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Title: Baseline prevalence and incidence and risk factors for new-onset drug induced hearing loss in adults receiving drug-resistant tuberculosis (DR-TB) treatment in Khayelitsha, South Africa

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Part 0: Preamble

DECLARATION

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

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Abstract

Introduction

Treatment for drug-resistant tuberculosis (DR-TB) is longer and associated with more significant side-effects than drug susceptible TB. Second line injectable therapy using kanamycin, amikacin or capreomycin is associated with irreversible hearing loss. There is a scarcity of literature regarding the frequency of hearing loss as well as associated risk factors, particularly with long term use. This study aimed to determine the incidence and risk factors for hearing loss among patients receiving second line injectable drugs.

Method

This was a retrospective cohort study that was conducted with patients from 11 primary health care clinics in Khayelitsha, Cape Town. All adult patients aged 18 years and above with a diagnosis of MDR-TB, XDR-TB or any other form of DR-TB receiving intramuscular amikacin or kanamycin or capreomycin as part of the DR-TB regimen were included. Subjects needed to have initiated DR-TB treatment between June 1, 2009 and Dec 31, 2010. Hearing was assessed at baseline and then monthly for a minimum period of 6 months. Hearing was measured objectively in a sound proof booth by an audiologist trained field worker using a pure tone audiometer (Interacoustics AS608) and recorded on a standardized audiometry form. Hearing was tested at the following frequencies: 250Hz, 500Hz, 1000Hz, 2000Hz, 4000Hz and 8000Hz. The exposure variables in this study included: age at DR-TB diagnosis, sex, starting aminoglycoside dose, previous aminoglycoside use, HIV status, CD4 count and NRTI use. The outcome variable in this study was hearing loss. Hearing loss was defined as a hearing threshold of >20 decibels at any test frequency in at least both ears. Hearing was measured by averaging the hearing thresholds at each visit for each ear separately from 250 Hz to 2000Hz for low frequency loss and from 4000 Hz to 8000 Hz for high frequency loss. Logistic regression was used to assess the bivariate and multivariate associations between hearing loss and explanatory variables.

Results

From June 1, 2009 to December 2010, 238 subjects meeting eligibility criteria started DR-TB treatment. Of these, 131 patients had at least one valid audiogram within 6 weeks of treatment initiation, and 47 (35.9%) patients had baseline hearing loss. In multivariable analysis, older age and HIV infection with CD4<100 cells/ μ l increased odds of baseline hearing loss. One hundred and nine patients had a second audiogram to assess deterioration. Of these, 37 (33.9%) patients had deterioration in hearing that was primarily mild but 9 patients (8.3%) had severe bilateral hearing loss. In multivariable analysis, older age and baseline hearing loss predicted deterioration of hearing.

Conclusion

Hearing loss at baseline and during treatment occurred frequently and was often severe. Increasing age and HIV infection with low CD4 count were associated with baseline hearing loss. Baseline hearing loss and older age predicted deterioration of hearing.

Key words: hearing loss, hearing impairment, ototoxicity, drug resistant tuberculosis, HIV/AIDS, South Africa

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University of Cape Town

PART A: Research Protocol

Title: Baseline prevalence and incidence and risk factors for new-onset drug induced hearing loss in adults receiving drug resistant tuberculosis (DR-TB) treatment in Khayelitsha, South Africa.

1. Introduction

1.1 Background

South Africa has a high burden of tuberculosis, and according to the 2012 World Health Organization (WHO) TB Global Report, it was ranked third among the 22 high burden countries, with a reported incidence of between 0.40 and 0.59 million cases in 2011.¹ It also has a high burden of multidrug-resistant tuberculosis (MDR-TB), and data from the WHO, ranked South Africa second amongst the 27 high burden MDR-TB countries with 10,085 reported cases in 2009.²

MDR-TB and XDR-TB pose a threat to effective TB control due to the difficulties in diagnosis, the requirement for prolonged chemotherapy for up to two years, increased cost (up to 100 times costlier than drug susceptible TB) and the use of more toxic second line drugs that are associated with increased adverse effects.²

Reported studies have shown that at least 60% of patients on MDR-TB therapy will experience adverse events.^{3,4} The most serious side effects are nephrotoxicity and ototoxicity which are caused by injectable second line agents such as amikacin, kanamycin and capreomycin,⁵ that are administered intramuscularly for a minimum period of 6 months as per the National Tuberculosis guidelines.⁶ Nephrotoxicity is reversible with discontinuation of therapy whilst ototoxicity is not. Ototoxicity comprises of damage to hearing and/or balance. This study will focus on the hearing loss component of ototoxicity.

The prevalence of hearing loss amongst DR-TB patients ranges from as low as 6%⁷ up to 50%.^{3,8,9,10,11} Variation in hearing loss data may be due to factors such as different patient demographics, dose of injectable, duration of injectable use, type of injectable used, method of measuring hearing loss or comorbid conditions.¹²

1.2 Rationale

Risk factors that are known to predict hearing loss include; older age, duration of injectable therapy, total cumulative dose,¹³ size of aminoglycoside dose, history of aminoglycoside use, noise exposure, presence of mitochondrial DNA mutations,⁹ HIV co-infection, opportunistic infections and use of nucleoside reverse transcriptase inhibitors (NRTIs).¹⁴ Despite this evidence, inconsistencies of data pertaining to risk factors for hearing loss remain.

Anecdotally, the prevalence of hearing loss due to second line injectable use in Khayelitsha is quite high and is therefore cause for concern. There is a scarcity of literature on aminoglycoside induced hearing loss, in particular, risk factors associated with this adverse effect in long term DR-TB studies.¹² Most of the literature on hearing loss is based on short term aminoglycoside use with limited studies assessing hearing loss during long term TB therapy. Furthermore, most of the studies have been conducted in developed countries where patient profiles would be expected to be different to those in a developing country. Hence, generalizability to the South Africa context may be a challenge. It is against this dearth of literature on hearing loss that this study will be conducted.

1.3 Aims/objectives of the research

The overall aim of this study is to determine the baseline prevalence of initial hearing loss and incidence of hearing deterioration during treatment as well as the risk factors associated with aminoglycoside/capreomycin induced hearing loss in adult patients with DR-TB.

Other secondary objectives of the study include:

- To measure the type and severity of hearing loss at start of treatment (baseline hearing loss)
- To measure the type and severity of deteriorating hearing loss during DR-TB treatment
- To determine overall prevalence of worsening hearing amongst those with baseline hearing loss
- To determine risk factors associated with baseline hearing loss and deterioration of hearing during DR-TB treatment

This study will focus on the following measurable risk factors: age at DR-TB diagnosis, sex, starting aminoglycoside dose, prior aminoglycoside use, HIV status, CD4 count, anti-retroviral therapy use, in particular use of nucleoside reverse transcriptase inhibitors (NRTIs). Injectable duration and total aminoglycoside dose will be collected but not included in the final analysis as the current dataset does not allow for us to determine the timing of hearing loss. This is because most patients had irregular audiograms with only a few individuals having at least 6 audiograms; thereby limiting our ability to determine timing of hearing loss with the current dataset.

2. Method

2.1 Study design and study setting

This is a retrospective cohort study that will be conducted with patients attending 11 primary health care clinics in Khayelitsha, Cape Town. This is a peri-urban sub-district within the Cape Metro area that has one of the highest TB incidence rates in the country with 60% to 70% of TB patients also co-infected with HIV.¹⁵ The Khayelitsha decentralized DR-TB programme was established in 2007 in response to the high DR-TB burden and lack of capacity of the local TB hospital to handle all cases resulting in treatment delays.¹⁵ Hence all DR-TB cases that do not have severe disease requiring hospitalization receive outpatient care and are followed up closely by treatment counselors.

2.2 Study Participants

All adult patients aged 18 years and above with a diagnosis of MDR-TB, XDR-TB or any other form of DR-TB who are receiving intramuscular amikacin, kanamycin or capreomycin as part of the DR-TB regimen will be included into the study. Subjects need to have initiated DR-TB treatment between June 1, 2009 and Dec 31, 2010. Our cohort period will begin in June 2009 and not earlier because audiometry testing only became standard of care in Khayelitsha from 2009.

2.3 Study Procedures

There will no formal recruitment process for study participants as collection of audiometry data has been standard of care since June 2009.

Baseline

At baseline, all patients who meet the inclusion criteria will be entered into the study. Upon treatment initiation, all demographic and clinical information is collected in a standardized form by the attending physician as part of standard of care (See Appendix 1 for standardized form). Thereafter, the patient will receive a referral to the audiometry testing centre based at Ubuntu Clinic (one of the 11 Khayelitsha primary health care clinics).

Hearing is measured objectively by an audiologist trained field worker using a pure tone audiometer (Interacoustics AS608) and recorded on a standardized audiometry form (See Appendix 2 for copy of audiometry form). This will be conducted in a sound proof booth at the following frequencies: 250Hz, 500Hz, 1000Hz, 2000Hz, 4000Hz and 8000Hz. Prior to doing the hearing assesment, the ear canal will be examined using an otoscope for any signs of obstruction such as inflammation, growths, foreign bodies or excessive cerumen because obstructions impair the transmission of sound.¹⁶

The picture below is an illustration of the sound proof booth that is used to perform audiometry testing at Ubuntu Clinic in Khayelitsha.



The reliability of the audiometry measurements will be assessed using test retest reliability at every frequency level on every audiogram and inter-rater reliability on a sub set of audiograms.

Hence, a valid audiogram will be defined as one that does not have any ear(s) obstructed and one that is classified as having good reliability.

Follow-up Visit

Patients in Khayelitsha are routinely followed up on a monthly basis until 2 months after the end of the injectable phase, typically a 6 to 8 month period. During the follow-up visits, ascertainment of hearing loss is measured quantitatively using audiometry. All audiometry data collected at follow-up visits is compared with baseline data (if available) to determine whether deterioration of hearing has occurred (See appendix 2 for copy of audiometry form).

2.4 Exposure and outcome variables

The exposure variables in this study include: age at DR-TB diagnosis, sex, starting aminoglycoside dose, previous history of aminoglycoside use, HIV status, CD4 count and NRTI use.

The outcome variable in this study is hearing loss. Hearing loss will be defined as a hearing threshold of >20 decibels at any test frequency in at least both ears.

Hearing loss will be categorized into three frequencies: i) low frequency hearing loss (250 Hz to 2000Hz) ; ii) high frequency hearing loss (4000Hz and 8000 Hz) and; iii) mixed hearing loss-hearing loss occurring in both low and high frequencies.

For low frequency hearing loss, hearing sensitivity will be measured by averaging the hearing thresholds across 250 Hz, 500Hz, 1000Hz and 2000Hz for each ear separately at each visit. For high frequency hearing loss, hearing sensitivity will be measured by averaging hearing thresholds across 4000 Hz and 8000 Hz for each ear separately at each visit. This method has been shown to be effective in assessing changes in hearing loss.¹⁷

The criteria for determining the presence of hearing loss and assessment of severity of hearing loss as defined by Gelfand (2009) will be used.¹⁸ However, we will modify the criteria slightly in order to have fewer categories. The criteria used will be as follows: i) Normal hearing: Hearing threshold of ≤ 20 decibels at either the low or high frequency test frequencies; ii) Mild hearing loss: Hearing threshold of between 21 and 40 decibels at either the low or high test frequencies;

iii) Moderate to severe hearing loss: Hearing threshold of between 41 and 70 decibels at either low or high test frequencies and iv) Severe to profound hearing loss: Hearing threshold of more than 70 decibels across the low or high frequencies. Hearing loss severity will be assessed using the WHO better ear criteria for grading hearing impairment.¹⁹ This stipulates that severity is categorized according to the ear with less severe hearing loss.

2.5 Assessment of hearing loss

Hearing severity will be measured semi-qualitatively. This will consist of plotting each patient's audiogram using calculated averages of hearing thresholds across low and high frequencies for each ear separately. This will be restricted to valid audiograms only. Hence, each subject will have four line graphs, for low and high frequency and for each ear separately. The line graphs will be assessed qualitatively by two independent assessors to determine whether hearing loss is present at baseline and whether deterioration has occurred with treatment. All discrepancies will be resolved by consensus. A qualitative assessment (without the use of statistical software) will be used because of the variation seen in audiometry readings making it difficult to do a quantitative assessment (See appendix 3 for examples of audiometry line graphs).

Audiograms for assessment of deterioration of hearing

The first ever audiogram for each patient will be classified relative to the start of TB treatment:

- i) Pre-treatment audiograms will be defined as audiograms taken within 10 days of initiating DR-TB treatment, prior to significant drug exposure
- ii) Early treatment audiograms will be defined as audiograms taken within 1.5 months of starting DR-TB treatment. Only patients with a pre-treatment audiogram or an early treatment audiogram will have deterioration of hearing measured. Hearing loss that occurs within 1.5 months of treatment start will be referred to as baseline hearing loss.

2.6 Other data sources

Electronic databases will be used to collect data on the following variables: i) Demographic variables- age, sex and weight; ii) Clinical variables- HIV status, CD4 count, type of DR-TB, previous TB history; iii) Treatment variables- type of aminoglycoside, starting injectable dose and injection duration, previous aminoglycoside use, NRTI use and ; iv) Outcome - hearing loss.

3 Statistical Analysis

The baseline characteristics of the cohort will be described using medians and inter quartile ranges for variables with a skew distribution and means and standard deviations for the normally distributed data. Wilcoxon rank-sum test and t tests will be used to measure differences between numerical variables where appropriate. The categorical variables will be analyzed using Fishers Exact Test and Chi-squared test where appropriate. Percentages will be used to describe the frequency, type and severity of hearing loss at start and during treatment.

Bivariate associations between hearing loss and explanatory variables will be assessed using logistic regression. Explanatory variables for inclusion into the model will be based on apriori assumptions derived from previous studies. Multivariable logistic models will used to test the association between hearing loss and the explanatory variables. All statistical analysis will be conducted using STATA version 11 (StataCorp, College Station, Texas).

4 Ethics

4.1 Privacy and Confidentiality

The patient's identity will be kept confidential by de-identifying the patient using unique patient identification numbers when data is transferred to the electronic database. The database will be password protected and only the investigators in the study will have access to this information. All information in hard copy will be stored in a locked cupboard in the Médecins Sans Frontières office in Khayelitsha.

4.2 Potential Risks and benefits

The potential risks in this study are minimal since it is an observational study and all procedures and data collection are part of standard of care. However, the results of the audiometry testing classify a person as having hearing loss and this may be considered a potential risk to the patient with regards to factors such as job security or social life. The patients are routinely referred for further management at Tygerberg Hospital if they experience moderate severe or severe profound hearing loss.

In terms of benefiting the wider community, the results of the research will enable health care providers to better understand the risk factors that are associated with hearing loss. This will enable them to implement early interventions in high risk patients e.g. more frequent monitoring, lower injectable dosage, or shorter duration of injectable therapy.

4.3 Feedback and dissemination of results

The results of this study will be disseminated to all staff members at Médecins Sans Frontières and medical personnel at the Khayelitsha clinics and Cape Town City Health. Wider dissemination to the scientific community will be in the form of a peer reviewed scientific paper.

4.4 Reimbursement for Participation and patient informed consent

There will be no reimbursement of participants because all procedures that will be carried out are all part of standard of care.

4.5 Patient Informed consent

This is not applicable in this study since the patient is receiving standard of care.

4.6 Ethical approval

Ethical approval will be sought from the University of Cape Town and Cape Town City Health (Appendix 4- Copy of ethical approval from University of Cape Town and City Health).

4.6 Budget

No funding was required for this project as it is sponsored by Médecins Sans Frontières.

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University of Cape Town

PART B: Literature review

Title: Baseline prevalence and incidence and risk factors for new-onset drug induced hearing loss in adults receiving drug resistant tuberculosis (DR-TB) treatment in Khayelitsha, South Africa.

1. Aims/objectives of the literature review

The overall aim of this literature review is to describe the frequency and risk factors of aminoglycoside/capreomycin induced hearing loss in adult patients with drug resistant tuberculosis (DR-TB).

Other secondary aims or objectives of the literature review include:

- To briefly describe the prevalence of pulmonary tuberculosis (TB) and DR-TB in South Africa
- To describe the mechanism of hearing loss caused by aminoglycosides or capreomycin
- To briefly describe the pharmacokinetics of aminoglycosides and capreomycin
- To describe the incidence /prevalence of aminoglycoside/capreomycin induced hearing loss in adults
- To describe the type of hearing loss

2. Search strategy

The literature search included all English language articles published until December 2012. Studies were primarily identified using key word searches of electronic databases such as Pubmed, Ebsco Host, Science Direct and Google Scholar. A few studies were identified from reference lists of published articles. The key words used to identify articles included: hearing loss, hearing impairment, ototoxicity, aminoglycosides, drug resistant tuberculosis, HIV/AIDS. Studies were included if they comprised of adult patients receiving aminoglycosides or capreomycin for short term use (5-7 days) or long term use (>1 month). There was no restriction on study design.

3. Background

South Africa is one of the highest tuberculosis (TB) burden countries in sub-Saharan Africa. According to the 2012 World Health Organization (WHO) Global TB report, South Africa was ranked third among the top five high TB burden countries, with

incident TB cases of between 0.4 million and 0.5 million and corresponding incident rate of 993 per 100,000.¹ South Africa also has a high burden of drug resistant tuberculosis (DR-TB). There has been an upward trend in the number of laboratory confirmed DR-TB cases in South Africa since 2006, possibly due to increased case detection and growth in the HIV epidemic.² In 2006, there were 464 laboratory confirmed MDR-TB cases² compared to 10,085 MDR-TB cases in 2011.¹ The 2011 estimates represented the second highest case notification amongst the 27 high burden MDR-TB countries.¹ There is also a high burden of extensively drug-resistant tuberculosis (XDR-TB) with a total of 573 XDR-TB cases reported in 2008, 10.5% of the MDR-TB burden.³

DR-TB poses a threat to effective TB control due to the difficulties in diagnosis, the requirement for prolonged chemotherapy for up to two years, increased cost (up to 100 times higher) and the use of more toxic second line drugs that may be associated with increased adverse effects.³ In addition, there are delays with DR-TB diagnosis, particularly in places where GeneXpert has not been rolled out and culture is still being used to detect DR-TB. Another problem with diagnosis of DR-TB in South Africa is that drug susceptibility testing is only done in patients who have failed first line treatment, and by time a diagnosis has been made, some patients may have already died.

Studies have shown that at least 60% of patients on MDR-TB chemotherapy will experience an adverse effect.^{4,5,6} Nephrotoxicity and ototoxicity are the two major dose-related adverse effects associated with aminoglycosides and capreomycin.⁷ Nephrotoxicity is reversible upon discontinuation of drug therapy whilst ototoxicity is irreversible and is defined as damage to hearing and/or balance functions of the ear.⁷ This literature review will be focusing primarily on the hearing loss component. The 2011 National South African guidelines for DR-TB that are based on the WHO guidelines recommend amikacin / kanamycin as the aminoglycosides of choice for the treatment of MDR-TB and capreomycin, a polypeptide for XDR –TB treatment.⁸

3.0 Pharmacokinetics and therapeutic dose of aminoglycosides and capreomycin

Amikacin and kanamycin are completely absorbed from IM injection sites and well distributed in extracellular fluids and body tissues.⁹ Capreomycin is also poorly absorbed orally and is administered intramuscularly. Excretion of these drugs is primarily through the renal route (70 to 95%) of dose, and accumulation occurs in renal impairment.⁹ The adult therapeutic dose of amikacin/kanamycin in DR-TB treatment is 15mg/kg to a maximum of 1g daily.⁹ The capreomycin dose is 750-1000mg/day to a maximum of 20mg/kg/day.⁹ Ototoxicity is a dose related side effect and will occur at the higher end of therapeutic doses or if the therapeutic doses are exceeded.

3.1 Mechanism of ototoxicity

Amikacin, kanamycin and capreomycin are chiefly cochleotoxic.¹⁰ The ototoxicity of aminoglycosides occurs mainly through the loss of cochlear or vestibular sensory cells (hair cells) or both.¹¹ The irreversible destruction of sensory cells in the cochlea leads to permanent hearing and begins with the outer hair cells at the base of the cochlea (responsible for high frequency sound) and then spreads to the apex (responsible for low frequency sound).¹¹ Hence; high frequency hearing loss generally occurs before low frequency hearing loss. Initially, it was thought that ototoxicity occurred through accumulation of aminoglycosides in the peri-lymph in the inner ear. It is now clear that aminoglycosides do not cause hearing loss through this mechanism, since pharmacokinetic studies have shown that the inner ear concentrations do not exceed plasma concentrations.^{11,12} Cumulative evidence has now established that reactive oxygen species (free radicals) are the mechanism behind aminoglycoside hearing impairment.^{11,12} It is postulated that aminoglycosides cause an imbalance in the inner ear through excessive formation of free radicals which triggers cell death pathways leading to cell apoptosis/ necrosis of sensory cells in the cochlea, which leads to permanent hearing impairment.¹¹

4. Incidence and type of hearing loss

The literature on the reported incidence of adult hearing loss due to aminoglycosides or capreomycin varies greatly. In one review, the reported incidence of hearing loss ranges

from a few percent to 33%,¹² whilst in another systematic review of 35 studies, hearing loss was reported to be as low as 10% in some studies, and approaching or exceeding 50% in other studies.¹³ Yet another systematic review of 15 studies, reported a hearing loss incidence ranging from 0% to 55%.¹⁴ In short term use (5-7 days) of aminoglycosides, the reported incidence of hearing loss is as high as 22%.¹⁵

The variation in hearing loss observed across studies is due to factors such as sensitivity of the testing methodology, study methodology, patient demographics, previous TB treatment, drugs used, dosages used, duration of aminoglycoside treatment or co-morbid diseases.¹³

Despite the variation in hearing loss incidence in the literature, there is consistency regarding the frequency range (Hz) at which hearing loss presents initially, with data from a systematic review of DR-TB patients reporting high frequency hearing loss occurring more frequently.¹⁴

5. Risk factors of hearing loss

The first aminoglycoside to be developed in the 1940's was streptomycin and it was isolated from *Streptomyces griseus* by Waksman and co-workers.¹² Subsequently, other aminoglycosides such as amikacin and kanamycin as well as capreomycin were developed. The ototoxicity profile of aminoglycosides has been well described in earlier studies describing short term use of these drugs for bacterial infections, but studies on long term use such as DR-TB are lacking. The risk factors that have been associated with hearing loss include older age, duration of aminoglycoside/capreomycin therapy, dose, prior exposure to aminoglycosides, noise exposure, specific mitochondrial mutations,⁷ HIV positive status, opportunistic infections and use of ototoxic medicines.¹⁶ This literature review will focus on the following risk factors: age, duration of aminoglycoside therapy, aminoglycoside dose, previous aminoglycoside use, genetic susceptibility, noise exposure, previous TB disease, HIV status and opportunistic infections and use of nucleoside reverse transcriptase inhibitors (NRTIs).

Table of studies of risk factors (age, duration of injectable and aminoglycoside dose)

Year	Author	Title and journal
1983	Moore et al	Risk factors for the development of auditory toxicity in patients receiving aminoglycosides. <i>J Infect Dis.</i> 1984; 149 (1): 23-30.
1987	Gatell et al	Univariate and multivariate analyses of risk factors predisposing to auditory toxicity in patients receiving aminoglycosides. <i>Antimicrob Agents Chemother.</i> 1987; 31(9): 1383-1387.
2004	Peloquin et al	Aminoglycoside Toxicity: Daily versus Thrice-Weekly Dosing for Treatment of Mycobacterial Diseases. <i>Clin Infect Dis.</i> 2004; 38: 1538-1544.
2002	De Jager et al	Hearing Loss and nephrotoxicity in long-term aminoglycoside treatment in patients with tuberculosis. <i>Int J Tuberc Lung Dis.</i> 2002; 6(7): 622-627.
2012	De Vasconcelos	Audiometric evaluation of patients treated for pulmonary tuberculosis. <i>J Bras Pneumol.</i> 2012; 38(1): 81-87.
2011	Sturdy et al	Multidrug-resistant tuberculosis (MDR-TB) treatment in the UK: a study of injectable use and toxicity in practice. <i>J Antimicrob Chemother.</i> 2011; 66 (8):1815-1820.

5.1 Age, duration of injectable therapy and aminoglycoside dose

Older age is considered a risk factor for hearing loss as it is associated with a decrease in hair cells in the cochlea or a reduction in endogenous protective mechanisms such as antioxidants; thereby exposure to ototoxic drugs increases one's susceptibility to this adverse event.¹² In addition, prolonged exposure to aminoglycosides (duration of therapy) and higher cumulative doses have a direct additive effect on the damage of hair cells of the cochlea, consequently leading to irreversible hearing loss.¹²

Despite these biological mechanisms explaining the effects of these variables on hearing loss, studies on both short term and long term use of aminoglycosides have reported conflicting data regarding age, duration of therapy and aminoglycoside dose.

Moore *et al.*¹⁵ determined the risk factors for auditory toxicity (hearing loss) using data from three randomized, double-blind trials in patients receiving either intravenous 2mg/kg of gentamicin/tobramycin or 8mg/kg of amikacin for bacterial infection. One hundred and thirty five patients were included if they had received at least nine doses of drug therapy. Hearing loss was defined as a decrease in consecutive audiograms of at least 15 decibels (dB) in auditory acuity at any frequency in the range of 1000-8000 Hz.¹⁵ The factors significantly associated with the development of hearing loss were total

aminoglycoside dose and duration of aminoglycoside therapy ($P < 0.05$) but age did not predict hearing loss.¹⁵

The findings by Moore *et al* conflict with another study of short term use of aminoglycosides. Gatell *et al.*¹⁷ randomized 187 patients to intravenous tobramycin or netilmycin 1.7mg/kg 8 hourly or amikacin 7.5 mg/kg 12 hourly for bacterial infection. Hearing loss was defined as a decrease in auditory threshold of at least 15 dB in at least two frequencies in the range of 250 to 8000Hz unilaterally or bilaterally. In the multivariate analysis, only increasing age was found to be significantly associated with auditory toxicity ($P < 0.00001$).¹⁷ Conversely the factors not significantly associated with auditory toxicity included duration of therapy and total aminoglycoside dose.¹⁷ The contrasting results observed in these two studies may be attributed to the different definitions of hearing loss used, type of aminoglycosides used and older patient population in the Gatell *et al* study. Furthermore, the duration of aminoglycoside therapy may also explain the differences. The duration of aminoglycoside therapy in patients with auditory toxicity was 7.9 and 9.1 days in the Gatell *et al* and Moore *et al* studies respectively.

A more recent study of aminoglycoside induced hearing loss in treatment of mycobacterial disease including MDR-TB had some findings consistent with the aforementioned studies. Peloquin *et al.*¹⁸ conducted a randomized trial of 87 adult patients based in US hospitals who received 25mg/kg three times weekly or 15mg/kg once daily of intravenous amikacin, streptomycin or kanamycin. Ototoxicity (hearing loss) was defined as ≥ 20 dB neurosensory hearing loss from baseline in either ear at any frequency from 250 to 8000Hz.¹⁸ Hearing loss occurred in 37% of the cohort and was significantly associated with increasing age, longer duration of aminoglycoside treatment, and greater total aminoglycoside dose received but not size of dose in mg/kg, dosing frequency or nephrotoxicity.¹⁸

In contrast to the study by Peloquin *et al*¹⁸, a retrospective study from the Netherlands failed to show any association between clinical or treatment risk factors and hearing loss.

De Jager *et al.*¹⁹ included 110 adults with a diagnosis of DR-TB and other mycobacteria infections who had received at least 14 days supply of kanamycin, amikacin or streptomycin. Hearing loss was defined as loss of 15 dB at two or more frequencies, or a minimum of 20 dB hearing loss of at least one frequency between 250 and 8000Hz.¹⁹ Older age, total duration of aminoglycoside treatment, total aminoglycoside dose and renal function were not associated with hearing loss. These contradicting results may be explained in part by the study design and the study population. This was a retrospective study and one of the limitations was the lack of baseline audiograms and variability in frequency of audiograms obtained, potentially underestimating the extent of hearing loss in this cohort. Furthermore, the study population was much younger with a mean age of 35.7 years compared to a median age of 49 years in the study by Peloquin *et al.*

Nonetheless, more recent retrospective studies of MDR-TB patients from Brazil and UK have consistently shown a relationship between demographic factors and hearing loss, in particular increasing age.^{20,21} The study from Brazil showed that increasing age almost trebled the odds of aminoglycoside induced hearing loss, (OR 2.8; 95%CI: 1.15-7.35).²⁰ The UK study also examined the relationship between injection duration and total aminoglycoside dose and hearing loss, but these variables failed to predict hearing loss.²¹ This may be explained by the premature discontinuation of aminoglycosides and adjustment of doses reported in a sub set of patients experiencing auditory toxicity.

From the reported literature, increasing age appears to be a more consistent risk factor for hearing loss in both short term and long term aminoglycoside use whilst injection duration and total aminoglycoside dose have mixed results and are less predictive. However, the comparison of the above studies is limited by the dearth of data on long term use of aminoglycosides in DR-TB, thereby necessitating the comparison of DR-TB studies with studies reporting on short term aminoglycoside use.

5.2 HIV infection

Hearing loss linked to HIV is classified as either, iatrogenic or non-iatrogenic. Non-iatrogenic causes may be varied, and may include factors such as the HIV infection as a

primary cause and opportunistic infections.²² On the other hand, iatrogenic causes are more specific and relate to effects of any ototoxic drugs such as nucleoside reverse transcriptase inhibitors (NRTIs) and anti-tuberculosis drugs.²²

Hearing changes have been identified as one of the presentations at any stage of HIV disease.¹⁶ However, there is a strong positive correlation between worsening hearing loss and progression of HIV disease. This suggests that the higher the viral load, the higher the frequency of HIV associated hearing loss. The exact mechanism of hearing loss due to HIV infection is unclear, but the HIV virus is considered to be a direct cause of hearing loss since it is neurotropic.¹⁶ There is heterogeneity of hearing loss manifestations in the HIV population which may be conductive, sensorineural or central. Sensorineural hearing loss, which is irreversible, is the most well documented form of hearing loss in this population and ranges from 20% to 50%, occurring more frequently at higher frequencies (4000-8000Hz) than at lower frequencies (<400Hz) with a greater tendency towards a bilateral gradual onset versus rapid onset.¹⁶ The hearing loss linked to HIV infection may be additive or even synergistic to that caused by aminoglycosides and capreomycin although these effects are unknown.¹⁶

There is a large body of evidence reporting on the frequency of HIV associated hearing loss. One extensive review of the literature, reports that the prevalence of HIV associated hearing loss was generally low in some studies but in a South African HIV positive cohort, this figure was as high as 23%.¹⁶ In another prospective study of 153 MDR-TB adults also conducted in South Africa, a hearing loss incidence of 39% amongst HIV positive subjects was observed.²³ This study also reported a more than threefold increased risk of hearing loss associated with HIV infection (OR 3.25; 95% CI: 1.65-6.37).²³

Table of references referring to antiretroviral (ART) and hearing loss

Year	Ref no.	Author	Title and journal
2000	24	Kakuda TN	Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. <i>Clinical Ther.</i> 2000; 22(6): 685-708.
2001	26	Simdon et al	Ototoxicity associated with use of nucleoside analog reverse transcriptase inhibitors: A report of 3 possible cases and review of the literature. <i>Clin Infect Dis.</i> 2001. 32: 1623-1627.
2002	27	Rey et al	Severe ototoxicity in a health care worker who received postexposure prophylaxis with stavudine, lamivudine and nevirapine after occupational exposure to HIV. <i>Clin Infect Dis.</i> 2002; 34 (3):418-419.
1997	28	Marra et al	Hearing loss and antiretroviral therapy in patients infected with HIV-1. <i>Arch Neurol.</i> 1997; 54(4): 407-410.
2006	29	Schouten et al	A prospective study of hearing changes after beginning zidovudine or didanosine in HIV-1 treatment naïve people. <i>BMC Infect Dis.</i> 2006; 6:28.

5.3 Anti-retroviral therapy (ART)

Anti-retroviral therapy (ART), in particular the nucleoside reverse transcriptase inhibitors (NRTIs) contribute to hearing loss in HIV infected people through mitochondrial toxicity.²⁴ NRTIs form the backbone of the antiretroviral programme in South Africa;²⁵ hence this is a potentially significant risk factor for hearing loss in this population. Most of the evidence supporting the association between NRTIs and hearing loss is based on case reports and cross-sectional studies. The major limitations of these study designs compared to prospective studies are that they provide weak evidence for causality, temporality cannot be ascertained and they are subject to selection biases. Moreover, the small sample size of case series makes it difficult to measure a relationship between the exposure and the outcome. Nevertheless, the findings from these study designs are still useful in helping us understand the relationship between NRTI use and hearing loss.

In one case series, 3 patients aged >45 years with a history of noise-induced hearing loss reported worsening hearing impairment confirmed by audiometry after initiation of NRTIs such as lamivudine (3TC), stavudine (d4T), zidovudine (AZT) or didanosine.²⁶ Another case report had similar findings when bilateral hearing loss was reported two weeks after a 23 year old female health care worker had received post exposure prophylaxis consisting of the NRTIs, d4T and 3TC.²⁷ A cross sectional study of 99 HIV

infected patients also reported NRTI use to be associated with hearing loss,²⁸ but this finding was not replicated in a prospective observational study of 33 HIV-1 treatment naïve adults exposed to AZT or didanosine after controlling for confounders such as noise, immune status and age.²⁹ The lack of association in the prospective study may be explained by the small sample size that was not sufficient to detect a significant difference. Alternatively, the results of the cross sectional study may be limited by the study design as temporality cannot be ascertained; hence hearing loss observed may have occurred before exposure to the NRTIs.

5.4 Previous aminoglycoside therapy

The aminoglycosides persist in the hair cells for extended periods (up to 6 months) after completion of injectable therapy.¹¹ This accumulation of aminoglycosides in addition to the pre-existent damage of hair cells of the inner ear may explain the association between previous aminoglycoside use and ototoxicity. There is limited literature reporting on the association between previous aminoglycoside therapy and hearing loss. One editorial states previous aminoglycoside therapy as a potential risk factor for hearing loss,⁷ whilst a prospective study reported no relationship between previous exposure to aminoglycosides and hearing loss.¹⁸

5.5 Genetic factors

Genetic susceptibility has been identified as a strong predictor of ototoxicity.^{7,11,30} A mutation in the mitochondrial DNA A1555G or C1494T in the 12S rRNA gene increases the susceptibility to aminoglycoside induced hearing loss.¹¹ It is hypothesized that this mutation causes the mitochondrial RNA gene in this region to resemble the bacterial ribosomal RNA gene; thereby leading to increased binding of aminoglycosides leading to altered protein synthesis of the mitochondria.¹¹ However; it has not been established whether altered protein synthesis occurs within cochlear cells *in vivo*, and why this toxicity is limited to the cochlea and not the vestibular system.¹¹

Data from three Chinese families reported that 15 members from those families with the A1555G genetic mutation developed hearing loss following exposure to aminoglycoside

therapy.¹¹ Data pertaining to South Africa is scarce with one reported study describing a family consisting of 18 members carrying the A1555G genetic mutation, and of these 11 of 18 members developed hearing loss following exposure to streptomycin during tuberculosis treatment.³¹

5.6 Previous Tuberculosis disease and noise exposure

There is only one reported study in the literature that describes the influence of previous TB treatment on hearing thresholds. Brits *et al.*³² conducted a retrospective study of gold miners in Johannesburg, South Africa to determine this relationship. The study reported that TB in itself was a risk factor for deterioration of hearing thresholds, particularly at higher frequencies (4000-8000Hz) compared to no history of TB disease. However, multiple episodes of TB compared to no history of TB disease was the most significant predictor of deterioration of hearing thresholds.³² This association was also attributed to the complex interaction between TB and TB regimens that may be potentially ototoxic as well as the associated risk profile including HIV infection.³²

Acoustic exposure to high intensity noise or prolonged noise may potentiate cochlear damage and subsequent administration of aminoglycosides is associated with synergistic potentiation of cochlear damage that may lead to hearing loss.³³ Brits *et al.* demonstrated that noise exposed individuals (drillers) had higher hearing thresholds compared to individuals not exposed to noise (administrative staff).³²

6. Gaps in the literature and study rationale

Anecdotally, the prevalence of hearing loss in Khayelitsha is quite high and this is cause for concern. The literature on hearing loss frequency is somewhat inconsistent and is primarily from developed countries whilst data from the South Africa context is limited. There is also a lack of standard definitions of hearing loss across the various studies making meaningful comparisons difficult. Furthermore, the risk profile of DR-TB patients in South Africa is likely to be vastly different to that of individuals from the developed world. This is primarily due to the high HIV burden in South Africa compared to more developed settings, with an estimated 5.7 million people infected with HIV by

2009.³⁴ TB/HIV co infection is high in South Africa, and data specific to Khayelitsha indicates an HIV/TB co infection rate of 60-70%.³⁵ These clinical differences in patient populations make generalizability to the South African context challenging.

In general, there is a scarcity of literature on risk factors for aminoglycoside induced hearing loss for long term use from both the developed and developing settings, in particular within South Africa. Furthermore, the available data is highly inconsistent, and some data is derived from low quality studies such as case series and case reports that provide weak evidence for causality.

In light of this, there is a need to conduct research to determine the prevalence and incidence as well as risk factors associated with aminoglycoside and capreomycin induced hearing loss in DR-TB patients within the South African context.

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University of Cape Town

Part C: Journal manuscript

1. Title: Baseline prevalence and incidence and risk factors for new-onset drug induced hearing loss in adults receiving drug resistant tuberculosis (DR-TB) treatment in Khayelitsha, South Africa.

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2. Abstract and key words

Introduction

Treatment for drug-resistant tuberculosis (DR-TB) is longer and associated with more significant side-effects than drug susceptible TB. Second line injectable therapy using kanamycin, amikacin or capreomycin is associated with irreversible hearing loss. There is a scarcity of literature regarding the frequency of hearing loss as well as associated risk factors, particularly with long term use. This study aimed to determine the incidence and risk factors for hearing loss among patients receiving second line injectable drugs.

Method

This was a retrospective cohort of all DR-TB patients aged 18 years and above initiated on second line injectable therapy in Khayelitsha clinics. DR-TB patients received a baseline audiometry screen followed by monthly screening using pure tone audiometry. Hearing loss was defined as a loss of hearing threshold of greater than 20 decibels at either high frequency (4000-8000Hz) or low frequency (250-2000Hz). Hearing loss was assessed at baseline and throughout treatment.

Results

From June 1, 2009 to December 2010, 238 subjects meeting eligibility criteria started DR-TB treatment. Of these, 131 patients had at least one valid audiogram within 6 weeks of treatment initiation, and 47 (35.9%) patients had baseline hearing loss. In multivariate analysis, older age and HIV infection with CD4<100 cells/ μ l increased odds of baseline hearing loss. One hundred and nine patients had a second audiogram to assess deterioration. Of these, 37 (33.9%) patients had deterioration in hearing that was primarily mild but 9 patients (8.3%) had severe bilateral hearing loss. In multivariate analysis, older age and baseline hearing loss predicted deterioration of hearing.

Conclusion

Hearing loss at baseline and during treatment occurred frequently and was often severe. Increasing age and HIV infection with low CD4 count were associated with baseline hearing loss. Baseline hearing loss and older age predicted deterioration of hearing.

Key words: drug resistant tuberculosis (DR-TB), hearing loss, aminoglycoside, adult, South Africa.

3. Introduction

3.1 Background

South Africa is a high tuberculosis (TB) burden country and was ranked third among the top 22 high TB burden countries, with incident TB cases of between 0.40 million and 0.50 million per year and an incidence rate of 993 per 100,000 person years [1]. South Africa also has a high burden of drug resistant TB (DR-TB), in particular multi-drug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB). WHO data from 2010, reported that South Africa had 10,085 reported cases of MDR-TB representing the second highest case notification amongst the 27 high burden MDR-TB countries [2].

MDR-TB (resistance to both rifampicin and isoniazid) poses a threat to effective TB control due to the difficulties in diagnosis, the requirement for prolonged chemotherapy for up to two years, increased cost (up to 100 times costlier than treating drug sensitive TB) and the use of more toxic second line drugs that are associated with increased adverse effects [2]

Studies have shown that at least 60% of patients on MDR-TB chemotherapy will experience an adverse effect [3-5]. Nephrotoxicity and ototoxicity are the two major adverse effects associated with the injectable second-line TB drugs, aminoglycosides and capreomycin [6]. Nephrotoxicity is reversible upon treatment discontinuation whilst ototoxicity is irreversible and is characterized by impairment of hearing and/or balance [6]. The 2011 South African TB guidelines recommend amikacin / kanamycin as the aminoglycosides of choice for the treatment of MDR-TB and capreomycin for XDR-TB [7]. Amikacin, kanamycin and capreomycin are routinely used to treat DR-TB in Khayelitsha, and are administered intramuscularly for a minimum duration of six months as per national guidelines.

A programme to provide decentralized care for DR-TB has been implemented in Khayelitsha since late 2007 [8]. Through this programme local clinicians have expressed concern about the observed prevalence of hearing loss associated with aminoglycosides and capreomycin. The literature on hearing loss frequency due to aminoglycosides and capreomycin is somewhat inconsistent with reported frequency in MDR-TB patients ranging from 6.7% [9] up to 50% in other cohorts [3,10-12]. Reported risk factors for hearing loss include longer duration of injectable treatment, older age, greater total injectable dose received [13], prior exposure to aminoglycosides, prior and current

noise exposure, presence of mitochondrial DNA mutations [6], drug interactions with other ototoxic drugs[14], HIV co-infection and use of anti-retroviral therapy in particular nucleoside reverse transcriptase inhibitors (NRTIs) [15].

However, most of these data are derived from developed countries that have low HIV/TB co-infection rates. South Africa has the largest HIV epidemic globally with an estimated 5.6 million people infected with HIV in 2009 [16], HIV co infection among TB patients in Khayelitsha is 70%, one of the highest in the country [8]. In addition, reported incidences of hearing loss in the literature are inconsistent and there is a dearth of data relating to risk factors for hearing loss in long term therapy such as for DR-TB. All these factors limit generalizability of existing data to many settings with high HIV and MDR-TB treatment burden. In light of this, there is a need to determine the incidence and risk factors associated with aminoglycoside and capreomycin induced hearing loss in DR-TB patients in the South African context.

Our aims of this study were to determine prevalence of hearing loss at treatment start and incidence of hearing deterioration with treatment, to describe type and severity of hearing loss at start of treatment and during DR-TB treatment; and to describe risk factors that are associated with hearing loss at start of treatment and deterioration during DR-TB treatment.

4. Methods

4.1 Study Design and setting

The retrospective cohort study was conducted with patients who received treatment at 11 clinics in Khayelitsha but only one clinic was used to do audiometry screening. Khayelitsha is a peri-urban sub-district within the Cape Town metropolitan area. The Khayelitsha decentralized DR-TB programme was established in late 2007 in response to the high DR-TB burden in Khayelitsha and lack of capacity of the local TB hospital to handle all DR-TB cases in a timely manner [8].

4.2 Study population

All adult patients aged 18 years and above with a diagnosis of rifampicin resistant TB (defined as DR-TB) who were receiving intramuscular amikacin, kanamycin or capreomycin as part of the DR-TB regimen were included in the cohort. In order to be included into the study, a patient must have been initiated on DR-TB treatment between June, 1 2009 and December, 31 2010. Ethical approval

was obtained from the University of Cape Town Ethics Committee and the study was approved by the City of Cape Town Research Committee. No informed consent was obtained from the study participants as audiometry testing was part of standard of care at Khayelitsha.

4.3 Data Collection

Upon initiation of DR-TB treatment, all demographic and clinical information was collected on a standardized form by the attending physician as standard of care. Thereafter, patients were referred to the audiometry testing centre based at one of the primary health care clinics in Khayelitsha.

Hearing thresholds were measured objectively by an audiologist trained field worker using a pure tone audiometer (Interacoustics AS608). The screening test was conducted in a sound proof booth at the following frequencies: 250Hz, 500Hz, 1000Hz, 2000Hz, 4000Hz and 8000Hz. Prior to doing the hearing assessment, the ear canal was examined using an otoscope for any signs of obstruction such as inflammation, growths, foreign bodies or excessive cerumen to prevent obscured hearing [17].

The reliability of the audiometry measurements was assessed using test retest reliability at every frequency level for every audiogram whilst inter-rater reliability was conducted on a sub set of audiograms. Test retest reliability was defined as consistent measurements made by the same rater. Hence, a valid audiogram was defined as one that did not have any ear(s) obstructed or one that was classified as having good test-retest reliability.

The first ever audiogram for each patient was classified relative to the start of DR-TB treatment: i) Pre-treatment audiograms were defined as audiograms taken within 10 days of initiating DR-TB treatment, prior to significant drug exposure ii) Early treatment audiograms were audiograms that were taken within 1.5 months of starting DR-TB treatment. Hearing loss that occurred within the first 1.5 months of treatment was referred to as baseline hearing loss. We used a less stringent definition that included audiograms taken up to 1.5 months into treatment because we had a limited number of individuals with pre-treatment audiograms. Furthermore, there was missing information with some of the variable which would have significantly reduced the precision of the estimates in the logistic regression if our definition of baseline was restricted to pre-treatment audiograms only.

Patients were routinely followed up monthly until 2 months after the end of the injectable phase, typically a 6 to 8 month period. During the follow-up visits, ascertainment of hearing loss was measured in the manner similar to at baseline. All audiometry data collected at follow-up visits was compared with baseline data (if available) to ascertain whether deterioration had occurred.

4.4 Outcome and explanatory variables

The main outcome of interest was hearing loss. Criteria from Gelfand (2009) were used to define hearing loss and severity but the criteria were modified in order to utilize fewer categories [18]. The criteria used were: *i) Normal hearing*: Average hearing threshold of ≤ 20 decibels at either the low or high frequency test frequencies ; *ii) Mild hearing loss*: Average hearing threshold of between 21 and 40 decibels at either the low or high test frequencies; *iii) Moderate to severe hearing loss*: Average hearing threshold of between 41 and 70 decibels at either low or high test frequencies and *iv) Severe to profound hearing loss*: Average hearing threshold of more than 70 decibels across the low or high frequencies. Severity of hearing loss was assessed using the better ear criteria which are based on WHO recommendations for assessment of hearing loss [19]. This means that hearing loss severity was categorized according to the ear with less severe hearing loss.

Hearing loss was categorized into three frequencies: *i) low frequency hearing loss* (250 Hz to 2000Hz) ; *ii) high frequency hearing loss* (4000Hz and 8000 Hz) and; *iii) mixed hearing loss*-hearing loss occurring in both low and high frequencies.

The measurement of hearing loss was done as follows: For low frequency hearing loss, averaging of hearing thresholds across 250 Hz, 500Hz, 1000Hz and 2000Hz was done for each ear separately at each visit; For high frequency hearing loss, averaging hearing thresholds across 4000 Hz and 8000 Hz for each ear separately at each visit [20].

Deterioration of hearing with treatment was assessed semi-qualitatively. This consisted of plotting each patient's valid audiograms over the treatment period using calculated averages of hearing thresholds across low and high frequencies for each ear separately. This produced four line graphs per patient which were assessed by two independent assessors to determine the presence of hearing loss and to categorize severity based on definitions described previously. All discrepancies were resolved by consensus. Assessors were used to assess the line graphs in preference of a statistical analysis due to highly varied patterns of baseline hearing function and subsequent changes.

The explanatory variables measured in this study were: age at DR-TB diagnosis, sex, history of aminoglycoside use, starting aminoglycoside dose, HIV status and CD4 count. The aminoglycosides (amikacin and kanamycin) and capreomycin were dosed as follows: i) weight < 33kg- 15mg/kg/day; ii) weight between 33 and 50 kg- 500-750mg ; and iii) weight >50kg- 1000mg.

5. Statistical Analysis

The baseline characteristics of the cohort were described using medians and inter quartile ranges for continuous variables with a skewed distribution and means and standard deviations for the normally distributed data. Wilcoxon rank-sum test and t tests were used to assess differences between continuous variables where appropriate. The categorical variables were assessed using Fishers Exact Test and Chi-squared test where appropriate.

Univariable and multivariable associations between hearing loss and explanatory variables were assessed using logistic regression. A multivariable logistic model was used to test the association between hearing loss and all the explanatory variables. The logistic regression models were built using a priori assumptions from the literature on risk factors for hearing loss. Sex was also included into the model as a potential confounder. Antiretroviral therapy could not be included into the final models due to co-linearity between this variable and HIV status. Due to the low proportion of patients with pre-treatment audiograms and the limited number of participants overall, restricting the analysis to patients with confirmed absence of hearing loss prior to TB treatment resulted in a substantially reduced sample size with limited power to determine associations with new onset hearing loss. We therefore looked for associations in models that were variously restricted to those with pre-treatment or early treatment first audiograms, and with and without restriction to normal hearing at the time of the first audiogram. This enabled us to confirm the consistency of associations across models and limit potential bias due to the selected inclusion criteria. Statistical analysis was conducted using STATA version 11.

6. Results

From June 1, 2009 to 31, Dec 2010, 238 eligible adult patients were started on DR-TB treatment in Khayelitsha primary health care clinics. Of those eligible for entry into the study, 175 (73.5%) patients had at least one valid audiogram but only 131 (74.9%) patients had at least one audiogram assessable within 1.5 months of starting DR-TB treatment and were included into the final analysis

(Figure 1). Sixty one (46.6%) and 70 (53.4%) patients had pre-treatment and early treatment audiograms respectively.

Baseline characteristics

There was no evidence of a difference between the patients with baseline audiogram within 1.5 months of treatment start and those with no audiometry done with regards to demographic characteristics (weight, age, sex), clinical characteristics (HIV status and CD4 counts), treatment factors (type of injectable) and treatment history (previous aminoglycoside use and previous TB treatment) (**Table 1**). However, fewer patients with no audiometry were already receiving ART (26.5%) at DR-TB diagnosis compared to those who had baseline assessable within 1.5 months of DR-treatment initiation (48%). Overall, in both groups, kanamycin was the most frequently used injectable.

Patients with a valid audiogram at baseline had median injection duration of 180 days, IQR [136-199] and a total injectable dose of 119.3 g, IQR [80.7-134.3] (data not shown). With regards to screening, only 13 (9.9%) patients had a complete set of 6 audiograms done during treatment. Overall, patients had a median of 4 audiograms IQR [2-6] during treatment and the median time to first audiogram was 11 days, IQR [6-21] (**Table 1**). The median time between the first and second audiogram was 32 days, IQR [20-42].

Frequency, type and severity of baseline hearing loss at treatment initiation

The prevalence of baseline hearing loss relating to pre-treatment (31.2%; 95% CI: 20-44.3) and early treatment audiograms (40%; 95%CI: 28.5-52.4) ($p=0.292$) was similar; therefore we combined both categories. Of 131 adults who had a valid audiogram within 1.5 months treatment start, 47 (35.9%) had baseline hearing loss (**Figure 2**). Mixed hearing loss occurred more frequently, 29 (22.1%) versus 18 (13.7%) patients with high frequency hearing loss. Overall, 25.2% (33/131) and 5.3% (7/131) of patients had mild and severe forms of hearing loss respectively. Seven patients (5.3%) had a combination of mild loss at low frequencies and severe loss at higher frequencies. The severe forms of hearing loss occurred more frequently in those with mixed hearing loss.

Frequency, type and severity of hearing deterioration during DR-TB treatment

Of 131 patients with a valid audiogram within 1.5 months of treatment start, 109 (83.2%) patients had at least two audiograms to assess deterioration (**Figure 3**). Of these, 38 (34.9%) had baseline hearing loss. In total, 37 (33.9%) patients had deterioration in hearing during DR-TB treatment that was bilateral in nature. Of the 71 subjects with no baseline hearing loss, only 12 (16.9%) patients had deterioration in hearing with treatment compared to 65.8% (25/38) in the group with baseline hearing loss.

Overall, hearing loss was predominately mixed in nature, 22% (24/109) patients had mixed loss versus 9.2% (10/109) and 2.8% (3/109) with high and low frequency loss respectively (**Figure. 3**). A similar pattern of deterioration in hearing was seen in patients with baseline hearing impairment. However, patients with normal hearing at treatment start, (n=71) had primarily deterioration in the high frequency ranges only.

Mild bilateral hearing loss occurred most frequently in the entire group with 20.2% (22/109) with mild hearing loss and 8.3% (9/109) patients with severe forms of bilateral hearing loss. Six patients (5.5%) had a combination of mild and severe loss. Mild hearing loss occurred more frequently in patients with normal hearing at treatment start whilst severe hearing impairment was more common amongst those with baseline hearing loss.

Univariable and multivariable analysis of factors associated with baseline hearing loss

In the univariable analysis, only increasing age (OR 2.2, 95% CI: 1.4-3.2) was associated with increased odds of baseline hearing loss within 1.5 months of treatment start (**Table 2**). In the multivariable data analysis, older age (OR 2.4; 95%CI: 1.5-3.8) and HIV infection and severity (CD4 count<100 cells/ μ l) (OR 4.1; 95%CI: 1.2-13.7) increased odds for baseline hearing loss.

Male sex, aminoglycoside history and positive HIV status did not predict hearing loss during DR-TB treatment in all three models (**Table 3**). Across a range of inclusion criteria for analyses of hearing deterioration (**Table 3**), there was evidence in both univariable and multivariable analyses in support of both age at diagnosis and baseline hearing loss being associated with deterioration. For example, amongst patients with normal pre-treatment or early treatment audiogram (n=71), increasing age for each ten year increment in age was associated with an increased odds of deterioration of hearing in both univariable (OR 2.5; 95% CI: 1.1-5.7) and multivariable analysis

(OR 3.1; 95% CI: 1.2-7.9) (**Table 3**). Baseline hearing loss amongst patients with a pre-treatment or early treatment audiogram (n=109) predicted deterioration of hearing (OR 8.5; 95% CI: 3.0-24.1).

7. Discussion

We report a high prevalence of baseline hearing loss (35.9%) and deterioration of hearing during DR-TB treatment (34%) that was primarily mixed and mild in severity. New onset hearing loss also occurred frequently, mostly affecting high frequencies and was mild in severity. In multivariable analysis, patients with older age and severe HIV infection with CD4<100 cells/ul were more likely to have baseline hearing loss. Amongst patients with a second audiogram to assess deterioration, older age and baseline hearing loss predicted hearing deterioration.

Hearing loss at first audiogram

We report an initial hearing loss prevalence of 35.9% that was bilateral, predominately mixed and mild in severity. In contrast, a study by Peloquin *et al* [13], reported a markedly lower prevalence of 5.7% of hearing loss at baseline. Differences in clinical characteristics, in particular HIV prevalence, a well-documented hearing loss risk factor [21-23], may account for the differences as the study by Peloquin *et al* was based in the US.

In multivariable analysis, increasing age and severe HIV disease (CD4<100 cells/ul) were associated with an increased odds of baseline hearing loss. Previous studies have not reported on the relationship between baseline characteristics and pre-existing hearing loss. Theoretical hypotheses suggest that a reduction in hair cells in the cochlea and/or reduction in endogenous protective mechanisms that occurs in older age [24] and HIV associated opportunistic infections such as otitis media associated with low CD4 counts increase the susceptibility for hearing loss [15].

Deterioration of hearing on treatment

Overall, we reported a cumulative incidence of 34% in deterioration of hearing. This is higher than estimates from previous studies primarily from developed settings that report hearing loss frequencies ranging from 6.7% to 21.3% [3,9,10].

We observed a new-onset hearing loss cumulative incidence of 16.9% during treatment in patients with normal hearing at first audiogram that mostly affected the high frequencies and was mild in severity. This pattern of selectively affecting high frequencies first before affecting conversational frequencies (low frequencies) is consistent with the mechanism of aminoglycoside induced hearing loss [14,25,26]. Our results are comparable to a previous study that reported an overall incidence of

18.75%, that was predominately high frequency loss [11]. However, our findings contrast with another South African DR-TB cohort that reported an incidence of 58% over a 3 month period [23], as well as data from developed settings that reported incidences ranging from 28% to 55% during DR-TB treatment [27-29]. These differences could be in part due to the inconsistent definitions of hearing loss across the studies. In particular, hearing loss definitions in previous studies included unilateral loss whilst our hearing loss definition restricted it to bilateral loss only. In addition, our cohort did not have regular audiometry monitoring possibly underestimating the true extent of hearing loss.

Theoretically, pre-existing hearing loss leads to deterioration of hearing due to fewer hair cells at the treatment start or a reduction in endogenous protective mechanisms [24,25]. Deterioration of hearing amongst those with baseline hearing loss occurred frequently in our cohort. In addition, multivariable analysis showed a positive association between baseline hearing loss and deterioration of hearing that has not been observed in a previously reported study of short term aminoglycoside use [30].

We observed that increasing age predicted new onset hearing loss, a finding that has been reported in previous DR-TB studies [13,27,28]. However, we failed to show an association between HIV infection and hearing loss during DR-TB treatment. This finding was unexpected as we had a high HIV prevalence and low median CD4 count at treatment start. This also conflicted with data from another Cape Town DR-TB cohort that demonstrated a strong positive association between HIV infection and development of hearing loss, [23] but this study did not control for potential confounders such as age and past aminoglycoside use.

Strengths and limitations

Our study findings are limited by missing information in some variables and small sample size that decreased the precision of estimates. In addition, audiograms were conducted at irregular intervals, thereby limiting our ability to measure timing of hearing loss; hence variables such as injection duration and total aminoglycoside dose could not be included into the analyses. Our results may also have been confounded by variables such as exposure to noise and concomitant ototoxic drugs that were not measured. We did not use a strict definition of baseline, and included audiograms that had been taken up to 1.5 months into treatment; hence baseline hearing loss may have been attributed to treatment exposure. In addition, a less stringent definition of baseline limited the

interpretation of our results as we may have underestimated the true incidence of hearing loss during DR-TB treatment as we considered individuals with an audiogram taken at 1.5 months as having existed prior to treatment start. The use of the better ear criteria also limited the analysis to bilateral hearing loss only, whilst previous studies include unilateral loss in their hearing loss definition. Another major limitation is that the audiometry tests are screening tests and we did not have any diagnostic hearing tests done by an audiologist to confirm whether hearing loss was sensorineural. Consequently, the true extent of hearing loss may have been overestimated due to other causes or underestimated as pure tone audiometry relies on the patient's ability to respond to hearing stimuli. Nonetheless, the findings of this study are strengthened by inclusion of all DR-TB patients, minimal selection bias in excluding subjects without audiometry, use of routine data, use of an objective measure to assess hearing loss as well as the rigorous method of assessing validity of audiograms.

8. Conclusion

The occurrence of hearing loss in DR-TB patients at baseline and deterioration during DR-TB treatment is common and often severe. Hearing loss on the first audiogram predicted subsequent deterioration of hearing. This finding underscores the value of conducting more frequent pre-treatment/ early treatment audiograms in patients commencing injectable therapy in order to minimize potential deterioration and implement prevention strategies such as dose reduction. There is limited data on hearing loss during long term aminoglycoside use from developing settings. More studies that measure exposure to ototoxic medication and noise exposure as well as measure timing of the onset of hearing deterioration are needed. Furthermore, studies on measures to avert deterioration of hearing such as changing the dose or stopping the aminoglycoside early are urgently needed.

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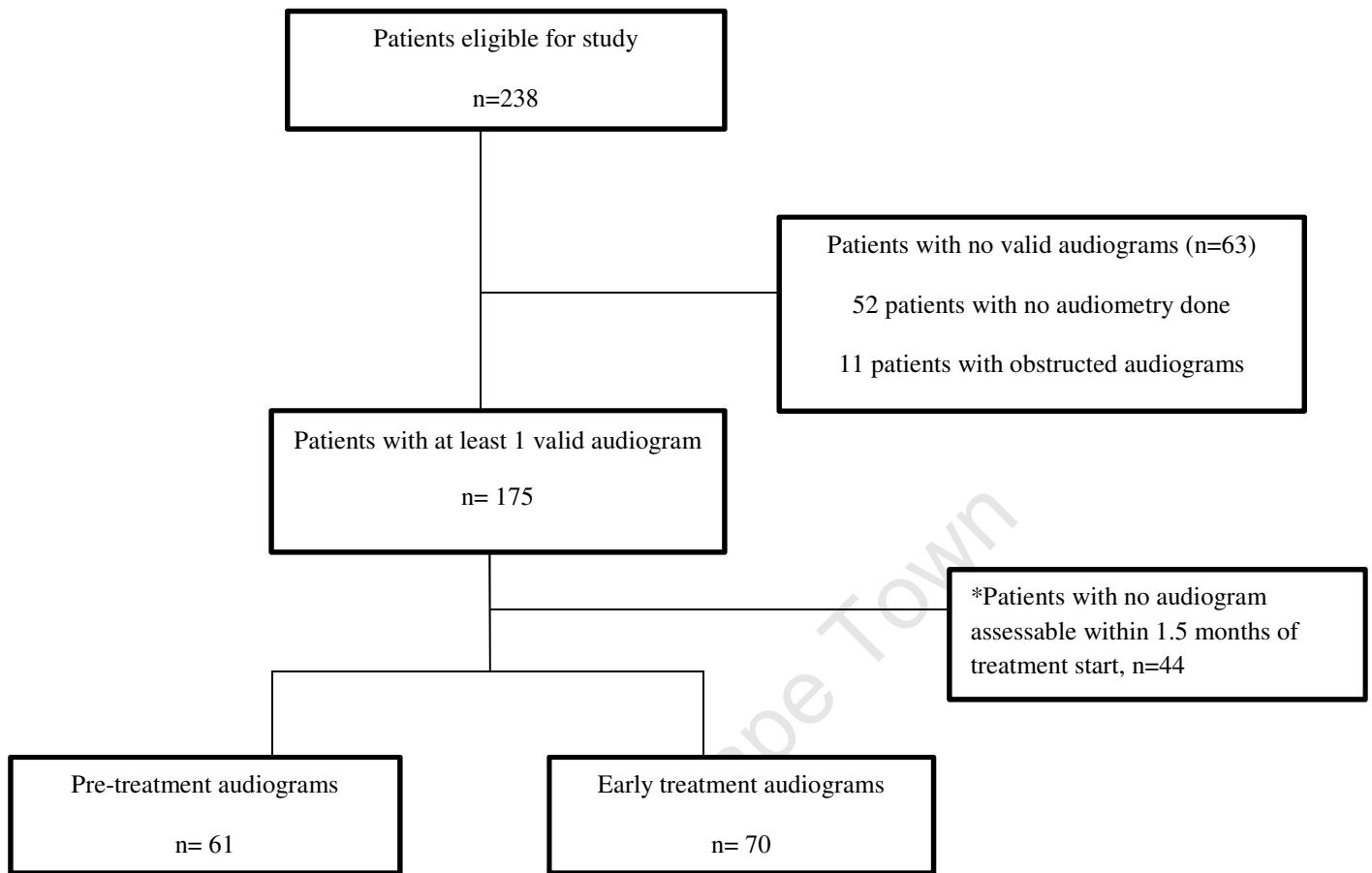


Figure 1. Flow chart of patients starting DR-TB treatment in Khayelitsha and those eligible for analysis.

*This refers to patients who did not have a pre-treatment audiogram or an early treatment audiogram.

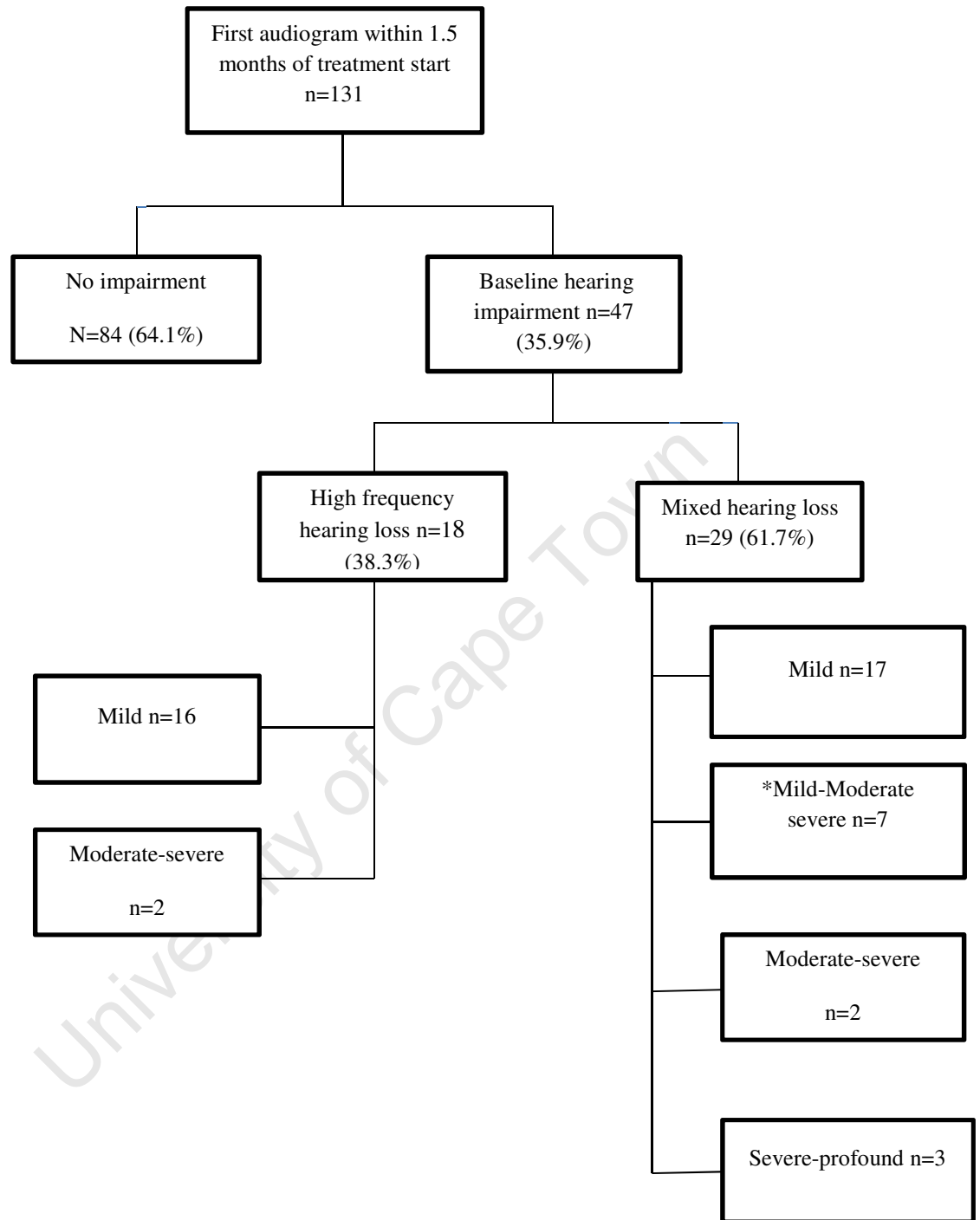


Figure 2. Frequency, type and severity of baseline hearing loss in patients initiated on DR-TB treatment.

*This reflects a combination of severity from low and high frequencies.

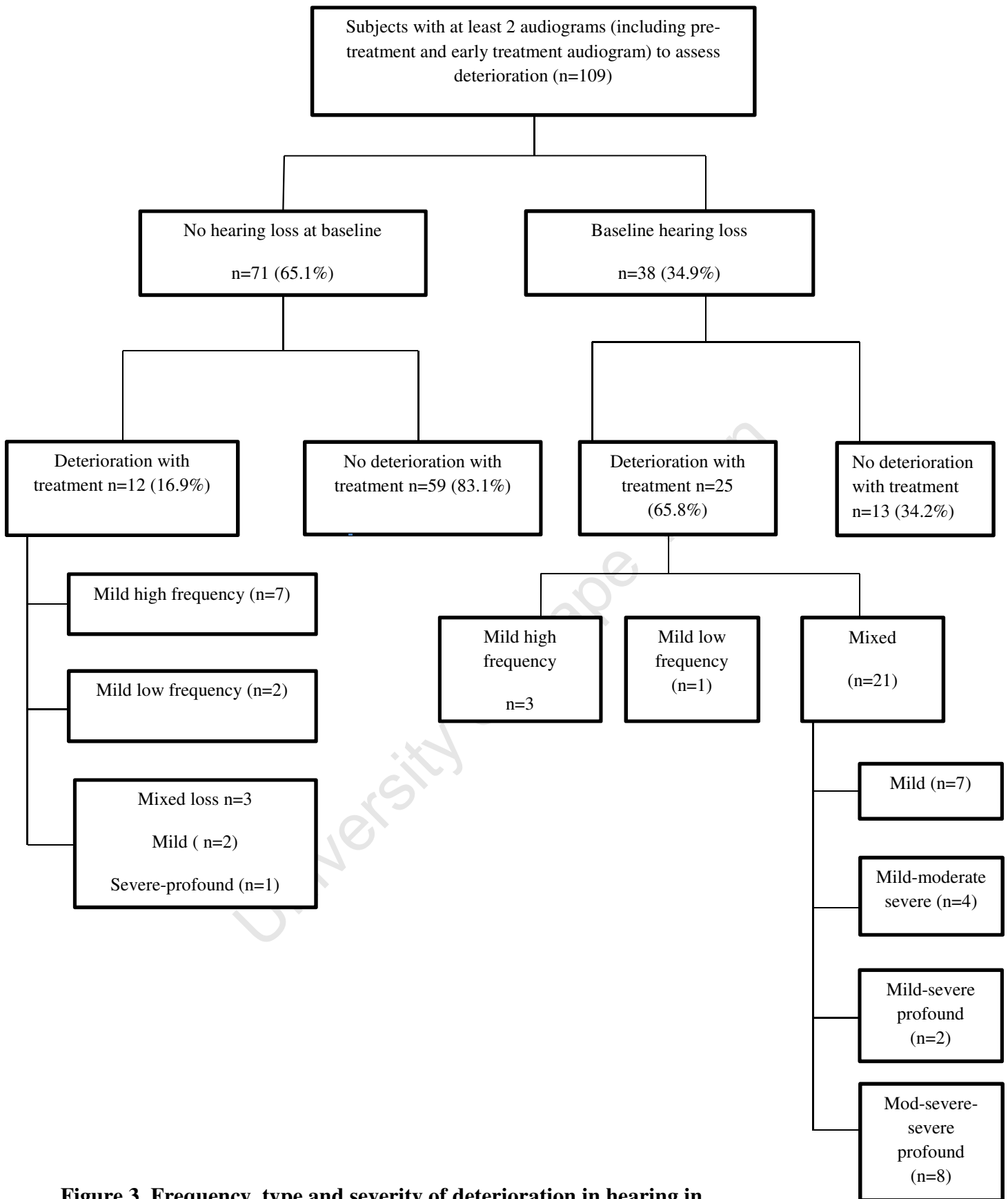


Figure 3. Frequency, type and severity of deterioration in hearing in patients with at least two audiograms.

Table 1. Baseline characteristics of patients with a valid baseline audiogram compared to those with no audiometry conducted.

Baseline characteristics	No audiometry (n=52)	Baseline audiogram within 1.5 months of treatment start (n=131)	P value*
Weight (kg), mean weight± sd	59± 8.5	55±9.7	0.13
Age, median years [IQR]	35.6 [28.7-39.9]	34.4 [27.6-40.7]	0.47
Sex			
Male	31 (59.6%)	65 (49.6%)	0.22
HIV status			
Positive	36 (69.2%)	98 (74.8%)	0.29
Negative	14 (26.9%)	32 (24.4%)	
Unknown	2 (3.8%)	1 (0.8%)	
CD4 , median cells/μl [IQR]	122 [48-174]	133 [54-228]	0.29
On ART at diagnosis€			
Yes	9 (26.5%)	47 (48.0%)	0.03
Previous TB treatment#			
Previous 1st line treatment	31 (60.8%)	86 (66.7%)	0.3
Previous 2nd line treatment	8 (15.7%)	10 (7.8%)	
No TB treatment	12 (23.5%)	33 (25.6%)	
Aminoglycoside history¥			
Yes	17 (42.5%)	48 (44.0%)	0.87
Type of injectable			
Kanamycin	51 (98.1%)	114 (87.0%)	0.07
Amikacin	1 (1.9%)	15 (11.5%)	
Capreomycin	0	2 (1.5%)	
Number of audiograms, median audiogram [IQR]	-	4 [2-6]	
Time to first audiogram, median days [IQR]	-	11 [6-21]	

ART- antiretroviral therapy; sd- standard deviation; IQR- interquartile range

*Wilcoxon rank sum test and t test for continuous variables, chi square tests and Fishers exact test where applicable for categorical variables.

€ Two patients in the no audiometry group had missing information.

One patient in the no audiometry group and 2 patients with a baseline audiogram had missing information.

¥ Twelve patients in the no audiometry group and 22 patients with a baseline audiogram had missing information.

Table 2. Univariable and multivariable analyses of risk factors for baseline hearing loss in patients initiating DR-TB treatment.

*N=131	Univariable analysis		Multivariable analysis	
Baseline characteristic	Odds ratio (OR)	95% CI	Odds ratio (OR)	95% CI
Age at diagnosis (yrs)-per 10 years	2.2	(1.4-3.2)	2.4	(1.5-3.8)
Male sex	1.6	(0.8-3.4)	1.4	(0.6-3.2)
Aminoglycoside history				
No				
Yes	0.7	(0.3-1.5)	0.8	(0.3-1.8)
HIV & CD4 category				
HIV negative (reference)	1.0		1.0	
HIV positive & CD4 \geq 100	1.1	(0.4-2.7)	2.5	(0.7-7.3)
HIV positive & CD4 <100	2.0	(0.7-5.2)	4.1	(1.2-13.7)

*The 131 patients refers to those who had at least one audiogram done (pre-treatment or early treatment audiogram) to assess baseline hearing loss.

Table 3. Associations with deterioration in hearing threshold on treatment categorized by first audiogram status

	*Pre-treatment AG available (n=53)				#Normal first AG pre-treatment or early treatment (n=71)				¥Any first AG pre-treatment or early treatment AG (n=109)			
	Univariable		Multivariable		Univariable		Multivariable		Univariable		Multivariable	
	OR	95%CI	OR	95% CI	OR	95%CI	OR	95% CI	OR	95%CI	OR	95% CI
Age at diagnosis (per 10 years)	1.5	(0.8-2.8)	1.4	(0.6-2.9)	2.5	(1.1-5.7)	3.1	(1.2-7.9)	1.9	(1.2-2.9)	1.3	(0.8-2.3)
Male sex	1.1	(0.3-3.4)	0.5	(0.1-2.3)	0.7	(0.2-2.7)	0.2	(0.0-1.4)	1.2	(0.6-2.8)	0.8	(0.3-2.2)
Aminoglycoside history	1.1	(0.3-3.7)	1.8	(0.4-7.6)	2.2	(0.5-8.0)	2.2	(0.5-1.0)	1.1	(0.5-2.4)	1.2	(0.5-3.3)
Aminoglycoside dose (per 100mg)	0.8	(0.6-1.2)	0.7	(0.5-1.1)	0.8	(0.5-1.2)	0.7	(0.4-1.1)	0.8	(0.6-1.0)	0.8	(0.6-1.1)
HIV status & CD4 count (cells/ μ l)												
HIV negative (reference)	1.0		1		1		1		1		1	
HIV positive & CD4 \geq 100	0.5	(0.1-1.7)	0.4	(0.1-1.8)	0.5	(0.1-2.3)	0.4	(0.1-2.1)	0.5	(0.2-1.3)	0.4	(0.1-1.5)
HIV positive and CD4<100	0.3	(0.1-2.0)	0.2	(0.0-1.4)	0.6	(0.1-3.8)	0.2	(0.0-2.6)	0.7	(0.2-2.1)	0.4	(0.1-1.7)
Baseline hearing loss	3.3	(0.9-12.1)	3.7	(0.7-18.1)					9.5	(3.8-23.6)	8.5	(3.0-24.1)

AG- audiogram; OR- Odds ratio

*Any pre-treatment audiogram plus a second audiogram available to assess deterioration. This included patients with hearing loss at baseline, so adjustment for baseline hearing loss included into the model

Only patients with a normal pre-treatment or normal early treatment audiogram plus a second audiogram to assess deterioration

¥ Any patient with a pre-treatment or an early treatment audiogram plus a second audiogram to assess deterioration. This included patients with hearing loss at baseline, so adjustment for baseline hearing loss included into the model.

Part D: Appendices

Appendix 1: Standardized initial audiometry referral form

Patients Surname:				Patients First Name:			
Patients address:							
DOB:				Pt. Telephone No.			
Referral Clinic:				Date referral made:			
Referral Doctor:				Folder No.			
Reason for Referral	Baseline		3 month Tx		6 month Tx		Other:
	1 month Tx		4 month Tx		7 month Tx		
	2 month Tx		5 month Tx		8 month Tx		
Date Current DR TB Treatment Started:				Patients current wt.			
Current dosages	€ Ka/Am _____ € Cm _____ € S _____ € EMB _____			€ Ofx _____ € Eto _____ € Z _____ € H _____		€ Mfx _____ € PAS _____ € Cys/Trd _____ € Other: _____	
Previous Treatment with Streptomycin, Kanamycin, Amikacin, Capreomycin							
Streptomycin		Kanamycin		Amikacin		Capreomycin	
Date start _____	Date start _____	Date start _____	Date start _____	Date start _____	Date start _____	Date start _____	Date start _____
Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____
Dose _____	Dose _____	Dose _____	Dose _____	Dose _____	Dose _____	Dose _____	Dose _____
Date start _____	Date start _____	Date start _____	Date start _____	Date start _____	Date start _____	Date start _____	Date start _____
Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____
Dose _____	Dose _____	Dose _____	Dose _____	Dose _____	Dose _____	Dose _____	Dose _____
Date start _____	Date start _____	Date start _____	Date start _____	Date start _____	Date start _____	Date start _____	Date start _____
Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____	Date stop _____
Dose _____	Dose _____	Dose _____	Dose _____	Dose _____	Dose _____	Dose _____	Dose _____
Previous Audiometry	€ Yes	€ No	Date:		Where?		
	Results:	€ Normal	€ Abnormal				
<i>Attach results of previous audiogram if not done at Ubuntu clinic</i>							
Clinical Assessment	<ul style="list-style-type: none"> • Patient complains of poor hearing • Family History of hearing loss to TB medication • Patient complains of vestibular symptoms Describe:				€ Yes	€ No	
					€ Yes	€ No	
					€ Yes	€ No	
Serum Creatinine:				Date serum creatinine:			
Appointment Date for Audiometry:				Appointment made by:			
				Appointment confirmed:			

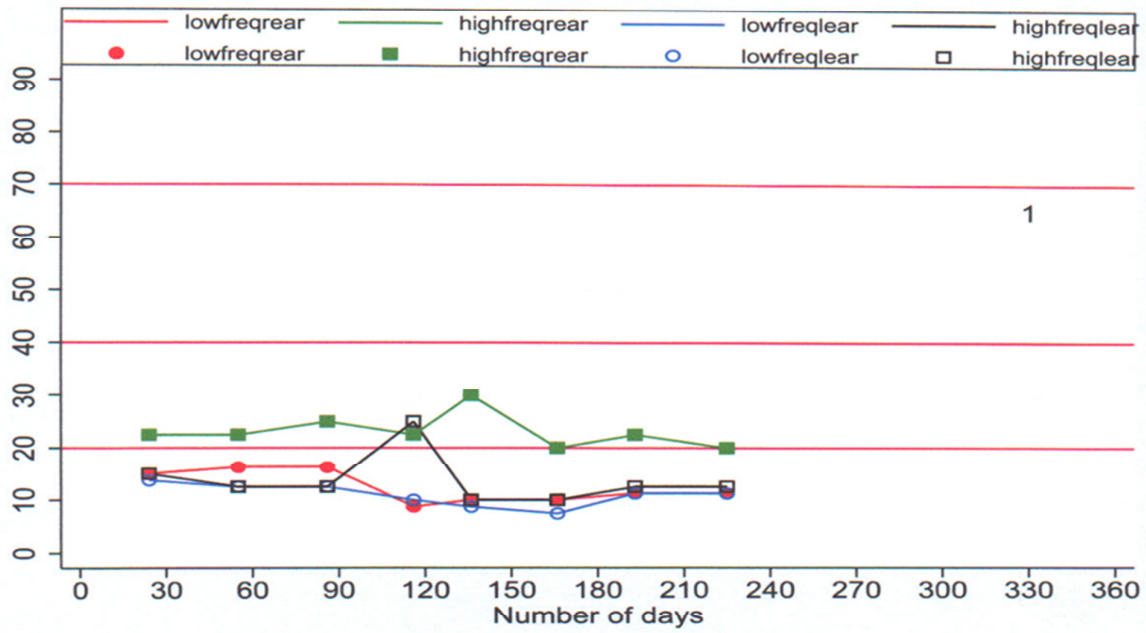
Appendix 2: Standardized data collection form for audiometry data

Patients Surname:		Patients First Name:							
Patients Address:									
DOB:		Pt. Telephone No.							
Referral Clinic:		Date referral made:							
Reason for Referral	Baseline	3 month Tx	6 month Tx						
	1 month Tx	4 month Tx	7 month Tx						
	2 month Tx	5 month Tx	8 month Tx						
			Other:						
Questions for Patient: <ul style="list-style-type: none"> • Ringing in ears • Hearing loss • Difficulty hearing in noise • Balance problems • Bouncing vision • Noise exposure • Family history: hearing loss from TB meds • 		Y	N	How Long?	Comments				
Date of Test:		Name of Screener:							
		Test Reliability:	<input type="checkbox"/> Good <input type="checkbox"/> Poor						
Hearing Levels (dB) Soft -10 0 10 20 30 40 50 60 Loud		Normal							
		Number of previous audiograms: _____							
		Low pitch	250	500	1000	2000	4000	8000	High pitch
		Frequency (Hz)							
				Response	No-Response				
		Right (red)		O	⊘				
		Left (blue)		X	X				
		Findings:							
			<input type="checkbox"/> Yes	<input type="checkbox"/> No	Ear Canal obstructed with _____				
			<input type="checkbox"/> Yes	<input type="checkbox"/> No	Abnormal ear drum (<i>describe</i>) _____				
	<input type="checkbox"/> Yes	<input type="checkbox"/> No	Abnormal audiogram						
	<input type="checkbox"/> Yes	<input type="checkbox"/> No	Significant change in hearing levels from baseline audiogram of _____						

	_____ (Date)		
	<i>If <u>yes</u> to any of the above, advise the patient to see his/her doctor urgently (not to wait until their next scheduled doctor's appt.</i>		
	€ Yes € No Patient referred to doctor urgently (if yes, put red sticker on this form)		
Follow up appt:		Signature :	

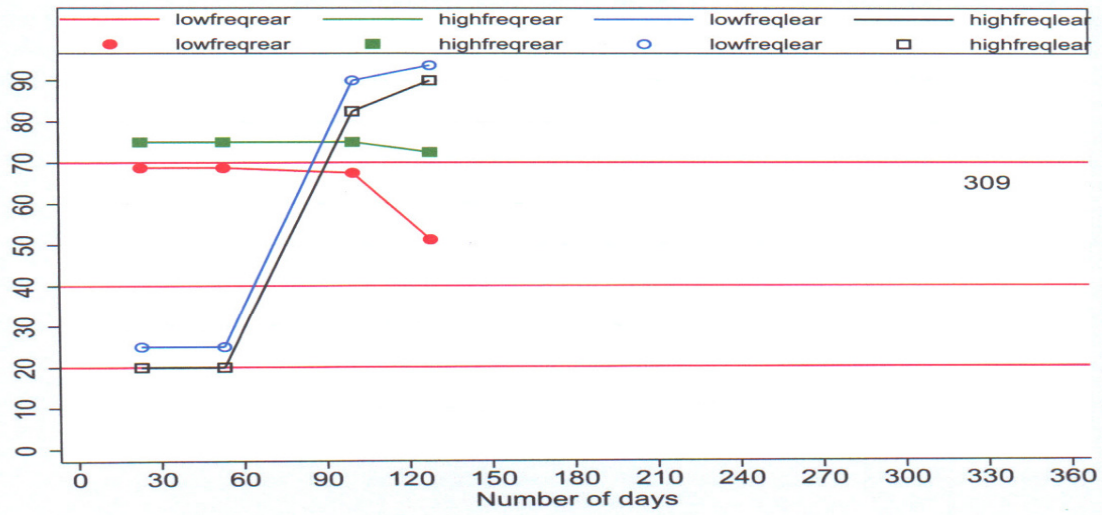
University of Cape Town

Appendix 3- Line graph of audiograms representing normal hearing



Y axis: Hearing levels in decibels (db)

Appendix 3: Line graph of audiograms representing severe hearing loss



Y axis: Hearing level in decibels (db)



Appendix 4: University of Cape Town ethics approval letter

10 December 2010

University of Cape Town

HREC REF: 503/2010

Ms C Njuguna
c/o Prof ABoulle
Public Health & Family Medicine

Dear Ms Njuguna

PROJECT TITLE: RISK FACTORS THAT PREDISPOSE TO AMINOGLYCOSIDE/CAPREOMYCIN INDUCED HEARING LOSS IN ADULT PATIENTS RECEIVING TREATMENT FOR MULTI-DRUG RESISTANT TUBERCULOSIS (MDR-TB) AND EXTENSIVE DRUG RESISTANT TUBERCULOSIS (XDR-TB) IN KHAYELITSHA, WESTERN CAPE

Thank you for submitting your new study to the Faculty of Health Sciences Human Research Ethics Committee. Thank you for your response to the queries raised by the HREC.

It is a pleasure to inform you that the FHS HREC has formally approved the above-mentioned study.

Approval is granted for one year until 15 December 2011.

Please send us an annual progress report (website form FHS 016) if your research continues beyond the approval period. Alternatively, please send us a brief summary of your findings so that we can close the research file.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator. Please quote the REC. REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN
ETHICS

Appendix 4: City Health study approval

CITY HEALTH — Specialised Health

2011-05-10

re: Research Request: Risk factors that predispose to aminoglycoside/capreomycin induced hearing loss in adult patients receiving treatment for multi-drug resistant tuberculosis (MDR-TB) and extensive drug resistant tuberculosis (XDR-TB) In Khayelitsha, Western Cape (ID HO: 10239)

Dear Dr Cox

Permission has been granted to do your research as per your protocol.

Khayelitsha Sub District

Contact People: Dr. V de Azevedo (Sub District Manager)

Tel: (021) 360 1258/083 629 3344

Mr Mhulbulwana (Head: PHC & Programmes)

Tel: (021) 360-1153/ 082 715 0147

Please note the following:

1. All individual patient information obtained must be kept confidential.
2. Access to the clinic and its patients must be arranged with the relevant Manager such that normal activities are not disrupted.
3. A copy of the final report must be sent to the City Health Head Office, P O Box 2815 Cape Town 8001, within 3 months of its completion and feedback must also be given to the clinics involved.
4. Your project has been given an ID Number (10239). Please use this in any future correspondence with us.

Thank you for your co-operation and please contact me if you require any further information or assistance.

Yours sincerely

DR G H VISSER

MANAGER: SPECIALISED HEALTH

cc. Dr Azevedo & Mr Mhulbulwana

Appendix 5- PLOS ONE AUTHOR INSTRUCTIONS

PLOS ONE Manuscript Guidelines

<http://www.plosone.org/static/guidelines>

1. Format Requirements

PLOS ONE does not consider presubmission inquiries. All submissions should be prepared with the following files:

- Cover letter
- Manuscript, including tables and figure legends
- Figures (guidelines for preparing figures can be found at the Figure and Table Guidelines)

Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use language-editing and copyediting services. Obtaining this service is the responsibility of the author, and should be done before initial submission. These services can be found on the web using search terms like "scientific editing service" or "manuscript editing service." Submissions are not copyedited before publication.

Submissions that do not meet the PLOS ONE Publication Criterion for language standards may be

1.1 Cover Letter

You should supply an approximately one page cover letter that:

- Concisely summarizes why your paper is a valuable addition to the scientific literature
- Briefly relates your study to previously published work
- Specifies the type of article you are submitting (for example, research article, systematic review, meta-analysis, clinical trial)
- Describes any prior interactions with PLOS regarding the submitted manuscript
- Suggests appropriate PLOS ONE Academic Editors to handle your manuscript (view a complete listing of our academic editors)
- Lists any recommended or opposed reviewers

Your cover letter should not include requests to reduce or waive publication fees. Should your manuscript be accepted, you will have the opportunity to include your requests at that time. See PLOS ONE Editorial Policy for more information regarding publication fees.

1.2 Manuscript Organization

PLOS ONE considers manuscripts of any length. There are no explicit restrictions for the number of words, figures, or the length of the supporting information, although we encourage a concise and accessible writing style. We will not consider monographs.

All manuscripts should include line numbers and page numbers.

Manuscripts should begin with the ordered sections:

- Title
- Authors
- Affiliations
- Abstract
- Introduction

and end with the sections of:

- Acknowledgments
- References
- Figure Legends
- Tables

Figures should not be included in the main manuscript file. Each figure must be prepared and submitted as an individual file. Find more information about preparing figures here. The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file. There are no explicit requirements for section organization between these beginning and ending sections. Articles may be organized in different ways and with different section titles, according to the authors' preference. In most cases, internal sections include:

- Materials and Methods
- Results
- Discussion
- Conclusions (optional)

PLOS ONE has no specific requirements for the order of these sections, and in some cases it may be appropriate to combine sections. Guidelines for individual sections can be found below. Abbreviations should be kept to a minimum and defined upon first use in the text. Non-standard abbreviations should not be used unless they appear at least three times in the text.

Standardized nomenclature should be used as appropriate, including appropriate usage of species names and SI units.

1.3 Manuscript File Type Requirements

Authors may submit their manuscript files in Word (as .doc or .docx), LaTeX (as .pdf), or RTF format. Only RTF and .doc files can be used during the production process.

LaTeX Submissions. If you would like to submit your manuscript using LaTeX, you must author your article using the PLOS ONE LaTeX template and BibTeX style sheet. Articles prepared in LaTeX may be submitted in PDF format for use during the review process. After acceptance, however, .tex files and formatting information will be required as a zipped file. Please consult our LaTeX guidelines for a list of what will be required.

Submissions with equations. If your manuscript is or will be in .docx format and contains equations, you must follow the instructions below to make sure that your equations are editable when the file enters production.

If you have not yet composed your article, you can ensure that the equations in your .docx file remain editable in .doc by enabling "Compatibility Mode" before you begin. To do this, open a new document and save as Word 97-2003 (*.doc). Several features of Word 2007/10 will now be inactive, including the built-in equation editing tool. You can insert equations in one of the two ways listed below.

If you have already composed your article as .docx and used its built-in equation editing tool, your equations will become images when the file is saved down to .doc. To resolve this problem, re-key your equations in one of the two following ways.

1. Use MathType to create the equation (recommended)
2. Go to Insert > Object > Microsoft Equation 3.0 and create the equation

If, when saving your final document, you see a message saying "Equations will be converted to images," your equations are no longer editable and PLoS will not be able to accept your file.

2 Guidelines for Standard Sections

2.1 Title

Manuscripts must be submitted with both a full title and a short title, which will appear at the top of the PDF upon publication if accepted. Only the full title should be included in the manuscript file; the short title will be entered during the online submission process.

The full title must be 150 characters or fewer. It should be specific, descriptive, concise, and comprehensible to readers outside the subject field. Avoid abbreviations if possible. Where appropriate, authors should include the species or model system used (for biological papers) or type of study design (for clinical papers).

Examples:

- Impact of Cigarette Smoke Exposure on Innate Immunity: A *Caenorhabditis elegans* Model
- Solar Drinking Water Disinfection (SODIS) to Reduce Childhood Diarrhoea in Rural Bolivia: A Cluster-Randomized, Controlled Trial

The short title must be 50 characters or fewer and should state the topic of the paper.

All author names should be listed in the following order:

- First names (or initials, if used),
- Middle names (or initials, if used), and
- Last names (surname, family name)

Each author should list an associated department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. If the article has been submitted on behalf of a consortium, all author names and affiliations should be listed at the end of the article.

This information cannot be changed after initial submission, so please ensure that it is correct.

To qualify for authorship, a researcher should contribute to all of the following:

- 1.Conception and design of the work, acquisition of data, or analysis and interpretation of data
- 2.Drafting the article or revising it critically for important intellectual content
- 3.Final approval of the version to be published

All persons designated as authors should qualify for authorship, and all those who qualify should be listed. Each author must have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. When a large group or center has conducted the work, the author list should include the individuals whose contributions meet the criteria defined above, as well as the group name.

One author should be designated as the corresponding author, and his or her email address or other contact information should be included on the manuscript cover page. This information will be published with the article if accepted.

See the PLOS ONE Editorial Policy regarding authorship criteria for more information.

2.2 Abstract

The abstract should:

- Describe the main objective(s) of the study
- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should not include:

- Citations
- Abbreviations, if possible

2.3 Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
- Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

2.4 Materials and Methods

This section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

We encourage authors to submit detailed protocols for newer or less well-established methods as Supporting Information. These are published online only, but are linked to the article and are fully searchable. Further information about formatting Supporting Information files, can be found here.

Methods sections of papers on research using human or animal subjects and/or tissue or field sampling must include required ethics statements. See the Reporting Guidelines for human research, clinical trials, animal research, and observational and field studies for more information.

Methods sections of papers with data that should be deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers and version numbers, if appropriate. Accession numbers should be provided in parentheses after the entity on first use. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication.

Methods sections of papers using cell lines must state the origin of the cell lines used. See the Reporting Guidelines for cell line research for more information.

Methods sections of papers adding new taxon names to the literature must follow the Reporting Guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

2.5 Results, Discussion and Conclusion

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labelled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn. Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the PLOS ONE Publication Criteria for more information.

2.6 Back to top Acknowledgments

People who contributed to the work but do not fit the PLOS ONE authorship criteria should be listed in the acknowledgments, along with their contributions. You must ensure that anyone named in the acknowledgments agrees to being so named.

Funding sources should not be included in the acknowledgments, or anywhere in the manuscript file. You will provide this information during the manuscript submission process.

2.7 Back to top References

Only published or accepted manuscripts should be included in the reference list. Manuscripts that have been submitted but not yet accepted should not be cited. Limited citation of unpublished work should be included in the body of the text only as “unpublished data.”

References must be listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, citations should be indicated by the reference number in brackets. Journal name abbreviations should be those found in the NCBI databases. A number of reference software companies supply PLOS style files (e.g., Reference Manager, EndNote).

Proper formatting of the references is crucial; some examples are shown below.

- Published papers. Hou WR, Hou YL, Wu GF, Song Y, Su XL, et al. (2011) cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpl9) of the giant panda (*Ailuropoda melanoleuca*). *Genet Mol Res* 10: 1576-1588.

Note: Use of a DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers.

- Accepted, unpublished papers. Same as above, but “In press” appears instead of the page numbers.

- Electronic journal articles. Huynen MMTE, Martens P, Hilderlink HBM (2005) The health impacts of globalisation: a conceptual framework. *Global Health* 1: 14. Available: <http://www.globalizationandhealth.com/content/1/1/14>. Accessed 25 January 2012.

- Books. Bates B (1992) *Bargaining for life: A social history of tuberculosis*. Philadelphia: University of Pennsylvania Press. 435 p.

- Book chapters Hansen B (1991) New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. *AIDS and the historian*. Bethesda: National Institutes of Health. pp. 21-28.

2.8 Table Guidelines

Tables submitted for publication should be included at the very end of the article file (.doc, .rtf, .tex). Supporting Information tables should be submitted as separate files in any of the following formats (although authors should aim to ensure that the file type is most appropriate to the information displayed): Word (.doc), Excel (.xls), PDF, PPT, JPG, EPS, or TIFF.

Title and Footnotes

Each table needs a concise title of no more than one sentence, placed above the table with the table number (e.g., Table 1). The legend and footnotes should be placed below the table. Footnotes may be used to explain abbreviations.

Specifications

Tables that do not conform to the following requirements may give unintended results when published. Problems may include the movement of data (rows or columns), loss of spacing, or disorganization of headings. Note: Multi-part tables with varying numbers of columns or multiple footnote sections should be divided and renumbered as separate tables.

In the published version, tables will be formatted in PLOS style. This includes alternate row shading, content left-aligned in cells, title above the table and legend/footnotes below the table.

Tables must:

- Be cell-based (e.g., created in Word with Tables tool (preferred) or in Excel).
- Be editable (i.e., not a graphic object).
- Have heading/subheading levels in separate columns.
- Be no larger than one printed page (7 in x 9.5 in). Larger tables can be published as online supporting information. Note: some wide tables may be printed sideways in the PDF.

Tables must not:

- Use returns or tabs within a cell.
- Have color or shading.
- Use lines, rules, or borders.
- Contain spaces within cells to align text.
- Have vertically merged cells; horizontally merged cells are fine.
- Have inserted text boxes or pictures.
- Have tables within tables.
- Include empty columns, rows, or cells to create spacing.
- Include hyperlinked text.

Figures should not be included in the manuscript file, but figure legends should be. Guidelines for preparing figures can be found here.

Figure legends should describe the key messages of a figure. Legends should have a short title of 15 words or less. The full legend should have a description of the figure and allow readers to understand the figure without referring to the text. The legend itself should be succinct, avoid lengthy descriptions of methods, and define all non-standard symbols and abbreviations.

Further information about figure legends can be found in the Figure Guidelines.

2.9 Figure preparation

Figures should not be included in the manuscript file, but figure legends should be. Guidelines for preparing figures can be found here. Figure legends should describe the key messages of a figure. Legends should have a short title of 15 words or less. The full legend should have a description of the figure and allow readers to understand the figure without referring to the text. The legend itself should be succinct, avoid lengthy descriptions of methods, and define all non-standard symbols and abbreviations.

File Size

Individual figure files should not exceed 10 MB. If you are having trouble reducing the size of your files, refer to the section below titled Reduce TIFF File Size with LZW Compression.

Figure Quality

A figure that looks good on screen may not be at optimal resolution. Test your figures by sizing them to their intended dimensions (see Quick Reference: Dimensions) and then printing them on your personal printer. The online version should look relatively similar to the personal-printer copy: it should not look fuzzy, jagged, pixelated, or grainy at the intended print size.

Note: The quality of your figures will be only as good as the lowest-resolution element placed in them. In other words, if you created a 72 ppi line graph and placed in it a 300 ppi TIFF, it will be upsampled, resulting in it looking blurred, jagged, or pixelated.

Figure Format

Figures for publication must only be submitted in high-resolution TIFF or EPS format. Some figure types should be submitted in TIFF only (see Figure Types below). If you have not made any annotations to your image, and you have a high-quality TIFF, there is no need to submit it embedded in an EPS, as there will be no increase in quality as a result. See How To: Convert Other File Types to TIFF below for more information on converting figure files to TIFF.

Color Mode

Figures containing color should be saved in either Grayscale or RGB (millions of colors), rather than Indexed Color, CMYK or any other color space. Grayscale or RGB files should be saved with a bit depth of 8 bits per channel, not 16. Bitmap (monochrome) images are not acceptable.

Layered TIFFs

TIFF files with multiple layers are not an accepted format for figures. Please make sure you provide us with a flattened version of your file. To flatten a layered TIFF file, open your figure in Photoshop. From the menu bar select Layer/Flatten Image and save the file. See also Combination Figures, below.

Multi-Page TIFFS

TIFF files with multiple pages are not an accepted format for figures.

Background Color

Create your figures using a white background. If you create figures using a transparent background, the figures may not display well in the online format.

Lines, Rules, and Strokes

Lines should be at least 0.5 point and no more than 1.5 point in order to reproduce well in a PDF file or web format.

Figure Dimensions

Figures for publication will be sized to fit 1, 1.5, or 2 columns of the final printable PDF of the article. Dimensions will also depend on the article type. Please follow the sizing recommendations below for your original submission to create high-quality, appropriately sized figures. See Figure Types below for descriptions and recommendations for line drawings, grayscale drawings, halftones, and combination figures. See below for sizing information.

Figure Alignment

Figures will be left-aligned on the page or column, so please design them accordingly.

Figure Width

Widths depend on article type layout and are listed in the tables below, but must be within the minimum of 3.27in/8.3cm wide and the maximum of 6.83in/17.35cm wide. Figures can have a maximum height of 9.19in/23.35 cm. If your figures have labels that are in 8 point type or if your figures are very detailed, it is recommended that your figure be created so that it will span two columns. Images will be published in a horizontal orientation, and cannot be rotated 90 degrees to have a vertical orientation. Please size your figure widths to one of the column sizes listed below.

Article Type

- 2-column: Research Article, Expert Commentary, Guidelines and Guidance, Learning Forum, Neglected Diseases, PLOS Medicine Debate, Primer, Review, Symposium.

- 3-column: Editorial, Education, Essay, Health In Action, Historical and Philosophical Perspectives, Historical Profiles and Perspectives, Interview, Message from ISCB, Opinion, Perspective, Policy Forum, Policy Platform, Research In Translation, Special Report, Viewpoint.

Figure Types

Line Art

Line art has sharp, clean lines and geometrical shapes against a white background. Line art is typically used for tables, charts, graphs, and gene sequences. You can use a program like Illustrator to create high-quality line art. A minimum resolution of 300 ppi will maintain the crisp edges of the lines and shapes.

- Format: TIFF or EPS
- Minimum Resolution: 300 ppi

Grayscale

Grayscale figures contain varying tones of black and white. They contain no color, so grayscale is synonymous with "black and white." The gray scale is divided into 256 sections with black at 0 and white at 255. Software for preparation of grayscale art includes Photoshop.

- Format: TIFF or EPS
- Minimum Resolution: 300 ppi

Halftones

The best example of a halftone is a photograph, but halftones include any image that uses continuous shading or blending of colors or grays, such as gels, stains, microarrays, brain scans, and molecular structures. To prepare and manipulate halftone images, use Photoshop or a comparable photo-editing program.

- Format: TIFF
- Minimum Resolution: 300 ppi

Combination Figures

Combination figures contain two or more types of images, for example, a halftone figure containing text. You should embed the images, group the objects, or flatten the layers, and flatten transparencies before saving as TIFF at a minimum of 300 ppi.

- Format: TIFF
- Minimum Resolution: 300 ppi

3. Specific Reporting Guidelines

3.1 Human Subject Research

Methods sections of papers on research using human subject or samples must include ethics statements that specify:

- The name of the approving institutional review board or equivalent committee(s). If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed
- Whether informed consent was written or oral. If informed consent was oral, it must be stated in the manuscript:
 - Why written consent could not be obtained
 - That the Institutional Review Board (IRB) approved use of oral consent
 - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: "Caucasian" should be changed to "white" or "of [Western] European descent" (as appropriate); "cancer victims" should be changed to "patients with cancer."

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal (PDF), which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about PLOS ONE policies regarding human subject research, see the Publication Criteria and Editorial Policies.

3.2 Observational and Field Studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

University of Cape Town