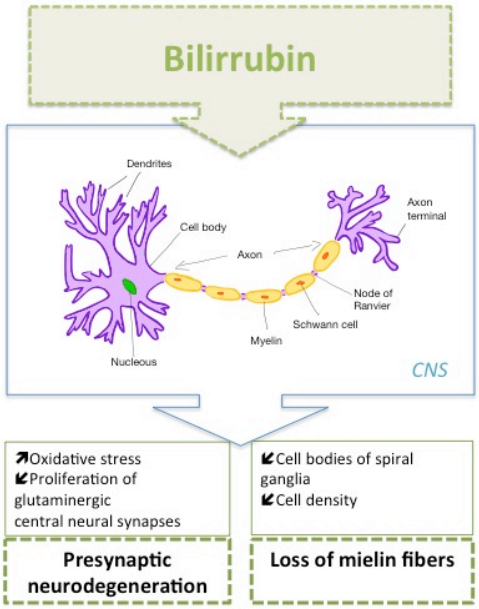

Figure 1.3-17 – Exposition of neurons to bilirubin

Simultaneously support cells release inflammatory mediators, which lead to an increased permeability of the blood-brain barrier (BBB) and therefore the entry of bilirubin's (Figure 1.3-18) (Olds & Oghalai 2015).

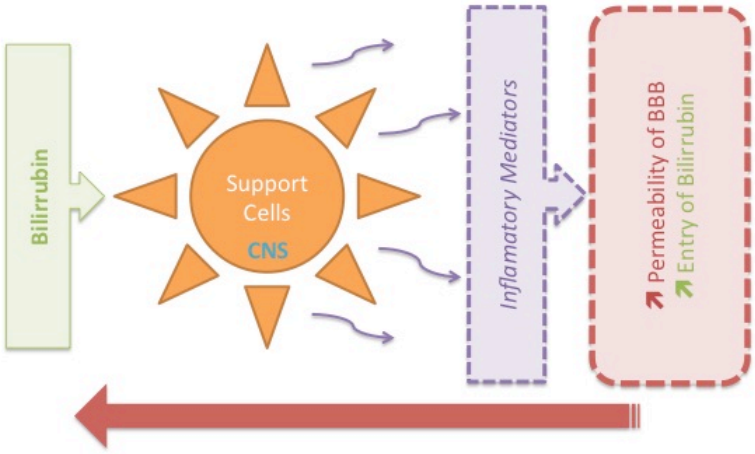

Figure 1.3-18 – Effect of bilirubin in CNS. (BBB- Blood-brain barrier)

Bilirubin at the level of the brainstem nuclei, will cause the main changes induced by hyperbilirubinemia and which are basically the auditory dyssynchrony. The cochlea is spared, however the injury may arise in the auditory nerve (VIII cranial nerve). In audiological field evaluation, waves III and V of the auditory brainstem potential have a diminished amplitude and interaural distance increased. Sometimes the substantial increase of the total bilirubin leads to loss of wave I (Olds & Oghalai 2015).

Cochlea is not directly affected by hyperbilirubinemia, however the cochlear injury can arise by the auditory nerve or injury of the cochlear nuclei of the brainstem (Olds & Oghalai 2015). It is estimated that the increase of both the conjugated bilirubin or not conjugated, leads to neurotoxic injury. Neonatal jaundice is often associated with a mutation on the X chromosome named – G6PD deficiency (Kuzniewicz et al. 2014; Nair & Al Khusaiby 2003). Although it is not pathognomonic that G6PD deficiency develops jaundice or kernicterus, the prevalence of cases associated with this change of blood is significant. This mutation leads to the development of a change in hemoglobin framing within the framework of hemoglobinopathies and characteristically confers some protection against infection by *plasmodium*, responsible for malaria. Is a very common hematological change in malaria endemic countries, and may represent an increase in cases of kernicterus and consequently auditory changes (Peters & Van Noorden 2009; Johnson et al. 2009).

The ANSD (Auditory Neuropathy Spectrum Disorder) is the entity that encompasses these diseases with abnormal neural auditory function (altered or missing ABR waveforms) in the presence of normal cochlear microphonics (the field potential emanating from the receptor potential of hair cells) and otoacoustic emissions (OAEs, i.e., sounds emanating from the ear due to non-linear force production by the outer hair cells) (Olds & Oghalai 2015). This disorder is characterized by tonal thresholds with moderate to profound loss that can vary on different days, associated with a decrease in discrimination, which is not predictable considering the tonal threshold and associated with difficulties in localization of sound and discrimination. More than 50% of children with ANSD have history of hyperbilirubinemia and/or anoxia during the neonatal period (Olds

& Oghalai 2015). A high total bilirubin and unconjugated bilirubin is associated with a low neurodevelopment and high incidence of hearing loss. In the study of Nickish *et al*, data changes in the brainstem auditory evoked potentials and hearing loss were observed in 10 to 37.5% of newborns with hyperbilirubinemia in which total bilirubin was less than 20 mg/dl. It was found in the same study that reversibility might be before the frame 12 months in 2:1000 newborns. The need for early and immediate action are important as well as the implementation of several procedures such as phototherapy, transfusion or even albumin infusion. It has been verified that the hyperbilirubinemia has an important social impact because it induces auditory changes (Olds & Oghalai 2015).

1.3.4.6 Hypoxia and asphyxia

The neonatal asphyxia is a situation that often occurs in underdeveloped countries and could be associated with cerebral hypoxia and metabolic changes that could lead to cell damage (Golubnitschaja et al. 2011).

In newborn infants with hypoxia or asphyxia, the spiral ganglion cells appear to be firstly affected. More severe hypoxia may cause irreversible cellular damage to the cochlea, particularly to the outer hair cells and stria vascularis. However, there is no clear threshold level of hypoxia defining the point at which hearing is at risk (Cristobal & Oghalai 2008). Other study, found that >50% of survivors of severe neonatal respiratory failure had sensorineural hearing loss at 4 years of age. Also, the use of extracorporeal membrane oxygenation has been found to increase the prevalence of sensorineural hearing loss among NICU survivors. In these patients, the prevalence of sensorineural hearing loss is in the range 3–26% (Cristobal & Oghalai 2008).

1.3.4.7 Neonatal Mechanical ventilation (≥5 days)

Exposure to the constant background noise generated by contemporary life-support equipment in the NICU can produce hearing loss (Cristobal & Oghalai 2008). Mechanical ventilation acts as risk factor, not only by the metabolic changes associated with the medical condition that led to mechanical ventilation, as well as

by acoustic trauma to which the newborn is submitted, leading to cochlear damage (Cristobal & Oghalai 2008).

Many studies point out that noise trauma can also produce damage to the inner hair cells, stria vascularis, spiral ganglion cells and supporting cells. The initial sign is outer hair cell damage. Suggesting that this could be due to free-radical formation (Cristobal & Oghalai 2008).

Environmental causes include infectious diseases and are of the utmost importance. In this chapter we included the classic group of pathologies-TORCH and other infectious diseases.

1.3.4.8 Infectious diseases

1.3.4.8.1 TORCH

New knowledge about the ways the maternal–fetal interface and placenta interact with the maternal immune system may explain these findings. Once thought to be 'immunosuppressed', the pregnant woman actually undergoes an immunological transformation, where the immune system is necessary to promote and support the pregnancy and growing fetus. When this protection is breached, as in a viral infection, this security is weakened and infection with other microorganisms can then propagate and lead to outcomes, such as preterm labor (Vesikari 1971).

Perinatal outcomes from viral infections during pregnancy can range from no effect to pregnancy, loss by spontaneous abortion due to fetal infection with resulting congenital viral syndromes (Vesikari 1971). In this group of infections we usually include Toxoplasmosis, Rubella, Cytomegalovirus, Herpes and other such as syphilis (TORCH). These diseases can present hematogenous, transplacental or ascendant transmission. The most frequent clinical manifestation in the fetus is diverse and can range from embryonic resorption, abortion, placental infection with fetal infection, intrauterine growth delay with fetal infection, stillbirth, newborn symptomatic or even asymptomatic. Among the different manifestations, the hearing is one of the most affected senses in this kind of infections.

*1.3.4.8.1.1 **T**oxoplasmosis*

Toxoplasmosis is a parasitic infection caused by *Toxoplasma gondii*, an obligate intracellular protozoan (Salviz et al. 2013). Cats are hosts of *T gondii* (Salviz et al. 2013). The infection is acquired when oocysts are ingested by consumption of contaminated food or water. Tachyzoites, a motile form of the parasite, enter nucleated cells and are disseminated through the blood stream. The tachzyoites elicit an inflammatory response (Salviz et al. 2013). The tachyzoites can form bradyzoites that are contained within cysts that may not elicit an immune response in the host (Salviz et al. 2013).

Acute infection with *Toxoplasma gondii* during pregnancy and its potentially tragic outcome for the fetus and newborn continue to occur in the United States, as well as worldwide, despite the fact that it can be prevented (Montoya & Remington 2008).

According the CDC data, the seroprevalence in United States is 22.5% and incidence is 1-10 per 10000. If infection occurs around the time of pregnancy transplacental transmission may occur. If fetal infection occurs early in gestation severe inflammation and necrosis can be observed particularly in the central nervous system. Untreated patients may go on to develop chorioretinitis, cognitive and motor dysfunction, seizures and hearing loss.

The frequency of vertical transmission increases with the gestational age. In contrast, severe clinical signs in the infected infant are more commonly observed in offspring of women whose infection was acquired early in gestation (Montoya & Remington 2008). The prevalence of sensorineural hearing loss is as high as 28% in children who do not receive treatment and this number can be reduced to 0% in patients that are identified early and are compliant with treatment (Salviz et al. 2013).

Because of the high transmission rates observed after 18 weeks of gestation, treatment with pyrimethamine, sulfadiazine, and folinic acid is also used for patients who have acquired the infection after 18 weeks of gestation, in an attempt to prevent fetal infection from occurring and, if transmission has occurred, to provide treatment for the fetus (Montoya & Remington 2008). Pyrimethamine is not used earlier because it is potentially teratogenic (Montoya & Remington 2008).

Educational measures should be done (e.g., books, magazines, or simple handouts), integrated into existing prenatal programs, visits, and classes trying to prevent this disease and his complications associated to fetal infection for example (Montoya & Remington 2008).

*1.3.4.8.1.2 **O**thers*

Congenital Syphilis is a condition caused by infection in utero with *Treponema pallidum* (CDC 2015b). Effective prevention and detection of congenital syphilis depends on the identification of syphilis in pregnant women and, therefore, on the routine serologic screening of pregnant women during the first prenatal visit (CDC 2015a). Congenital syphilis (CS) is a disease that occurs when a mother with syphilis transmit /passes the infection on to her baby during pregnancy.

CS can cause miscarriage, stillbirth or death shortly after birth. Up to 40% of babies born from women with untreated syphilis may be stillborn, or die from the infection as a newborn. Babies born with CS can have: deformed bones, severe anemia, enlarged liver and spleen, jaundice, nerve problems, like blindness or deafness, meningitis, and skin rashes (CDC 2015a).

This is a preventable disease, which we can be treated, when suspected.

*1.3.4.8.1.3 **R**ubella*

Rubella was initially considered to be a variant of measles or scarlet fever and was called “third disease” (Centers for Disease Control and Prevention 2015). Rubella virus is classified as a togavirus, genus Rubivirus. It is an enveloped RNA virus, with a single antigenic type that does not cross-react with other members of the togavirus group (Centers for Disease Control and Prevention 2015). Rubella is a contagious, generally mild viral infection that occurs most often in children and young adults (WHO Media centre 2015b). Pregnancy outcomes as a result of maternal rubella infection include spontaneous abortion, fetal infection, stillbirths or fetal growth restriction, and the congenital rubella syndrome (CRS). Maternal infection occurs in the first trimester, fetal infection rates are up to 50%, dropping to <1% after 12 weeks. Peripartum maternal infection does not seem to increase the risk of CRS (Vesikari 1971).

Children with CRS can suffer hearing impairments, eye and heart defects and other lifelong disabilities, including autism, diabetes mellitus and thyroid dysfunction – many of which require costly therapy, surgeries and other expensive care. CRS rates are highest in the WHO African and South-East Asian regions where vaccine coverage is lowest (WHO Media centre 2015b).

Worldwide, over 100,000 babies are born with CRS every year. There is no specific treatment for rubella but the disease is preventable by vaccination (WHO Media centre 2015b).

Prevention of CRS is the main objective of rubella vaccination programs in the United States (Centers for Disease Control and Prevention 2015). Rubella is one of the preventable causes of hearing loss.

*1.3.4.8.1.4 **C**ytomegalovirus*

Cytomegalovirus (CMV) is a ubiquitous virus with variable clinical manifestations. CMV infects 60% of women of childbearing age in developed countries and 90% in developing countries (Vesikari 1971). Person-to-person transmission occurs by contact with infected nasopharyngeal secretions, urine, saliva, semen, cervical and vaginal secretions, breast milk, tissue, or blood. Primary maternal infection occurs in 1–4% of susceptible women, and reactivation may occur in approximately 10% of seropositive women. Vertical transmission occurs most commonly after maternal primary infection, usually by the following mechanisms (Vesikari 1971): transplacental after the virus infects the placenta, intrapartum via ingestion or aspiration of cervicovaginal secretions during delivery, postpartum via breastfeeding, and ascending from the maternal genital tract antepartum (rare). CMV is the most common congenital viral infection, with a birth prevalence of about 0.5% (range 0.2–2.5%) and affects the vestibule, the organ of Corti and the neurons of the eighth cranial nerve, which explains why it is the leading cause of congenital hearing loss (Vesikari 1971).

CMV burden has been shown to be associated with the development of non-genetic sensorineural hearing loss. Early identification of children with CMV burden in early stages of development, as they are in increased risk for development the

SNHL, is very important for early intervention and better outcome (Devdariani et al. 2011).

While the rate of transmission correlates with advancing gestation, the severity of the disease is inversely related to gestational age. Prenatal diagnosis of fetal CMV is based on amniocentesis performed 6 weeks after the presumed time of infection and after 21 weeks of gestation, while sonographic findings usually imply a poor prognosis (Vesikari 1971).

1.3.4.8.1.5 ***H****erpes*

The high prevalence of hearing impairment among children due to herpes simplex virus infection was described (al Muhaimeed & Zakzouk 1997).

Genital herpes simplex virus (HSV-2) is the most common sexually transmitted infection among the adult female population of the United States. Herpes Simplex Virus 1 (HSV-1) and 2 (HSV-2) are part of a large family of DNA viruses of which eight are known to be infectious in humans. They are transmitted across epithelial mucosal cells as well as through skin interruptions and migrate to nerve tissues where they persist latent. Factors that affect a woman's risk of infection before pregnancy include ethnicity, poverty, cocaine abuse, earlier onset of sexual activity, number of lifetime sexual partners, sexual behavior and the presence of bacterial vaginosis. Overall seroprevalence of HSV among pregnant women is 72%. During pregnancy, HSV infection has been associated with spontaneous abortion, intrauterine growth restriction, preterm labor, and congenital and neonatal herpes infections. The clinical management revolves around decreasing vertical transmission to the fetus, thereby decreasing the risk of neonatal herpes infection. Primary or first genital HSV infection late in pregnancy carries a 30–50% risk of neonatal infection, while early pregnancy infection carries a risk of <1%. Transplacental or ascending transmembranal transmission of HSV from mother to fetus during pregnancy is uncommon; 80–90% of perinatal transmission occurs during labor and delivery (Vesikari 1971).

1.3.4.8.2 Malaria

Another infection relevant to the hearing, but still somehow in research, is malaria. This disease, caused by the bite of the anopheles mosquito transmits the *Plasmodium* spp protozoarium. There are 5 different kinds of *Plasmodium*: *falciparum*, *vivax*, *berghei*, *malariae* and *ovale* that triggers immune processes and relevant hemodynamic responses (OMIM 2012). Among the relevant pathophysiological phenomena, sequestration in cerebral microcirculation, which lead to loss of function anywhere from the auditory cortex to the cochlear hair cells, the release of inflammatory mediators for circulation and on the other hand the effect caused by the antimalarial therapy instituted are speculative theories to explain the exact mechanism of how parasite damages hearing (Zhao & Mackenzie 2011). The most severe course of the human disease is caused by *Plasmodium falciparum*, leading to multi-organ disease. In particular cerebral malaria (CM) is a potentially leading cause to a wide range of neurocognitive sequelae.

Ototoxicity from antimalarial is a well-publicized cause of deafness and a great deal of time and resources are spent assessing it in relation to new drugs. The effect of the malaria parasite itself on hearing is, however, poorly documented (Zhao & Mackenzie 2011).

Malaria induced by *Plasmodium falciparum* has been suspected to cause hearing loss (Schmutzhard et al. 2010). Developmental, cognitive and language disorders have been observed in children, surviving to cerebral malaria. Furthermore, it has been shown that severe *falciparum* malaria may lead to an acquired language disorder (Schmutzhard et al. 2010). The study of Schmutzhard et al. show, that malaria leads to a significant hearing impairment in mice. When comparing the results of the different subgroups an affection at all measured frequencies could been shown in the CM group, but only at the lower and middle frequencies in the non-CM group. Malaria retinopathy has been suggested to be the result of adherence and sequestration of *P. falciparum* - infected erythrocytes in the microvascular system leading to impaired microcirculation. It seems conceivable that impairment of cerebral microcirculation within the central nervous system is very likely to include both organs (Schmutzhard et al. 2010). The spiral modular artery enters the cochlea at the basal turn and ends at the apical turn. Therefore, disturbances of the microvascular system and impairment of perfusion of the

cochlea should effect the apical turns more than the basal (Schmutzhard et al. 2010). With a significant hearing loss at frequency that is located more apically in the cochlea - the hypothesis of microvascular affection in the CM group is supported. The non-CM group with a significant impairment of the lower and middle frequencies suggests an equivalent interpretation possibility. Other pathologic alterations are known to affect the inner ear with various inflammatory mediators (Schmutzhard et al. 2010). In 2011, Schmutzhard et al. suggest that the severity of the disease has an impact on the development of hearing impairment (Schmutzhard et al. 2011). ICAM 1 findings suggest that various systemic alterations may lead to a disturbance of the endocochlear potential, causing hearing impairment (Schmutzhard et al. 2011).

1.3.4.8.3 Meningitis

Meningitis is inflammation of the meninges (membranes) that surround and protect the brain and spinal cord. It is usually caused by a bacterial or viral infection (NDCS 2015). Meningitis can affect anyone, of any age, at any time. However, babies, toddlers and children under five years are the major risk group for meningitis, and young people aged 15–19 years the next most likely to suffer meningitis(NDCS 2015).

In meningitis, the infection may spread to the inner ear and irreversibly damage the hair cells of the cochlea thus causing deafness (Desai et al. 2015).

Several different bacteria can cause meningitis. Meningococcal disease, also referred to as cerebrospinal meningitis is a contagious bacterial disease caused by the meningococcus *Neisseria meningitidis* the one with the potential to cause large epidemics (WHO Media centre 2015c). The disease occurs sporadically throughout the world with seasonal variations and accounts for a proportion of endemic bacterial meningitis. However, the highest burden of the disease is due to the cyclic epidemics occurring in the African meningitis belt (Figure 1.3-19) (WHO 2014b). The onset of symptoms is sudden and death can follow within hours. In as many as 10-15% of survivors, there are persistent neurological defects, including hearing loss, speech disorders, loss of limbs, mental retardation and paralysis (WHO Media centre 2015c)(WHO 2014b).

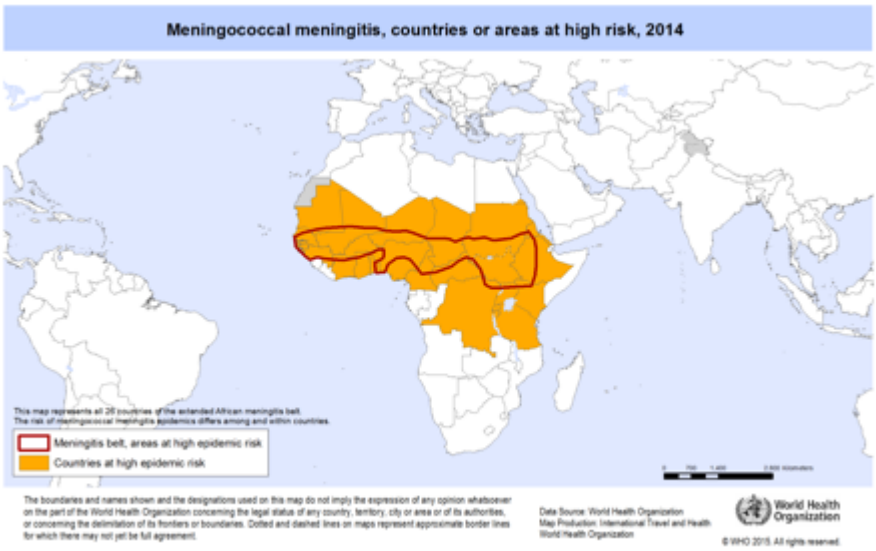

Figure 1.3-19 - Highest burden of the disease in the African meningitis belt.(WHO 2014b)

1.3.4.8.4 HIV infection

HIV is widespread in sub-Saharan Africa and the evidence suggests that it is linked to disabilities, affecting a range of body structures and functions (Banks et al. 2015). All three studies using a HIV-control group found a significantly higher prevalence of hearing impairment among people living with HIV (PLHIV)(Banks et al. 2015). Although more research is needed to fill the gaps in knowledge, in the review of Banks et al. data HIV is strongly linked to disability, and HIV-related disability is common in this region (Banks et al. 2015).

1.3.4.9 Ototoxicity

The high occurrence of hearing loss requires the implementation of monitoring program for children receiving ototoxic medication (Ghafari et al. 2015).

Aminoglycosides (AGs) are a well-known and successful class of antibiotics. Currently, nine AGs (streptomycin, neomycin, tobramycin, kanamycin, paromomycin, spectinomycin, gentamicin, netilmicin, and amikacin) are approved by the Food and Drug Administration (FDA) (Huth et al. 2011). In addition to their potent antimicrobial efficacy, all AGs can cause toxic side effects to the kidneys and

inner ear (Huth et al. 2011). While damage inflicted by AGs on the kidney is usually reversible, damage to the inner ear is permanent (Huth et al. 2011). Symptoms of cochleotoxicity include hearing loss and/or tinnitus, while those of vestibulotoxicity consist of disequilibrium and dizziness. AGs cochleotoxicity typically affects first the high frequency and then extends towards the lower frequency and ranges over time in a dose-dependent manner (Huth et al. 2011). One main susceptibility factor (17%–33% of patients with reported ototoxic damage) is the genetic predisposition to AG ototoxicity. This increased susceptibility was inherited maternally suggesting mitochondrial involvement, since several mutations in mitochondrial DNA has been linked to increased susceptibility to AG ototoxicity (Huth et al. 2011). The mutation A155G in mtDNA is described as increasing susceptibility risk to AG ototoxics (Fan et al. 2017).

Gentamicin is still widely used, and the neonatal population and young adult women are at especially high risk for gentamicin-induced ototoxicity (Kushner et al. 2016). In countries like Tanzania, gentamicin is widely used and is a known ototoxic agent especially in less well-controlled medical facility (Freeland, Jones & N. K. Mohammed 2010)

Others therapeutics we know that could lead to ototoxicity are, for example, the antimalarials as quinine. These are often associated to hearing loss (Claessen et al. 1998). At effective doses quinine causes considerable but reversible cochlear hearing losses in both healthy persons and in patients (Claessen et al. 1998)..

Quinine usually causes a reversible deafness but in an infant given an overdose the outcome may be different (Freeland, Jones & N. Mohammed 2010; Schmutzhard et al. 2011).

The use of artemisinin derivatives has increased exponentially with the use of artemisinin combination therapy (ACT) in all malaria's areas (Carrara et al. 2008a). They are highly effective and are considered safe, but in animal studies artemisinin derivatives produce neurotoxicity targeting mainly the auditory and vestibular pathways. In the study of Carrara et al (2008) neither audiometric or the ABR tests showed clinical evidence of auditory toxicity seven days after receiving oral artesunate and mefloquine (Carrara et al. 2008b).

1.3.4.10 Acoustic trauma

Some 1.1 billion of teenagers and young adults are at risk of hearing loss due to the unsafe use of personal audio devices, including smartphones, and exposure to damaging levels of sound at noisy entertainment venues such as nightclubs, bars and sporting events, according to WHO data. Data from studies in middle- and high-income countries indicate that among individuals aged 12-35 years, nearly 50% are exposed to unsafe levels of sound from the use of personal audio devices and around 40% are exposed to potentially damaging levels of sound at entertainment venues (WHO 2013d). Unsafe levels of sounds can be, for example, exposure to in excess of 85 dB for eight hours or 100dB for 15 minutes (WHO Media centre 2015a).

Exposure to loud, prolonged sounds (acoustic trauma, AT) leads to the death of both inner and outer hair cells (IHCs and OHCs), death of neurons of the spiral ganglion and degeneration of the auditory nerve (Baizer et al. 2015).
Hearing impairment may be induced by a single impulsive noise or after repetitive exposure to moderate or high intensity noise (Sanz et al. 2015) The cumulative damaging effects on the inner ear depend on the noise characteristics (frequency, level), chronicity and individual susceptibility to noise. Inner and particularly outer hair cells especially those located in the basal turn of the cochlea in mammals, are very sensitive to noise damage (Sanz et al. 2015).
Susceptibility to noise trauma could be genetic. Xing-Xing Zhou et al data, recently had verified that a Cx26 KD mice with acoustic trauma experienced higher hearing loss than simple noise exposure siblings and nearly had no recovery. Additionally, this study had revealed that reduced Cx26 expression in the mature mouse cochlea may increase susceptibility to noise-induced hearing loss and facilitate the cell degeneration in the organ of Corti (Zhou et al. 2016). Cx 26 is a protein clearly related to genetic HL as discussed further.

1.3.4.11 Anemia

Anemia is characterized by a decreased quantity of red blood cells, often accompanied by diminished hemoglobin levels or altered red blood cell

morphology (Kassebaum et al. 2014). Different biological, socioeconomic and environmental factors are reported to affect the dietary intake and nutrient absorption capacity of individuals (Degarege et al. 2015). Hypoproliferative anemia's are the most common anemia, and in the clinic, iron deficiency anemia is the most common of these followed by the anemia of inflammation or intestinal parasitosis. In anemia there is a reduced O_2 delivery to tissue (Kasper et al. 2015).

Anemia is pathophysiological diverse and often multifactorial. Symptoms result from impaired tissue oxygen delivery and may include weakness, fatigue, difficulty concentrating, or poor work productivity. Children may have issues with mental and motor development (Kassebaum et al. 2014).

Global anemia prevalence in 2010 was 32.9%, (Kassebaum et al. 2014) causing 68.36 million years lived with disability (8.8% of total for all conditions). Prevalence dropped for both sexes from 1990 to 2010, although more for males. Prevalence in females was higher in most regions and age groups. South Asia and Central, West, and East sub-Saharan Africa had the highest burden, while East, Southeast, and South Asia saw the greatest reductions (Kassebaum et al. 2014).

Iron-deficiency anemia was the top cause globally, although 10 different conditions were among the top 3 in regional rankings. Malaria, schistosomiasis, and chronic kidney disease–related anemia were the only conditions to increase in prevalence burden (Figure 1.3-20) (Kassebaum et al. 2014).

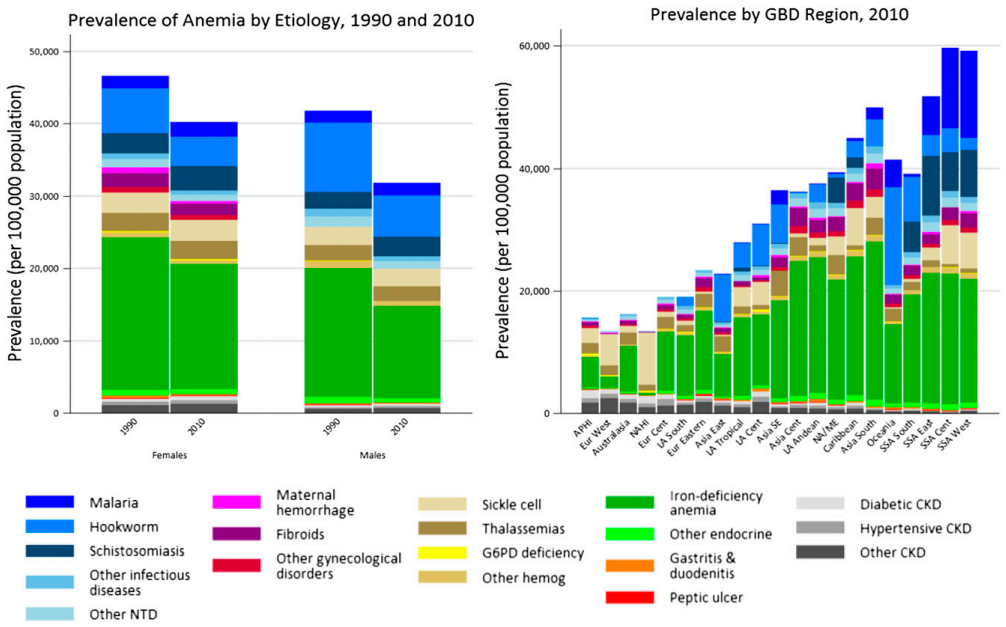

Figure 1.3-20 - Global and regional cause-specific anemia prevalence for 1990 and 2010.(Kassebaum et al. 2014)

Hemoglobinopathies made significant contributions in most populations, and are especially common in areas in which malaria is endemic (Kasper et al. 2015). This clustering of hemoglobinopathies is assumed to reflect a selective survival advantage for the abnormal RBC, which presumably provides a less hospitable environment during the obligate RBC stages of the parasitic life cycle (Kasper et al. 2015).

Sickle cells lose the pliability needed to pass through small capillaries. They possess altered "sticky" membranes that are abnormally adherent to the endothelium of small venules (Kasper et al. 2015). These abnormalities provoke unpredictable episodes of microvascular vasoocclusion and premature RBC destruction (hemolytic anemia). Hemolysis occurs because the spleen destroys the abnormal RBC. The rigid adherent cells clog small capillaries and venules, causing tissue ischemia, acute pain, and gradual end-organ damage. This venoocclusive component usually dominates the clinical course (Kasper et al. 2015).

A finite life span is a distinct characteristic of red cells. Hence, a logical, time-honorand classification of anemia is in three groups: (1) decreased production of red cells, (2) increased destruction of red cells, and (3) acute blood loss.

In Hemolytic anemia it should be classified as hereditary or acquired; in what hereditary intracorpuscular defects include hemoglobinopathies, enzymopathies or membrane cytoskeletal defects and extracorpuscular defects in Familial (atypical) hemolytic-uremic syndrome. Acquired hemolytic anemia include paroxysmal nocturnal hemoglobinuria (PNH) as intracorpuscular defect and as extracorpuscular: toxic agents, drugs, infections, autoimmune and mechanical (microangiopathic) destruction (Kasper et al. 2015).

G6PD deficiency is a prime example of an HA due to interaction between an intracorpuscular cause and an extracorpuscular cause, because in the majority of cases hemolysis is triggered by an exogenous agent (Kasper et al. 2015).

G6PD deficiency, an X-linked disorder, is the most common enzymatic disorder of red blood cells in humans, affecting more than 400 million people worldwide (Glader 2016).

With the most prevalent G6PD variants (G6PD A^- and G6PD Mediterranean), hemolysis is induced in children and adults by the sudden destruction of older, more deficient erythrocytes after exposure to drugs having a high redox potential (including the antimalarial drug primaquine and certain sulfa drugs) or to fava beans, selected infections, or metabolic abnormalities. However, in the neonate with G6PD deficiency, decreased bilirubin elimination may play an important role in the development of jaundice (Glader 2016).

Heterozygous females are genetic mosaics as a result of X-chromosome inactivation (in any cell, one X chromosome is inactive, but different cells randomly inactivate one chromosome or the other) and the abnormal cells of a heterozygous female can be as deficient for G6PD as those of a G6PD-deficient male: therefore, such females can be susceptible to the same pathophysiological phenotype. Although heterozygous women, on average, have less severe clinical manifestations than G6PD-deficient males, some develop severe acute hemolytic anemia (Cappellini & Fiorelli 2008).

The worldwide distribution of malaria is remarkably similar to that of mutated G6PD alleles, making the malaria hypothesis of G6PD deficiency—that G6PD deficiency is protective against malaria, revealed in some studies that parasite

growth is slowest in G6PD-deficient cells. Luzzatto *et al.* data showed that red blood cells with normal G6PD activity, taken from G6PD A^- heterozygous females (who underwent random X-chromosome inactivation), had 2–80 times more parasitic growth than G6PD-deficient red-blood cells. G6PD-deficient red-blood cells infected with parasites undergo phagocytosis by macrophages at an earlier stage of parasite maturation than do normal red-blood cells with parasitic infection, which could be a further protective mechanism against malaria.

Fortunately, most G6PD-deficient individuals are asymptomatic throughout their life, and unaware of their status (Cappellini & Fiorelli 2008). The illness generally manifests as acute hemolysis, which usually arises when red blood cells undergo oxidative stress triggered by agents such as drugs, infection, or the ingestion of fava beans. G6PD deficiency does not seem to affect life expectancy, quality of life, or the activity of affected individuals (Cappellini & Fiorelli 2008).

1.3.4.12 Genetic

The genetic influence in hearing loss is a known cause. Not only hearing loss is influenced by disease in the ear, but also, could have origin in another genetic cause and this is responsible for the hearing loss (Smith et al. 2005).

In developed countries the genetic study reveals important, as hearing loss due to genetic causes has increased on the decrease of environmental causes (Smith et al. 2005).

About 1.4 \ 1000 newborns have clinically relevant hearing loss and 3.5 \1000 by adolescence (Lafferty et al. 2014). Approximately 50% is explained genetically(Lafferty et al. 2014). Among these, ≈70% hearing loss is the only clinical sign and the remaining ≈30% can be syndromic hearing loss (Lafferty et al. 2014). The forms of nonsyndromic hearing loss, autosomal recessive are genetically heterogeneous and are the most common form of hereditary hearing loss, accounting for about ≈77% of cases (Lafferty et al. 2014). This number is followed by autosomal dominant inheritance (≈22%) and the mitochondrial

heredity or X-linked, which represents ≈1% of cases of genetic hearing loss (Figure 1.3-21) (Lafferty et al. 2014).

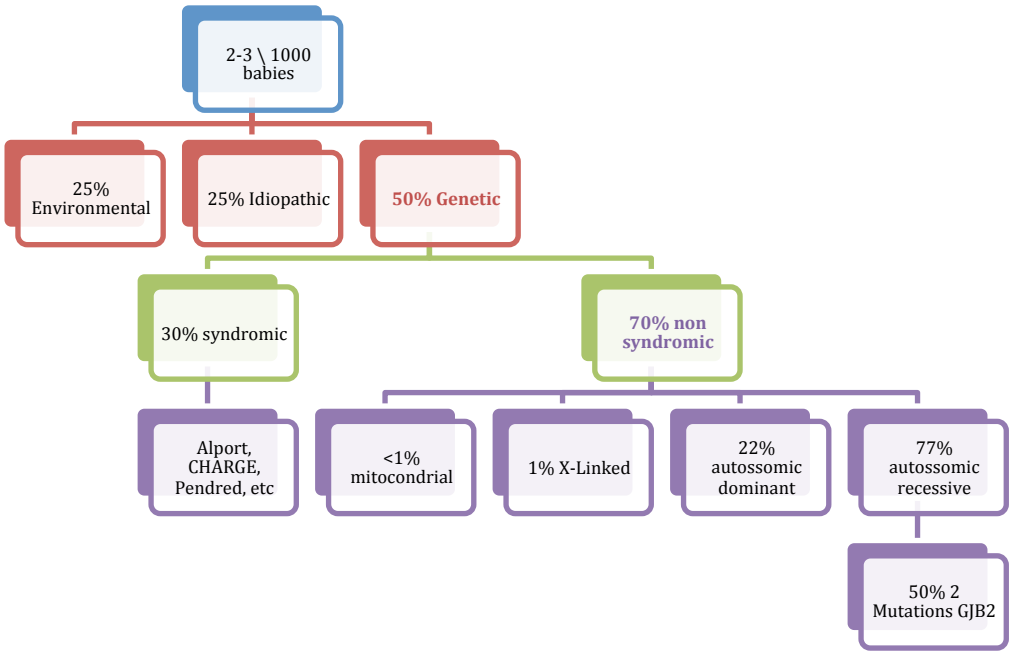

Figure 1.3-21 – Prevalence scheme about hearing loss causes(Lafferty et al. 2014).

The most recent advances in molecular genetics have allowed associate hearing loss more than 400 syndromes, while more than 140 loci related to non-syndromic hearing loss have been mapped (Matos et al. 2013). To date, 179 deafness loci have been reported in the literature: 67 DFNA loci, 106 DFNB loci, and 6 X-linked loci (http://hereditaryhearingloss.org/).

The GJB2 (Gap Junction β-2) forms with the GJB6 gene (Gap Junction β-6) DFNB1 *locus* located on chromosome 13q11-q12, and encoding the protein connexin-26 (Cx26) and connexin-30 (Cx30), respectively (Nickel & Forge 2008). The connexin proteins have a transmembrane location and can associate in a hexamer forming connexons resulting from the dock of both hexamers on the surface of adjacent cells (Martínez et al. 2009). The connexons are designated homomeric when consist of a single type of connexin or heteromeric, when they are formed by different connexins (Matos et al. 2013). The cells connected by gap junctions use such connexons to transfer ions and other small molecules among themselves

(Nickel & Forge 2008). In the cochlea, Cx26 and Cx30 are co-located and co-expressed, contributing to cochlear homeostasis, through the formation of gap junctions heteromeric (Nickel & Forge 2008). The maintenance of this homeostasis is achieved through the recirculation of ion K^+ in endolymph after stimulation of hair cells in the inner ear (Nickel & Forge 2008).

The *GJB2* is about 5500 bp and one coding exon, in a total of two exons (Kenneson et al. 2002). More than 100 mutations (http://davinci.crg.es/deafness/index.php) have been identified in this gene and, depending on the population, account for about 10-40% of cases of hereditary hearing loss, representing the most common cause of non-syndromic autosomal recessive hereditary hearing loss (Matos et al. 2011). The most common mutation in the Caucasian population is the c.35delG, which results in premature termination of the polypeptide chain of Cx26 after a change in reading frame leading to a premature STOP codon. The allelic frequency for this mutation was estimated at 2.5% in the general Caucasian population, in a total of 3.0% allele frequency for all mutations in *GJB2* (Matos et al. 2011). The c.167delT mutation, in turn, has a high frequency in Ashkenazi Jewish population (7.5%) and c.235delC mutation is the most common in Japanese and Korean populations, allele frequencies estimated at 0.5 and 1%, respectively.(Tang et al. 2006; Falah et al. 2011).

The *GJB6* gene, contrary to *GJB2* gene, has today described a few mutations, only six that have a pattern of recessive inheritance. These six mutations, four are deletions (del Castillo et al. 2005). The most common are the deletion Δ (GJB6-D13S1830), and Δ (GJB6-D13S1854), which, while at the same time they truncate *GJB6*, inactivate the gene *CRYL1*, eliminating the region between them. The *CRYL1* gene coding for λ-crystalline and to date, no case of individuals possessing either the Δ deletion (GJB6-D13S1830), whether Δ deletion (GJB6-D13S1854), has been reported with ocular disorders (Rodriguez-Paris et al. 2011).

2 Objectives

2.1 General objective

The overall objective will be to investigate possible etiological factors in the origin of sensorineural hearing loss in a sample of individuals observed in humanitarian missions from February 2012 to January 2014, a total of 2 full years of Humanitarian Missions in São Tomé and Príncipe. The selected sample will be stratified by:

- Hearing Loss complain
- Degree of hearing loss, acceded according to WHO classification,
- Hemoglobinopathy trait, considering HbS and G6PD deficiency,
- Study of connexin 26 and 30 genes, for study of DFNB1 locus,
- Immune status for rubella.

Determination of the prevalence of HbS trait and G6PD deficiency will be important as they may be associated with hearing loss. Both factors are associated to thromboembolic events triggered by the deformation of the erythrocytes or by their hemolysis. In presence of infection with *Plasmodium falciparum*, this erythrocyte's deformation, which represents a protection factor to severe malaria, can also be responsible to thromboembolic events.

The study of *DFNB1 locus* will always be relevant by the fact that this *locus* is strongly associated to HL but also because São Tomé and Príncipe is a country that had been colonized by the Portuguese and other European countries. In this kind of study, we are trying to determine the influence of other people on the genetic characterization of the population. São Tomé and Príncipe is an island, and as such it is expected that in-breeding might be one of the causes of the perpetuation of genetic deafness. In the study of *DFNB1* gene, the most frequent genetic deafness, mutations of *GJB2* and *GJB6* are those that classically are more prevalent. Thus, and to think of our genetic influence, it would be interesting to evaluate these changes.

The evaluation of the immune status of rubella is important first to analyse the existence or not of this disease in the community. Knowing that there isn't

vaccination for this disease, in this country, as well they don't have possibility of diagnosis.

2.2 Specific Objective

The specific objectives are:

- Analyse specificity and sensitivity of HL complain
- Study the association of HbS traits and G6PD deficiency with cases of sensorineural hearing loss,
- Determine the prevalence of hearing loss associated with HbS traits and G6PD deficiency in the sample,
- Determine the prevalence of hearing loss caused by genetic factors associated with *DFNB1 locus* in the sample,
- Determine the immune status for rubella in the sample and if there is any association to hearing loss.

These objectives would allowed to answer the following questions:

- Did the population of STP recognize the complain of HL?
- Is the sensorineural hearing loss in São Tomé and Príncipe associated with traits of the malaria resistance (HbS or G6PD deficiency)?
- Is genetic deafness, namely the mutations of GJB2 and GJB6 responsible for sensorineural deafness in São Tomé and Príncipe?
- Do they have rubella in the population of São Tomé and Príncipe? Could it be associated to hearing loss?
- What epidemiological factors may be acting as risk factors for hearing loss in this sample?

3 Methods

3.1 Subjects

We will present here a compilation of all the material and methods' information's that will be described in the next chapters, as full part of each published papers presenting the results.

The missions that took place between 2011 and 2014, formed by the surgery group and audiologic group, allowed contact with the population in order to recruit individuals who are part of this study.

For the study of hearing loss, 2 samples were obtained in the period from 2011 to 2014, one consisted of individuals who have agreed to participate in the study and answered to the question: "Do you think you have a hearing loss?" (section 4.1.); and another sample in which accepted not only answer the question, but also accepted blood collection to DBS (or Guthrie) card (Cap 4.2 – 4.6.).

To analyze the hearing complain by the question "Do you think you have a hearing loss?", we analyzed all the subjects that answered the question and have been evaluated by audiometric exams.

For the second part of this study, we selected a sample of natural individuals of STP that was based on data collected during otolaryngology missions carried out between February 2012 and January 2014. We selected the individuals who met the following inclusion criteria:

- Natural and resident in São Tomé and Príncipe, the subject and their parents,
- Aged between 2 and 35,

- Normal otoscopy - tympanogram type A,

- Having data of pure tone audiogram (PTA) or Auditory Brainstem Response (ABR) in both ears,

- Agreed to participate in the study answering a survey on identification of risk factors for hearing loss and agreed to give a blood sample to DBS card to be processed for genetic and virological laboratorial tests.

This project is based on the work done during the Otolaryngology missions - Project Health for all specialties, with which the ENT department of the Hospital CUF Infante Santo has collaborated since 2011. The sample consisted of data collected during the years 2012-2014. The establishment of temporal limitation to February 2012 was because it was the point by which was possible to hold the hearing study to all subjects (cooperating and non-cooperating). Individuals undergoing the study were those who resorted to ENT assessment during missions or were institutions that were selected to perform auditory screening (schools and local hotel unit). The selection of schools and hotel unit was based on the fact that electricity and otherwise offered less likely to run out of power during the screening. All individuals evaluated by ENT missions was conducted a clinical questionnaire and recorded the audiometric tests.

The selection of individuals between 2 and 35 years is based on the fact that this research project is partially conducted with the humanitarian project and hearing rehabilitation as described (chapter 6.1.2). The age groups contemplated for hearing rehabilitation (supply of hearing aids) were the children and young adults, having advantage in adapting hearing aids, in which were studying or working, in order to provide better integration into society and improving self-esteem. On the other hand, thinking that hearing loss may have a degenerative evolution over the years, these age classes are less exposed to risk factors and are protected from a degenerative hearing loss, being better for a study about etiologic factors of hearing loss.

The sampling method used for this research project, considering the above, it was a convenience sample.

In São Tomé and Príncipe there is no way to assess auditory function, except during missions. The individuals in this project are those who voluntarily from the different geographical areas of São Tomé and Príncipe, resorted to ENT assessment and those who were selected during the missions for screening (schools and hotel unit with electricity and less likely to electrical failure during tracking).

The size of the sample obtained was 316 individuals, 144 males and 172 women. Described in detail in 4th Chapter (Results and discussion).

All individuals with middle ear pathology, or mixed conduction hearing loss were excluded as those presenting psychomotor developmental delays or probable cause of hearing loss identified.

To all individuals was done: otoscopy, medical history collection, application of a questionnaire on general health to himself or to family or caretaker that accompanying (Appendix 4), audiological assessment and blood draw for DBS card (Figure 3.1-1).

Figure 3.1-1 – Material to collect the sample, collecting blood to DBS card and storing. (Personal photo)

3.2 Ethic Commission

The study was submitted and approved by the Medical Ethics Committee of São Tomé and Príncipe and Ethics Research Committee NMS|FCM-UNL (nº02/2014/CEFCM). The Ethics Research Committee is aligned with the Declaration of Helsinki for the Protection of Human Subjects. A full consenting process was applied in respect of all participants. Consent to use the survey data was also obtained. (Appendix 1-3)

3.3 Study methodology

The methodology adopted was a case-control study based on subjects who voluntarily agreed to participate in the study and signed the Informed Consent (Appendix 3).

The organization of subjects in the sample (n = 316 individuals) was performed according to the audiological data being integrated in a control or case group being always considered the respective age groups:

- **Control Group**: includes subjects with normal hearing (both ears are normal)

- **Case Group**: have at least one ear with sensorineural hearing loss

From all subjects included in the study was collected a blood sample for DBS card and stored at room temperature. Later, it will be analyzed in the laboratory, in Portugal (Figure 3.3-1). Genomic DNA was obtained from each blood sample using a commercially available kit (QIAamp® DNA micro kit; Qiagen) according to the manufacturer's instructions. All DNA samples were stored at –20 °C until genetic study of the β*-globin* gene, *G6PD* deficiency, *GJB2* and *GJB6* genes.

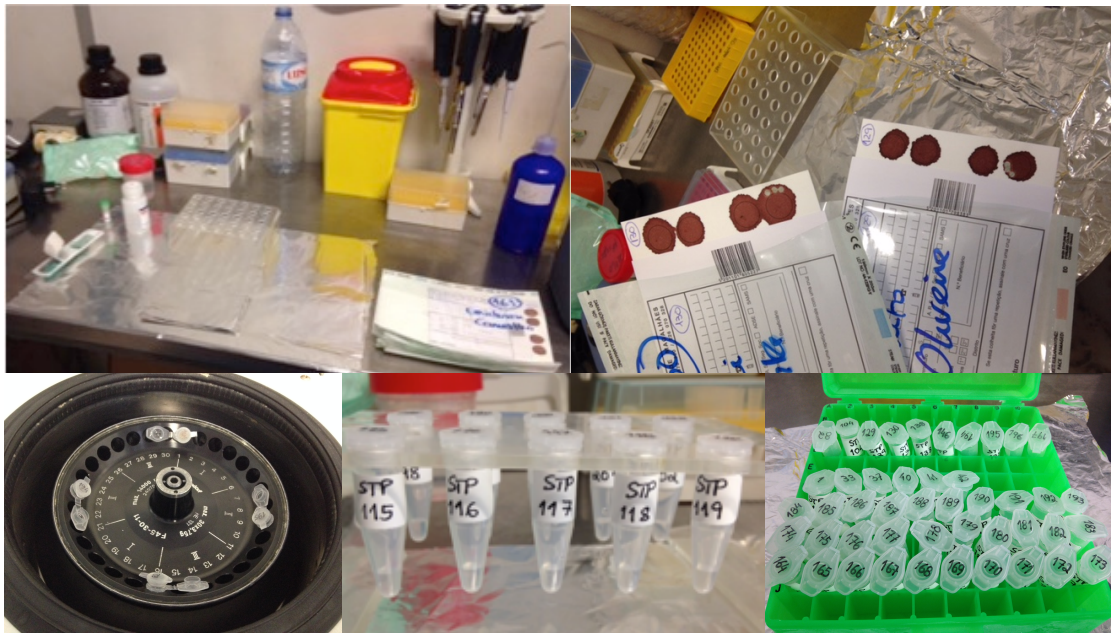

Figure 3.3-1 – Some views of the collecting to DNA Extraction. (Personal photo)

3.4 Audiologic Evaluation

To evaluate and quantify the hearing loss will be held tympanogram, pure tone audiogram and if there is no cooperation, in young children or in case of doubt, we opted to analyze the hearing status by Auditory Brainstem Response.

The equipment used was:

- impedanciometer - GSI 39 Timp V2

  The GSI 39 Auto Tymp provides a flexible screening product for tympanometry and acoustic reflex measurements. It is a lightweight and portable equipment, designed to make detection and documentation of middle ear pathologies, fast and accurate. The ipsilateral and contralateral reflex measurements may be performed along with 226 Hz tympanometry with the frequencies available on 500, 1000, 2000 and 4000 Hz (GSI 2016).

- audiometer - Madsen Midimate 622

  The MIDIMATE 622 is the second member of the new generation of audiometers from GN Otometrics, which started with the Midimate 602. This dual-channel clinical/diagnostic audiometer features a unique multilingual menu-driven display and five user-programmable test setups for optimal operating efficiency during daily routine examinations. New features incorporated into the MIDIMATE 622 include, amongst others, data interface, 1 dB attenuator resolution, ipsilateral masking, and simultaneous presentation of stimulus and masking binaurally (Madsen 2011).

- Auditory Evoked Potentials - Vivosonic Integrity V500

  Vivosonic Integrity V500 is the #1 Awake ABR system for clinics specializing in pediatric audiology, while providing equally valuable benefits for clinics serving general and special needs adults, as well as the geriatric population.

  Integrity™ combines new signal processing technologies, improved amplifier design and wireless capabilities to produce clear recordings with or without sedation. With this innovative technology, Integrity™ minimizes patient risk, expedites the early detection of hearing loss, significantly

reduces loss to follow-up issues for newborn hearing screening programs, and demonstrates reliable performance in less-than-ideal clinical environments (Vivosonic 2017).

Audiological tests are performed without audiometric cabin with TDH 39 headphones in a closed room, with noise levels below 30 dB measured by *iPhone* application *SchabelDoesIT GbR, Munich, Germany* (version 1.0.0) (Figure 3.4-1).

Figure 3.4-1– Pure Tone Audiogram done in a closed room. (Personal photo)

The audiometric equipment is calibrated according to the calibration standards for free field audiometry Delta-K01-2002, ISO 389-7 Standard[2].

WHO classification will be adopted to quantify hearing loss, and so is considered for calculation of the degree of hearing impairment the average of pure tone threshold at 500 Hz, 1000 Hz, 2000 Hz and 4000 Hz (Table 3.4-1) in the best ear (WHO 2016).

[2] DELTA. Guidelines for the Set-up and Calibration of Equipment Employed in Free-Field Audiometry. *AV 01/02-2002*. 2002:1–20

Table 3.4-1 – Classification of Hearing Loss by degree (WHO 2016).

Degree of Hearing Loss	Intensity of Sound (dB)
Normal	<=25
Mild	26-40
Moderate	41-60
Severe	61-80
Profound	>=81

If the subject doesn't collaborate in pure tone audiogram, we adopted the electrophysiological threshold with Auditory Brainstem Response (Figure 3.4-2).

Figure 3.4-2 – Determination of electrophysiological threshold by Auditory Brainstem Response (ABR). (Personal photo)

The determination of electrophysiological threshold with Auditory Brainstem Response was based on the study of Gorga *et al.* where the use of ABR could be

adapted to predict the pure tone thresholds in a wide range of frequencies, and in some cases underestimate the hearing threshold (Gorga et al. 2006).

3.5 HbS

The *HbS* gene point mutation (Glu6Val) was determined by PCR-RFLP. The primers and PCR conditions for the mutation site of this gene is shown in Table 3.5-1. For all of them the nucleotide mutated resulted in either gain or loss of restriction site, which therefore allowed the wild-type (HbAA) and variant alleles to be discriminated by RFLP after appropriate restriction enzyme digestion.

Table 3.5-1 - PCR-RFLP conditions for identification of HbAS (rs334) polymorphism.

Gene	Primers	PCR fragment	Patterns after restriction enzyme digestion
HbAS codon 6 (rs334)	Rv 5'- AGG GTG GGA AAA TAG ACC AA -3' FW 5'- CGG CTG TCA TCA CTT AGA CCT -3'	395 bp	HbAA: 202, 180, 13 bp HbAS: 382, 202,180,13 bp HbSS: 382, 13 bp

PCR was carried out with 50 ng of DNA in 50 ml reaction volume, containing 1.3x ImmoBuffer, 1.5 mM MgCl2, 0.6 mM dNTP, 1.0 mM of each primer, and 0.75 U of Immolase (Bioline). The amplification started with an initial denaturation step at 95 °C for 7 min, cycling parameters were 35 cycles of 95 °C for 30 s, specific annealing temperature (60 °C) for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. After amplification, 10 ml of each PCR products were digested with appropriate restriction enzymes (*DdeI*) for 3 h at 37 °C followed by an inactivation step at 65 °C for 20 min and electrophoresed in 2% agarose gel with ethidium bromide (0.5 mg/ml) for visualization under ultraviolet light (Figure 3.5-1). The expected products for each genotype of the tested gene are shown in Table 3.5-1. All the genotype determinations were carried out twice in independent experiments and inconclusive samples were reanalyzed.

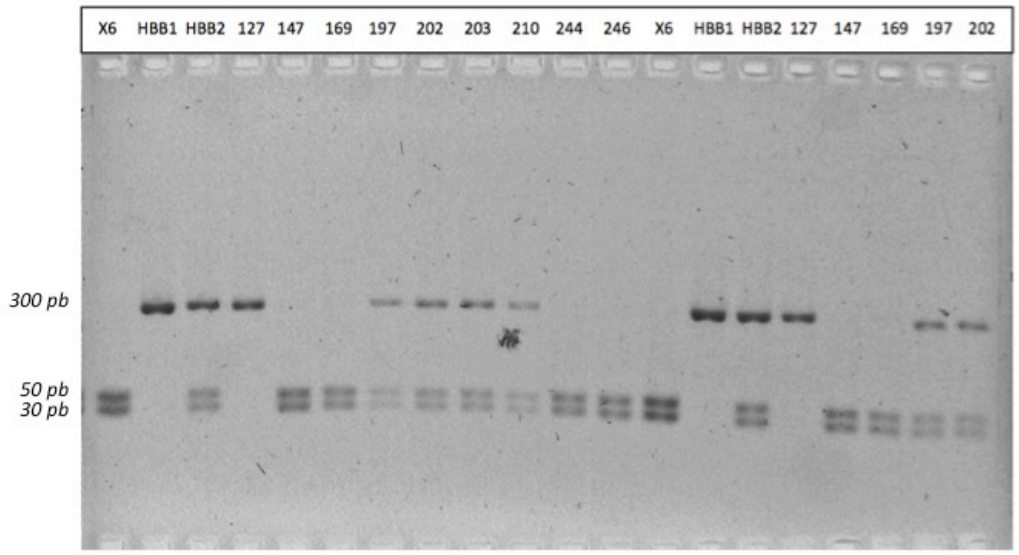

Figure 3.5-1 - Agarose gel obtained in HbS study. (Lane 1 - HbAA, Lane 2 - HbSS, Lane 3 - HbAS).

3.6 G6PD deficiency

We selected for this study two polymorphisms in *G6PD* gene between the most. prevalent SNPs described (rs1050828 and rs1050829) in Sub-Saharan Africa (Howes et al. 2013; Manco et al. 2007).

The SNPs selected were genotyped using real-time PCR (RT-PCR 7300 Applied Biosystem), through TaqMan® SNP genotyping assays (Life Technology), according to manufacturer instructions (Figure 3.6-1). Real-Time PCR genotype duplicate validations were carried in 20% of randomly selected samples in independent experiments and all the inconclusive samples were reanalyzed.

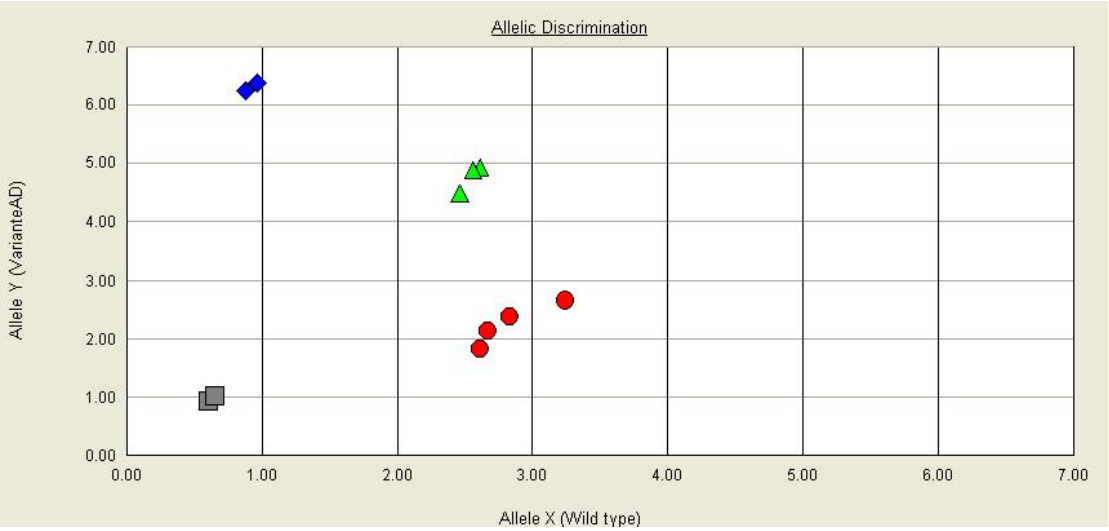

Figure 3.6-1 – Real-time PCR through TaqMan® SNP genotyping assays for G6PD

3.7 GJB2 gene

For *GJB2* gene the DNA sample was analyzed by sequencing regarding the *GJB2* coding region.

PCR amplification and sequencing of the coding region of the *GJB2* gene was performed using previously described primers (Matos et al. 2010). The *GJB2* fragment that was amplified comprises the coding region and flanking noncoding regions including the acceptor splice site. However, the extension of the sequence obtained beyond the coding region was variable not allowing results from the acceptor splice site for all the subjects.

All electrophoretograms were visually inspected, the low-quality extremities were trimmed off and heterozygosities were marked, using the Chromas Lite software (v.2.01). The resulting analysed and edited sequences were copied from Chromas Lite in Fasta format and blasted against the reference sequence NG_008358.1 using NCBI's Blast program (suite 2-sequences) (Figure 3.7-1). All the variants here described were named according to Human Genome Variation Society's recommendations.

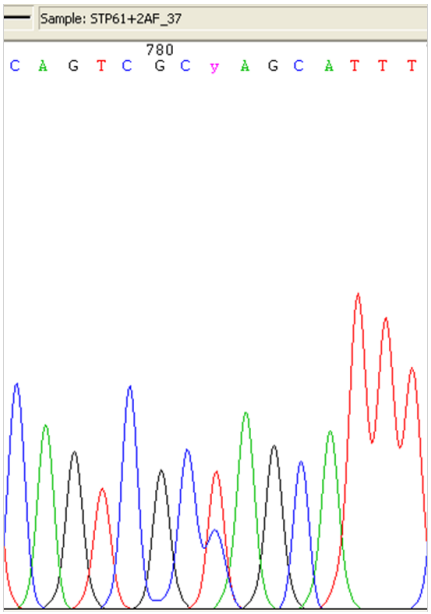

Figure 3.7-1 – Example of an electrophoretogram of GJB2 sequences obtained, for a small part of the sequence showing the quality of the process and the type of results

3.8 GJB6 gene

GJB6 gene was analyzed by multiplex PCR (del Castillo et al. 2005) regarding the presence of the two *GJB6* large deletions, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) in agarose gel (Figure 3.8-1).

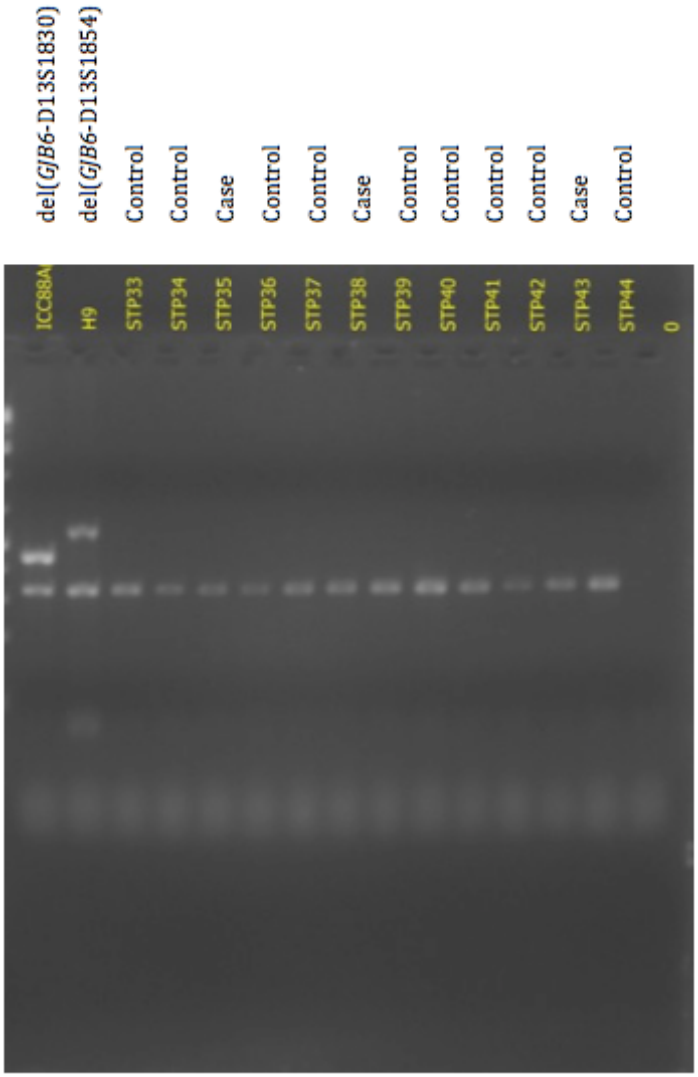

Figure 3.8-1 – Agarose gel detecting GJB6 deletions

3.9 Rubella

Rubella IgG extraction: For IgG extraction it was added 400 µL of SERION ELISA kit dilution solution to ¼ of the DBS, corresponding to 32 mm^2. The extraction was carried out for 60 minutes at 600 rpm at room temperature and 18 hours at 4ºC.

Rubella IgG determination: For the IgG determination we used SERION ELISA classic rubella virus IgG kit and 100 µL of extracted. The protocol performed was recommended by SERION.

The results were interpreted according to the algorithm of Figure 3.9-1.

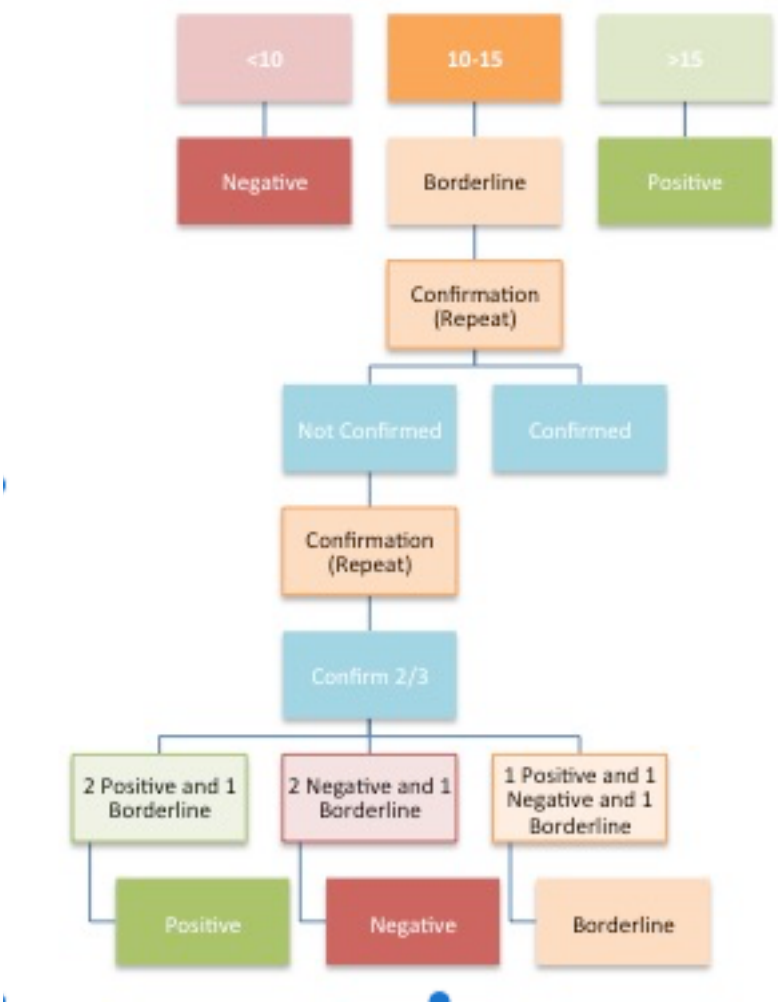

Figure 3.9-1 – Algorithm for interpretation of results of IgG rubella. Results are expressed in international units (U/I).

The procedure of IgG determination from DBS was first validated and optimized, evaluating the correlation of IgG achieved by our extraction method *versus* the immune status (vaccinated / unvaccinated) (work done by Vera Vicente – master student - IgG prevalence study for rubella virus in São Tomé and Príncipe with resource to DBS cards)

3.10 Bioinformatics and statistical analysis

All analyses were performed using the Statistical Package for the Social Sciences for Mac 20.0 version (SPSS).

Description of the sample was made with Descriptive Statistics, using Frequency Analysis, Means and Standard Deviation (SD).

The Hardy Weinberg Principle was tested with Qui-square test for one sample.

To study the association between variables we used Qui-square test.

To identify risk factors of HL, we adopted a Multiple Binary Logistic Regression.

4 Results and Discussion

Results were subject to publication and therefore will be presented as articles as published or submitted and under review.

4.1 Sensitivity and specificity of self-reported hearing loss in a sub-Saharan African country. Do they recognize the disability?

Submitted to BMJ Open

Cristina Caroça, Paula Campelo, Diogo Ribeiro, Helena Caria, Susana Nunes Silva, João Paço

ABSTRACT

Objective:

Hearing loss is an important problem in today's society. It is responsible for social isolation, depression, low education and thus leads to low social productivity and low quality of life. Self-identification of this disability, especially at a young age, is essential to the early implementation of social and educational measures necessary to promote an integrative society.

The aim of this study is to compare the prevalence of self-reported hearing loss with the prevalence of hearing loss obtained from audiometric testing in a sample with different age groups from the population of the Democratic Republic of São Tomé and Príncipe, observed during the humanitarian project "Health for all specialties".

Methods:

This is a retrospective study of data collected from a clinical audiology assessment of patients in São Tomé and Príncipe. All patients were asked about their hearing status with the following questions: "Do you feel you have a hearing loss?" and "which ear? one or both?" and were submitted to an audiometric evaluation (Pure Tone Audiogram or Auditory Brainstem Response).

Results:

A total of 567 patients answered the questions. Considering the hearing loss in one or both ears, we obtained a sensitivity of 87.01% and a specificity of 64.48%, when compared to the full range of audiometric tests. The youngest group revealed a lower sensitivity (65.22%) and a higher specificity (73.58%) when compared to the oldest group.

Conclusions:

The subjective perception of hearing loss continues to be a form of deafness identification and may be useful in epidemiological studies, especially in least developed countries where hearing tests are not always available. In this study, based on the sensitivity, more than 30% of children with hearing loss were not detected, meaning that the opportunity was lost for early intervention during critical periods of language acquisition, which could be problematic.

Key words: Hearing Loss; Questionnaires; São Tomé and Príncipe; Self-Report; Sensitivity; Specificity

Acronyms and abbreviations: Auditory Brainstem Response (ABR), hearing loss (HL), Instituto Marquês de Valle Flor (IMVF), least developed countries (LDC), Pure Tone Audiogram (PTA), unilateral hearing loss (UHL), World Health Organization (WHO)

ARTICLE SUMMARY

Strengths and limitations of this study

- Hearing loss (HL) is an important problem in today's society. Approximately 5% of the world's population has disabling HL. This loss is responsible for social isolation, depression, low education and thus leads to low social productivity and low quality of life. Auditory evaluation was not available in São Tomé and Príncipe unless it was part of humanitarian missions of otolaryngology.
- The application of questionnaires was useful in least developed countries (LDC), where there is a lack of equipment to facilitate the early detection of hearing impairment in children. In São Tomé and Príncipe, the questionnaire was applied to patients and tutors. The auditory evaluation was performed only during missions, 3 to 4 weeks a year. The first limitation was the location of the exam, which was in a quiet room without a soundproof booth (none are available on the island). Attempts were made to limit the sound, which was tested by an iPhone app, The Auditory Brainstem Response (ABR) was used to assess all patients who did not cooperate with the Pure Tone Audiogram (PTA). To perform the ABR we chose to use the click instead of tone bursts because it allowed us to obtain more information in a short time, taking into account the limitations. If the ABR thresholds were normal for the click, we assumed that the low frequencies were normal.
- For younger children, the questions were answered by the caretakers, and the self-report presented a high specificity despite the low sensitivity. Based on the sensitivity, more than 30% of children with HL were not detected, and opportunities for early intervention during critical periods of language acquisition were missed, which could be problematic for the younger group.

INTRODUCTION

Hearing loss (HL) is an important problem in today's society. Approximately 5% of the world's population has disabling HL,(WHO 2013a) expecting that the prevalence of HL is higher if the criteria such as "worse than 25 dB" are used.

This loss is responsible for social isolation, depression, low education and thus leads to low social productivity and low quality of life.

The application of questionnaires is useful in least developed countries (LDC), where there is a lack of equipment to facilitate the early detection of hearing impairment in children.

The knowledge of HL in a community should lead to implementation of some measures to reduce inequalities and achieve an integrative society, which is one of the goals for sustainable development according to the World Health Organization (WHO)(United Nations 2016). According to the United Nations, a country such as São Tomé and Príncipe in Sub-Saharan Africa belongs to the LDC. With scarce healthcare resources in these countries, there is no possibility to identify HL by audiometric exams.

During humanitarian missions of the Instituto Marquês de Valle Flor (IMVF) and the *Instituto Camões* for the project "Health for all specialties", a group of otolaryngologists and audiologists came to this equatorial island and evaluated the audiologic status of the population.

While standardized audiometric assessment of HL could be considered the gold standard for estimating its prevalence, large studies are often constrained by limited budget, expertise and the logistic difficulty of performing audiometric screening on a large scale(Sindhusake et al. 2001). Moreover, some countries, such as those that are among the LDC, do not conduct audiometric exams nor have professionals available to identify this disability unless suspicions are raised from a self-report of HL.

A self-reported questionnaire can give HL indicators, and it is a fast and economic way of getting information for large epidemiological studies(Sindhusake et al. 2001).

A large number of questionnaires are available for the assessment of HL, such as the following: Social Hearing Handicap Index (SHHI), Hearing Performance Inventory (HPI), Hearing Handicap Inventory for the Elderly: Screening Version (HHIE-S) and National Health and Nutrition Examination Survey (NHANES). In these tests, we can find questions on hearing such as “Do you have a problem with your hearing?” or “Do you feel you have a hearing problem?”.

The validation of the question, confirmed through the results of the audiometric testing, is important and necessary to assess the HL status, and although it is a “subjective” exam, it is considered a gold standard assessment when characterizing hearing.

According to Peter Torre III et al., there have been epidemiological studies in which different questions about audiology assessment are rated on adult patients over forty years of age (Torre et al. 2006).

The study of Marini et al. investigated the value of the hearing and non-hearing complaints reported on the clinical history and compared them with the results of the audiometric exams using the Pure Tone Audiogram (PTA). A sensitivity of 80.9% and a specificity of 69.7% was reported for the HL complaints in all age groups (Marini et al. 2005).

Newton et al. evaluated the use of a questionnaire to detect HL in Kenyan pre-school children and demonstrated that it could be usefully applied at the primary health care level (Newton et al. 2001).

The necessity of knowledge concerning the HL status in the population of São Tomé and Príncipe, who do not have access to audiometric examination, is important in the early identification and effective treatment of hearing loss and implementing social and educational measures. In this study, we aimed to evaluate the sensitivity and specificity of the self-reported question “Do you feel you have a hearing loss?”, which was asked during the audiology appointment associated with the humanitarian project “Health for all Specialties” in São Tomé and Príncipe with a focus on young age groups.

MATERIALS AND METHODS

Subjects

We performed a retrospective study using medical charts of individuals who were observed at the audiology appointment during the humanitarian missions in São Tomé and Príncipe (Hospital Ayres de Menezes, schools and a local hotel). Only individuals or caretakers (if children or older individuals could not answer the question) answered a self-reported question "Do you feel you have a hearing loss?". If the answer was "yes", follow-up questions "Which ear? One or both?", were asked. The audiometric or electrophysiological threshold was recorded for all individuals who answered both questions.

We included in this study all individuals who were accepted to participate, answered the clinical oral inquiry about previous history (familial history of HL; personal history of ear infections and infectious diseases such as malaria and meningitis; head trauma; medications; and for young children, questions were asked about the history of infections and medications in the mother during pregnancy and delivery and post-delivery complications), were observed using otoscopy, and had performed an audiological evaluation.

Methods - audiometric and electrophysiological threshold

An audiologist performed the audiometric exams (PTA and ABR).

To determine the audiometric thresholds, the equipment used was the Otometrics Madsen Midimate 622 (audiometer). The audiometric exams were performed without an audiometric cabin, with earphones-TDH39, in a closed room, and with a level of noise measured by *iPhone SchabelDoesIT GbR, Munich, Germany* (version 1.0.0). The noise did not exceed 30 dBSPL, based on ANSI S3.1-1999 (R2013). The audiometric equipment was calibrated according to the calibration ISO389 1975/Oslo recommendation.

The procedure used to determine the audiometric thresholds was the bottom-up method with the familiarization of the test signal according to the American National Standards 2004[a]. To perform the masking, when necessary, we used air

conduction in the non-test ear. The stimulus used was a narrow band noise centred around the test frequency.

The electrophysiological estimation of the thresholds was performed when the PTA threshold results were not available or when subjects did not cooperate during the PTA exams (younger or deaf individuals).

Electrophysiological thresholds were translated into the audiometric thresholds for the frequencies of 2000 Hz and 4000 Hz. No correction factor was applied as is advocated in studies conducted by Jerger and Mauldin, 1978;(Jerger & Mauldin 1978) Gorga et al., 1985;(Gorga et al. 1985) van der Drift et al., 1987;(van der Drift et al. 1987) and Gorga et al., 2006,(Gorga et al. 2006) in which a strong correlation was established between the electrophysiological thresholds with the "click" and the audiometric thresholds at 2000 and 4000 Hz. Moreover, a simple correction applied to the electrophysiological thresholds did not increase the prediction of audiometric thresholds.

The Vivosonic Integrity™ V500 audiometer system was used to collect the ABR and determine the electrophysiological thresholds. This modular equipment is comprised of four main components as follows: the computer, the VivoLink (SN: VL0026), the Amplitrode (SN: AJ0270) and the earphones. The earphones used were the ER-3A (ER-3A Left SN:63762 and, ER-3A Right SN: 63763), calibrated according to ANSI S3.6-1996. The click stimulus was used to evoke the ABR, calibrated in decibel peak-equivalent to the sound pressure level (dBpeSPL) according to the procedure IEC 60645-3 to the calibration of short duration stimuli.

Electrophysiological thresholds were determined according to the protocol established by the American Academy of Audiology in the Audiologic Guidelines for the Assessment of Hearing in Infants and Young Children (Dworsack-dodge et al. 2012). The descendant method was used for determining the electrophysiological thresholds.

To determine the electrophysiological thresholds, we used a fixed mask (wideband noise) in the non-test ear with a 30 dBnHL difference from the stimulus presented in the test ear. The maximum mask that we used was 50 dBnHL.

For both exams (PTA and ABR), the better ear was always chosen to start the test. If this information was not provided, conventionally the right ear was chosen to initiate the exam.

According to the hearing exams, we adopted two classifications of HL.

Both classifications are based on the mean value of PTA at 500, 1000, 2000 and 4000 Hz air conduction thresholds. One classification is the WHO classification, which defines HL as a hearing threshold (mean value PTA) higher than 25 dB in the "better ear", or the electrophysiological threshold of the "better ear" correlated to audiological thresholds. This classification includes patients who suffer unilateral hearing loss (UHL) in the normal hearing group and patients who suffer bilateral disability in the HL group (WHO 2013e).

The other classification of HL considers the same mean value of PTA at 500, 1000, 2000 and 4000 Hz air conduction thresholds, but considers the "worse ear", which means that at least one or both ears with HL is/are included in the HL group. In the normal hearing group, only individuals with bilateral normal hearing are included.

In this study, we adopted the self-reports of HL in one or both ears and adopted self-reports of HL in both ears, the latter of which is more likely a WHO classification.

The sensitivity of a test is defined as the probability that a test result will be positive when the disease is present (true positive rate). Moreover, the specificity is the probability that a test result will be negative when the disease is not present (true negative rate)(MedCalc 2014).

The study of HL in São Tomé and Príncipe was submitted and approved by the ethics committee of São Tomé e Príncipe and the Ethics Research Committee of Nova Medical School|FCM-UNL. All patients or their caregivers consented to participate in the study.

RESULTS

A total of 567 individuals were analyzed, aged 1 to 83 years with a mean age of 20.74±16.148 years.

Otoscopy was performed on all subjects, and a normal tympanic membrane was the most prevalent observation, except in cases of chronic otitis media.

The sample was divided into three age groups as follows: 17.5% of the subjects were younger than 7 years of age (99\567); 38.1% of subjects ranged from 7 to 19 years of age (216\567); and 44.4% of subjects were older than 20 years of age (252\567).

The younger group had an age distribution that was higher at 6 years (39\99) than at 2 years (22\99), 3 years (15\99), 4 years (13\99), 5 years (9\99) and 1 year (1\99).

Regarding gender, 52.2% of the subjects were female (296\567), and 47.8% of the subjects were male (271\567).

The audiometric tests used to validate the question were the PTA in 81.3% (461\567) of the cases and the ABR in 18.7% (106\567) of the subjects. The use of each type of exam depended on the age and cooperation of the individual. In individuals under the age of 7, the ABR was adopted in 60.6% (60\99) of the cases compared to 18.0% (39\216) of subjects in the age group 7 to 19 years of age and 2.8% (7\253) in those above the age of 20 years. The decision to adopt the PTA or ABR was dependent on individual cooperation.

There was a high statistical difference between the age groups and the applied audiometric exams (PTA and ABR), showing that the ABR was preferred in the youngest group, and the PTA was the preferred method of testing in the older group (p=0.0001).

Using the criteria of the mean value of PTA at 500, 1000, 2000 and 4000 Hz air conduction thresholds higher than 25 dB, audiometry revealed HL in 46.56% of subjects in the right ear (264\567) and in 44.44% of subjects in the left ear (252\567) (Table 1). The sensitivity and specificity observed were 74.62% and 76.90%, respectively, for the right ear and 79.76% and 73.02, respectively, for the

left ear.

When we stratified the subjects by age groups, the specificity for normal hearing in the youngest group (for which the tutors or parents answered the questions) was 81.67% for the right ear and 75.81% for the left ear. Sensitivity for the identification of HL in the youngest group was lower in the right ear (58.97%) than in the left ear (70.27%) (Table 4.1-1).

Table 4.1-1 - Crosstab between ear complaint and HL by ear, sensitivity and specificity in the total sample and by age group.

			Hearing Loss (HL)		Sensitivity	Specificity
		n	Yes	No		
Total Sample	**Right Ear Complaint**	567	264	303	74.62%	76.9%
	Yes	267	197	70		
	No	300	67	233		
	Left Ear Complaint	567	252	315	79.76%	73.02%
	Yes	286	201	85		
	No	281	51	230		
<7 Years	**Right Ear Complaint**	99	37	62	58.97%	81.67%
	Yes	41	23	11		
	No	58	16	49		
	Left Ear Complaint	99	37	62	70.27%	75.81%
	Yes	41	26	15		
	No	58	11	47		
≥7 <20 Years	**Right Ear Complaint**	216	103	113	79.61%	69.03%
	Yes	117	82	35		
	No	99	21	78		
	Left Ear Complaint	567	252	315	88.24%	64.04%
	Yes	286	201	85		
	No	281	51	230		

≥20 Years	**Right Ear Complaint**	252	122	130	75.41%	81.54%
	Yes	116	92	24		
	No	136	30	106		
	Left Ear Complaint	252	113	139	75.22%	79.14%
	Yes	114	85	29		
	No	138	28	110		

HL – PTA mean value at 500, 1000, 2000 and 4000 Hz air conduction thresholds > 25 dB

HL classification based on "worse ear" or at least one or both ears having HL

Considering the classification of HL, 63.5% of individuals (360\567) complained of HL. Of these subjects, HL was confirmed by audiologic testing (268\360) in 74.44% of subjects (Table 4.1-2).

Table 4.1-2 - Total sample description by HL complaint: HL classification in one or both ears – "worse ear"

			Hearing Loss Complaint (HL in at least one ear)		**Sensitivity**	**Specificity**
		n	**Yes**	**No**		
Age Group		567	360	207	87.01%	60.29%
<7Y		99	44	55	65.22%	73.58%
HL	Yes	44	(30)	(14)		
	No	55	(16)	(39)		
≥7 <20 Years		216	159	57	90%	46.88%
HL	Yes	159	(108)	(51)		
	No	57	(12)	(45)		
≥20 Years		252	157	95	91.55%	75.45%
HL	Yes	157	(130)	(27)		
	No	95	(12)	(83)		

Gender		567	360	207	74.44%	80.68%
Male		271	107	164	75.61%	82.24%
HL	Yes	143	(124)	(19)		
	No	128	(40)	(88)		
Female		296	196	100	73.47%	79%
HL	Yes	165	(144)	(21)		
	No	131	(52)	(79)		
Oral Language		520	330	190	67.90%	77.74%
Yes		438	268	170	67.16%	85.29%
HL	Yes	205	(180)	(25)		
	No	233	(88)	(145)		
No		82	62	20	98.39%	55%
HL	Yes	70	(61)	(9)		
	No	12	(1)	(11)		

It was observed that the probability of the question diagnosing HL in individuals who had HL confirmed by audiologic testing was 87.01% (sensitivity), while the probability of the answer to the question being negative and the individual not presenting with any HL was 64.48% (specificity) (Table 4.1-2).

According to age range, it was observed that the sensitivity increases as the age increases, reaching its highest in individuals over the age of 20 with a 91.55% sensitivity. The specificity was lower in the 7 to 19 years of age group (Table 4.1-2).

In analysing the gender groups, a higher sensitivity (75.61%) and specificity (82.24%) was observed in males compared to females (73.47% and 79%, respectively) (Table 4.1-2).

Of all individuals, some of them did not have oral language because they were younger. In the total sample, sensitivity between oral language and complaint of HL was 67.9%, and specificity was 77.74% (Table 4.1-2).

HL classification based on the "better ear" or both ears having HL

When the self-report only considered HL in the "better ear" or in "both ears", it was found that the complaint of HL had decreased, being present in 34.6% of individuals (196\567) that had answered affirmatively to the question. HL was validated through the diagnostic exams in 71.4% of individuals (140\196) (Table 4.1-3).

Table 4.1-3- Total sample description by HL complaint: HL classification in which both ears have HL – "better ear" (WHO classification)

			Hearing Loss Complaint (HL in both ears)		**Sensitivity**	**Specificity**
		n	**Yes**	**No**		
Age Group		567	196	371	71.43%	80.05%
<7Y		99	31	68	67.74%	88.76%
HL	Yes	30	(21)	(9)		
	No	69	(10)	(59)		
≥7 <20 Years		216	89	127	74.16%	83.46%
HL	Yes	87	(66)	(21)		
	No	129	(23)	(106)		
≥20 Years		252	76	176	69.74%	75%
HL	Yes	97	(53)	(44)		
	No	155	(23)	(132)		
Gender		567	196	371	71.43%	80.58%
Male		271	90	181	68.89%	79.01%
HL	Yes	100	(62)	(38)		
	No	171	(28)	(143)		
Female		296	106	190	73.58%	81.05%

HL	Yes	114	(78)	(36)		
	No	182	(28)	(154)		
Oral Language		520	169	351	67.82%	80.34%
Yes		438	107	331	56.07%	80%
HL	Yes	124	(60)	(64)		
	No	314	(47)	(267)		
No		82	62	20	93.55%	75%
HL	Yes	63	(58)	(5)		
	No	19	(4)	(15)		

A high specificity (88.76%) was observed in the youngest group, which means that they recognized when they had normal hearing, but nearly 30% of individuals with disability were not detected (sensitivity 67.74%) (Table 4.1-3). Especially in this young group, we verified difficulty in identification of which ear had the HL.

The stratification by gender showed a higher sensitivity (73.58%) and specificity (81.05%) in the female group through the evaluation of the "better ear" compared to the male group where they obtained 68.89% sensitivity and 79.01% specificity (Table 4.1-3).

The presence of oral language had a specificity of 80.34% and a sensitivity of 67.82% in the total sample. The absence of oral language was associated with HL (p=0.0001) (Table 4.1-3).

DISCUSSION

Epidemiological studies often report the prevalence of HL worldwide. However, few studies validate the subjective complaint with gold standard audiometric tests.

This type of validation is useful in cases where the subjective complaint is the only method to evaluate HL because of a lack of audiometric exams to determine hearing status.

There have been studies on the evaluation of self-reported HL in individuals from older age groups, usually older than 40 years of age. In our study, different age groups are included, with subjects younger than 20 years of age being more prevalent. Only Marini et al. also evaluated self-reported HL in individuals from different age groups.

In the literature, the definition of HL varies according to which ear ("better" or "worse") is used to classify the individual as well as to which frequencies are included in the calculation of the hearing thresholds (Valete-Rosalino & Rozenfeld 2005).

Classifying HL by the "worse ear" (at least one or both ears with HL) leads to an increase in the prevalence of HL (Valete-Rosalino & Rozenfeld 2005). In these cases, the group of individuals with HL included UHL, thus a high prevalence of HL was reported.

The diagnosis of UHL is important for oral development in children. Detection of UHL before school entrance means that the school teacher can provide the child with a favourable class position and reduce the possibility of a handicap (Newton et al. 2001). Children with UHL have been found to have lower language scores, higher grade failures and needs for individualized education plans (IEPs) (Lieu et al. 2012).

In our study, we found a prevalence of HL of 63.49% in the total sample when considering the "worse ear" (at least one or both ears with HL) compared to 37.74% when considering the "better ear" (both ears with HL).

Considering HL by the "worse ear", the sensitivity of self-report was quite high (87.01%); however, the same was not true for specificity (64.48%). Self-report has the strength to identify those with HL; however, it does not correctly recognize the normal-hearing individuals.

When considering HL by the "better ear", the specificity was high at 84.14%, showing that the question was capable of identifying the normal hearing population. Sensitivity, although presenting lower values, was above 60%. At this rate, the self-report HL may be reduced, identifying normal hearing through the "better ear".

In children with HL in both ears, the answer to the question about HL was sometimes difficult, resulting in a lower sensitivity (67.74%) and a high specificity of 88.76%. In this younger group, considering HL in both or one/both ears, the complaint was always minor compared to the diagnostic determination of HL. The question was posed mostly to the parents or tutors of the children, and it was difficult for them to identify the affected ear. As cited in Newton et al. (2001) "parents may also be in denial". In Robertson et al. (1995), 58% of parents noted abnormal behaviour in their children, whereas some parents went through a process of denial (Newton et al. 2001; Robertson et al. 1995).

To achieve early identification of HL, it is essential to become educated about health; therefore, parents and tutors should learn how to identify HL early to act properly and reduce HL effects (Marini et al. 2005).

The probability of identifying real deaf and real normal hearing children under 7 years of age improves when hearing is evaluated in both ears with HL (a sensitivity of 67.74% and specificity of 88.76%). Newton et al. implemented a questionnaire in children and obtained 100% sensitivity for the questionnaire when a HL of >40 dB in both ears was considered, but the specificity was lower at 75% (Newton et al. 2001).

As age increased, when evaluating both ears with HL, the sensitivity was higher in the middle age group, and the specificity decreased. The prevalence of HL was higher in the middle age group. The reasons for these findings are still being investigated in a study by Caroça et al.

The sensitivity in the older group was lower because it was hard to recognize one's HL as it is considered a sign of ageing. Additionally, if the HL is gradual, there is no perception of the HL. It is even more difficult to identify HL when both ears present with gradual disability, the typical pattern of presbycusis.

Considering results in the "better ear" classification, men appear to underestimate their hearing impairment with a lower specificity (68.89%) as already described in the study by Ushida et al (Uchida et al. 2003).

A study by Torre P et al.,(Torre et al. 2006) which evaluated the perception of self-report in adult Latin-American individuals, showed that females presented with a

higher sensitivity to the hearing complaint (80%) and a specificity of 83.3%. In contrast, in a study by Marini et al (Marini et al. 2005), in which different age groups were included, both the sensitivity and the specificity were higher in the male group (82.6% and 69.7%, respectively), and the female group revealed a higher sensitivity (68.72%) and specificity (84.62%). The results from this study were similar to a study considering the "better ear"(Marini et al. 2005).

Oral acquisition depends on age maturity and hearing status. In this study, we found a positive association of HL with the absence of oral language (p=0.0001).

In Kenya, a questionnaire based upon a child's responses to environmental sounds and speech development can potentially be useful for detecting HL in developing countries (Newton et al. 2001).

Even without a complete questionnaire about HL, the use of the question "Do you feel you have hearing loss?" in São Tomé and Príncipe has revealed itself useful.

CONCLUSION

Although audiometric testing still remains the gold standard, the subjective perception of HL continues to be an important method of identifying HL, especially in epidemiologic studies and where the audiometric exams are nonexistent. The question "Do you feel you have hearing loss?" has been demonstrated to be efficient in identifying HL in older individuals and has become a useful question on audiological screening in this population.

In younger children, where mostly the caretakers answer the questions, the self-report presents a high specificity despite a low sensitivity. Based on the sensitivity, more than 30% of children with HL will not be detected, and opportunities for early intervention during critical periods of language acquisition will be missed for them. This can be considered problematic for the younger group. We recognize the need for efforts to identify this disability as soon as possible, particularly in LDC, as HL is one disability responsible for a lower social and economic development in the community.

4.2 Sickle cell trait, Malaria and Sensorineural Hearing Loss – results from several Humanitarian Missions in São Tomé and Príncipe.

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Sickle Cell Trait, Malaria and Sensorineural Hearing Loss–A Case-Control Study from São Tomé and Príncipe

Cristina Caroça[*1-3], **João Pereira de Lima**[3], **Paula Campelo**[2], **Elisabete Carolino**[4], **Helena Caria**[5,6], **João Paço**[1,2] **and Susana Nunes Silva**[3]

[1]*Department of Otolaryngology, NOVA Medical School, Universidade Nova de Lisboa, Campo Mártires da Pátria 130, 1169-056 Lisboa, Portugal*

[2]*Hospital CUF Infante Santo, 34, 6º, 1350-079 Lisboa, Portugal*

[3]*Centre for Toxicogenomics and Human Health (ToxOmics), Faculty of Medical Sciences, NOVA Medical School, Universidade Nova de Lisboa, Campo Mártires da Pátria 130, 1169-056 Lisboa, Portugal*

[4]*Escola Superior de Tecnologia da Saúde de Lisboa, Av. Dom João II Lote 4.69.01, 1990-096 Lisboa*

[5]*BioISI - Biosystems & Integrative Sciences Institute, Faculty of Science of the University of Lisbon, R. Ernesto de Vasconcelos, 1749-016 Lisboa, Portugal*

[6]*ESS/IPS, School of Health, Polytechnic Institute of Setúbal, Campus do IPS - Estefanilha, 2910-761 Setúbal, Portugal*

[*]**Corresponding author:** Cristina Caroça, MD, Department of Otolaryngology, Nova Medical School, Universidade Nova de Lisboa, Campo Mártires da Pátria 130, 1169-056 Lisboa, Portugal, E-mail: cristinacaroca@netcabo.pt

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Abstract

Background: Hearing loss is a problem with higher incidence in South Asia, Asia Pacific and sub-Saharan Africa. In these countries there is also associated history of anemia and malaria.

Objective: This study aims to identify a putative role of Beta globin mutation - sickle cell trait and HL in São Tomé and Príncipe population.

Methods: A retrospective case-control study of a convenience sample was collected during Otolaryngologist Humanitarian Missions in São Tomé and Príncipe. Control group includes individuals with normal hearing in both ears, and the case group has participants presenting bilateral or unilateral HL. It was evaluated the potential risk factors and sickle cell trait with HL, as well self-report of malaria infection, consanguinity, familial history of HL. The HbS gene point mutation (Glu6Val) was determined by PCR-RFLP.

Results: Our results showed a statistical significance between HL - oral language and self-report of HL. Taken altogether, our data did not reveal association between sickle cell trait and HL. However, a statistical association between HL and self-report of malaria was found.

Conclusion: No association between sickle cell trait and the high prevalence of HL was found. Self-report of Malaria was found as a risk factor for the development of HL in São Tomé and Príncipe population. The multifactorial profile of HL shall not exclude the relevance of other etiologic factors than Malaria to justify the high prevalence of HL in São Tomé and Príncipe and further investigation must be applied.

Keywords Hearing loss; Sensorineural hearing loss; São tomé and príncipe; Sickle cell trait; Sickle cell disease; Malaria; Hemoglobinopathies

Introduction

More than 360 million people in the World have disabling hearing loss (HL). According to new World Health Organization (WHO) Global Estimates on prevalence of the HL [1,2], there is a higher incidence in South Asia, Asia Pacific and sub-Saharan Africa [1-3]. Sub-Saharan Africa holds 10% of the world's population and two thirds of the world's least developed nations.

More than 1.2 million of the children living in sub-Saharan Africa aged 5 to 14 years old have moderate to severe bilateral HL [3]. The consequences of hearing problems are well known. HL in children can result in developmental delays and lead to significant inability to engage in oral/aural communication.

Anemia has been proposed as an etiologic factor in sensorineural deafness for many years, but there is little supporting evidence [4]. In these African countries there is a high prevalence of anemia and this could be associated with infections, hemoglobinopathies or stunting [5].

Several gene mutations and polymorphisms in the human hosts confer survival advantage and have increased in frequency through natural selection over generations. These include hemoglobinopathies like Sickle Cell Disease (SCD), thalassemias and glucose-6-phosphate dehydrogenase (G6PD) deficiency. Sickle Cell Disease (SCD) refers to a group of symptomatic disorders associated with a specific mutation on the Beta globin (HBB) gene [6]. Sickle hemoglobin (HbS), a structural variant of normal adult hemoglobin, results in the substitution of glutamic acid with valine in the sixth position of β-chain of the hemoglobin (β6Glu-Val) [7]. HbS is the most common pathological hemoglobin variant worldwide [7].

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Several studies have suggested that HbS has an effect of protection against Plasmodium falciparum, the etiologic agent of malaria [6], showing that heterozygous people carrying the sickle-cell trait (HbAS) are protected against severe malaria (prevalence of HbAS, in some populations, is above 90%) [6,8].

This mechanism act as natural selection and is co-responsible for the high prevalence of HbS in malaria endemic regions as a result of natural selection over generations [9].

The red cells of individuals with the mutant homozygous gene (HbSS) become sickle shaped in low oxygen tension pressure with reduced oxygen-carrying capacity. The basal turn of the cochlea is particularly sensitive to anoxia, due to the high oxygen consumption rate of the stria vascularis and poor capacity for anaerobic metabolism. In this cases, SNHL start with the loss on the higher frequencies, then the lower frequencies, resulting from the damage at the apical region (low frequencies) and finally loss of the function throughout the entire cochlea [8].

SNHL is one of the several complications of this disease and has been found to occur in 8% of SCD children in Nigeria, 12% in USA, 22% in Jamaica, 36.5% in Kenya and 60% in Ghana [3,8].

In some studies, HbAS in malaria exposure could be also a cause of SNHL [10].

São Tomé and Príncipe it's an archipelago in western equatorial Africa, near Gabon, Equatorial Guinea, Camaroon and Nigeria, with Portuguese as official language [11]. São Tomé and Príncipe was discovered by Portuguese explorers in 1470. The resident population is about 187,000 inhabitants who have a low average age distribution (17-18 years) with low socioeconomic conditions and poor sanitary conditions, as well as a public health infection problem–malaria [12–14].

Since 2011 an otolaryngologist group formed by 2 doctors, 2 nurses and 1 audiologist began humanitarian missions in São Tomé and Príncipe (Project "Health for all"–Instituto Marquês de Valle Flor (IMVF)). Apparently according their clinical registries, this was the first hearing evaluation performed in these islands. Upon the first mission, a high prevalence of SNHL in the population was identified and it was observed upon subsequent missions.

As far as we know this is the first study developed in São Tomé and Príncipe population to evaluate the causes inherent to the high incidence of HL in this population. Such way, this study proposes to answer the question: will it be possible to establish an association between the sickle cell trait (HbAS) and the high incidence of SNHL in São Tomé and Príncipe?

Materials and Methods

Subjects

We present a case-control study, with a convenience sample where the control group include individuals with normal hearing in both ears, and patients with unilateral or bilateral HL compose the case group. The project was submitted and approved by the Medical Ethics Committee of São Tomé and Príncipe and Ethics Research Committee NMS|FCM-UNL.

A total of 316 individuals (136 HL patients and 180 controls with bilateral normal hearing), ranging from 2 to 35 years old, agreed to participate in this study. The limitation to 35 years was chosen to avoid action of other risk factors of HL, like acoustic trauma, age and the effect of other diseases. We organized into two age groups based on WHO: 1) below 14 years old (2–14 years) as children group and 2) above 15 years old (15–35 years) as adults group. The patients were recruited during consultation provided by the humanitarian missions in São Tomé and Príncipe over a period from February 2012 to May 2014. The controls (normal hearing bilateral) were recruited at consultation at health services, primary schools from São Tomé and local hotel.

All patients signed an informed consent and answered a clinical questionnaire recording risk factors and clinical history. An otolaryngologist observed all. The risk factors included were family history of HL, consanguinity, self-report of malaria infection, pre-natal and perinatal history and history of infections.

All 316 individuals were evaluated regarding their hearing status with Pure Tone Audiogram (PTA) or Auditory Brainstem Response (ABR) depending on collaboration to participate on the audiometric exams.

Audiometric exams were carried out by an audiologist without an audiometric cabin, with earphones-TDH39, in a closed room, with a level of noise measured by iPhone using SchabelDoesIT GbR, Munich, Germany (version 1.0.0), considered acceptable (ANSI S3.1-1999) (R2013). The equipment used was the Madsen Midimate 622 and Vivosonic Integrity V500 audiometer (auditory brainstem response), calibrated according to calibration ISO389 1975/Oslo Recommendation. The Integrity^{TM} V500 system used to collect auditory brainstem is a modular equipment comprised by 4 main components: the computer, the VivoLink (SN: VL0026), the Amplitrode (SN: AJ0270) and the earphones. The earphones used were the ER-3A (ER-3A Left SN:63762 e ER-3A Right SN: 63763) and were calibrated according to ANSI S3.6-1996 and the stimulus used was the CLICK, calibrated in dB equivalent to the sound pressure level (dBpeSPL) according to the procedure IEC 60645-3 for the calibration of short duration stimulus.

Electrophysiological thresholds were translated into the audiometric thresholds for frequencies 2000 Hz and 4000 Hz. No correction factor was applied as is advocated in studies conducted by Jerger and Mauldin [15]; Gorga et al. [16]; van der Drift et al. [17]; Gorga et al. [18] in which establish a strong correlation between the electrophysiological thresholds with the "click" and the audiometric thresholds at 2000 and 4000 Hz.

The hearing loss of each patient was determined based on the better ear and following the WHO classification [19].

Patients with less than 2 years and more than 35 years of age, conductive hearing loss, syndromic hearing loss, obvious environmental causes such as meningitis, cerebral malaria, intra-uterine or neonatal complications, ototoxicity, severe head trauma or developmental delay were excluded.

DNA extraction

Blood samples of all patients and controls were collected into Guthrie cards. Genomic DNA was obtained from each sample using a commercially available kit (QIAamp® DNA micro kit, Qiagen) according to the manufacturer's instructions. All DNA samples were stored at –20°C.

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SNP screening

The HbS gene point mutation (Glu6Val) was determined by PCR-RFLP. The primers and PCR conditions for the mutation site of this gene is shown in Table 1. For all of them the nucleotide mutated resulted in either gain or loss of restriction site, which therefore allowed the wild-type (HbAA) and variant alleles to be discriminated by RFLP after appropriate restriction enzyme digestion.

PCR was carried out with 50 ng of DNA in 50 ml reaction volume, containing 1.3x ImmoBuffer, 1.5 mM $MgCl_2$, 0.6 mM dNTP, 1.0 mM of each primer, and 0.75 U of Immolase (Bioline). The amplification started with an initial denaturation step at 95°C for 7 min, cycling parameters were 35 cycles of 95°C for 30 s, specific annealing temperature (60°C) for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min. After amplification, 10 ml of each PCR products were digested with appropriate restriction enzymes (DdeI) for 3 h at 37°C followed by an inactivation step at 65°C for 20 min and electrophoresed in 2% agarose gel with ethidium bromide (0.5 mg/ml) for visualization under ultraviolet light. The expected products for each genotype of the tested gene are shown in Table 1. All the genotype determinations were carried out twice in independent experiments and inconclusive samples were reanalyzed.

Gene	Primers	PCR fragment	Patterns after restriction enzyme digestion
HbAS codon 6 (rs334)	Rv 5'- AGG GTG GGA AAA TAG ACC AA -3'	395 bp	HbAA: 202, 180, 13 bp
	FW 5'- CGG CTG TCA TCA CTT AGA CCT -3'		HbAS: 382, 202,180,13 bp
			HbSS: 382, 13 bp

Table 1: PCR-RFLP conditions for identification of HbAS (rs334) polymorphism.

Statistical Analysis

The Hardy-Weinberg equilibrium of HbS was assessed by Qui-square test, calculated by HW calculator™ - Michael H Court (2005-2008).

Description of the sample was made with descriptive statistics, considering frequency analysis, means and standard deviation (SD).

To study the association between HL and each of the following parameters as district origin, oral language, perception of HL, sex and history of malaria infection, have been used the Qui-squared test.

The association between HbS genotype and the degree of HL of each ear and was analysed with Qui-square test by Monte Carlo Simulation.

To identify risk factors of HL we adopted a Binary Logistic Regression, where HL is a response variable and independent variables were HbS, age groups and self-report of Malaria infection.

All analyses were performed using the IBM, SPSS 20 version.

Results

We evaluated 316 subjects (Table 2) of whom 146 (45.6%) were men and 172 (54.4%) were women, with an age mean of 17.4 ± 9.74 years and a median of 15 years.

	Control-180	Case-136	Case Unil HL	Case Bil HL	p-Value
Age range					0.361
[2-14]	82 (45.6%)	69 (50.7%)	15 (34.1%)	54 (58.7%)	
[15-35]	98 (54.4%)	67 (49.3%)	29 (65.9%)	38 (41.3%)	
Mean Age SD	17.8±9.77	16.9±9.73	20.1±9.29	15.4±9.61	
Sex					0.256
Male:	87 (48.3%)	57 (41.9%)	17 (38.6%)	40 (43.5%)	
Female:	93 (51.7%)	79 (58.1%)	27 (61.4%)	52 (56.5%)	
Oral Language					0.0001
No:	4 (2.2%)	33 (24.3%)	1 (2.3%)	32 (34.8%)	
Yes:	170 (94.5%)	85 (62.5%)	42 (95.4%)	43 (46.7%)	
Indefined:	6 (3.3%)	18 (13.2%)	1 (2.3%)	17 (18.4%)	
Family History Hearing Loss					0.278
No:	140 (77.8%)	115 (84.6%)	40 (90.9%)	75 (81.5%)	
Yes:	34 (18.9%)	20 (14.7%)	4 (9.1%)	16 (17.4%)	
Missing:	6 (3.3%)	1 (0.7%)		1 (1.1%)	
Consanguinity					0.443
No:	171 (95%)	127 (93.4%)	43 (97.7%)	84 (91.3%)	
Yes:	3 (1,7%)	4 (2.9%)	1 (2.3%)	3 (3.3%)	
Missing:	6 (3.3%)	5 (3.7%)		5 (5.4%)	
Malaria					0.07
No:	72 (40%)	41 (30.1%)	11 (25%)	30 (32.6%)	

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Yes:	103 (57.2%)	91 (66.9%)	33 (75%)	58 (63.1%)	
Missing	5 (2.8%)	4 (3%)		4 (4.3%)	
Hearing Loss					
Normal:	180 (100%)	44 (32.3%)	44 (100%)		
Mild:		16 (11.8%)		16 (17.4%)	
Moderate:		19 (14%)		19 (20.7%)	
Severe:		17 (12.5%)		17 (18.5%)	
Profound:		40 (29.4%)		40 (43.5%)	
Right Ear					
Normal:	180 (100%)	19 (14%)	19 (43.2%)		
Mild:		26 (19.1%)	13 (29.5%)	13 (14.1%)	
Moderate:		17 (12.5%)	3 (6.8%)	14 (15.2%)	
Severe:		17 (12.5%)	2 (4.5%)	15 (16.3%)	
Profound:		57 (41.9%)	7 (15.9%)	50 (54.3%)	
Left Ear					
Normal:	180 (100%)	25 (18.4%)	25 (56.8%)		
Mild:		18 (13.2%)	8 (18.2%)	10 (10.9%)	
Moderate:		19 (14%)	1 (2.3%)	18 (19.6%)	
Severe:		16 (11.8%)	2 (4.5%)	14 (15.2%)	
Profound:		58 (42.6%)	8 (18.2%)	50 (54.3%)	
Hb					0.743
HbAA:	142 (78.9%)	112 (82.3%)	35 (79.5%)	77 (83.7%)	
HbAS:	35 (19.4%)	22 (16.2%)	9 (20.5%)	13 (14.1%)	
HbSS:	3 (1.7%)	2 (1.5%)	0	2 (2.2%)	
SD – Standard Deviation; HbAA - Wild Type; HbAS - Trait; HbSS - Homozygotic					

Table 2: General characteristics for convenience sample of São Tomé and Príncipe population.

Both groups (case-control) are homogeneous to gender (p=0.256) and age groups (p=0.361).

According to our results, HL is not associated with the district of origin (p=0.058) of each individual.

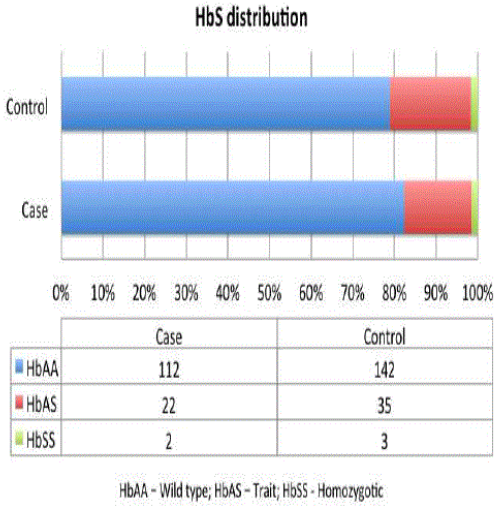

Figure 1: Case/control group distribution by HL grade.

Among the 316 subjects, we found 180 (57.0%) individuals with bilateral normal hearing, 44 (13.9%) unilateral HL (UHL) and 92 (29.1%) with bilateral HL (Figure 1). The individuals with UHL and bilateral HL were included in the case group.

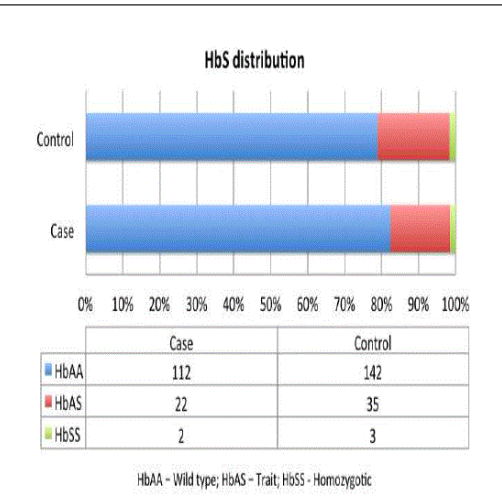

Figure 2: The Hardy-Weinberg Principle for HbS genotype was confirmed (p>0.05).

The Hardy-Weinberg Principle for HbS genotype was confirmed based on the distribution of HbS genotype in control and case group (Figure 2).

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When we calculate the media of the tone threshold, we found the left ear (40.02 dB) slight the same than the right (39.85 dB). The audiometric curve in the global sample has a horizontal shape, around mild HL.

The self-reported HL was confirmed by the audiometric evaluation, showing a positive association ($p<0.0001$) between both parameters. The tendency for self-reported HL was confirmed by the exams ($\phi=0.553$; $p<0.0001$).

Considering oral language for communication, this was absent in 33 (24.3%) of the individuals from case group and in 4 (2.2%) of the control groups these showing a negative association ($\phi=-0.379$; $p<0.0001$) between oral language and HL ($p<0.0001$). There was a tendency to have oral language in the control group.

A familial history of HL was found to be more prevalent in the control group than in the case group, respectively with 34 controls (18.9%) and 20 patients (14.7%). In the case group, 16 (80%) had bilateral HL and 4 (20%) unilateral HL.

From our sample 3 individuals (1.7%) in the control group and 4 patients (2.9%) in case group confirmed the existence of consanguinity in their families. Thus, no association between HL and consanguinity was performed.

In São Tomé and Principe we did not found any registry about history of malaria infection. At same time, we found that malaria infection is a public health problem and all individuals enrolled recognize malaria infection and can answer about their clinical history regarding this endemic infection. Self-report of malaria infection was more prevalent in the case group with 91 patients reporting a history of malaria (66.9%), being reported by 103 individuals (57.2%) of the control group.

The genotype distribution in our population was shown in Table 2. Concerning our results the HbAS showed no significant predisposition to HL (Table 3).

	Cases n (%)	Controls n (%)	P-value	Crude OR (95% CI)	P-value	Adjusted OR (95% CI)
Age group			0.362		0.07	
[2-14]	69 (45.7%)	82 (54.3%)		Reference		Reference
[15-35]	98 (59.4%)	98 (59.4%)		0.812 [0.520-1.269]		0.633 [0.386-1.038]
Malaria infection			0.071		0.021	
No:	41 (30.1%)	72 (40%)		Reference		Reference
Yes:	91 (66.9%)	103 (57.2%)		1.552 [0.964-2.497]		1.840 [1.097-3.085]
HbS			0.744		0.839	
(HbAA)	112 (82.3%)	142 (78.9%)		Reference		Reference
(HbAS)	22 (16.2%)	35 (19.4%)	0.449	0.797 [0.443-1.435]	0.573	0.842 [0.463-1.530]
(HbSS)	2 (1.5%)	3 (1.7%)	0.855	0.845 [0.139-5.145]	0.832	0.821 [0.133-5.063]

Table 3: Binary logistic regression between HbS and HL without and with risk factors (age group and history of malaria infection), HL: Normal hearing–0–Reference Category is a response variable; independent variables: HbS (0–HbAA wild type, 1- HbAS trait, 2–HbSS homozygotic), age groups (0–[2-14] years, 1–[15-35] years) and history of Malaria infection (0–no, 1–yes).

By applying the model of binary logistic regression the self-report of malaria was identified as a risk factor for HL (Table 3). The history of malaria infection almost doubled the risk of HL (OR=1.840; CI 95% [1.097-3.085]). We also found that, even not statistically significant, the oldest age group presented a decreased risk of HL in 36.7%.

For sickle cell trait we did not verify a significant predisposition to develop HL (OR=0.842; CI95% [0.463-1.530]) as sickle cell disease (OR=0.821; CI95% [0.133-5.063]).

Discussion

The results of this study revealed a prevalence of bilateral SNHL in 92 (29.1%) individuals and unilateral SNHL in 44 (13.9%) of subjects from our convenience sample. The unilateral and bilateral SNHL, accounting for 43% of the total sample, were included in the case study group since the unilateral SNHL also contribute to worse language acquisition and decreased school performance [20].

The horizontal audiologic curve in tonal audiogram was not specific or characteristic to a specific risk factor, meaning that inumerous causes may be responsible for SNHL in São Tomé and Príncipe and not one in particular.

Analysing the risk factors, the history family of HL and consanguinity were excluded from the analysis because they have a small number of cases. They haven't significant association in contrast to what we are expecting, since has been reported the relevance of these factors in HL [21, 22].

The individuals included in this study rarely reported to have consanguineous parents, even knowing that São Tomé and Príncipe are small islands, with high probability of consanguinity.

São Tomé and Príncipe is a population of Sub-Saharan Africa in which several haemoglobinopathies have been identified, including HbSS as the most prevalent [7,23]. It would be expected to obtain an increased prevalence of HbS homozygous (HbSS) and increased HbAS [4,8,10,24] in the São Tomé and Príncipe population. However in our

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results, HbSS genotype were found only in 16% and 18% for HbAS of the sample.

The HbAS has been reported in many studies as a factor of protection from malaria, reducing its severity [6]. The mechanism that contribute for this protection is the accelerated sickling of parasite-infected HbAS erythrocytes, low growth rates and parasite invasion in HbAS erythrocytes (in low oxygen conditions) and increased phagocytosis of infected HbAS erythrocytes [6]. Recently, had been show that intra-erythrocytic parasite growth is greatly inhibited by HbS polymerization when oxygen levels drop below 5% and, higher parasite-infected sickle erythrocyte phagocytosis by host immune cells has been observed when compared to infected normal erythrocytes [6]. Moreover, HbS erythrocytes infected by P. falciparum lower the surface expression of PfEMP-1, which results in a reduction of cytoadherence and thus protection against severe malaria [6]. This haemoglobin alteration otherwise may lead to thromboembolic events which sought being the source of SNHL [10].

The study model adopted did not confirm this assumption that the HbAS can promote SNHL. In our study we found a significant association between SNHL and a self-report of malaria. Although the patient provides the clinical malaria episode information, we considered their report valid. We must also consider that in the São Tomé Príncipe population exists a strong public awareness of Malaria infection as public health problem and standard procedures and guidelines for infection evaluation are followed, including the collection of thick blood film Plasmodium sp. in the presence of fever and illness.

Our results are supported by others, suggesting also that mild clinical malaria may also be associated with SNHL [25,26].

The malaria infection may trigger the onset of SNHL by pathophysiological processes, thromboembolism and release of inflammatory mediators [26,27]. On the other hand, malaria treatment adopted may be ototoxic. Some studies report reversibility of ototoxicity of some antimalarial [28–32]. In the specific case of children it does not apply [33] especially when is not performed a drug weight adjustment, and children ingest high doses of antimalarial therapy which may induce irreversible ototoxicity [32,33]. Eventually the higher association of the youngest patients of the case group with HL could be supported by the report of children malaria infection in this country during last 14 years [34,35].

The epidemiological profile of Malaria in São Tomé and Príncipe reveals a significant decrease of malaria admissions and deaths over 2006-2007 [34]. Since then, the children were most affected, representing a high proportion relatively to all patients. In 2003 they started some measures to control the disease witch include indoor residual spraying (IRS). In 2004 initiated the intermittent preventive treatment (IPT) with and sulfadoxine and pyrimethamine and changed the antimalarial policy with association of artesunate (AS) and amodiaquine (AQ), as first line treatment, in 2005 implemented the use of insecticide-treated nets (ITN) [34].

Our results support the hypothesis that the sickle cell trait (HbAS) acts as a protective role against malaria and SNHL in São Tomé and Príncipe.

Moreover, although São Tomé and Príncipe region is also affected by other HBB mutations, their prevalence is not relevant [23], justifying no screening for them in our study. There are others haemoglobin diseases, like deficiency of glucose-6-phosphate dehydrogenase, which ototoxicity is well known when combined with primaquine antimalarial therapy [36,37].

Conclusion

No association between sickle cell trait (HbAS) and the high prevalence of HL was found. However, our study suggests that in this sample, HbAS is preventing HL because is protecting against malaria. Malaria was found as a risk factor for the development of HL in São Tomé and Príncipe population. The multifactorial profile of HL and the horizontal audiologic curve, highly suggests the relevance of other etiologic factors than Malaria to justify the high prevalence of HL in São Tomé and Príncipe and further investigation must be applied.

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Competing Interests

There are no conflicts of interest.

Ethics Approval and Consent to Participate

The project was submitted and approved by the Medical Ethics Committee of São Tomé and Príncipe and Ethics Research Committee NMS|FCM-UNL (nº02/2014/CEFCM). The Ethics Research Committee is aligned with the Declaration of Helsinki for the Protection of Human Subjects. A full consenting process was applied in respect of all participants. Consent to use the survey data was also obtained.

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4.3 G6PD variants, malaria and Sensorineural Hearing Loss in São Tomé and Príncipe: a case-control study

Submitted to International Health

Cristina Caroça, Paula Campelo, Helena Caria, João Paço, Susana Nunes Silva

ABSTRACT

Background: São Tomé and Príncipe (STP) is a Least Developed Country (LDC) on Sub-Saharan Africa, in which was detected a high prevalence of sensorineural Hearing Loss (SNHL). HL is a common condition with both genetic and environmental causes, and it greatly impacts on global health. STP population has leading with additional health problems over the years, such as anemia and malaria infection.

The present study aims to identify the correlation between the most prevalent G6PD variants and the high prevalence of HL in STP population.

Methods: A sample of 316 individuals collected during Humanitarian Missions in STP, was retrospectively studied in a case-control approach to evaluate the role of *G6PD* gene variants in individual susceptibility to HL and it correlation with other potential risk factors.

Results: The results obtained showed an increased risk for those cases that have reported malaria infection (OR 1.867, CI 95% [1.107 – 3.48]) in global population. The same effect of increased risk was found after stratification for male gender (OR 3.721 CI 95% [1.631 – 8.489]).

Conclusions: Our results did not allowed us to correlate any specific variant of *G6PD* gene with HL. However, emphasize the hypothetical correlation between malaria infection and the increased risk for HL.

Keywords: Anemia, G6PD deficiency, Hearing loss, Malaria, São Tomé and Príncipe, Sub-Saharan Africa,

INTRODUCTION

The Hearing loss (HL) is a condition that could be related to genetic and environmental factors. The high prevalence of such condition in under developing countries, such as African countries has been described (Tucci et al. 2010).

São Tomé and Príncipe (STP) is a Least Developed Country (LDC) on Sub-Saharan Africa, which has been receiving humanitarian help from Portugal through a program called "Health for all – specialties". In this program, a team of otolaryngologists, nurses, audiologists and speech therapist collaborate in STP.

During the humanitarian action, the clinical team was faced with a high prevalence of HL, mainly sensorineural hearing loss (SNHL) in STP population (Caroça et al. 2013; Caroça, Campelo, et al. 2016). Anemia and malaria infection were two additional health problems detected in this population(Instituto Nacional de Estatística et al. 2010).

Anemia can be caused by several factors as such hemoglobinopathies or enzymophaties which can act by themselves as a protector effect to malaria infection (Cappellini & Fiorelli 2008; Luzzatto & Seneca 2014; Nguetse et al. 2016; Ruwende et al. 1995). Previous case control studies reported to the hypothesis of the existence of enzymopathies which act as selectively advantageous in malarial endemic areas (Nguetse et al. 2016; Petit et al. 2016).

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most common genetic enzymopathy in humans. This pathology is an X-linked disorder with recessive genetic trait.

G6PD is a cytoplasmic enzyme that catalyzes the first step in the hexose monophosphate pathway leading to synthesis of pentose phosphate. It also catalyzes conversion of nicotinamide adenine dinucleotide phosphate (NADP) to its reduced form (NADPH), thus protecting erythrocytes from oxidative damage. G6PD activity has been shown to be reduced in infected erythrocytes when

compared to uninfected ones (Petit et al. 2016). Clinical symptoms include acute or chronic hemolytic anemia, neonatal jaundice or hyperbilirubinemia, but also can remain asymptomatic and is rarely mortal (Cappellini & Fiorelli 2008).

The reduced capacity of cell defense against oxidative damage induced by the G6PD deficiency, has been correlated to a high resistance against malaria infection by *Plasmodium falciparum,* especially in populations living in malaria endemic regions, as sub-Saharan Africans (Cappellini & Fiorelli 2008; Manco et al. 2007).

More than 400 different variants of *G6PD* gene have been described to date (López et al. 2010), from which at least 186 have been characterized as mutations (Howes et al. 2013). The most common variants described are 376A (G6PD type B, normal phenotype), 376G (G6PD type A^+, moderately deficient phenotype), both variants generated by G6PD (Asn126Asp) polymorphism and characterized by c.376A>G; and 202A (G6PD type A^-, severely deficient phenotype) generated by G6PD (Val68Met) and characterized by c.202G>A (Cappellini & Fiorelli 2008; Nguetse et al. 2016; Petit et al. 2016). However, the type A^- is heterogeneous since it can result from a combination of the Asn126Asp replacement with any of three additional mutations, the most common of which is Val68Met (Luzzatto & Seneca 2014; Manco et al. 2007).

The present exploratory study aims to identify the correlation between the most prevalent G6PD variants and the high prevalence of HL in STP population.

MATERIAL AND METHODS

Study subjects

A convenience sample of 316 individuals (136 HL patients and 180 controls), was collected during the humanitarian missions from February 2012 to May 2014. The individuals who agreed to participate were attended in medical consultation and were included individuals from 2 to 35 years old.

All enrolled population answered a clinical questionnaire identifying risk factors, clinical history and clinical observation. The risk factors included were: family history of HL, consanguinity, self-report of malaria infection, pre-natal and

perinatal history, and history of infections. In Table 4.3-1 can be find the general characteristics of the global population under study.

Table 4.3-1 – Global distribution and characterization of the individuals enrolled in this study (n=316).

	Control n (%)	Cases n (%)	P Value*
Age range			
[2-14]	82 (45.6)	69 (50.7)	0.361
[15-35]	98 (54.4)	67 (49.3)	
Sex			
Male	87 (48.3)	57 (41.9)	0.256
Female	93 (51.7)	79 (58.1)	
Oral Language			
Yes	170 (94.4)	85 (62.5)	**<0.0001**
No	4 (2.2)	33 (24.3)	
Missing	6 (3.3)	18 (13.2)	
Familial History HL			
Yes	34 (18.9)	20 (14.7)	0.278
No	140 (77.8)	115 (84.6)	
Missing	6 (3.3)	1 (0.7)	
Consanguinity			
Yes	3 (1.7)	4 (2.9)	0.443
No	171 (95)	127 (93.4)	
Missing	6 (3.3)	5 (3.7)	
Self-report of malaria			
Yes	103 (57.2)	91 (66.9)	0.070
No	72 (40)	41 (30.1)	
Missing	5 (2.8)	4 (2.9)	
G6PD (202G>A)			
GG	137 (76.1)	99 (72.8)	
GA	28 (15.6)	24 (17.6)	0.857
AA	15 (8.3)	11 (8.1)	
Missing	---	2 (1.5)	
G6PD (376A>G)			
AA	95 (52.8)	69 (50.7)	
AG	42 (23.3)	32 (23.5)	0.952
GG	43 (23.9)	34 (25)	
Missing	---	1 (0.7)	

***** P-value determined by $\chi 2$ test.

To all was evaluated hearing status with Pure Tone Audiogram (PTA) or Auditory Brainstem Response (ABR) depending collaboration. There was collected a sample of blood to *Guthrie* paper after an informed consent and Medical Ethics Committee

approval, to diagnosis G6PD deficiency, demonstrating specific mutations by DNA studies.

The audiometric exams were carried out by an audiologist. The equipment used was the Madsen Midimate 622 and Vivosonic Integrity V500 audiometer (auditory brainstem response). The audiometric exams were carried out without an audiometric cabin, with earphones-TDH39, in a closed room, with a level of noise measured by *iPhone* de *SchabelDoesIT GbR, Munich, Germany* (version 1.0.0), considered acceptable, based on ANSI S3.1-1999 (R2013). The audiometric equipment was calibrated according to calibration ISO389 1975/Oslo Recommendation. The Integrity™ V500 system used to collect auditory brainstem is a modular equipment comprised by 4 main components: the computer, the VivoLink (SN: VL0026), the Amplitrode (SN: AJ0270) and the earphones. The earphones used were the ER-3A (ER-3A Left SN:63762 e ER-3A Right SN: 63763) are calibrated according to ANSI S3.6-1996 and the stimulus used was the CLICK, calibrated in dB equivalent to the sound pressure level (dBpeSPL) according to the procedure IEC 60645-3 to the calibration of short duration stimulus .

Hearing loss is classified in mild (26-40dB), moderate (41-60dB), severe (61-80dB) and profound (>80dB) according pure tone averages in 500; 1000; 2000 and 4000 Hz. Based on the better ear, each patient was classified, according to the WHO classification.

The exclusion factors used were: individuals with less than 2 years and more than 35 years of age, conductive hearing loss, and history of cranial trauma, intra-uterine, neonatal complications and obvious mental retardation.

This is a case-control study, where control group include patients with both ears normal, and case group have patients with one or both ears with HL, being a homogeneous group for gender and age.

The project was submitted and approved by the Medical Ethics Committee of STP and Ethics Research Committee NMS|FCM-UNL. The Ethics Research Committee is aligned with the Declaration of Helsinki for the Protection of Human Subjects. A full consenting process was applied in respect of all participants. Consent to use the survey data was also obtained.

DNA Extraction

A drop of peripheral blood samples of all enrolled population was collected into *Guthrie* paper by qualified assistants. Genomic DNA was obtained from each blood sample using a commercially available kit (QIAamp® DNA micro kit; Qiagen) according to the manufacturer's instructions. All DNA samples were stored at –20 °C until analysis.

SNP Selection

We selected for this study two polymorphisms in *G6PD* gene between the most prevalent Single Nucleotide Polymorphisms (SNPs) described in this gene (rs1050828 and rs1050829) in Sub-Saharan Africa (Manco et al. 2007; Howes et al. 2013).

The SNPs selected were genotyped using Real-Time Polymerase Chain Reaction (RT-PCR 7300 Applied Biosystem), through TaqMan® SNP genotyping assays (Life Technology), according to manufacturer instructions. Real-Time PCR genotype duplicate validations were carried in 20% of randomly selected samples in independent experiments and all the inconclusive samples were reanalyzed.

Statistical Analysis

All analyses were performed using the Statistical Package for the Social Sciences for Mac 20.0 version (SPSS).

The Hardy Weinberg Principle was tested with Qui-square test for one sample.

Description of the sample was made with Descriptive Statistics, using Frequency Analysis, Means and Standard Deviation (SD).

To study the association between Oral Language and HL, self-report of HL and HL, Sex and HL, self-report of malaria and District we used Qui-square test. The Hardy-Weinberg Equilibrium was carried out using exact probability tests available in SNPStat software (Sole et al. 2006).

To identify risk factors of HL, we adopted a Binary Logistic Regression, where HL is a response variable and independent variables were age, *G6PD* variants and self-report of Malaria infection. In Table 4.3-2 could be find the genotype combination for both SNPs under study. The G6PD deficiency is an X-linked recessive disorder occurring much often in males, so deficient variants are expressed more commonly in males than in females. (Tables 4.3-2 and 4.3-3)

Table 4.3-2 – Genotype distribution of both variants of *G6PD*: rs1050828 (202G>A) and rs1050829 (376A>G) by gender in global population of São Tomé and Principe

G6PD rs1050828 (202G>A)		
	Female, n (%)	Male, n (%)
G6PD WT (GG)	112 (65.1)	124 (86.1)
G6PD Heterozygotic (GA)	52 (30.2)	--
G6PD Homo/Hemizygotic (AA)	7 (4.1)	19 (13.2)
Missing	1 (0.6)	1 (0.7)
G6PD rs1050829 (376A>G)		
G6PD WT (AA)	72 (41.9)	92 (63.9)
G6PD Heterozygotic (AG)	74 (43)	--
G6PD Homo/Hemizygotic (GG)	25 (14.5)	52 (36.1)
Missing	1 (0.6)	--
Total	172 (100)	144 (100)

WT – Wild-Type.

Table 4.3-3 **–** Allelic Frequencies of both *G6PD* SNPs under study stratified by gender.

SNP ID	Nucleotide substitution	Variant group	Allele Frequency (%)	
			Male	Female
rs1050829	c.376A>G	B (376A)	63.9	63.7
	c.376A>G	A^{+} (376G)	36.1	36.3
rs1050828	c.202G>A	A^{-} (202A)	13.2	19.3

RESULTS

This study comprised 316 individual, from which 136 were included in HL case group while the other 180 represent the control group. The most representative *G6PD* gene variants from Sub-Saharan Africa were studied, carrying-out their genotyping to evaluate a possible association between them and the high incidence of hearing loss in this country. Table 4.3-1 describe the main characteristics of the populations under study. No significant differences were found between both groups, exception for oral language stratification ($P<0.0001$).

Concerning the genotype distribution for each variant under study, the results obtained are shown in Table 4.3-2. These results were stratified by gender and can be observed the higher frequency of each allele in men, the main reason is because men are hemizygotic (only have one allele of each variant, since they only have one X-chromossome). However, the allelic frequency determined for each allele was very similar between both genders, except for c.202 G>A variant (Table 4.3-3).

Our data was analyzed according the most common sub-Saharan *G6PD* variants, which could be correlated accordingly their phenotypic relevance (B – normal; A^+ - intermediate; A^- - deficient). Table 4.3-4 presents the G6PD variants distribution. The normal phenotype described by B variant group corresponds to the most frequent one (51.1% in control group and 50.7% in case group), while the intermediate phenotype correspond to around 12% in both populations. Full enzymatic-deficient individuals, represented by A^- group, which included males and homozygous females for both SNPs under study, correspond to 7.2% and 8.2% in control and cases populations, respectively.

Table 4.3-4 **–** Distribution of G6PD variants between STP populations under study.

G6PD Variants	Control n (%)			Case n (%)		
	Global	Male	Female	Global	Male	Female
B (376A)	**92 (51.1)**	57 (65.5)	35 (37.6)	**68 (50.7)**	33 (58.9)	35 (44.9)
A^+ (376G)	**22 (12.2)**	19 (21.8)	3 (3.2)	**17 (12.7)**	15 (26.8)	2 (2.6)
A^- (202A+376G)	**13 (7.2)**	10 (11.5)	3 (3.2)	**11 (8.2)**	8 (14.3)	3 (3.8)
Heterozygous group*	**51 (28.3)**	-	51 (54.8)	**38 (28.4)**		38 (48.7)

B include AA genotyped women for 376 variant and A genotyped men; A^+ include GG genotyped women and G men for variant 376; A^- include women carrying both AA genotype for 202 variant and GG for 376 variant and men carrying both variant alleles; *All heterozygous women were grouped together independently.

Our results were stratified according clinical or phenotypic relevance of G6PD variants (Table 4.3-5, table 4.3-6 and table 4.3-7).

Table 4.3-5 – *G6PD* Variant Groups distribution in case (n=136) and control (n=180) populations.

	Crude OR (95%) CI	Adjusted OR (95% CI)[a]
Gender Group		
Female	1 (Reference)	1 (Reference)
Male	0.771 [0.492 – 1.208]	0.717 [0.397 – 1.296]
Age Range		
[2-14]	1 (Reference)	1 (Reference)
[15-35]	0.812 [0.520 – 1.269]	0.650 [0.394 – 1.072]
Malaria Infection		
NO	1 (Reference)	1 (Reference)
YES	1.552 [0.964 – 2.497]	**1.867 [1.107 – 3.148]***
G6PD Variant Groups		
B	1 (Reference)	1 (Reference)
A^+	1.045 [0.516 – 2.119]	1.259 [0.598 – 2.649]
A^- (376A/202G)	1.145 [0.483 – 2.711]	1.328 [0.547 – 3.222]
Heterozygous group	1.008 [0.597 – 1.703]	0.904 [0.481 – 1.701]

[a]ORs were adjusted for age, malária and gender. Data in bold highlights the statistic significant results (P<0.05)
*PAdjusted = 0.019 (P values are adjusted by unconditional multiplicative logistic regression).

Table 4.3-6 – *G6PD* Variant Groups distribution in male population.

	Crude OR (95%) CI	Adjusted OR (95% CI)
Age Range		
[2-14]	1 (Reference)	1 (Reference)
[15-35]	0.881 [0.452 – 1.721]	0.516 [0.234 – 1.139]
Malaria Infection		
NO	1 (Reference)	1 (Reference)
YES	**2.798 [1.351 – 5.798]†**	**3.721 [1.631 – 8.489]‡**
G6PD Variant Groups		
B	1 (Reference)	1 (Reference)
A⁺	1.364 [0.612 – 3.039]	1.378 [0.596 – 3.184]
A⁻ (376A/202G)	1.382 [0.496 – 3.847]	1.424 [0.485 – 4.184]

Data in bold highlights the statistic significant results (P<0.05)

†PCrude = 0.006 (P values are adjusted by unconditional multiplicative logistic regression).

‡PAdjusted = 0.002 (P values are adjusted by unconditional multiplicative logistic regression).

Table 4.3-7 – *G6PD* Variant Groups distribution in case (n=136) and control (n=180) populations.

	Crude OR (95%) CI	Adjusted OR (95% CI)[a]
Age Range		
[2-14]	1 (Reference)	1 (Reference)
[15-35]	0.763 [0.418 – 1.393]	0.765 [0.393 – 1.487]
Malaria Infection		
NO	1 (Reference)	1 (Reference)
YES	0.914 [0.478 – 1.748]	1.065 [0.529 – 2.141]
G6PD Variant Groups		
B	1 (Reference)	1 (Reference)
A⁺	0.667 [0.105 – 4.238]	0.783 [0.120 – 5.107]
A⁻ (376A/202G)	1.000 [0.189 – 5.299]	1.093 [0.203 – 5.870]
Heterozygous group	0.745 [0.397 – 1.398]	0.857 [0.447 – 1.642]

[a]ORs were adjusted for age, malaria and gender.

Table 4.3-5 presents the observed results after logistic distribution analysis of each G6PD variant group, as well as the distribution of the potential risk factors inherent to this study: gender, age and malaria infection.

The genotypic analysis was also performed between each population, however the results did not reveal any association between each G6PD genotype and the incidence of HL (data not shown). None of the two SNPs were in agreement with the expectation of the Hardy-Weinberg law (P < 0.0001, exact probability test).

The global analysis present on table 4.3-5 showed an increased risk for those cases that have reported malaria infection (OR 1.867, CI 95% [1.107 – 3.48], P value = 0.019). However, and after stratification by gender, in male population the same effect of increased risk was found (OR 3.721 CI 95% [1.631 – 8.489], P value = 0.002).

DISCUSSION

The enrolled population was recruited during humanitarian missions occurred during 2012 – 2014. The high prevalence of hearing impairment in STP open the door to study the possible risk factors inherent to HL disorder in this country. To answer questions about the most relevant risk factors were considered to be studied in this population: 1) their genetic background (Caroça, de Matos, et al. 2016) through the study of the role of the DFNB1 locus (*GJB2* and *GJB6* genes); 2) the putative role of Beta globin mutation - sickle cell trait and HL (Caroca & de Lima 2016); and 3) to estimate the rate of infected people with rubella and its correlation with HL (Caroça et al. 2017). These previous studies helped us to characterize STP population, and especially for rubella infection, allowed to implement the rubella vaccination in this country (Caroca & de Lima 2016; Caroça et al. 2017; Caroça, de Matos, et al. 2016).

The high frequency of polymorphic variants has arisen because G6PD deficiency gives a relative protection against severe malaria hypothesizing about its selective advantageous in malaria endemic areas consequence of evolutionary pressure (Cappellini & Fiorelli 2008; Ruwende et al. 1995; Petit et al. 2016). Many variants have been describe, however the main focus of this work was on G6PD type B, type A^+ and type A^- described as the most prevalent in Africa by several studies (Luzzatto & Seneca 2014; Nguetse et al. 2016; Ruwende et al. 1995; Petit et al. 2016; Manco et al. 2007; Beutler et al. 1989).

The distribution and prevalence of G6PD variants have shown a distinct geographical pattern, reflecting correlations with epidemiology of malaria, such as malaria-endemic and malaria eliminating countries (Luzzatto & Seneca 2014; Howes et al. 2013). G6PD A^- is a common variant in sub-Saharan Africa that

reaches frequencies of 20% in populations living in malarial areas. This variant is characterized by two nonsynonymous changes relative to the normal allele (G6PD B), which decrease enzyme activity ≈12% of normal and confer ≈50% reduction in risk of severe malaria in both females and males (Ruwende et al. 1995). It follows that the G6PD A^- variant is beneficial in the presence of malaria caused by *Plasmodium falciparum*, while in the absence of malaria this variant is deleterious (Saunders et al. 2005). For G6PD A^+ the prevalence is almost the same as G6PD A^-, but with a decreased enzymatic activity, with 80% of normal activity and without reduction of risk of severe malaria (Saunders et al. 2005).

In fact, our results reveal an increased risk to develop HL in global STP population for those who have stated to have had a malaria infection episode (OR: 1.867, 95% CI [1.107 – 3.148]). We also found an association between malaria infection and HbS in STP population (Caroca & de Lima 2016).

The epidemiological profile of Malaria in São Tomé and Príncipe reveals a significant decrease of malaria admissions and deaths over 2006-2007 (WHO 2015). Since then, the high proportion of all patients pointed to children as the most affected. Some preventive actions to control the disease have started in 2003, including indoor residual spraying (IRS) to intermittent preventive treatment (IPT) and also through change the antimalarial policy (WHO 2015).

The G6PD deficiency is an X-linked trait, and is often stated as being more common in males, however, this is not correct. As a result of X-chromosome inactivation, heterozygous females are genetic mosaics. However, could not be state the mathematical average of alleles' distribution, 50% G6PD normal and 50% G6PD deficient, in females. The random distribution of heterozygous genotype define females in which the enzyme activity phenotype overlaps with normal, whereas in others it overlaps with the G6PD deficiency prevalent in homozygous. Of course this mosaicism interferes in clinical implications (Luzzatto & Seneca 2014).

G6PD deficiency is normally associated to hemolytic anemia, which could manifest during infections, after treatment with certain drugs or after eating fava beans (known as favism) and also Kernicterus. Mechanism of protection is more prone to induce acute hemolytic anemia and early phagocytosis of infected cells. Males have

more severe hemolytic anemia than female (Bope et al. 2010; Cardoso et al. 1992), this can probably increase the risk of having HL when associated to malaria and G6PD, as we have when analysing the male gender. In our sample, male group presents a high risk to develop HL (OR: 3.721 95% CI [1.631 – 8.489]). This could be explained by the fact that, male gender is usually more exposed to malaria infection as described by Cardoso et al. and Prettz et al. inherent to their job activity being more exposed to *P. falciparum* infection, leading to hemolytic anemia (Cardoso et al. 1992; Prettz et al. 2015). In opposite, female gender, even with more susceptibility to anemia (Kasper et al. 2015) is probably more protected to severe anemia with malaria and G6PD even because female gender presents more G6PD variants which randomly interfered with enzymatic alterations.

In the general sample of São Tomé and Príncipe we expected a high prevalence of G6PD variants and because of it, we expected a high incidence in hyperbilirubinemia neonatal and HL consequently.

Prevention should be emphasized, screening newborns in regions with a high frequency of G6PD deficiency, educating the population to avoid the drugs and fava beans that precipitate attacks of hemolytic anemia. At the same time, it's essential being alert to kernicterus in newborns, preventing sequel in future, as HL and mental retardation (Mason et al. 2007).

The effect of the G6PD deficiency in the ear is not known unless during neonatal period (Nair & Al Khusaiby 2003; Olds & Oghalai 2015). It is well known that G6PD deficiency is associated with hyperbilirubinemia and Kernicterus, which is a Bilirrubin Induced Neurologic Disfunction (BIND), associated with Auditory Neuropathy Spectrum Disorder (ANSD), acute and chronic hemolytic anemia (Nair & Al Khusaiby 2003; Johnson & Bhutani 2011; Kuzniewicz et al. 2014).

Hyperbilirubinemia occurs commonly in neonates and is usually mild and transient, with no long-lasting sequel. However, bilirubin-induced neurologic damage may occur in some children (Kuzniewicz et al. 2014). The auditory pathway is the most sensitive part of the central nervous system to bilirubin-induced toxicity, and permanent sequel may result from only moderately elevated total serum/plasma bilirubin levels. The damage to the auditory system occurs

primarily within the brainstem and cranial nerve VIII, and manifests clinically as auditory neuropathy spectrum disorder. Around 30% of jaundice infants who have permanent neurological damage are G6PD deficiency (Mason et al. 2007).

CONCLUSIONS

The results reveal an adaptive response of population of São Tomé and Príncipe, to an increase in virulence of *P. falciparum* in sub-Saharan Africa. In the sample, we found that malaria infection has significant implication in developing HL increasing the risk in general population, being more representative in male gender. Our results did not allowed us to correlate any specific variant of *G6PD* gene with HL. However, they emphasize the hypothetical correlation between malaria infection and the increased risk for HL.

This work alerts the need in screening these variants in early age (neonatal), with the objective to prevent serious complications during life which are not only HL.

4.4 Genetic Basis of Nonsyndromic Sensorineural Hearing loss in the Sub-Saharan African Island Population of São Tomé and Príncipe: The role of the *DFNB1* locus?

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Research Articles

Genetic Basis of Nonsyndromic Sensorineural Hearing Loss in the Sub-Saharan African Island Population of São Tomé and Príncipe: The Role of the *DFNB1* Locus?

Cristina Caroça,[1,2] Tiago Morim de Matos,[3] Diogo Ribeiro,[2] Vera Lourenço,[2] Tânia Martins,[2] Paula Campelo,[2] Graça Fialho,[3] Susana Nunes Silva,[4] João Paço,[1,2] and Helena Caria[3,5]

Abstract

Hearing loss (HL) is a common condition with both genetic and environmental causes, and it greatly impacts global health. The prevalence of HL is reportedly higher in developing countries such as the Sub-Saharan African island of São Tomé and Príncipe, where the deaf community is estimated to be less than 1% of the population. We investigated the role of the *DFNB1* locus (*GJB2* and *GJB6* genes) in the etiology of nonsyndromic sensorineural hearing loss (NSSHL) in São Tomé and Príncipe. A sample of 316 individuals, comprising 136 NSSHL patients (92 bilateral, 44 unilateral) and 180 controls, underwent a clinical and audiological examination. Sequencing of the *GJB2* coding region and testing for the (*GJB6*-D13S1830) and del(*GJB6*-D13S1854) *GJB6* deletions were performed. A total of 311 out of 316 individuals were successfully analyzed regarding the *GJB2* and *GJB6* genetic variations, respectively. The frequency of the *GJB2* coding mutations in patients and controls was low. Some of those coding mutations are the most commonly found in Eurasian and Mediterranean populations and have also been identified in Portugal. None of the *GJB6* deletions was present. The presence of certain coding variants in São Tomé and Príncipe suggests a non–Sub-Saharan genetic influx and supports the previously reported genetic influx from European (mainly Portuguese) ancestors. In summary, *DFNB1* locus does not appear to be a major contributor to NSSHL in São Tomé and Príncipe. However, the presence of both pathogenic and likely pathogenic mutations in *GJB2* suggests that *GJB2*-related NSSHL might still occur in this population, warranting further research on *GJB2* testing in NSSHL cases.

Introduction

Hearing loss (HL) is a condition that is an outcome of both environmental and genetic factors. The prevalence of HL is higher in certain developing countries such as many African countries. In São Tomé and Príncipe, the deaf community is estimated to comprise less than 1% of the population based on the National Institute of Statistic of São Tomé and Príncipe (INE, 2014). Mutations in the *GJB2* gene (encoding connexin 26) are responsible for a significant proportion of nonsyndromic hearing loss (NSHL) cases in several populations. Two large deletions, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854), truncating the *GJB6* gene (encoding connexin 30), are also responsible for NSHL in some populations, being mostly found in *trans* with *GJB2* mutations (del Castillo et al., 2003, 2005). These genes map to 13q11-q12, and both of them are located within the *DFNB1* locus, with the first locus defined for nonsyndromic autosomal recessive HL. Loci for nonsyndromic autosomal recessive HL are designated by DFNB followed by a suffix integer (Smith and Van Camp, 1998). Mutations in *GJB2* reportedly do not play a significant role in the etiology of HL in Sub-

[1]Otolaryngology Department, Nova Medical School/Faculty of Medical Sciences, Universidade Nova de Lisboa, Lisboa, Portugal.
[2]Hospital CUF Infante Santo, Otolaryngology Department, Hospital CUF Infante Santo, Lisboa, Portugal.
[3]Deafness Research Group, Biomedicine and Translational Research, BioISI, Faculty of Sciences, University of Lisbon, Lisbon, Portugal.
[4]Centre for Toxicogenomics and Human Health (ToxOmics), NOVA Medical School, Faculty of Medical Sciences, Universidade Nova de Lisboa, Lisboa, Portugal.
[5]School of Health, Polytechnic Institute of Setubal, Setubal, Portugal.

Saharan African populations or their descendant populations (Bosch et al., 2014b; Javidnia et al., 2014; Lasisi et al., 2014; Shan et al., 2010). The role of del(*GJB6*-D13S1830) *GJB6* large deletion in NSHL in these populations is so far null (Bosch et al., 2014a; Kabahuma et al., 2011; Samanich et al., 2007; Shan et al., 2010). Regarding the del(*GJB6*-D13S1854) *GJB6* deletion, its presence has been investigated in Nigerian HL patients (Lasisi et al., 2014) and in HL patients of a predominantly Caribbean Hispanic and African descent (Shan et al., 2010), without positive results.

São Tomé and Príncipe, a former Portuguese colony, was formerly settled first by people from different regions of Sub-Saharan Africa, mostly slaves from the Gulf of Guinea, Congo, and Angola, brought to work in local plantations, and, to a minor extent, Portuguese who were involved in the slave trade between Africa and the Americas. In the first centuries after the discovery of São Tomé and Príncipe, besides the Portuguese, other Europeans were involved in the slave trade along the coast of Africa, namely the French, Spanish, Dutch, and English (Neves, 1989). In fact, São Tomé and Príncipe's population has been shown to present $10.7\% \pm 0.9\%$ of European (mainly Portuguese) admixture (Tomás et al., 2002). Therefore, a putative role of *GJB2*, or even of the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) large *GJB6* deletions, in the HL observed in São Tomé and Príncipe might potentially be anticipated.

The aim of the present study was to examine the genetic basis of HL in this understudied Sub-Saharan African island population, and so as to obtain a broader insight on the role of genetic contributions to this important disease impacting global health.

Methods and Materials

Subjects

A total of 316 individuals (136 HL patients and 180 controls), all of whom were born in São Tomé and Príncipe, ranging from 2 to 35 years old, participated in this study. The subjects were recruited during consultation provided by the humanitarian missions in São Tomé and Príncipe, at hospitals, schools, and a hotel, over a period from February 2012 to May 2014, constituting a convenience sample. All patients and controls answered a clinical questionnaire identifying risk factors (family history of HL, consanguinity, malaria infection, prenatal and perinatal history, and history of other infections), clinical history, and otolaryngology observation.

The patients presented with mild to profound nonsyndromic sensorineural hearing loss (NSSHL), which was bilateral HL in 92 individuals and unilateral HL in 44 individuals. All the control individuals had normal hearing in both ears. The patients and control samples did not display a significant difference in gender ($p = 0.233$) and age ($p = 0.271$).

The classification of HL was adopted from the World Health Organization (WHO, 2013). It considers the mean value of hearing threshold (considering the 0.5, 1, 2, and 4 kHz frequencies) in the better ear. The individual has HL when the best ear presents a hearing threshold higher than 25 dB and is graded after that with mild (26–40 dB), moderate (41–60 dB), severe (61–80 dB), and profound (81 dB or greater) (WHO, 2013).

The project was reviewed and approved by the Medical Ethics Committee of São Tomé and Príncipe and the Ethics Research Committee NMS|UNL (n°02/2014/CEFCM).

Audiological examination

All 316 individuals were evaluated regarding their hearing status with a pure tone audiogram—Madsen Midimate 622 or auditory brainstem response—Vivosonic Integrity V500 audiometer depending on collaboration. The audiometric exams were carried out without an audiometric cabin, with earphones TDH39, in a closed room, with a level of noise measured by iPhone de SchabelDoesIT GbR, Munich, Germany (version 1.0.0), considered acceptable, based on ANSI S3.1-1999 (R2013). Electrophysiological thresholds were translated into the audiometric thresholds for frequencies 2 and 4 kHz, without applying any correction factor (Gorga et al., 1985, 2006; Jerger and Mauldin, 1978; van de Drift et al., 1987).

DFNB1 molecular analysis

Peripheral blood samples were collected in Guthrie paper cards after informed consent had been signed. Approval of the Medical Ethics Committee was also obtained. Genomic DNA was extracted from each blood sample by using a commercially available kit (QIAamp® DNA micro kit; Qiagen) according to the manufacturer's instructions. All DNA samples were stored at −20°C until analysis.

Of the 316 subjects, 311 were successfully analyzed by sequencing regarding the *GJB2* coding region, and by multiplex polymerase chain reaction (PCR) (del Castillo et al., 2005) regarding the presence of the two *GJB6* large deletions, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854). These 311 individuals comprise 134 patients (90 bilateral and 44 unilateral) and 177 controls, matched by sex and age. PCR amplification and sequencing of the coding region of the *GJB2* gene was performed using previously described primers (Matos et al., 2010). The *GJB2* fragment that was amplified comprises the coding region and flanking noncoding regions, including the acceptor splice site. However, the extension of the sequence obtained beyond the coding region was variable, not allowing results from the acceptor splice site for all the subjects.

All electrophoretograms were visually inspected; the low-quality extremities were trimmed off; and heterozygosities were marked, using the Chromas Lite software (v.2.01). The resulting analyzed and edited sequences were copied from Chromas Lite in Fasta format and blasted against the reference sequence NG_008358.1 using NCBI's Blast program (suite 2-sequences). All the variants described here were named according to the recommendations of the Human Genome Variation Society.

Statistical analyses

A chi-square test was performed, and *p*-values were calculated using the SPSS v.20 software. When chi-square was not possible, we adopted Fisher's exact test. Hardy–Weinberg equilibrium test was performed using Court Lab's HW calculator. This test was only performed for c.*84T>C, c.*104A>T, and c.*111C>T, since these were the only variants whose respective genotypes were all observed in five or more individuals.

Results

Insofar as the *GJB2* sequencing is concerned, all the analyzed subjects were sequenced between c.-2 and c.*6 positions, although most sequences extended, with quality, several nucleotides before c.-2 and after c.*6. The sequencing results

Table 1. G*JB*2 Coding Variants Identified in Patients and Controls (All in Heterozygosity) and Respective Carrier Frequencies

GJB2 *coding variants*	*Bilateral HL*	*Unilateral HL*	*Controls*
c.35delG (p.Gly12Valfs)	0/90	0/44	**1/177 (0.28%)**
c.101T>C (p.Met34Thr)	**1/90 (0.56%)**	0/44	0/177
c.109G>A (p.Val37Ile)	0/90	0/44	**1/177 (0.28%)**
c.186C>T (p.Asn62=)	0/90	0/44	**1/177 (0.28%)**
c.225G>T (p.Arg75=)	**1/90 (0.56%)**	0/44	**5/177 (1.41%)**
c.380G>A (p.Arg127His)	**1/90 (0.56%)**	0/44	**1/177 (0.28%)**
c.457G>A (p.Val153Ile)	0/90	0/44	**1/177 (0.28%)**
c.499G>A (p.Val167Met)	**1/90 (0.56%)**	**1/44 (1.14%)**	**1/177 (0.28%)**
Frequency of mutated alleles	4/180 (2.22%)	1/88 (1.13%)	11/354 (3.11%)

HL, hearing loss.
Boldface = carrier frequencies > 0.

allowed for the identification of eight coding variants (Table 1) and 10 noncoding variants (Table 2) in patients and/or controls.

Coding variants of GJB2

Eight different coding variants, all in heterozygosity, have been identified in this study, in patients and/or control individuals (Table 1). None of these patients harbored any mutation in the acceptor splice site.

Noncoding variants of GJB2

We have identified 10 noncoding variants in the subjects of this study, being the genotyping results presented in Table 2. The most commonly identified variant in bilateral and unilateral HL patients and controls was c.*84T>C, presenting in the latter group an allelic frequency of 51.36%. This variant as well as c.*104A>T and c.*111C>T are in Hardy–Weinberg equilibrium in the control group.

Table 2. G*JB*2 Noncoding Variants Identified in the Subjects and Respective Genotypic Frequencies

GJB2 *noncoding variants*	*Genotypes*	*Bilateral HL*	*Unilateral HL*	*Controls*
c.-22-12C>T	CC	59.4% (19/32)	60% (6/10)	66.7% (26/39)
	CT	31.3% (10/32)	40% (4/10)	30.8% (12/39)
	TT	9.4% (3/32)	0% (0/10)	2.6% (1/39)
c.-15C>T	CC	82.7% (67/81)	85.7 (36/42)	88% (146/166)
	CT	16% (13/81)	14.3% (6/42)	11.4% (19/166)
	TT	1.2% (1/81)	0% (0/42)	0.6% (1/166)
c.-14G>A	GG	98.8% (80/81)	100% (43/43)	99.4% (165/166)
	GA	1.2% (1/81)	0% (0/43)	0.6% (1/166)
	AA	0% (0/81)	0% (0/43)	0% (0/166)
c.-7G>A	GG	100% (88/88)	100% (44/44)	99.4% (175/176)
	GA	0% (0/88)	0% (0/44)	0.6% (1/176)
	AA	0% (0/88)	0% (0/44)	0% (0/176)
c.-6T>A	TT	100% (89/89)	100% (44/44)	97.7% (172/176)
	TA	0% (0/89)	0% (0/44)	2.3% (4/176)
	AA	0% (0/89)	0% (0/44)	0% (0/176)
c.*78A>T	AA	97.6% (80/82)	100% (40/40)	100% (152/152)
	AT	2.4% (2/82)	0% (0/40)	0% (0/152)
	TT	0% (0/82)	0% (0/40)	0% (0/152)
c.*84T>C	TT	21.3% (17/80)	30% (12/40)	24.5% (36/147)
	TC	36.3% (29/80)	47.5% (19/40)	48.3% (71/147)
	CC	42.5% (34/80)	22.5% (9/40)	27.2% (40/147)
c.*96A>G	AA	100% (54/54)	100% (29/29)	99.2% (122/123)
	AG	0% (0/54)	0% (0/29)	0.81% (1/123)
	GG	0% (0/54)	0% (0/29)	0% (0/123)
c.*104A>T	AA	57.7% (30/52)	79.3% (23/29)	68.1% (79/116)
	AT	40.4% (21/52)	17.2% (5/29)	29.3% (34/116)
	TT	1.9% (1/52)	3.4% (1/29)	2.6% (3/116)
c.*111C>T	CC	57.1% (28/49)	79.3% (23/29)	67% (75/112)
	CT	42.9% (21/49)	17.2% (5/29)	30.4% (34/112)
	TT	0% (0/49)	3.4% (1/29)	2.7% (3/112)

Table 3. Genotypic Distribution Regarding the c.*84T>C Variant in Controls and Patients with Bilateral, Severe, or Profound Hearing Loss (Chi-Square Test; $p = 0.005$)

	*c.*84T>C*			
	TT	*TC*	*CC*	*Total*
Controls	36	71	40	147
Expected count	38.4	61.3	47.3	
Patients	16	12	24	52
Expected count	13.6	21.7	16.7	
Total	52	83	64	199

We have observed a statistically significant difference in the distribution of genotypes regarding c.*84T>C between bilateral HL patients and controls when considering only the cases with severe and profound deafness (Table 3; $p = 0.005$). When also including the moderate bilateral HL patients, the difference in genotypic distribution did not remain statistically significant for the c.*84T>C variant ($p = 0.101$).

GJB6 *deletions*

The del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) *GJB6* deletions have not been identified in the 134 patients (90 bilateral +44 unilateral) or 177 controls analyzed.

Table 4. All *GJB2* Variants Identified in This Study and the Populations, Per the 1000 Genomes Project (Phase 3), in Which the Attendant Variants Have Been Observed

GJB2 *variant*	*dbSNP ID*	*Change at protein level/ Location*	*Effect*	*1000 genomes project (phase 3) populations*[a]
c.-22-12C>T	rs9578260	Intron	Polymorphism[b]	ACB, ASW, CLM, ESN, ITU, LWK, MSL, MXL, PEL, PUR, YRI
c.-15C>T	rs72561725	5′UTR	Polymorphism[b]	ACB, ASW, CEU, CLM, ESN, GWD, LWK, MSL, MXL, PUR, YRI
c.-14G>A	rs367567291	5′UTR	Unknown	n.a.
c.-7G>A	rs398123813	5′UTR	Unknown	n.a.
c.-6T>A	rs148136545	5′UTR	Unknown	ASW, CLM, ESN, LWK, MSL, PUR
c.35delG	rs80338939	p.Gly12Valfs	Pathogenic[c]	BEB, CEU, CLM, FIN, GBR, IBS, MXL, TSI
c.101T>C	rs35887622	p.Met34Thr	Pathogenic[d]	ASW, CEU, CLM, FIN, GBR, IBS, MXL, PUR, TSI
c.109G>A	rs72474224	p.Val37Ile	Pathogenic[c]	CDX, CHB, CHS, CLM, JPT, KHV, LWK, MXL
c.186C>T	rs397516869	p.Asn62=	Unknown	n.a.
c.225G>T	rs149137695	p.Arg75=	Unknown	n.a.
c.380G>A	rs111033196	p.Arg127His	Controversial	BEB, GBR, GIH, ITU, KHV, PJL, STU, TSI
c.457G>A	rs111033186	p.Val153Ile	Controversial	BEB, CLM, GIH, ITU, PJL, STU, TSI
c.499G>A	rs111033360	p.Val167Met	Likely pathogenic[e]	LWK
c.*78A>T	rs576671031	3′UTR	Unknown	ACB
c.*84T>C	rs3751385	3′UTR	Polymorphism[f]	All populations
c.*96A>G	rs188027627	3′UTR	Unknown	ESN, LWK
c.*104A>T	rs7337074	3′UTR	Polymorphism[b]	ACB, ASW, CLM, ESN, GWD, IBS, LWK, MSL, MXL, PEL, PUR, YRI
c.*111C>T	rs7329857	3′UTR	Polymorphism[b]	ACB, ASW, CLM, ESN, GIH, GWD, IBS, LWK, MSL, MXL, PEL, PUR, YRI

Unknown—The authors consider that there are still insufficient data in the literature, including this study, for inferring benignity or pathogenicity of the variant.

Controversial—In view of the conflicting data in the literature regarding the pathogenicity of the variant, the authors are unable to classify it as either benign (polymorphism) or pathogenic.

[a]One thousand Genome Project's Populations: ACB, African Caribbeans in Barbados; ASW, Americans of African Ancestry in SW USA; BEB, Bengali from Bangladesh; CDX, Chinese Dai in Xishuangbanna, China; CEU, Utah Residents (CEPH) with Northern and Western European Ancestry; CHB, Han Chinese in Bejing, China; CHS, Southern Han Chinese; CLM, Colombians from Medelin, Colombia; ESN, Esan in Nigera; FIN, Finnish in Finland; GBR, British in England and Scotland; GIH, Gujarati Indian from Houston, Texas; GWD, Gambian in Western Divisions in The Gambia; IBS, Iberian population in Spain; ITU, Indian Telugu from the UK; JPT, Japanese in Tokyo, Japan; KHV, Kinh in Ho Chi Minh City, Vietnam; LWK, Luhya in Webuye, Kenya; MSL, Mende in Sierra Leone; MXL, Mexican Ancestry from Los Angeles USA; PEL, Peruvians from Lima, Peru; PJL, Punjabi from Lahore, Pakistan; PUR, Puerto Ricans from Puerto Rico; STU, Sri Lankan Tamil from the UK; TSI, Toscani in Italy; YRI, Yoruba in Ibadan, Nigera.

[b]Significant allelic frequencies in some 1000 Genome Project's populations and homozygous genotypes have been observed in normal-hearing controls.

[c]The pathogenicity of the variant is well established in the literature.

[d]In spite of OMIM considering this variant as one of unknown significance (www.omim.org/entry/121011), we considered it as pathogenic based on several reports from the literature.

[e]Based on *in silico* analytic tools as assessed at the Deafness Variation Database (http://deafnessvariationdatabase.org/).

[f]This variant is clearly a polymorphism based on the genotypic frequencies available for several populations (dbSNP at NCBI).

n.a., not available.

Discussion

HL remains an important global health burden. We highlight and contextualize the salient findings and conclusions from the present study.

Coding variants of GJB2

Pathogenic and controversial variants. In this study, with regard to São Tomé and Príncipe's population, we have identified the pathogenic c.35delG, p.Met34Thr and p.Val37Ile mutations, and the controversial p.Arg127His and p.Val153Ile variants (Table 4). These five sequence changes are the most commonly found in patients from Eurasian and Mediterranean populations, and all have been previously found in Portuguese HL patients (Matos et al., 2013). Thus, a non–Sub-Saharan genetic influence in São Tomé and Príncipe's population is suggested, and the European genetic influx reported by Tomás et al. (2002), most likely due to admixture with the Portuguese, is supported by our results.

p.Val167Met: a likely recessive pathogenic variant. As regards the p.Val167Met variant, it has been previously found, in heterozygosity, in four Kenyans with prelingual, nonsyndromic HL (Gasmelseed et al., 2004) and in one Cameroonian HL patient (Bosch et al., 2014b). This variant has also been observed in the Luhya (Webuye, Kenya) population (Table 4), from the 1000 genomes project (www.1000genomes.org/), in heterozygosity and with an allelic frequency of 0.51%. The p.Val167Met variant, carried by one control individual from our sample, was also present in 1 out of 188 African-American control chromosomes (Samanich et al., 2007). The p.Val167Met variant has also been identified, in heterozygosity, in two studies, including African-American subjects, but the ethnicities of the carriers were not disclosed (Putcha et al., 2007; Ross et al., 2007). *In silico* analytic tools, as accessed at the Deafness Variation Database (http://deafnessvariationdatabase.org/), suggest the pathogenicity of p.Val167Met. Taking all together, p.Val167Met seems to be a recessive pathogenic variant of Sub-Saharan African origin.

Synonymous variants. We have identified two synonymous variants as well. The p.Arg75= variant was identified in one bilateral HL patient and in five controls (5/177 alleles = 1.41%), and the p.Asn62= variant was found in one control individual (1/354 alleles = 0.28%). Noteworthy, these two synonymous *GJB2* coding variants, of as yet unknown significance (Table 4), are present in Sub-Saharan African and Eastern Asian populations (Bosch et al., 2014b; Chen et al., 2014; Gasmelseed et al., 2004; Han et al., 2008; Mingkun et al., 2007; Trotta et al., 2011).

Noncoding variants of GJB2

As expected, the São Tomé and Príncipe's population harbors some *GJB2* noncoding variants that are shared mainly with Sub-Saharan African populations and populations of a Sub-Saharan African ancestry. A statistically significant difference in genotypic distribution regarding the c.*84T>C variant was observed between controls and patients with severe or profound bilateral HL ($p = 0.005$).

The most common of the 10 noncoding variants genotyped in the subjects of our sample, c.*84T>C, is also found in the project's populations of all the 1000 genomes (Table 4) and was observed in the control sample with an allelic frequency close to those of the Sub-Saharan populations as well as the Americans of African ancestry, Han Chinese and Japanese populations.

The c.*84T>C variant (rs3751385) has been previously found to be significantly associated with HL (Dickson et al., 2010), in the context of demonstrating the creation of synthetic genome-wide associations by rare variants. In our sample, both allele T and C at the c.*84 are common but a synthetic association between one or both c.*84 alleles and HL cannot be excluded. A larger sample would be necessary to further investigate a putative association of variants at position c.*84 with HL in São Tomé and Príncipe's population.

As regards the noncoding variants c.-22-12C>T and c.-15C>T, they were identified in controls with allelic frequencies of 17.95% and 6.33%, respectively. These polymorphisms are the most frequent in Sub-Saharan African populations and in populations of a Sub-Saharan African ancestry (www.1000genomes.org/; Table 4).

The c.-6T>A variant, observed in controls with an allelic frequency of 1.14%, is also the most frequent in Sub-Saharan African populations and in populations of a Sub-Saharan African ancestry (www.1000genomes.org/). To the extent of our knowledge, this variant, of an unknown effect (Table 4), has only been described in the heterozygous state (Al-Qahtani et al., 2010; Shan et al., 2010; Tang et al., 2006; www.1000genomes.org/).

Some other noncoding variants, of an unknown effect (Table 4), were rarely observed in this study. The c.-7G>A, c.-14G>A, c.*78A>T, and c.*96A>G variants were each observed in our sample only once or twice, in heterozygosity. The c.*96A>G variant was observed only in the Luhya (Webuye, Kenya) and Essan (Nigeria) populations, in the heterozygous form. The c.*78A>T variant has been observed only in the African Caribbean (Barbados) population (Table 4), also in the heterozygous form. No populational data are available for the c.-7G>A and c.-14G>A variants (Table 4).

GJB6 *deletions*

None of the two large *GJB6* deletions has been identified in São Tomé and Príncipe. These results are similar to those obtained in previous studies on HL patients from Sub-Saharan African populations and Sub-Saharan African ancestry populations.

The del(*GJB6*-D13S1854) is not particularly frequent in Portuguese NSSHL patients (0.4% of the patients' alleles), and the del(*GJB6*-D13S1830) seems to be fairly rare in these patients (Chora et al., 2010; Matos et al., 2013). Given the relatively low degree of European (mainly Portuguese) admixture of São Tomé and Príncipe's population (Tomás et al., 2002), it is likely that none of the *GJB6* deletions plays a relevant role in NSSHL in São Tomé and Príncipe's population, as suggested by our data.

Conclusions

The role of *GJB2* coding mutations in NSSHL in São Tomé and Príncipe seems to be of little significance. Our study, however, suggests the existence of pathogenic, and likely pathogenic, coding variants in São Tomé and Príncipe's population. Thus, although no biallelic HL patients have been

identified in our sample, the eventual occurrence of *GJB2*-related HL in this population should not be disregarded. The role of the *GJB6* large deletions in NSSHL in São Tomé and Príncipe, if any, is predicted by this study to be small.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Address correspondence to:
Helena Caria, PhD
School of Health
Polytechnic Institute of Setubal
ESS/IPS—Campus do IPS
Estefanilha
Setubal 2914-503
Portugal

E-mail: helena.caria@ess.ips.pt

Abbreviations Used

ANSI = American National Standards Institute
DNA = deoxyribonucleic acid
GJB2 = gap junction protein beta 2
GJB6 = gap junction protein beta 6
HL = hearing loss
NCBI = National Center for Biotechnology Information
NMS|UNL = NOVA Medical School | Universidade Nova de Lisboa
NSHL = nonsyndromic hearing loss
NSSHL = nonsyndromic sensorineural hearing loss
PCR = polymerase chain reaction
WHO = World Health Organization

4.5 Rubella in Sub-Saharan Africa and sensorineural hearing loss: a case control study.

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Rubella in Sub-Saharan Africa and sensorineural hearing loss: a case control study

Cristina Caroça[1,2,3*], Vera Vicente[4], Paula Campelo[2], Maria Chasqueira[4], Helena Caria[5,6,7], Susana Silva[1,3], Paulo Paixão[4,8] and João Paço[1,2]

Abstract

Background: Rubella infection can affect several organs and cause birth defects that are responsible for congenital rubella syndrome (CRS). Congenital hearing loss is the most common symptom of this syndrome, occurring in approximately 60% of CRS cases. Worldwide, over 100 000 babies are born with CRS every year. There is no specific treatment for rubella, but the disease is preventable by vaccination. Since 1969, the rubella vaccine has been implemented in many countries, but in Africa, only a few countries routinely immunize against rubella. The aim of this study was to estimate the rate of infection from the wild-type rubella virus in São Tomé and Príncipe by determining rubella seroprevalence with a DBS method. The goal of this study was to reinforce the need for implementation of the rubella vaccine in this country. As secondary objectives, the validation of a DBS method was first attempted and an association between seroprevalence and hearing loss was assessed.

Methods: We collected samples from individuals observed during humanitarian missions in São Tomé and Príncipe. All individuals underwent an audiometric evaluation, and a drop of blood was collected for the dried blood spot (DBS). We define two groups: the case group (individuals with unilateral or bilateral hearing loss (HL)) and the control group (individuals with two normal ears). Patients were excluded if they suffered from conductive HL, if they showed evidence of possible causes of HL, if they had developmental delay or if they refused to participate in the study.

Results: Among the 315 subjects, we found 64.1% individuals with IgG for the rubella virus, 32.1% without immunity for the rubella virus and 3.8% who were borderline.
In the control group, 62.6% were positive for the rubella IgG, whereas in the case group, 72% were positive. Analyzing both groups, with ages ranging from 2 to 14 years of age and from 15 to 35 years of age, we found a seroprevalence of 50.3% to rubella in the younger group and 82.1% in the older group, with a significant difference between cases and control group noted within the younger patients ($p = 0.025$).

Conclusions: Rubella is a disease that can be prevented. Rubella infections are still very common in São Tomé and Príncipe, and women of child-bearing age are still at risk for rubella infection during pregnancy, justifying the urgency of vaccination against rubella.
A statistically significant association between the group of children under 14 years of age with HL and immunity for rubella was observed in this country, although this study did not allow us to establish a cause-effect relationship between rubella infection and SNHL.

Keywords: Rubella, Hearing loss, Sub-Saharan Africa, Congenital Rubella Syndrome, World Health Organization

* Correspondence: cristinacaroca@netcabo.pt
[1]Otolaryngology Department, NOVA Medical School/Faculty of Medical Sciences, Universidade Nova de Lisboa, Campo dos Mártires da Pátria, 130, 1169-056 Lisboa, Portugal
[2]Hospital CUF Infante Santo, Avenida Infante Santo, 34, 6°, 1350-079 Lisboa, Portugal
Full list of author information is available at the end of the article

Background

Primary rubella infection during pregnancy, particularly during the first trimester, can affect several organs and cause birth defects that are responsible for congenital rubella syndrome (CRS) [1]. The most common defects of CRS are hearing impairment (unilateral or bilateral sensorineural), eye defects (e.g., cataracts, congenital glaucoma, or pigmentary retinopathy), and cardiac defects (e.g., patent ductus arteriosus or peripheral pulmonic stenosis). Congenital hearing loss is the most common sequela, occurring in approximately 60% of cases, especially when infection occurs in the 4th month of pregnancy [2]. In a Brazilian study, congenital rubella was thought to be the cause of hearing loss in 32% of patients with deafness [3], and in studies conducted in sub-Saharan Africa, rubella was considered to be one of the causes of HL [4].

The Global Measles and Rubella Strategic Plan (2012–2020) included goals to eliminate rubella and CRS in at least two WHO regions by 2015 as well as in at least five WHO regions by 2020. However, in this plan, the African region does not have a specific target. The number of rubella cases reported from 2000 to 2014 increased in the African region (from 865 cases in seven countries to 7402 cases in 44 countries). Although the rubella vaccine has been implemented in many countries since 1969, worldwide coverage is still a distant goal, particularly in Africa, where only a few countries routinely immunize against rubella [5, 6].

The efficacy of the vaccine is approximately 95%, without significant side effects [7]. In 2015, the Americas region was the world's first region to eliminate rubella and CRS. In Europe, all 53 Member States of the WHO European Region committed in 2010 to the goal of interrupting the endemic transmission of measles and rubella by 2015, which was not yet achieved in all regions. However, many states, including Portugal, have already interrupted the endemic transmission of rubella [8].

The São Tomé and Príncipe islands are within the Atlantic in sub-Saharan Africa, located at the level of the Equator. It is an underdeveloped country with few economic resources that survives through external support, including humanitarian service. As part of a humanitarian project on the islands of São Tomé and Príncipe, the "Health for All" system of Institute Marquês Valle Flor (IMVF) has been implemented to improve the primary and secondary health care of the population. This project includes doctors of several specialties, including otolaryngology.

During these tasks, an increased prevalence of sensorineural hearing loss (SNHL) cases was initially noted, particularly in the younger age group [9]. CRS cannot be excluded as a possible etiology of SNHL and is one of several possible causes.

The epidemiology of rubella is not known in this country, and there is no vaccine implementation [10] nor is there the possibility of diagnosis through laboratory tests.

The aim of this study is to estimate the rate of infected people with wild-type rubella virus in São Tomé and Príncipe by determining rubella seroprevalence through the DBS method to reinforce the need for vaccine implementation in this country. As secondary objectives, the validation of a DBS method was first attempted and an association between seroprevalence and hearing loss was also evaluated.

Methods

Subjects

The samples studied were collected between January and May of 2014 from individuals who presented for an audiology consultations at the Hospital Ayres de Menezes on São Tomé Island and the Hospital Dr Manuel Quaresma Dias da Graça on Principe Island during humanitarian missions. Samples were also collected from students and workers from a hotel. All participants in the study were natives and residents of the islands. In total, we analyzed 315 samples collected from individuals 2 to 35 years old. Of these, 171 individuals were female and 144 were male. All individuals underwent audiometric evaluation (tonal audiogram or auditory brainstem response measurements) according to the degree of collaboration, having adapted the results of the auditory brainstem response (ABR) to audiometric thresholds according to standards [11].

We defined two groups based on the WHO classification [12]. The case group was composed of individuals with hearing loss, in which we included individuals with unilateral HL or both ears with HL. The control group included individuals with two normal ears.

All patients answered a questionnaire about self-reported HL and clinical history. There are no clinical registries about these patients in any hospital from São Tomé or Príncipe.

Patients were excluded if they suffered from conductive deafness, showed evidence of possible causes of HL, had developmental delay, or did not give consent to participate in the study.

In addition to the audiometric evaluation, a drop of blood was collected via venous or capillary puncture and blotted onto filter paper. After collection, the blood spots were dried at room temperature for 24 h (Dried blood spot—DBS). The IgG measurement was carried out in Portugal after 9 months (during which the samples were stored at room temperature).

The project was submitted to and approved by the Medical Ethics Committee of STP and Ethics Research Committee NMS|FCM-UNL (n°02/2014/CEFCM). The

Ethics Research Committee is aligned with the Declaration of Helsinki for the Protection of Human Subjects. A full consent process was applied for all participants. Consent to use the survey data was also obtained.

Technical validation of the rubella IgG determination

The procedure of IgG determination from DBS was validated and optimized by two approaches: 1) First, samples were collected from 20 pregnant women at the Hospital da Luz, Portugal. We simultaneously collected blood samples for DBS and for serum. The specificity and sensitivity of the IgG determination from DBS were evaluated compared to the standard method (IgG determination from serum). 2) Second, 15 DBS samples were collected in 10 children between 1 and 10 years of age (who were vaccinated against rubella) and 5 children between 9 and 12 months of age (who were not vaccinated against rubella). In this group, IgG determination from DBS was correlated with the immune status (vaccinated / unvaccinated).

The extraction protocol for IgG in DBS was tested with three different volumes of diluent (200 μL, 400 μL, and 800 μL) in this validation step, while the protocol for the determination of the serum IgG was recommended by the SERION ELISA classic rubella virus IgG kit. Both the extraction and IgG ELISA protocols are described below.

Rubella IgG determination from the São Tomé and Príncipe population

Rubella IgG extraction: For the IgG extraction, we added 400 μL of SERION ELISA kit dilution solution to ¼ of the DBS, corresponding to 32 mm^2. The extraction was carried out for 60 min at 600 rpm at room temperature and 18 h at 4 °C.

Rubella IgG determination: The SERION ELISA classic rubella virus IgG kit was used for this determination. We performed the protocol recommended by SERION. Briefly, 100 μL of the control, standard and extracted samples were pipetted into a 96 well microplate (only one sample per patient was tested). The microplate was then incubated at 37 °C for 60 min in a humid chamber and washed 3 times with wash solution (300 μl). Then, the IgG conjugate (100 μl) was added and the microplate was incubated under the same conditions, after which the washing process was repeated. Subsequently, the substrate (100 μl) was added and the incubation process was repeated. Finally, we added a stop solution (100 μl), and the optical density was read at 415 nm against 630 nm. The optical densities were converted into UI through the Serion Activity V11 program, with the following interpretation: negative: <10UI; borderline; 10–15UI;positive >15UI.

The results were interpreted according to the algorithm shown in Fig. 1.

Statistical processing of the data

Sample descriptions were made using descriptive statistics, considering the frequency analysis, means and standard deviations (SDs).

To study the association between IgG rubella in the case/ control group and each of the following parameters, age group, district origin, oral language, gender and HL, the chi-square test was used by Monte Carlo Simulation.

To identify the risk factors of HL, we adopted a Binary Logistic Regression, where HL is a response variable. The independent variables were the IgG rubella and age groups.

All analyses were performed using the Statistical Package for the Social Sciences for Mac version 20.0 (SPSS).

Results

Technical validation of the rubella IgG determination

The sensitivity and specificity for each of the different volumes of diluent tested, when compared with the standard method were, 100 and 50% (200 μL), 89 and 100% (400 μL) and 72 and 100% (800 μL). According to these results, a volume diluent of 400 μL was chosen for the determination of Rubella IgG in the São Tomé and Príncipe populations.

The DBS method also showed a good correlation (100%) with the immune status (vaccinated/unvaccinated). The ten vaccinated children were positive for the IgG while the five unvaccinated children were negative for IgG.

Rubella IgG determination from São Tomé and Príncipe population

We evaluated 315 subjects (Table 1), from 2 to 35 years of age, of whom 144 (45.7%) were men and 171 (54.3%) were women, with a mean age of 17.36 ± 9.734 years.

Among the 315 subjects, we found 202 (64.1%) individuals with IgG for the rubella virus. Of these, 101 (32.1%) did not have immunity to rubella and 12 (3.8%) were borderline. Borderline cases were excluded from the study.

In the sample, we did not find any statistical significance for gender ($p = 0.391$), resident district ($p = 0.061$, or chi-squared test by Monte Carlo simulation), family history of HL ($p = 0.207$), or consanguinity ($p = 0.461$).

We established two groups concerning hearing status: a control group with patients with normal hearing in both ears and a case group with at least one ear with sensorineural hearing loss (SNHL).

In the control group, we found 62.6% of positive immunity to rubella, whereas in the case group, positive

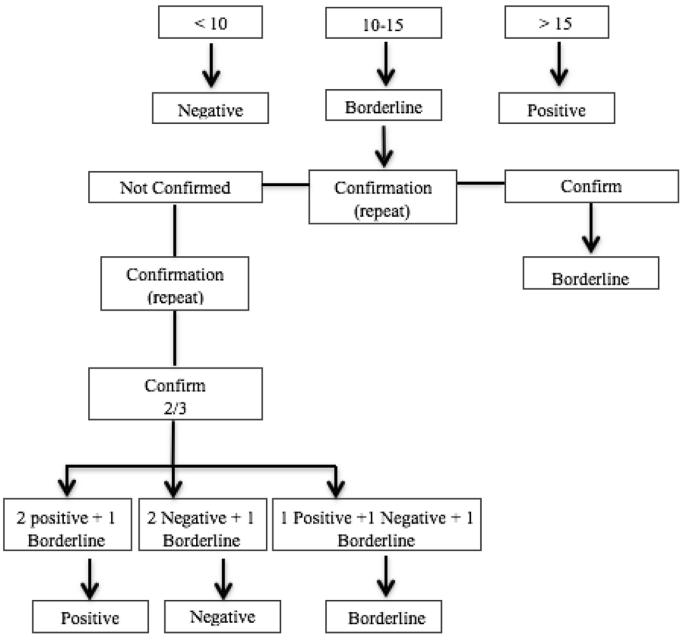

Fig. 1 Algorithm for the interpretation of the results. The results are expressed in international units (U/l)

immunity was 72%. There was not a significant difference between the case and control groups ($p = 0.085$).

Analyzing both the 2-to-14 years of age group and the 15-to-35 years of age group, we found a seroprevalence of 50.3% to rubella in the younger group and 82.1% in the older group, with a significant difference between the case and control groups within the younger group ($p = 0.025$) but not within the older group ($p = 0.528$) (Table 2).

By applying the binary logistic regression model, positive immunity to rubella was identified as a risk factor for HL (Table 3). Positive immunity to rubella almost doubled the risk of HL (OR = 1.776; CI 95% [1.050–3.004]) when analyzed with the age groups, but without any statistical significance in these age groups. Therefore, immunity to rubella was associated with HL.

A higher prevalence of "no oral language" was found in the case group compared to the control group ($p = 0.0001$).

Discussion

Since their first diagnostic application almost five decades ago, DBSs have been employed in many research areas and in clinical applications for several viruses, including Human immunodeficiency virus, Hepatitis C virus, Hepatitis B virus, Hepatitis A virus, Hepatitis E virus, Human cytomegalovirus, Epstein-Barr virus, Herpes simplex viruses, and measles-, dengue- and rubella-viruses. Although it is not expected that current wet sampling techniques will be replaced by the use of DBSs, this method allows sampling in individuals and settings with difficult access and/or a lack suitable storage facilities [13]. Indeed, the DBS method is a minimally invasive and more economic sampling method that is readily available and that facilitates sample collection and storage. It involves the collection of capillary blood from a fingerstick onto a protein-saver card, which is then air-dried and stored until processing. DBS samples can be stored and transported for testing at a later date, which may also provide enhanced surveillance in resource-limited settings [14].

In the current study, the accuracy of the IgG determination from DBS samples was assessed before the determination of rubella seroprevalence. The main limitations of this evaluation were the number of samples tested, which were lower than initially planned, and the fact that immunity by vaccination may not be similar to immunity by natural infection. In fact, children from São Tomé and Príncipe had higher average IgG results than Portuguese children (respectively 89.35 and 50.9; $p = 0.034$, data not presented). Despite these limitations, the results suggest that the determination of IgG for the rubella virus in DBS had a good correlation with the standard method. These results are in accordance with a previous publication that showed no significant differences in the antibody concentrations in paired serum-

Table 1 General characteristics of the São Tomé and Príncipe population

	Total ($n = 303$)	Control Gr (171–56.4%)	Case Gr (132–43.6%)	*p-Value*
Age range				*0.359*
[2–14]	**147 (48.5%)**	79 (46.2%)	68 (51.5%)	
[15–35]	**156 (51.5%)**	92 (53.8%)	64 (48.5%)	
Mean Age SD	**17.31 ± 9.722**	17.73 ± 9.729	16.77 ± 9.723	
Gender				*0.391*
Male	**137 (45.2%)**	81 (47.4%)	56 (42.4%)	
Female	**166 (54.8%)**	90 (52.6%)	76 (57.6%)	
Resident District				*0.061**
Água Grande	**174 (57.4%)**	110 (64.3%)	64 (48.5%)	
Mezochi	**47 (15.5%)**	17 (9.9%)	30 (22.7%)	
Cantagalo	**17 (5.6%)**	8 (4.7%)	9 (6.8%)	
Caué	**23 (7.6%)**	12 (7%)	11 (8.3%)	
Lemba	**6 (2%)**	3 (1.8%)	3 (2.3%)	
Lobata	**19 (6.3%)**	11 (6.4%)	8 (6.1%)	
Príncipe	**17 (5.6%)**	10 (5.8%)	7 (5.3%)	
Spoken Language				***0.0001***
Yes	**242 (86.7%)**	161 (97.6%)	81 (71.1%)	
No	**37 (13.3%)**	4 (2.4%)	33 (28.9%)	
Undefined	**24**	6	18	
Family History of HL				*0.207*
Yes	**52 (17.5%)**	33 (20%)	19 (14.4%)	
No	**245 (82.5%)**	132 (80%)	113 (85.6%)	
Missing	**6**	6		
Consanguinity				*0.461*
Yes	**7 (2.4%)**	3 (1.8%)	3 (3.1%)	
No	**285 (97.6%)**	162 (98.2%)	123 (96.9%)	
Missing	**11**	6	5	
Rubella IgG				*0.085*
Positive	**202 (66.7%)**	107 (62.6%)	95 (72%)	
Negative	**101 (33.3%)**	64 (37.4%)	37 (28%)	
Borderline	**12**			

Abbreviations: *SD* standard deviation
*Chi-square Test by Monte Carlo Simulation; Bold: Total sample; Italic: *p*-Value; Italic bold: statistic significant *p*-Value

DBS samples, [15] although in our study, only the qualitative correlation is presented. Actually, quantitative DBS samples were also previously tested for the diagnosis of rubella during an outbreak, with an excellent correlation with the determination in serum, provided that the DBS indeterminate results were positive [16]. In addition, this methodology was used for the diagnosis of congenital rubella syndrome in another study, by detecting rubella IgM and IgG (the last in ≥ 6 months old infants) in DBS, although in this study, the comparison was not performed with the reference sample, serum, but with oral fluid samples [17]. Therefore, using DBS for serology determinations seems to be an appropriate and useful approach, particularly in countries where routine immunization is not performed, to estimate the rate of infection with the wild-type rubella virus.

Table 2 Sample description of the case/control group and rubella IgG in the different age ranges

	Total ($n = 303$)	IgG Pos (117–58.8%)	IgG Neg (82–41.2%)	*p-Value*
Age range				***0.025***
[2–14]	**147 (48.5%)**	74	73	
Control group	79	33 (44.6%)	46 (63%)	
Case group	68	41 (55.4%)	27 (37%)	
Age range				*0.528*
[15–35]	**156 (51.5%)**	128	28	
Control group	92	74 (57.8%)	18 (63.3%)	
Case group	64	54 (42.2%)	10 (35.7%)	

Bold: Total sample; Italic: *p*-Value; Italic bold: statistic significant *p*-Value

The prevalence of IgG in the population from São Tomé and Príncipe of 303 individuals from 2 to 35 years of age was 66.7%, confirming that rubella infections are still very common in this country. In the group of children under 14 years of age, the prevalence of immunity to rubella was 50.3% (74/147 subjects), while in the age group between 15 and 35 years of age, the prevalence increased by up to 82.1% (128/156 subjects. This increase

Table 3 Binary Logistic Regression between Rubella and HL with and without factors (age group)

	Cases *n* (%)	Controls *n* (%)	*P*-value	Crude OR (95% CI)	*P*-value	Adjusted OR (95% CI)
Age group						
			0.359		*0.111*	
[2–14]	68 (51.5%)	79 (46.2%)		Reference		Reference
[15–35]	64 (48.5%)	92 (53.8%)		0.808 [0.513–1.274]		0.672 [0.412–1.096]
Rubella IgG						
			0.086		***0.032***	
Negative	37 (28%)	64 (37.4%)		Reference		Reference
Positive	95 (72%)	107 (62.6%)		1.536 [0.941–2.507]		1.776 [1.050–3.004]

Abbreviations: *HL* normal hearing – 0 – Reference Category is a response variable, *independent variables* Rubella IgG (0 –Negative, 1- Positive), age groups (0 – [2–14] years, 1 – [15–35] years); Italic: *p*-Value; Italic bold: statistic significant *p*-Value

suggests that women of child-bearing age are still suffering from rubella primary infections, and 18% of these women are at risk for rubella infection during pregnancy and subsequent CRS/birth defects in their children.

Interestingly, an association between seroprevalence and SNHL was observed in the younger group (children <14 years, $p = 0.025$), but in the older group (>14 years, $p = 0.528$). While congenital rubella infection is a known cause of deafness, the relationship between postnatal acquired infection and hearing loss is not proven, although a few reports suggest that it may occasionally occur [18, 19]. In this study, it was not possible to conclude how many cases of HL are attributable to rubella infection because the time of infection cannot be determined. Therefore, the rate of positive IgG infection resulting from congenital infections is not known, although the probability of infection during the gestational period is higher in the youngest group. The detection of rubella IgM in DBS from newborns will help to clarify this key question. This will be part of a future project of our team.

The development of oral language is dependent of the hearing status. Therefore, we found an expected higher prevalence of "no oral language" in the case group than in the control group.

According to the CDC, the rubella vaccine may be administered in combination with the mumps vaccine or the measles and mumps vaccine [20]. These should be administered at 12 to 15 months of age, with a second dose given at 4 to 6 years of age. However, given the need to control the transmission of rubella during pregnancy, vaccination of female children between 10–12 years of age and women of childbearing age is also recommended. The rubella vaccine has been shown to be effective without significant side effects and should thus be quickly implemented in the population of São Tomé and Príncipe as well as other African countries in which the rubella vaccine is not currently implemented and where there is an increase of SNHL [4].

The combined vaccine with the measles and mumps vaccine would be advantageous because these two pathologies can also unleash SNHL in the course of the disease [20].

Conclusion

Rubella is a preventable disease. Currently, most of the African countries do not use this vaccine.

Rubella infections are still very common in São Tomé and Príncipe, and women of child-bearing age are still at risk of rubella infection during pregnancy and subsequent CRS/birth defects of their children, justifying the urgency of vaccination against rubella.

According to the results obtained, a statistically significant association between the group of children under 14 years of age with SNHL and immunity for rubella was observed in this country. However, this study did not permit us to establish a cause-effect relationship between rubella infection and SNHL. Therefore, another study aiming to screen newborns for congenital rubella infection and to follow them for audiometric assessment is critical to determine the real impact of this infection in this African country.

Abbreviations

CDC: Centre of Diseases Control; CI: Confidence interval; CRS: Congenital Rubella Syndrome; DBS: Dried blood spot; HL: Hearing loss; IMVF: Institute Marquês Valle Flor; OR: Odds ratio; SNHL: Sensorineural hearing loss; SPSS: Statistical package for the social sciences; WHO: World Health Organization

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Authors' contributions
CC, PP, MJC, and JP designed the study. CC, VV and PC analysed the data and wrote the manuscript. PC, HC, SNS, MJC, PP and JP gave essential feedback on all versions of the manuscript. All authors have read and approved the final version of the manuscript.

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Author details
[1]Otolaryngology Department, NOVA Medical School/Faculty of Medical Sciences, Universidade Nova de Lisboa, Campo dos Mártires da Pátria, 130, 1169-056 Lisboa, Portugal. [2]Hospital CUF Infante Santo, Avenida Infante Santo, 34, 6°, 1350-079 Lisboa, Portugal. [3]Centre for Toxicogenomics and Human Health (ToxOmics), NOVA Medical School / Faculty of Medical Sciences, Universidade Nova de Lisboa, Campo dos Mártires da Pátria, 130, 1169-056 Lisboa, Portugal. [4]CEDOC, NOVA Medical School/ Faculty of Medical Sciences, Universidade Nova de Lisboa, Lisboa, Portugal. [5]Escola Superior de Tecnologia da Saúde de Lisboa, Avenida D. João II, Lote 4.69.01, 1990-096 Lisboa, Portugal. [6]BioISI-Biosystems and Integrative Sciences Institute, Faculty of Science of the University of Lisbon, Lisbon, Portugal. [7]ESS/IPS, School of Health, Polytechnic Institute of Setúbal, Setúbal, Portugal. [8]Clinical Pathology Laboratory—Labco, Hospital da Luz, Avenida Lusíada, 100, 1500-650 Lisboa, Portugal.

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4.6 Global Analysis of potential risk factors in Sensorineural Hearing Loss in sub-Saharan Africa – São Tomé and Príncipe

Background

More than 360 million people in the World have disabling hearing loss with a higher incidence in South Asia, Asia Pacific and sub-Saharan Africa(WHO 2013c; Tucci et al. 2010,Olusanya et al. 2014). According to new Global Estimates on prevalence of the HL there is a higher incidence in South Asia, Asia Pacific and sub-Saharan Africa (WHO 2013c; Tucci et al. 2010; Olusanya et al. 2014). Sub-Saharan Africa holds 10% of the world's population and two thirds of the world's least developed nations.

More than 1.2 million of the children living in sub-Saharan Africa aged 5 to 14 years old have moderate to severe bilateral HL (Tucci et al. 2010). Infections of the ear are the leading cause of the hearing impairment, especially in low and middle-income countries (Li et al. 2015), but others infectious diseases such as rubella, meningitis, measles and mumps can also lead to HL. Most of these infectious diseases can be prevented through vaccination in the countries where they exist.

The consequences of hearing problems are well known. Mild to moderate HL in children can result in developmental delays and profound HL can lead to significant inability to engage in oral/aural communication.

During the humanitarian missions in São Tomé and Principe with the Project "Health for all" – Instituto Marquês de Valle Flôr (IMVF) a group of otolaryngologists and audiologists started the audiometric evaluation and verify a high prevalence of sensorineural hearing loss (SNHL) (Caroça, Campelo, et al. 2016; Caroça et al. 2013). After this discover the question about the causes of the high prevalence of SNHL was a challenge, with the objective to minimize the effects of this disability as to treat and control if possible.

The causes of SNHL probably are not only one, but resulting from a group of risk factors that can act isolated or in-group and lead to HL.

São Tomé and Principe is a small country in Africa Sub-Saharan in the middle of the Atlantic sea, near Gabon, Equatorial Guinea, Camaroon and Nigeria. The population is young, with about 187000 inhabitants, poor socioeconomics and sanitary conditions with a public health infectious problem – malaria (Instituto Nacional de Estatística 2013; Instituto Nacional de Estatística et al. 2010).

Also associated to malaria infections they have a high prevalence of anemia, not only associated to malaria but also to hemoglobinopathies and deficiency anemia, more frequent in woman and child (Instituto Nacional de Estatística et al. 2010; STP & Unicef 2015).

Trying to identify possible causes of HL we found that in this country they don't have implemented rubella vaccine. Rubella infection during pregnant woman has a potential risk factor not only for HL as for eye or cardiac diseases.

There are low resources and lab test are limited to simple tests, being malaria test the most frequent test done in these islands.

Hemoglobinopathies should be the cause of the high prevalence of anemia, and considering the history of malaria infections, sickle cell disease probably it was one of the most prevalent, as G6PD deficiency (Manco et al. 2007; López et al. 2010).

More than 400 different variants of *G6PD* gene have been described to date,(López et al. 2010) from which at least 186 have been characterized as mutations (Howes et al. 2013). The most common variants described are 376A (G6PD type B, normal phenotype), 376G (G6PD type A^+, moderately deficient phenotype), both variants generated by G6PD (Asn126Asp) polymorphism and characterized by c.376A>G; and 202A (G6PD type A^-, severely deficient phenotype) generated by G6PD (Val68Met) and characterized by c.202G>A (Cappellini & Fiorelli 2008; Nguetse et al. 2016; Petit et al. 2016). However, the type A^- is heterogeneous since it can result from a combination of the Asn126Asp replacement with any of three additional mutations, the most common of which is Val68Met (Luzzatto & Seneca 2014; Manco et al. 2007).

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most common genetic enzymopathy in humans. This pathology is an X-linked disorder with recessive genetic trait. Heterozygous females are genetic mosaics as a result of X-chromosome inactivation (in any cell, one X chromosome is inactive, but different

cells randomly inactivate one chromosome or the other) and the abnormal cells of a heterozygous female can be as deficient for G6PD as those of a G6PD-deficient male: therefore, such females can be susceptible to the same pathophysiological phenotype (Cappellini & Fiorelli 2008).

Considering that São Tomé and Príncipe are islands, colonized by several European and African countries, especially the Portuguese and Jews, the consanguinity associated with a genetic cause caused by mutations of GJB2 and GJB6 could be significant.

This study projected during humanitarian missions, pretends to identify some possible risk factors of HL in São Tomé and Príncipe.

In a sample of 316 individuals, witch 18 were excluded because not presented all the laboratory tests, we had obtained a sample of 298 subjects.

The sample was stratified according to the audiometric results. Control group with bilateral normal hearing, while the case group met individuals with at least one ear with sensorineural hearing loss (Table 4.6-1).

Table 4.6-1- General characteristics in São Tomé and Príncipe sample.

	Total (n=298)	**Control Gr (169-56.7%)**	**Case Gr (129-43.3%)**	***p-Value***
Age range				*0.275*
[2-14]:	144 (48.3%)	77 (45.6%)	67 (51.9%)	
[15-35]:	154 (51.7%)	92 (54.4%)	62 (48.1%)	
Mean Age SD	17.36±9.724	17.83±9.726	16.74±9.724	
Gender				*0.346*
Male:	134 (45%)	80 (47.3%)	54 (41.9%)	
Female:	164 (55%)	89 (52.7%)	75 (58.1%)	
Resident District				*0.051**
Água Grande	173 (58.1%)	109 (64.5%)	64 (49.6%)	
Mezochi	46 (15.4%)	16 (9.5%)	30 (23.3%)	
Cantagalo	17 (5.7%)	8 (4.7%)	9 (7%)	
Caué	21 (7%)	12 (7.1%)	9 (7%)	
Lemba	5 (1.7%)	3 (1.8%)	2 (1.6%)	
Lobata	19 (6.4%)	11 (6.5%)	8 (6.2%)	
Príncipe	17 (5.7%)	10 (5.9%)	7 (5.4%)	
Oral Language				***0.0001***
Yes:	239 (86.9%)	160 (97.6%)	79 (71.2%)	
No:	36 (13.1%)	4 (2.4%)	32 (28.8%)	
Indefined:	23	5	18	
Family History HL				*0.221*
Yes:	52 (17.8%)	33 (20.2%)	19 (14.7%)	
No:	240 (82.2%)	130 (79.8%)	110 (85.3%)	
Missing:	6	6		
Consanguinity				*0.451*

Yes:	7 (2.4%)	3 (1.8%)	4 (3.2%)	
No:	280 (97.6%)	160 (98.2%)	120 (96.8%)	
Missing:	11	6	5	
Malaria				*0.113*
Yes:	184 (63.7%)	98 (58%)	86 (66.7%)	
No:	105 (36.3%)	66 (40.2%)	39 (30.2%)	
Missing:	9	5	4	
Audiometric exam				***0.0001***
TA:	252 (84.6%)	157 (92.9%)	95 (73.6%)	
ABR:	46 (15.4%)	12 (7.1%)	4 (26.4%	
Self-report of HL				***0.0001***
Yes	170 (58%)	58 (34.5%)	112 (89.6%)	
No	123 (42%)	110 (65.5%)	13 (10.4%)	
HL degree				
Normal:	209 (70.1%)	169 (100%)	40 (31%)	
Mild:	15 (5%)		15 (11.6%)	
Moderate:	19 (6.4%)		19 (14.7%)	
Severe:	16 (5.4%)		16 (12.4%)	
Profound:	39 (13.1%)		39 (30.2%)	
Right Ear				
Normal:	186 (62.4%)	169 (100%)	17 (13.2%)	
Mild:	24 (8.1%)		24 (18.6%)	
Moderate:	17 (5.7%)		17 (13.2%)	
Severe:	15 (5%)		15 (11.6%)	
Profound:	56 (18.8%)		56 (43.4%)	
Left Ear				
Normal:	192 (64.4%)	169 (100%)	23 (17.8%)	
Mild:	16 (5.4%)		16 (12.4%)	
Moderate:	19 (6.4%)		19 (14.7%)	
Severe:	16 (5.4%)		16 (12.4%)	
Profound:	55 (18.5%)		55 (42.6%)	
Hb				*0.463*
AA:	239 (80.2%)	133 (78.7%)	106 (82.2%)	
AS:	56 (18.8%)	35 (20.7%)	21 (16.3%)	
SS:	3 (1%)	1 (0.6%)	2 (1.6%)	
G6PD (202G>A)				*0.926*
GG:	224 (75.2%)	128 (75.7%)	96 (74.4%)	
GA (F):	48 (16.1%)	26 (15.4%)	22 (17.1%)	
AA (F)/ GA/AG (M):	26 (8.7%)	15 (8.9%)	11 (8.5%)	
G6PD (376A>G)				*0.963*
AA:	155 (52%)	89 (52.7%)	66 (51.2%)	
AG (F):	71 (23.8%)	40 (23.7%)	31 (24%)	
GG (F)/ AG/GA (M):	72 (24.2%)	40 (23.7%)	32 (24.8%)	
G6PD variants				*0.782*
B & B/B:	152 (51%)	86 (50.9%)	66 (51.2%)	
A & A/A:	36 (12.1%)	19 (11.2%)	17 (13.2%)	
A- & A-/A-:	24 (8.1%)	13 (7.7%)	11 (8.5%)	
B/A:	36 (12.1%	23 (13.6%)	13 (10.6%)	
B/A-:	35 (11.7%)	17 (10.1%)	18 (14%)	
A/A-:	12 (4%)	8 (4.7%)	4 (3.1%)	
202G>A:	3 (0.9%)	3 (1.8%)		
G6PD variants				*0.962*
B	152 (51%)	86 (50.9%)	66 (51.2%)	
Others (non-B)	146 (49%)	83 (49.1%)	63 (48.8%)	
GJB2 reg codificant				*0.425*
Yes:	15 (5%)	10 (5.9%)	5 (3.9%)	
No:	283 (95%)	159 (94.1%)	124 (96.1%)	
GJB6 del				
Yes:	0	0	0	
No:	298 (100%)	169 (100%)	129 (100%)	
Rubella IgG				*0.110*
Positive	200 (67.1%)	107 (63.3%)	93 (72.1%)	
Negative	98 (32.9%)	62 (36.7%)	36 (27.9%)	

SD – Standard Deviation;*Qui-squared Test by Monte Carlo Simulation; F – female; M – male

The groups were homogeneous, with no statistically significant difference when assessed for gender (p=0.346) or for age groups (p=0.275).

All administrative districts of São Tomé and Príncipe were covered by audiology consultation, and the Água Grande district presented the highest percentage of individuals (58.1%), with no statistically significant difference between case-control groups (p=0.051), verifying a higher prevalence (65.2%) of HL in Mé-Zóchi district.

The hearing threshold was determined in 84.6% of subjects by pure tone audiogram (PTA) and the remaining 18.6% by auditory brainstem response (ABR).

It was evaluated the self-report of hearing loss by the question "do you feel you have a hearing loss?" and there has been confirmed by the audiometric tests (p=0.0001). The prevalence of persons with hearing complaints was 58% and that effectively presented hearing loss was 42.7%.

We evaluated hearing loss by degrees according to the WHO classification, the global sample presents with 70.1% of subjects normal hearing, followed in descending order: profound hearing loss (13.1%), moderate (6.4%), severe (5.4 %) and finally mild (5%).

The development of oral language in the total sample revealed that 13.1% did not present oral and 86.9% had oral with a statistically significant difference (p=0.0001). However, 23 individuals did not show enough maturity to have orality.

Potential Risk Factors

Consanguinity

When asked about the existence of HL in the family, only 17.8% answered affirmatively and 6 individuals do not remember or do not know. The remaining 82.2% reported not having a family history of HL. When applying the $\chi 2$ test it was found that in this sample, there is no statistical significance between family history of HL and HL (p=0.221).

The relationship between HL and consanguinity in this sample showed that only 2.4% respond affirmatively to the question, with 11 subjects chosen to answer that they did not know. There was no statistical significance by χ2 test (p=0.451).

Self-report of Malaria Infection

By questioning about the history of clinical malaria infection, about 63.7% answered affirmatively that at some point in life suffered infection by malaria, having performed antimalarial therapy. The association between episodes of malaria and HL have no statistically significant significance (p=0.113).

The antimalarial therapy ranged from quinine to the latest therapy artesunate and amodiaquine, but not all were able to answer the question about treatment carried out, having been excluded from the analysis the type of antimalarial treatment instituted.

Hemoglobinopathies - HbS

In the analysis of hemoglobinopathies, hemoglobin S was expected with a high prevalence. In this sample hemoglobin S was in Hardy-Weinberg equilibrium, with no deviations in the genotypes. There was obtained few cases of homozygous (1%) and heterozygous (18.8%).

G6PD

The deficit of glucose-6-phosphate dehydrogenase was evaluated by assessing two mutations (202G>A and 376A>G) and was found at Hardy-Weinberg disequilibrium with an increase of the mutant allele. The prevalence of this disease is increased in the sample, as we expected. At the 202G>A (χ2 test, p=0.926) and 376A>G (χ2 test, p=0.963) there was no statistically significant difference between case-control groups. Analyzing the G6PD variants based on this mutations we organized the sample in variant B and non-variant B. G6PD B was found in 152 subjects (51%) and others variants 146 subjects (49%) with no statistical significance (p=0.962) between case-control groups.

Conexins – Genetic penetrance

Evaluating the impact of DFNB1 in sensorineural hearing loss on an island, whereas the information collected during the investigation about consanguinity does not have revealed significant and recalling the historical facts about the colonization of the island, this did not prove significant. For GJB2 gene (Cx26) it was found 5% with heterozygous mutations, most of mutations in the control group (66.7%), with no statistical significance (p=0.425). For GJB6 (Cx30) none mutation was identified.

Rubella Infection

The immunity to rubella, is a known risk factor for gestational age, and is not known in this community. There is no availability to make the diagnosis by laboratory tests on the island, being an often-overlooked disease. The results showed that in this sample it is confirmed that the population is in contact with the virus, showing positive IgG results in 67.1%, with a higher prevalence in the case group. Although not presenting a statistically significant relevance (p=0.110), we find in the analysis by age group that the group of young individuals shows a statistically significant difference with p=0.028, while in the age group between 15 and 35 years is not significant (p=0588).

Global Analysis of potential risk factors for sensorineural hearing loss in sub-Saharan Africa – São Tomé and Príncipe

It was performed multiple binary logistic regression (Table 4.6-2) where the dependent variable is HL (case-control) and the independent variables are age groups, gender, clinical malaria history, Hemoglobin S, G6PD variants and immunity to rubella. They are still considered as independent variables associations between malaria & age groups, malaria & gender, G6PD variant & gender, G6PD variant & malaria, G6PD variant & HbS and HbS & Malaria. It was

excluded from this study the existence of mutation for GJB2 and GJB6 because it represents a small universe.

Table 4.6-2 - Binary Logistic Regression between independent variables and HL without and with risk factors and interactions.

	Cases 129(43.3%)	**Controls 169(56.7%)**	***P*-value**	**Crude OR (95% CI)**	***P*-value**	**Adjusted OR (95% CI)**
Gender Group			*0.347*		***0.024***	
Female	54 (41.9%)	80 (47.3%)		Reference		Reference
Male	75 (58.1%)	89 (52.7%)		0.801 [0.505-1.272]		0.312 [0.113-0.860]
Age group			*0.275*		*0.130*	
[2-14]:	67 (51.9%)	77 (45.6%)		Reference		Reference
[15-35]:	62 (48.1%)	92 (54.4%)		0.774 [0.489-1.226]		2.047 [0.809-5.181]
Malaria infection			*0.114*		*0.053*	
No:	86 (66.7%)	98 (58%)		Reference		Reference
Yes:	39 (30.2%)	66 (40.2%)		1.485 [0.909-2.425]		2.885 [0.987-8.435]
HbS			*0.475*		*0.768*	
AA:	106 (82.2%)	133 (78.7%)		Reference		Reference
AS:	21 (16.3%)	35 (20.7%)	*0.352*	0.753 [0.414-1.369]	*0.939*	0.931 [0.266-3.403]
SS:	2 (1.6%)	1 (0.6%)	*0.455*	2.509 [0.224-28.051]	*0.523*	2.470 [0.154-39.633]
G6PD variants			*0.962*		*0.884*	
B	66 (51.2%)	86 (50.9%)		Reference		Reference
Non-B	63 (48.8%)	83 (49.1%)		0.989 [0.625-1.564]		1.079 [0.388-3.004]
Rubella IgG			*0.111*		***0.023***	
Neg:	36 (27.9%)	64 (37.4%)		Reference		Reference
Pos:	93 (72.1%)	107 (62.6%)		1.497 [0.912-2.458]		1.986 [1.100-3.585]
Malaria infection & Age groups			*0.084*		***0.0001***	
No & [2-14]				Reference		Reference
				0.658 [0.409-1.059]		0.123 [0.039-0.385]
Malaria infection & Gender			*0.168*		***0.046***	
No & Fem				Reference		Reference
				1.448 [0.856-2.451]		3.001 [1.021-8.817]
G6PD variants & Gender			*0.672*		*0.258*	
No & Fem				Reference		Reference
				1.141 [0.620-2.101]		1.804 [0.649-5.014]
G6PD variants & Malaria infection			*0.844*		*0.459*	
B & No				Reference		Reference
				1.051 [0.639-1.729]		0.669 [0.230-1.942]
G6PD variants & HbS*			*0.436*		*0.643*	
B & HbAA				Reference		Reference
				0.749 [0.362-1.550]		0.738 [0.204-2.668]
HbS* & Malaria infection			*0.960*		*0.506*	
HbAA & No				Reference		Reference
				0.982 [0.481-2.005]		1.264 [0.634-2.522]

HL: Normal hearing – 0 – Reference Category is a response variable; independent variables: Gender Group (0 – Female, 1 – Male), age groups (0 – [2-14] years, 1 – [15-35] years), history of Malaria infection (0 – no, 1 – yes), Immunity Rubella (0 – Negative, 1 – Positive), HbS (0 – HbAA wild type, 1- HbAS trait, 2 – HbSS homozygotic), G6PD variants (0 – G6PD B, 1 – G6PD A/ G6PD A-/ 202G>A) and HbS* (0 – HbAA, 1 – HbAS/HbSS)

The results showed statistical significance for gender, with males being protected for HL (p=0.024, OR=0.312; 95% [0.113-0.860]) and immunity to rubella doubled

the risk of hearing loss (p=0.023; OR=1.986; 95% [1.100-3.585]). Malaria even not significant, increase two and half times the possibility of having hearing loss (p=0.053; OR=2.885; 95% [0.987-8.435]). The interactions between malaria and age groups, malaria and gender have a statistical significance. Malaria with gender interaction has an increased risk for HL in males (p=0.046; OR=3.001; 95% [1.021-8.817]). Malaria with age groups interaction shows a decreased risk of developing hearing loss in older age group (p=0.0001; OR=0.123; 95% [0.039-0385]).

The remaining analysis of the model, although no statistical significance in this sample the older age group is at higher risk for hearing loss (p=0.130; OR=2.047; 95% [0.809-5.181]) and sickle cell disease (HbSS) is associated with an increased risk for hearing loss (p=0.523; OR=2.470; 95% [0.154-39.633]) with high confidence interval due to the small number of cases. On the other hand, the sickle cell trait (HbAS), is acting as a protective factor for HL (p=0.939; OR=0.952; 95% [0.266-3.403]).

Analyzing interactions without statistical significance, we find that the G6PD non-B variant in association with the male has a higher risk of developing hearing loss (p=0.258; OR=1.804; 95% [0.649-5.014]), instead interaction of G6PD non-B variant with malaria infection is associated to a lower risk of hearing loss (p=0.459; OR=0.669; 95% [0.230-1.942]). To interaction of G6PD non-B and HbS non-wild type it is found that there is lower risk of hearing loss (p=0.643; OR=0.738; 95% [0.204-2.668]) and interaction of HbS non-wild type with malaria infection increase the risk of HL (p=0.6.91; OR=1.306; 95% [0.350-4.876]).

5 Discussion and Conclusion

During these missions, we highlight an increased prevalence of SNHL (35.7%). Contrary to expectations the conduction HL (2.9%) and mixed (1.9%) in São Tomé and Príncipe, are underrepresented (Caroça, Campelo, et al. 2016).

With the high prevalence of HL we verify the social isolation of this children, bad oral language and low schooling, which is associated to a least developed country.

The oral language reflects the effect of hearing loss on the acquisition of correct oral language, therefore it is important to identify cases that do not have oral or have poorly. As expected before the prevalence of increased SNHL, the existence of oral language is also reduced, in this sample, showing a statistically significant association (p=0.0001).

Although we think that the consanguinity in an island is a reality, which is not confirmed analyzing the sample for genetic deafness recessive non-syndromic. (DFNB1). Identifying mutations of GJB2 (Cx26) and GJB6 (Cx30) genes in the sample, it tried to also check the influence of the island's colonization and the contribution to the genetic HL. Genetic deafness by GJB2 mutation and GJB6 did not proved significance in this sample. Consanguinity in these cases would lead to an increase in homozygosity and emergence of pathogenic mutations and this did not happen (Zakzouk 2002).

Family history did not prove significance, and also there was no association between family history and GJB2 and GJB6 mutations. In any case, it stresses the importance of family history in the clinical history, especially for direct relatives (parents and siblings) knowing that only 15% of those with a positive family history, have genetic HL, and should therefore target of audiometric surveillance (Driscoll et al. 2015).

In São Tomé and Príncipe, health care is limited, with no additional tests of laboratory diagnosis available. For this reason the infectious causes in the population are not fully known. Malaria is the main pathology known and

diagnosed. If there is no possibility of serological tests to assist in the identification of infectious diseases that may be involved in the diagnosis of hearing loss, especially diseases that are part of TORCH (toxoplasmosis, rubella, cytomegalovirus and herpes) (Shet 2011).

Rubella has been decreasing its etiologic role in most of worldwide due to the implementation of the vaccine(WHO 2015b; da Silva et al. 2006), but in the case of São Tomé and Príncipe, this may be an important cause, because it is not implemented this vaccine (WHO 2013b) this study revealed the existance of disease in STP population, with high prevalence and statistical significance stratified by age group, doubling the risk of SNHL (as described in Section/Chapter 4.5) This may be another important cause of congenital HL, preventable and therefore should be implemented vaccination as soon as possible (Hulbert et al. 1991). There are also particular cases of heart and eye disease on the island that can be framed in cases of gestational rubella.

The infectious framework of meningitis is under diagnosed due to the high prevalence of malaria and sometimes diagnosed as cerebral malaria, and there is therefore history of HL associated with the diagnosis of meningitis.

There are also several factors related to the environment and the individual himself that may be associated with the development of HL, of which stand out: the malaria (Zhao & Mackenzie 2011; Schmutzhard et al. 2010); the ototoxicity (Freeland, Jones & N. Mohammed 2010) and antimalarial therapy.

In antimalarial therapy that, although in some studies not considered as ototoxic (Gürkov et al. 2008; Gürkov et al. 2008), in child (Hutagalung et al. 2006) in case of dose is often not appropriate for the child's weight, can cause irreversible HL.

Malaria is a disease that affects this country for several years, being responsible for a high rate of mortality and morbidity. Recently, the incidence of severe clinical malaria has decreased as the mortality due to this pathology. There are no medical records in hospital about the diagnosis of this disease, so the questionnaire was based only on the subjects or tutors response. The established antimalarial therapy also draws attention as co-responsible for HL (Freeland, Jones & N.

Mohammed 2010), but in this study, there were small number of subjects that knew the treatment they had done and so no relation was possible to achieve.

In STP, for several years, it is implemented prophylactic antimalarial therapy during pregnancy and has been found through the questionnaire that has had strong adhesion. Clinical malaria on the island of Príncipe is considered at this time as eradicated, but on the island of São Tomé is still decreasing cases (WHO 2014a).

In this study, malaria increase the risk of HL, around three times more. This risk increases when associated with younger age groups and males. The justification for this may be because this is the gender that is more susceptible to infection by malaria, more exposed and usually developing hemolytic anemia triggered by infection that could be more severe in males (Bope et al. 2010; Mason et al. 2007). On the other hand, the youngest age group, the effects of the disease, not only are more exuberant due to the immaturity of the immune status of children, who are less fit for the disease, but also by the performed treatment which usually is not adjusted to the weight, leading to an irreversible effect by ototoxic effect (Freeland, Jones & N. Mohammed 2010).

On the other hand, Sub-Saharan hemoglobinopathies are prevalent in this region, normally associated with malaria endemic areas and occasionally are associated with HL in homozygous form (Burch-Sims & Matlock 2005; Mgbor & Emodi 2004). In this sample this was demonstrated that sickle cell disease (HbSS) have almost two and half of risk to develop HL and sickle cell trait, is protecting to HL. This protection of the sickle cell trait to HL may result from the fact that this variant confers protection against malaria, reducing the risk of HL.

The variants non-wild type (non-B) of Glucose-6-phosphate dehydrogenase (G6PD) deficiency in part is associated with the increase of severe neonatal jaundice cases and acute hemolytic anemia and consequent SNHL neonatal (Kuzniewicz et al. 2014; Worley et al. 1996).

When evaluating the G6PD non-B variant in association with gender, it was found that these were not statistically significant, but almost duplicating the risk to develop HL in male gender. The increased risk of hearing loss associated with G6PD deficiency in male, may be related to the fact that the male has a more severe

form of the disease, manifesting a more aggressive hemolytic anemia (Mason et al. 2007).

From the interaction between G6PD non-B variant and malaria infection, even not significant, we found a low risk of develop HL, probably is associated to more malaria resistance attributable to variant A^- of G6PD (Glader 2016; Guindo et al. 2007).

The interaction between sickle cell disease or trait and non-B variant of G6PD both knowing as protectors to malaria infection, and usually in some communities with an inverse relationship between both, presented a low risk to have HL. Instead that, sickle cell disorders with malaria infection increased the risk of HL in this sample, probably because of the association of pathologic effect of malaria infection and vascular disease in sickle cell disorders.

Common to sickle cell disorders, malaria infection and G6PD deficiency we have a common diagnostic – anemia. Anemia is a frequent diagnostic in São Tomé and Príncipe, being more prevalent in child and women (ICF Macro Inc. 2015), and women could be associated to iron deficiency (ICF Macro Inc. 2015; Instituto Nacional de Estatística et al. 2010). This anemia prevalence in woman could be responsible for higher risk of HL in female group. In male the principal cause of anemia should be hemolytic anemia, by G6PD deficiency or even malaria infection. Usually hemolytic anemia is more exuberant in male (Mason et al. 2007).

Analyzing the main effects of the risk factors evaluated, it was found that rubella have an increased risk for hearing loss, whereas males are protected. Malaria was almost a risk factor with around three times more prone to HL. For the interactions observed, malarial infection and male are at increased risk for hearing loss, while the presence of malaria in older age group presents a reduced risk for hearing loss. Malaria infection has a great impact in younger age and should carried out more control of this disease. Younger group are immunologically more fragile and susceptible to ototoxicity. The hemoglobinopathies that confer protection to malaria infection (HbS and G6PD) revealed that HgS is not prevalent in the sample, with a few cases of sickle cell disease (HbSS) and sickle cell trait

(HbAS). G6PD deficiency, assessing two mutations more frequent and responsible for the variants B, A^+ and A^- revealed an increased prevalence of non-B variants, being in Hardy Weinberg disequilibrium, which means that this population it is being under selective pressure. G6PD deficiency is often associated with neonatal jaundice and acute or chronic hemolytic anemia, in some cases triggered by drug therapies that have the potential to induce oxidative stress, and may be responsible to hearing loss. Eventually the inclusion and exclusion criteria have undergone some influence on the results of G6PD deficiency, and therefore not significant. In this country preventive measure should be taken, particularly perinatal screening, greater control of neonatal jaundice and therapies used to prevent hemolysis and peripartum complications.

No doubt that rubella is a significant and preventable risk factor for which preventive measures should be implemented, including the implementation of the vaccine in São Tomé and Príncipe.

The genetic causes of this sample showed not play a significant role in the origin of SNHL in STP population.

This is an original work done during humanitarian missions, trying to identify some possible causes of HL, it is important to recognize that cannot be involved only one single cause, but a set of factors that synergistically lead to the onset of HL. Must be carried out more studies to evaluate possible causes of HL in São Tomé and Príncipe.

6 Final Considerations

The study here present is a pioneer work in the specialty of Otolaryngology. Until the first mission (February 2011), there was not the possibility to carrying out hearing assessment in the population of STP. This project allowed not only the identification, as well as the guidance of deaf individuals to a better social integration and identification of possible risk factors for the onset of deafness.

The study of contributing causes for hearing loss was determined by the contact with high prevalence of sensorineural hearing loss in a country, where we feel like "parents".

As reported by WHO, in countries like São Tomé and Principe the high prevalence of hearing loss is preventable.

During these 6 years of humanitarian missions, even without knowing what is responsible for the disability in this Country, we have promoted integrated projects during the study with sessions of public and health education to alert this situation, minimize the disability and try to treat and control the problem.

According the results, we can answer the questions purposed on objectives chapter:

- Did the population of STP recognize the existence of hearing loss?
  Yes, the global population of STP recognize the existence of hearing loss. But, based on the sensitivity, more than 30% of children with hearing loss will not be detected, and opportunities for early intervention during critical periods of language acquisition will be missed for them.

- Is the sensorineural hearing loss in São Tomé and Príncipe associated with traits of the malaria resistance (HbS or G6PD deficiency)?
  Hemoglobinopathies that confer protection to malaria didn't show any significant association to hearing loss.

- Is genetic deafness, namely the mutations of *GJB2* and *GJB6* responsible for sensorineural deafness in São Tomé and Príncipe?
  GJB2 and *GJB6* mutations revealed that these mutations aren't responsible for hearing loss in São Tomé and Príncipe

- Do they have rubella in the population of São Tomé and Príncipe? Could it be associated to hearing loss?
  Yes, they have a high prevalence of rubella infection and has a significant increased risk for hearing loss which is preventable.

- What epidemiological factors may be acting as risk factors for hearing loss in São Tomé e Príncipe?
  - ✓ Rubella infection duplicate the risk to hearing loss
  - ✓ Males are protected to HL, but in presence of malaria infection they have a high risk to have hearing loss
  - ✓ Malarial infection is a potential risk factor and representing a higher risk in younger people, not only associated to the pathophysiology of the disease, but also probably because of therapeutics, and males (who are more exposed to the disease because of activity)

- What epidemiological factors may be acting as risk factors for hearing loss in this sample?
  - ✓ Older age group normally has higher risk to have HL. Because they are exposed to more risk to develop HL
  - ✓ History of Malaria infection is almost a significant risk factor, increasing the risk near 3 times. Malaria infection should lead to hemolytic anemia and inflammatory mediators that can induce HL. Also antimalarial therapeutics' can lead to HL
  - ✓ HbS is a hemoglobinophathy that usually is associated to malaria protection. HbS trait is acting as protector to HL, instead HbS disease increase the risk of HL, almost 2,5 times.

- ✓ G6PD Def is another genetic disease from hemoglobin that confers protection for malaria infection. In this sample we didn't state an increased risk impact.
- ✓ G6PD def is more prevalent as disease manifestation in male because they are hemizygotic, having more exuberant hemolytic anemia, with higher risk to HL
- ✓ G6PD def has a protector effect to develop severe malaria infection in this sample G6PD variant non-B is acting as protector to HL because is protecting to malaria
- ✓ G6PD def and HbS have a protector effect to develop severe malaria infection, in this sample the presence of G6PD variant non-B and HbS are acting as protectors to HL since they are protecting for malaria
- ✓ HbS have a protector effect to develop severe malaria infection, but also reveal mechanisms that can induce HL in this sample, increasing the risk to develop HL.

This work tried to identify the possible factors that are contributing to hearing loss and also, promote some measures not only to prevent, but also to treat, or even as early identification marker of the disability and promotes educational programs for public and health services.

6.1 Integrated projects developed during the study

6.1.1 Health education

From 2011, seminars during missions on warning signs to identify deaf children: "Taking care of your ears" (Figure 6.1-1)

During these six years we try to promote educational programs not only for health services but also for general public.

Figure 6.1-1 – Seminars during humanitarian missions to promote health education to general public. (Personal photo)

6.1.2 Adaptation of hearing aids

Trying to reduce the disability and improve the social integration of some children and young adults, since February 2012 some of them were adapted to hearing aids. (Figure 6.1-2).

Figure 6.1-2 – Child with hearing aids adapted during missions. (Personal photo)

6.1.3 Speech therapy

At the same time we started the adaptation of hearing aids, those children with difficulty in speaking, have speech therapist sessions, during the missions to promote and help to reach a better oral language (Figure 6.1-3).

Figure 6.1-3 – Speech therapy during humanitarian mission, trying to increment oral language. (Personal photo)

Because there isn't a speech therapist in this country, we try to have parents during the session, to teach them to make some exercises with children between missions.

6.1.4 STP – Sign language

The need of communication with deaf was imperative, so it was created the conditions to promote the emergence of their own sign language.

Since February 2013 integration in an element to guide the emergence of sign language of São Tomé and Príncipe and in July 2014, publication of the first dictionary of Sign Language in São Tomé and Príncipe. ("Emerging Linguistic Features of São Tomé and Príncipe Sign Language - Sign Language and Linguistics") (Figure 6.1-4).

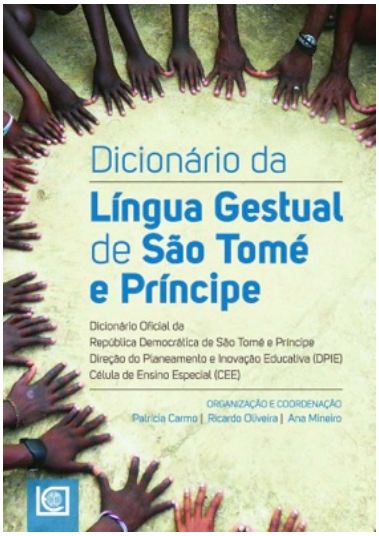

Figure 6.1-4 – The born of Sign language of São Tomé and Principe during these missions, with creation of a sign language dictionary

The needs of their own sign language was because they have some objects, fruits, habits different from other countries.

6.1.5 Neonatal hearing screening STP

Associated to all this work, it was created the neonatal screening, to early identification of hearing loss, and at the same time try to understand if hearing loss is congenital or acquired during life.

Since November 2014 in São Tomé and February 2015 in Príncipe we have tried to identify early cases of deafness in order to act in accordance (Figure 6.1-5).

Depending on the results we get it will be interesting to adapt the genetic tests to etiological screening.

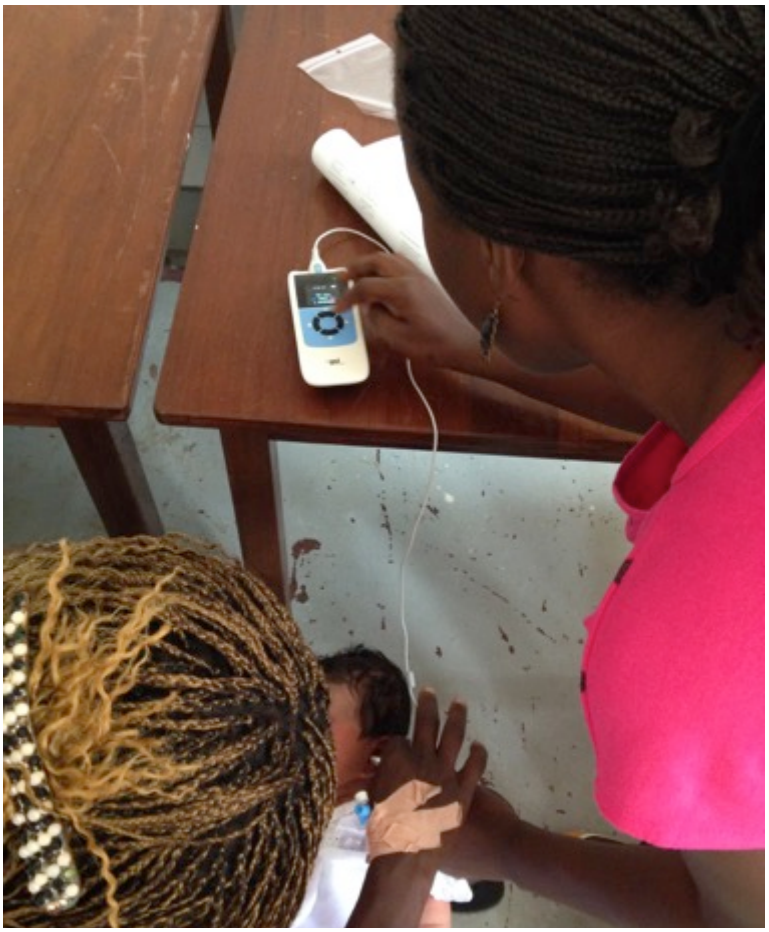

Figure 6.1-5 – The need to identify hearing loss as soon as possible, trying to prevent social isolation.

6.1.6 Rubella vaccine STP implementation

Within the framework of this study concerning the etiologic factors of hearing loss in STP and associated to the humanitarian missions, there was a high prevalence of rubella in the sample population under study.

In August 2016, WHO was started a vaccine campaign for rubella in this country. At the end of 2016 rubella vaccine was integrated on the vaccine program of STP.

6.2 Future perspectives...

Considering the results achieved in this study and in the health and quality of life of the population of STP, various aspects can be considered:

6.2.1 Early identification of hearing loss, follow-up and identification of other risk factors

With implementation of Neonatal Hearing Screening (Acustic Otoemissions), early identification of hearing loss could be possible. In these situations, and with Computerized Tomography in São Tomé and Principe, we are creating favorable conditions to adapt cochlear implant. Nowadays they need at least an operating room with good conditions, a speech therapist, an audiologist and a psychotherapist composing a small team to adapt a cochlear implant.

At the same time, there is the needing of follow-up hearing loss cases in order to integrate them in society, as also the evaluation and identification of other risk factors, trying to screen other deafness genes and infections.

6.2.2 G6PD STP screening

Deficiency of G6PD frequently associated to hemolysis and neonatal jaundice has a higher prevalence in this country, even without association to hearing loss, should be early identified to protect child from detrimental effect of hyperbilirubinemia during neonatal period

6.2.3 Promote health education

Promote health education including early diagnosis of infections and other conditions that may trigger hearing loss.

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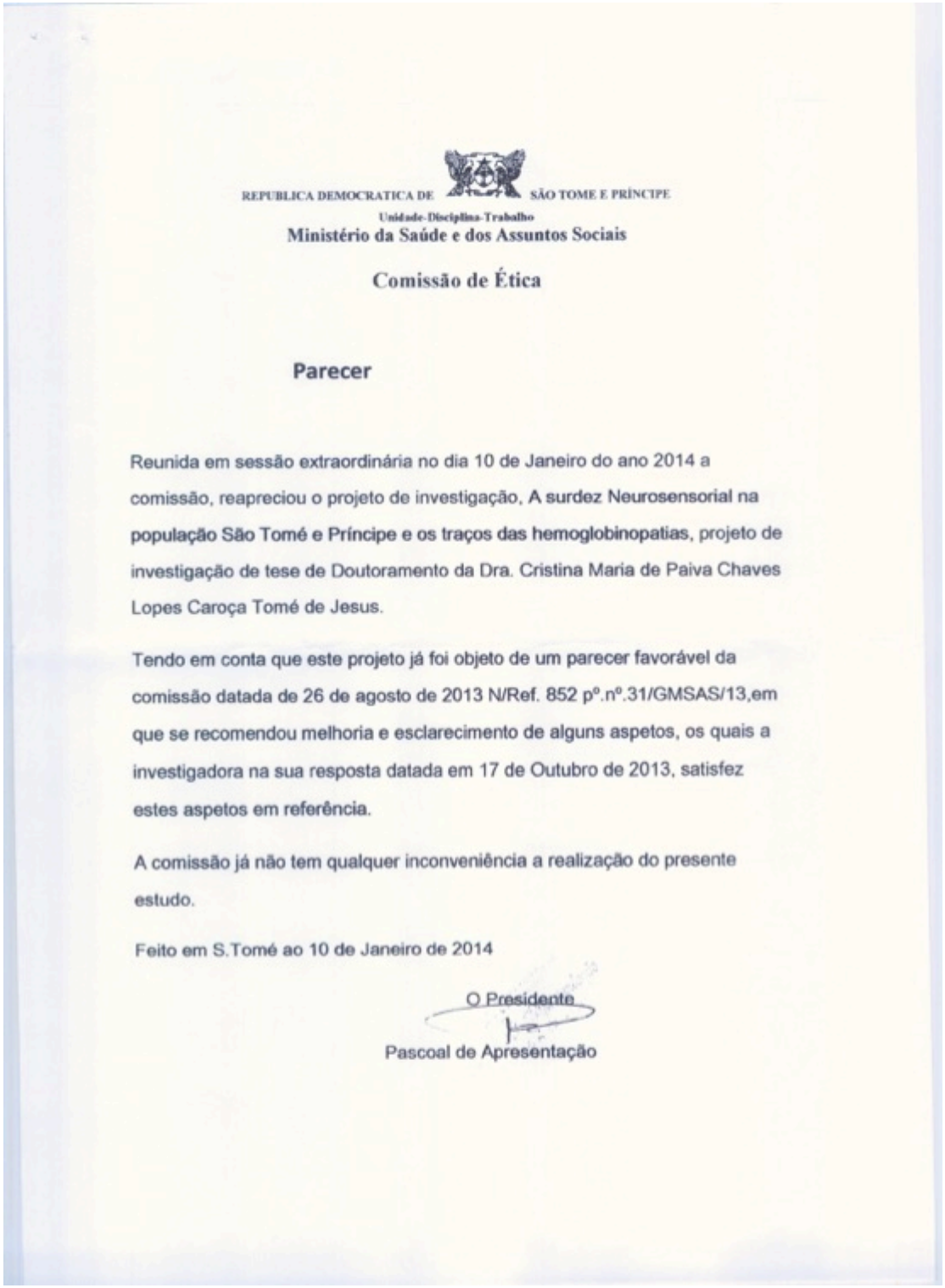
Appendix

Appendix 1



REPUBLICA DEMOCRATICA DE SÃO TOME E PRÍNCIPE

Unidade-Disciplina-Trabalho

Ministério da Saúde e dos Assuntos Sociais

Comissão de Ética

Parecer

Reunida em sessão extraordinária no dia 10 de Janeiro do ano 2014 a comissão, reapreciou o projeto de investigação, **A surdez Neurosensorial na população São Tomé e Príncipe e os traços das hemoglobinopatias**, projeto de investigação de tese de Doutoramento da Dra. Cristina Maria de Paiva Chaves Lopes Caroça Tomé de Jesus.

Tendo em conta que este projeto já foi objeto de um parecer favorável da comissão datada de 26 de agosto de 2013 N/Ref. 852 pº.nº.31/GMSAS/13,em que se recomendou melhoria e esclarecimento de alguns aspetos, os quais a investigadora na sua resposta datada em 17 de Outubro de 2013, satisfez estes aspetos em referência.

A comissão já não tem qualquer inconveniência a realização do presente estudo.

Feito em S.Tomé ao 10 de Janeiro de 2014

O Presidente

Pascoal de Apresentação

Appendix 2

Decisão final sobre o projeto "O que poderá estar associado à surdez neurosensorial em São Tomé e Príncipe?"

A Comissão de Ética da NMS|FCM-UNL (CEFCM) decidiu, por unanimidade, aprovar o projeto de investigação intitulado " O que poderá estar associado à surdez neurosensorial em São Tomé e Príncipe?" (nº02/2014/CEFCM), submetido pela Dra. Cristina Caroça.

Lisboa, 21 de Outubro de 2015

O Presidente da Comissão de Ética,

(Prof. Doutor Diogo Pais)

TO WHOM IT MAY CONCERN

The Ethics Research Committee NMS|FCM-UNL (CEFCM) has unanimously approved the Project entitled "O que poderá estar associado à surdez neurosensorial em São Tomé e Príncipe?" (nr.02/2014/CEFCM), submitted by Dr. Cristina Caroça.

Lisbon, October 21th, 2015

The Chairman of the Ethics Research Committee,

(Diogo Pais, MD, PhD)

Campo dos Mártires da Pátria, 130 | 1169-056 Lisboa | Portugal | **T.** +351 218 803 000 – Ext. 20447 E-mail cefcm@fcm.unl.pt

Appendix 3

Termo de consentimento livre e informado

"O que poderá estar associado à surdez neurosensorial na População de São Tomé e Príncipe?"

O estudo tem por objectivo a elaboração de uma tese para obtenção do grau de Doutor em Otorrinolaringologia, a decorrer na Universidade Nova de Lisboa, sob a orientação do Prof. Doutor João Paço do Centro Clínico e Universitário de Otorrinolaringologia do Hospital CUF Infante Santo – FCML (co-orientadoras: Dra Susana Nunes Silva; Prof. Helena Caria).

Pesquisa: Estabelecer uma relação entre traços de hemoglobinopatias e surdez neurosensorial em São Tomé e Príncipe.

Investigadora: Cristina Paiva Chaves Caroça, Otorrinolaringologista do Centro Clínico e Universitário de Otorrinolaringologia do Hospital CUF Infante Santo - FCML

Objectivo: Recolher os dados de uma amostra de população de São Tomé e Príncipe, com indivíduos dos 2 aos 35 anos.

São realizados testes Audiológicos: Audiograma Tonal e/ou Potenciais Evocados Auditivos. Simultaneamente ao estudo auditivo, será realizada colheita de 4 gotas de sangue para estudo de traço de hemoglobinopatia, viral e genético.

Os testes audiológicos consistem na apresentação de sons, através de auscultadores e/ou elétrodos, que o indivíduo deverá assinalar, ou serão registadas as ondas eléctricas correspondentes à resposta ao estímulo. Os testes audiológicos não são invasivos, não requerem a administração de qualquer fármaco, não provocam qualquer dor ou desconforto ao indivíduo avaliado.

A colheita de sangue para papel de filtro será de pequena quantidade, mas poderá ser dolorosa.

Eu, abaixo assinado, declaro que tomei conhecimento dos objectivos do trabalho de investigação intitulado **"O que poderá estar associado à surdez neurosensorial na População de São Tomé e Príncipe?"** realizado por Cristina Paiva Chaves Caroça, que frequenta o Doutoramento em Otorrinolaringologia da Universidade Nova de Lisboa. Acrescento que estou informado de que todos os dados recolhidos serão tratados de modo estritamente confidencial, aceitando, por isso, fazer parte da amostra do referido trabalho.

□ Indivíduo <18 anos:

Após ter sido devidamente informado, _____, pai/mãe de _____, declaro que tomei conhecimento dos objectivos do estudo e que aceito que o(a) meu (minha) filho(a) colabore no mesmo.

□ Indivíduo ≥18 anos:

Após ter sido devidamente informado, declaro que tomei conhecimento dos objectivos do estudo e que aceito a minha colaboração no mesmo.

_____(Indivíduo / Representante Legal)

_____(Investigadora / Enfermeiro)

_____, ____ de _____ de 201__

Appendix 4

Questionário São Tomé e Príncipe **Nº**_____

Nome: _____**Nome de casa:** _____
Idade: _____**Sexo:** □ F□ M **Data Nascimento:**_____**Contacto**_____
Distrito:_____**Subdistrito:**_____**Localidade:**_____
□ **Estuda Escola:**_____**Ano Escolar:**_____
☐ **Trabalha Profissão**_____**Habilitações**_____
Fala ☐ Sim ☐ Não ☐ Pouco **História Famílar Surdez** ☐ Sim ☐ Não ☐ NS/NR
Cosanguinidade ☐ Sim ☐ Não ☐ NS/NR

Gravidez
Mãe Gesta (Gravidezes)_____Para (Partos)_____Idade da mãe no Parto_____
Gravidez ☐ Pré-termo (<36sem) ☐ Termo (38sem) ☐ Pós –termo (>40sem)
Local ☐ Casa ☐ Hospital Parto Eutócito ☐ Sim ☐ Não Apgar _____
TORSH: □ Rubéola □ Toxoplasmose □ Herpes □ Citomegalovirus □ Outras
Complicações Periparto: ☐ Icterícia Neonatal ☐ Prematuridade ☐ Anoxia Cerebral

Malária/Paludismo Profilaxia na gravidez ☐ Sim (Fansidar ou Quinino) ☐ Não ☐ NS/NR
Profilaxia Paludismo periparto ☐ Sim (Fansidar ou Quinino) ☐ Não ☐ NS/NR
Teve Paludismo?_____Idade_____Quanto tempo ☐ <6 meses ☐ >6 meses
Internamento por paludismo ☐ Sim ☐ Não ☐ NS/NR Idade _____
Outros internamentos:_____Idade: _____
Terapêutica anti-palúdica efectuada: ☐ Artenusato/Amodiaquina ☐ Artemeter/Lumefantrine
☐ Quinino Oral ☐ Quinino EV ☐ NS/NR
Quantas vezes teve Paludismo:_____

História Pessoal
Otites de repetição: ☐Sim ☐Não ☐NS/NR Otorreia ☐Sim ☐Não ☐NS/NR
Fístulas Préauriculares ☐ Sim (Unilateral_____/Bilateral) ☐ Não
Traumatismo Craneano ☐ Sim ☐ Não ☐ NS/NR
Acha que ouve mal? ☐ Sim (OD/OE) ☐ Não ☐ NS/NR

Observação
CAE OD☐ Normal ☐ Cerumen ☐ Otorreia ☐ Otorragia ☐ Otomicose ☐ Exostoses
CAE OE☐ Normal ☐ Cerumen ☐ Otorreia ☐ Otorragia ☐ Otomicose ☐ Exostoses
Timpano OD ☐ Normal ☐ Não visível ☐ OMD ☐ OMC ☐ OMC Col ☐ Disf TE
Timpano OE ☐ Normal ☐ Não visível ☐ OMD ☐ OMC ☐ OMC Col ☐ Disf TE

Exames Audiométricos – Impedanciometria
OD Tipo ☐ A ☐ B ☐ C1 ☐ C2 OE Tipo ☐ A ☐ B ☐ C1 ☐ C2
OD Reflexos ☐ Presentes ☐ Ausentes OEReflexos ☐ Presentes ☐ Ausentes

Exames Audiométricos – Audiograma Tonal

OD	250	500	1000	2000		4000		Média
VO								
VA								

OE	250	500	1000	2000		4000		Média
VO								
VA								

Exames Audiométricos – Potenciais Evocados Auditivos (PEA)
Limiar OD_____Limiar OE_____

Destino
☐ Alta ☐ C. ORL ☐ Cirurgia ☐ C. Aud ☐ PEA ☐ Próteses ☐ Terapia Fala ☐ Lingua Gestual