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Advanced Phenotyping of Otosclerosis in an Ontario Population and Two Large Newfoundland Families

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Abstract

Otosclerosis is a relatively common hearing loss disorder characterized by abnormal bone growth in the otic capsule leading to stapes fixation. In approximately half of cases, otosclerosis is inherited as an autosomal dominant trait. Typically, gene discovery efforts rely on surgical confirmation, audiometry and occasionally acoustic reflexes to identify affected cases of otosclerosis within families, requiring that the otosclerosis was at an advanced stage to be detected. This makes it difficult to identify individuals with early otosclerosis. The use of advanced phenotyping to identify cases of otosclerosis was tested in an Ontario otosclerotic population as well as in two large Newfoundland families, one with otosclerosis due to a newly discovered deletion in the *FOXL1* gene. Family history questionnaires revealed that approximately two-thirds of Ontario probands had a significant family history of non-congenital hearing loss with almost half of those probands reporting another family member with otosclerosis. Furthermore, all Ontario probands were screened for the *FOXL1* deletion identified in the NL family, with one testing positive, providing evidence that *FOXL1* may underlie cases of otosclerosis in other populations.

The otosclerotic phenotype of prospective data obtained in a surgically-confirmed Ontario cohort was quite variable with 30% of subjects presenting with unilateral otosclerosis and 9% presenting with sensorineural hearing loss (SNHL) in their non-surgical ear. Results suggest that distortion product otoacoustic emissions and acoustic reflex thresholds are absent in all surgical ears, SNHL ears and ears with a conductive hearing loss. To further enhance the advanced phenotyping of otosclerosis, power absorbance (PA) was analyzed to determine its utility as a phenotyping tool. Results suggest that PA has a valid test-retest reliability, but that instrument and stimulus effects are present.

Advanced phenotyping was used to develop a predictive model for *FOXL1*-associated otosclerosis suggesting a progressive mixed hearing loss. Phenotyping in a second large, non-*FOXL1* family identified members with suspected early disease progression. Early identification of otosclerosis, without having to wait for a surgical confirmation, will aid future gene discovery research. Furthermore, insights gained from advanced phenotyping in sub-clinical gene carriers can provide a deeper understanding of the natural history of otosclerosis.

Keywords

immittance, middle ear analysis, power absorbance, reflectance, wideband acoustic immittance, genetics, phenotyping, advanced phenotyping, otosclerosis, hearing disorder

Summary for Lay Audience

Otosclerosis is a heritable bone disorder characterized by abnormal bone growth within the ear occurring later-in-life. When the abnormal bone growth invades the middle ear, it results in a conductive hearing loss, however, it can cause a sensorineural hearing loss if the abnormal bone growth invades the cochlea. Recently, the first causative gene (*FOXL1)* for the disorder was identified in a large Newfoundland family. In an Ontario otosclerosis population, *FOXL1* was screened, and one subject was identified with the mutation, providing evidence that the *FOXL1* mutation occurs outside of the family where it was originally found. Advanced phenotyping, using clinical tools to measure the physiological changes of a trait, was used in an Ontario otosclerotic population, as well as in two large families with otosclerosis. Results from the Ontario otosclerotic population confirmed that the clinical presentation of otosclerosis is variable. Subjects had hearing loss in one or both ears, as well as the conductive or sensorineural form of the hearing loss, or a combination of the two referred to as a mixed hearing loss. A normative study was conducted to investigate whether, power absorbance (PA), a measure of how sound can travel through the middle ear, is a valid phenotyping tool for otosclerosis. Results suggest that PA is different in otosclerotic ears compared to typical ears and could be used for future phenotyping studies. Advanced phenotyping of a large Newfoundland family with family members carrying the mutation in *FOXL1* gene suggests that the clinical features of otosclerosis can present quite variably even when the same gene is responsible for the hearing loss. A predictive model of otosclerosis caused by *FOXL1* was also created which suggests a progressive mixed hearing loss in affected gene carriers. Finally, advanced phenotyping was conducted in a separate large otosclerotic Newfoundland family of unknown genetic cause. Results suggest that using an advanced phenotyping approach has the possibility of improving future genetic and

clinical studies of otosclerosis by possibly identifying early indicators of otosclerosis progression.

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Chapter 1

1 Overview of Thesis

1.1 Rationale

Otosclerosis (OTSC) is a common hearing disorder associated with abnormal sclerotic bone growth within the otic capsule and is found in approximately 2.5% of the population (Crompton et al., 2019; Declau et al., 2001; Sakihara & Parving, 1999). One feature of otosclerosis is hearing loss due to the sclerotic bone growth around the stapes, causing stapes fixation, or invading the cochlea causing sensorineural hearing loss (Batson & Rizzolo, 2017). In many cases, both conductive hearing loss and sensorineural hearing loss can be present, a clinical phenomenon called mixed hearing loss (Crompton et al., 2019; Ishai, Halpin, Shin, McKenna, & Quesnel, 2016; Sakihara & Parving, 1999; Schuknecht & Barber, 1985). However, hearing loss due to otosclerosis is not present in all cases with otosclerosis. Hearing loss caused by otosclerosis is estimated to be present in 0.3-0.4% of the Caucasian population (Declau et al., 2001), while the presence of histological otosclerosis without hearing loss is much higher, around 3.5% as determined by post-mortem investigations of temporal bones (Declau et al., 2001; Schuknecht & Barber, 1985). Otosclerosis is heritable in approximately half of all cases and transmitted in an autosomal dominant manner, requiring one copy of the affected allele to exhibit the hearing loss (Thys & Camp, 2009). Research into the genetic etiology of otosclerosis has identified ten regions within the human genome, named OTSC 1-10, which may be separately responsible for monogenic (caused by mutations in a single gene) forms of the disorder. These genetic loci, OTSC1-10, have been identified in large multiplex families, where multiple 1st and 2nd degree family members present with otosclerosis. Currently OTSC 6 and 9 are reserved but not yet published (Bel Hadj Ali et al., 2008; Brownstein, Goldfarb, Levi,

Frydman, & Avraham, 2006; Chen et al., 2002; Pauw et al., 2006; Schrauwen et al., 2011; Thys, Van Den Bogaert, et al., 2007; Tomek et al., 1998; Van Den Bogaert et al., 2001). Despite years of research, none of these OTSC loci yielded a causative gene until Abdelfatah (2014) identified *FOXL1*, the first gene underlying autosomal dominant otosclerosis in a large Newfoundland family.

It is important to characterize the phenotypic features of otosclerosis, both in genetic and sporadic forms (unknown etiology). Currently, genetic studies of families with otosclerosis have relied on surgical confirmation of otosclerotic bone growth or audiometric thresholds showing significant conductive or mixed hearing loss, in order to identify family members affected by otosclerosis (Bel Hadj Ali et al., 2008; Brownstein et al., 2006; Chen et al., 2002; Thys, Van Den Bogaert, et al., 2007; Tomek et al., 1998; Van Den Bogaert et al., 2001; Weegerink et al., 2011), with few considering additional phenotyping measurements of middle ear muscle reflexes or otoacoustic emissions (Bel Hadj Ali et al., 2008; Van Den Bogaert, 2004; Weegerink et al., 2011), or advanced temporal bone imaging (Bel Hadj Ali et al., 2008). Comprehensive phenotyping beyond pure tone audiometry or surgical confirmation could facilitate the discovery of other gene mutations causing otosclerosis. Physiological measures of auditory system function, such as middle ear muscle reflexes or otoacoustic emissions (Bel Hadj Ali et al., 2008; Van Den Bogaert, 2004; Weegerink et al., 2011) and temporal bone imaging using high resolution computed tomography (Bel Hadj Ali et al., 2008), have been used clinically and in otosclerosis research, but not systematically applied in genetic studies of otosclerosis.

Advanced phenotyping of otosclerosis can also have a significant impact on the discovery of other genetic mutations responsible for the development of otosclerosis in families inheriting an autosomal dominant form. This is achieved by identifying individuals with otosclerotic

hearing loss in the family versus those unaffected or with hearing loss due to another etiology. The current gold standard for a confirmed diagnosis of otosclerosis is through corrective surgery (Quesnel et al., 2013). However, not all cases of otosclerotic hearing loss require, or can be resolved by corrective surgery, as is the case with cochlear otosclerosis (Cureoglu, Yildirim, & Paparella, 2010; Doherty & Linthicum, 2004; Schuknecht & Kirchner, 1974). The use of advanced phenotyping measurements promises to improve the accuracy of otosclerosis diagnosis in families with suspected otosclerosis to ensure the proper diagnosis is given to each case.

Furthermore, the early identification of genetic mutations causing otosclerosis can improve the clinician's ability to identify individuals at risk of developing this progressive disease. Once a causative genetic mutation is identified, a clinical genetic test could be developed to identify whether the mutation is present within an individual or within a family (Abdelfatah, 2014; Ealy & Smith, 2010; Ealy, 2011; Morrison, 1967). Advanced phenotyping can be used in conjunction with genetic testing to determine whether carriers of the mutation demonstrate any early indicators or sub-clinical features of the development of the hearing loss. Since otosclerosis causes a later onset hearing loss, typically developing between the third and fifth decade of life (Crompton et al., 2019; Gordon, 1989; Rudic et al., 2015), incorporating advanced phenotyping with genetic testing could improve clinical care by monitoring the progression of the disorder, and choosing the appropriate clinical interventions or future treatments based on this progression.

As the genes underlying genetic forms of otosclerosis are identified, so too will the creation and implementation of treatments to slow, stop or reverse hearing loss associated with otosclerosis. Advanced phenotyping will aid in the early identification of patients with sub-clinical symptoms and be used to track progression of the disease, which are important for

patient management and monitoring treatment efficacy. In the case of known genetic mutations, advanced phenotyping will contribute to our understanding of the natural history and clinical course of the disorder.

Up to this point, the use of various treatments for otosclerosis have been investigated with no substantial evidence of success. For example, fluoride was proposed as a treatment method to slow the progression of otosclerosis-associated hearing loss due to its ability to decrease osteoclast activation and therefore slow the bone resorption pathway (Cruise, Singh, & Quiney, 2010; House & Linthicum, 1974; Liktor, Szekanecz, Batta, Sziklai, & Karosi, 2013). With advances in knowledge about the genetic etiology of otosclerosis and the underlying pathological mechanisms of otosclerosis revealed, there will undoubtedly be new treatment options for otosclerosis beyond a surgical one. Advanced phenotyping measures will be useful outcome measures for investigating the efficacy of novel gene therapy or pharmaceutical treatments for preventing or treating otosclerosis.

The main purpose of this research is to improve the advanced phenotyping of otosclerosis in order to facilitate future gene discovery studies of otosclerosis and enhance our understanding of the natural history and variability of otosclerosis.

The specific aims are to:

1. Investigate the value of advanced phenotyping features in genetic studies of otosclerosis, by investigating a clinical population diagnosed with otosclerosis, and two families with heritable forms of otosclerosis.

2. Address whether the gene mutation causing otosclerosis in a large multigenerational family in Newfoundland (NL) is present in a clinical population from Ontario (Canada).

1.2 Organization of Thesis

The thesis is organized into seven chapters including this introductory chapter. Chapter 2 reviews the literature covering relevant topics of this thesis. Relevant topics include the clinical presentation of otosclerosis, middle ear measurements including wideband acoustic immittance and the genetics of otosclerosis. The advanced phenotype, family history and genotype of otosclerosis are explored in an Ontario population with clinical diagnosis of otosclerosis in Chapter 3. In Chapter 4, wideband acoustic immittance (WAI) will be evaluated as a potential phenotypic measurement of middle ear function, focusing on instrument and stimulus level effects, and test-retest reliability, in a normal hearing population and an otosclerotic cohort. The utility of WAI as an advanced phenotypic measurement in conjunction with other auditory phenotyping tools will then be applied to two families with otosclerosis. The first family study, the large NL family with the known mutation causing otosclerosis is presented in Chapter 5. Phenotyping of this family will provide the natural history and clinical course of otosclerosis due a known genetic mutation. The second family presented in Chapter 6 is another large family from NL with heritable otosclerosis of unknown etiology. An advanced phenotyping approach was used to characterize the auditory phenotype of family members to facilitate segregation for the purpose of future gene discovery research.

1.3 References

Abdelfatah, N. (2014). The Genetic Aetiology of Otosclerosis in the Population of Newfoundland and Labrador. Memorial University of Newfoundland.

Batson, L., & Rizzolo, D. (2017). Otosclerosis: An update on diagnosis and treatment. Journal of the American Academy of Physician Assistants, 30(2), 17–22. https://doi.org/10.1097/01.JAA.0000511784.21936.1b

Bel Hadj Ali, I., Thys, M., Beltaief, N., Schrauwen, I., Hilgert, N., Vanderstraeten, K., … Van Camp, G. (2008). A new locus for otosclerosis, OTSC8, maps to the pericentromeric region of chromosome 9. Human Genetics, 123(3), 267–272. https://doi.org/10.1007/s00439-008-0470-3

Brownstein, Z., Goldfarb, A., Levi, H., Frydman, M., & Avraham, K. B. (2006). Chromosomal mapping and phenotypic characterization of hereditary otosclerosis linked to the OTSC4 locus. Archives of Otolaryngology--Head & Neck Surgery, 132(4), 416–424. https://doi.org/10.1001/archotol.132.4.416

Chen, W., Campbell, C. A., Green, G. E., Van Den Bogaert, K., Komodikis, C., Manolidis, L. S., … Smith, R. J. H. (2002). Linkage of otosclerosis to a third locus (OTSC3) on human chromosome 6p21.3-22.3. Journal of Medical Genetics, 39(7), 473–477. https://doi.org/10.1136/jmg.39.7.473

Crompton, M., Cadge, B. A., Ziff, J. L., Mowat, A. J., Nash, R., Lavy, J. A., … Dawson, S. J. (2019). The Epidemiology of Otosclerosis in a British Cohort. Otology & Neurotology, 40(1), 22–30. https://doi.org/10.1097/MAO.0000000000002047

Cruise, a S., Singh, a, & Quiney, R. E. (2010). Sodium fluoride in otosclerosis treatment: review. The Journal of Laryngology and Otology, 124(6), 583–586. https://doi.org/10.1017/S0022215110000241

Cureoglu, S., Yildirim, M. B., & Paparella, M. M. (2010). Cochlear Otosclerosis. Curr Opin Otolaryngol Head Neck Surg, 18(5), 357–362. https://doi.org/10.1097/MOO.0b013e32833d11d9.Cochlear

Declau, F., Van Spaendonck, M., Timmermans, J. P., Michaels, L., Liang, J., Qiu, J. P., & Van de Heyning, P. (2001). Prevalence of Otosclerosis in an Unselected Series of Temporal Bones. Otology & Neurotology, 22(5), 596–602. https://doi.org/10.1097/00129492-200109000-00006

Doherty, J. K., & Linthicum, F. H. (2004). Spiral Ligament and Stria Vascularis Changes in Cochlear Otosclerosis: Effect on Hearing Level. Otology & Neurotology, 25(4), 457–464. https://doi.org/10.1097/00129492-200407000-00010

Ealy, Megan, & Smith, R. J. H. (2010). The genetics of otosclerosis. Hearing Research, 266(1–2), 70–74. https://doi.org/10.1016/j.heares.2009.07.002

Ealy, ML. (2011). Otosclerosis-identifying genetic contributions to a complex hearing disorder. University of Iowa. Retrieved from http://ir.uiowa.edu/etd/956/

Gordon, M. (1989). The Genetics of Otosclerosis: A Review. The American Journal of Otology, 10, 426–438.

House, H. P., & Linthicum, F. H. (1974). Sodium fluoride and the otosclerotic lesion. Archives of Otolaryngology (Chicago, Ill. : 1960), 100(6), 427–430. https://doi.org/10.1001/archotol.1974.00780040441004

Ishai, R., Halpin, C. F., Shin, J. J., McKenna, M. J., & Quesnel, A. M. (2016). Long-term Incidence and Degree of Sensorineural Hearing Loss in Otosclerosis. Otology & Neurotology, 37(10), 1489–1496. https://doi.org/10.1097/MAO.0000000000001234

Liktor, B., Szekanecz, Z., Batta, T. J., Sziklai, I., & Karosi, T. (2013). Perspectives of pharmacological treatment in otosclerosis. European Archives of Oto-Rhino-Laryngology : Official Journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : Affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery, 270(3), 793–804. https://doi.org/10.1007/s00405-012-2126-0

Morrison, A. W. (1967). Genetic factors in otosclerosis. Annals of the Royal College of Surgeons of England, 41(2), 202–237. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2311999/

Pauw, R. J., De Leenheer, E. M. R., Van Den Bogaert, K., Huygen, P. L. M., Van Camp, G., Joosten, F. B. M., & Cremers, C. W. R. J. (2006). The phenotype of the first otosclerosis family linked to OTSC5. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 27(3), 308–315. Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/lary.21463/full

Quesnel, A. M., Moonis, G., Appel, J., O'Malley, J. T., McKenna, M. J., Curtin, H. D., & Merchant, S. N. (2013). Correlation of computed tomography with histopathology in otosclerosis. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 34(1), 22–28. https://doi.org/10.1097/MAO.0b013e318277a1f7

Rudic, M., Keogh, I., Wagner, R., Wilkinson, E., Kiros, N., Ferrary, E., … Zarkovic, N. (2015). The pathophysiology of otosclerosis: Review of current research. Hearing Research, 330, 51–56. https://doi.org/10.1016/j.heares.2015.07.014

Sakihara, Y., & Parving, A. (1999). Clinical otosclerosis, prevalence estimates and spontaneous progress. Acta Oto-Laryngologica, 119(4), 468–472. https://doi.org/10.1080/00016489950181017

Schrauwen, I., Weegerink, N., Fransen, E., Claes, C., Pennings, R., Cremers, C., … Van Camp, G. (2011). A new locus for otosclerosis, OTSC10, maps to chromosome 1q41-44. Clinical Genetics, 79(5), 495–497. https://doi.org/10.1111/j.1399-0004.2010.01576.x

Schuknecht, H. F., & Barber, W. (1985). Histologic variants in otosclerosis. The Laryngoscope, 95(11), 1307–1317. https://doi.org/10.1288/00005537-198511000-00003

Schuknecht, H. F., & Kirchner, J. C. (1974). Cochlear otosclerosis: fact or fantasy. The Laryngoscope, 84(5), 766–782. https://doi.org/10.1288/00005537-197405000-00008

Thys, M., & Camp, G. Van. (2009). Genetics of Otosclerosis. Otology & Neurotology, 30(8), 1021–1032. https://doi.org/10.1097/MAO.0b013e3181a86509

Thys, M., Van Den Bogaert, K., Iliadou, V., Vanderstraeten, K., Dieltjens, N., Schrauwen, I., … Van Camp, G. (2007). A seventh locus for otosclerosis, OTSC7, maps to chromosome 6q13–16.1. European Journal of Human Genetics, 15(3), 362–368. https://doi.org/10.1038/sj.ejhg.5201761

Tomek, M. S., Brown, M. R., Mani, S. R., Ramesh, A., Srisailapathy, C. R., Coucke, P., … Smith, R. J. (1998). Localization of a gene for otosclerosis to chromosome 15q25-q26. Human Molecular Genetics, 7(2), 285–290. https://doi.org/10.1093/hmg/7.2.285

Van Den Bogaert, K. (2004). A fifth locus for otosclerosis, OTSC5, maps to chromosome 3q22-24. Journal of Medical Genetics, 41(6), 450–453. https://doi.org/10.1136/jmg.2004.018671

Van Den Bogaert, Kris, Govaerts, P. J., Schatteman, I., Brown, M. R., Caethoven, G., Offeciers, F. E., … Van Camp, G. (2001). A second gene for otosclerosis, OTSC2, maps to chromosome 7q34-36. American Journal of Human Genetics, 68(2), 495–500. https://doi.org/10.1086/318185

Weegerink, N. J. D., Schrauwen, I., Huygen, P. L. M., Pennings, R. J. E., Cremers, C. W. R. J., Van Camp, G., & Kunst, H. P. M. (2011). Phenotype of the first otosclerosis family linked to OTSC10. The Laryngoscope, 121(4), 838–845. https://doi.org/10.1002/lary.21463

Chapter 2

2 Literature Review

2.1 Pathology of Otosclerosis

Otosclerosis, first described by Politzer (1894), is a bone remodeling disorder and is characterized by the abnormal hardening of bone growth within the otic capsule. The term otosclerosis can be broken down to the root words *oto,* meaning “in the ear”, and *sclerosis,* referring to abnormal hardening of tissue. The hardened bone frequently occurs around the stapes resulting in fixation of the stapes footplate causing an increase in the stiffness of the middle ear system. The stiffening of the middle ear system is responsible for the characteristic conductive hearing loss associated with the disorder (Cherukupally, Merchant, & Rosowski, 1998; Vittorio Colletti, Fiorino, Sittoni, & Policante, 1993; Hannley, 1993; Shahnaz & Polka, 1997; Fei Zhao et al., 2002), however there are variations to the profile of the disorder, which can include sensorineural, mixed, or no hearing loss (Declau et al., 2001; Schuknecht & Barber, 1985; Schuknecht & Kirchner, 1974; Uppal, Bajaj, Rustom, & Coatesworth, 2009). It was proposed by Schuknecht & Kirchner (1974) that three separate definitions of otosclerosis be used. These include clinical otosclerosis, cochlear otosclerosis and histological otosclerosis. Clinical otosclerosis represents a form of the disease-causing stapes fixation resulting in conductive hearing loss. Cochlear otosclerosis is the form of otosclerosis where the otosclerotic foci has replaced a portion of the endosteal layer of bone of the cochlea resulting in a sensorineural hearing loss (Cureoglu et al., 2010; Quesnel et al., 2013; Schuknecht & Kirchner, 1974; Shambaugh, 1965). Finally, histological otosclerosis is the form where the sclerotic foci do not invade the stapes or the cochlea, yet, there is one or more otosclerotic foci located in the bony labyrinth. The cases with histological otosclerosis do not exhibit any associated hearing loss, or

clinical symptoms, since the sclerotic foci are found in the bony labyrinth (McKenna & Merchant, 2010; Schuknecht & Barber, 1985).

Otosclerosis is a common type of hearing loss with prevalence rates at 2.5% of the general population with some form of histological otosclerosis (based on temporal bone histology), and 0.30-0.38% of who exhibit the symptoms of clinical otosclerosis (Cawthorne, 1955; Declau et al., 2001; Shambaugh, 1965). The disorder is highly heritable, whereby it is estimated that approximately 50% of cases are caused by an inherited genetic change (Crompton et al., 2019; Shin et al., 2001). The average age of onset of otosclerosis is typically in the third and fourth decade (Gordon, 1989), however, the actual age of onset is difficult to recognize due to a number of factors. The progression of otosclerosis varies greatly between individuals, and the onset of hearing loss can be sudden, or progress slowly over time (Crompton et al., 2019; Sakihara & Parving, 1999). When the hearing loss progresses slowly over time, an individual may be less likely to notice their hearing loss, and therefore delay diagnosis of clinical otosclerosis until well after the first signs of hearing loss have appeared.

There is no observable osteoclast or osteoblast activity within the normal otic capsule, suggesting slow bone resorption and slow bone turnover rates (Frisch & Overgaard, 2000). However, in the case of otosclerosis, there is an imbalance to the normal bone resorption pathway, resulting in an increase in osteoblast and osteoclast activity (Chole & McKenna, 2001; Declau et al., 2001). The etiopathogenesis of otosclerosis remains a mystery. There are a number of studies investigating the pathogenesis of the disorder, suggesting many contributing factors which include genetic predisposition, autoimmune factors, hormonal pathways, metabolic influences, and inflammatory mechanisms (Karosi & Sziklai, 2010).

The progression of otosclerosis has been described in three phases; early phase, transitional phase and late phase (reviewed by Rudic et al., 2015). Early phase otosclerosis is characterized as the active phase of the disease involving otospongiosis. Otospongiosis is the formation of spongy bone in the bony labyrinth of the ear. The formation of spongy bone occurs due to active osteoclasts resorbing bone, causing lesions around the otic capsule. The lesions become highly vascularized which open the way for increased osteocyte activity (Karosi, Csomor, & Sziklai, 2012; Parahy & Linthicum, 1984; Schuknecht & Barber, 1985). Areas of highly vascularized lesions and increased osteocyte activity become deprived of mature collagen, and thus give rise to spongy bone growth (Rudic et al., 2015).

The late phase of otosclerosis involves the formation of sclerotic or dense bone in the previously spongy bone growth areas of the otic capsule which characterize early phase otosclerosis (Rudic et al., 2015). The term sclerosis simply refers to the hardening of tissue. It is the advancement of sclerotic bone that causes stapes fixation and the classic form of conductive hearing loss associated with clinical otosclerosis. However, there is variation in the localization of sclerotic bone within the otic capsule, resulting in different types of hearing loss (conductive, mixed, sensorineural).

When the otosclerotic foci invade the cochlear endosteum, a sensorineural component to the hearing loss can arise (Schuknecht & Kirchner, 1974). It is suggested that otosclerotic foci can cause atrophy of the spiral ligament and stria vascularis, which can prevent ion recycling and alter the endocochlear potential required for proper cochlear hair cell function (Doherty & Linthicum, 2004; McKenna & Merchant, 2010). Therefore, when the invasion of otosclerotic foci reach the cochlea, a sensorineural component to the hearing loss can arise, resulting in a mixed or sensorineural hearing loss in patients with otosclerosis. It is estimated that cochlear

otosclerosis is responsible for approximately 1% of pure sensorineural hearing losses (Quesnel et al., 2013; Schuknecht & Kirchner, 1974).

2.2 Clinical Presentation of Otosclerosis

The diagnosis of otosclerosis can only be *confirmed* at the time of corrective surgery for stapes fixation but can be *inferred* clinically based on clinical symptoms. However, other hearing disorders can clinically mimic otosclerosis, such as superior canal dehiscence (Merchant, Rosowski, & McKenna, 2007) or enlarged vestibular aqueduct syndrome (Wieczorek, Anderson, Harris, & Mikulec, 2013). An *inferred* diagnosis of stapes fixation due to otosclerosis is based on the profile of otoscopy, tympanometry, pure-tone hearing thresholds and acoustic reflexes (Emmett, 1993).

Otoscopy is conducted by looking into the ear canal towards the tympanic membrane using an otoscope. In otosclerosis, the tympanic membrane may appear normal, however early in the progression of the disorder, a reddish hue may be seen through the tympanic membrane (Figure 1). This reddish hue, sometimes referred to as the Schwartz sign, suggests increased vascularization within the middle ear associated with early stages of otosclerosis (Emmett, 1993; Hannley, 1993; Purohit, Hermans, & Op de beeck, 2014).

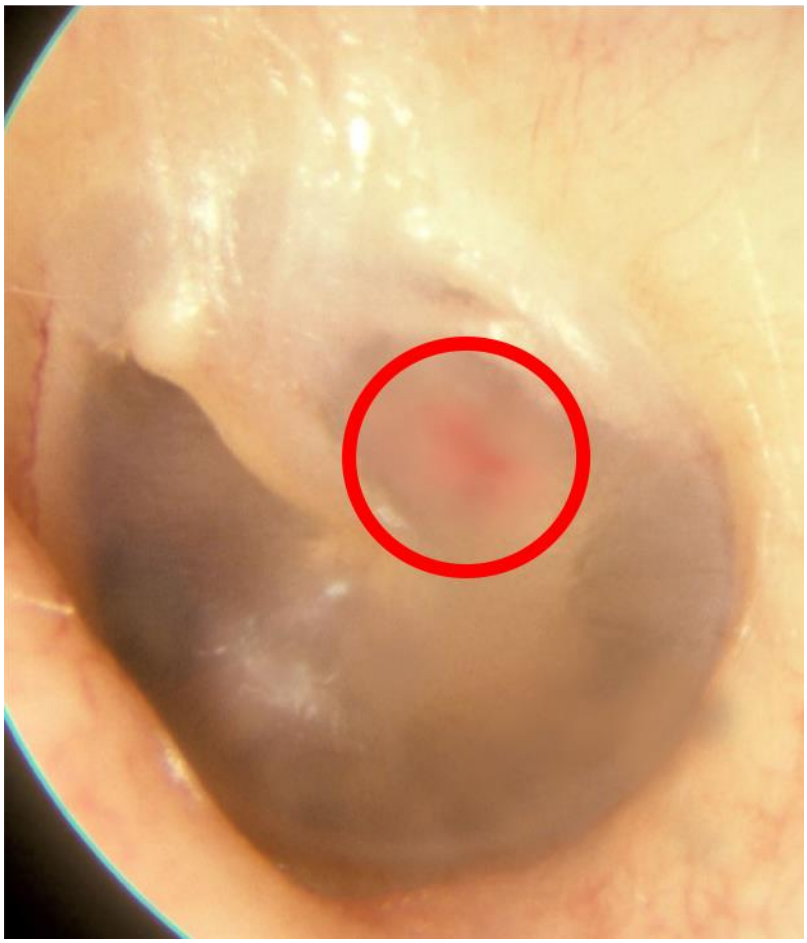


Figure 1. Otoscopy image of the reddish hue referred to as the "Schwartz Sign". Schwartz sign is circled in red. Image edited from: https://commons.wikimedia.org/wiki/File:Normal_Left_Tympanic_Membrane.jpg#globalusage

Otosclerotic ears tend to have the typical 'A' or a shallower 'As' shape tympanogram. An 'A' shape tympanogram suggests maximum tympanic membrane compliance, or mobility, around 0 dekapascals, daPa, or equal pressure in the external and middle ear. An 'As' tympanogram may be present in otosclerotic ears suggesting stiffness of the middle ear system. This increased stiffness can therefore translate to a shallower compliance peak. However, conventional tympanometry using a 226 Hz stimulus is not sensitive enough to distinguish otosclerotic ears from normal ears (Shahnaz, Bork, et al., 2009; Shahnaz & Polka, 1997).

Early in the development of otosclerosis, a low frequency conductive loss may begin to appear below 2 kHz (Emmett, 1993; Hannley, 1993; Meranger, David, Beigner, Charpiot, & Tavernier, 2019). A conductive hearing loss is diagnosed when air-conduction thresholds are elevated compared to bone-conduction thresholds. The difference between air-conduction thresholds and bone-conduction thresholds are referred to as the air-bone gap (ABG). Clinically, a conductive hearing loss is when the ABG is greater than 10 dB at three neighbouring frequencies. Otosclerosis may also cause a mixed or a sensorineural hearing loss (SNHL) depending on the histological presentation of the disorder which can progress into advanced otosclerosis, with air-conduction thresholds reaching the profound level (Calmels et al., 2007). Mixed hearing loss is diagnosed when there is an ABG of greater than 10 dB at three neighbouring frequencies and the bone-conduction thresholds are elevated compared to normal, above 20 dB HL. A SNHL occurs when there is no ABG or an ABG of less than 10 dB, however the bone-conduction thresholds are above the normal threshold level of 20 dB HL. Clinical otosclerosis can be present either bilaterally or unilaterally, with bilateral otosclerosis occurring in between 47-76% of otosclerosis cases (Hueb, Goycoolea, Paparella, & Oliveira, 1991; Khorsandi, Jalali, & Shoshi, 2018).

Otosclerotic ears fail to have a stapedius muscle contraction in the presence of a loud stimulus because of the increased stiffness of the middle ear. This increase stiffness results in absent or elevated acoustic reflex thresholds (ARTs) (Hannley, 1993; Keefe et al., 2017; Terkildsen, Osterhammel, & Bretlau, 1973). ARTs are a measure of the movement of the tympanic membrane in response to an ipsilateral or contralateral stimulus. When a loud stimulus is presented (85-110 dB SPL), the stapedius muscle is stimulated, causing contraction.

The variability of clinical features of otosclerosis has been well known for decades. Extensive histological studies have demonstrated the variability of otosclerotic foci within large populations of otosclerotic subjects (Lagleyre et al., 2009; Schuknecht & Barber, 1985; Schuknecht & Kirchner, 1974). These histological variations have been linked to differences in the clinical presentation of the disorder, where patients have demonstrated unilateral or bilateral hearing loss, either as a purely conductive hearing loss, mixed hearing loss, or sensorineural hearing loss (Cherukupally et al., 1998; Ramsay & Linthicum, 1994; Shambaugh, 1965). Reports also indicate that the disorder can be present without any notable clinical features, described as histological otosclerosis (Schuknecht & Barber, 1985). The accurate diagnosis of otosclerosis is crucial for genetic studies of otosclerosis to appropriately identify affection status (affected versus unaffected).

There is an approximate 2:1 ratio in the incidence of otosclerosis in females to males (Cawthorne, 1955; Ishai et al., 2016; Khorsandi et al., 2018; Marchese et al., 2009). There is evidence suggesting that hearing thresholds in females may become worse following pregnancy (Cawthorne, 1955; Crompton et al., 2019; Morrison, 1967). The increased incidence of otosclerosis in females, specifically following pregnancy, has led to the hypothesis that hormone pathways may influence the etiology of the disorder (Crompton et al., 2019; Imauchi et al., 2008). However, recent case-controlled cohort studies comparing hearing thresholds of otosclerotic women with children to those without children found no effects of pregnancy on pre-surgical hearing thresholds (Lippy, Berenholz, Schuring, & Burkey, 2005) or pre and post-operative functional outcomes (Marchese et al., 2009). Furthermore, Crompton et al. (2019) compared age of onset and hearing levels between a cohort of female otosclerosis patients who had a history of pregnancy (n=313) and those who had no history of pregnancy (n=96). They

found no significant difference between groups for age of onset or hearing levels, thus providing further evidence that pregnancy does not elevate the risk of developing otosclerosis. Therefore, while the reported number of women affected with otosclerosis is approximately 2:1 compared to males, and there are reports of otosclerosis occurring post-pregnancy, the evidence is still mixed. It is possible that there is a sub-group of otosclerotic females where the hormonal changes associated with pregnancy may increase the progression of hearing loss. In this case, genes involved in the hormone pathways may contribute to the development and progression of otosclerosis. The genetics of sex-specific differences in otosclerosis development is an interesting area for further study.

2.3 Genetics of Otosclerosis

Familial conductive hearing loss was first described in the 19th century by Toynbee (1841). In familial cases, otosclerosis is usually reported as an autosomal dominant pattern. A century after the initial investigation of otosclerosis by Toynbee, a retrospective study conducted by Cawthorne (1955) revealed that 54% of British otosclerotic patients disclosed that they had one or more family member affected by a similar type of hearing impairment. More recently, 24% of an otosclerotic cohort from France, comprised of 211 surgically-confirmed patients, reported having a family history of otosclerosis (Shin et al., 2001). Patients were asked via a questionnaire whether they had a family member who was also diagnosed with otosclerosis. Another 27% of their cohort reported a family history of hearing loss of unknown pathology. This would suggest that approximately half of otosclerosis cases would be sporadic.

Genome wide association studies have been used to identify genes associated with sporadic cases and use a case-control approach to determine if there are significant differences in variant frequencies between cases and controls. Several genes identified as potentially important

include: *COL1A1*, *RELN*, *TGFB1*, *BMP2*, *BMP4*, *AGT* and *ACE* (Rodríguez et al. 2004; Imauchi et al. 2008; Schrauwen et al. 2009; Khalfallah et al. 2011; Schrauwen et al. 2012; Ealy et al. 2014; Sommen et al. 2014; Mowat et al. 2018). A summary of the associated genes of otosclerosis are presented in Table 1 and are also reviewed by Ealy & Smith (2010) and Thys & Van Camp (2009).

Table 1. Summary of associated genes of otosclerosis identified through genome-wide association studies. Summary of review by Ealy & Smith (2010).

Associated Gene	Replicated	Expression/Function	Reference
COL1A1	Yes	Codes for alpha-1 type 1 collagen protein which is a component of type 1 collagen. Hypothesized to change bone remodeling process in otic capsule.	McKenna et al. 1998; Rodríguez et al. 2004; Khalfallah et al. 2011; Schrauwen et al. 2012; Ertugay et al. 2013; Mowat et al. 2018
RELN	Yes	Codes protein called reelin. Reelin is an extracellular matrix protein which is involved in the regulation of interactions between neurons and glia. Reelin is also hypothesized to be important for varying cell adhesion.	Schrauwen et al. 2009; Sommen et al. 2014; Mowat et al. 2018
TGFB1	Yes	Encodes TGFB1 protein which is involved in the embryogenesis in the otic capsule. Involved in stimulating matrix protein synthesis.	Schrauwen et al. 2008; Khalfallah et al. 2011; Sommen et al. 2014; Mowat et al. 2018
BMP2	Yes	Part of the TGF-β superfamily. *BMP2* involved in the recruitment and activation of transcription factors of the SMAD family. Expressed in the otic vesicle	Schrauwen et al. 2008; Khalfallah et al. 2011; Sommen et al. 2014; Mowat et al. 2018
BMP4	Yes	Part of the TGF-β superfamily. *BMP4* involved in the recruitment and activation of transcription factors of the SMAD family. Expressed in the otic vesicle.	Schrauwen et al. 2008; Khalfallah et al. 2011; Sommen et al. 2014; Mowat et al. 2018
AGT	No	Part of the renin-angiotensin system. Codes for protein termed angiotensinogen. Angiotensinogen involved in the regulation of blood pressure and body fluid salinity.	Imauchi et al. 2008; Schrauwen et al. 2009; Sommen et al. 2014
ACE	No	Part of the renin-angiotensin system. Codes for an angiotensin-converting enzyme. involved in the regulation of blood pressure and body fluid salinity.	Imauchi et al. 2008; Schrauwen et al. 2009

Otosclerosis is reported as a heritable disorder in approximately half of otosclerosis cases in French and British otosclerotic populations (Crompton et al., 2019; Shin et al., 2001). When a trait is inherited in an autosomal dominant manner, only one copy of the affected allele is

required in order to express the altered phenotype. A summary of the eight chromosomal regions (loci) harboring candidate genes for monogenic forms is presented in Table 2.

Genetic variants in *SERPINF1* were reported as causing otosclerosis in four otosclerotic families: three of European descent and one of European and Caribbean descent (Ziff et al., 2016). However, a re-evaluation of the pathogenic mutations of *SERPINF1* by Valgaeren et al., (2019) using a larger otosclerotic population of 126 patients from 63 families yielded conflicting results raising into the question the pathogenicity of mutations in *SERPINF1*.

Abdelfatah (2014) reported the first gene causing otosclerosis using a large multigenerational family from Newfoundland, Canada. Seven family members with otosclerosis (confirmed via corrective stapes surgery) inherited a 15bp in-frame deletion in *FOXL1*, predicted to remove five highly conserved amino acids from the expressed protein. Functional analysis revealed that the deletion caused down-regulation of several downstream genes, including *ILIA*, *CXCL10, IL29, IFNB1, IFIT1, FEN1* and *SP4.* The identification of the causative gene in familial otosclerosis, *FOXL1*, provides supportive evidence that molecular pathways involved with inflammation and immunity along with anti-angiogenic activity play a role in the development of otosclerosis. Abdelfatah (2014) concluded that candidate genes involved in these pathways should be considered in order to identify other otosclerosis genes.

Table 2. Summary of a selection of candidate genes located within each genetic locus segregating with familial otosclerosis. Summary table created from information reviewed by Ealy & Smith (2010) along with gene information from Ziff et al. (2016) and Abdelfatah (2014).

Locus	Chromosomal Location	Population	# of Genes in Loci	Candidate Genes	Expression/Function	References
OTSC1	15q26.1-qter	India	33	*aggrecan*	Expressed in bony labyrinth, causes hearing loss in mice.	Yoo, Cho, & Yamada, 1991, Tomek et al. 1998
OTSC2	7q34-q36	Belgium	152	*TIFIa*	Involved in bone remodeling.	Van Den Bogaert et al. 2001
				PLOD3	Involved in collagen synthesis.	
OTSC3	6p21.3-22.3	Cyprus	488	HLA	Human leukocyte antigen (HLA) is region of chromosome 6 containing genes for major histocompatibility complex (MHC). Involved in immune system function.	Ealy & Smith 2010
				COL11A2	Expressed in bony labyrinth and previously associated with autosomal dominant hearing loss.	Chen et al. 2002 McGuirt et al. 1999
OTSC4	16q21-23.2	Israel	74	*COG8, COG4*	Involved with cell trafficking.	Brownstein et al. 2006
				DDX19, DDX28, DHX38	Involved in transcription and other RNA activity.	
				ZNF19, ZNF23, ZNRF1, ZFP1	Genes coding zinc finger proteins. Zinc finger proteins are multifunctional proteins.	
OTSC5	3q22-q24	Netherlands	*59*	*PCOLCE2*	Expressed in non-ossified cartilage of developing tissu	Van Den Bogaert et al. 2004
				CHST2	Golgi-associated sulfotransferase. Role in intercellular communication.	
OTSC7	6q13-16.1	Greek Netherlands	*66*	*COL12A1*	Collagen type XII, expressed in the cochlea.	Thys et al., 2007
OTSC8	9p13.1-9q21.11	Tunisia	*24*	*TJP2*	Encodes for a tight junction protein.	Bel Hadj Ali et al. 2008
				TRMP3	Involved in osteoclast function.	
				KLF9	Believed to regulate cranial facial development.	
OTSC10	1q41-44	Netherlands	306	*TGFB2*	Involved in bone remodeling pathway, previously associated with otosclerosis.	Schrauwen et al. 2011
				AGT		
Gene						
SERPINF1	17p13.3	European/ European-Caribbean			Codes for PEDF protein which is involved in anti-angiogenic activity. PEDF inhibits angiogenic activity, reducing formation of new blood vessels.	Ziff et al., 2016
FOXL1	16q24.1	Canada			Transcription factor. Deletion was found to cause down-regulation of several genes involved with cytokine regulation and inflammation.	Abdelfatah, 2014

2.3.1 Challenges in gene identification

Determining the genetic origin of otosclerosis has been difficult. One major reason for the difficulty of identifying pathogenic mutations is the scarcity of multiplex families. Since linkage analysis studies require large multiplex, multigenerational families, and otosclerosis typically develops in the 3rd decade (Schrauwen & Van Camp 2010), identifying families with affected individuals spanning multiple generations can be difficult.

There is also the challenge of penetrance, or the percentage of gene carriers which present with the affected phenotype. When not all gene-carriers express the altered phenotype, such as hearing loss, then the allele is defined as demonstrating incomplete penetrance. Early work by Fowler (1966) studying the presence of otosclerosis in 40 pairs of monozygotic twins, suggests incomplete penetrance as a result of his findings of two twin pairs where one twin does not exhibit a hearing loss. Around the same time, Morrison (1967) calculated penetrance by comparing family histories of otosclerosis and hearing loss of affected probands to the expected number of affected family members assuming complete penetrance. His estimate is a penetrance of approximately 40%. Further, more recent genetic studies have incorporated estimates of penetrance in their analysis of large families with otosclerosis in order to account for the potential reduced penetrance of otosclerosis (Brownstein et al., 2006; Van Den Bogaert et al., 2001). Caution should be used when interpreting these estimates of penetrance of otosclerosis, since the true penetrance level remains unknown until the pathogenic mutations are identified. The challenge of penetrance is further complicated by the unknown variability of age. Since the age of onset of otosclerosis, like many autosomal dominant conditions, can be variable, calculations of penetrance should take into consideration the potential for a delayed onset of the condition.

A further complication affecting the identification of pathogenic mutations for otosclerosis is the potential presence of phenocopies. Phenocopies refer to cases presenting the same phenotype caused by a different etiology. In the case of otosclerosis, this may include tympanosclerosis, Paget's disease, osteogenesis imperfecta, superior canal dehiscence and enlarged vestibular aqueduct syndrome. All of these disorders can present as a conductive hearing loss with absent acoustic reflexes, which is also the standard clinical presentation for otosclerosis. Therefore, most genetic family studies of otosclerosis favour surgical diagnosis to confirm the presence or absence of the disorder, at which point the otosclerotic disease is at an advanced stage (Tomek et al. 1998; Van Den Bogaert et al. 2001; Chen et al. 2002; Brownstein et al. 2006; Pauw et al. 2006; Thys et al. 2007; Bel Hadj Ali et al. 2008; Schrauwen et al. 2011; Weegerink et al. 2011; Abdelfatah 2014).

The variability of clinical presentation in patients with otosclerosis can also be challenging for genetic studies of this disease. Since the location of otosclerotic foci can affect both middle ear and cochlear function, a spectrum of different clinical features is possible. The use of advanced phenotyping has the potential to improve the accuracy of identifying "affected" versus "unaffected" individuals for the purpose of genetic discovery.

2.4 Phenotyping Measurements

2.4.1 Acoustic Immittance

The auditory system is a complex system, wherein physical acoustic stimuli must travel through the external auditory canal, reach the tympanic membrane, pass through and be amplified by the middle ear system. The stimuli are transmitted to the inner ear and transferred into an electrical signal by the cochlea which then propagates through the brainstem and up to

the auditory cortex for processing. The audiological test battery must therefore be sensitive enough to test for deterioration of the auditory signal along the entirety of the auditory system, including the peripheral structures. Distal to the auditory brainstem, the peripheral auditory system can be subdivided into the external ear canal, middle ear and inner ear. The middle ear cavity refers to the tympanic membrane and the ossicles; the incus, the malleus and the stapes. The role of the middle ear is to transform vibrations of the air entering the ear canal into vibrations of the fluid-filled cochlea. As sound travels through the ear canal, the physical vibrations are collected by the tympanic membrane that then sets the ossicles in motion behind it. The hinge-like movement of the ossicles then transmit the energy from the stapes footplate through the oval window of the cochlea. The transmission of sound through the middle ear system occurs in a frequency specific manner, with a maximum mean sound pressure gain of approximately 24 dB at 1.2 kHz and gain slopes of 6 dB/octave below 1.2 kHz and -6 dB/octave above 1.2 kHz (Aibara, Welsh, Puria, & Goode, 2001).

Immittance testing has been widely used for decades to measure the movement of the tympanic membrane to acoustic stimuli (Sanford, Schooling, & Frymark, 2012; Terkildsen & Thomsen, 1959; Wiley et al., 1996; Zwolan, 2010). Immittance is the general term of the mobility of the tympanic membrane that includes impedance (Z), admittance (Y), reflectance (R) and absorbance (A) (reviewed by Rosowski, Stenfelt, & Lilly, 2013). Acoustic impedance, simply stated, refers to the amount of resistance the acoustic source will encounter as it flows through the middle ear system. Alternatively, admittance is the inverse of impedance, and refers to the ease of the acoustic signal to flow through the middle ear system. This concept can be broken down further where acoustic admittance (Y_a) is defined by the relationship $Y_a=G_a+jB_a$, where G_a is the acoustic conductance, and B_a is the acoustic susceptance. Conductance refers to

the ease with which the acoustic stimulus can pass through the system, while susceptance relates to how susceptible the middle ear system is to receiving acoustic stimuli. There are two main components to susceptance, mass susceptance (B_m) and stiffness susceptance (B_s). Mass susceptance is the admittance within the middle ear system due to mass, while stiffness susceptance is the admittance within the middle ear system due to stiffness (Shahnaz, 2007).

Tympanometry is the measure the admittance of the middle ear system. Since the probe is placed within the ear canal, the admittance of the ear canal is also included in the measurement. To overcome this issue, tympanometry is conducted by sweeping pressure, typically from positive (+400 daPa) to negative (-400 daPa). Under extreme pressure situations, the tympanic membrane becomes extremely stiff, causing a decrease in admittance. At these extreme pressures, it is assumed that the measured admittance of the system is that of the ear canal volume because the tympanic membrane acts as a hard wall cavity and the tympanic membrane is not contributing to the overall admittance of the system (Shanks & Lilly, 1981). By subtracting the admittance at extreme positive pressure, the peak compensated static admittance (Y_{tm}), or more simply static admittance is calculated. The static admittance is the admittance of the middle ear system without the inclusion of admittance of air of the ear canal space.

Admittance of the middle ear system changes with respect to frequency. A tone of 226 Hz is typically used for tympanometry. The reason for this is that at 226 Hz a 1.0 cm^3 hard-walled cavity has an admittance of 1.0 mmhos. Conventional 226 Hz tympanometry has been used for decades, where the output of the tympanogram results in values of tympanometric peak pressure (TPP), equivalent ear canal volume (ECV), static compensated acoustic admittance (Y_{tm}) and tympanometric shape as outlined by Jerger (1970) (Figure 2). Tympanograms can be qualitatively described as falling into one of three categories; A, B or C. Type ‘A’ represents a

tympanogram with “normal” admittance, and an observable peak within normal limits. Type ‘A’ tympanograms can be subdivided into types A, As, or Ad. ‘A’ would be classified as a normal tympanogram, however As, would refer to a type ‘A’ looking tympanogram, just with a shallow peak, below normal limits (Margolis & Heller, 1987). Type ‘Ad’ would also have a peak pressure around normal limits with a normal peak, however, the admittance peak would extend above normal limits. Type ‘B’ or a flat tympanogram, is typically characteristic of a middle ear pathology involving fluid or infection. Due to the fluid in the middle ear, the tympanic membrane is more immobile, and does not form the characteristic peak. Type ‘C’ tympanograms have the characteristic peak, but the peak occurs in extreme negative pressure. This type of tympanogram is typically associated with Eustachian tube dysfunction, as the TPP occurs at the pressure which matches the pressure of the middle ear. Therefore, there is a significant negative pressure found in the middle ear.

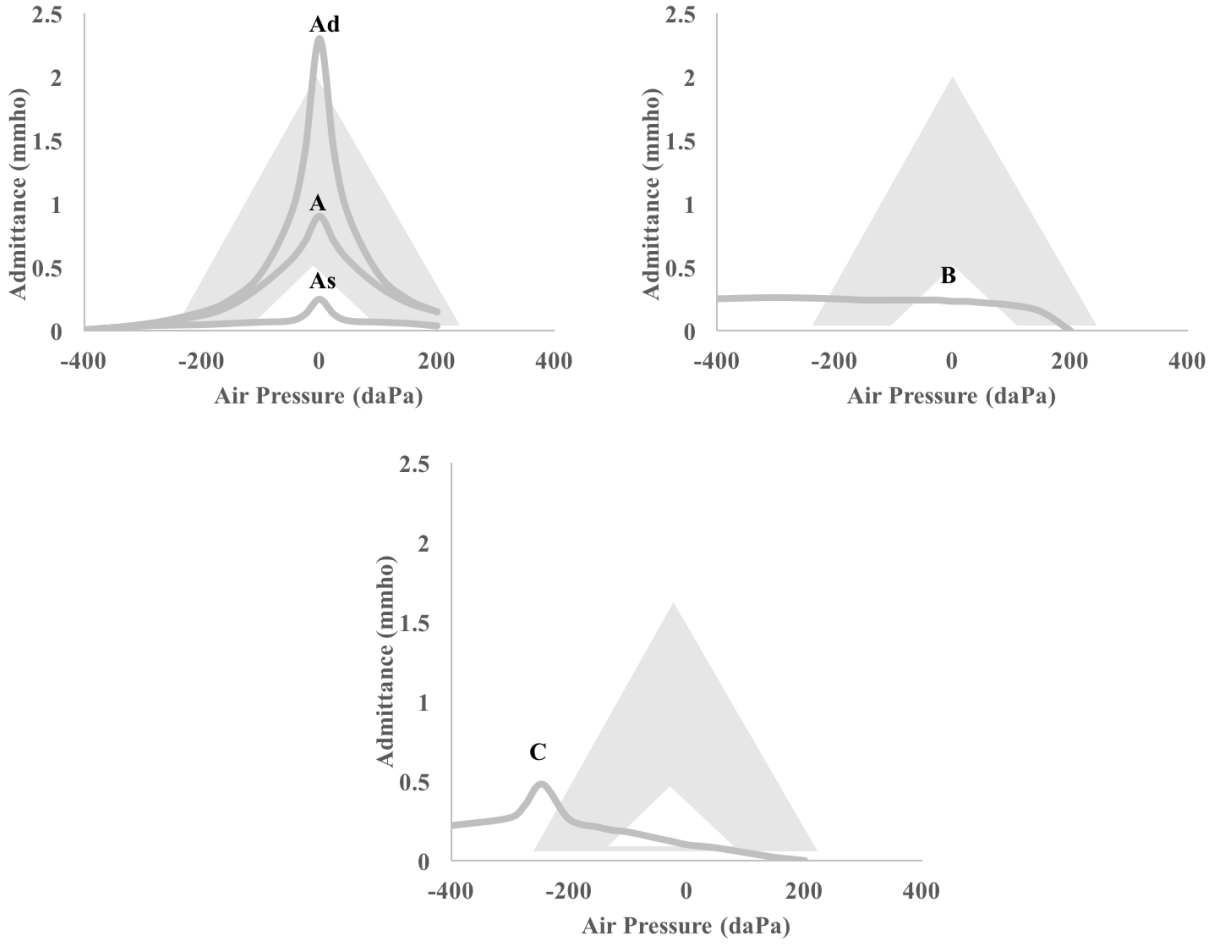


Figure 2. Diagram demonstrating the various qualitative types of tympanograms as described by Jerger (1970). "normal" type 'A' tympanogram, with TPP close to zero, and admittance within normal limits. B) Flat type 'B' tympanogram with no observable peak. C) type 'C' tympanogram with TPP in extreme negative range.

Conventional tympanometry using a 226 Hz probe tone is insensitive to many middle ear pathologies, including otosclerosis (Colletti, 1975; Shahnaz & Polka, 1997). Over the past several decades, wideband stimuli in the measurement of immittance have become more widely used. Improvements to immittance procedures have demonstrated success, specifically in the identification of otosclerosis (Feeney, Grant, & Marryott, 2003; Nakajima, Rosowski, Shahnaz, & Voss, 2013; Ogut, Serbetcioglu, Kirazli, Kirkim, & Gode, 2008; Shahnaz, Bork, et al., 2009; Shahnaz & Polka, 1997) and superior canal dehiscence (Nakajima et al., 2012).

2.4.2 Multifrequency Tympanometry

Tympanometry has been conducted for decades using a probe tone of 226Hz (Lilly, 1984), however a single probe tone of 226Hz fails to capture differences between normal ears and ears with middle ear pathologies affecting the ossicular chain (Lilly, 1984; Shahnaz & Polka, 1997). The use of multifrequency tympanometry has been proposed as a method to increase sensitivity of tympanometric measurements to identify middle ear pathologies (Korres, 2014; Shahnaz, Bork, et al., 2009; Shahnaz & Polka, 1997; Vanaja & Manjula, 2003). One of the objectives of multifrequency tympanometry (MFT) is to measure resonance frequency (RF) which is the frequency where stiffness and mass contribute equally to admittance of the middle ear system. The use of MFT has been demonstrated as a method to differentiate pathologically affected ears from normal ears. For example, in a sample of 68 normal hearing subjects, Shahnaz & Polka (1997) calculated the resonance frequency using a sweep frequency positive tail compensation strategy. They obtained a mean resonant frequency in normal ears of 0.894 kHz (SD = 0.166), compared to 1.142 kHz (SD=0.393) in a group of 4 otosclerotic cases. Since otosclerotic stapes fixation results in the increased stiffness of the middle ear system, the otosclerotic population has an increased resonant frequency compared to the normal population, albeit with a large overlap in values (Shahnaz & Polka, 1997).

Zhao et al. (2002) suggest that this overlap of resonant frequency values between otosclerotic ears and normal ears occurs partly because of the development stage of otosclerosis. When otosclerosis is in the early stage of spongy bone growth referred to otospongiosis, the middle ear system is believed to have lower stiffness compared to later stage otosclerosis when the spongy bone growth hardens to more stiff sclerotic bone. Zhao et al., (2002) discovered approximately 10% of their otosclerotic ears fell into a low stiffness category, characterized by

resonant frequency values of <800 Hz. This overlap of resonant frequency values between otosclerotic ears and normal ears limits the clinical utility of multifrequency tympanometry as a single technique for diagnosing otosclerosis.

2.4.3 Wideband Acoustic Immittance

Wideband acoustic immittance (WAI), like tympanometry is a measurement of acoustic admittance of the middle ear system. However, it differs from tympanometry as it uses wideband chirp or a click stimulus to simultaneously determine the physical properties of the middle ear system across a broad range of sound frequencies. By treating the ear canal as a rigid tube, and presenting a wideband stimulus, the impedance of the middle ear system can be quickly calculated in either a static or dynamic pressure environment.

Power reflectance (PR), also known as energy reflectance, uses a probe placed in the external ear canal to estimate acoustic vibration at the tympanic membrane when set in motion by a wideband acoustic probe stimulus. The power is measured across the frequency range of the wideband probe signal and is measured by the same ear canal probe. Overall, the basic premise behind power reflectance is that residual acoustic energy measured in the ear canal represents the acoustic energy not absorbed by the middle ear. Power reflectance therefore involves measuring the reflected power of the acoustic stimulus across frequencies, and dividing it by the power of the original acoustic stimulus introduced at the probe tip, giving the simple equation:

$$PR = \text{Reflected power/Incident power}$$

This is achieved by relating the *measured* impedance of the ear canal (Z) to the *characteristic* impedance of the ear canal (Z_o). The measured impedance is the impedance measured by the probe tip, whereas the characteristic impedance is the impedance calculated by

dividing the phase velocity of sound from the area of the tube (Keefe, 1992; Voss & Allen, 1994). The assumption with PR is that the ear canal between the probe tip and the tympanic membrane is a rigid, hollow tube. The measured power reflectance results from the ratio between the acoustic power of the wideband acoustic probe signal used to stimulate the middle ear system, and the acoustic power that is reflected from the tympanic membrane and measured at the probe tip. The PR is squared to avoid negative values, and a value between 0-1 is achieved. The absolute amplitude of PR is defined as:

$$PR = ([(Z/Z_{o)} - 1] / [(Z/Z_o)+1])^2$$

A value of 1 represents a situation where all acoustic power is reflected back into the ear canal, whereas a value of 0 represents a situation where no acoustic power is reflected back into the ear canal.

PR is related to power absorbance (PA), as PA=1-PR. PA then refers to the amount of acoustic energy that is absorbed by the middle ear system. The remainder of this thesis will utilize the term power absorbance when referring to reflectance/absorbance of the middle ear system.

Similar to the case of tympanometry, WAI is the technique of using an acoustic stimuli and movement of the TM to estimate the ability of the middle ear to move in response to the stimulus, and thus help differentiate between various middle ear pathologies (Prieve, Feeney, Stenfelt, & Shahnaz, 2013; Shahnaz, Bork, et al., 2009; Shahnaz, Longridge, & Bell, 2009). Keefe, Ling, & Bulen (1992) developed a system to measure PA in humans based on a similar system used by Allen (1986) in cats. Energy reflectance (or 1-PA), was measured at ambient pressure using a wideband stimulus. These early studies set the stage for the two commercial

systems currently available, the Mimosa HearID (Figure 3) and Interacoustics Titan (Figure 4). The Titan allows users to conduct three-dimensional tympanometry measurements in a pressurized environment. A tight-fitting rubberized tip is required with the Interacoustics Titan ear canal probe in order to maintain a hermetic seal while pressurized ear canal measurements are acquired. This system uses a wideband chirp stimulus to acquire WAI data across frequencies of 0.26-8kHz, and across pressures from +200 to -300 daPa, including ambient or tympanometric peak pressure. The Mimosa HearID system is a non-pressurized system capable of measuring PA across the frequency spectrum but at ambient pressure only. The non-pressurized Mimosa HearID system uses an ER-10C foam tip connected to the ear canal probe in order to deliver chirp or tone stimuli and record responses.

When calculating absorbance of the middle ear, the Thévenin equivalent method is adopted in both the Mimosa HearID and the Interacoustics Titan. This method has two main assumptions: 1) there is no loss of acoustic energy along the length of the ear canal wall, and 2) the cross-sectional area of the tympanic membrane at the end of the ear canal is known and does not change. There is a slight difference in the Thévenin calibration process of the Mimosa HearID and Interacoustics Titan. The HearID is conducted using 4 calibration tubes of known length and diameter, whereas the Titan only uses 2. Another difference between the two systems is the way they compensate for ear-canal area. The Mimosa HearID estimates ear-canal area by the size of the probe used, whereas the Interacoustics Titan uses a human average value when compensating for ear-canal area in PA measurements.

The Mimosa HearID system also allows the user to select a stimulus level for the WAI measurement. This is useful for improving the signal-to-noise ratio (SNR) of the measurement by increasing the level of stimulus, and therefore the level of the measured output. Theoretically,

improving the SNR of the measurement should allow for a more reliable response by minimizing the effect of noise on the WAI measurement which in turn could improve the accuracy of WAI.

Figure 3. Image of the Mimosa HearID hardware including the USB audiobox, processing unit, MEPA calibration cavity set and Etymotic ER10C probe. Photo retrieved from http://www.mimosaacoustics.com/product.html with permission from Mimosa Acoustics Inc.

Figure 4. Interacoustics Titan hardware including the Titan handheld device, wideband tympanometry probe attachment and Titan Suite software. Image retrieved from http://www.interacoustics.com/titan with permission from Interacoustics Inc.

Because WAI is a relatively new tool, it is unclear whether the 2 WAI instruments currently available, and their associated protocols yield similar results for the same subject. Shahnaz et al. (2013) made wideband measurements using the Mimosa HearID system and the Interacoustics wideband acoustic immittance tympanometry (WAIT) system, an older research version of the Interacoustics Titan. Although differences in absorbance values between the two systems were found, these were much smaller than changes caused by middle ear pathology. The further investigation of instrument differences in WAI is important to determine whether WAI results obtained with currently available commercial systems are comparable. WAI research utilized different systems, either the clinically available instruments or modified research prototypes. Inter-instrument investigations of WAI using the 2 commercial systems are needed to determine their clinical utility of previous WAI research.

2.4.4 Variability within WAI

The value of WAI is based on the assumption that this measure of middle ear physiology will reflect changes caused by pathological processes that are greater than the normal variability in WAI associated with ear canal size and middle ear mobility. Likewise, measurements in the same individual should be stable across consecutive measurements despite minor procedural changes like differences in insertion depth of the WAI probe in the ear canal. Voss et al. (2008) investigated the effects of ear canal volume, probe placement, and ear-canal cross-sectional area on PA measurements. Their findings suggest that middle-ear cavity volume had the largest effect on PA, while ear-canal volume, probe placement, and area all had negligible effects on PA.

Age has been shown to be partially responsible for inter-subject variability of PA. In three separate groups of adults, young (20-38 years), middle age (42-64 years) and older (65-82 years), Mazlan et al. (2015) demonstrated that between PA values between 0.4 and 0.56 kHz were lowest in the younger group than the middle and older adults. In higher frequencies between 2.24 and 5.04 kHz, PA in the young adults was higher than the middle age and older groups. In a cross-sectional analysis, Feeney et al. (2014) found a minimal effect of age between three age groups of 20-29 years (n=37), 30-39 years (n=118) and 40-59 years (n=32). Slight differences in PA occurred between frequencies of 0.5 and 1.6 kHz. The youngest group had significantly lower PA compared to the middle age group from 0.5 to 1.3 kHz, while the oldest group had significantly lower PA compared to the middle group from 0.63 to 1.6 kHz. No significant difference in PA between the young and older groups was reported. Therefore, it appears as though in the low frequencies (0.5 to 1.6 kHz) PA tends to increase in the fourth decade, before decreasing later in the life.

There are conflicting reports on the effect of sex on WAI measurements. No significant difference in PA between males and females was reported in adults (Werner, Levi, & Keefe, 2010) and children (Beers, Shahnaz, Westerberg, & Kozak, 2010; Hunter, Tubaugh, Jackson, & Propes, 2008). However, pooled data of Shahnaz & Bork (2006) and the unpublished thesis of Shaw (2009) reports a significantly lower PA between 4 and 5 kHz in males compared to females in a sample of 186 adults (Shahnaz et al., 2013). In another large sample of 112 adults, Feeney et al. (2014) reported that females had lower PA below 0.5 kHz and higher PA above 4 kHz than males. Although, of the 112 participants in their study, only 24 were female. A similar result was found by Feeney & Sanford (2004), where PA was lower in females between 0.794-1 kHz, and higher in females above 5.04 kHz.

2.4.5 Test-retest Reliability of WAI

In order for PA measurements to be reliable at diagnosing middle ear pathologies, it is important that they have good test-retest reliability. Test-retest reliability has been investigated in WAI research instruments to determine how WAI measurements from the same instrument vary over time. Vander Werff, Prieve, & Georgantas (2007) found good test-retest reliability in a sample of 127 infants and 10 adults using a research reflectance system by Mimosa Acoustics Inc. Following probe reinsertion, the mean absolute differences in reflectance measurements and 90^{th} percentile were less than 0.1.

The test-retest reliability of the Mimosa HearID and earlier Reflectance Measurement prototype systems have been investigated in a number of methods in normal hearing populations. Vander Werff, Prieve, & Georgantas, (2007) calculated the absolute difference between two trials in order to investigate the test-retest reliability of energy reflectance (1-PA) in a population of adults (n=10) and infants (n=127). The mean absolute difference between two trials in a

sample of 10 adults was approximately 0.02 with the probe reinserted during the same testing session, and slightly lower than 0.02 when the probe was left in place. They report no significant difference between the two different probe conditions, suggesting similar test-retest reliability of ER both when the probe is left in place and when measurements were recorded following probe reinsertion.

Rosowski et al. (2012) tested the reliability of the Mimosa HearID system in a subset of 7 adults measured 4 times separated by 1 week between ear measurements. Mean absolute difference in energy reflectance (inverse of PA) was below 0.1 at all frequencies, with the test-retest standard deviation measured below the population standard deviation for energy reflectance, suggesting the variation in repeated testing of an individual is less than the variation in energy reflectance of the broader population. Werner et al. (2010) also investigated the test-retest reliability of wideband measurements using a research system and an ER-7C microphone similar to that of the Mimosa HearID, at different time intervals through the use of absolute differences. In their sample of 210 adults, they found a mean absolute difference in energy reflectance of approximately 0.1 in the low frequency range increasing in the higher frequencies to approximately 0.2 when measured approximately two weeks apart. These suggest that the Mimosa HearID and the research prototype systems yields appropriate test-retest reliability in the normal population.

Similar to the reports of reliability of the Mimosa HearID and prototype systems, the test-retest reliability of the earlier prototype system from Interacoustics has been investigated. The absolute differences and test-retest reliability of absorbance was investigated by Feeney et al. (2017) in a group of 33 individuals using an Interacoustics Wideband Research System, a more flexible prototype system than the commercially available Titan system. Results from Feeney et

al. (2017) show that absorbance measurements obtained approximately 1 month apart had a mean absolute difference of between 0.04 and 0.1 depending on frequency, suggesting the research prototype system yields appropriate test-retest reliability in their population.

2.4.6 WAI and Middle Ear Pathologies

WAI has recently been used in an attempt to better identify and differentiate various middle ear pathologies based on the physical characteristics of the middle ear system (Feeney et al., 2003; Hunter et al., 2008; Keefe, Sanford, Ellison, Fitzpatrick, & Gorga, 2012; Merchant, Merchant, Rosowski, & Nakajima, 2016; Nakajima et al., 2012; Hideko Heidi Nakajima et al., 2013; Prieve et al., 2013; Sanford et al., 2012; Shahnaz, Bork, et al., 2009; Vander Werff et al., 2007). These include otosclerosis (Allen, Jeng, & Levitt, 2005; Nakajima et al., 2013; Shahnaz, Longridge, et al., 2009; Shahnaz, Bork, et al., 2009), ossicular disarticulation (Feeney et al., 2003), third window disorders (Merchant et al., 2015), and otitis media with effusion (Keefe, Sanford, Ellison, Fitzpatrick, & Gorga, 2012).

PA measurements in otosclerotic ears have consistently shown lower PA (higher reflectance) below 1 kHz compared to normal ears (Feeney et al., 2003; Sanford et al., 2012; Shahnaz, Bork, et al., 2009; Shahnaz, Longridge, et al., 2009). Reduced PA values in the low frequencies may be explained by the increased stiffness of the middle-ear system caused by abnormal otosclerotic bone growth with reduced mobility or fixation of the stapes in advanced stages of the disease (Feeney et al., 2003; Nakajima et al., 2012; Nakajima et al., 2013; Shahnaz, Bork, et al., 2009). Shahnaz et al. (2009) evaluated the number of otosclerotic ears with energy reflectance above the 90th percentile at 0.5 kHz and found that 23/28 (82%) met this criterion. The approximate value of their 90th percentile energy reflectance at 0.5 kHz is 0.8, which would equate to PA of 0.2 at this frequency. A reduction in energy absorption by the middle ear system

at these low frequencies is compatible with the characteristic low frequency conductive hearing loss typically associated with otosclerosis. Nakajima et al. (2012) compared the sensitivity and specificity of PA values in 14 ears with stapes fixation. When combined with average air-bone gaps between 1-4 kHz, the absorbance level averaged over 0.6-1 kHz, these combined measures had a sensitivity of 86% and specificity of 100%. PA combined with the audiometric conductive component is a useful tool for identifying middle ear pathology in ears with otosclerosis.

Recently, Niemczyk, Lachowska, Tataj, Kurczak, & Niemczyk (2018) proposed that PA differences exist across the frequency range, and identified five PA profiles in otosclerotic ears, labelled types I-V. These profiles differ with respect to the number of absorbance peaks, the frequency of the peaks as well as the depth of the peaks. Type I reflects PA profiles with two moderate distinct peaks reaching moderate to high levels, Type II reflects PA profiles with a one distinct peak at higher frequencies reaching a high value, type III reflects PA profiles with an overall moderate PA value while demonstrating a reduction of PA below 1000 Hz, Type IV reflects PA profiles with low PA values across the frequency bandwidth, and finally Type V reflects PA profiles with lower PA limited to frequencies above 2000 Hz. Overall, these PA profile types are categorized based on the subjective categorization of PA peak heights and overall morphology. Therefore, while research has shown that in large otosclerotic populations there is an overall lower PA in the low frequencies of otosclerotic ears, a variation in the profiles across the frequency range should also be considered.

2.4.7 WAI and Advanced Phenotyping

In order for a measurement to be useful for phenotyping, it must be considered reliable and should accurately portray known features of the phenotype of interest (Lanktree, Hassell, Lahiry, & Hegele, 2010). In this thesis PA was compared across two new WAI instruments.

WAI has been shown to be a reliable measurement (Feeney et al., 2014; Vander Werff et al., 2007; Werner et al., 2010), while also capable of accurately identifying middle ear dysfunction associated with various middle ear pathologies (Allen et al., 2005; Keefe et al., 2012; Merchant et al., 2015; Nakajima et al., 2013; Shahnaz, Longridge, et al., 2009; Shahnaz, Bork, et al., 2009).

In otosclerosis gene discovery research WAI holds promise as a new phenotyping tool to aid in the segregation of affected family members from those unaffected by middle ear disease, as well as confirm the etiology of the hearing loss. Due to the subjective nature of PA classification by Niemczyk et al. (2018), and the lack of detailed information for the classification of PA profiles in their five sub-types, the classification system reported in their study was not used for phenotyping purposes in this thesis. Rather, the recommendations put forth by Nakajima et al. (2013), Shahnaz et al. (2009) and Merchant et al. (2015) mentioned above, served as a guideline for using WAI procedures to study middle ear pathology in families with inherited hearing loss.

2.5 References

Abdelfatah, N. (2014). The Genetic Aetiology of Otosclerosis in the Population of Newfoundland and Labrador. Memorial University of Newfoundland.

Aibara, R., Welsh, J. T., Puria, S., & Goode, R. L. (2001). Human middle-ear sound transfer function and cochlear input impedance. Hearing Research, 152(1–2), 100–109. https://doi.org/10.1016/S0378-5955(00)00240-9

Allen, J B. (1986). Measurement of Eardrum Acoustic Impedance. In J B Allen, J. L. Hall, A. E. Hubbard, S. T. Neely, & A. Tubis (Eds.) (pp. 44–51). Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-50038-1_6

Allen, Jont B, Jeng, P. S., & Levitt, H. (2005). Evaluation of human middle ear function via an acoustic power assessment. Journal of Rehabilitation Research and Development, 42(4 Suppl 2), 63–78. https://doi.org/10.1682/JRRD.2005.04.0064

Beers, A. N., Shahnaz, N., Westerberg, B. D., & Kozak, F. K. (2010). Wideband reflectance in normal Caucasian and Chinese school-aged children and in children with otitis media with effusion. Ear and Hearing, 31(2), 221–233. https://doi.org/10.1097/AUD.0b013e3181c00eae

Bel Hadj Ali, I., Thys, M., Beltaief, N., Schrauwen, I., Hilgert, N., Vanderstraeten, K., … Van Camp, G. (2008). A new locus for otosclerosis, OTSC8, maps to the pericentromeric region of chromosome 9. Human Genetics, 123(3), 267–272. https://doi.org/10.1007/s00439-008-0470-3

Brownstein, Z., Goldfarb, A., Levi, H., Frydman, M., & Avraham, K. B. (2006). Chromosomal mapping and phenotypic characterization of hereditary otosclerosis linked to the OTSC4 locus. Archives of Otolaryngology--Head & Neck Surgery, 132(4), 416–424. https://doi.org/10.1001/archotol.132.4.416

Calmels, M. N., Viana, C., Wanna, G., Marx, M., James, C., Deguine, O., & Fraysse, B. (2007). Very far-advanced otosclerosis: Stapedotomy or cochlear implantation. Acta Oto-Laryngologica, 127(6), 574–578. https://doi.org/10.1080/00016480600987768

Cawthorne, T. (1955). Otosclerosis. Journal of Laryngology and Otology, 69, 437–456.

Chen, W., Campbell, C. A., Green, G. E., Van Den Bogaert, K., Komodikis, C., Manolidis, L. S., … Smith, R. J. H. (2002). Linkage of otosclerosis to a third locus (OTSC3) on human chromosome 6p21.3-22.3. Journal of Medical Genetics, 39(7), 473–477. https://doi.org/10.1136/jmg.39.7.473

Cherukupally, S. R., Merchant, S. N., & Rosowski, J. J. (1998). Correlations between pathologic changes in the stapes and conductive hearing loss in otosclerosis. The Annals of Otology, Rhinology, and Laryngology, 107(4), 319–326. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9557767

Chole, R. A., & McKenna, M. (2001). Pathophysiology of Otosclerosis. Otology & Neurotology, 22(2), 249–257. https://doi.org/10.1097/00129492-200103000-00023

Colletti, V. (1975). Methodologic Observations on Tympanometry with Regard to the Probe Tone Frequency. Acta Oto-Laryngologica, 80(1–6), 54–60. https://doi.org/10.3109/00016487509121300

Colletti, Vittorio, Fiorino, F. G., Sittoni, V., & Policante, Z. (1993). Mechanics of the Middle Ear in Otosclerosis and Stapedoplasty. Acta Oto-Laryngologica, 113(5), 637–641. https://doi.org/10.3109/00016489309135877

Crompton, M., Cadge, B. A., Ziff, J. L., Mowat, A. J., Nash, R., Lavy, J. A., … Dawson, S. J. (2019). The Epidemiology of Otosclerosis in a British Cohort. Otology & Neurotology, 40(1), 22–30. https://doi.org/10.1097/MAO.0000000000002047

Cureoglu, S., Yildirim, M. B., & Paparella, M. M. (2010). Cochlear Otosclerosis. Curr Opin Otolaryngol Head Neck Surg, 18(5), 357–362. https://doi.org/10.1097/MOO.0b013e32833d11d9.Cochlear

Declau, F., Van Spaendonck, M., Timmermans, J. P., Michaels, L., Liang, J., Qiu, J. P., & Van de Heyning, P. (2001). Prevalence of Otosclerosis in an Unselected Series of Temporal Bones. Otology & Neurotology, 22(5), 596–602. https://doi.org/10.1097/00129492-200109000-00006

Doherty, J. K., & Linthicum, F. H. (2004). Spiral Ligament and Stria Vascularis Changes in Cochlear Otosclerosis: Effect on Hearing Level. Otology & Neurotology, 25(4), 457–464. https://doi.org/10.1097/00129492-200407000-00010

Ealy, M., Meyer, N. C., Corchado, J. C., Schrauwen, I., Bress, A., Pfister, M., … Smith, R. J. H. (2014). Rare variants in BMP2 and BMP4 found in otosclerosis patients reduce Smad signaling. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 35(3), 395–400. https://doi.org/10.1097/MAO.0000000000000244

Ealy, M., & Smith, R. J. H. (2010). The genetics of otosclerosis. Hearing Research, 266(1–2), 70–74. https://doi.org/10.1016/j.heares.2009.07.002
Emmett, J. R. (1993). Physical examination and clinical evaluation of the patient with otosclerosis. Otolaryngologic Clinics of North America, 26(3), 353–357.
Ertugay, O. C., Ata, P., Kalaycik Ertugay, C., Kaya, K. S., Tatlipinar, A., & Kulekci, S. (2013). Association of COL1A1 polymorphism in Turkish patients with otosclerosis. American Journal of Otolaryngology, 0505468291(5), 2–5. https://doi.org/10.1016/j.amjoto.2013.02.001
Feeney, M. P., Grant, I. L., & Marryott, L. P. (2003). Wideband energy reflectance measurements in adults with middle-ear disorders. Journal of Speech, Language, and Hearing Research : JSLHR, 46(4), 901–911. https://doi.org/10.1044/1092-4388(2003/070)
Feeney, M. P., Keefe, D. H., Hunter, L. L., Fitzpatrick, D. F., Garinis, A. C., Putterman, D. B., & McMillan, G. P. (2017). Normative Wideband Reflectance, Equivalent Admittance at the Tympanic Membrane, and Acoustic Stapedius Reflex Threshold in Adults. Ear and Hearing, 38(3), e142–e160. https://doi.org/10.1097/AUD.0000000000000399
Feeney, M. P., & Sanford, C. a. (2004). Age effects in the human middle ear: wideband acoustical measures. The Journal of the Acoustical Society of America, 116(6), 3546–3558. https://doi.org/10.1121/1.1808221
Feeney, M. P., Stover, B., Keefe, D. H., Garinis, A. C., Day, J. E., & Seixas, N. (2014). Sources of Variability in Wideband Energy Reflectance Measurements in Adults. Journal of the American Academy of Audiology, 25(5), 449–461. https://doi.org/10.3766/jaaa.25.5.4
Fowler, E. P. (1966). Otosclerosis in Identical Twins: A Study of 40 Pairs. Archives of Otolaryngology - Head and Neck Surgery, 83(4), 324–328. https://doi.org/10.1001/archotol.1966.00760020326006
Frisch, T., & Overgaard, S. (2000). Estimation of volume referent bone turnover in the otic capsule after sequential point labeling. The Annals of Otology, Rhinology, and Laryngology, 109(1), 33–39. Retrieved from http://aor.sagepub.com/content/109/1/33.short
Gordon, M. (1989). The Genetics of Otosclerosis: A Review. The American Journal of Otology, 10, 426–438.
Hannley, M. T. (1993). Audiologic characteristics of the patient with otosclerosis. Otolaryngologic Clinics of North America, 26(3), 373–387.
Hueb, M. M., Goycoolea, M. V, Paparella, M. M., & Oliveira, J. A. (1991). Otosclerosis: the University of Minnesota temporal bone collection. Otolaryngology--Head and Neck Surgery : Official Journal of American Academy of Otolaryngology-Head and Neck Surgery, 105(3), 396–405. https://doi.org/10.1177/019459989110500308
Hunter, L. L., Tubaugh, L., Jackson, A., & Propes, S. (2008). Wideband Middle Ear Power Measurement in Infants and Children. Journal of the American Academy of Audiology, 19(4), 309–324. https://doi.org/10.3766/jaaa.19.4.4
Ishai, R., Halpin, C. F., Shin, J. J., McKenna, M. J., & Quesnel, A. M. (2016). Long-term Incidence and Degree of Sensorineural Hearing Loss in Otosclerosis. Otology & Neurotology, 37(10), 1489–1496. https://doi.org/10.1097/MAO.0000000000001234
Jerger, J. (1970). Clinical Experience With Impedance Audiometry. Archives of Otolaryngology - Head and Neck Surgery, 92(4), 311–324. https://doi.org/10.1001/archotol.1970.04310040005002

Karosi, T., Csomor, P., & Sziklai, I. (2012). The value of HRCT in stapes fixations corresponding to hearing thresholds and histologic findings. Otology and Neurotology, 33(8), 1300–1307. https://doi.org/10.1097/MAO.0b013e31826352ad

Karosi, T., & Sziklai, I. (2010). Etiopathogenesis of otosclerosis. European Archives of Oto-Rhino-Laryngology, 267(9), 1337–1349. https://doi.org/10.1007/s00405-010-1292-1

Keefe, D. H. (1992). Method to measure acoustic impedance and reflection coefficient. The Journal of the Acoustical Society of America, 91(1), 470. https://doi.org/10.1121/1.402733

Keefe, D. H., Ling, R., & Bulen, J. C. (1992). Method to measure acoustic impedance and reflection coefficient. The Journal of the Acoustical Society of America, 91(1), 470–485. https://doi.org/10.1121/1.402733

Keefe, D. H., Sanford, C. a., Ellison, J. C., Fitzpatrick, D. F., & Gorga, M. P. (2012). Wideband aural acoustic absorbance predicts conductive hearing loss in children. International Journal of Audiology, 51(12), 1–12. https://doi.org/10.3109/14992027.2012.721936

Keefe, D., L Archer, K., Schmid, K., Fitzpatrick, D., Feeney, P., & Hunter, L. (2017). Identifying Otosclerosis with Aural Acoustical Tests of Absorbance, Group Delay, Acoustic Reflex Threshold, and Otoacoustic Emissions. Journal of the American Academy of Audiology, 28. https://doi.org/10.3766/jaaa.16172

Khalfallah, A., Schrauwen, I., Mnejja, M., HadjKacem, H., Dhouib, L., Mosrati, M. A., … Masmoudi, S. (2011). Association of COL1A1 and TGFB1 polymorphisms with otosclerosis in a Tunisian population. Annals of Human Genetics, 75(5), 598–604. https://doi.org/10.1111/j.1469-1809.2011.00665.x

Khorsandi A., M. T., Jalali, M. M., & Shoshi D., V. (2018). Predictive factors in 995 stapes surgeries for primary otosclerosis. The Laryngoscope, 128(10), 2403–2407. https://doi.org/10.1002/lary.27160

Korres, S. G. (2014). The Profile of Otoacoustic Emissions and Multifrequency Tympanometry in Otosclerotic Patients Undergoing Two Types of Stapes Surgery: Small Fenestra and Microtraumatic Stapedotomy. Medical Science Monitor, 20, 1613–1620. https://doi.org/10.12659/MSM.890755

Lagleyre, S., Sorrentino, T., Calmels, M.-N., Shin, Y.-J., Escude, B., Deguine, O., & Fraysse, B. (2009). Reliability of high-resolution CT scan in diagnosis of otosclerosis. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 30(8), 1152–1159. https://doi.org/10.1097/MAO.0b013e3181c2a084

Lanktree, M. B., Hassell, R. G., Lahiry, P., & Hegele, R. a. (2010). Phenomics: expanding the role of clinical evaluation in genomic studies. Journal of Investigative Medicine : The Official Publication of the American Federation for Clinical Research, 58(5), 700–706. https://doi.org/10.231/JIM.0b013e3181d844f7

Lilly, D. J. (1984). Multiple Frequency, Multiple Component Tympanometry. Ear and Hearing, 5(5), 300–308. https://doi.org/10.1097/00003446-198409000-00007

Lippy, W. H., Berenholz, L. P., Schuring, A. G., & Burkey, J. M. (2005). Does pregnancy affect otosclerosis? The Laryngoscope, 115(10), 1833–1836. https://doi.org/10.1097/01.MLG.0000187573.99335.85

Marchese, M. R., Conti, G., Cianfrone, F., Scorpecci, A., Fetoni, A. R., & Paludetti, G. (2009). Predictive role of audiological and clinical features for functional results after stapedotomy. Audiology and Neurotology, 14(5), 279–285. https://doi.org/10.1159/000212105

Margolis, R., & Heller, J. (1987). Screening Tympanometry: Criteria for Medical Referral. International Journal of Audiology, 26(4), 197–208. https://doi.org/10.3109/00206098709081549

Mazlan, R., Kei, J., Ya, C. L., Yusof, W. N. H. M., Saim, L., & Zhao, F. (2015). Age and Gender Effects on Wideband Absorbance in Adults With Normal Outer and Middle Ear Function. Journal of Speech Language and Hearing Research, 58(4), 1377. https://doi.org/10.1044/2015_JSLHR-H-14-0199

McGuirt, W. T., Prasad, S. D., Griffith, A. J., Kunst, H. P., Green, G. E., Shpargel, K. B., … Smith, R. J. (1999). Mutations in COL11A2 cause non-syndromic hearing loss (DFNA13). Nature Genetics, 23(4), 413–419. https://doi.org/10.1038/70516

McKenna, M. J., Kristiansen, A. G., Bartley, M. L., Rogus, J. J., & Haines, J. L. (1998). Association of COL1A1 and otosclerosis: evidence for a shared genetic etiology with mild osteogenesis imperfecta. The American Journal of Otology, 19(5), 604–610. https://doi.org/10.1017/S0022215100136606

McKenna, M. J., & Merchant, S. N. (2010). Disorders of bone. In Schuknecht's Pathology of the Ear. Shelton, CT: People's Medical Pub. House-USA Inc (pp. 716–719).

Meranger, A., David, A., Beigner, B. M., Charpiot, A., & Tavernier, L. (2019). Audiometric Results of Stapedotomy Surgery for Otoscelorsis. Otology & Neurotology, 40(2), e75–e81. https://doi.org/10.1097/MAO.0000000000002109

Merchant, G. R., Merchant, S. N., Rosowski, J. J., & Nakajima, H. H. (2016). Controlled exploration of the effects of conductive hearing loss on wideband acoustic immittance in human cadaveric preparations. Hearing Research, 341, 19–30. https://doi.org/10.1016/j.heares.2016.07.018

Merchant, G. R., Röösli, C., Niesten, M. E. F., Hamade, M. A., Lee, D. J., McKinnon, M. L., … Nakajima, H. H. (2015). Power Reflectance as a Screening Tool for the Diagnosis of Superior Semicircular Canal Dehiscence. Otology & Neurotology, 36(1), 172–177. https://doi.org/10.1097/MAO.0000000000000294

Merchant, S. N., Rosowski, J. J., & McKenna, M. J. (2007). Superior Semicircular Canal Dehiscence Mimicking Otosclerotic Hearing Loss. In Otosclerosis and Stapes Surgery (Vol. 3096, pp. 137–145). Basel: KARGER. https://doi.org/10.1159/000098790

Morrison, A. W. (1967). Genetic factors in otosclerosis. Annals of the Royal College of Surgeons of England, 41(2), 202–237. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2311999/

Mowat, A. J., Crompton, M., Ziff, J. L., Aldren, C. P., Lavy, J. A., Saeed, S. R., & Dawson, S. J. (2018). Evidence of distinct RELN and TGFB1 genetic associations in familial and non-familial otosclerosis in a British population. Human Genetics, 137(5), 357–363. https://doi.org/10.1007/s00439-018-1889-9

Nakajima, Hideko H, Pisano, D. V, Roosli, C., Hamade, M. a, Merchant, G. R., Mahfoud, L., … Merchant, S. N. (2012). Comparison of ear-canal reflectance and umbo velocity in patients with conductive hearing loss: a preliminary study. Ear and Hearing, 33(1), 35–43. https://doi.org/10.1097/AUD.0b013e31822ccba0

Nakajima, Hideko Heidi, Rosowski, J. J., Shahnaz, N., & Voss, S. E. (2013). Assessment of Ear Disorders Using Power Reflectance. Ear and Hearing, 34(1), 48s-53s. https://doi.org/10.1097/AUD.0b013e31829c964d

Niemczyk, E., Lachowska, M., Tataj, E., Kurczak, K., & Niemczyk, K. (2018). Wideband tympanometry and absorbance measurements in otosclerotic ears. The Laryngoscope, ePub ahead. https://doi.org/10.1002/lary.27747

Ogut, F., Serbetcioglu, B., Kirazli, T., Kirkim, G., & Gode, S. (2008). Results of multiple-frequency tympanometry measures in normal and otosclerotic middle ears. International Journal of Audiology, 47(10), 615–620. https://doi.org/10.1080/14992020802178656

Parahy, C., & Linthicum, F. H. (1984). Otosclerosis And Otospongiosis: Clinical and histological comparisons. The Laryngoscope, 94(4), 508–512. https://doi.org/10.1288/00005537-198404000-00015

Pauw, R. J., De Leenheer, E. M. R., Van Den Bogaert, K., Huygen, P. L. M., Van Camp, G., Joosten, F. B. M., & Cremers, C. W. R. J. (2006). The phenotype of the first otosclerosis family linked to OTSC5. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 27(3), 308–315. Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/lary.21463/full

Prieve, B. a, Feeney, M. P., Stenfelt, S., & Shahnaz, N. (2013). Prediction of conductive hearing loss using wideband acoustic immittance. Ear and Hearing, 34 Suppl 1, 54S-59S. https://doi.org/10.1097/AUD.0b013e31829c9670

Purohit, B., Hermans, R., & Op de beeck, K. (2014). Imaging in otosclerosis: A pictorial review. Insights into Imaging, 5(2), 245–252. https://doi.org/10.1007/s13244-014-0313-9

Quesnel, A. M., Moonis, G., Appel, J., O'Malley, J. T., McKenna, M. J., Curtin, H. D., & Merchant, S. N. (2013). Correlation of computed tomography with histopathology in otosclerosis. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 34(1), 22–28. https://doi.org/10.1097/MAO.0b013e318277a1f7

Ramsay, H. A., & Linthicum, F. H. (1994). Mixed hearing loss in otosclerosis: indication for long-term follow-up. The American Journal of Otology. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8588610

Rodríguez, L., Rodríguez, S., Hermida, J., Frade, C., Sande, E., Visedo, G., … Zapata, C. (2004). Proposed association between the COL1A1 and COL1A2 genes and otosclerosis is not supported by a case-control study in Spain. American Journal of Medical Genetics Part A, 128A(1), 19–22. https://doi.org/10.1002/ajmg.a.30074

Rosowski, J. J., Nakajima, H. H., Hamade, M. A., Mahfoud, L., Merchant, G. R., Halpin, C. F., & Merchant, S. N. (2012). Ear-Canal Reflectance, Umbo Velocity, and Tympanometry in Normal-Hearing Adults. Ear and Hearing, 33(1), 19–34. https://doi.org/10.1097/AUD.0b013e31822ccb76

Rosowski, J. J., Stenfelt, S., & Lilly, D. (2013). An overview of wideband immittance measurements techniques and terminology: you say absorbance, I say reflectance. Ear and Hearing, 34 Suppl 1(0 1), 9S-16S. https://doi.org/10.1097/AUD.0b013e31829d5a14

Rudic, M., Keogh, I., Wagner, R., Wilkinson, E., Kiros, N., Ferrary, E., … Zarkovic, N. (2015). The pathophysiology of otosclerosis: Review of current research. Hearing Research, 330, 51–56. https://doi.org/10.1016/j.heares.2015.07.014

Sakihara, Y., & Parving, A. (1999). Clinical otosclerosis, prevalence estimates and spontaneous progress. Acta Oto-Laryngologica, 119(4), 468–472. https://doi.org/10.1080/00016489950181017

Sanford, C., Schooling, T., & Frymark, T. (2012). Determining the Presence or Absence of Middle Ear Disorders: An Evidence-Based Systematic Review on the Diagnostic Accuracy of Selected Assessment. American Journal of Audiology, 21(December), 251–268. https://doi.org/10.1044/1059-0889(2012/11-0029)a

Schrauwen, I, Ealy, M., Huentelman, M. J., Thys, M., Homer, N., Vanderstraeten, K., … Van Camp, G. (2009). A Genome-wide Analysis Identifies Genetic Variants in the RELN Gene Associated with Otosclerosis. The American Journal of Human Genetics, 84(3), 328–338. https://doi.org/10.1016/j.ajhg.2009.01.023

Schrauwen, Isabelle, Khalfallah, A., Ealy, M., Fransen, E., Claes, C., Huber, A., … Van Camp, G. (2012). COL1A1 association and otosclerosis: a meta-analysis. American Journal of Medical Genetics. Part A, 158A(5), 1066–1070. https://doi.org/10.1002/ajmg.a.35276

Schrauwen, Isabelle, Thys, M., Vanderstraeten, K., Fransen, E., Dieltjens, N., Huyghe, J. R., … Van Camp, G. (2008). Association of Bone Morphogenetic Proteins With Otosclerosis. Journal of Bone and Mineral Research, 23(4), 507–516. https://doi.org/10.1359/jbmr.071112

Schrauwen, Isabelle, Thys, M., Vanderstraeten, K., Fransen, E., Ealy, M., Cremers, C. W. R. J., … Van Camp, G. (2009). No Evidence for Association Between the Renin-Angiotensin-Aldosterone System and Otosclerosis in a Large Belgian-Dutch Population. Otology & Neurotology, 30(8), 1079–1083. https://doi.org/10.1097/MAO.0b013e3181ab3058

Schrauwen, Isabelle, & Van Camp, G. (2010). The etiology of otosclerosis: a combination of genes and environment. The Laryngoscope, 120(6), 1195–1202. https://doi.org/10.1002/lary.20934

Schrauwen, Isabelle, Weegerink, N., Fransen, E., Claes, C., Pennings, R., Cremers, C., … Van Camp, G. (2011). A new locus for otosclerosis, OTSC10, maps to chromosome 1q41-44. Clinical Genetics, 79(5), 495–497. https://doi.org/10.1111/j.1399-0004.2010.01576.x

Schuknecht, H. F., & Barber, W. (1985). Histologic variants in otosclerosis. The Laryngoscope, 95(11), 1307–1317. https://doi.org/10.1288/00005537-198511000-00003

Schuknecht, H. F., & Kirchner, J. C. (1974). Cochlear otosclerosis: fact or fantasy. The Laryngoscope, 84(5), 766–782. https://doi.org/10.1288/00005537-197405000-00008

Shahnaz, N. (2007). Multi-frequency Tympanometry and Evidence-based Practice Immittance Principles, 11(1), 2–12.

Shahnaz, N., & Bork, K. (2006). Wideband reflectance norms for Caucasian and Chinese young adults. Ear and Hearing, 27(6), 774–788. https://doi.org/10.1097/01.aud.0000240568.00816.4a

Shahnaz, N., Bork, K., Polka, L., Longridge, N., Bell, D., & Westerberg, B. D. (2009). Energy reflectance and tympanometry in normal and otosclerotic ears. Ear and Hearing, 30(2), 219–233. https://doi.org/10.1097/AUD.0b013e3181976a14

Shahnaz, N., Feeney, M. P., & Schairer, K. S. (2013). Wideband Acoustic Immittance Normative Data: Ethnicity, Gender, Aging, and Instrumentation. Ear and Hearing, 34, 27s-35s. https://doi.org/10.1097/AUD.0b013e31829d5328

Shahnaz, N., Longridge, N., & Bell, D. (2009). Wideband energy reflectance patterns in preoperative and post-operative otosclerotic ears. International Journal of Audiology, 48(5), 240–247. https://doi.org/10.1080/14992020802635317

Shahnaz, N., & Polka, L. (1997). Standard and Multifrequency Tympanometry in Normal and Otosclerotic Ears. Ear and Hearing, 18(4), 326–341. https://doi.org/10.1097/00003446-199708000-00007

Shambaugh, G. E. (1965). Clinical diagnosis of cochlear (labyrinthine) otosclerosis. The Laryngoscope, 75(10), 1558–1562. https://doi.org/10.1002/lary.5540751009
Shanks, J. E., & Lilly, D. J. (1981). An Evaluation of Tympanometric Estimates of Ear Canal Volume. Journal of Speech, Language, and Hearing Research, 24(4), 557–566. Retrieved from http://dx.doi.org/10.1044/jshr.2404.557
Shaw, J. (2009). Comparison of Wideband Energy Reflectance and Tympanometric Measures Obtained With Reflwin Interacoustics, Mimosa Acoustics and GSI Tympstar Systems. University of British Columbia, Vancouver, Canada.
Shin, Y. J., Calvas, P., Deguine, O., Charlet, J. P., Cognard, C., & Fraysse, B. (2001). Correlations between computed tomography findings and family history in otosclerotic patients. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 22(4), 461–464. https://doi.org/10.1097/00129492-200107000-00008
Sommen, M., Van Camp, G., Liktor, B., Csomor, P., Fransen, E., Sziklai, I., … Karosi, T. (2014). Genetic association analysis in a clinically and histologically confirmed otosclerosis population confirms association with the TGFB1 gene but suggests an association of the RELN gene with a clinically indistinguishable otosclerosis-like phenotype. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 35(6), 1058–1064. https://doi.org/10.1097/MAO.0000000000000334
Terkildsen, K., & Thomsen, K. A. (1959). The Influence of Pressure Variations on the Impedance of the Human Ear Drum. The Journal of Laryngology & Otology, 73(7), 409–418. https://doi.org/10.1017/S002221510005550X
Terkildsen, Knud, Osterhammel, P., & Bretlau, P. (1973). Acoustic Middle Ear Muscle Reflexes in Patients with Otosclerosis. Archives of Otolaryngology, 98(3), 152–155. https://doi.org/10.1001/archotol.1973.00780020160003
Thys, M., & Camp, G. Van. (2009). Genetics of Otosclerosis. Otology & Neurotology, 30(8), 1021–1032. https://doi.org/10.1097/MAO.0b013e3181a86509
Thys, M., Van Den Bogaert, K., Iliadou, V., Vanderstraeten, K., Dieltjens, N., Schrauwen, I., … Van Camp, G. (2007). A seventh locus for otosclerosis, OTSC7, maps to chromosome 6q13–16.1. European Journal of Human Genetics, 15(3), 362–368. https://doi.org/10.1038/sj.ejhg.5201761
Tomek, M. S., Brown, M. R., Mani, S. R., Ramesh, A., Srisailapathy, C. R., Coucke, P., … Smith, R. J. (1998). Localization of a gene for otosclerosis to chromosome 15q25-q26. Human Molecular Genetics, 7(2), 285–290. https://doi.org/10.1093/hmg/7.2.285
Toynbee, J. (1841). Pathological and Surgical Observations on the Diseases of the ear. Medico-Chirurgical Transactions, 24, 190–211. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20895731
Uppal, S., Bajaj, Y., Rustom, I., & Coatesworth, A. P. (2009). Otosclerosis 1: The aetiopathogenesis of otosclerosis. International Journal of Clinical Practice, 63(10), 1526–1530. https://doi.org/10.1111/j.1742-1241.2009.02045.x
Van Den Bogaert, K. (2004). A fifth locus for otosclerosis, OTSC5, maps to chromosome 3q22-24. Journal of Medical Genetics, 41(6), 450–453. https://doi.org/10.1136/jmg.2004.018671
Van Den Bogaert, Kris, Govaerts, P. J., Schatteman, I., Brown, M. R., Caethoven, G., Offeciers, F. E., … Van Camp, G. (2001). A second gene for otosclerosis, OTSC2, maps to

chromosome 7q34-36. American Journal of Human Genetics, 68(2), 495–500. https://doi.org/10.1086/318185

Vanaja, C., & Manjula, P. (2003). Middle Ear Resonant Frequency in Normal and Otosclerotic Ears: Effect of Procedural Variation. JOURNAL OF …, 27(3), 158–162. Retrieved from http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Middle+Ear+Resonant+Frequency+in+Normal+and+Otosclerotic+Ears:+Effect+of+Procedural+Variation#3

Vander Werff, K. R., Prieve, B. A., & Georgantas, L. M. (2007). Test-retest reliability of wideband reflectance measures in infants under screening and diagnostic test conditions. Ear and Hearing, 28, 669–681. https://doi.org/10.1097/AUD.0b013e31812f71b1

Voss, S. E., & Allen, J. B. (1994). Measurement of acoustic impedance and reflectance in the human ear canal. The Journal of the Acoustical Society of America, 95(1), 372–384. https://doi.org/10.1121/1.408329

Voss, S. E., Horton, N. J., Woodbury, R. R., & Sheffield, K. N. (2008). Sources of variability in reflectance measurements on normal cadaver ears. Ear and Hearing, 29(4), 651–665. https://doi.org/10.1097/AUD.0b013e318174f07c

Weegerink, N. J. D., Schrauwen, I., Huygen, P. L. M., Pennings, R. J. E., Cremers, C. W. R. J., Van Camp, G., & Kunst, H. P. M. (2011). Phenotype of the first otosclerosis family linked to OTSC10. The Laryngoscope, 121(4), 838–845. https://doi.org/10.1002/lary.21463

Werner, L. A., Levi, E. C., & Keefe, D. H. (2010). Ear-canal wideband acoustic transfer functions of adults and two- to nine-month-old infants. Ear and Hearing, 31(5), 587–598. https://doi.org/10.1097/AUD.0b013e3181e0381d

Wieczorek, S. S., Anderson, M. E., Harris, D. A., & Mikulec, A. A. (2013). Enlarged vestibular aqueduct syndrome mimicking otosclerosis in adults. American Journal of Otolaryngology, 34(6), 619–625. https://doi.org/10.1016/j.amjoto.2013.07.015

Wiley, T. L., Cruickshanks, K. J., Nondahl, D. M., Tweed, T. S., Klein, R., & Klein, B. E. (1996). Tympanometric measures in older adults. Journal of the American Academy of Audiology, 7(4), 260–268. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8827920

Yoo, T. J., Cho, H., & Yamada, Y. (1991). Hearing impairment in mice with the cmd/cmd (cartilage matrix deficiency) mutant gene. Annals of the New York Academy of Sciences, 630, 265–267. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1749-6632.1991.tb19600.x/abstract

Yutaka Imauchi, Xavier Jeunemaître, Magali Boussion, Evelyne Ferrary, Olivier Sterkers, & Alexis Bozorg Grayeli. (2008). Relation Between Renin-Angiotensin-Aldosterone System and Otosclerosis. Otology & Neurotology, 29(3), 295–301. https://doi.org/10.1097/MAO.0b013e318164d12c

Zhao, F., Wada, H., Koike, T., Ohyama, K., Kawase, T., & Stephens, D. (2002). Middle ear dynamic characteristics in patients with otosclerosis. Ear and Hearing, 23(2), 150–158. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11951850

Ziff, J. L., Crompton, M., Powell, H. R. F., Lavy, J. A., Aldren, C. P., Steel, K. P., … Dawson, S. J. (2016). Mutations and altered expression of SERPINF1 in patients with familial otosclerosis, 25(12), 2393–2403. https://doi.org/10.1093/hmg/ddw106

Zwolan, T. a. (2010). Diagnostic audiology. Cummings Otolaryngology Head and Neck Surgery (Fifth Edit). Copyright © 2010, 2005, 1998, 1993, 1986 by Mosby, Inc. All Rights Reserved. https://doi.org/10.1016/B978-0-323-05283-2.00134-8

Chapter 3

3 Advanced Phenotyping and *FOXL1* Screening in an Ontario Otosclerotic Population

3.1 Introduction

Recently, the first genetic mutation for otosclerosis was identified in a large Newfoundland family, a 15 bp deletion in *FOXL1* (Abdelfatah, 2014). It remains unclear whether this mutation occurs in otosclerotic populations outside of the family, or founder's population where it was discovered.

The previous chapter describes the phenotyping methods currently used for genetic studies of otosclerosis families, which include audiometry, acoustic reflexes, surgical confirmation and rarely, high-resolution CT imaging (Bel Hadj Ali et al., 2008; Brownstein, Goldfarb, Levi, Frydman, & Avraham, 2006; Chen et al., 2002; Pauw et al., 2006; Schrauwen et al., 2011; Thys, Van Den Bogaert, et al., 2007; Tomek et al., 1998; Van Den Bogaert et al., 2001). There have been reports of multifrequency tympanometry (Shahnaz & Polka, 1997; Vanaja & Manjula, 2003), acoustic reflex thresholds (Hannley, 1993; Knud Terkildsen et al., 1973), and occasionally otoacoustic emissions (Herzog, Shehata-Dieler, & Dieler, 2001; Keefe et al., 2017; Singh, Gupta, & Verma, 2012) in clinical cohorts with otosclerosis. However, no studies have investigated the advanced auditory phenotype of otosclerosis in addition to family history in a clinical population data.

3.1.1 Aims of this Study

In this study, a cohort of patients with otosclerosis from the province of Ontario were evaluated. There were 3 aims in this study:

Specific Aim 1: To determine whether the newly discovered deletion in *FOXL1* is present outside the family from the province of Newfoundland and Labrador;

Specific Aim 2: To assess the family history and detailed phenotype in a cohort of otosclerotic patients residing in southwestern Ontario and finally

Specific Aim 3: To create an in-depth clinical profile for both pre-operative and post-operative otosclerotic ears.

3.2 Methods

3.2.1 Subjects

Participants from Ontario, Canada were recruited from the H.A. Leeper Speech and Hearing Clinic and the Department of Otolaryngology (London Health Sciences Centre – University Hospital) at Western University. The was study approved by the Health Science Research Ethics Board at The University of Western Ontario (HSREB #103679). Participants qualified for the study if diagnosed with otosclerosis based on either surgical confirmation, or clinical presentation, including the hallmark features of low-frequency conductive hearing loss present either in isolation or as part of a mixed hearing loss, and absent acoustic reflex thresholds. Ears that met the audiometric criteria for diagnosis of otosclerosis had air-conduction thresholds greater than 25dB HL and an air-bone gap greater than 10dB at frequencies of 250, 500 and 1000 Hz. Exclusion criteria for the current study included a history of noise exposure, exposure to ototoxic drugs potentially causing a sensorineural hearing loss (SNHL), or a history of ear surgery besides corrective stapes surgery. Of the forty individuals recruited, 35 participants met the inclusion criteria. Of these, 33 subjects that had undergone either unilateral

or bilateral stapes surgery, and two participants with a clinical diagnosis of otosclerosis but who did not have corrective surgery were included in the final subject sample (Table 3).

Table 3. Summary of demographic information from 35 otosclerotic subjects including average age (standard deviation), total number of males and females, and total number that had undergone corrective surgery.

	Ave Age (SD)	Unilateral Surgery		Bilateral Surgery	No Surgery	Overall
		Left	Right			
Females	53.76 (13.05)	4	12	6	2	24
Males	54.85 (7.91)	5	3	3	0	11
Total	54.11 (11.52)	9	15	9	2	35

3.2.2 *FOXL1* Screening

Saliva samples were collected using Oragene-DNA kits (DNA Genotek, Canada), and genomic DNA extracted in the laboratory of Dr. Terry-Lynn Young at Memorial University of Newfoundland (St. John's, NL, Canada). DNA samples were screened at the Young Laboratory at Memorial University by using Sanger sequencing for the newly found heterozygous deletion c.976_990het_del within the *FOXL1* gene. To do this, 4 primer sets were used to sequence the entire *FOXL1* gene (Table 4) and the Polymerase Chain Reaction (PCR) amplification protocol was employed, where the amplified products were subsequently bidirectionally sequenced using the Big Dye terminator Sanger sequencing, described in Abdelfatah (2014) and reported in Appendix A.

Table 4. List of PCR sequencing primers spanning the deletion in *FOXL1*. Modified table from PhD thesis of Nelly Abdelfatah 2014.

FOXL1 (NM_005250)			
Exon	**Primer ID**	**Sequence**	**Amplicon Size (bp)**
1a	NM_005250-Ex1aR NM_005250-Ex1aR	GGAGGGAAAAGCTTGGAGTT TGTCGTGGTAGAAGGGGAAG	579
1b	NM_005250-Ex1bF NM_005250-Ex1bR	GCCTCCCTACAGCTACATCG GTCACCAGCGTCCTCGTT	514
1c	NM_005250-Ex1cF NM_005250-Ex1cR	GGAAGAGGAAGCCCAAG GCAGGGGGAAATAAGAGAGG	585
1d	NM_005250-Ex1dF NM_005250-Ex1dR	AACGAGGACGCTGGTGAC CCCAGGCAAAGATCATTTTA	585

The pathogenicity of the 15bp deletion in *FOXL1* was investigated by using the variant interpretation process recommended by the American College of Medical Genetics and Genomics reported by Richards et al. (2015) with the assistance of Dr. Darren O'Reilly, Director of Molecular Genetics Lab at Memorial University and Dr. Terry-Lynn Young, Professor, Discipline of Genetics at Memorial University and Co-Principal investigator. The process is reported in Appendix B.

3.2.3 Family History Analysis

Participants were asked about hearing loss among their relatives through a structured interview centered around family history (Appendix C). The questionnaire was designed to target information pertaining to potential confounding effects on hearing loss including complications at birth, history of hearing issues or vision issues in the participant and their family members. In conjunction with the family history questionnaire, a family pedigree was created for each participant to highlight whether any family member dating back three generations could be identified as having hearing loss. As much detail as possible was obtained, including age of onset, the potential etiology of the hearing loss, or a potential diagnosis of the hearing loss.

3.2.4 Phenotyping Procedures

Audiometry

Participants had air conduction (AC) and bone conduction (BC) thresholds tested using a Grason Stadler (GSI 61) audiometer calibrated to American National Standards Institute standards (re: S3.6.2010). Measurements were conducted in a sound-booth using ER3 insert earphones for air conduction, and a bone oscillator for bone conduction thresholds. Air conduction thresholds were measured at octaves between 0.25 – 8 kHz, while bone conduction

thresholds were measured at octaves between 0.5-4 kHz, using a modified Hughson-Westlake technique. Four frequency pure tone averages (PTA4) were calculated by averaging the thresholds of 500, 1000, 2000 and 4000 Hz.

Acoustic Immittance

Acoustic immittance measurements including standard 226 Hz tympanometry, multifrequency tympanometry and acoustic reflex thresholds were measured in both ears of each participant. Acoustic immittance data was obtained for 67 of the 70 ears. One participant had a sensitivity to the pressurized measurement and therefore immittance data could not be tested on either ear, while the left ear of another participant could not be tested due to inability to acquire a hermetic seal.

Conventional Immittance

Single component tympanometry, multifrequency tympanometry and acoustic reflexes were measured in each ear using the GSI Tympstar v.2 tympanometer calibrated to ANSI standard (re: S3.39.1989). For single component tympanometry, a probe tone of 226 Hz was used, and tympanograms were classified for shape, ear volume, peak pressure and compliance.

Multifrequency Tympanometry

Utilizing the multifrequency tympanometry function of the Tympstar v.2, resonant frequency was measured in each ear. Resonant frequency is calculated within the GSI Tympstar v2 by calculating where the delta B crosses zero in a sweep frequency tympanogram. The resonant frequency was calculated automatically within the Tympstar v.2 software and was rounded to the nearest 50 Hz.

Acoustic Reflex Threshold

Acoustic reflexes were calculated by presenting activation stimuli of 0.5, 1, 2, 4 kHz and a broadband noise (BBN) in the presence of a 226 Hz probe tone. Thresholds were identified when the compliance of the eardrum in the presence of the stimulus reached a minimum of 0.02 mL in two of three trials. If a reflex did not reach the criteria within three trials, the stimulus was increased by 5 dB until an acoustic reflex threshold was obtained. No response was recorded if there was no measurable acoustic reflex in two of three trials at the maximum stimulus level of 110 dB HL. Ipsilateral acoustic reflex thresholds represent the reflex obtained with the probe and stimulus tone originating from the same ear, while contralateral acoustic reflex thresholds are obtained with the stimulus tone and probe tone presented to different ears. The conventional clinical nomenclature for acoustic reflexes is to name the ear specific reflex based on the stimulus tone. However, since we are characterizing each ear separately, the reflex will be named based on the location of the probe, while the terms ipsilateral and contralateral will be used to describe the origin of the stimulus. Ipsilateral stimulation denotes a setup whereby the stimulation tone and the probe are situated in the same ear, and contralateral stimulation denotes a setup where the stimulation is occurring in the opposite ear to the probe.

Distortion Product Otoacoustic Emissions

Distortion product otoacoustic emissions (DPOAEs) were elicited using the Intelligent Hearing Systems (IHS) DPOAE (v.4.54) system. Emissions were evoked using two tones, F1 and F2, where a F2/F1 ratio of 1.22 was used to elicit the DPOAE. The DPOAE was measured at the frequency of 2F1-F2. F1 was presented at 65 dB SPL and f2 was presented at 55 dB SPL (summary of frequencies in Table 5). A total of 17 DPOAEs were recorded spanning a 2F1-F2

frequency range of 357-5649 Hz. At each DPOAE frequency, the noise-floor is measured, as well as the distortion product level. The difference between the noise-floor and distortion product is calculated and reported as the signal-to-noise ratio (SNR).

A screening protocol was used to determine whether a given ear had present or absent DPOAEs across the broad frequency range. For an ear to pass DPOAE screening, there must be at least 50% DPOAEs passed at all frequencies, 50% DPOAEs passed in every octave and 80% passed between 1000 and 2000 Hz. A passed DPOAE was defined as a distortion product greater than -10 dB SPL, and a signal-to-noise ratio (SNR) greater than 6 dB SPL between the noise floor and the distortion product.

Table 5. Stimulus frequencies 1 and 2 (F1 and F2 respectively) along with their associated distortion product frequency (2F1-F2) collected using the Intelligent Hearing Systems (IHS) system. In total, 17 distortion products were elicited across an f2 frequency range of 553-8837 Hz.

F2 (Hz)	F1 (Hz)	2F1-F2 (Hz)
553	455	357
655	538	421
783	641	499
929	763	597
1105	905	704
1316	1076	836
1560	1281	1003
1858	1521	1184
2211	1810	1409
2626	2152	1677
3125	2563	2000
3717	3047	2377
4416	3619	2822
5253	4309	3365
6250	5120	3991
7429	6089	4749
8837	7243	5649

Ear Status

Ears were separated into four groups; surgical ears, clinical otosclerotic ears, normal ears and sensorineural hearing loss ears (SNHL). Any ear that had previously undergone corrective surgery for otosclerosis was classified as “surgical ear”. Ears that met the audiometric criteria for diagnosis of otosclerosis (air-conduction thresholds greater than 25dB HL and air-bone gap greater than 10dB at frequencies of 250, 500 and 1000 Hz) were classified as “clinical otosclerotic”. Any ears that had air-conduction thresholds greater than 25dB HL at three or more octave frequencies but did not exhibit ABGs greater than 10dB at frequencies of 250, 500 and 1000 Hz were classified as SNHL. Finally, any ear with air-conduction thresholds below 25dB HL were classified as “normal”.

3.3 Results

3.3.1 Ear Status

In the total sample, 70 ears were included in the analysis. Forty-two ears from 33 participants underwent corrective surgery for otosclerosis and were therefore categorized as “surgical”. The remaining 28 ears were categorized based on audiometric results into either “clinical otosclerosis” (n=14 ears), “SNHL” (n=3 ears), or “normal” (n=11 ears).

3.3.2 Phenotyping

Audiometry

Surgical ears (n=42) had a mean air conduction PTA4 value of 31.33 dB (SD = 18.17), with mean thresholds of each tested octave ranging from 28.12 to 49.07 dB HL. The surgical ears had a PTA4 air-bone gap of 12.76 dB (SD = 11.83), with mean thresholds from 8.60 to 25.38 dB HL. The surgical ears had an overall mean air conduction threshold with thresholds

being best at the low-frequencies, and thresholds becoming more elevated in the higher frequencies. This is represented by smaller air-bone gaps in the higher frequencies indicating an overall average of a mixed hearing loss (Figure 5).

In the cases of clinical otosclerosis (n=14), mean air conduction PTA4 was 37.86 dB (SD = 12.46). Octave specific mean thresholds ranged from 33.57 to 45.71 dB HL, with mean air-conduction thresholds lowest at 2000 Hz, and highest at 8000Hz. PTA4 for air-bone gaps was 17.69 dB (SD = 7.87), with mean air-bone gaps at each octave frequency ranging from 9.64 to 33.85 dB. Air-bone gaps were largest in the lower frequencies, with the lowest mean ABG measured at 2000 Hz. This profile is consistent with the classic audiometric profile of otosclerosis of a low-frequency conductive hearing loss and the presence of a Carhartz notch at 2000 Hz.

Normal ears (n=11) had a PTA4 via air conduction of 9.66 dB HL (SD = 5.88) with mean octave thresholds between 8.18- and 20-dB HL. Mean thresholds between 250-4000 Hz were all under 15 dB HL, with the highest mean threshold measured at 8000 Hz measured at 20 dB HL. Mean ABG measurements at octave frequencies ranged from 0 dB to 16 dB HL. The mean PTA4 ABG was 5.11 (SD = 5.31).

The ears identified as SNHL (n=3) had a mean PTA4 via air conduction of 39.17 dB HL (SD = 15.73). Mean thresholds at octave frequencies ranged from 36.67 dB HL and 45 dB HL. The highest mean thresholds were measured at 8000 Hz. The mean PTA4 air-bone gap was 5.42 dB (SD = 6.05) with octave frequency means ranging between 1.67 to 8.33.

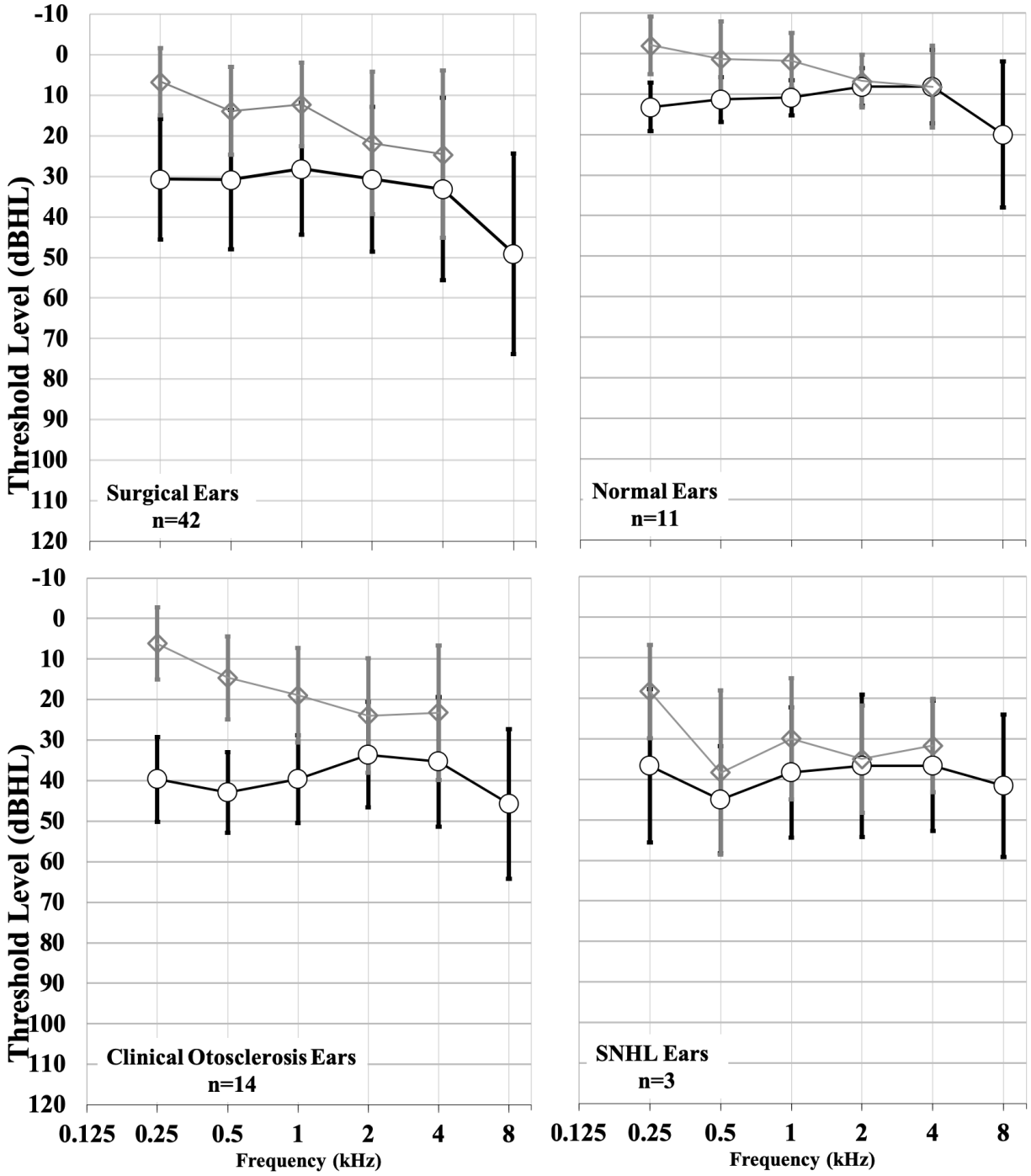


Figure 5. Mean audiometric thresholds separated based on ear status for 70 ears from 35 individuals diagnosed with otosclerosis. Ears separated into four groups: Surgical (n=42), Normal (n=11), Clinical Otosclerosis (n=14) and SNHL (n=3). Circles represent mean air conduction thresholds, and grey diamonds represent mean bone conduction thresholds. Error bars represent ± 1 standard deviation.

Acoustic Immittance

Mean ECV, TPP, Ytm (admittance) and RF (resonant frequency) were calculated based on ear status (Table 6). Multivariate analysis of variance (MANOVA) was conducted to determine whether there was a significant effect of ear status, gender and ear on measurements of ear canal volume, mean admittance, tympanometric peak pressure and resonant frequency (Table 7). Due to the small sample size of SNHL ears, these ears were removed from analysis. Significant multivariate effects were identified for ear status, $F(8, 100) = 2.179$, $p = 0.035$, Wilk's $\Lambda = 0.725$, partial $\eta^2 = 0.148$, and gender, $F(4, 50) = 5.945$, $p = 0.001$, Wilk's $\Lambda = 0.678$, partial $\eta^2 = 0.322$. Post-hoc analysis using a Bonferroni correction revealed a significant difference in RF between surgical and clinical otosclerosis ears, as well as surgical and normal ears. There was no significant difference in RF between clinical otosclerosis and normal ears. A test of between subject effects revealed that RF was significantly higher among female ears compared to males $F(1, 53) = 22.479$, $p < 0.001$, partial $\eta^2 = 0.298$.

Table 6. Mean (M) and standard deviation (SD) values for ear canal volume (ECV), Tympanometric peak pressure (TPP), admittance (Ytm), and resonant frequency (RF) for surgical ears (n=40), clinical otosclerotic ears (n=14), normal ears (n=11) and SNHL ears (n=2).

	Surgical (n=40)		**Clinical Otosclerosis (n=14)**		**Normal (n=11)**		**SNHL (n=3)**	
Variable	**M**	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
ECV	1.27	0.49	1.33	0.55	1.28	0.33	1.85	1.34
TPP	-13.63	56.15	-7.14	43.27	12.73	8.17	-32.50	81.32
Ytm	1.10	0.56	0.72	0.37	0.87	0.53	1.20	0.85
RF	676.92	134.19	921.43	306.16	840.00	225.83	950.00	N/A

Table 7. Summary of MANOVA for four dependent variables of Ytm, ECV, TPP and RF between three different ear statuses (normal, clinical otosclerosis, and surgical). Statistically significant results are bolded.

Variable	Wilks Λ	F	df	Error df	Sig.
Ear	.992	.104	4	50	.981
Ear Status	**.725**	**2.179**	**8**	**100**	**.035**
Gender	**.678**	**5.945**	**4**	**50**	**.001**
Ear Status * Ear	.893	.728	8	100	.667
Ear Status * Gender	.791	1.554	8	100	.148
Ear * Gender	.971	.378	4	50	.823
Ear Status * Ear * Gender	.906	.629	8	100	.752

Distortion Product Otoacoustic Emissions

Mean DPOAE amplitudes and noise floor based on ear status are plotted in Figure 6. To determine any significant difference in distortion product amplitude, a mixed model ANOVA was conducted with gender and ear status (surgical, clinical otosclerosis and normal) as between subject factors and frequency (17 frequencies) as a within-subject factor. Given the low number of SNHL ears (n=3), they were removed from analysis. Following a Greenhouse-Geisser correction to account for a violation of Mauchly's test of sphericity (Greenhouse & Geisser, 1959), there was a significant interaction between ear status and frequency [$F(20.824, 614.304) = 2.3.278$, $p < 0.001$]. Post-hoc analysis was conducted using a Bonferroni correction to examine the nature of this interaction. Results suggest that ears classified as normal demonstrated a higher distortion product amplitude compared to surgical ears at frequencies of 0.5-4 kHz, while normal ears had a higher distortion product amplitude compared to clinical otosclerosis ears at frequencies of 0.6-4 kHz ($p < 0.05$). There was no significant difference in distortion product amplitude between surgical and clinical otosclerosis ears at any frequency.

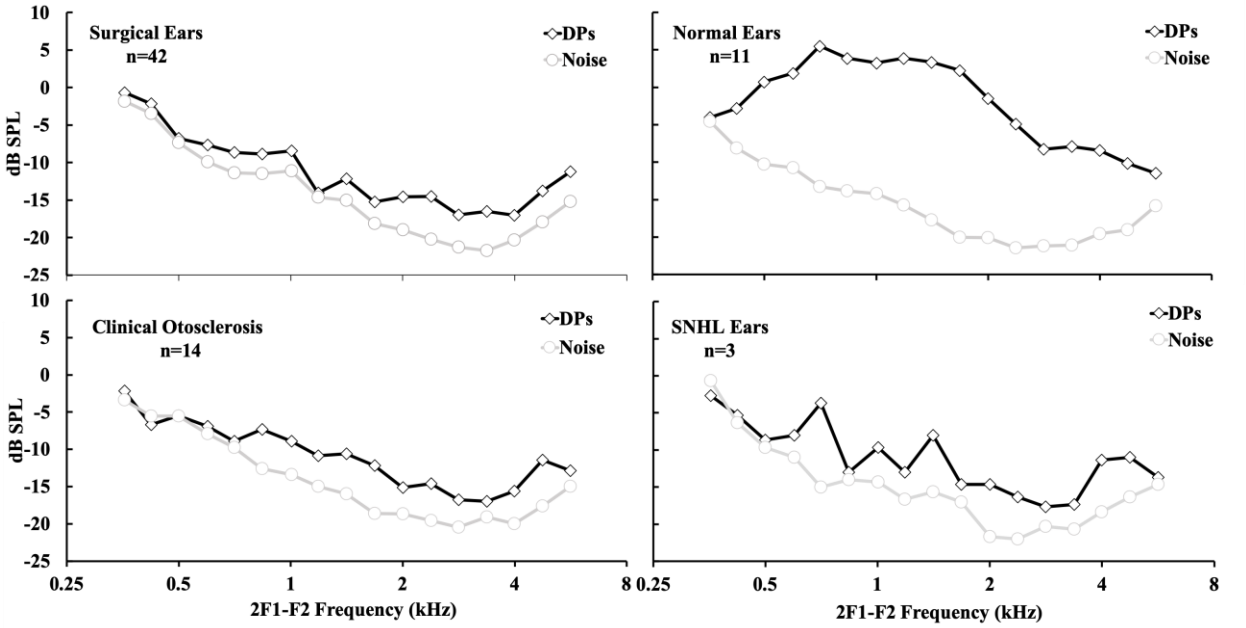


Figure 6. Mean distortion product (DP) otoacoustic emission amplitudes and noise floor amplitudes in dB SPL for ears classified as Surgical (n=41), Normal (n=11), Clinical Otosclerosis (n=14) and SNHL (n=3).

Results of the DPOAE screening procedure indicated that no post-surgical ears (n=41) passed the DPOAE screen. Similarly, no ears with clinical otosclerosis (n=14) passed the DPOAE screening. All of the ears categorized as normal (n=11) based on their audiometric thresholds passed the DPOAE screening criteria.

Acoustic Reflex Thresholds

Acoustic reflex thresholds were evaluated for 67 ears of the 34 otosclerotic participants. One participant (2 ears) could not be tested due to sensitivity of the pressurization and loud stimuli, while one ear of another participant could not be tested due to the inability to obtain a hermetic seal. There were no measurable acoustic reflex thresholds at any frequency tested in ears classified as clinical otosclerotic (n=13). In the cases classified as normal, 100% of the ears (11/11) had measurable ipsilateral acoustic reflex thresholds at frequencies of 500, 1000 and 2000 Hz with mean acoustic reflex thresholds of 86.8, 85.9 and 88.2dB SPL, respectively. In

general, mean acoustic reflex thresholds were higher for contralateral presentation with mean reflex thresholds ranging from 75-90dB SPL for ipsilateral presentation and 86.7-103.1dB SPL for contralateral presentation. A summary of percent present and mean acoustic reflex thresholds for the normal ears are reported in Table 8.

Table 8. Percent present and mean (SD) acoustic reflex thresholds for normal ears (n=11).

Normal Ears (n=11)	**500 Hz**		**1000 Hz**		**2000 Hz**		**4000 Hz**		**BBN**	
	Ipsi	**Contra**	**Ipsi**	**Contra**	**Ipsi**	**Contra**	**Ipsi**	**Contra**	**Ipsi**	**Contra**
% Present	100	72.7	100	81.8	100	90.9	100	63.6	90	90
Mean (SD)	86.8 (5.6)	103.1 (8.4)	85.9 (5.8)	98.3 (9.0)	88.2 (6.4)	98 (9.2)	90 (7.1)	100.8 (7.4)	75 (6.1)	86.7 (9.0)

3.3.3 *FOXL1* Screening

Of the 35 participants, only 1 participant was identified with a single copy (heterozygous) of the *FOXL1* (c.976_990het_del) deletion identified in the NL family segregating with autosomal dominant otosclerosis.

Based on the guidelines for interpretation of variants recommended by the American College of Medical Genetics (ACMG) (Richards et al., 2015), the 15bp deletion in *FOXL1* is determined to be classified as “pathogenic” (Appendix B). Pathogenicity of the deletion is based on the deletion meeting four criteria for pathogenicity recommended by the ACMG guidelines: Supporting computational and predictive data (PP3), moderate computational and predictive data (PM4), strong functional evidence (PS3) and strong segregation evidence (PPI-S). There is computational evidence for the deleterious effect of the deletion meeting criteria for ACMG guideline PP3 (see Abdelfatah 2014). The 15bp deletion results in a change in protein length of 5 amino acids meeting criteria for ACMG guideline PM4. To meet criteria of PS3, there is reported strong functional evidence that the 15bp deletion has a damaging effect on gene

expression (see Abdelfatah 2014). Finally, the criteria of PPI-S is met due the cosegregaton of otosclerosis in family members with the *FOXL1* deletion (see Abdelfatah 2014).

3.3.4 Family History

Family history questionnaires were conducted for the 35 participants across 33 families (included two sets of sisters). Of the 33 family histories, 22 families had at least two relatives with a hearing loss that developed between the ages of 20 and 60 years (Figure 7). Of these 22 families, 9 reported that an otosclerosis diagnosis was confirmed by an otolaryngologist.

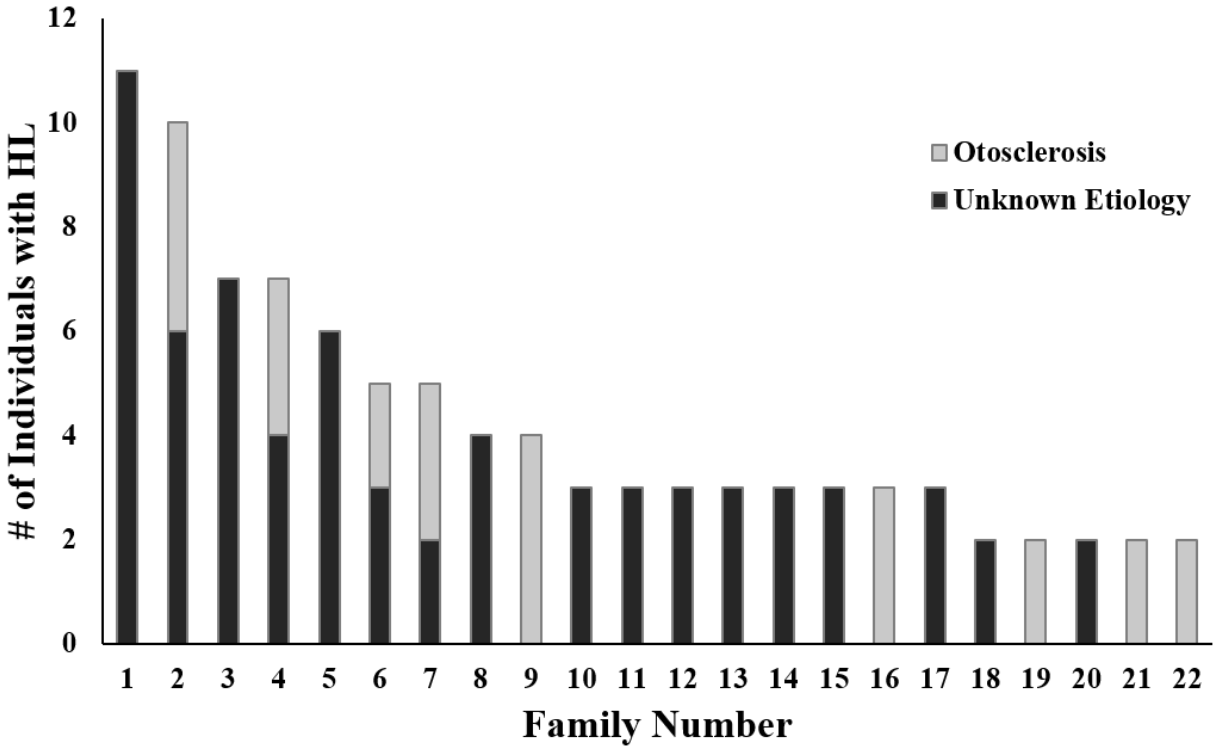


Figure 7. Number of affected family members in each of the 22 families with a family history of adult onset hearing loss. Cases were reported by the proband and identified as either otosclerosis specifically (grey line) or unknown etiology (black line).

3.3.5 *FOXL1* Phenotype Case Study

One participant out of 35 was heterozygous (carrier) for the *FOXL1* (c.976_990het_del) mutation. This section will serve as an individual case study outlining the auditory phenotype of

this subject. Two retrospective pre-surgical audiological records were obtained for the proband, as well as two post-surgical audiological tests.

Audiometric Thresholds

Audiometric thresholds were obtained retrospectively (3 audiograms) and prospectively (1 audiograms) for the *FOXL1* carrier. Audiograms were obtained using ER3 insert earphones and were reported to have good reliability. A summary of the audiometric thresholds is presented in Figure 8. Two pre-surgical thresholds obtained at 61 years of age (5 months apart) reveal a severe to profound mixed hearing loss in the right ear and a moderate rising to mild sensorineural hearing loss in the left ear. Following stapedotomy surgery on the patient's right ear (London Health Sciences) air conduction thresholds improved to a moderate rising to mild sensorineural hearing loss in the right ear. Similar results were obtained approximately 5 years later when the subject was recruited into this study. Improvement of the air-conduction thresholds in her right ear following successful stapedotomy surgery were consistent with otosclerosis. The presence of stapes fixation and the diagnosis were confirmed by the surgeon at the time of surgery.

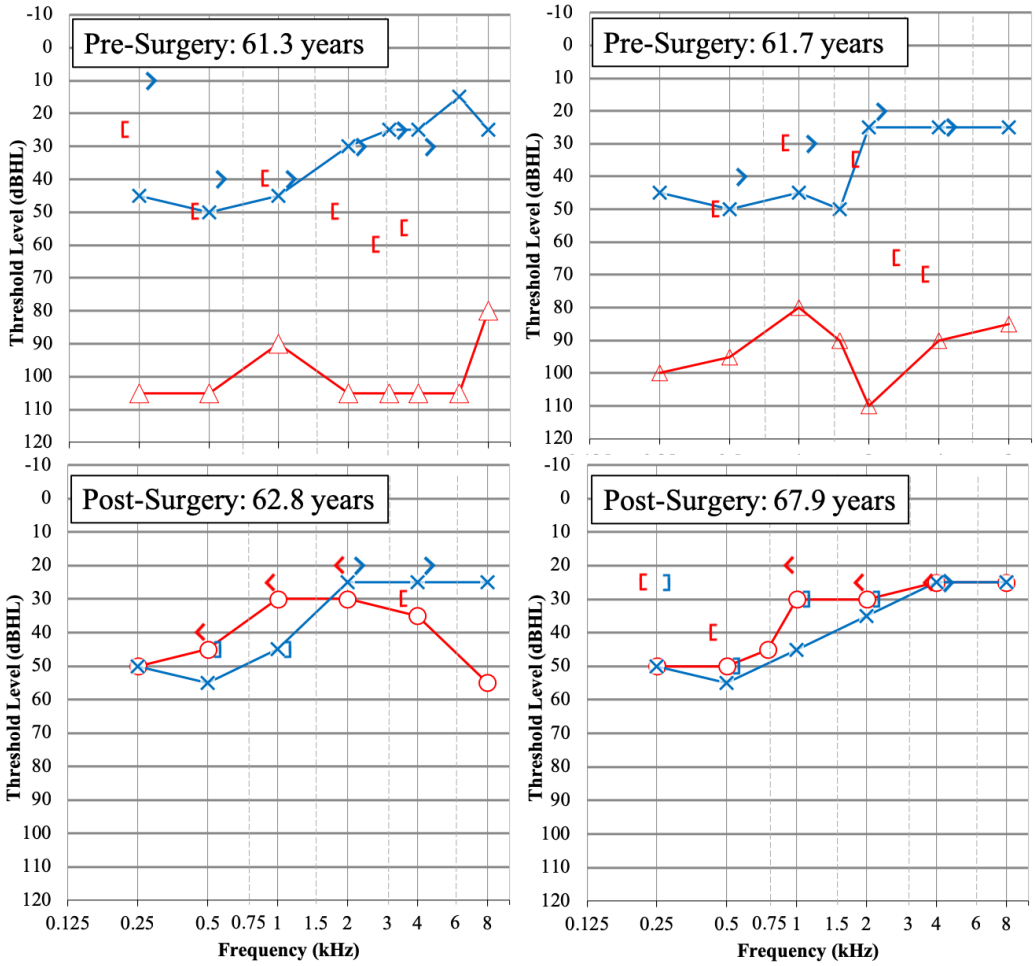


Figure 8. Pre and post-surgery audiometric thresholds for the *FOXL1* gene carrier. Left ear air conduction thresholds denoted by blue X while left bone conduction thresholds denoted by blue >. Right masked air conduction thresholds denoted by red triangles, while right masked bone conduction thresholds denoted by red [.

Due to patient discomfort to pressurization, standard tympanometry, multifrequency tympanometry and acoustic reflex thresholds could not be performed at the time of testing. DPOAE testing was conducted without any discomfort to the subject. DPOAEs were considered absent bilaterally based on the criteria provided in the Methods section, DPOES for both ears are presented in Figure 9. However, present DPOAEs using the criteria of DP > -10dB SPL and SNR > 6dB were present at 0.5 and 0.6 kHz in the right ear, and 0.4, 0.7 and 1.4 kHz in the left ear.

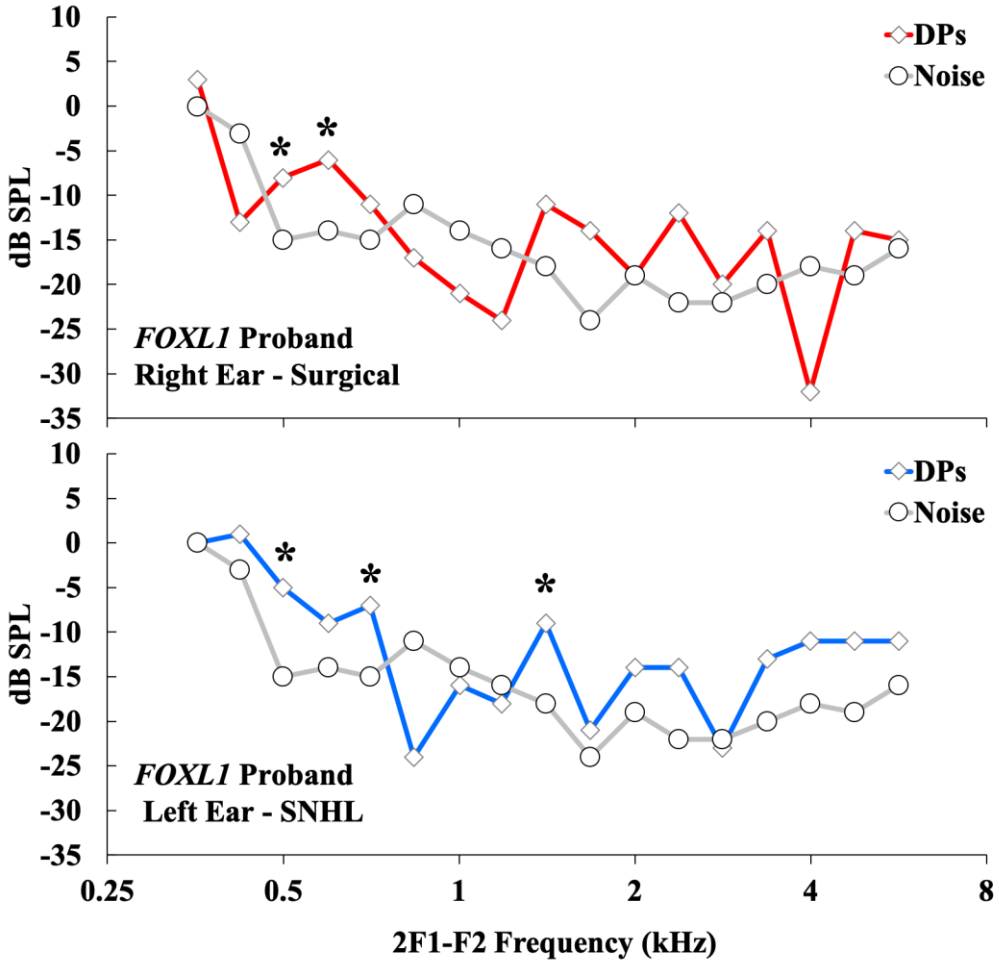


Figure 9. Distortion product (DP) otoacoustic emission amplitudes and noise floor amplitudes in dB SPL for the proband heterozygous for *FOXL1* deletion. Present DPOAEs represented by asterisks (*). Right ear classified as Surgical ear, while left ear represents Non-surgical ear classified as SNHL.

From the family history questionnaire, a pedigree was drawn (Figure 10). The family of the proband were unavailable for study recruitment due to limitations of the research ethics. The proband, individual IV-3, described a positive family history for late onset hearing loss. Although there was no hearing loss reported on her mother's side, she reported that her sister, father, two paternal uncles and her paternal aunt had hearing loss. This pattern of transmission (Figure 10) from one generation to the next, affecting both males and females, suggests a positive family history consistent with an autosomal dominant trait. An analysis of the pedigree was conducted to confirm an autosomal dominant inheritance pattern. The analysis for

inheritance pattern was guided by recommendations reported by Shearer et al. (2017). The proband was heterozygous for the *FOXL1* deletion, suggesting a dominant transmission pattern where only one copy of the affected allele is required to exhibit the phenotype. In cases of recessive transmission patterns, two copies of the alleles must be inherited for the individual to inherit the trait. Secondly, the family exhibits a later onset hearing loss restricted to the proband's paternal side of the pedigree. This suggests that the genetic trait for hearing loss is being inherited from the paternal lineage. Finally, to rule out a situation of sex-linked dominant inheritance pattern, individual IV-4 does not exhibit any signs of hearing loss. It would be expected that in the case of sex-linked dominant transmission through the paternal lineage that individual IV-4 would be an obligatory carrier of the allele because she would inherit the affected allele from her father (III-4). This would be the case, because III-4 would carry the affected allele on the X chromosome and would pass the affected X chromosome to all of his daughters. Therefore, in the proband's family, an autosomal dominant inheritance pattern is most likely.

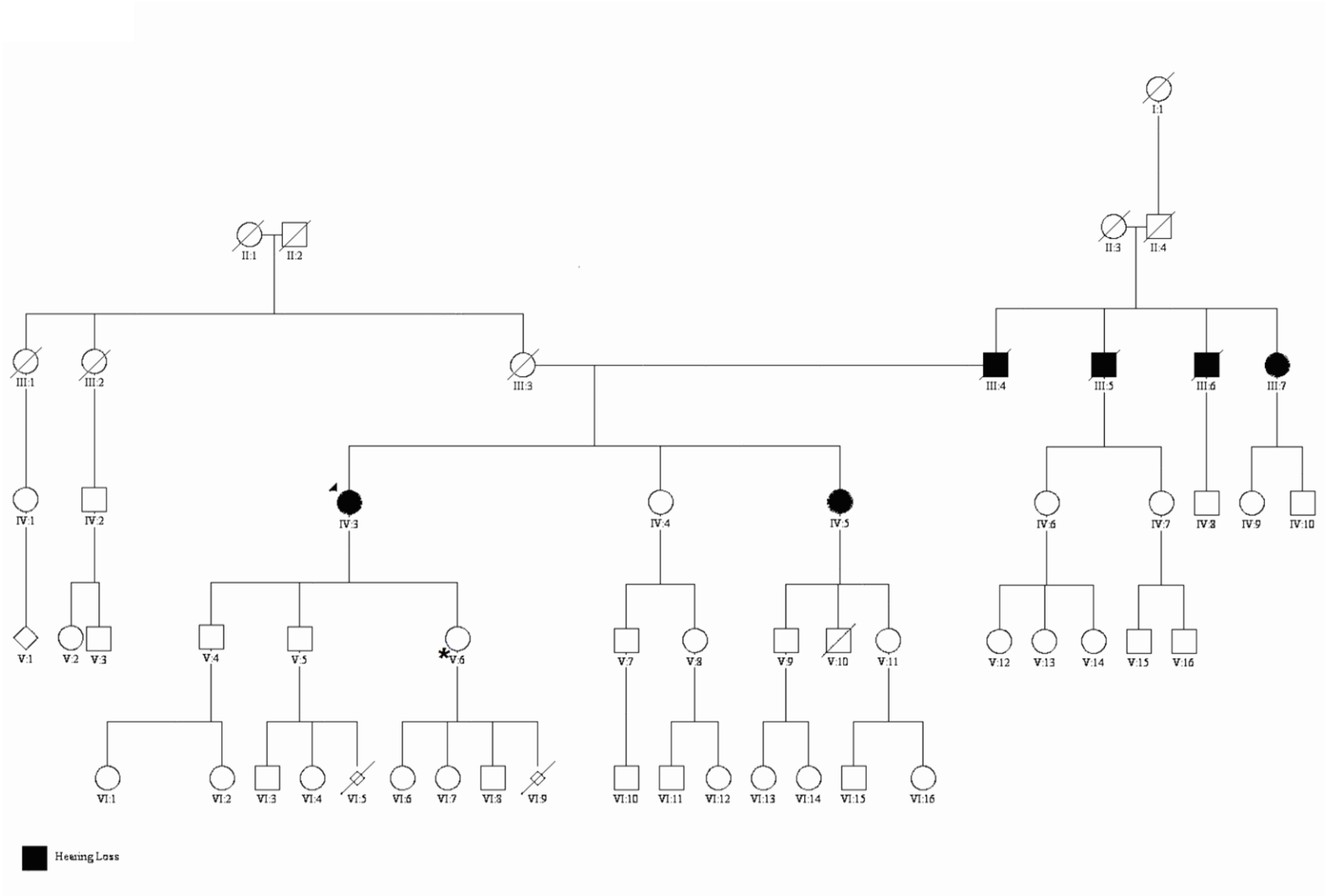


Figure 10. Pedigree of Ontario proband identified as carrying *FOXL1* c.976_990het_del deletion (Individual IV-3). Proband identified by arrow in upper left. * represents hearing status unknown but reported as being suspected early onset hearing loss.

3.4 Discussion

3.4.1 Phenotype

With respect to the ear asymmetry of otosclerosis and hearing loss, the phenotype of the presented Ontario otosclerotic cohort appears to be quite variable. Of the thirty-five otosclerotic subjects, eleven (31.4%) presented with true unilateral hearing loss, where their unaffected ear had audiometric thresholds within normal clinical limits. These findings are consistent with previous reports of approximately 53% of otosclerosis cases having true unilateral hearing loss in an Iranian otosclerotic population (Khorsandi et al., 2018) and unilateral otosclerosis in approximately 21% of cases in a Hungarian population (Karosi et al., 2012).

Fourteen of the seventy ears were classified as clinical otosclerosis when they presented with a significant air-bone gap, greater than 10 dB at three frequencies while also presenting with absent acoustic reflexes in the probe ear. In these ears the mean bone conduction thresholds at octave frequencies between 250 to 4000 Hz ranged between 21.74 to 24.53 dB HL which fall within the clinical criteria for “normal hearing” of 25 dB HL. Therefore, we would consider at the population level that the clinical otosclerotic ears present with the conductive hearing loss. It is possible that the clinical otosclerosis ears have a high-frequency sensorineural hearing loss component to their hearing loss, as air-conduction thresholds at 8 kHz have a mean threshold of approximately 50 dB HL. However, due to limitations of audiometry, air-bone gaps can only be measured up to 4 kHz.

In total there were three ears from three different subjects with sensorineural hearing loss in their non-surgical ear, representing 8.6% of ears in the study sample. These three ears would be classified as cochlear otosclerosis cases. This is consistent with previous reports of

otosclerosis that suggest that over the course of this disorder, hearing loss can progress to become sensorineural or mixed in nature (Schuknecht & Barber, 1985).

The status of the middle ear is determined clinically by an otoscopic exam and acoustic immittance tests; a well aerated ear shows no otoscopic evidence of middle ear pathology (for example middle ear fluid), with a normal tympanogram and normal static compliance results which suggest that the middle ear system is mobile and that sound conductance through the middle ear system is possible. However, it has been reported that the use of traditional tympanometric measures of static compliance may not be adequate in the differential diagnosis of otosclerosis (Shahnaz, Bork, et al., 2009). This is due to the variability of static compliance in the normal population, whereby a reduction in static compliance due to otosclerosis may not be great enough to overcome the population variability.

An abnormal air-bone gap (i.e. conductive hearing loss component) combined with absent acoustic reflexes and an otherwise aerated middle ear are therefore considered hallmark features of otosclerosis. The results of this study were consistent with these findings. There was no significant difference for Ytm with regards to gender, ear or ear status. However, there was a significant difference in RF between ears with clinical otosclerosis and those which had undergone stapes surgery. Post-surgical ears had an overall lower RF than ears with clinical otosclerosis. Previous reports investigating tympanometric measurements in cases of otosclerosis have suggested that otosclerotic ears have a significantly higher RF compared to normal ears (Ogut, Serbetcioglu, Kirazli, Kirkim, & Gode, 2008; Shahnaz & Polka, 1997). The results of this study indicate no significant difference in Ytm and RF between the ears categorized as normal and those with clinical otosclerosis. These results suggest that ears with otosclerosis can

demonstrate values within normal limits for ECV, Ytm, TPP and RF and that multifrequency tympanometry alone is not sensitive enough to distinguish otosclerotic ears from normal ears.

In summary, normal sensorineural hearing thresholds, indicated by normal bone conduction thresholds, in the presence of a conductive hearing loss component are the hallmark features of otosclerosis. Likewise, abnormal acoustic reflexes in a well-aerated ear are also considered to be a clinical indication of otosclerosis. In this study, when stimulated ipsilaterally or contralaterally, there were no measurable ipsilateral or contralateral acoustic reflex thresholds in the probe ear for ears classified as surgical, which is expected since the stapedius muscle is cut during stapedotomy surgery. ARTs were also absent in all clinical otosclerotic ears. This supports the clinical concept of using absent ARTs as a method of differentially diagnosing otosclerosis clinically. Absent acoustic reflex thresholds for detecting otosclerosis in individuals with aerated middle ears, who also exhibit a significant conductive component (i.e. conductive or mixed hearing loss), are clinical criteria used for the differential diagnosis of otosclerosis from other middle ear disorders. In family studies, some members may not have surgically confirmed otosclerosis, and this criterion is a valuable addition for phenotyping purposes.

This current study also utilized DPOAEs as a phenotyping tool for otosclerosis. Previous research has been mixed in terms of the presence of OAEs either prior to or following stapes surgery for otosclerosis (Riad, El-Rahman, Abdel Latif, Fawzy, & El-Anwar, 2017; Singh et al., 2012). The results of this study provide evidence that in 42 ears that have undergone corrective surgery for otosclerosis, none passed the DPOAE screening criteria. Sample mean data for all 17 frequencies also showed that the mean amplitude of DPOAEs for post-surgical otosclerosis was significantly lower than amplitudes in the normal ears. Although the current study does not provide any patient specific pre-surgical DPOAE measurements, 100% of ears suspected of

having otosclerosis (n=14) did not pass DPOAE screening and had mean DP amplitudes significantly lower than normal ears. The results of this study suggest that DPOAEs will also be helpful for phenotyping otosclerosis.

Superior semicircular canal dehiscence (SSCD) can mimic several of the hallmark features of otosclerosis – specifically a conductive or mixed hearing loss combined with a normal tympanogram, static compliance and otoscopic exam indicating a normally aerated middle ear (Merchant, Rosowski, & McKenna, 2007; Keefe et al. (2017). In this regard, the use of middle ear reflex and DPOAE testing may be particularly useful in genetic research. A conductive hearing loss caused by SSCD can present with acoustic reflexes (Merchant & Rosowski, 2008). Since we were unable to obtain any acoustic reflex thresholds in the probe ear of the clinical otosclerotic ears, the use of acoustic reflex thresholds in normally aerated ears should be used to better distinguish between conductive hearing losses due to stapes fixation versus those with dehiscence of the semicircular canals. Furthermore, OAEs can be present in ears with a conductive hearing loss and superior canal dehiscence (Merchant & Rosowski, 2008; Thabet, 2011). In conclusion, both acoustic reflexes and OAEs are valuable physiological procedures for detecting otosclerosis, and differentiating this disease from SSCD which can mimic otosclerosis in many respects, particularly in those with an audiometric threshold profile of low-frequency conductive hearing loss (Merchant, Rosowski, & McKenna, 2007).

The combination of audiometry, single or multifrequency tympanometry, acoustic reflexes, wideband acoustic immittance and otoacoustic emissions may be particularly useful for the advanced phenotyping of families with conductive and mixed hearing loss. Utilizing advanced clinical phenotyping tools for the purpose of genetic studies can aid in the differential diagnosis of various hearing disorders like SSCD. Another value of using DPOAEs and ARTs in

genetic studies would be the identification and longitudinal investigation of non-penetrant cases. Non-penetrant cases are individuals carrying the genetic mutation for a particular disorder, but who do not exhibit any clinical features of the disorder. However, these non-penetrant cases may be in the early stages of the disorder progression, and while they do not exhibit overt symptoms, they may have sub-clinical features when sensitive methods are used to assess auditory function. In the case of otosclerosis, using DPOAEs and ARTs may be useful physiological phenotyping measures for this purpose. For example, in the case of pre-clinical otosclerosis, it is possible to have poor or absent DPOAEs due to an abnormal middle ear system that is in the beginning stages of developing stapes fixation. Likewise, a middle ear system that is disrupted due to early otosclerosis may exhibit elevated or absent ARTs as the acoustic reflex is not strong enough to overcome the increased stiffness of the early-otosclerotic middle ear. Additionally, newer techniques of measuring the transmission of acoustic stimuli through the middle ear, such as wideband acoustic immittance (WAI), may also aid in future genetic research studies. Subsequent chapters of this thesis will focus on the use of these techniques, including WAI, as advanced phenotyping tools following gene discovery, for the purpose of gene discovery.

3.4.2 Family History

Otosclerosis is known to be a highly heritable hearing disorder typically developing later in life, around the 3rd decade and beyond. Results from the family history questionnaire identified 22 families which were positive for a family history of non-congenital hearing loss. Out of these 22 families, 9 were positive for multiple family members diagnosed with otosclerosis, representing 27.3% of the families. These results are consistent with findings from Shin et al. (2001) who reported 24.2% of their French otosclerotic population were positive for a family history of otosclerosis. However, the high proportion of families with multiple cases of

hearing loss in this study should be interpreted with caution. The recruitment letter clearly stated that this was a study into the genetic causes of otosclerosis. Therefore, it is feasible that patients with a family member diagnosed with a hearing loss were more likely to respond to the recruitment letter than patients without any family history of hearing loss; this is a recognised form of ascertainment bias.

3.4.3 *FOXL1* Screening

Through genetic screening for the mutation in the *FOXL1* gene, one participant in the Ontario cohort carried the deletion (c.976_990het_del). The discovery of the Ontario participant with the *FOXL1* c.976_990het_del genetic confirms that this causative mutation is present in other populations outside of Newfoundland. The onset of hearing loss, along with a positive family history of autosomal dominant otosclerosis in this Ontario proband, is consistent with findings from the Newfoundland *FOXL1* families. The Ontario proband reports no known familial connection to the island of Newfoundland.

The 15 bp deletion in *FOXL1* is predicted to result in a loss of five amino acids within the protein product and cosegregates with otosclerosis within a large Newfoundland family. Functional microarray analysis of expression of the mutated *FOXL1* gene confirmed that the mutation results in a change in the transcription levels of many genes involved in inflammation or bone remodelling, such as *IL29* and *CCXL10* (Abdelfatah, 2014). The *FOXL1* deletion is therefore considered pathogenic based on guidelines from the American College of Medical Genetics (Richards et al., 2015).

3.4.4 *FOXL1* Proband Phenotyping

The Ontario gene carrier has unilateral stapes fixation caused by otosclerosis as well as a positive family history, with vertical transmission, affecting both males and females, which is suggestive of an autosomal dominant inheritance. An autosomal dominant inheritance with an adult-onset hearing loss is consistent with previous reports of monogenic forms of otosclerosis (Ealy & Smith, 2010). Additional phenotype and genetic information should be collected in both affected and non-affected individuals of this family. This would allow for more comprehensive phenotyping of *FOXL1*-related otosclerosis in the Ontario population.

Based on retrospective and prospective assessments, the proband presents with a severe to profound mixed hearing loss in her right ear, and a moderate rising to mild sensorineural hearing loss in her left ear, consistent with a clinical diagnosis of otosclerosis. Retrospective data revealed absent ipsilateral and contralateral reflexes at all previous test dates and tympanometric immittance within the normal range of 0.3 to 2.1 mL. However, due to the proband's sensitivity to the pressurized testing required for acoustic immittance measures, we were unable to obtain acoustic reflex, tympanometry and resonant frequency measurements prospectively. Having the proband undergo successful surgery, as well as receiving a surgical diagnosis of otosclerosis, was an important step in confirming the presence of otosclerosis. As reviewed above, other hearing disorders, such as superior canal dehiscence or enlarged vestibular aqueduct syndrome, can mimic otosclerosis (Merchant et al., 2007; Wieczorek, Anderson, Harris, & Mikulec, 2013). In addition to showing improved air conductive thresholds post-operatively, the proband's bone-conduction hearing thresholds at 4 kHz in her right ear also improved following her surgery. It is expected that her air-conduction thresholds would improve following surgery, however on two separate pre-surgery audiometric thresholds, her bone conduction thresholds at 4 kHz showed a

moderate sensorineural loss, while two post-surgery audiometric thresholds confirmed a 40-45 dB improvement to a mild sensorineural hearing loss component at this frequency. There are reports of an improvement in bone conduction thresholds following stapes surgery (Manuele & Francesco, 2015; Quaranta, Besozzi, Fallacara, & Quaranta, 2005; Sperling, Sury, Gordon, & Cox, 2013; Vijayendra & Parikh, 2011). The mean improvement of bone conduction thresholds at 4 kHz reported by Sperling et al. (2013) was 6.3 dB in their cohort of 81 cases and the maximum improvement of bone conduction thresholds at 4 kHz of 55 dB, however they did not report how many individuals experienced such a drastic improvement. Bone conduction hearing is thought to require three different pathways; the osseotympanic route, the displacement of cochlear fluid, and the ossicular and cochlear fluid inertia (Tonndorf, 1966). Stiffening the stapes has been shown to increase the resonant frequency of the middle ear system, result in a change in the ossicular inertia, as well as change the physical characteristics of the oval window. This can contribute to changes to the cochlear fluid flow by removing the stapes footplate's contribution to cochlear fluid inertia (Stenfelt, Hato, & Goode, 2002). The subsequent post-operative improvement in bone conduction at 4 kHz may be due to changes to the inertia of the ossicles and cochlear fluid and may be contributing factors to the reduced bone conduction thresholds prior to stapes surgery.

3.4.5 Limitations and Future Directions

The identification of an otosclerotic proband outside of Newfoundland carrying the *FOXL1* deletion provides supporting evidence that the *FOXL1* gene discovered by Abdelfatah (2014) is truly causative. Further research in larger cohorts of otosclerosis patients will help clarify the proportion of otosclerosis caused by this or other pathogenic variants in *FOXL1*.

In terms of phenotyping of otosclerosis, the utilization of resonant frequency to distinguish otosclerotic from normal ears may have been limited by the frequency resolution of the GSI Tympstar v2 which sweeps across frequencies of 250-2000 Hz in 50 Hz intervals (Vanaja & Manjula, 2003). This low frequency resolution could explain why no significant interaction between RF and ear status was discovered in the current study. A newer technique, wideband acoustic immittance (WAI) is now available for physiological analysis of middle ear function and may be valuable in the advanced phenotyping of otosclerosis (Shahnaz, Bork, et al., 2009). The subsequent chapters of this dissertation will address the value of WAI for advanced phenotyping of otosclerosis.

Another limitation of the current study was the investigation of only the 15bp deletion in *FOXL1*. The aim was to identify whether this deletion was present in an outbred population, outside of the Newfoundland founder population where it was identified. However, without assessing the rest of the genome, the currents study cannot comment on concomitant genetiac abnormalities. One Ontario proband was identified with this *FOXL*1 deletion, however there were 22 additional families with later-onset hearing loss, of which 9 report having otosclerosis specifically. Additional genotyping work on these Ontario families may identify other genetic mutations responsible for monogenic forms of otosclerosis at unique or previously reported genetic loci (Bel Hadj Ali et al., 2008; Brownstein, Goldfarb, Levi, Frydman, & Avraham, 2006; Chen et al., 2002; Pauw et al., 2006; Schrauwen et al., 2011; Thys, Van Den Bogaert, et al., 2007; Tomek et al., 1998; Van Den Bogaert et al., 2001). Future genetics work may include the application of whole exome sequencing in order to identify new causative genes.

As more genes underlying the OTSC loci are identified, detailed phenotyping measures could be used to delineate the clinical and sub-clinical course of this hearing loss, particularly the

early development of otosclerosis. Early diagnosis of the disorder will become imperative as new preventative or rescue treatments are developed. The natural history of otosclerosis may be best studied in gene carriers before clinical symptoms are apparent. Advanced phenotyping should include a combination of behavioural and physiological techniques (wideband energy reflectance, OAEs, audiometry, acoustic reflex thresholds, and basic or multifrequency tympanometry) to investigate auditory system function in genetically confirmed pre-clinical or non-penetrant otosclerotic individuals.

3.5 References

Abdelfatah, N. (2014). The Genetic Aetiology of Otosclerosis in the Population of Newfoundland and Labrador. Memorial University of Newfoundland.

Bel Hadj Ali, I., Thys, M., Beltaief, N., Schrauwen, I., Hilgert, N., Vanderstraeten, K., … Van Camp, G. (2008). A new locus for otosclerosis, OTSC8, maps to the pericentromeric region of chromosome 9. Human Genetics, 123(3), 267–272. https://doi.org/10.1007/s00439-008-0470-3

Brownstein, Z., Goldfarb, A., Levi, H., Frydman, M., & Avraham, K. B. (2006). Chromosomal mapping and phenotypic characterization of hereditary otosclerosis linked to the OTSC4 locus. Archives of Otolaryngology--Head & Neck Surgery, 132(4), 416–424. https://doi.org/10.1001/archotol.132.4.416

Chen, W., Campbell, C. A., Green, G. E., Van Den Bogaert, K., Komodikis, C., Manolidis, L. S., … Smith, R. J. H. (2002). Linkage of otosclerosis to a third locus (OTSC3) on human chromosome 6p21.3-22.3. Journal of Medical Genetics, 39(7), 473–477. https://doi.org/10.1136/jmg.39.7.473

Greenhouse, S. W., & Geisser, S. (1959). On methods in the analysis of profile data. Psychometrika, 24(2), 95–112. https://doi.org/10.1007/BF02289823

Hannley, M. T. (1993). Audiologic characteristics of the patient with otosclerosis. Otolaryngologic Clinics of North America, 26(3), 373–387.

Herzog, M., Shehata-Dieler, W. E., & Dieler, R. (2001). Transient evoked and distortion product otoacoustic emissions following successful stapes surgery. European Archives of Oto-Rhino-Laryngology : Official Journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : Affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery, 258(2), 61–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11307607

Karosi, T., Csomor, P., & Sziklai, I. (2012). The value of HRCT in stapes fixations corresponding to hearing thresholds and histologic findings. Otology and Neurotology, 33(8), 1300–1307. https://doi.org/10.1097/MAO.0b013e31826352ad

Keefe, D., L Archer, K., Schmid, K., Fitzpatrick, D., Feeney, P., & Hunter, L. (2017). Identifying Otosclerosis with Aural Acoustical Tests of Absorbance, Group Delay,

Acoustic Reflex Threshold, and Otoacoustic Emissions. Journal of the American Academy of Audiology, 28. https://doi.org/10.3766/jaaa.16172

Khorsandi A., M. T., Jalali, M. M., & Shoshi D., V. (2018). Predictive factors in 995 stapes surgeries for primary otosclerosis. The Laryngoscope, 128(10), 2403–2407. https://doi.org/10.1002/lary.27160

Manuele, C., & Francesco, C. (2015). Bone Conduction after Stapes Surgery in Otosclerotic Patients with Mixed Hearing Loss : Fact or Fiction ?, 2(2), 2–5.

Merchant, S.N., & Rosowski, J. J. (2008). Conductive hearing loss caused by third-window lesions of the inner ear. Otology & Neurotology, 29(3), 282–289. https://doi.org/10.1097/mao.0b013e318161ab24.Conductive

Merchant, Saumil N., Rosowski, J. J., & McKenna, M. J. (2007). Superior Semicircular Canal Dehiscence Mimicking Otosclerotic Hearing Loss. In Otosclerosis and Stapes Surgery (Vol. 3096, pp. 137–145). Basel: KARGER. https://doi.org/10.1159/000098790

Pauw, R. J., De Leenheer, E. M. R., Van Den Bogaert, K., Huygen, P. L. M., Van Camp, G., Joosten, F. B. M., & Cremers, C. W. R. J. (2006). The phenotype of the first otosclerosis family linked to OTSC5. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 27(3), 308–315. Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/lary.21463/full

Quaranta, N., Besozzi, G., Fallacara, R. A., & Quaranta, A. (2005). Air and Bone Conduction Change after Stapedotomy and Partial Stapedectomy for Otosclerosis. Otolaryngology–Head and Neck Surgery, 133(1), 116–120. https://doi.org/10.1016/j.otohns.2005.03.011

Riad, H., El-Rahman, H., Abdel Latif, S., Fawzy, A., & El-Anwar, M. (2017). Dynamic characteristics of the middle ear after stapes surgery: a distortion product otoacoustic emission study. The Egyptian Journal of Otolaryngology, 33(1), 1. https://doi.org/10.4103/1012-5574.199397

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., … Rehm, H. L. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genetics in Medicine, 17(5), 405–423. https://doi.org/10.1038/gim.2015.30

Schrauwen, I., Weegerink, N., Fransen, E., Claes, C., Pennings, R., Cremers, C., … Van Camp, G. (2011). A new locus for otosclerosis, OTSC10, maps to chromosome 1q41-44. Clinical Genetics, 79(5), 495–497. https://doi.org/10.1111/j.1399-0004.2010.01576.x

Schuknecht, H. F., & Barber, W. (1985). Histologic variants in otosclerosis. The Laryngoscope, 95(11), 1307–1317. https://doi.org/10.1288/00005537-198511000-00003

Shahnaz, N., Bork, K., Polka, L., Longridge, N., Bell, D., & Westerberg, B. D. (2009). Energy reflectance and tympanometry in normal and otosclerotic ears. Ear and Hearing, 30(2), 219–233. https://doi.org/10.1097/AUD.0b013e3181976a14

Shahnaz, N., & Polka, L. (1997). Standard and Multifrequency Tympanometry in Normal and Otosclerotic Ears. Ear and Hearing, 18(4), 326–341. https://doi.org/10.1097/00003446-199708000-00007

Shin, Y. J., Calvas, P., Deguine, O., Charlet, J. P., Cognard, C., & Fraysse, B. (2001). Correlations between computed tomography findings and family history in otosclerotic patients. Otology & Neurotology : Official Publication of the American Otological Society,

American Neurotology Society [and] European Academy of Otology and Neurotology, 22(4), 461–464. https://doi.org/10.1097/00129492-200107000-00008
Singh, P. P., Gupta, N., & Verma, P. (2012). Transient evoked and distortion product otoacoustic emission profile in patients of otosclerosis: a preliminary report. Indian Journal of Otolaryngology and Head and Neck Surgery : Official Publication of the Association of Otolaryngologists of India, 64(1), 25–30. https://doi.org/10.1007/s12070-011-0148-3
Sperling, N. M., Sury, K., Gordon, J., & Cox, S. (2013). Early postoperative results in stapedectomy. Otolaryngology--Head and Neck Surgery : Official Journal of American Academy of Otolaryngology-Head and Neck Surgery, 149(6), 918–923. https://doi.org/10.1177/0194599813507232
Stenfelt, S., Hato, N., & Goode, R. L. (2002). Factors contributing to bone conduction: The middle ear. The Journal of the Acoustical Society of America, 111(2), 947–959. https://doi.org/10.1121/1.1432977
Terkildsen, K., Osterhammel, P., & Bretlau, P. (1973). Acoustic Middle Ear Muscle Reflexes in Patients with Otosclerosis. Archives of Otolaryngology, 98(3), 152–155. https://doi.org/10.1001/archotol.1973.00780020160003
Thabet, E. M. (2011). Transient evoked otoacoustic emissions in superior canal dehiscence syndrome. European Archives of Oto-Rhino-Laryngology, 268(1), 137–141. https://doi.org/10.1007/s00405-010-1313-0
Thys, M., Van Den Bogaert, K., Iliadou, V., Vanderstraeten, K., Dieltjens, N., Schrauwen, I., … Van Camp, G. (2007). A seventh locus for otosclerosis, OTSC7, maps to chromosome 6q13–16.1. European Journal of Human Genetics, 15(3), 362–368. https://doi.org/10.1038/sj.ejhg.5201761
Tomek, M. S., Brown, M. R., Mani, S. R., Ramesh, A., Srisailapathy, C. R., Coucke, P., … Smith, R. J. (1998). Localization of a gene for otosclerosis to chromosome 15q25-q26. Human Molecular Genetics, 7(2), 285–290. https://doi.org/10.1093/hmg/7.2.285
Tonndorf, J. (1966). Bone conduction. Studies in experimental animals. Acta Oto-Laryngologica, Suppl 213:1+.
Van Den Bogaert, K., Govaerts, P. J., Schatteman, I., Brown, M. R., Caethoven, G., Offeciers, F. E., … Van Camp, G. (2001). A second gene for otosclerosis, OTSC2, maps to chromosome 7q34-36. American Journal of Human Genetics, 68(2), 495–500. https://doi.org/10.1086/318185
Vanaja, C., & Manjula, P. (2003). Middle Ear Resonant Frequency in Normal and Otosclerotic Ears: Effect of Procedural Variation. JOURNAL OF …, 27(3), 158–162. Retrieved from http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Middle+Ear+Resonant+Frequency+in+Normal+and+Otosclerotic+Ears:+Effect+of+Procedural+Variation#3
Vijayendra, H., & Parikh, B. (2011). Bone conduction improvement after surgery for conductive hearing loss. Indian Journal of Otolaryngology and Head and Neck Surgery : Official Publication of the Association of Otolaryngologists of India, 63(3), 201–204. https://doi.org/10.1007/s12070-011-0130-0

Chapter 4

4 Evaluation of Wideband Acoustic Immittance as an Advanced Phenotyping Tool for Otosclerosis: Effect of Instrument, Stimulus Level and Otosclerosis on Power Absorbance

4.1 Introduction

Wideband acoustic immittance (WAI) is an umbrella term referring to different methods which use a wideband acoustic stimulus to assess the acoustic immittance of the middle ear system. WAI is an emerging clinical method for detecting middle ear dysfunction and differentiating between pathological conditions of the middle ear causing conductive hearing loss (Merchant et al., 2015; Nakajima et al., 2012; Prieve, Feeney, Stenfelt, & Shahnaz, 2013; Shahnaz, Bork, et al., 2009; Shahnaz, Longridge, & Bell, 2009).

One parameter of WAI, power absorbance (PA), is calculated between 0 and 1, where 0 represents no acoustic energy absorbed by the middle ear system, and 1 represents all the acoustic energy absorbed by the middle ear system. There are reports of significant differences in PA between ears with otosclerosis, semicircular canal dehiscence and normal ears (Merchant et al., 2016, 2015; Nakajima et al., 2012; Prieve et al., 2013). Since PA has demonstrated the ability to differentially diagnose middle ear pathologies, it has potential for improving auditory phenotyping for genetic studies of otosclerosis.

WAI measurements are now available in 2 commercial systems: The Interacoustics Titan and the Mimosa HearID. The Titan allows users to conduct three-dimensional tympanometry measurements in a pressurized environment. A tight-fitting rubberized tip is required with the Interacoustics Titan ear canal probe in order to maintain a hermetic seal while pressurized ear canal measurements are acquired. This system uses a wideband chirp stimulus to acquire WAI

data across frequencies of 0.26-8kHz, and across pressures from +200 to -300 daPa, including ambient or tympanometric peak pressure. The Mimosa HearID system is a non-pressurized system capable of measuring PA across the frequency spectrum but at ambient pressure only. The non-pressurized Mimosa HearID system uses an ER-10C foam tip connected to the ear canal probe in order to deliver chirp or tone stimuli and record responses. Research focusing on the diagnostic value of WAI has used different WAI systems and protocols (Feeney et al., 2003; Keefe & Simmons, 2003; Merchant et al., 2016; Prieve et al., 2013; Robinson, Thompson, & Allen, 2016; Shaver & Sun, 2013). Although PA has been measured for the Mimosa HearID and Interacoustics Titan, or their prototype systems, (Feeney et al., 2017; Jaffer, 2016; Vander Werff, Prieve, & Georgantas, 2007), the PA results collected with these 2 instruments have not been compared under the same experimental conditions in the same ear.

Both WAI systems also allow for the measurement of ear-canal volume (ECV). ECV is a useful clinical tool, as it indicates the overall size of the ear-canal and can be sensitive to help identify the presence of a perforation of the eardrum, or evaluate the patency of ventilation tubes in children (MRC Multicentre Otitis Media Study Group, 2008; Shanks, Stelmachowicz, Beauchaine, & Schulte, 1992). ECV also has implications for the measurement of PA. Stepp & Voss (2005) suggest that variability in ECV in the normal population may contribute to variations in absorbance measurements. The effect of middle ear cavity volume on absorbance was further investigated by Voss et al. (2008) who report an increase in absorbance with an increase in middle-ear cavity volume. As highlighted in Chapter 2, the two systems use different methods to calculate ear-canal area, which is an important component for the Thevénin calibration process. Therefore, it is important to compare instrument effects on ECV for two reasons. The first, is to determine whether ECV from one system can be directly compared to

another for clinical purposes, and the second is to determine whether differences in ECV may be contributing to instrument differences in PA.

The value of PA for detecting and differentiating middle ear pathology also depends, in part, on within and between subject variability. Factors that can contribute to test-retest reliability in normal ears include placement and reinsertion of the measurement probe (Abur, Horton, & Voss, 2014), time delay between recordings (Mahoney, McFarland, Carpenter, Rizvi, & Cacace, 2013; Vander Werff et al., 2007; Werner et al., 2010), and changes in middle ear pressure with repeated measurement (Burdiek & Sun, 2014). The test-retest reliability of WAI measurements has been investigated for different test instruments and populations (Feeney et al., 2017; Jaffer, 2016; Rosowski, Nakajima, & Hamade, 2012; Vander Werff et al., 2007). Findings from previous studies of the test-retest reliability of PA suggest that PA exhibits good test-retest reliability. However, no direct comparison of test-retest reliability has been conducted between the two systems.

4.1.1 Aims of Study 1:

Although PA and its test-retest reliability has been measured for the Mimosa HearID and Interacoustics Titan, or their prototype systems (Feeney et al., 2017; Jaffer, 2016; Vander Werff, Prieve, & Georgantas, 2007), WAI measurement outcomes for these 2 instruments have not been directly compared. Both the absolute PA and test-retest reliability of this measure will be compared for the Mimosa HearID and Interacoustics Titan under the same experimental conditions. The Mimosa HearID has the option to record PA using different wide band stimulus levels between 60 – 80 dB SPL. Since the effect of stimulus level on PA has yet to be reported in the literature, this study will also investigate the stimulus level effects on PA outcomes using the Mimosa system. There were two specific aims for Study 1:

Specific Aim 1. Conduct an inter-instrument comparison of two commercially available instruments, the Interacoustics Titan and the Mimosa HearID. More specifically:

Specific aim 1a: Compare PA outcomes for the two systems using the same stimulus level.

Specific aim 1b: Compare PA test-retest reliability between the two systems.

Specific aim 1c: Compare ECV outcomes for the two systems.

Specific aim 1d: Compare ECV test-retest reliability between the two systems.

Specific Aim 2. Compare PA measurements obtained at various stimulus levels using the Mimosa HearID. More specifically:

Specific aim 2a: Compare PA measurements obtained at four stimulus levels using the Mimosa HearID.

Specific aim 2b: Compare test-retest reliability of PA measurements obtained at four stimulus levels using the Mimosa HearID.

4.2 Study 1 Methods

Normal hearing subjects were invited and consented to participate in the study. An initial assessment of the ear and hearing thresholds was performed to determine that subjects met additional inclusion criteria. Those that passed the initial assessment phase proceeded to the experimental procedures evaluating the WAI and were asked to sit quietly in a sound-treated booth for one session lasting approximately 1.5 hours.

4.2.1 Subjects

Subjects were invited to participate in the study if they were between the ages of 18-75 and had self-reported normal hearing. Individuals with a hearing impairment related to injury or

disease or history of previous ear surgeries or conditions (i.e. chronic ear infections) were excluded from participating in the study. In order to pass inclusion criteria for the study, subjects were required to have a clear otoscopic examination, free of occluding cerumen and normal tympanic membrane, normal hearing thresholds and normal middle ear status using conventional acoustic immittance measures. Normal hearing levels in subjects were defined as air conduction thresholds of 25 dB HL or below at octave frequencies between 0.25 – 8 kHz and no air-bone gap greater than 10 dB at octave frequencies between 0.25 – 4 kHz. Subjects were also considered for the study if they had normal tympanometric measures, which included 226 Hz tympanometry, where compliance was within 0.2-2.1 mL and tympanometric peak pressure was between +50 and -100 daPa.

A total of 52 subjects were recruited as study participants. However, 12 subjects did not participate in the experimental procedures for the following reasons: failing audiometry inclusion criteria (n=5), failing tympanometry inclusion criteria (n=1) or inability to complete testing due to limitations in schedule and incomplete data (n=6). An additional 15 subjects underwent experimental procedures but were removed from the study due to poor seal of the probe in the ear canal (n=12) or a noisy measurement (n=3). In total the final sample included twenty-five subjects (14 females, 11 males; 50 ears) with an average age of 38.8 (ranging from 22.5 to 68.2 years old) representing 50 ears.

4.2.2 Instrumentation

Pure tone audiometry was conducted using the Interacoustics AC40 audiometer calibrated according to ANSI standards (re: S3.6.2010). Both air and bone conduction thresholds were measured. Measurements were conducted in a sound booth using ER3 insert earphones for air conduction, and a bone oscillator for bone conduction thresholds. Air conduction thresholds were

measured between 0.25 – 8 kHz, while bone conduction thresholds were measured between 0.25 – 4 kHz. A standard bracketing method was used to conduct audiometry.

Standard 226 Hz tympanometry was conducted using the Interacoustics Titan system (version 3.3.0) and pressures between +200 daPa to -300 daPa. Tympanometric peak pressure, and compliance were collected. Tympanometric peak pressure and compliance served as confirmation of normal tympanic membrane function and normal middle ear pressure. All ears in the study had tympanometric peak pressures between -100 and +50 daPa, and compliance values between 0.2 and 2.1cc.

Power absorbance and ECV were collected using the two systems; the Mimosa HearID and Interacoustics Titan. PA and ECV was collected with the Mimosa HearID (version 5.1.7.1) using the middle ear power analysis (MEPA3) protocol while PA and ECV were collected with the Interacoustics Titan using the 3D Tympanometry protocol (version 3.3.0.).

4.2.3 Experimental Procedures

Power absorbance and ECV measurements were collected twice for each system providing two trials per instrument and for the right and left ears of each subject. The ear order was randomly selected. Trial 2 of each ear was measured only after the probe was reinserted. For each instrument, the acquisition order of ears and trials went as follows: Ear 1-Trial 1, Ear 2-Trial 1, Ear 1-Trial 2, Ear 2-Trial 2. To minimize any potential pressurization effects on the middle ear measurements, data was first acquired using the Mimosa HearID followed by measurements with the Interacoustics Titan.

Titan measurements using the 3D tympanometry protocol were conducted with a wideband chirp stimulus spanning 0.2 kHz – 8 kHz at a stimulus level of 100 dB peak equivalent

SPL, which is approximately 65 dB SPL. During stimulus presentation, pressure was swept across +200daPa to -300 daPa.

HearID measurements were conducted using a wideband stimulus spanning 0.2 kHz to 6 kHz at stimulus levels of 60, 65, 70, and 80 dB SPL. PA and ECV measurements collected by the Mimosa HearID and used for comparison to the Interacoustics Titan were achieved under the 65 dB SPL stimulus condition. This level was chosen to match the stimulus level of the Interacoustics Titan since the Titan does not allow for the manipulation of stimulus level.

4.2.4 Data Analyses

For the inter-instrument comparison of absolute PA, measurement results were exported from each instrument and processed as follows. PA measurements for the Titan were extracted from the 3D tympanogram by exporting PA values obtained at atmospheric pressure. PA measurements from trial 1 and trial 2 of each condition were averaged together across the full frequency bandwidth of the measurement. PA measurements obtained at 4 stimulus levels were exported from the Mimosa system and processed in a similar manner. At each stimulus level (60, 65, 70 and 80 dB SPL), PA measurements obtained over two trials were averaged together for each participant. Following this calculation, the PA measurements were then divided into 1/3rd octave bands. Once the mean PA was obtained, the full bandwidth measurement was divided into fourteen 1/3rd octave bands. The upper limit of 1/3rd octave bands was chosen at 5 kHz. This allowed for the full 1/3rd octave bandwidth to be included in the average for both systems.

In order to evaluate the test-retest reliability of PA measurements, and to compare the test-retest reliability between systems, the absolute difference in PA was calculated between trials. First, the PA of each trial was averaged into 1/3rd octave bands. The absolute difference

between Trial 1 and 2 was then calculated for each octave band. The absolute difference between trials was measured for the Titan at 65 dB SPL and for the Mimosa at 60, 65, 70 and 80 dB SPL.

For the inter-instrument comparison of ECV, measurement results were exported from each instrument and processed as follows. PA measurements for the Titan were extracted from the 3D tympanogram. ECV measurements from trial 1 and trial 2 of each condition were averaged together. ECV measurements obtained at 65 dB SPL with the Mimosa were extracted. ECV measurements obtained over two trials were averaged together for each participant.

For the analysis of the test-retest reliability of ECV measurements, and to compare the test-retest reliability between systems, the absolute difference in ECV was calculated between trials. The absolute difference between trials was measured for the Titan at 65 dB SPL and for the Mimosa at 65 dB SPL.

4.2.5 Statistical Analysis

For the inter-instrument comparison of absolute PA, a mixed model ANOVA was conducted with frequency (14 levels), instrument (2 levels) and ear (2 levels) as the within-subject factor while sex (2 levels) served as a between-subject factor. When appropriate, *post-hoc* analysis was conducted by completing a pairwise comparison following Bonferroni correction to determine the frequencies where PA was significantly different between the two systems. The inter-instrument difference in test-retest reliability of PA measurements was also investigated using a mixed model ANOVA to test for mean absolute differences in PA with instrument (2 levels), ear (2 levels) and frequency (14 levels) as within-subject factors and sex (2 levels) as a between-subject factor. Further analysis of the test-retest reliability of each system was carried out by calculating the Pearson correlation coefficient between Trial 1 and Trial 2 for each system.

An inter-system comparison of ECV measures was conducted using a Bland-Altman approach (Bland & Altman, 1999). The difference between ECVs obtained with each system was plotted on the y-axis, while the mean ECV obtained with the two systems was plotted on the x-axis. A one-sample t-test was used to determine if there were significant differences between the two systems. Further analysis of the instrument differences of ECV was conducted using a mixed model ANOVA where instrument (2 levels), ear (2 levels) served as within-subject factors and sex (2 levels) as a between-subject factor.

For the analysis of ECV test-retest reliability, a similar Bland-Altman approach was conducted separately for each instrument. The difference between ECVs obtained at each trial was plotted on the y-axis, while mean ECV obtained with the two trials was plotted on the x-axis. A one-sample t-test was used to determine if there were significant differences between the two systems. A comparison of the test-retest reliability of ECV was conducted using a mixed model ANOVA for absolute mean differences between Trial 1 and Trial 2, where instrument (2 levels), ear (2 levels) served as within-subject factors and sex (2 levels) as a between-subject factor.

For the Mimosa Hear ID system, a mixed-model ANOVA was conducted to determine whether stimulus presentation level had a significant effect on absolute PA measurement. Stimulus level (4 levels), frequency (14 levels) and ear (2 levels) were used as within-subject factors while sex served as a between-subject factor. When appropriate, *post-hoc* analysis was conducted by completing a pairwise comparison following Bonferroni correction.

Stimulus level effects on test-retest reliability were also examined for the Mimosa HearID. A mixed model ANOVA was completed for mean absolute difference of PA using

frequency (14 levels), stimulus level (4 levels) and ear (2 levels) as within-subject factors and sex (2 levels) as a between subject factor. When appropriate, *post-hoc* analysis was conducted by completing a pairwise comparison following Bonferroni correction.

4.3 Study 1 Results

4.3.1 PA System Comparison

Power absorbance collected using the two systems was compared to determine whether there were instrument effects on PA measurements. The results of the mixed model ANOVA analyzing the inter-instrument differences in PA revealed a violation of sphericity using Mauchly's test of sphericity for frequency $\chi^2(90) = 642$, $p < 0.001$, and therefore a Greenhouse-Geisser correction was used. It was then determined there was a significant main effect for frequency [F (2.163, 51.903) = 97.803, $p < 0.001$] and instrument [F (1, 24) = 9.425, $p = 0.005$]. There was also a significant interaction between ear and frequency [F (2.461, 59.063) = 3.222, $p = 0.037$] and instrument and frequency [F (2.569, 61.647) = 25.247, $p < 0.001$]. There was no significant effect of sex on PA measurements ($p > 0.05$). *Post-hoc* analysis suggests there is a significant difference in PA measured from different instruments at all frequencies except 1587, 2000 and 5040 Hz ($p < 0.05$). Mean PA values for both systems are plotted in Figure 11 and mean PA values along with their 95% confidence interval are reported in Appendix E. *Post-hoc* analysis suggests there is a significant difference in PA between ears, where right ears had a lower PA at frequencies of 1587 Hz and 2000 Hz, and a higher PA at frequencies of 2520, 3175 and 4000 Hz ($p < 0.05$).

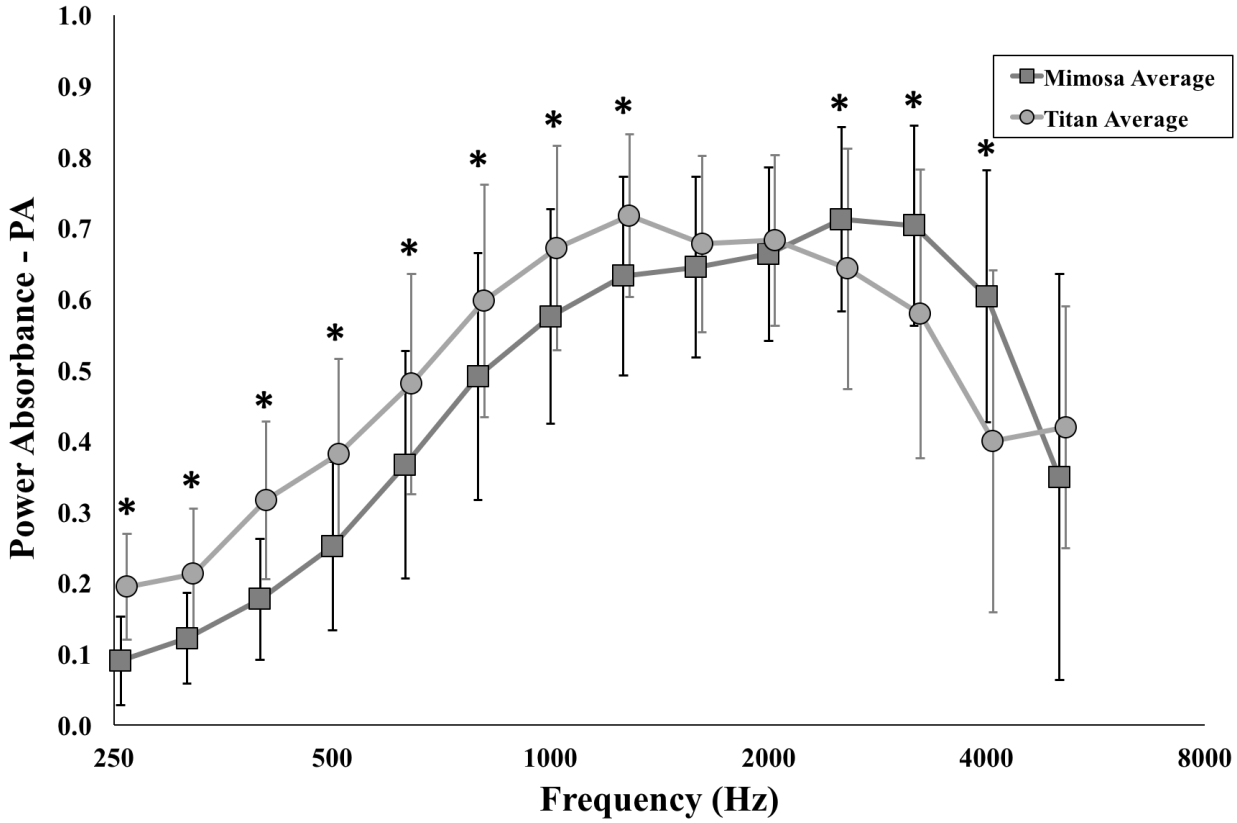


Figure 11. Mean power absorbance (PA) for Mimosa HearID and Interacoustics Titan (n=50 ears). Error bars represent standard deviations. Significant differences in PA between instruments are denoted by asterisks (*).

4.3.2 PA System Comparison Test-retest Reliability

An inter-system comparison of test-retest reliability of PA measurements was carried out by comparing the absolute difference in PA between the two trials of the two systems (Figure 12). This was conducted using a mixed model ANOVA approach. A violation of sphericity using Mauchly's test of sphericity was found for frequency $\chi^2(90) = 329.574$, $p < 0.001$, therefore a Greenhouse-Geisser correction was used to determine significance which revealed a significant effect of frequency on test-retest reliability (F (4.168, 95.868) = 3.701, p = 0.007) and a significant interaction of instrument and frequency (F (4.80, 110.394) = 4.591, p = 0.001). A paired samples t-test with a Bonferroni correction indicated that the Titan had a significantly lower inter-trial difference in absorbance at 0.25 kHz compared to the Mimosa (p = 0.014), while

the Mimosa had significantly lower inter-trial differences in absorbance between 3.2 - 4.0 kHz, as shown in Figure 12 ($p < 0.05$).

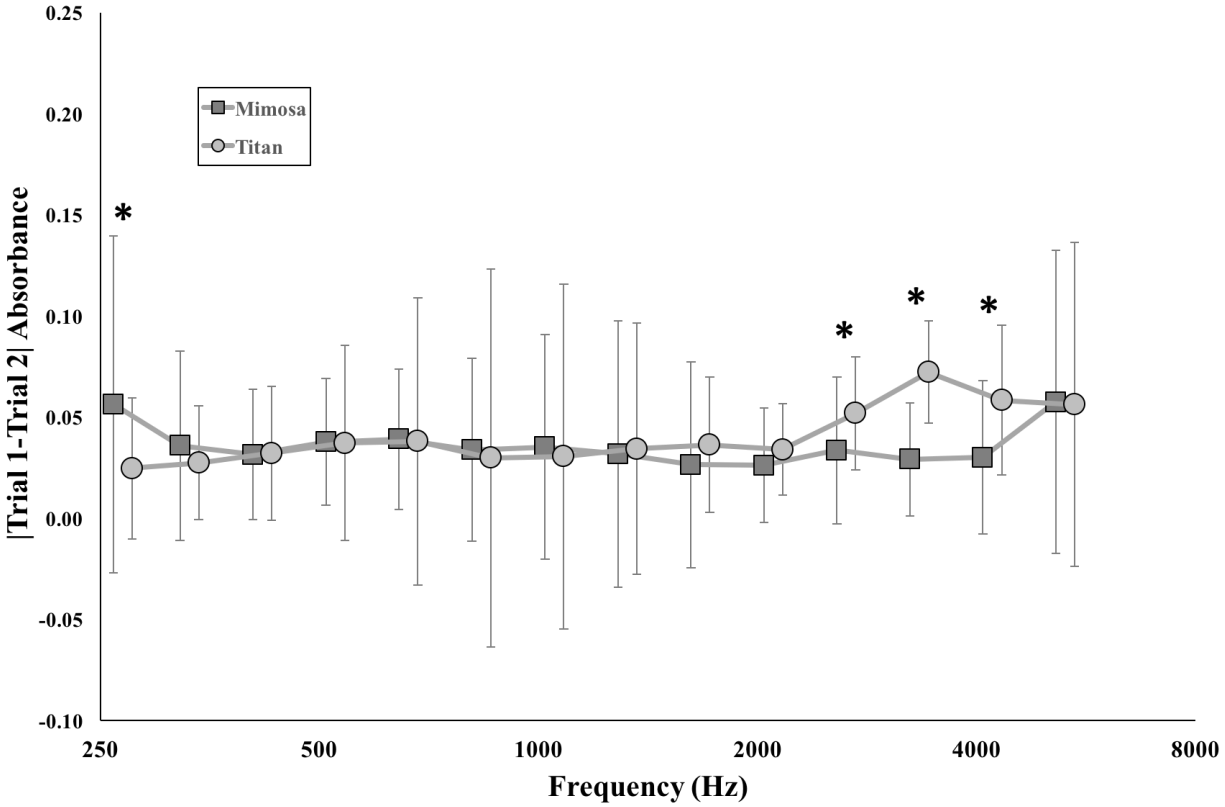


Figure 12. Mean absolute difference between trial 1 and trial 2 of power absorbance (PA) using the 65 dB SPL stimulus level of the Mimosa HearID and the PA measurement at ambient pressure of the Interacoustics Titan 3D tympanometry measurement (n=50 ears). Error bars represent standard deviations. Significant differences in absolute difference of PA between instruments are denoted by asterisks (*).

The test-retest reliability of PA measurements was analyzed separately for both systems using Pearson correlation coefficients of trial 1 versus trial 2 (Figures 13 and 14). The Pearson correlation coefficients of PA measurements obtained using the Interacoustics Titan were significant across all octave bands, while all Pearson correlation coefficients of PA measurements obtained using the Mimosa HearID were significant across octave bands with the exception of the 250 Hz 1/3rd octave band (r = .250, N = 50, p = .053).

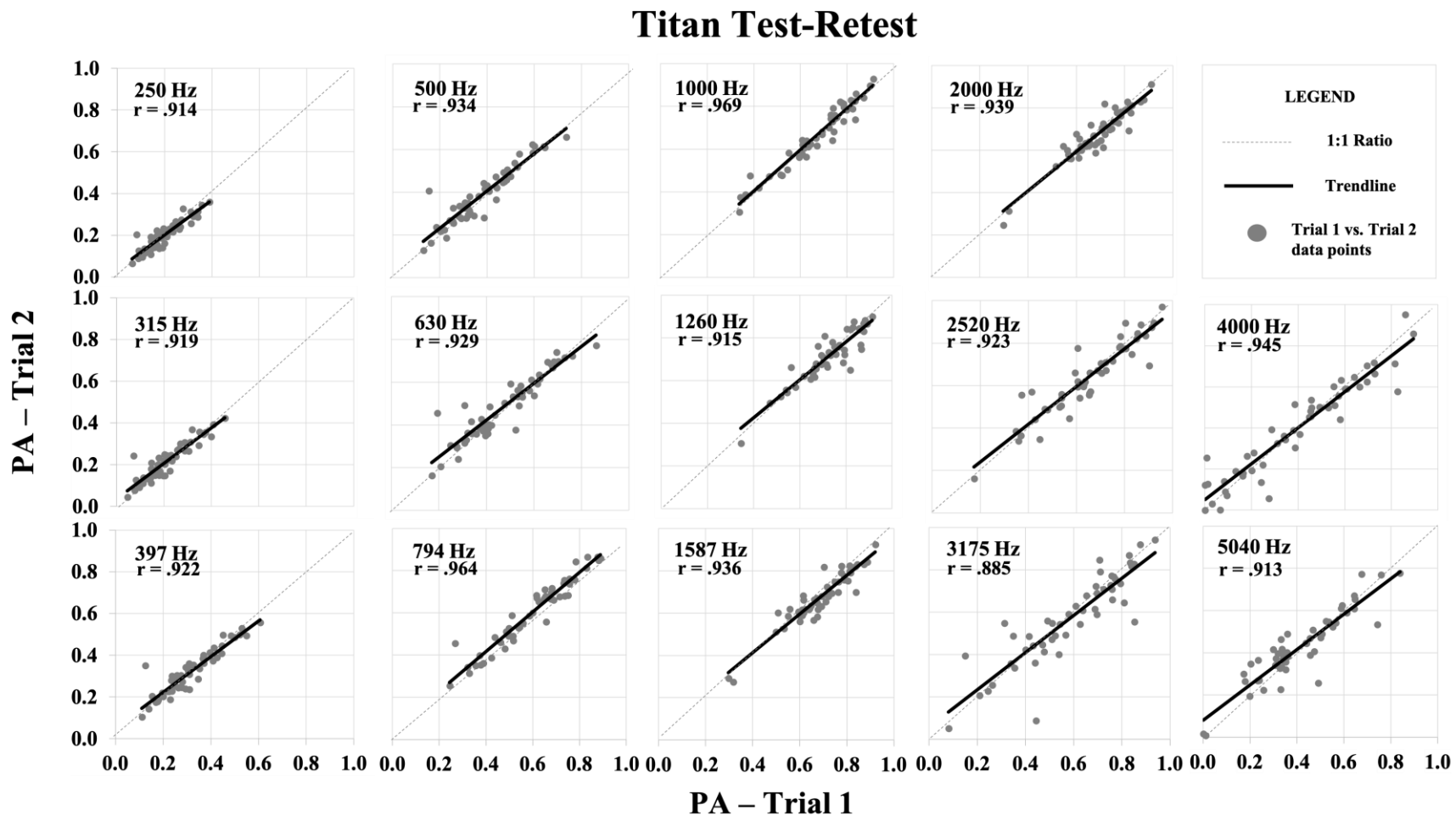


Figure 13. Test-retest reliability of power absorbance of the Interacoustics Titan measured by Pearson Correlation Coefficients (n = 50 ears). X-axis represents trial 1, and y-axis represents trial 2. Solid black line represents the trend line while diagonal dashed line represents a 1:1 ratio. Pearson correlation coefficients (r) are also reported.

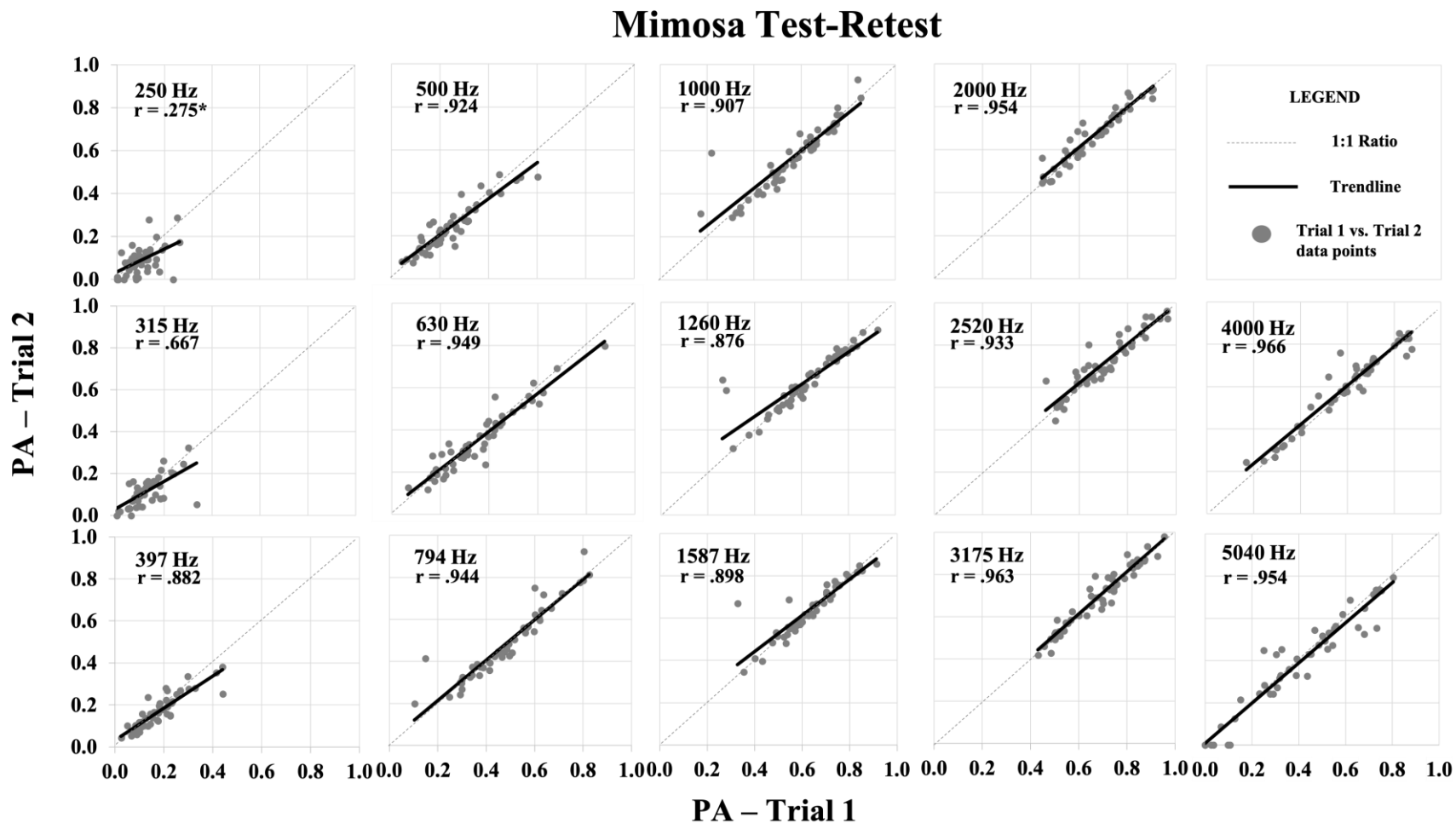


Figure 14. Test-retest reliability of power absorbance (PA) of the Mimosa HearID measured by Pearson Correlation Coefficients (n = 50 ears). X-axis represents trial 1, and y-axis represents trial 2. Solid black line represents the trend line while diagonal dashed line represents a 1:1 ratio. Pearson correlation coefficients (r) are also reported. * indicates a Pearson r value with a $p > 0.001$.

4.3.3 ECV System Comparison

The Bland-Altman plot comparing ECV measurements obtained with the Titan and Mimosa are presented in Figure 15. The mean difference was -0.046 with a limit of agreement of -0.56 to 0.46. A one-sample T-test was carried out to test whether mean ECV difference was significantly greater than zero. Results indicated that there was no significant difference in mean ECV difference between the two systems [t (49) = -1.250, p = .217]. This suggests that the ECV measurements conducted with the two systems are similar. To confirm these results, a linear regression analysis to predict mean ECV on ECV difference was performed. Results suggest that there is a significant regression between mean ECV and mean ECV difference with the two systems [$F(1,48) = 4.148$, $p = .047$] with an R^2 of .08, suggesting that ECV measurements obtained with the two systems are significantly different. On careful observation of the data, it was observed that three measurements fell outside of 2 standard deviations (clinically more stringent) from the group mean difference (shown in filled black in Figure 15). These data points were removed, and re-analysis was done where no significant regression between mean ECV and mean ECV difference of the two systems was identified [$F(1,48) = 2.573$, $p = .116$] with an R^2 of .054.

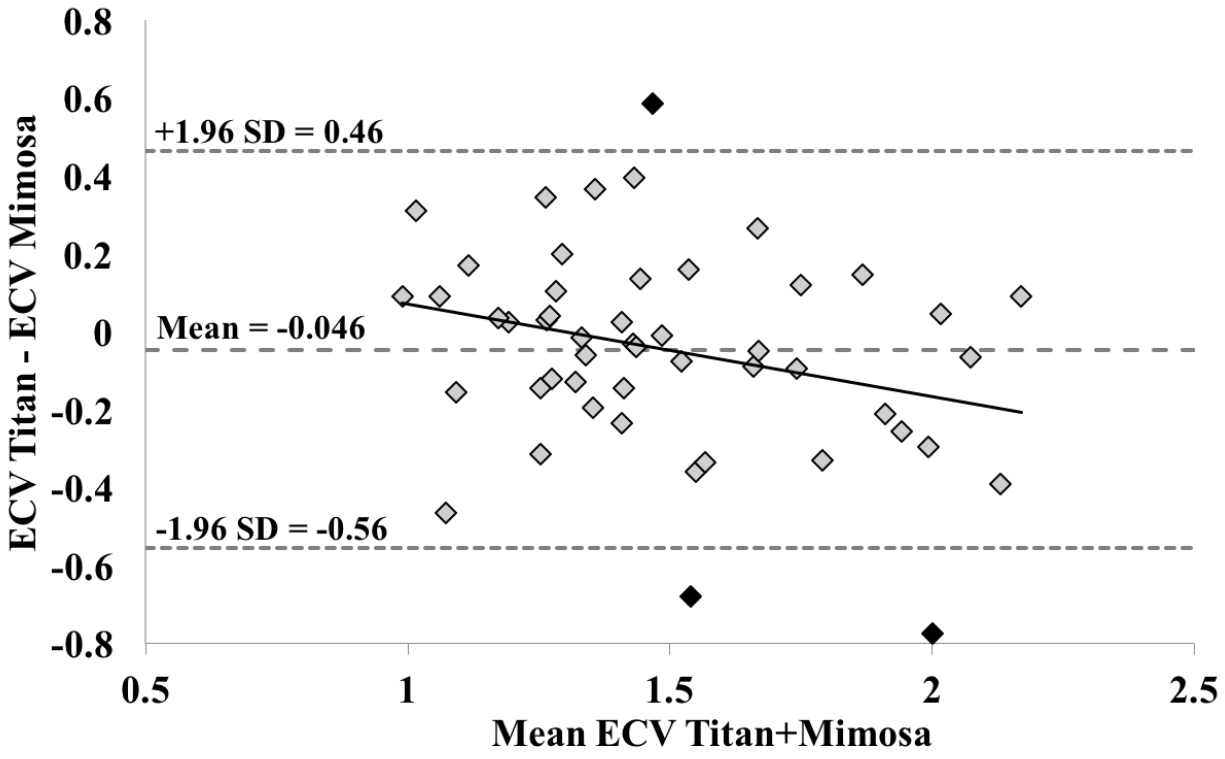


Figure 15. Bland-Altman plot for the comparison of ear canal volume (ECV) measurements obtained with the Titan and the Mimosa. Solid black line represents the trend line, while dashed grey lines indicate mean of the difference between the two systems and ±1.96 SD around the mean. Filled black data points represent data points falling outside of 2 SD from the group mean.

To compare the ECV between systems, a mixed model ANOVA was performed comparing mean ECV obtained via the Interacoustics Titan (M = 1.47, SD = .30) to ECV values obtained using the Mimosa (M = 1.51, SD = .37). Results indicate no significant difference in mean ECV collected between the Titan and Mimosa [F (1,23) = 1.710, p = .204]. This suggests that at a population level, there does not appear to be a significant difference in ECV obtained by either system.

4.3.4 ECV Test-retest Reliability

The distribution of ECV measurements obtained from each system were assessed for normality using the Shapiro-Wilks test, whereby it was determined that measurements obtained with the Titan and Mimosa were normally distributed ($p > 0.05$). The Bland-Altman plot for

ECV obtained with the Titan and Mimosa are reported in Figure 16. For the Titan, the mean difference was 0.035 with a limit of agreement of -0.24 to 0.31. A one-sample T-test was carried out to test whether mean ECV difference was significantly greater than zero. Results indicated that there was no significant difference in ECV mean difference between trial 1 and trial 2 of the Titan [t (49) = 1.769, p = 0.083]. This suggests that the measurements conducted at two different time intervals were similar for the Titan. These results were confirmed with a linear regression analysis to predict mean ECV on ECV difference. There was no significant regression between mean ECV and ECV difference with the Titan [F(1,48) = 2.182, p = 0.146] with an R^2 of .043. A similar analysis was carried out for the Mimosa, where a one-sample T-test suggests that mean ECV mean difference between the two trials was not significantly different from zero [t(49) = 1.359, p = .180]. This suggests that the two ECV measurements conducted with the Mimosa were similar. These results were also confirmed with a linear regression analysis to predict mean ECV on ECV difference, where there was no significant regression [F(1,48) = .031, p = .861] with an R^2 of .001.

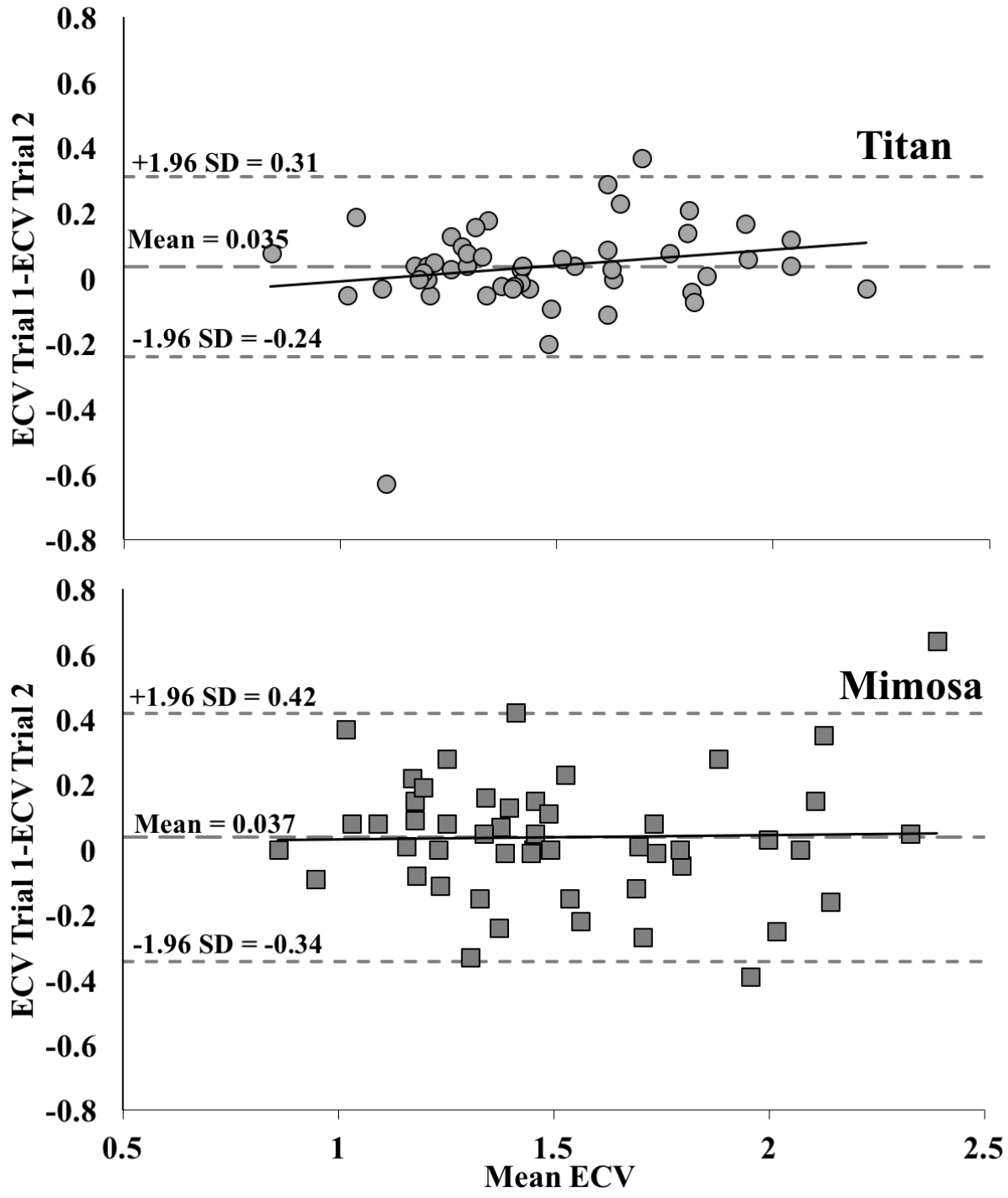


Figure 16. Bland-Altman plot for the test-retest reliability of ear canal volume (ECV) measurements obtained with the Titan and the Mimosa. Dashed black lines represent the trend lines, while solid grey lines indicate means of the difference between trial 1 and trial 2. Dashed grey lines represent ±1.96 SD around the mean.

The results of the mixed model ANOVA indicate the absolute difeerence between consecutive ECV measures was greater when using the Mimosa HearID than the Interacoustics Titan ($F(1,23) = 5.819$, $p = 0.024$), suggesting the Titan had better test-retest reliability Figure 17).

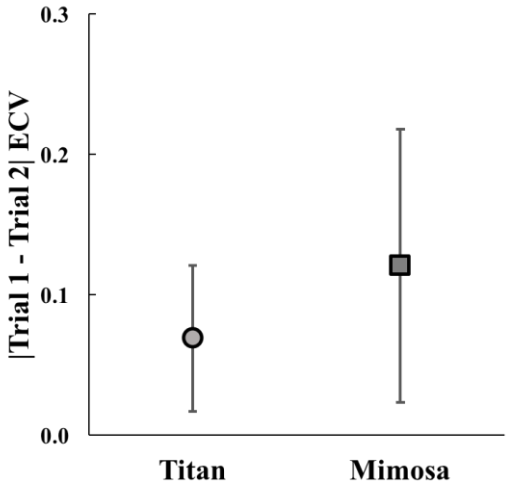


Figure 17. Mean absolute difference in ear canal volume (ECV) collected in two trials using the Interacoustics Titan and Mimosa HearID. Error bars represent 5th and 95th percentile values.

4.3.5 Stimulus Effects on PA

Mean PA within each 1/3rd octave band was calculated for each of the four stimulus levels, as shown in Figure 18. The results of the mixed model ANOVA revealed a violation of sphericity using Mauchly's test of sphericity for frequency $\chi^2(90) = 580.860$, $p < 0.001$ and stimulus level $\chi^2(5) = 24.992$, $p < 0.001$. A Greenhouse-Geisser correction was used to determine significance. There was a significant main effect of stimulus level [F (1.699,39.07) = 5.110, p = .014] and frequency [F (2.41,55.426) = 96.106, p < .001], and for the interaction between stimulus level and frequency [F (6.683,153.718) = 2.339, p = .029]. *Post hoc* analysis with a Bonferroni correction for multiple comparisons are also presented in Figure 18. The 60 dB SPL stimulus level had significantly higher PA at 314 Hz compared to the 80 dB SPL condition (p = .013). At 397 Hz, PA was significantly higher in the 60dB SPL condition compared to the 70 (p

= .030) and 80 dB SPL ($p < .001$) conditions. At 2000 Hz the 60 dB SPL condition had significantly higher PA compared to the 65 dB SPL condition ($p = .024$) and the 80dB SPL condition ($p = .009$), while the 65dB SPL condition had significantly lower PA compared to the 70dB SPL condition ($p = .031$). Also, at 2000Hz, the 70dB SPL condition had significantly higher PA compared to the 80dB SPL condition ($p = .034$). At 3174Hz, the 60dB SPL condition had significantly higher PA compared to the 80dB SPL condition ($p = .024$). Finally, at 5039 Hz, the 60dB SPL condition had significantly lower PA compared to the 65dB SPL condition (p = .023).

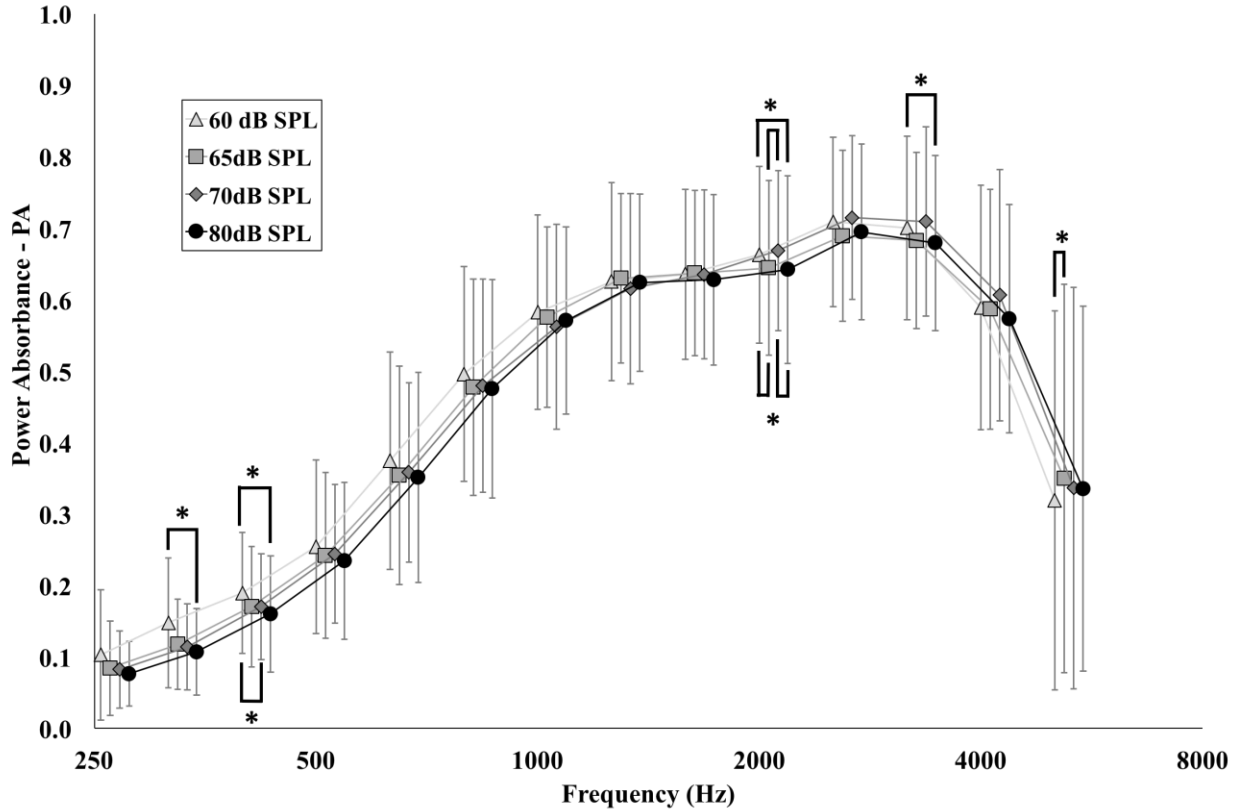


Figure 18. Mean power absorbance (PA) measured in 50 ears using 4 stimulus levels (60, 65, 70, and 80 dB SPL) with the Mimosa HearID. Error bars represent standard deviations. Significant differences in absolute difference of PA between instruments are denoted by asterisks (*).

4.3.6 Stimulus Effects on test-retest reliability of PA

There were three events where significance was not reached with regards to Pearson correlation coefficients (Table 9). The 60dB SPL measurement of PA using the Mimosa HearID did not have a significant correlation coefficient at 1/3rd octave bands of 250 Hz (r = -.141, N = 50, p = .33) and 315 Hz (r = .011, N = 50, p = .941) while the 65dB SPL PA measurement did not have significant Pearson correlation at the 250 Hz octave band (r = .275, N = 50, p = .053).

Table 9. Effect of stimulus level on test-retest reliability of power absorbance (PA) of the Mimosa HearID measured by Pearson Correlation Coefficients (n = 50 ears) at four different stimulus levels. Correlation coefficients with P values of >0.001 are bolded*.

	Frequency (Hz)													
	250	**315**	**397**	**500**	**630**	**794**	**1000**	**1260**	**1587**	**2000**	**2520**	**3175**	**4000**	**5040**
Stim Level														
60dB SPL	**-.141***	**.011***	0.689	0.768	0.792	0.837	0.745	0.907	0.922	0.959	0.929	0.968	0.951	0.917
65dB SPL	**.275***	0.677	0.882	0.924	0.949	0.944	0.907	0.876	0.898	0.954	0.933	0.963	0.966	0.954
70dB SPL	0.527	0.811	0.865	0.857	0.851	0.817	0.823	0.896	0.946	0.968	0.954	0.967	0.963	0.94
80dB SPL	0.665	0.797	0.843	0.867	0.87	0.819	0.835	0.905	0.962	0.967	0.96	0.956	0.961	0.94

The stimulus effects on test-retest reliability of PA revealed a violation of sphericity using Mauchly's test of sphericity for frequency $\chi^2(90) = 480.757$, $p < 0.001$, and stimulus level $\chi^2(5) = 37.025$, $p < 0.001$, therefore a Greenhouse-Geisser correction was used to determine significance. There was a significant main effect of frequency [F (2.964,68.163) = 4.161, p = .009] and stimulus level [F (11.743, 40.098) = 13.966, p < .001] and a significant interaction of frequency and stimulus level on the inter-trial difference of absorbance [F (4.435,102.007) = 11.418, p < .001]. *Post-hoc* analysis with a Bonferroni correction for multiple comparisons revealed the 60 dB SPL stimulus level had significantly higher absolute differences in absorbance compared to the other three stimulus level conditions at 0.25 kHz and 0.314 kHz (p < 0.001) and for the 65 dB SPL condition at 0.63 kHz (p = .044). It was also discovered that the 80 dB SPL condition had a significantly smaller absolute difference in absorbance at 0.25 kHz

compared to the 70 dB SPL condition (p = 0.022). Between trial differences in absorbance for the 1/3rd octave bands are plotted in Figure 19 and statistically significant differences are indicated.

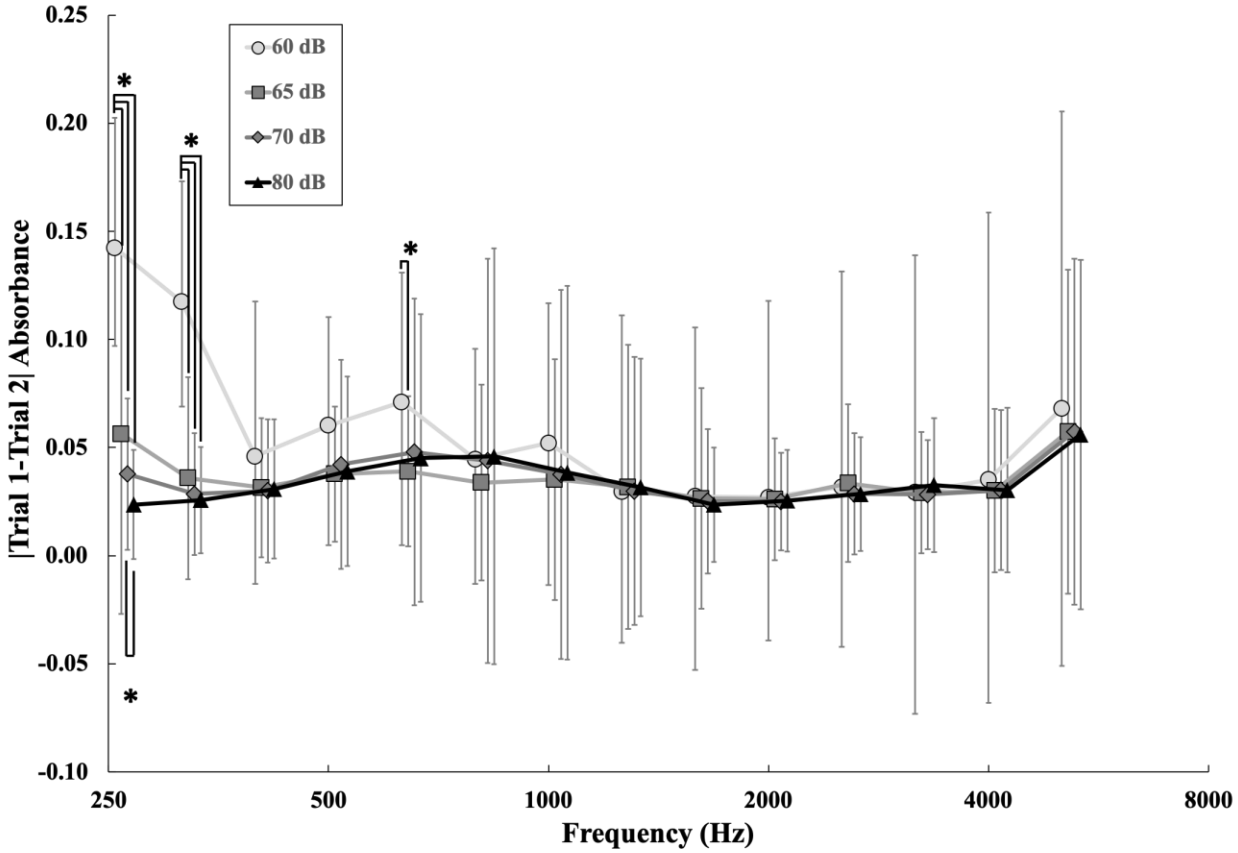


Figure 19. Mean absolute difference between trial 1 and trial 2 of power absorbance (PA) using various stimulus levels (60, 65, 70- and 80-dB SPL) with the Mimosa Hear ID (n = 50 ears). Error bars represent standard deviations. Significant differences in absolute difference of power absorbance between instruments are denoted by asterisks (*).

4.4 Study 1 Discussion

WAI has the potential to be a powerful tool for the identification and differential diagnosis of middle ear pathologies. Commercial systems, Interacoustics Titan and Mimosa Hear ID have been designed for clinical use, but the data produced by these 2 systems may not be

comparable. Standards for these instruments do not exist, and research focusing on WAI has used different instruments and protocols.

In this study, absolute PA and test-retest reliability were directly compared for the two commercial systems, the Mimosa HearID and the Interacoustics Titan systems under the same experimental conditions. Frequency-specific differences were found, with PA measurements using the Mimosa HearID significantly lower between 250-1260 Hz and significantly higher between 2520-4000 Hz compared to the Interacoustics Titan. This inter-instrument difference in PA is greater than that reported by Shahnaz et al. (2013), who found a significant difference only at 5000 Hz, with the commercial Mimosa Hear ID having lower PA at this frequency compared to the prototype WAIT (wideband acoustic immittance tympanometry) a non-commercial Interacoustics research system. Shahnaz et al. (2013) suggests that instrumental differences were minor, and that differences in pathology would be large enough to overcome these instrument effects. Differences in PA between the two systems may be in part due to calibration technique, the method to calculate ear canal area or the type of probe tip used (Shanaz et al, 2013). Results of the inter-instrument effects on ECV volumes suggest that there was no significant difference in ECV between the two instruments, providing evidence that instrument differences in PA are not likely due to differences in ECV calculations between the two systems. Additional work should be conducted to investigate these instrument effects on PA.

These results have implications for the clinical application of PA research in disordered populations. Recent studies report significantly different PA profiles in conductive hearing losses compared to normal ears (Merchant, Merchant, Rosowski, & Nakajima, 2016; Nakajima et al., 2012; Nakajima et al., 2013; Niemczyk, Lachowska, Tataj, Kurczak, & Niemczyk, 2018; Sanford, Schooling, & Frymark, 2012; Shahnaz et al., 2009). Clinical diagnostic criteria for an

abnormal PA result, and algorithms for the differential diagnosis of specific pathologies, including otosclerosis and superior canal dehiscence (Merchant et al., 2015; Nakajima et al., 2012; Sanford et al., 2012) have been developed. Since there are instrument-specific effects on PA, the use of WAI for evaluating middle ear pathology must be interpreted accordingly using instrument specific normative data. Additional work should continue to investigate the inter-instrument differences in PA, as well as work toward developing a standard in PA measurements.

The inter-instrument comparison of test-retest reliability under the same experimental conditions also revealed a difference between the two systems. The Interacoustics Titan demonstrated significantly better test-retest reliability in the lowest 1/3rd octave band (250 Hz) and worse test-retest reliability in higher frequency octave bands of 2520, 3175 and 4000 Hz compared to the Mimosa HearID,

Results of this study are consistent with previous investigations of test-retest reliability using either current or research prototype versions of the Interacoustics system (Feeney et al., 2017) and the Mimosa system (Feeney et al., 2014; Vander Werff et al., 2007). The test-retest reliability of 0.025-0.072 found here for the Interacoustics Titan are consistent with Feeney et al. (2017), with mean between-trial differences of 0.04-0.1 identified for a research prototype of the Interacoustics system. Furthermore, the mean between-trial differences from 0.026 - 0.056 for the Mimosa HearID found in this study are similar to the test-retest differences < 0.1 reported for a research prototype system of the Mimosa, with PA recorded during the same session (Vander Werff et al., 2007). Werner et al. (2010) report that when measurements were obtained approximately 2 weeks apart, wideband measurements had a mean absolute difference between 0.1- 0.2, also using a research prototype similar to the Mimosa system. These findings indicate

that more variability in PA measurements occur when separated by a longer period of time between recording sessions. Longitudinal variability of PA measurements obtained with the Interacoustics Titan and Mimosa Hear ID systems over significantly longer timescales are required before WAI can be used to track the natural course of middle ear diseases.

For both systems, the variability of repeated measurements in this study was lower than the variability in the normative population, and similar to the findings of Rosowski et al. (2012). However, frequency-specific differences in inter-trial reliability were also identified in this study. Specifically, the Interacoustics Titan exhibits slightly better test-retest reliability at the very lowest frequency band (250 Hz) compared to the Mimosa HearID, while the Mimosa exhibits slightly better test-retest reliability in the mid-frequency bands (2520 - 4000 Hz).

Users of the Mimosa system have the flexibility to record PA measurements at 4 stimulus levels between 60- 80 dB SPL. A stimulus effect on PA below 2000Hz, and also in higher frequency bands (3175 and 5040 Hz) was identified in Study 1. A low frequency reduction in PA below 2000 was found at 80 dB SPL only, and may be explained by induction of the acoustic stapedius-muscle reflex at this high presentation level. Using a research prototype WAI system similar to the Mimosa HearID, Feeney & Sanford (2005) identified decrease reduction in PA below 1000 Hz following contralateral activation of the acoustic reflex, which decreased as a function of increasing stimulus level. A similar increase in middle ear stiffness with activation of the ipsilateral acoustic reflex could also explain the PA findings of study 1.

Test-retest reliability below 315 Hz was significantly worse at 60 dB SPL and significantly better at 80 dB SPL compared to the other stimulus levels. In the 60 dB SPL condition PA may be contaminated by low frequency noise generated by the body (Buss, Porter,

Leibold, Grose, & Hall, 2016) resulting in a poor signal-to-noise ratio (Liu et al., 2008). Using stimulus level between 65 dB SPL – 80 dB SPL should improve SNR and provide a more reliable PA measurement, however changes introduced by activation of the acoustic reflex may be introduced as the stimulus intensity increases.

4.5 Study 2: Evaluation of PA Variability and Effects of Otosclerosis on PA

Differences in absolute PA have been reported for ears with otosclerosis compared to normal ears. Otosclerotic ears have a lower absorbance in frequencies below 1000 Hz (Feeney et al., 2003; Merchant et al., 2016; Nakajima et al., 2012; Sanford et al., 2012; Shahnaz, Bork, et al., 2009). Several methods have been used to investigate the differences in PA between normal and otosclerotic ears, or otosclerotic ears and other middle ear pathologies. Shahnaz, Bork, et al. (2009) compared energy reflectance (inverse of PA) between normal and otosclerotic ears using group differences. The effect of otosclerosis on PA has also been investigated using cadaveric ears and controlling for the middle ear pathology (Merchant et al., 2016). Results of this controlled study suggest that inducing stapes fixation results in a decrease in PA of greater than 0.2 below frequencies of 1000 Hz. Results by Nakajima et al. (2012) report the PA profile of various middle ear disorders, where the PA profile of four otosclerotic ears were reported and no statistical analysis was conducted. Three of the four ears demonstrated the characteristic low-frequency low-absorbance compared to normal ears, however one ear demonstrated a PA peak, around 800 Hz. This variability of PA of otosclerotic ears was recently investigated in a large otosclerotic population of 77 ears (Niemczyk et al., 2018). By visually examining each PA plot, they propose that otosclerotic ears can be categorized into five subgroups based on their PA plot characteristics. These characteristics include the number of peaks in the plot, the frequency of the

peaks and the height of the peaks. Their preliminary findings suggest that PA plots of otosclerotic ears are quite variable and that the variability of these PA plots should be investigated further. Overall, on a population level, otosclerotic ears have low PA below 1000 Hz, yet there is some evidence to suggest that there is individual variability with a more complex PA profile across otosclerotic ears.

4.5.1 Aim of Study 2:

The aim of Study 2 was to compare PA measurements between normal ears and otosclerotic ears.

> ***Specific aim 2:*** Compare PA measurements obtained in the normal hearing control group to a cohort of otosclerotic ears.

4.6 Study 2 Methods

4.6.1 Subjects

The normal hearing control group for Study 2 were the same subjects presented in Study 1 of this chapter. The normal hearing control group consisted of 25 subjects (50 ears). The otosclerotic cohort was recruited from two sources. All otosclerosis subjects had surgically confirmed otosclerosis in at least one ear, and all ears met the criteria for clinical otosclerosis with a significant air-bone gap and absent acoustic reflexes (same criteria as outlined in Chapter 3). The first source were pre-surgical cases from University Hospital in London, Ontario, later confirmed otosclerotic subjects following successful stapes surgery. Five subjects, with eight otosclerotic ears, were included from this group. Five other otosclerotic subjects were recruited to the study in the province of Newfoundland. These five subjects had previously undergone successful stapes surgery, confirming otosclerosis, and presented with clinical otosclerosis in their non-surgical ear. These non-surgical ears presenting with a conductive hearing loss were

considered as clinical otosclerotic ears and included in the study. In total, 13 otosclerotic ears from nine patients were included for analysis.

4.6.2 Instrumentation

PA was obtained in both the control group and the otosclerotic cohort using the Mimosa HearID at a stimulus level of 80 dB SPL. This instrument and stimulus level were selected based on the results of study 1 which reports a significantly better test-retest reliability of the Mimosa HearID at 80 dB SPL stimulus level. Additional information about the instrumentation is reported in Study 1.

4.6.3 Data Analyses

PA data was analysed in the same manner as reported in Study 1.

4.6.4 Statistical Analyses

The effects of otosclerosis on PA were examined using 1/3rd octave band PA measurements from the normative group and the otosclerotic cohort. A mixed model ANOVA was completed for mean PA using frequency (14 levels) as within-subject factor and sex (2 levels) and ear status (2 levels) as a between-subject factor. When appropriate, post-hoc analysis was conducted by completing a pairwise comparison following Bonferroni correction.

4.7 Study 2 Results

Mean PA values for normal ears and otosclerotic ears are plotted in Figure 20. The effect of otosclerosis on PA revealed a violation of sphericity using Mauchly's test of sphericity for frequency $\chi^2(90) = 1509.777$, $p < 0.001$, therefore a Greenhouse-Geisser correction was used to determine significance. There was a significant main effect of frequency [F (3.114,183.728) =

161.475, $p < .001$]. The main effect of ear status approached by did not reach statistical significance of $p < 0.05$ [F (1,59) = 3.447, p = .068].

Overall, seven of thirteen otosclerotic ears demonstrate PA values below 1 SD of the normative mean at frequencies below 1000 Hz (Figure 20), while 6 of 13 demonstrate PA values above the normative mean at these low frequencies, with four of these ears demonstrating the unusual peak pattern. In total, 10/13 otosclerotic ears demonstrate PA profiles of either low-absorbance (n=6) in the low-frequencies or the unusual peak between 800-1000 Hz (n=4). A review of PA profile plots of the normative ears revealed the 800 -1100 Hz PA peak to be present in 4/50 ears, with two of those ears having elevated compliance values greater than 1.7 mL based on standard 226 Hz acoustic admittance.

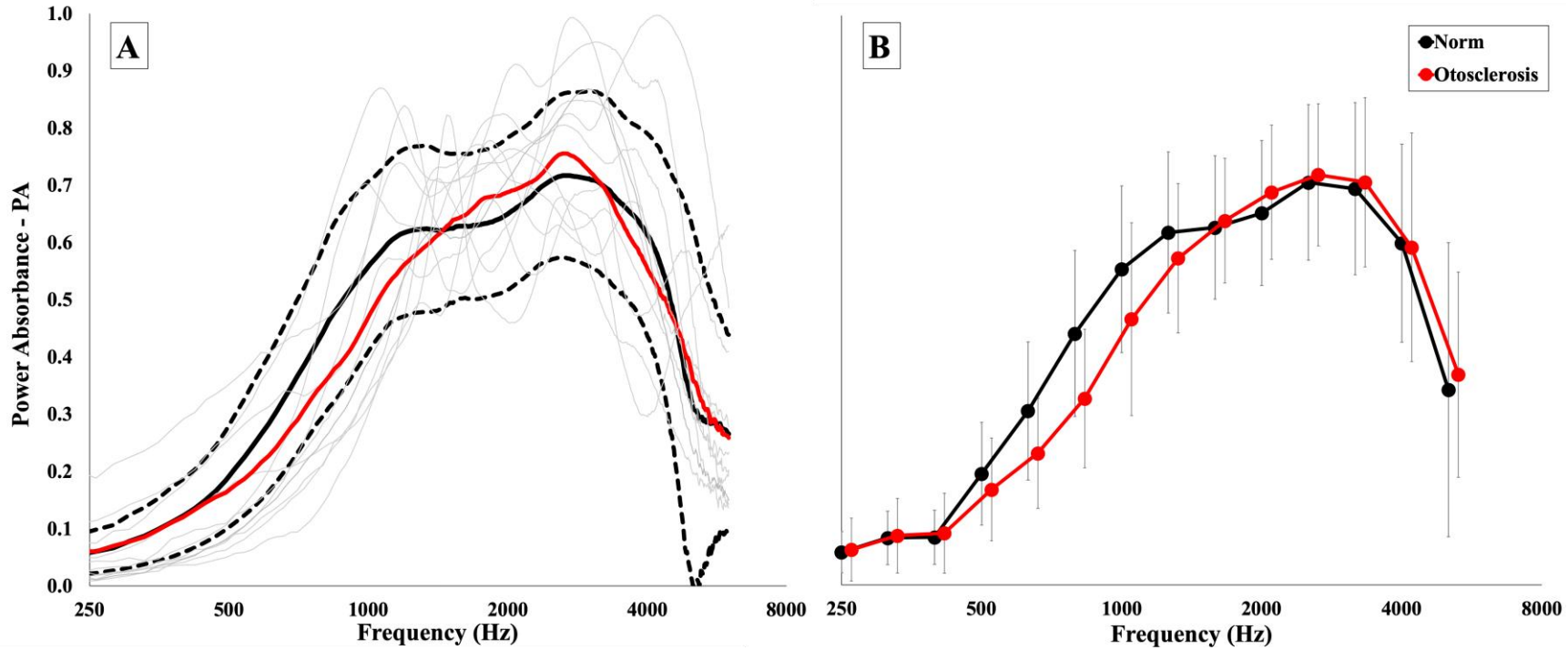


Figure 20. A) Mean (±1 SD) power absorbance (PA) measured in normal ears (n=50) represented by black line, and otosclerotic ears (n=13), represented by red line using 80 dB SPL stimulus level with the Mimosa HearID. Grey lines represent individual otosclerotic ear PA measurements. B) Mean power absorbance (PA) averaged into 1/3rd octave bands, measured in normal ears (n=50), and otosclerotic ears (n=13). Error bars represent standard deviations.

4.8 Study 2 Discussion

In Study 2 of this chapter, there were no statistically significant differences in power absorbance between otosclerotic and normal ears, however results were approaching significance. A lack of statistical significance was likely due to the low number of otosclerotic ears available to the study. Previous studies comparing otosclerotic ears to normal ears, where a significant decrease in PA (increase in energy reflectance) in low frequencies are reported, which is explained by increased stiffness of the middle ear. Sclerotic bone growth around the stapes results in a less efficient middle ear system, particularly for low-frequency stimuli which results in less acoustic energy absorption by the middle ear (Feeney et al., 2003; Merchant et al., 2016; Nakajima et al., 2012; Sanford et al., 2012; Shahnaz, Longridge, et al., 2009).

An interesting finding of our otosclerotic cohort revealed 4/13 demonstrating a second PA profile, presenting with a PA peak around 800-1100 Hz. This peak profile is similar to the reported subgroup IV reported by Niemczyk, Lachowska, Tataj, Kurczak, & Niemczyk (2018). A low frequency PA peak was also reported in one otosclerotic ear by Nakajima et al. (2012) while a similar PA profile can also be seen in the results from Shahnaz, Bork, et al. (2009), who provide individual PA plots for an otosclerotic population of 28 ears. Two otosclerotic ears in their cohort also exhibit this characteristic PA peak occurring around 800 Hz. The same profile has been reported in ears with normal hearing thresholds, but who demonstrate elevated compliance values of greater than 1.7 mL (Feeney et al., 2014). A review of PA profile plots of the normative ears presented in Study 1 revealed the 800 -1100 Hz PA peak to be present in 4/50 ears, with two of those ears having elevated compliance values greater than 1.7 mL. Therefore,

when interpreting the presence of the abnormal PA peak, standard tympanometry will be considered, as ears with elevated compliance values may exhibit the same PA profile.

Differences in the PA profile of otosclerotic ears may reflect changes associated with disease progression and/or location of sclerotic bone growth. Early work from Schuknecht & Barber (1985) based on temporal bone histology reveal variability in location as well as size of sclerotic bone growth. Future work should focus on investigating whether there are any correlations between PA sub-types or profiles and the location of the sclerotic bone growth or the disease progression using advanced imaging methods, such as high-resolution CT (Maraghy et al., 2015; Quesnel et al., 2013), or by measuring PA pre-surgically and correlating the PA profiles to the size and location of sclerotic bone growth at the time of corrective surgery. During the natural course of otosclerosis, abnormal bone growth may induce changes in middle ear mass and/or stiffness which in turn differentially affect the PA. Since otosclerosis begins with the formation of spongy vascularized bone growth (otospongiosis) before transitioning to harder sclerotic bone growth (Parahy & Linthicum, 1984), it is possible that PA may be sensitive enough to detect differences in the various stages of disease progression. PA has shown to be sensitive to middle ear disorders with altered mass and stiffness characteristics. In a controlled study using cadaveric ears to measure PA in mass-dominated and stiffness dominated middle ear conditions, the PA profile in a more mass-dominated middle ear system (ie. ossicular discontinuity), has a PA profiles where a peak in PA is present in the low-frequencies around 800-1000 Hz, while stiffening the middle ear by fixating the stapes (ie. otosclerosis) resulted in a lower absorbance in the low frequencies similar to our mean PA in the entire otosclerotic cohort (Merchant et al., 2016).

Previous work has attempted to determine criteria for the determination of presence or absence of otosclerosis using wideband acoustic immittance (Shahnaz, Bork, et al., 2009), however recent work by Keefe et al. (2017) suggests that WAI be included as part of an overall test battery including acoustic reflexes and otoacoustic emissions in order to improve the overall diagnostic accuracy of otosclerosis compared to normal ears. Since there is currently no standard for determining normal versus abnormal PA profiles, it is recommended that an abnormal PA profile be considered when PA is 1 SD below the normative mean at frequencies below 1000 Hz, or if the PA plot demonstrates a peak falling outside of 1 SD at frequencies between 800-1100 Hz. Both of these characteristic PA plots have been reported in otosclerotic ears (Nakajima et al., 2012; Niemczyk et al., 2018; Shahnaz, Bork, et al., 2009). It is also recommended that an abnormal PA profile only be considered as part of a greater test battery, where an altered PA profile plot will only be considered as evidence of otosclerosis or potential early onset otosclerosis if it is companioned with another clinical indicator, such as hearing loss (conductive, mixed or SNHL), absent reflexes or absent otoacoustic emissions (Chapter 3, Keefe et al., 2017).

4.8.1 Overall Conclusions

In conclusion, Study 1 identified an inter-instrument difference in PA and test-retest reliability between the two commercially available systems. Since there is no current standard for the measurement of PA, clinicians and researchers should use of the same equipment and measurement protocols when collecting data and interpreting results. Study 2 reports that within individual ear data, a PA profile with a characteristic peak in the low frequencies was identified in 31% of otosclerotic ear and 8% of normal control ears. These results support previous reports of an otosclerotic PA peak (Nakajima et al., 2012; Niemczyk et al., 2018; Shahnaz, Bork, et al., 2009), possibly due to a change in mass (Merchant et al., 2016) and should also be considered

when classifying PA profiles. Otosclerosis is typically associated with low compliance due to the increased middle ear stiffness (Hannley, 1993; Ogut et al., 2008; Zhao et al., 2002). However, the presence of high compliance (greater than 1.75 mL) should be considered when interpreting this PA peak profile (Feeney et al., 2014), as a highly compliant middle ear, as measured by standard tympanometry, has demonstrated the same PA peak profile described in the present otosclerotic sub-group. Limitations of Study 2 include the low number of otosclerotic ears available for recruitment, a likely contributor to the lack of statistical significance of PA between normal and otosclerotic ears. Recruitment numbers for pre-operative research on otosclerotic ears was low due to the limited recruitment window between when an individual is seen by the otolaryngologist to determine surgical candidacy and when they are scheduled for corrective stapes surgery. Future work should focus on the recruitment of additional pre-surgical ears to determine further PA differences between normal ears and otosclerotic ears, as well as to investigate the variability of PA among pre-surgical otosclerotic ears.

4.9 References

Abur, D., Horton, N. J., & Voss, S. E. (2014). Intrasubject Variability in Power Reflectance. Journal of the American Academy of Audiology, 25(5), 441–448. https://doi.org/10.3766/jaaa.25.5.3

Bland, J. M., & Altman, D. G. (1999). Measuring agreement in method comparison studies. Statistical Methods in Medical Research, 8(2), 135–160. https://doi.org/10.1177/096228029900800204

Burdiek, L. M., & Sun, X.-M. (2014). Effects of consecutive wideband tympanometry trials on energy absorbance measures of the middle ear. Journal of Speech, Language, and Hearing Research : JSLHR, 57(5), 1997–2004. https://doi.org/10.1044/2014_JSLHR-H-13-0344

Buss, E., Porter, H. L., Leibold, L. J., Grose, J. H., & Hall, J. W. (2016). Effects of Self-Generated Noise on Estimates of Detection Threshold in Quiet for School-Age Children and Adults. Ear and Hearing, 37(6), 650–659. https://doi.org/10.1097/AUD.0000000000000337

EL Maraghy, A., Aboelwafa, W., Abdel Monem, M., Abdul Ghany, A., Sheref, H., & Mahmoud, A. (2015). Role of high resolution multislice CT scan in otosclerosis. Egyptian Journal of Ear, Nose, Throat and Allied Sciences, 16(3), 247–254. https://doi.org/10.1016/j.ejenta.2015.07.008

Feeney, M. P., Grant, I. L., & Marryott, L. P. (2003). Wideband energy reflectance measurements in adults with middle-ear disorders. Journal of Speech, Language, and Hearing Research : JSLHR, 46(4), 901–911. https://doi.org/10.1044/1092-4388(2003/070)

Feeney, M. P., Keefe, D. H., Hunter, L. L., Fitzpatrick, D. F., Garinis, A. C., Putterman, D. B., & McMillan, G. P. (2017). Normative Wideband Reflectance, Equivalent Admittance at the Tympanic Membrane, and Acoustic Stapedius Reflex Threshold in Adults. Ear and Hearing, 38(3), e142–e160. https://doi.org/10.1097/AUD.0000000000000399

Feeney, M. P., Stover, B., Keefe, D. H., Garinis, A. C., Day, J. E., & Seixas, N. (2014). Sources of Variability in Wideband Energy Reflectance Measurements in Adults. Journal of the American Academy of Audiology, 25(5), 449–461. https://doi.org/10.3766/jaaa.25.5.4

Feeney, P. M., & Sanford, C. A. (2005). Detection of the Acoustic Stapedius Reflex in Infants Using Wideband Energy Reflectance and Admittance. Journal of the American Academy of Audiology, 16(5), 278–290. https://doi.org/10.3766/jaaa.16.5.3

Hannley, M. T. (1993). Audiologic characteristics of the patient with otosclerosis. Otolaryngologic Clinics of North America, 26(3), 373–387.

Keefe, D., L Archer, K., Schmid, K., Fitzpatrick, D., Feeney, P., & Hunter, L. (2017). Identifying Otosclerosis with Aural Acoustical Tests of Absorbance, Group Delay, Acoustic Reflex Threshold, and Otoacoustic Emissions. Journal of the American Academy of Audiology, 28. https://doi.org/10.3766/jaaa.16172

Liu, Y.-W., Sanford, C. a, Ellison, J. C., Fitzpatrick, D. F., Gorga, M. P., & Keefe, D. H. (2008). Wideband absorbance tympanometry using pressure sweeps: system development and results on adults with normal hearing. The Journal of the Acoustical Society of America, 124(6), 3708–3719. https://doi.org/10.1121/1.3001712

Mahoney, M. J., McFarland, D. J., Carpenter, M. C. S., Rizvi, S., & Cacace, A. T. (2013). Reliability of broadband middle-ear power reflectance in younger and older adults: Application of generalizability theory. American Journal of Audiology, 22(2), 241–251. https://doi.org/10.1044/1059-0889(2013/12-0063)

Merchant, G. R., Merchant, S. N., Rosowski, J. J., & Nakajima, H. H. (2016). Controlled exploration of the effects of conductive hearing loss on wideband acoustic immittance in human cadaveric preparations. Hearing Research, 341, 19–30. https://doi.org/10.1016/j.heares.2016.07.018

Merchant, G. R., Röösli, C., Niesten, M. E. F., Hamade, M. A., Lee, D. J., McKinnon, M. L., … Nakajima, H. H. (2015). Power Reflectance as a Screening Tool for the Diagnosis of Superior Semicircular Canal Dehiscence. Otology & Neurotology, 36(1), 172–177. https://doi.org/10.1097/MAO.0000000000000294

MRC Multicentre Otitis Media Study Group. (2008). An extension of the Jerger classification of tympanograms for ventilation tube patency--specification and evaluation of equivalent ear-canal volume criteria. Ear and Hearing, 29(6), 894–906. https://doi.org/10.1097/AUD.0b013e3181824d15

Nakajima, Hideko H, Pisano, D. V, Roosli, C., Hamade, M. a, Merchant, G. R., Mahfoud, L., … Merchant, S. N. (2012). Comparison of ear-canal reflectance and umbo velocity in patients with conductive hearing loss: a preliminary study. Ear and Hearing, 33(1), 35–43. https://doi.org/10.1097/AUD.0b013e31822ccba0

Nakajima, Hideko Heidi, Rosowski, J. J., Shahnaz, N., & Voss, S. E. (2013). Assessment of Ear Disorders Using Power Reflectance. Ear and Hearing, 34(1), 48s-53s. https://doi.org/10.1097/AUD.0b013e31829c964d

Niemczyk, E., Lachowska, M., Tataj, E., Kurczak, K., & Niemczyk, K. (2018). Wideband tympanometry and absorbance measurements in otosclerotic ears. The Laryngoscope, ePub ahead. https://doi.org/10.1002/lary.27747

Ogut, F., Serbetcioglu, B., Kirazli, T., Kirkim, G., & Gode, S. (2008). Results of multiple-frequency tympanometry measures in normal and otosclerotic middle ears. International Journal of Audiology, 47(10), 615–620. https://doi.org/10.1080/14992020802178656

Parahy, C., & Linthicum, F. H. (1984). Otosclerosis And Otospongiosis: Clinical and histological comparisons. The Laryngoscope, 94(4), 508–512. https://doi.org/10.1288/00005537-198404000-00015

Prieve, B. a, Feeney, M. P., Stenfelt, S., & Shahnaz, N. (2013). Prediction of conductive hearing loss using wideband acoustic immittance. Ear and Hearing, 34 Suppl 1, 54S-59S. https://doi.org/10.1097/AUD.0b013e31829c9670

Quesnel, A. M., Moonis, G., Appel, J., O'Malley, J. T., McKenna, M. J., Curtin, H. D., & Merchant, S. N. (2013). Correlation of computed tomography with histopathology in otosclerosis. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 34(1), 22–28. https://doi.org/10.1097/MAO.0b013e318277a1f7

Sanford, C., Schooling, T., & Frymark, T. (2012). Determining the Presence or Absence of Middle Ear Disorders: An Evidence-Based Systematic Review on the Diagnostic Accuracy of Selected Assessment. American Journal of Audiology, 21(December), 251–268. https://doi.org/10.1044/1059-0889(2012/11-0029)a

Schuknecht, H. F., & Barber, W. (1985). Histologic variants in otosclerosis. The Laryngoscope, 95(11), 1307–1317. https://doi.org/10.1288/00005537-198511000-00003

Shahnaz, N., Bork, K., Polka, L., Longridge, N., Bell, D., & Westerberg, B. D. (2009). Energy reflectance and tympanometry in normal and otosclerotic ears. Ear and Hearing, 30(2), 219–233. https://doi.org/10.1097/AUD.0b013e3181976a14

Shahnaz, N., Feeney, M. P., & Schairer, K. S. (2013). Wideband Acoustic Immittance Normative Data: Ethnicity, Gender, Aging, and Instrumentation. Ear and Hearing, 34, 27s-35s. https://doi.org/10.1097/AUD.0b013e31829d5328

Shahnaz, N., Longridge, N., & Bell, D. (2009). Wideband energy reflectance patterns in preoperative and post-operative otosclerotic ears. International Journal of Audiology, 48(5), 240–247. https://doi.org/10.1080/14992020802635317

Shanks, J. E., Stelmachowicz, P. G., Beauchaine, K. L., & Schulte, L. (1992). Equivalent Ear Canal Volumes in Children Pre- and Post-Tympanostomy Tube Insertion. Journal of Speech & Hearing Research, 35(4), 936. Retrieved from http://10.0.4.20/jshr.3504.936

Stepp, C. E., & Voss, S. E. (2005). Acoustics of the human middle-ear air space. The Journal of the Acoustical Society of America, 118(2), 861–871. https://doi.org/10.1121/1.1974730

Vander Werff, K. R., Prieve, B. A., & Georgantas, L. M. (2007). Test-retest reliability of wideband reflectance measures in infants under screening and diagnostic test conditions. Ear and Hearing, 28, 669–681. https://doi.org/10.1097/AUD.0b013e31812f71b1

Voss, S. E., Horton, N. J., Woodbury, R. R., & Sheffield, K. N. (2008). Sources of variability in reflectance measurements on normal cadaver ears. Ear and Hearing, 29(4), 651–665. https://doi.org/10.1097/AUD.0b013e318174f07c

Werner, L. A., Levi, E. C., & Keefe, D. H. (2010). Ear-canal wideband acoustic transfer functions of adults and two- to nine-month-old infants. Ear and Hearing, 31(5), 587–598. https://doi.org/10.1097/AUD.0b013e3181e0381d

Zhao, F., Wada, H., Koike, T., Ohyama, K., Kawase, T., & Stephens, D. (2002). Middle ear dynamic characteristics in patients with otosclerosis. Ear and Hearing, 23(2), 150–158. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11951850

Chapter 5

5 Advanced Phenotyping of a Large Otosclerotic *FOXL1* Newfoundland Family

5.1 Introduction

The purpose of this study is to investigate the auditory phenotype of otosclerosis in *FOXL1* gene carriers in a large Newfoundland otosclerotic family. Family design methodologies are beneficial over population studies because they help reduce the effects of population stratification and heterogeneity (Laird & Lange, 2006). Earlier in this thesis, the auditory phenotype of an Ontario otosclerotic population was reported, suggesting a broad mix of auditory phenotypes in that heterogenous population. Analysis of the auditory phenotype in a more homogenous population, a family with the *FOXL1* mutation, allows for an improved understanding of the phenotype and phenotypic variation due to changes in the same gene.

For the family design methodology, a cross-sectional analysis of Family 2081 was carried out. Investigation of audiometry, ARTs, DPOAEs, and WAI improve our understanding of variability within a family context. Audiometric thresholds were used to develop a predictive model for the *FOXL1*-associated hearing loss, which will lead to the better understanding of the natural course of *FOXL1*-specific otosclerosis.

5.1.1 Aims of this Study

The aims of this study are to conduct a phenotypic analysis on gene carriers of the *FOXL1* deletion using clinically available tools. More specifically, the aims are:

Specific aim 1: Develop a predictive model for *FOXL1*-associated hearing loss.

Specific aim 2: Compare audiometric and non-audiometric phenotypes of ARTs, DPOAEs and WAI of carriers with non-surgical family members with the *FOXL1* deletion.

Specific aim 3: Summarize the phenotypes of all *FOXL1* gene carriers.

5.2 Methods

The test battery for participants in the research study consisted of audiometry, tympanometry, DPOAEs, ART testing and WAI measurements. Conventional immittance testing as well as ARTs were obtained using the Interacoustics Titan, whereas wideband acoustic immittance measurements were conducted using the Mimosa HearID. The testing protocol for this study is an abbreviated test battery of the protocol presented in Chapter 4. The test battery for this phenotyping study was selected based on test-retest reliability, phenotypic relevance, as well as limiting the protocol to an acceptable length of time.

5.2.1 Family 2081

Family 2081 has thirteen family members carrying the 15bp deletion in the *FOXL1* gene. A pedigree of the family was constructed (Figure 21). Only family members heterozygous for the deletion were recruited for this study in collaboration with Memorial University (MUN Ethics #01.186).

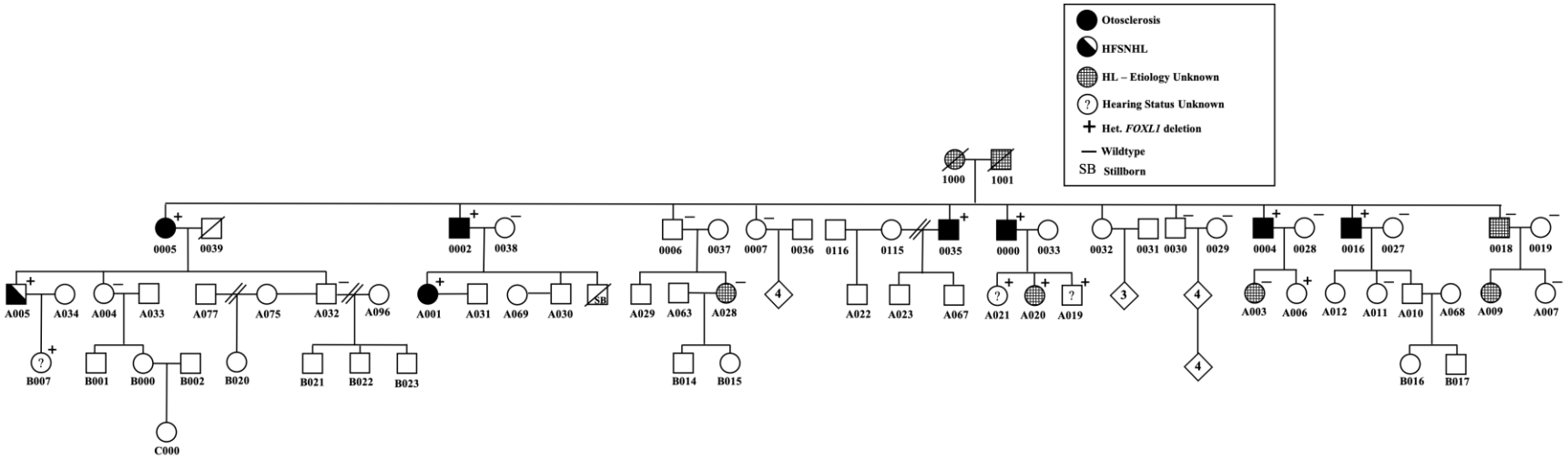


Figure 21. Pedigree for Family 2081, a large Newfoundland otosclerotic family with *FOXL1* deletion co-segregating with otosclerosis.

5.2.2 Retrospective Data

Retrospective audiometric data (per-surgery) was collected from affected family members carrying the *FOXL1* deletion (N=8) who consented to the study. Patient histories were also retrospectively collected to obtain age of onset data. Audiograms were analysed for air conduction hearing threshold levels at 0.25, 0.5, 1, 2, 4 and 8 kHz, while bone conduction thresholds were analyzed for frequencies of 0.5, 1, 2, and 4 kHz. Air-bone gaps were calculated by subtracting bone conduction thresholds from air conduction thresholds at their given frequencies.

5.2.3 Prospective Data

Prospective data was collected on two of nine *FOXL1* deletion carriers, representing the two family members who had not undergone corrective surgery for otosclerosis. Corrective stapes surgery alters the physical properties of the middle ear by replacing the stapes with a prosthetic piston. This physical change to the middle ear will significantly change the non-audiometric data such as tympanometry and WAI. As reviewed in Chapter 3, otosclerotic ears that have undergone surgery do not have measurable ARTs or present DPOAEs.

5.2.3.1 Audiometry

Pure tone audiometry was conducted using the Interacoustics AC40 audiometer calibrated according to ANSI standards (re: S3.6.2010). Both air and bone conduction thresholds were measured. Measurements were conducted in a sound-booth using ER3 insert earphones for air conduction, and a bone oscillator for bone conduction thresholds. Air conduction thresholds were measured between 0.25 – 8 kHz, while bone conduction thresholds were measured between 0.5-4 kHz. A standard bracketing method was used to conduct audiometry.

To measure the rate of progression of the hearing loss within Family 2081, retrospective and prospective audiometric thresholds (air conduction and bone conduction), along with air-bone gaps were plotted with respect to age. All available pre-surgical retrospective hearing thresholds were included in the analysis. Prior to analysis, ears were separated into two categories: worse ear and better ear. Ears were classified into these two groups based on pure-tone averages of 500, 1000 and 2000 Hz (PTA3). Linear regression was then conducted between age and threshold for air-conduction thresholds, bone-conduction thresholds and air-bone gaps for the categories of worse and better ear. A model was calculated to determine the frequency-specific progression reported in dB per decade. Linear regression was also used to generate a predicted audiogram for *FOXL1* deletion carriers. The predicted audiogram was generated by using linear regression from air conduction and bone conduction thresholds of the worse ears for eight family members heterozygous for the *FOXL1* deletion. The worse ear was used to generate the hearing loss model since otosclerosis can appear as a unilateral hearing loss or develop asymmetrically.

5.2.4 Acoustic Immittance

5.2.4.1 Tympanometry (conventional)

Standard 226 Hz tympanometry was conducted using the Interacoustics Titan hardware version 1.0 and Titan Suite version 3.2.2.5 software. Tympanometry was conducted using a 226 Hz probe tone between pressures of +200 daPa to -300 daPa. Tympanometric peak pressure, compliance, and ear canal volume were all collected.

5.2.4.2 Acoustic Reflex Thresholds

Acoustic reflex thresholds were measured using the Interacoustics Titan system. ARTs were measured both ipsilaterally and contralaterally at generator tone frequencies of 0.5, 1, 2, 3,

4 kHz, along with a wideband noise (WBN). A probe tone of 226 Hz was used at all frequencies. To pass the criteria for the ART to be considered present, an amplitude of 0.02 mL or greater must be measured in two out of three presentations. Failure to meet criteria at the given stimulus level would result in an increase in stimulus by 5 dB. An initial stimulus level of 80 dB HL was used for all measurements, with a maximum stimulus presentation level of 100 dB HL. Failure to meet the amplitude criteria at all stimulus levels up to and including 100 dB HL resulted in the classification of "absent" acoustic reflex. The same nomenclature for ARTs in Chapter 3 was used in this chapter. The designation of the ear represents the probe ear and not the stimulus ear as is conventional in audiology clinics. For example, in cases of right contralateral reflexes, the stimulating tone is generated from the left ear, and the reflex is measured in the right.

5.2.4.3 Wideband Acoustic Immittance

Power absorbance was measured using the Mimosa HearID system at a stimulus level of 80 dB SPL. This level was chosen based on results from the previous chapter of this dissertation. 80 dB SPL generated the best test-retest reliability in the low frequency octave band (0.25 kHz). Methods for PA acquisition are described in Chapter 4. PA measurements were compared to the normative data set reported in Chapter 4 for all 50 ears. The normative mean was subtracted from PA measurements of each individual to calculate the PA difference from the mean.

PA measurement for an individual family member was considered abnormal if their PA measurement below 1000 Hz was outside 1 SD of the normative mean as previously reported in the literature and in Chapter 4 (Feeney et al., 2003; Shahnaz, Bork, et al., 2009; Shahnaz, Longridge, et al., 2009). PA profiles were interpreted for the presence of the classical PA profile associated with otosclerosis, where PA is expected to be lower than the normative mean in the low frequencies (below 1000 Hz). PA profiles were also considered abnormal if they

demonstrated a peak between 800-1100 Hz which has also been associated with otosclerosis (Chapter 4, Nakajima et al., 2012; Niemczyk et al., 2018; Shahnaz, Bork, et al., 2009).

5.2.5 Distortion Product Otoacoustic Emissions (DPOAEs)

Distortion product otoacoustic emissions were elicited using the Mimosa HearID system. The F2 primary tone was presented at 65 dB SPL, while the F1 tone was presented at 55 dB SPL. The frequency ratio of F2/F1=1.22 was used to elicit the distortion product. The DP was measured as the amplitude of the response at 2F2-F1. The normative data set from Gorga et al. (1997) was used, whereby minimum DPOAE levels were chosen based on the 10^{th} percentile of their normative ears. A list of F1, F2 and the associated 2F2-F1 distortion product, along with the 10^{th} percentile of their normal population from the normative data set were used as criteria for the presence/absence of a distortion product at each F2 frequency (Table 10). For a DPOAE to be considered present, it must have a minimum signal-to-noise ratio of 6 dB and meet the frequency specific minimum DP level.

Table 10. F2, F1, 2F1-F2 and minimum distortion product level values for measured DPOAEs collected using the Mimosa HearID system. In total, 12 distortion products were elicited across an F2 frequency range of 984-8016 Hz.

F2 (Hz)	F1 (Hz)	2F1-F2 (Hz)	Min DP level (dB)
984	844	703	-8
1500	1266	1031	-7
2016	1688	1359	-10
2484	2062	1641	-11
3000	2484	1969	-12
3516	2906	2297	-9
3984	3328	2672	-6
5016	4172	3328	-7
5484	4594	3703	-7
6000	5016	4031	-8
6984	5812	4641	-15
8016	6556	5297	-22

5.3 Results

Overall, nine family members of 2081 have been identified as heterozygous for the *FOXL1* deletion causing otosclerosis. Of these nine individuals, seven have been identified as being affected by otosclerosis by an otolaryngologist on the basis of surgical confirmation. Since these individuals have undergone middle ear surgery to correct for their stapes fixation due to otosclerosis, and thus altering the physical characteristics of their middle ear, they were removed from any prospective data analysis. Individuals 0002, 0016 and A001 (Figure 21) were unavailable to participate in the prospective data collection. These individuals represent cases of unilateral surgical intervention, and had they been available to participate, their non-surgical ear would have been included in the analysis.

5.3.1 *FOXL1* deletion carriers

Audiometric thresholds were available for eight family members of Family 2081 heterozygous for the *FOXL1* deletion. Three family members presented with bilateral conductive hearing loss prior to stapes surgery (individuals 0000, 0004 and A001). Only one family member presented with a bilateral mixed hearing loss (0005). Two family members presented with a unilateral mixed hearing loss, accompanied with a sensorineural hearing loss on the non-surgical ear (0002, 0016). Finally, two carriers of the deletion exhibited normal, or essentially normal hearing thresholds and were classified as non-penetrant carriers (A005, A006). All affected members of the family reported an onset of hearing loss within the second or third decade of life.

To investigate the rate of progression of the hearing loss in this family, air-conduction and bone-conduction thresholds along with air-bone gaps were plotted based on age for each participants' worse ear (Figure 22) and for their better ear (Figure 23). A linear model was

created based on available retrospective audiometric data to determine the approximate rate of progression of the hearing loss. Results of the linear regression are reported for the worse ear (Table 11) and for the better ears (Table 12). R^2 values of the worse ears were calculated between .194 and .508 for air-conduction and bone-conduction thresholds and age, while the better ears had R^2 value between .068 and .619. All linear regression models were statistically significant with the exception of BC thresholds versus age at frequencies of 500 and 1000 Hz in the better ears.

The overall progression of otosclerosis in this family is quite variable (Figures 22 and 23). In general, AC and BC thresholds and measured ABGs increase over the lifespan at all frequencies. There is a difference in the rate of progression between the conductive component and sensorineural component of the hearing loss, with the conductive component progressing more rapidly at frequencies of 500, 1000 and 2000 Hz and the sensorineural component to the hearing loss progressing more rapidly at 4000 Hz.

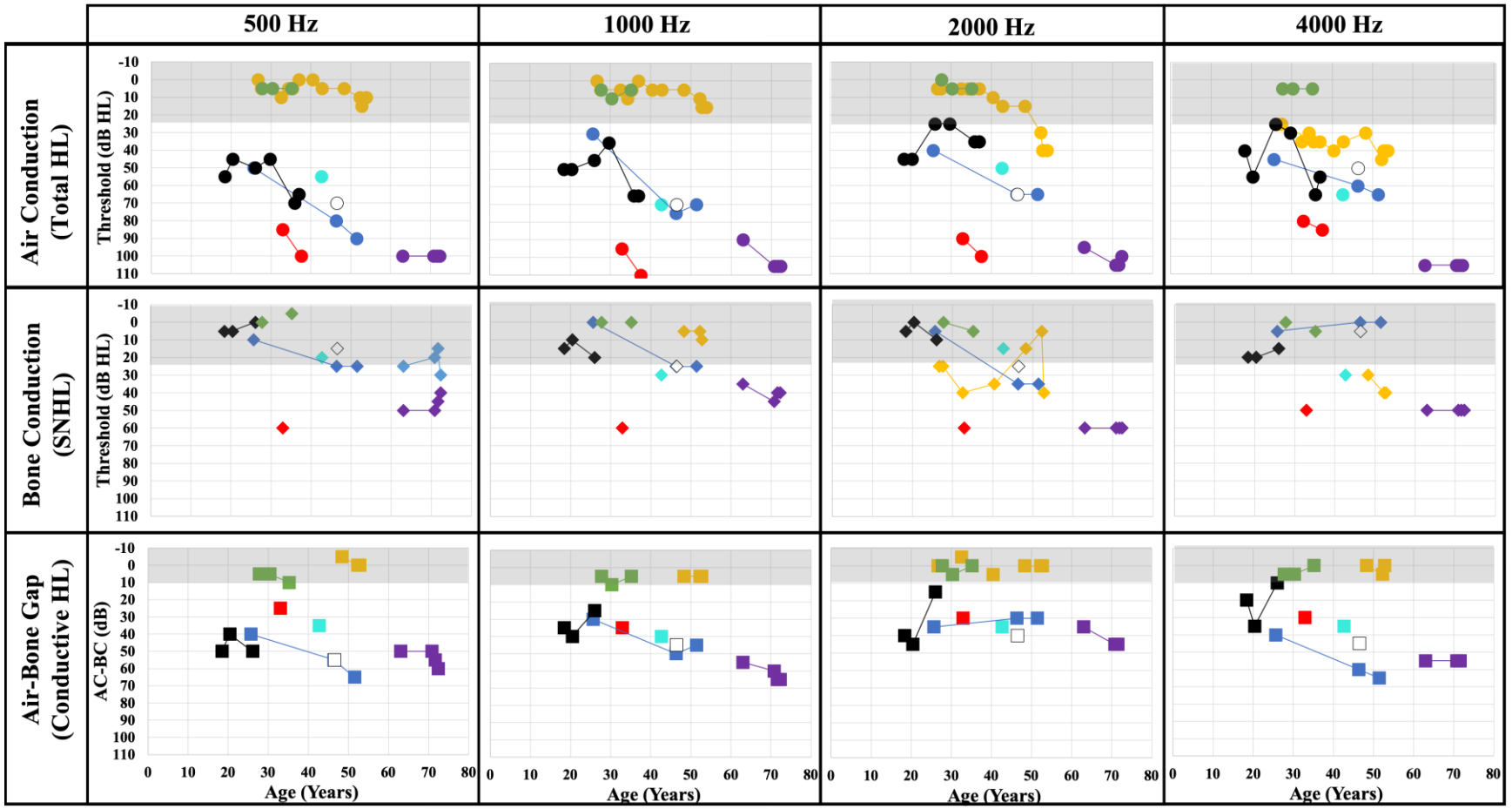


Figure 22. Audiometric thresholds of participants' worse ear as a function of age for eight family members of Family 2081 identified as heterozygous for *FOXL1* deletion. Each colour represents data from a different family member. Data points connected with a line represent longitudinal data from the same individual ear. Audiometric thresholds broken down into air conduction (total hearing loss), bone conduction (sensorineural hearing loss component) and air-bone gap (conductive hearing loss component).

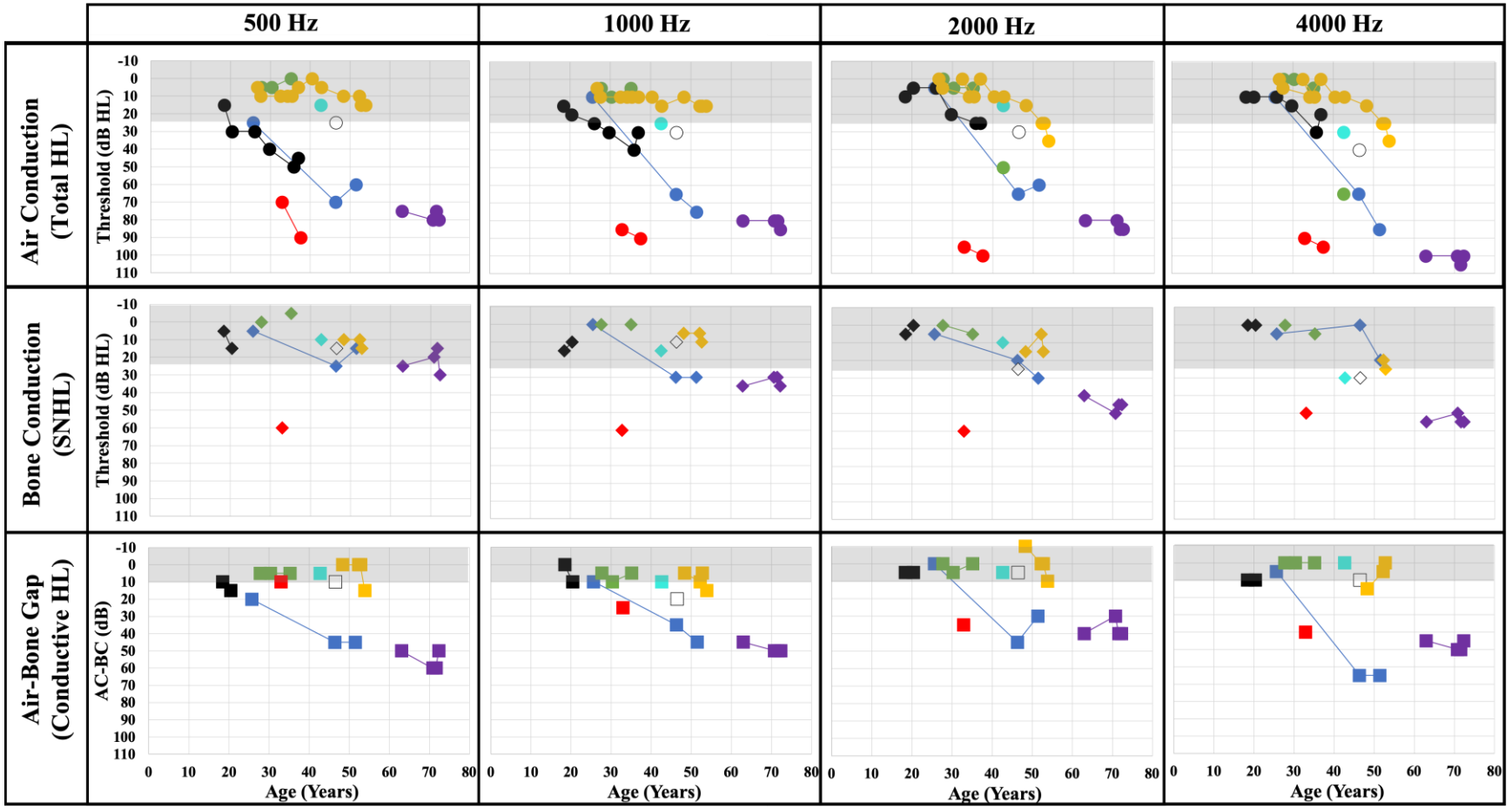


Figure 23. Audiometric thresholds of participants' better ear as a function of age for eight family members of Family 2081 identified as heterozygous for *FOXL1* deletion. Each colour represents data from a different family member. Data points connected with a line represent longitudinal data from the same individual ear. Audiometric thresholds broken down into air conduction (total hearing loss), bone conduction (sensorineural hearing loss component) and air-bone gap (conductive hearing loss component).

Table 11. Linear regression analysis for relationship between age and worse ear hearing thresholds for eight family members heterozygous for the *FOXL1* deletion.

	Worse Ear					
Source	***B***	***SE B***	**β**	***t***	***p***	R^2
Air Conduction						
250 Hz	.959	.357	.440	2.686	.012	.194
(Constant)	-.652	15.409		-.042	.967	
500 Hz	1.200	.429	.454	2.794	.009	.206
(Constant)	-6.886	18.534		-.371	.713	
1000 Hz	1.271	.425	.479	2.988	.006	.229
(Constant)	-10.09	18.362		-.549	.587	
2000 Hz	1.517	.341	.631	4.452	.000	.398
(Constant)	-21.84	14.709		-1.485	.148	
4000 Hz	1.361	.265	.685	5.143	.000	.469
(Constant)	-6.413	11.422		-.562	.579	
8000 Hz	1.204	.404	.478	2.982	.006	.229
(Constant)	-10.01	17.425		-.575	.570	
Bone Conduction						
500 Hz	.743	.214	.656	3.477	.003	.430
(Constant)	-12.08	10.214		-1.182	.254	
1000 Hz	.502	.217	.500	2.311	.035	.250
(Constant)	-.770	10.390		-.074	.942	
2000 Hz	.927	.204	.712	4.541	.000	.508
(Constant)	-11.04	9.259		-1.193	.247	
4000 Hz	.694	.228	.605	3.039	.008	.366
(Constant)	-5.43	10.910		-.497	.626	

Table 12. Linear regression analysis for relationship between age and better ear hearing thresholds for eight family members heterozygous for the *FOXL1* deletion.

	Better Ear					
Source	***B***	***SE B***	**β**	***t***	***p***	R^2
Air Conduction						
250 Hz	.980	.263	.563	3.730	.001	.317
(Constant)	-11.21	11.348		-.988	.331	
500 Hz	1.031	.312	.517	3.307	.002	.267
(Constant)	-11.10	13.464		-.825	.416	
1000 Hz	1.214	.301	.592	4.027	.000	.351
(Constant)	-17.61	13.015		-1.353	.186	
2000 Hz	1.379	.339	.596	4.070	.000	.356
(Constant)	-29.35	14.627		-2.006	.054	
4000 Hz	1.855	.317	.730	5.852	.000	.533
(Constant)	-42.36	13.688		-3.095	.004	
8000 Hz	1.415	.408	.540	3.459	.002	.292
(Constant)	-26.47	17.617		-1.502	.144	
Bone Conduction						
500 Hz	.217	.208	.261	1.046	.312	.068
(Constant)	5.937	10.134		.586	.567	
1000 Hz	.389	.226	.407	1.726	.105	.166
(Constant)	1.009	11.003		.092	.928	
2000 Hz	.739	.217	.661	3.412	.004	.437
(Constant)	-11.78	10.569		-1.115	.283	
4000 Hz	.971	.203	.787	4.774	.000	.619
(Constant)	-19.27	9.924		-1.942	.073	

A summary of the rate of progression in dB/decade is reported in Table 13 for AC and BC thresholds in the worse and better ear. In the worse ears, progression of hearing loss occurs most rapidly in 2000 Hz AC thresholds, while the better ears have a most rapid progression in 4000 Hz AC thresholds. In both the worse and better ears, BC thresholds had an increase in the rate of progression with increasing frequency, indicative of a progressive SNHL component to the hearing loss. In the worse ear, air-conduction thresholds were estimated to deteriorate at a rate between 9.6-15.2 dB/dec, while bone-conduction thresholds were estimated to deteriorate at

a rate between 5.0-18.6 dB/dec depending on frequency. Similar rates of progression were identified in the better ears, however linear regression analysis was not significant for bone-conduction thresholds at 500 and 1000 Hz.

Table 13. Rate of progression of air-conduction (AC) and bone-conduction (BC) thresholds reported in dB per decade (dB/dec) for eight family members of Family 2081 identified as heterozygous for *FOXL1* deletion. Numbers bolded with * represent non-significant linear regression analysis.

		Frequency (Hz)					
		250	**500**	**1000**	**2000**	**4000**	**8000**
Worse	**AC**	9.6	12.0	12.7	15.2	13.6	12.0
Ear	**BC**		7.4	5.0	9.3	18.6	
Better	**AC**	9.8	10.3	12.1	13.8	18.6	14.2
Ear	**BC**		**2.2***	**3.9***	7.4	9.7	

5.3.1.1 Predicted Audiogram

A separate linear regression analysis was carried out following removal of the non-penetrant cases for the worse ears of *FOXL1* gene carriers (Table 14). Analysis from the linear regression of the worse ear was used to generate a predicted audiogram based on three ages of 20, 40 and 60 years (Figure 24). Based on the linear regression predictive model, it is estimated that *FOXL1* deletion carriers will exhibit a moderate conductive hearing loss around the age of 20. This is consistent with phenotypic results of family members carrying the mutation who report their hearing loss being identified in their 20s (Table 15). Predictive modeling at the age of 40 reveals a moderately-severe mixed hearing loss, with predicted bone conduction thresholds demonstrating the beginning of the associated sensorineural component to the *FOXL1* hearing loss. By the age of 60, the predicted audiogram suggests a profound mixed hearing loss. This is consistent with participant 0002 who underwent surgery for a cochlear implant. All other family members with such severe hearing loss had undergone corrective stapes surgery to resolve the conductive component to their hearing loss.

Table 14. Linear regression analysis for relationship between age and worse ear hearing thresholds for six family members heterozygous for the *FOXL1* deletion who exhibit clinical signs of otosclerosis. The two non-penetrant cases of Family 2081 were removed from analysis.

	Worse Ear					
Source	***B***	***SE B***	**β**	***t***	***p***	R^2
Air Conduction						
250 Hz	.828	.155	.809	5.338	.000	.655
(Constant)	29.273	7.155		4.091	.001	
500 Hz	1.003	.178	.825	5.647	.000	.680
(Constant)	31.174	8.198		3.803	.002	
1000 Hz	1.100	.241	.762	4.559	.000	.581
(Constant)	25.555	11.133		.295	.037	
2000 Hz	1.273	.273	.770	4.668	.000	.592
(Constant)	9.591	12.590		.762	.458	
4000 Hz	1.248	.212	.836	5.896	.000	.699
(Constant)	13.611	9.772		1.393	.184	
8000 Hz	1.160	.252	.765	4.598	.000	.585
(Constant)	17.683	11.645		1.519	.150	
Bone Conduction						
500 Hz	.724	.214	.713	3.377	.006	.509
(Constant)	-5.813	10.513		-.553	.591	
1000 Hz	.485	.193	.604	2.517	.029	.365
(Constant)	6.526	9.453		.690	.504	
2000 Hz	1.044	.203	.841	5.150	.000	.707
(Constant)	-14.123	9.941		-1.421	.183	
4000 Hz	.583	.269	.547	2.169	.053	.299
(Constant)	.181	13.183		.014	.989	

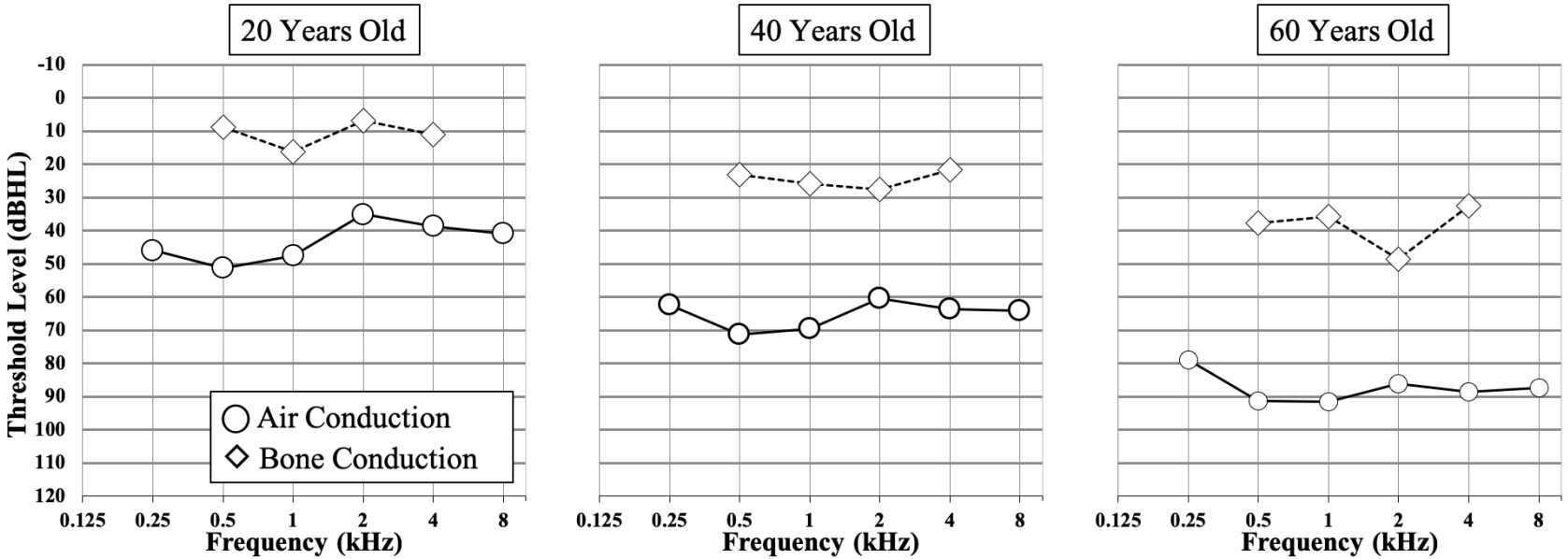


Figure 24. Predicted audiogram generated from linear regression results for the worse ear of *FOXL1* deletion carriers for three ages: 20, 40 and 60 years old.

5.3.2 Non-Surgical Case Studies

Within Family 2081, there have currently been two *FOXL1* mutation carriers (individual A005, A006) recruited with no evidence of the clinical features of otosclerosis. These clinical features would include evidence of a conductive hearing loss, or evidence of cochlear otosclerosis. The only exceptions to this would be the absence of acoustic reflexes using a high frequency probe tone in both cases, the presence of a high-frequency SNHL in individual A005, and the abnormal PA measurement in individual A006. A summary of the advanced phenotyping in these two non-surgical cases is reported.

Individual A005

Audiometric thresholds for A005 reveal a mild high-frequency sensorineural hearing loss at 3 and 4 kHz at the age of 26.7 years in his left ear (Figure 25). Over the next two and a half decades, the hearing loss in the high frequencies increases gradually, progressing to a moderate high-frequency sensorineural hearing loss between 2000 - 4000 Hz, rising back to normal hearing above 4000 Hz. Hearing thresholds in A005's right ear also demonstrates a sensorineural hearing loss notch around 4 kHz which is present by the age of 52.3 years. The advanced phenotypic procedure was conducted at the age of 52.8 years and is presented in Figures 26 and 27.

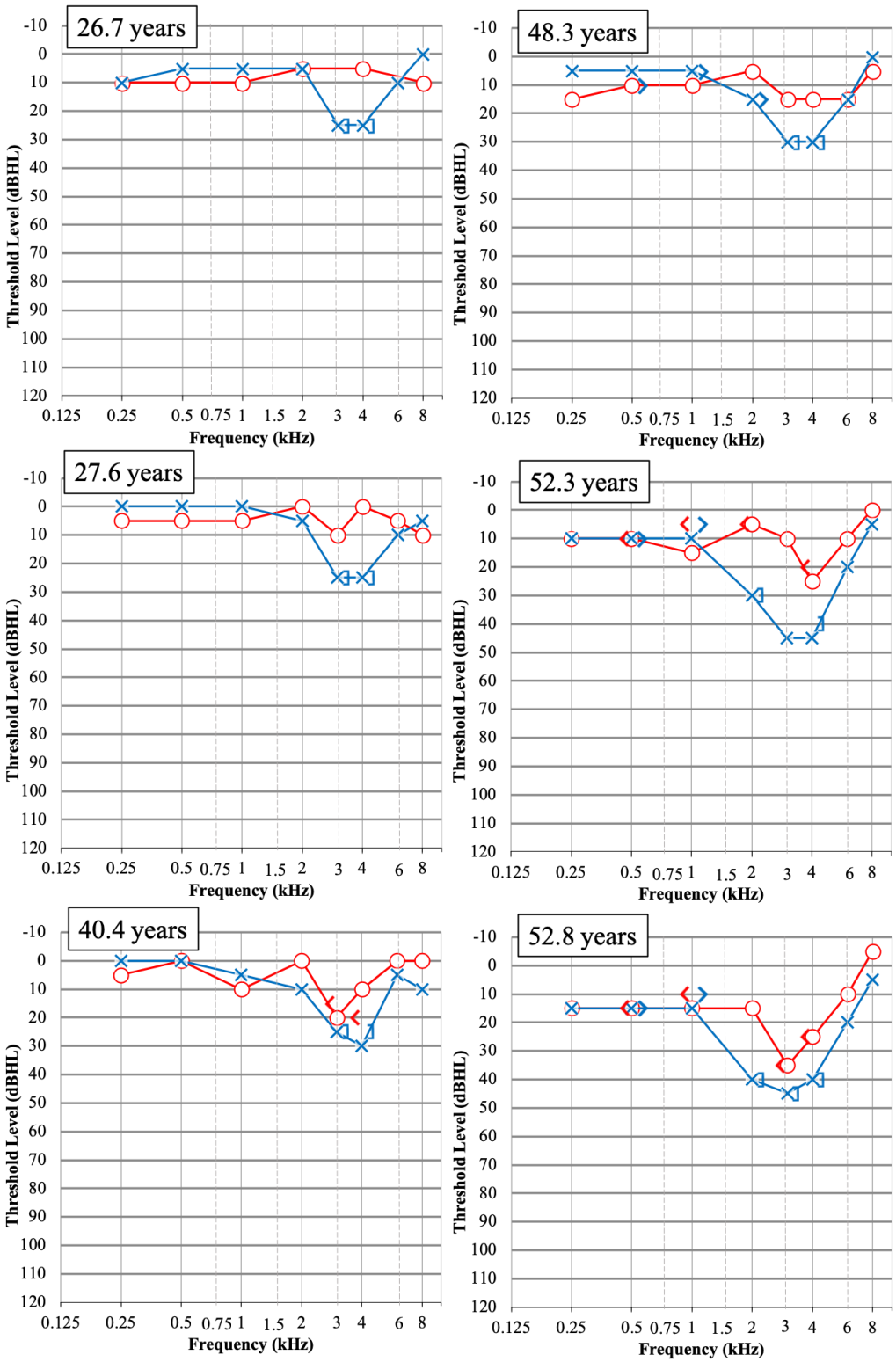


Figure 25. Audiometric thresholds obtained for family member A005. Conventional audiometric symbols are used where x = left air conduction, o=right air conduction, <=right unmasked bone conduction, and >=left unmasked bone conduction.

A005 had measurable reflexes in the left ear (Figure 26). An elevated reflex was identified at 4000 Hz, with absent contralateral reflexes at 500 and 4000 Hz in the left ear. However, in A005's right ear, acoustic reflexes were elevated in the low frequencies, and absent at 4000 Hz and using a wideband noise in the right (Figure 27). There were also no measurable contralateral reflexes with the probe in the right ear. Distortion product otoacoustic emissions were recorded at twelve frequencies spanning 2F1-F2 frequencies of 700 to 5300 Hz. In A005's left ear, all DPOAEs were considered absent with the exception of distortion products at frequencies of 1031 and 4641 Hz (Figure 27). DPOAEs are mainly present in the right ear, with the exception of 2F1-F2 frequencies of 703 and 5297 Hz (Figure 27). Power absorbance for individual A005 falls within 1 standard deviation of the norm for frequencies up to approximately 1800 Hz, where PA in both the left (Figure 26) and right (Figure 27) ear fall just outside of 1 standard deviation of the normative range.

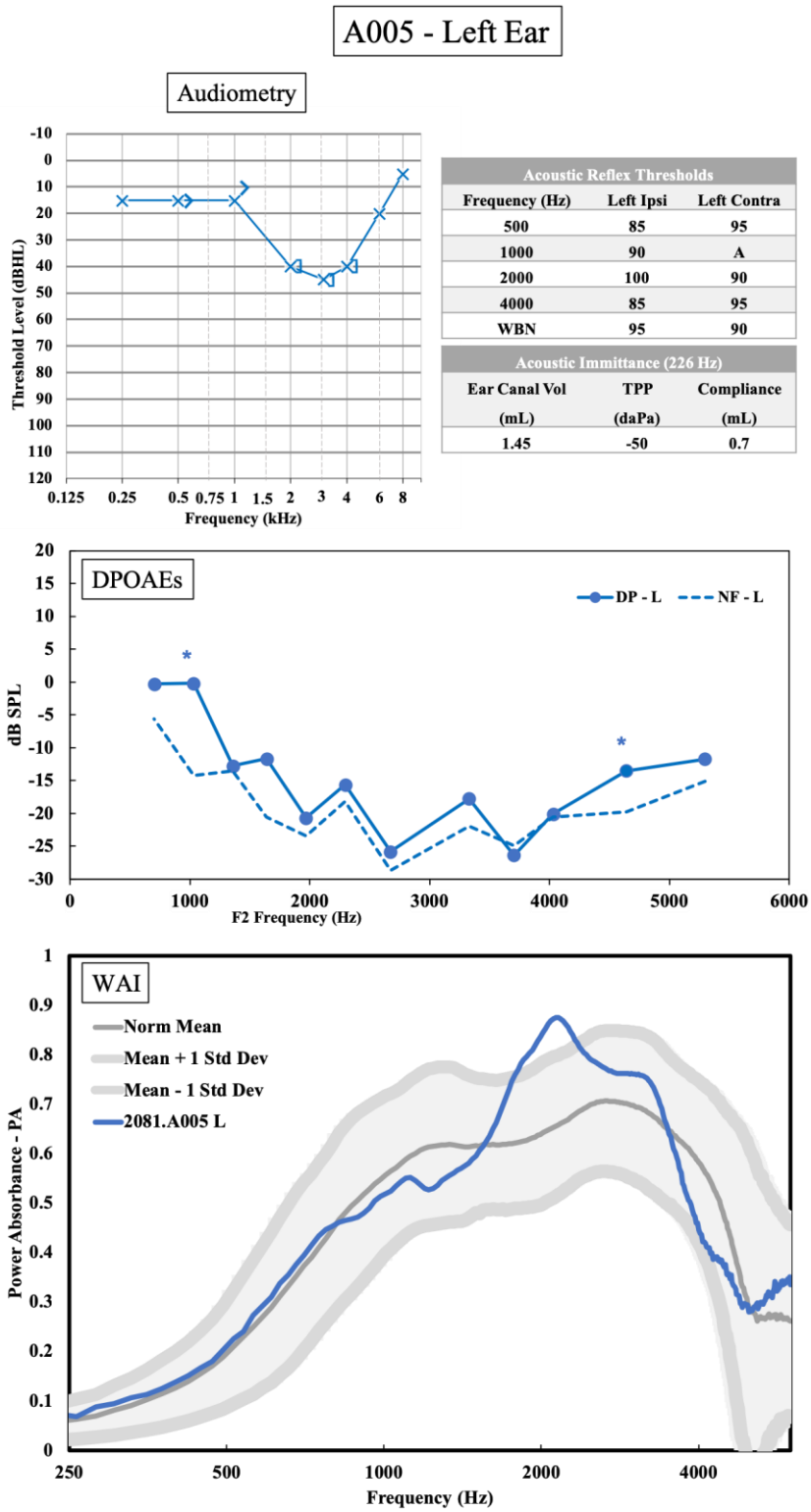


Figure 26. Phenotypic results for left ear of individual A005. Results show most recent audiogram, acoustic reflex thresholds (ARTs), standard tympanometry, distortion product otoacoustic emissions (DPOAEs) and power absorbance (PA). DPOAEs meeting criteria to be considered present are denoted by an asterisks (*).

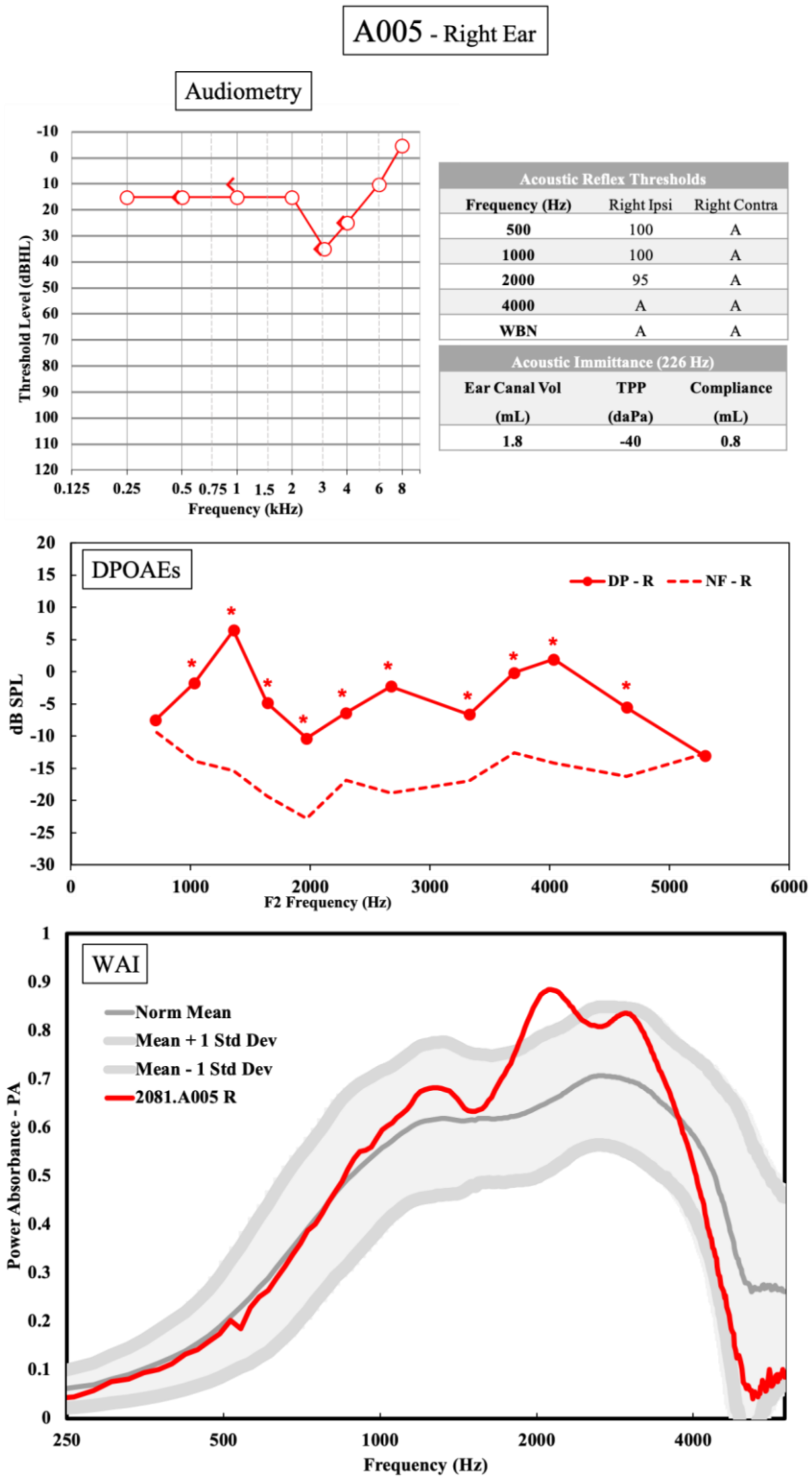


Figure 27. Phenotypic results for right ear of individual A005. Results show most recent audiogram, acoustic reflex thresholds (ARTs), standard tympanometry, distortion product otoacoustic emissions (DPOAEs) and power absorbance (PA). DPOAEs meeting criteria to be considered present are denoted by an asterisks (*).

Overall, the phenotype of A005 suggests a possible case of non-penetrance, given that he was 53.8 years of age at the time of recruitment. The case of non-penetrance is difficult to determine given there are some findings indicating possible onset of otosclerosis. Audiometric thresholds, while consistent with industrial workplace noise exposure, could be a result of cochlear otosclerosis. Distortion product otoacoustic emissions were mostly absent in the left ear. Acoustic reflexes were absent in or elevated in the better right ear, and present in the worse ear. Finally, PA in both ears would be classified as normal since the PA profile does replicate those presented in Chapter 4.

Individual A006

Audiometric thresholds for A006 were retrospectively collected from a previous hearing test conducted at age 27.9 years, along with two audiometric tests collected as part of the hearing research study; ages 30.5 and 34.1 years of age (Figure 28). All three tests reveal AC thresholds below 15 dB HL at all frequencies tested suggestive of normal hearing thresholds. Bone conduction thresholds were only tested at the earliest test date which revealed no air-bone gaps greater than 5 dB. These results suggest no sensorineural or conductive hearing loss present. The results also support that over the 8 years between the first and last test, there is no progression in hearing loss as thresholds obtained at the age of 34.1 years of age were obtained at 5dB HL and below at all audiometric frequencies tested. The advanced phenotyping procedures were conducted at the age of 34.1 years.

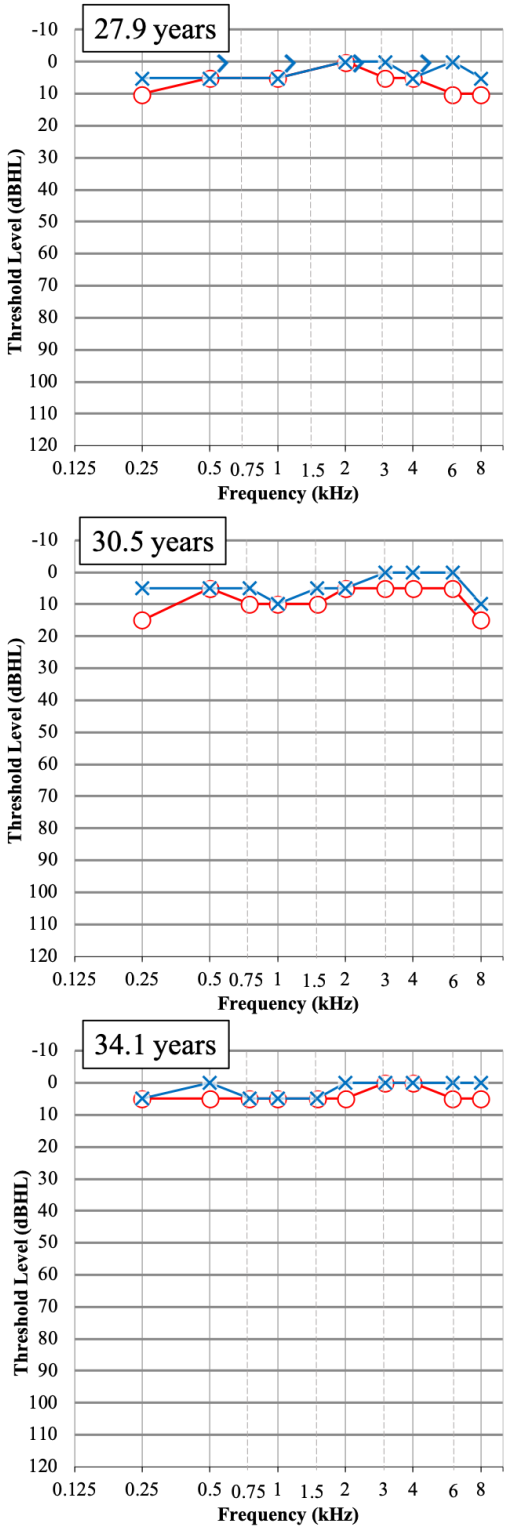


Figure 28. Audiometric thresholds obtained for family member A006. Conventional audiometric symbols are used where x = left air conduction, o=right air conduction, <=right unmasked bone conduction, and >=left unmasked bone conduction.

Acoustic reflexes were present in all conditions when the probe was situated in A006's left ear (Figure 29). In A006's right ear, reflexes were present in all conditions except at 4000 Hz with ipsilateral and contralateral stimulation (Figure 30). A006 has measurable DPOAEs in both ears and at all frequencies, with the exception of the distortion product at 4641 Hz in their left ear (Figure 29). Power absorbance measurements for individual A006's right ear fall outside of 1 standard deviation of the normative data reported in Chapter 4 and is considered abnormal given the absence of acoustic reflexes also in the right ear. PA in A006's right ear also demonstrates the unusual peak around 960 Hz (Figure 30).

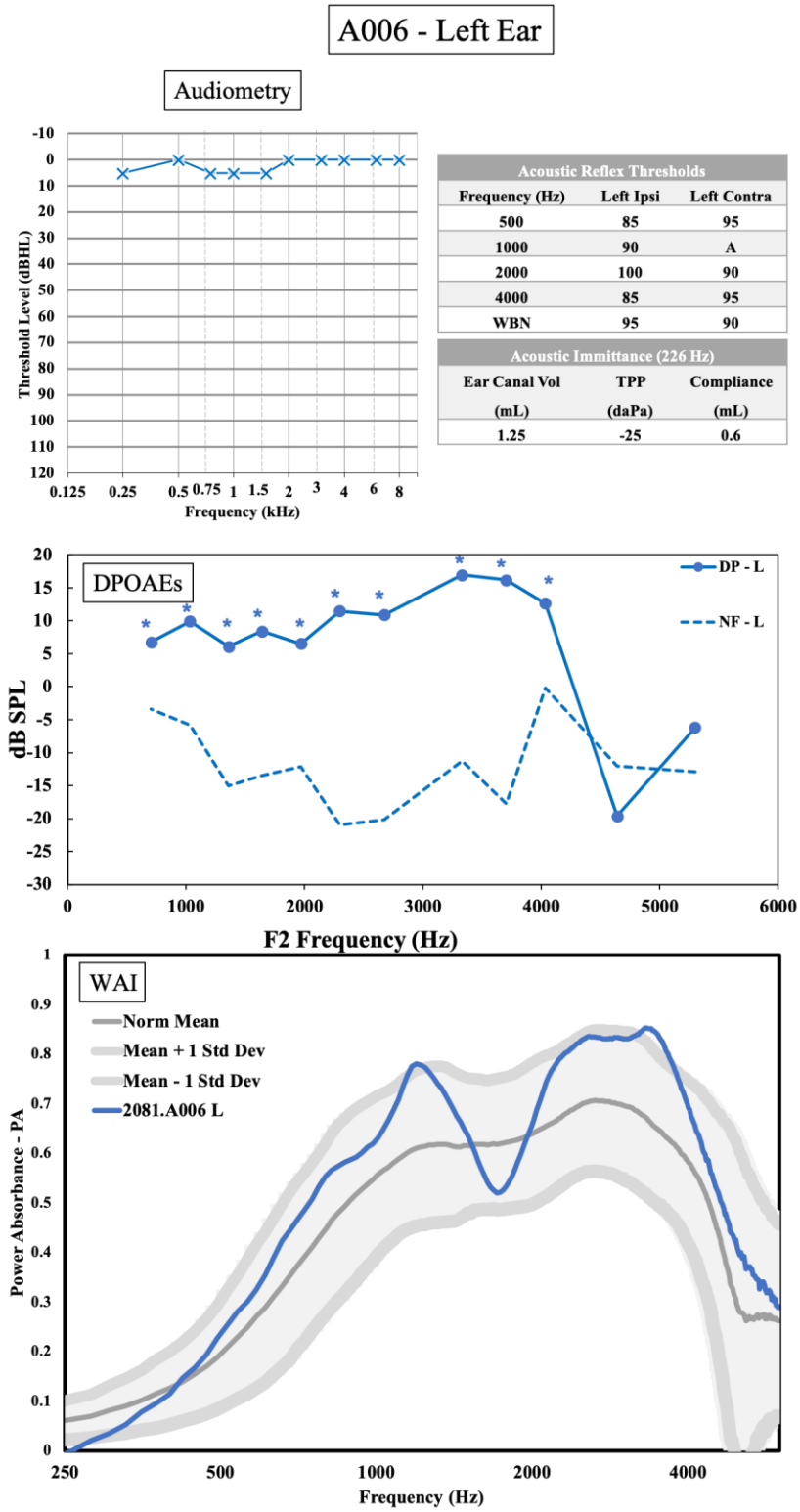


Figure 29. Phenotypic results for left ear of individual A006. Results show most recent audiogram, acoustic reflex thresholds (ARTs), standard tympanometry, distortion product otoacoustic emissions (DPOAEs) and power absorbance (PA). DPOAEs meeting criteria to be considered present are denoted by an asterisks (*).

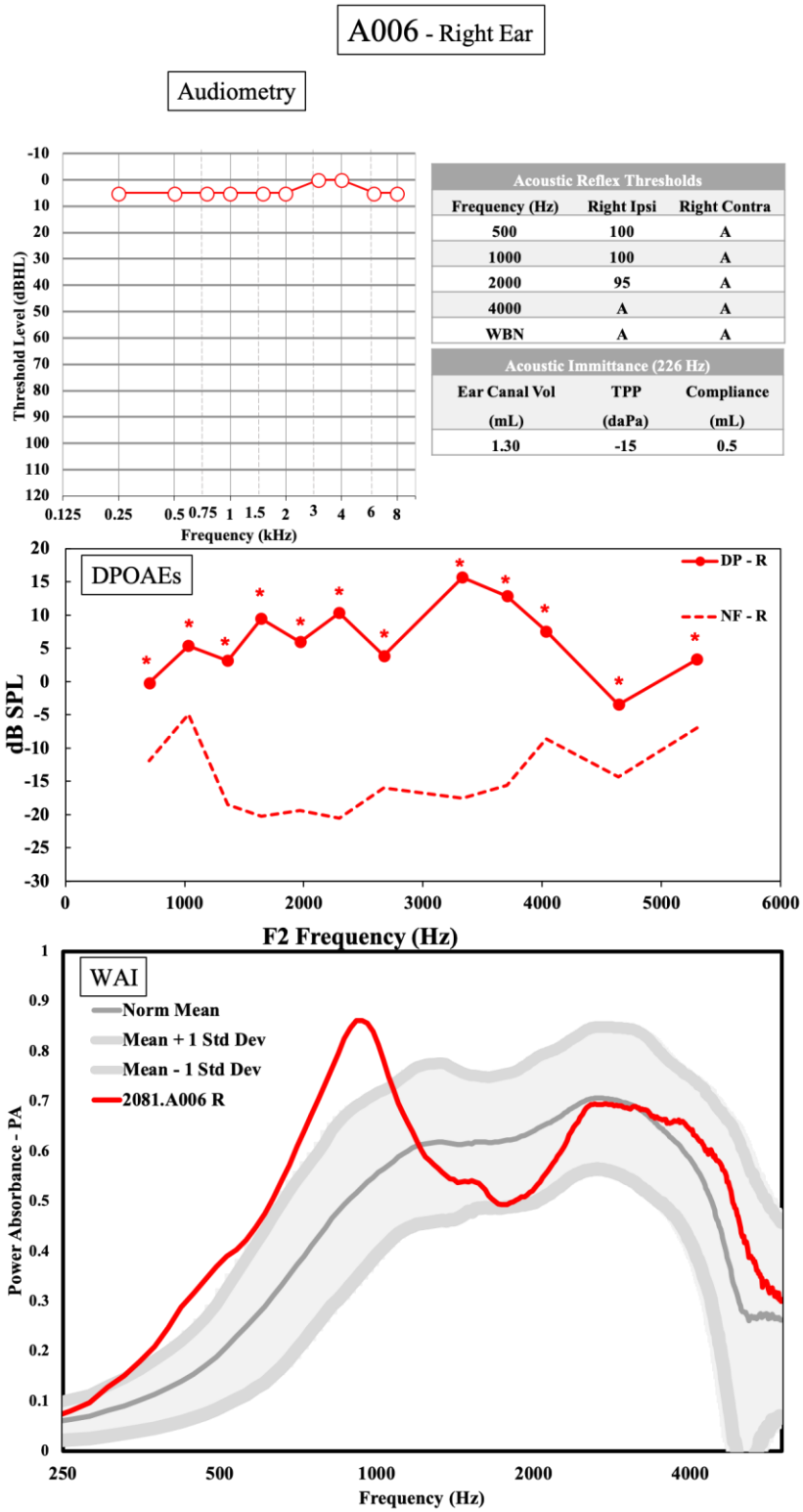


Figure 30. Phenotypic results for right ear of individual A006. Results show most recent audiogram, acoustic reflex thresholds (ARTs), standard tympanometry, distortion product otoacoustic emissions (DPOAEs) and power absorbance (PA). DPOAEs meeting criteria to be considered present are denoted by an asterisks (*).

Overall, the phenotype of A006 also suggests a case of non-penetrance, however there may be some clinical signs of potential otosclerosis. Individual A006 was in her fourth decade at the time of testing, which is older than the age of onset reported by the seven affected *FOXL1* carriers. Therefore, we would expect her to start showing clinical signs of otosclerosis. While her audiometric thresholds are normal, the DPOAE in her left ear at 4641 Hz was absent. Her right ear is considered more abnormal as acoustic reflexes were also absent at 4000 Hz in her right ear and she demonstrated a PA peak around 950 Hz which fell well outside of 1 standard deviation of the normative values while demonstrating compliance 0.5 mL. This PA profile has been linked with otosclerosis previously by Niemczyk, Lachowska, Tataj, Kurczak, & Niemczyk (2018) as well as presented in the results of the subgroup presented in Chapter 4. Given these sub-clinical signs of absent high-frequency DPOAEs and abnormal PA profile, we expect that individual A006 may demonstrate early stages of otosclerotic development. Longitudinal studies of this individual would be required in order to confirm the development of otosclerosis.

5.3.3 Phenotypic Summary

Two *FOXL1* mutation carriers do not exhibit any typical clinical features of otosclerosis (conductive hearing loss or evidence of cochlear otosclerosis). Results of the advanced phenotyping may suggest the presence of some sub-clinical evidence of later onset otosclerosis development. Individual A005 presents with a SNHL, however, the clinical profile, as well as a history of workplace noise, suggest the presence of noise-induced SNHL. The other non-surgical case, Individual A006, has audiometric thresholds within the clinically defined normal range of 20 dB HL at all frequencies tested, however she demonstrates a PA peak previously seen in four of our otosclerotic ears in Chapter 4.

A summary of the surgical interventions and clinical phenotype for all *FOXL1* deletion carriers is presented in Table 15. The interventions for these family members are quite variable, where two family members, 0005 and 0002 have undergone unilateral cochlear implants to correct their hearing loss. Four family members (0000, 0005, 0004 and 0035) have undergone surgical intervention to treat bilateral conductive components. Two family members (0002 and 0016) present with mixed loss in one ear, and sensorineural hearing loss in the other, with both undergoing stapes surgery to correct the conductive hearing loss component of their hearing loss.

Table 15. Phenotypic summary of all family members in Family 2081 heterozygous for the *FOXL1* deletion.

Individual	Diagnosis (affected ear)	Age of Onset (decade)	Hearing Status before Surgical Intervention		Surgical Interventions (age in years)	
			Right Ear	Left Ear	Right Ear	Left Ear
Affected Family Members						
0000	Otosclerosis	Early 3rd	Conductive	Conductive	Stapedectomy (51)	Stapedectomy (52)
0005	Otosclerosis	Late 2nd - early 3rd	Mixed	Mixed	Stapedectomy (36)	Canaloplasty (71) Middle Ear Implant (75) Cochlear implant
0002	Otosclerosis (possibly cochlear)	2nd	Mixed	Sensorineural	Stapedectomy (60) Cochlear Implant (61)	None
0004	Otosclerosis	Early 3rd	Conductive	Conductive	Stapedectomy (47)	Stapedectomy (22)
0016	Otosclerosis (left)	Early 3rd	Sensorineural	Mixed	None	Stapedectomy (42)
0035	Otosclerosis	Early 3rd	No data	No data	Stapedectomy (27)	Stapedectomy (52)
A001	Otosclerosis (bilateral) Cholesteatoma (right)	Mid 2nd	Conductive	Conductive	Tympanotomy with stapes mobilization (17) Mastoidectomy (17) Tympanoplasty with ossicular chain re-mobilization (18)	None
Non-Penetrant Cases						
A005	Hearing Loss (left); possibly noise-induced	3rd	Normal	Sensorineural	None	None
A006	Normal Hearing	NA	Normal	Normal	None	None

5.4 Discussion

The rationale of this chapter was to investigate the advanced phenotype of Family 2081 in order to better understand the natural course of *FOXL1*-associated otosclerosis and to develop a model of the associated hearing loss. Eleven family members of this family were recently identified as gene carriers for the otosclerosis causing mutation in *FOXL1*, nine of which were available for recruitment to this study.

5.4.1 Phenotypic Variability

Phenotypic analysis of Family 2081 revealed that although the *FOXL1* deletion is causative of otosclerosis in all affected family members, considerable variability in the auditory phenotype exists for this family. Different audiometric profiles including bilateral conductive hearing losses, unilateral mixed hearing loss, sensorineural hearing loss, and hearing within normal limits was found for all gene mutation carriers. These findings are consistent with other family studies of otosclerosis in which affected family members present with a broad range of hearing loss, including unilateral or bilateral conductive hearing losses, mixed hearing loss, or sensorineural hearing loss (Brownstein et al., 2006).

The identification of genetic causes of otosclerosis has been a challenge for researchers, with the first causative genes identified just recently. While family studies are powerful in the identification of genetics causes of disorders, they require an accurate identification of affected versus unaffected family members for gene discovery. Family 2081 is a good example of how the clinical features of an otosclerotic family can vary even in a monogenic inheritance of the disorder. Traditionally, the gold standard for diagnosis of otosclerosis is confirmation of stapes fixation at the time of stapes surgery. In the case of Family 2081, seven family members have

been diagnosed with otosclerosis following surgery (Abdelfatah, 2014). This large number of family members who underwent stapes surgery allowed for the identification of the *FOXL1* mutation.

In future genetic studies of families with heritable otosclerosis, the incorporation of non-surgically confirmed cases using a more comprehensive battery of auditory phenotyping measures may be feasible since large families are rare. Future hypotheses of "affected" versus "unaffected" family members should also account for the presence of cases without clinical presentations of otosclerosis. This chapter reports the phenotypic analysis of two family members, A005 and A006, who would not receive a surgical or clinical diagnosis of otosclerosis since A005 demonstrates a unilateral sensorineural hearing loss which is hypothesized to be due to noise exposure, and A006 has hearing thresholds within normal limits at the age of 34.

Previous genetic research of otosclerosis has estimated the penetrance of otosclerosis between 40-90% (Brownstein et al., 2006; Morrison, 1967; Van Den Bogaert et al., 2001). While the true penetrance of otosclerosis remains unknown, larger studies into the prevalence of the *FOXL1* mutation in the otosclerotic population will help determine the true value of penetrance of otosclerosis due to the *FOXL1* mutation. The case study of individual A006 provides evidence that although no diagnosis of otosclerosis would be given, there may be sub-clinical signs of otosclerosis present in gene carriers. Therefore, the use of advanced phenotyping including the use of DPOAEs and PA should be incorporated in future genetic studies of otosclerosis as a method to potentially identify family members with normal hearing thresholds, but who may be early in disease progression.

5.4.2 Predictive Model for *FOXL1*-Associated Otosclerosis Progression

While the natural history of *FOXL1*-associated otosclerosis is quite variable, it is still worth developing a predictive model for the hearing loss as a starting point to understand the progression of *FOXL1*-associated hearing loss. Individuals of Family 2081 who present with clinical otosclerosis, report their hearing loss beginning around the 3rd decade. The *FOXL1*-associated hearing loss is predicted to progress into a mixed hearing loss, with air-conduction thresholds in the severe to profound range by the age of 60, with air-conduction thresholds progressing between 9.6-15.2 dB/dec and bone-conduction thresholds progressing between 5.0-18.6 dB/dec in the worse ear. The results of our analysis are similar to the progression results reported in another large otosclerotic family, where air-conduction thresholds were estimated to deteriorate at a rate of 8.1-13.2 dB/dec and bone-conduction thresholds at a rate of 3.7-8.2 dB/dec (Declau et al., 2007). The results of the predictive model can be used by audiologists and clinicians as a guide to counsel any future individuals identified with the *FOXL1* mutation about the anticipated progression of the conductive and sensorineural components of their hearing loss. A similar approach is used to predict the course of autosomal dominant forms of sensorineural hearing losses caused by different gene mutations and made available to clinicians and researchers by Smith and colleagues through their AudioGene website (Hildebrand et al., 2009). The evidence from this Newfoundland pedigree with *FOXL1*-associated otosclerosis will support counselling for individual family members regarding the progressive sensorineural component of this auditory phenotype, allowing them to prepare for appropriate interventions which may include the use of amplification devices, or cochlear implantation if the sensorineural loss deteriorates substantially.

Prospective research is recommended, with comprehensive, longitudinal monitoring of the behavioural and physiological phenotype using ARTs, DPOAEs and PA in conjunction with audiometric thresholds and thorough medical history questionnaires, providing insight into the course of this genetic disorders over the lifespan. A limitation of using linear regression analysis for the purpose of modeling *FOXL1*-associated hearing loss in Family 2081 is the analysis assumes that the hearing loss progresses in a linear fashion. Reports of the pathology of otosclerosis however suggest that the disorder progresses in stages, which are characterized by varying enzymatic and bone remodeling (Crompton et al., 2019; Rudic et al., 2015). Therefore, as additional types of data are collected in individuals carrying the *FOXL1* mutation, a more accurate model can be developed taking into account the various stages of the disorder.

5.4.3 Non-Surgical Cases

Studies investigating the genetic cause of otosclerosis have long suggested there are cases of non-penetrance in dominantly inherited otosclerosis. There are two individuals in the presently studied family who may be non-penetrant cases of the inherited form of otosclerosis in their family; individuals A005 and A006. In the case of Individual A005, there is the appearance of what might be described as noise-induced hearing loss appearing around 4 kHz, especially in the left ear. This high-frequency hearing loss appears to begin by the age of 26 years and continues to worsen by the age of 53 years. The use of high-resolution imaging might offer some clarity as to whether this case of non-penetrant otosclerosis might be a case of histological otosclerosis. There are some potential sub-clinical features of otosclerosis present in the case of A005. There is no measurable acoustic reflex with the probe in the participant's right ear, and the stimulation of the left ear (described in this study as "right contralateral"). A005 also has absent otoacoustic emissions, which can be a feature of otosclerosis, either in the

sensorineural form of the disorder or in the conductive form of the disorder (see Chapter 3 of this thesis). In Chapter 3, DPOAEs were absent in otosclerotic ears with sensorineural hearing loss (n=3), or ears with a conductive hearing loss (n=14).

A006 is also a carrier of the deletion in *FOXL1,* but by the age of 34, does not exhibit any audiometric signs of otosclerosis. Despite normal pure tones thresholds, acoustic reflexes were absent with the probe in her left ear with the stimulus presented in her right (left contra) at frequencies of 500 and 4000 Hz. Acoustic reflexes were also absent with the probe in her right ear for 4000 Hz. Wideband acoustic immittance testing revealed a PA peak around 950 Hz, a profile which is linked to otosclerosis by Niemczyk et al. (2018) as and results of this thesis as discussed in Chapter 4.

Without confirming the presence or absence of otosclerotic foci affecting the otic capsule in these non-surgical cases of otosclerosis, it is difficult to determine whether these are cases of true non-penetrance. Temporal bone studies suggest that histological otosclerosis is more common than clinical or cochlear otosclerosis (Crompton et al., 2019; Karosi et al., 2012; Schuknecht & Barber, 1985). Therefore, using advanced phenotyping in future genetic studies that is sensitive to detect otosclerotic foci in histological otosclerosis cases, may improve the chance of identifying causative genes. This study supports this idea since presented in this chapter are 2 family members, heterozygous for the *FOXL1* deletion, yet who would not meet the standard clinical criteria of diagnosis for otosclerosis.

5.4.4 Conclusion and Future Direction

This large Newfoundland family presents as the first otosclerotic family with a known mutation causing their heritable form of otosclerosis. This chapter describes in detail the

phenotype of the individuals in this family and provides a predictive model based on audiometric thresholds of family members presenting with *FOXL1*-associated hearing loss.

There are three family members of great importance for prospective study recruitment (Individuals; A001, 0016 and B007). These three individuals are carriers of the *FOXL1* deletion but were unavailable to participate in the study at the time of prospective data collection. Only retrospective audiometric thresholds were available for 0002, 0016 and A001. All three of these individuals have had unilateral stapes surgery. Audiometric thresholds for 0002 and 0016 revealed a sensorineural hearing loss in their non-surgical ear, while A001 presents with a conductive hearing loss in their non-surgical ear. At present, there is no retrospective audiometric thresholds available for B007. Focusing future recruiting efforts for these individuals will provide additional data for the physiological phenotype measurements of WAI, ARTs and DPOAEs in non-surgical *FOXL1* confirmed ears. B007 also serves as the first identified carrier of the deletion in the younger generation. Therefore, they would serve as an excellent case study to determine the longitudinal changes in audiometric thresholds as well as the longitudinal changes in non-audiometric phenotyping measures in *FOXL1* carriers.

Future research focused on gene discovery of mutations causing otosclerosis should therefore look to broaden their definition of otosclerosis. The identification of gene carriers solely based on surgical confirmation of stapes fixation could result in additional difficulty in identifying the causative genetic mutations for the disorder since there is significant phenotypic variability in otosclerosis, and specifically within a family with otosclerosis caused by the same genetic mutation.

5.5 References

Abdelfatah, N. (2014). The Genetic Aetiology of Otosclerosis in the Population of Newfoundland and Labrador. Memorial University of Newfoundland.

Brownstein, Z., Goldfarb, A., Levi, H., Frydman, M., & Avraham, K. B. (2006). Chromosomal mapping and phenotypic characterization of hereditary otosclerosis linked to the OTSC4 locus. Archives of Otolaryngology--Head & Neck Surgery, 132(4), 416–424. https://doi.org/10.1001/archotol.132.4.416

Crompton, M., Cadge, B. A., Ziff, J. L., Mowat, A. J., Nash, R., Lavy, J. A., … Dawson, S. J. (2019). The Epidemiology of Otosclerosis in a British Cohort. Otology & Neurotology, 40(1), 22–30. https://doi.org/10.1097/MAO.0000000000002047

Declau, F., Van Den Bogaert, K., Van De Heyning, P., Offeciers, E., Govaerts, P., & Van Camp, G. (2007). Phenotype-genotype correlations in otosclerosis: Clinical features of OTSC2. Advances in Oto-Rhino-Laryngology, 65, 114–118. https://doi.org/10.1159/000098745

Gorga, M. P., Neely, S. T., Ohlrich, B., Hoover, B., Redner, J., & Peters, J. (1997). From laboratory to clinic: a large scale study of distortion product otoacoustic emissions in ears with normal hearing and ears with hearing loss. Ear and Hearing, 18(6), 440–455.

Hildebrand, M. S., DeLuca, A. P., Taylor, K. R., Hoskinson, D. P., Hur, I. A., Tack, D., … Smith, R. J. H. (2009). A Contemporary Review of AudioGene audioprofiling: A machine-based candidate gene prediction tool for autosomal dominant nonsyndromic hearing loss. The Laryngoscope, 119(11), 2211–2215. https://doi.org/10.1002/lary.20664

Karosi, T., Csomor, P., & Sziklai, I. (2012). The value of HRCT in stapes fixations corresponding to hearing thresholds and histologic findings. Otology and Neurotology, 33(8), 1300–1307. https://doi.org/10.1097/MAO.0b013e31826352ad

Laird, N. M., & Lange, C. (2006). Family-based designs in the age of large-scale gene-association studies. Nature Reviews Genetics, 7(5), 385–394. https://doi.org/10.1038/nrg1839

Morrison, A. W. (1967). Genetic factors in otosclerosis. Annals of the Royal College of Surgeons of England, 41(2), 202–237. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2311999/

Nakajima, H. H., Pisano, D. V, Roosli, C., Hamade, M. a, Merchant, G. R., Mahfoud, L., … Merchant, S. N. (2012). Comparison of ear-canal reflectance and umbo velocity in patients with conductive hearing loss: a preliminary study. Ear and Hearing, 33(1), 35–43. https://doi.org/10.1097/AUD.0b013e31822ccba0

Niemczyk, E., Lachowska, M., Tataj, E., Kurczak, K., & Niemczyk, K. (2018). Wideband tympanometry and absorbance measurements in otosclerotic ears. The Laryngoscope, ePub ahead. https://doi.org/10.1002/lary.27747

Rudic, M., Keogh, I., Wagner, R., Wilkinson, E., Kiros, N., Ferrary, E., … Zarkovic, N. (2015). The pathophysiology of otosclerosis: Review of current research. Hearing Research, 330, 51–56. https://doi.org/10.1016/j.heares.2015.07.014

Schuknecht, H. F., & Barber, W. (1985). Histologic variants in otosclerosis. The Laryngoscope, 95(11), 1307–1317. https://doi.org/10.1288/00005537-198511000-00003

Shahnaz, N., Bork, K., Polka, L., Longridge, N., Bell, D., & Westerberg, B. D. (2009). Energy reflectance and tympanometry in normal and otosclerotic ears. Ear and Hearing, 30(2), 219–233. https://doi.org/10.1097/AUD.0b013e3181976a14

Van Den Bogaert, K., Govaerts, P. J., Schatteman, I., Brown, M. R., Caethoven, G., Offeciers,

F. E., … Van Camp, G. (2001). A second gene for otosclerosis, OTSC2, maps to chromosome 7q34-36. American Journal of Human Genetics, 68(2), 495–500. https://doi.org/10.1086/318185

Chapter 6

6 Advanced Phenotyping of an Otosclerosis Family of Unknown Genetic Etiology

6.1 Introduction

The purpose of this chapter is to apply advanced phenotyping methodology to a family with suspected hereditary otosclerosis. Advanced auditory phenotyping, using a comprehensive battery of audiometry, DPOAEs, ARTs and PA was analyzed in order to generate an auditory phenotype profile for each family member. Hierarchical cluster analysis was used to develop a phenotype segregation map of auditory features. These methods were used to develop a phenotype segregation hypothesis for this multiplex family. Phenotypic segregation and the accurate determination of affection status (affected versus unaffected) is essential in order to determine the most likely mode of inheritance in order to successfully identify new otosclerosis genes.

In the population cohort of surgically confirmed cases, otosclerosis presented as a unilateral or bilateral hearing loss and as conductive, mixed or sensorineural hearing loss (Chapter 3). Acoustic reflexes and DPOAEs were absent in cases of conductive or mixed hearing losses, while DPOAEs were absent in cases with SNHL. Although there was insufficient data regarding the presence of ARTs in SNHL cases of otosclerosis in the study presented in Chapter 3, the literature suggests that ARTs could be present in cases with SNHL of unknown etiology, up to approximately 60 dB HL (Gelfand, 1994). It is unclear, beyond the report of Chapter 3, regarding the presence or absence of DPOAEs in cases of cochlear otosclerosis.

In Chapter 4, another phenotyping tool, WAI, showed that PA was lower in otosclerotic ears compared to normal ears. However, individual PA data from otosclerotic ears suggests ears can be quite variable (Chapter 4, Niemczyk et al., 2018). Chapter 4 presents otosclerotic ears with a peak in the PA measurement between 800-1100 Hz, as well as a more typical PA profile of reduced absorbance in low frequencies, as previously reported in the literature (Keefe et al., 2017; Shahnaz, Bork, et al., 2009). This variability could reflect various stages of otosclerotic progression, as well as differences in the size and location of sclerotic foci in the otic capsule, for example around the stapes footplate as opposed to the cochlea. One factor for the presence of the peak, is the reported increase in PA around 1000 Hz correlated with ears demonstrating a high compliance value of greater than 1.75 mL (Feeney et al., 2014). Therefore, the presence of the PA peak will be considered as a potential indicator for otosclerosis provided the individual presents with compliance values of less than 1.75 mL.

6.1.1 Aims of this Study

The aims of this study are to conduct a phenotypic analysis of members of Family 2143 using clinically available tools. More specifically, the aims are:

> ***Specific aim 1:*** Collect advanced phenotyping and family history data on a multiplex family (Family 2143) and develop a pedigree with segregation of family members as ***affected, unaffected*** and ***possibly affected*** for otosclerosis.
>
> ***Specific aim 2***: Analyze auditory phenotype segregation on Family 2143 using hierarchical cluster analysis.

6.2 Methods

The test battery for participants in the previous studies consisted of audiometry, tympanometry, DPOAEs, ARTs and PA measurements. The testing protocol for this study in this chapter is a modified version based on the outcomes of the study presented in Chapter 4 and is described below.

6.2.1 Family 2143

A second multiplex family (2143) with suspected hereditary otosclerosis was identified in NL (Figure 31). Twelve members spanning three generations, affected with various hearing status, were recruited to this study (MUN Research Ethics Board #01.186). Their ages range from 19 to 82 years at the time of testing and represent one individual from generation II, five individuals from generation III and six individuals from generation IV (Figure 31). The family pedigree was generated based on family history and audiometric thresholds obtained retrospectively and/or prospectively. Genetic screening of recruited family members for the *FOXL1* deletion was conducted in the lab of Dr. Terry-Lynn Young at Memorial University which confirmed that family members of Family 2143 are not carriers of the *FOXL1* mutation described in the previous two chapters.

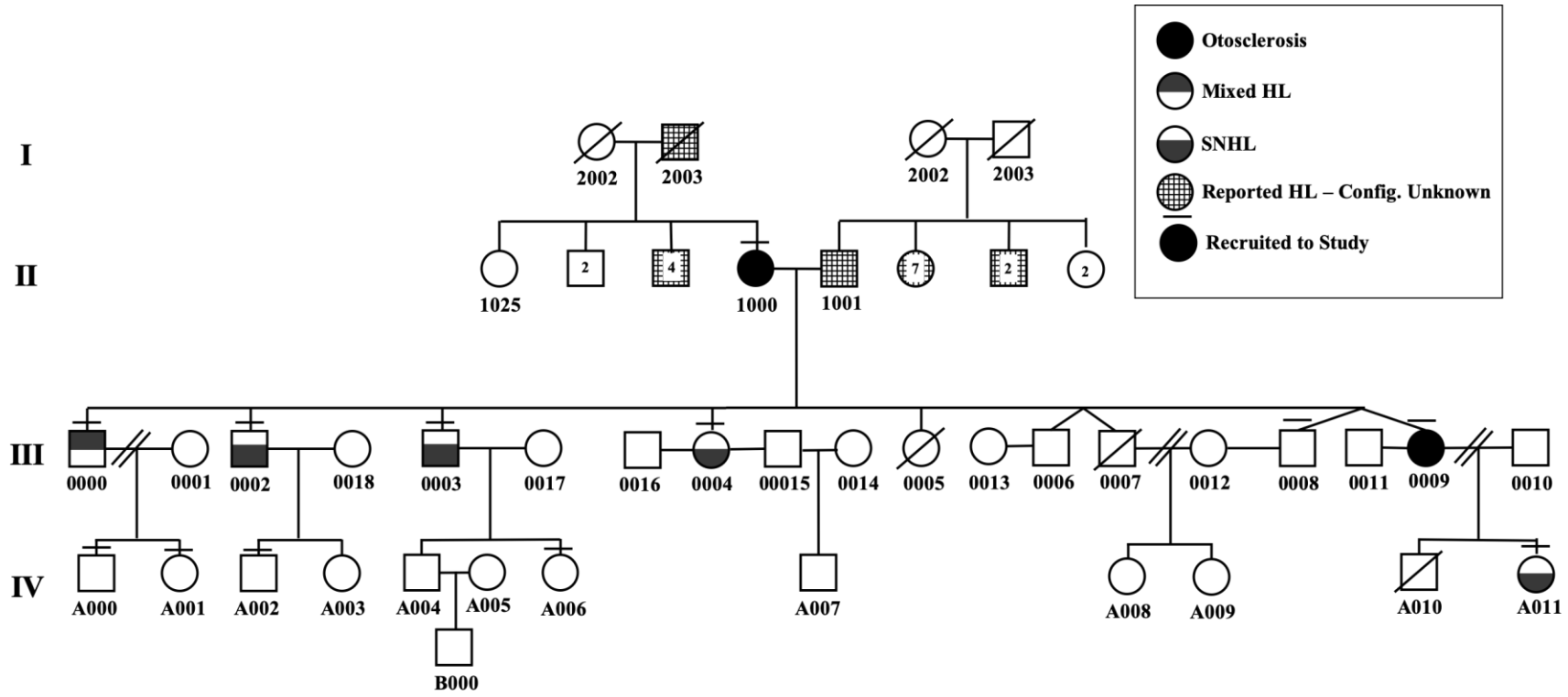


Figure 31. Family 2143 with suspected hereditary otosclerosis from NL. Pedigree based on audiometric thresholds and surgical confirmation of otosclerosis.

6.2.2 Advanced Phenotyping Protocol

The same advanced phenotyping protocol was used in this chapter as reported in Chapter 5. The advanced phenotyping protocol included the use of audiometry, tympanometry, acoustic reflex thresholds, DPOAEs and WAI, specifically PA. The rationale for determine normal versus abnormal PA is also reported in Chapters 4 (Study 2) and Chapter 5.

6.2.3 Statistical Analyses

A hierarchical cluster analysis using data from 11 members of Family 2143 with the software R v.3.3.3 using the clustering of mixed variables (Szepannek, 2019). The hierarchical cluster analysis and dendrogram was conducted with the assistance of Shailendra Singh, research assistant at the University of Western Ontario, who developed appropriate R code. In order to assess the fit of the individual data into the final clusters, a Silhouette width approach was conducted as a method of validation of the final clusters (Lengyel & Botta-Dukát, 2019; Rousseeuw, 1987). The silhouette width was calculated based on Euchlidean distance for each cluster, where values of silhouette width will range from -1 to +1. A value closer to +1 idicates euchlidean distance is closest to that of its own cluster compared to another cluster, while a value closer to -1 indicates that the Euchlidean distance is more similar to that of a different cluster. It is therefore suggested that a positive silhouette width is indicative of a good clustering result (Lengyel & Botta-Dukát, 2019). Subject 1000, the proband, was removed from the analysis due to bilateral stapes surgery. Cluster analysis was carried out to determine whether the clinical features of family members cluster into distinct groups. Air-conduction thresholds, bone-conduction thresholds, acoustic reflex thresholds, DPOAE amplitudes and associated SNRs were used for analysis. Data was extracted at frequencies of 500, 1000, 2000 and 4000 Hz for all measurements except DPOAE amplitudes and SNR at 500 Hz (data not elicited). Prior to

analysis, ART, DP amplitudes and SNR data were normalized by subtracting the minimum value of each measurement and dividing by the range of the data, allowing for a better visualization on the phenotypic heatmap. A hierarchical dendrogram, representing the phenotypic heatmap, was then created demonstrating Euclidean distances of individual ears based on their phenotype features. A dendrogram was created based on the clustering analysis. The height of the vertical bars, or leaves, of the dendrogram represents how similar or dissimilar each individual ear in the cluster is from one another.

For the statistical analysis, a cutoff of three clusters was used for the repeated measures ANOVA to determine whether specific auditory phenotype features were significantly different between each cluster. The raw data was used to conduct three repeated measures ANOVA to test for differences in clinical measures between clusters. The first repeated measures ANOVA was conducted for air and bone conduction thresholds where Frequency (4 levels) and measurement (2 levels) were used as within-subject factors, while cluster group (3 levels) was used as between subject factor. The second repeated measures ANOVA was conducted for ipsilateral and contralateral acoustic reflex thresholds where Frequency (4 levels) and measurement (2 levels) were used as within-subject factures, while cluster group (3 levels) was used as a between-subjects factor. Finally, the third repeated measures ANOVA was conducted for DPOAE amplitude and SNR, where Frequency (3 levels) and measurement (2 levels) were used as within-subject factors, while cluster group (3 levels) was used as a between-subject factor. When appropriate, *post-hoc* analysis was conducted by completing a pairwise comparison following Bonferroni correction.

6.2.4 Individual Phenotyping and Genotyping Hypothesis

The rationale for determining whether the family member is considered ***affected, possibly affected***, or ***unaffected*** was based on the criteria shown in Table 16 and Figure 32. If a family member presented with a hearing loss, then they were considered ***affected***. As reported in this thesis and by others, otosclerosis can present as conductive, mixed, or SNHL. Therefore, we categorized all members with conductive, mixed, or SNHL as ***affected***. Family members with normal hearing thresholds and age-appropriate sub-clinical features were considered ***unaffected*** (Figure 32). DPOAE amplitudes have been reported to decrease with age (Uchida et al., 2008), and therefore, absence of DPOAEs in the highest frequencies in family members above the age of 50 years were considered part of the normal aging process. Finally, a family member with normal hearing and sub-clinical feature(s), either absent ARTs, absent DPOAEs or an altered PA profile, was classified as ***possibly affected***, based on our assumption that sub-clinical features may be associated with otosclerosis.

Table 16. Criteria for the classification of hearing status of family members based on bone conduction (BC) thresholds, air-conduction (AC) thresholds and air-bone gaps (ABG).

Hearing Classification	BC Thresholds	AC Thresholds	ABG
Mixed Hearing Loss	> 20 dB HL at 2 or more frequencies	> 20 dB HL at 2 or more frequencies	> 10 dB at 2 or more frequencies
High-Frequency Sensorineural Hearing Loss	> 20 dB HL at 2 or more frequencies at or above 4000 Hz	> 20 dB HL at 2 or more frequencies at or above 4000 Hz	≤ 10 dB at all frequencies
Mid-Frequency Sensorineural Hearing Loss	> 20 dB HL at 2 or more frequencies between 1000 and 4000 Hz	> 20 dB HL at 2 or more frequencies between 1000 and 4000 Hz	≤ 10 dB at all frequencies
Low-Frequency Sensorineural Hearing Loss	>20 dB HL at 2 or more frequencies at or below 1000 Hz	>20 dB HL at 2 or more frequencies at or below 1000 Hz.	≤ 10 dB at all frequencies
Clinically Unaffected	≤ 20 dB HL at all frequencies	≤ 20 dB HL at all frequencies	≤ 10 dB at all frequencies

These sub-clinical signs include elevated (above 95 dB HL) or absent acoustic reflex thresholds, absent otoacoustic emissions, reduced (below 0.3 mL) or elevated (above 1.4 mL) static immittance, or compliance, measured via conventional 226 Hz tympanometry and abnormal PA profile (as reported in Chapters 4 and 5).

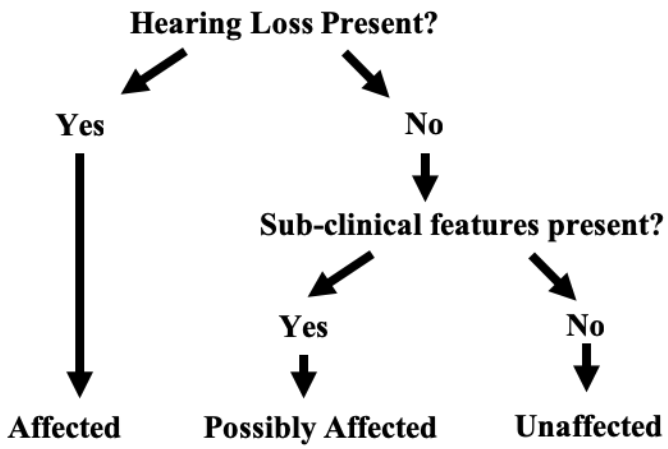


Figure 32. Decision tree for determining affection status of family members.

6.3 Results

6.3.1 Hierarchical Cluster Analysis

Results of the hierarchical cluster analysis reveal three distinct clusters (Figure 33). Cluster 1 is comprised of cases with mixed hearing loss, consisting of both ears from individuals 0000 and 0009. Cluster 2 is comprised of all ears showing a SNHL, with both ears from individuals 0002, 0003, 0004, and A011. Cluster 3 is comprised of all ears with family members who audiometrically have thresholds within normal limits. This third cluster consists of both ears of family members 0008, A000, A001, A002 and A006. Sihouette widths of Cluster 1, 2 and 3 generated values of 1, 0.314, and 0.0297 respectively, validating the clustering results. An overall Sihouette score of 0.413 was calculated across all Clusters, further validating the stabile configuration of clusters showing good intra-cluster cohesion and inter-cluster separation of data points.

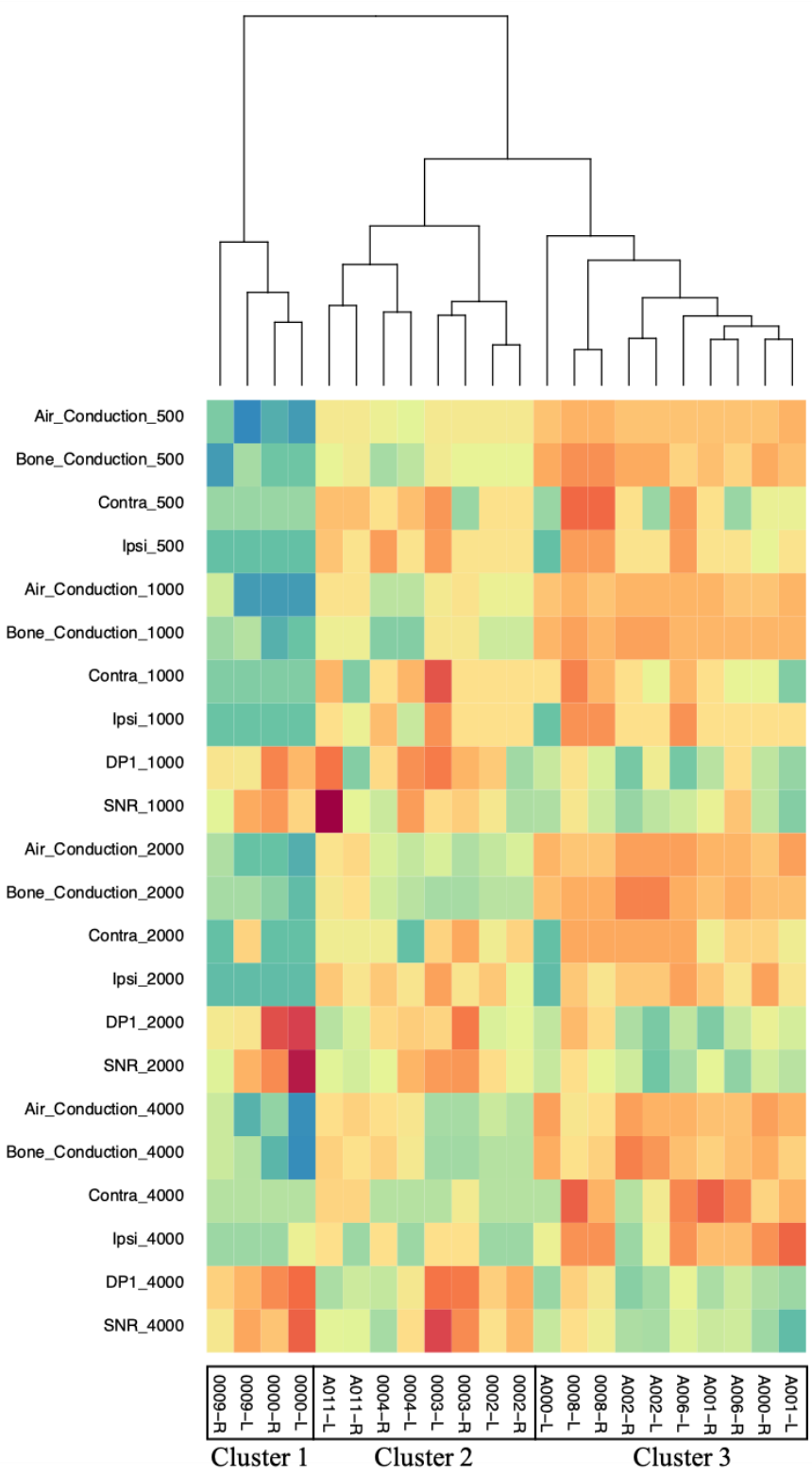


Figure 33. Hierarchical dendrogram and phenotypic heatmap of auditory phenotype features for members of Family 2143.

Three repeated measures ANOVAs were performed to identify the measurements and frequencies significantly different between clusters. The first repeated measures ANOVA comparing differences in air and bone conduction thresholds was carried out using measurement (2 levels), frequency (4 levels) as within-subject factors and cluster group (3 levels) as between-subject factors. Mauchly's Test of Sphericity indicated the assumption of sphericity was violated for the within-subject factor of frequency $\chi^2(5) = 32.867$, $p < .001$ and for the frequency*measurement interaction $\chi^2(5) = 12.016$, $p = 0.035$. Therefore, a Greenhouse-Geisser correction was carried out for this factor. There was a significant effect of measurement [$F(1, 19) = 36.310$, $p < .001$], as well as the interactions of measurement*cluster [$F(2,19) = 8.301$, $p = .003$], measurement*frequency [$F(2.283,43.371 = 9.544$, $p < .001$], and measurement*frequency*cluster [$F(4.565,43.371) = 5.252$, $p = .001$]. *Post-hoc* analysis was conducted using Bonferroni correction to determine the pairwise comparison of each measurement at each frequency for each cluster. Results of the pairwise comparison (Table 17) suggest a significant difference in air and bone conduction thresholds between all three clusters at all four frequencies ($p < .05$). The second repeated measures ANOVA comparing differences in ipsilateral and contralateral acoustic reflexes was carried out using measurement (2 levels), frequency (4 levels) as within-subject factors and cluster group (3 levels) as between-subject factors. Mauchly's Test of Sphericity indicated the assumption of sphericity was not violated for the within-subject factor of frequency ($p = .177$) or measurement*frequency ($p = .443$. There was a significant effect of measurement [$F(1, 19) = 10.565$, $p = .004$], frequency [$F(3, 57) = 3.719$, $p = .016$], as well as the interaction of frequency*cluster [$F(6,57) = 4.495$, $p = .001$]. *Post-hoc* analysis was conducted using Bonferroni correction to determine the pairwise comparison of each frequency for each cluster. Results of the pairwise comparison (Table 17) suggest a

significant difference in acoustic reflex thresholds between Cluster 1 and Cluster 2 at frequencies of 500, 1000 and 2000 Hz ($p < .05$), a significant difference in ARTs between Cluster 1 and Cluster 3 at all frequencies ($p < .05$), and a significant difference in ARTs between Cluster 2 and Cluster 3 at 4000 Hz ($p = .016$). The final repeated measures ANOVA comparing differences in DPOAE amplitude and SNRs was carried out using measurement (2 levels), frequency (3 levels) as within-subject factors and cluster (3 levels) as between-subject factors. Mauchly's Test of Sphericity indicated the assumption of sphericity was violated for the within-subject factor of frequency $\chi^2(2) = 6.722$, $p = .035$ and therefore, a Greenhouse-Geisser correction was carried out for this factor. There was a significant effect of measurement [$F(1, 19) = 323.897$, $p < .001$], as well as the interactions of measurement*frequency [$F(1.942,36.907) = 47.605$, $p < .001$], measurement*frequency*cluster [$F(3.885,36.907 = 3.330$, $p = .021$]. *Post-hoc* analysis was conducted using Bonferroni correction to determine the pairwise comparison of each frequency for each cluster. Results of the pairwise comparison (Table 17) suggest a significant difference in DPOAE amplitude and SNR between Cluster 1 and Cluster 3 at frequencies of 1000 and 2000 Hz ($p < .05$) and a significant difference in DPOAE amplitude and SNR between Cluster 2 and Cluster 3 at 4000 Hz ($p < .05$).

Table 17. Pairwise comparison of auditory phenotype features in Family 2143 following Bonferroni correction for 3 repeated measures ANOVA for six measurements at four frequencies between the three distinct clusters. Significant differences are highlighted in bold.

Pairwise Comparison										
		Cluster 1 - 2			Cluster 1 - 3			Cluster 2 - 3		
Measurement	**Frequency (Hz)**	**Mean Diff**	**Std Error**	**Sig.**	**Mean Diff**	**Std Error**	**Sig.**	**Mean Diff**	**Std Error**	**Sig.**
AC	**500**	**44.375**	**2.695**	**.000**	**62.750**	**2.604**	**.000**	**18.375**	**2.088**	**.000**
	1000	**36.875**	**5.878**	**.000**	**59.250**	**5.679**	**.000**	**22.375**	**4.553**	**.000**
	2000	**24.375**	**4.753**	**.000**	**53.000**	**4.591**	**.000**	**28.625**	**3.681**	**.000**
	4000	**30.625**	**8.488**	**.006**	**58.250**	**8.201**	**.000**	**27.625**	**6.575**	**.001**
BC	**500**	**23.125**	**3.991**	**.000**	**48.000**	**3.855**	**.000**	**24.875**	**3.091**	**.000**
	1000	**17.500**	**5.167**	**.009**	**46.500**	**4.991**	**.000**	**29.000**	**4.002**	**.000**
	2000	**15.000**	**5.162**	**.027**	**50.000**	**4.987**	**.000**	**35.000**	**3.998**	**.000**
	4000	**23.125**	**7.829**	**.024**	**47.250**	**7.564**	**.000**	**24.125**	**6.064**	**.002**
ARTs	**500**	**29.056**	**5.876**	**.000**	**25.00**	**5.676**	**.001**	-4.063	4.551	1.00
	1000	**27.500**	**4.448**	**.000**	**26.500**	**4.297**	**.000**	-1.00	3.446	1.00
	2000	**24.688**	**5.416**	**.001**	**26.00**	**5.232**	**.000**	1.312	4.195	1.00
	4000	8.125	6.604	.701	**24.250**	**6.380**	**.004**	**16.125**	**5.116**	**.016**
DP	**1000**	-1.226	4.289	1.000	-10.362	4.144	.065	**-9.136**	**3.322**	**.038**
	2000	-8.815	4.410	.180	**-14.525**	**4.261**	**.009**	-5.708	3.416	.333
	4000	-12.651	7.399	.311	**-28.107**	**7.148**	**.003**	**-15.456**	**5.731**	**.043**
SNR	**1000**	-1.273	4.319	1.000	-8.868	4.172	.141	-7.595	3.345	.105
	2000	-6.241	3.413	.250	**-13.072**	**3.297**	**.002**	-6.831	2.644	.055
	4000	-5.271	4.978	.909	**-16.389**	**4.810**	**.009**	**-11.118**	**3.856**	**.029**

6.3.2 Phenotypic Segregation

A summary of the phenotype segregation of family members based on criteria in Figure 32 is reported in Table 18. The results of the hierarchical cluster analysis are also shown in the far-right column of Table 18.

Seven family members were identified as ***affected*** based on the clinical definition of audiometric hearing loss; three with mixed hearing loss and four with SNHL. Hierarchical cluster analysis of these 7 family members revealed three distinct sub-phenotypes, Clusters 1, 2 and 3 based on the combined hearing threshold and physiological measures of the auditory phenotype. This subdivision of the 7 ***affected*** family members into Clusters 1 and 2, is based on significantly different patterns of threshold loss and acoustic reflexes (see Table 17) and correlates with the clinical categories of mixed versus sensorineural hearing loss. Five family members had normal pure-tone hearing thresholds with no significant conductive component and were identified by the hierarchical analyses as a single cluster - Cluster 3. By applying the additional criteria shown in Figure 32 to these five family members. three were designated as ***affected*** (A000, A001, and A006) and two ***unaffected*** (0008 and A002). A detailed breakdown of all data for each individual in Family 2143 are reported in the appendices (Appendices F-CC). A summary of the phenotypic breakdown for all family members is reported in Table 18.

Table 18. Phenotypic summary and associated cluster of Family 2143 members including diagnosis of otosclerosis via surgical confirmation (*). Family members subcategorized into their segregated phenotype category of affected, possibly affected and unaffected, based on phenotypic presentation. RE = right ear; LE = left ear; MFSNHL = mid-frequency sensorineural hearing loss; HFSNHL = high-frequency sensorineural hearing loss; LFSNHL = low-frequency sensorineural hearing loss; HF = high-frequencies.

			Standard Phenotyping				**Advanced Phenotyping**				**Cluster**
Individual	**Onset**	**Age**	**Hearing thresholds**		**ARTs**		**DPOAEs**		**PA**		
	(decade)		RE	LE	RE	LE	RE	LE	RE	LE	
Affected Family Members											
1000*	3rd	82	Mixed	Mixed	Absent	Absent	DNT	DNT	DNT	DNT	N/A
0000	3rd	54	Mixed	Mixed	Absent	Absent	Absent	Absent	Abnormal	Abnormal	1
0009*	3rd	55	Mixed	Mixed	Absent	Absent	Absent	Absent	Abnormal	Abnormal	1
0004	6th	57	MFSNHL	MFSNHL	Present	Present	Absent	Absent	Normal	Abnormal	2
0002	6th	58	HFSNHL	HFSNHL	Present	Present	Absent	Absent	Normal	Normal	2
0003	6th	57	HFSNHL	HFSNHL	Present	Present	Absent	Absent	Normal	Normal	2
A011	3rd	25	LFSNHL	LFSNHL	Present	Present	Present	Present	Abnormal	Abnormal	2
Possibly Affected Family Members											
A000		26	Normal	Normal	Present	Absent	Present	Present	Abnormal	Abnormal	3
A001		19	Normal	Normal	Present	Present	Absent in HF	Absent in HF	Normal	Normal	3
A006		32	Normal	Normal	Present	Present	Absent in HF	Absent	Abnormal	Abnormal	3
Unaffected Family Members											
0008		54	Normal	Normal	Present	Present	Present	Present	Normal	Normal	3
A002		20	Normal	Normal	Present	Present	Present	Present	Normal	Normal	3

6.3.2.1 Mixed hearing loss

Three members of Family 2143, IDs 1000, 0000 and 0009 were diagnosed with mixed hearing loss (Figure 34). Hierarchical cluster analyses also grouped these individuals into Cluster 1, although ID 1000 was not included in the cluster analysis. The proband (ID 1000) had stapes surgery reportedly around the age of 50, and no pre-operative hearing tests were available for this study. The proband's offspring, 0000 and 0009, also with bilateral mixed hearing loss, with self-reported onset in their 3rd or 4th decade, and both siblings describe the development of their hearing losses as progressive. Individual 0009 was confirmed to have otosclerosis during corrective stapes surgery of her left ear following the most recent audiogram.

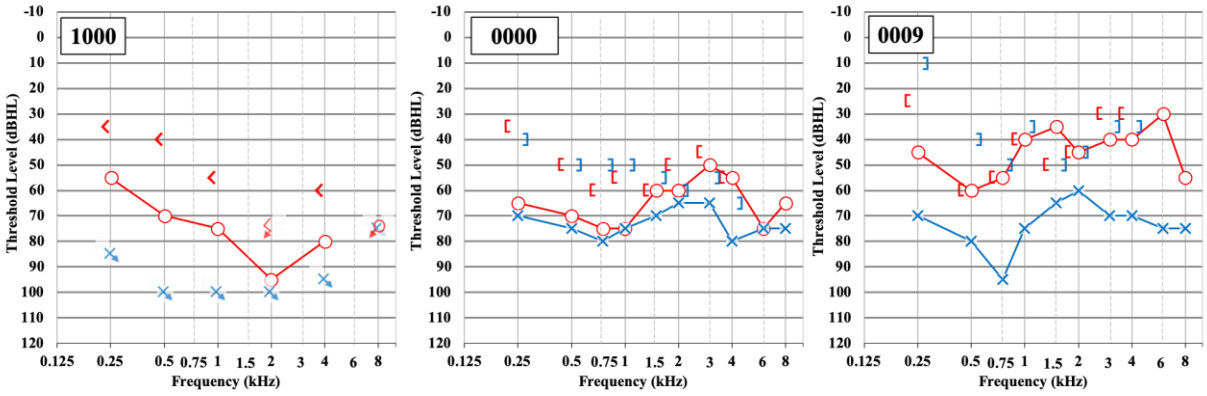


Figure 34. Pre-operative audiograms for individuals in Family 2143 presenting with mixed hearing loss.

6.3.2.2 SNHL

The second audiometric profile, SNHL, is present in four family members, also offspring of the proband ID1000 (Figure 35). Two (IDs 0002, 0003) present with a high-frequency SNHL (HFSNHL), one (ID 0004) with a mid-frequency SNHL (MFSNHL), and one (ID A011) presents with a borderline low-frequency SNHL (LFSNHL). These 4 individuals were identified statistically as Cluster 2.

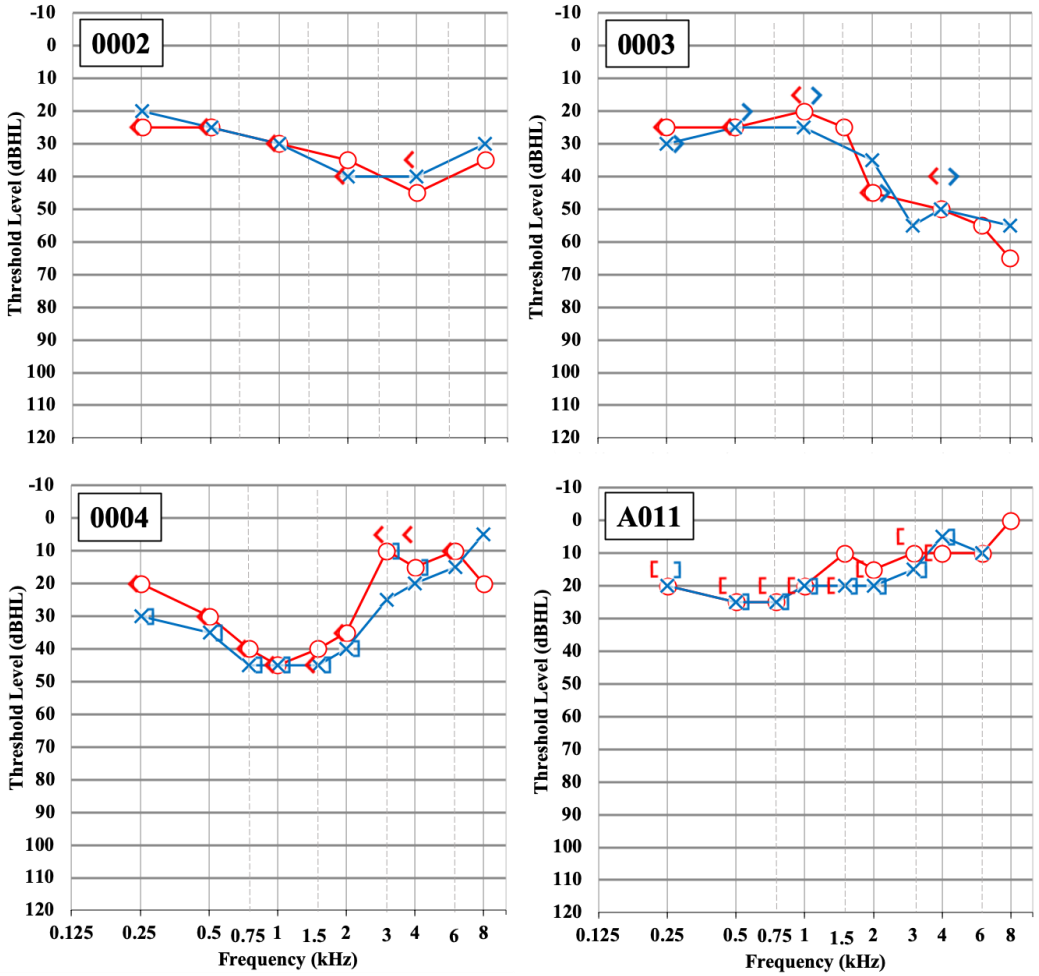


Figure 35. Audiograms for individuals in Family 2143 presenting with SNHL.

6.3.2.3 Normal Hearing

In total, five family members had measurable audiometric thresholds within the normal range (IDs A000, A001, A002, A006 and 0008) and were grouped into Cluster 3. Individuals A000, A001, A002 and A006 were all between the ages of 19-33 at the time of testing. Given the reported later onset of hearing loss in this family, between the 3rd and 6th decade of affected individuals, these four individuals may be at risk of developing the familial hearing loss. Individual 0008 however had measurable thresholds within the normal range at the age of 54.6 years. All of Individual 0008's affected siblings had measurable hearing loss by the 6th decade,

meaning that Individual 0008 is considered unaffected since it is hypothesized that the heritable hearing loss would have developed by this age. Results of the physiologic phenotypic analysis suggest that individual A002 and individual 0008 do not exhibit any sub-clinical features of early onset of hearing loss. Their audiometric thresholds are reported in Figure 36.

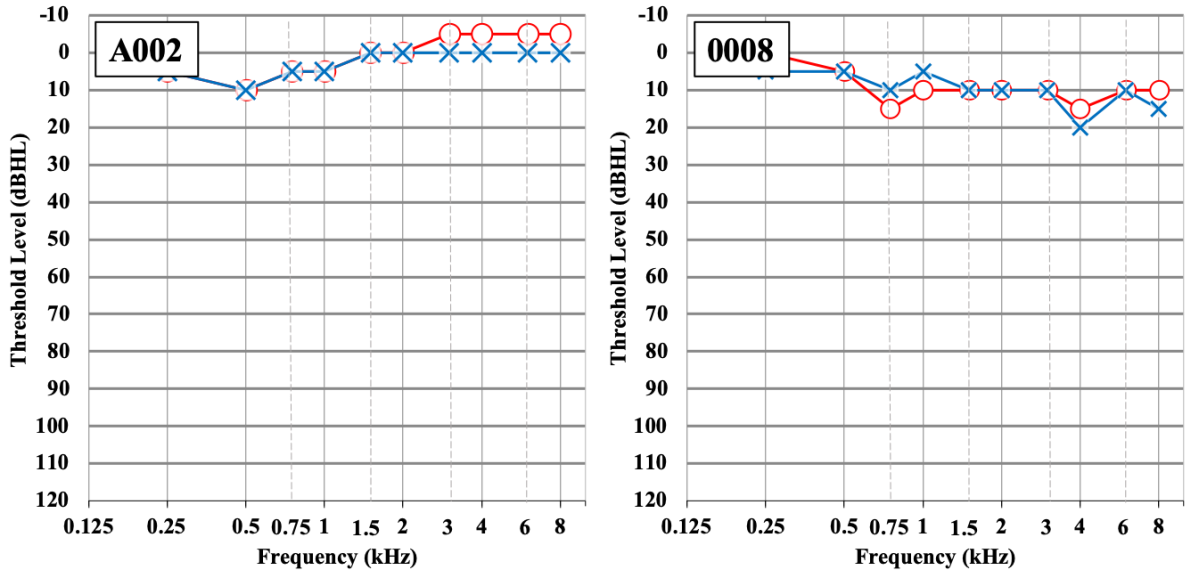


Figure 36. Audiograms for individuals in Family 2143 presenting with normal hearing and who do not exhibit any sub-clinical features of early disease progression.

Physiological phenotypic analysis suggests that Individuals A000, A001 and A006 all present with sub-clinical features of heritable hearing loss. They present with either absent ARTs, absent DPOAEs or abnormal PA (Table 18). Therefore, we considered these individuals to be ***possibly affected***, as these physiological measures may represent early signs of either conductive otosclerosis or cochlear otosclerosis.

Individual A000 presents with normal hearing thresholds, but absent ARTs with the probe in their left ear and demonstrate a PA profile similar to otosclerotic ears where the PA in the low-frequencies is below 1 SD of the normative mean (Figure 37).

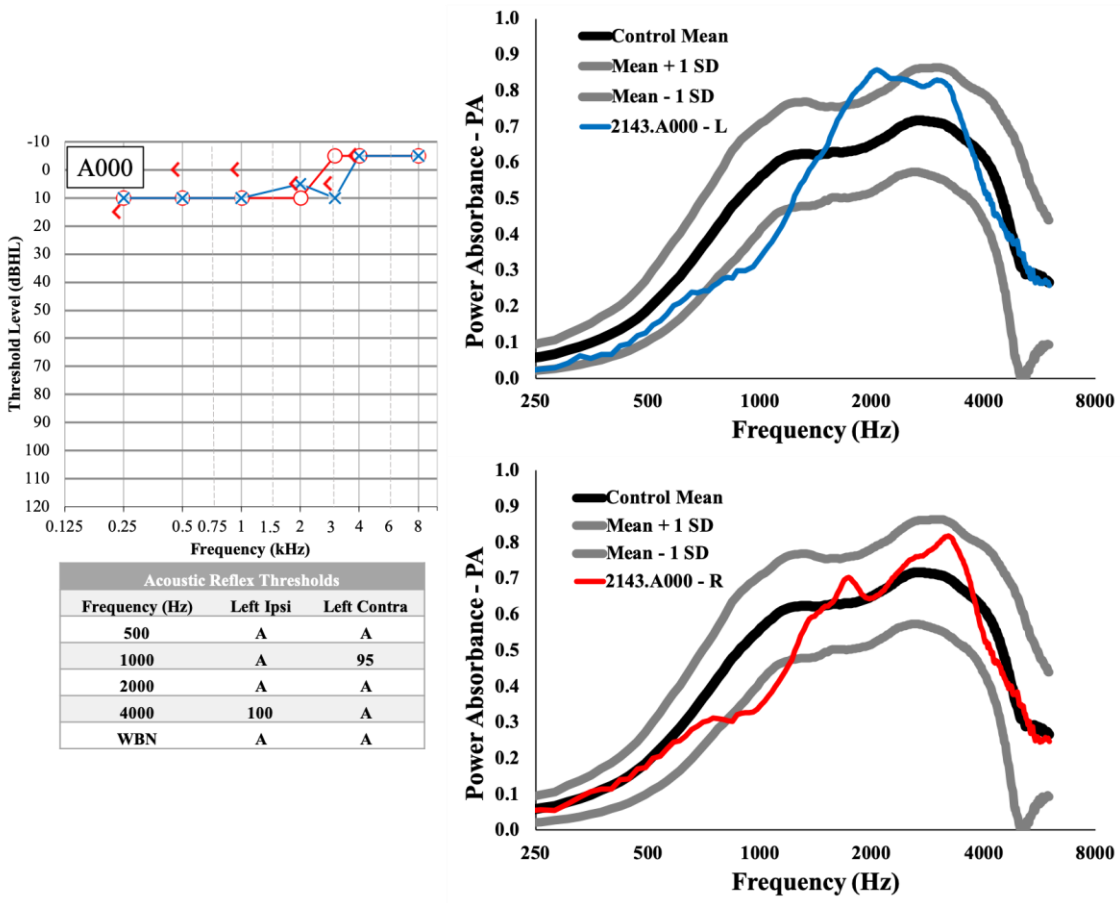


Figure 37. Audiometric thresholds for individual A000 along with sub-clinical features hypothesized of indicating early stages of disease progression. Sub-clinical features include absent acoustic reflex thresholds in left ear, and PA outside of 1 SD from normative mean below 1000 Hz demonstrating a low-absorbance profile in the low frequencies.

Phenotypic analysis of individual A001 suggests normal hearing thresholds, but sub-clinical feature of absent DPOAEs in the higher frequencies in her left ear (Figure 38).

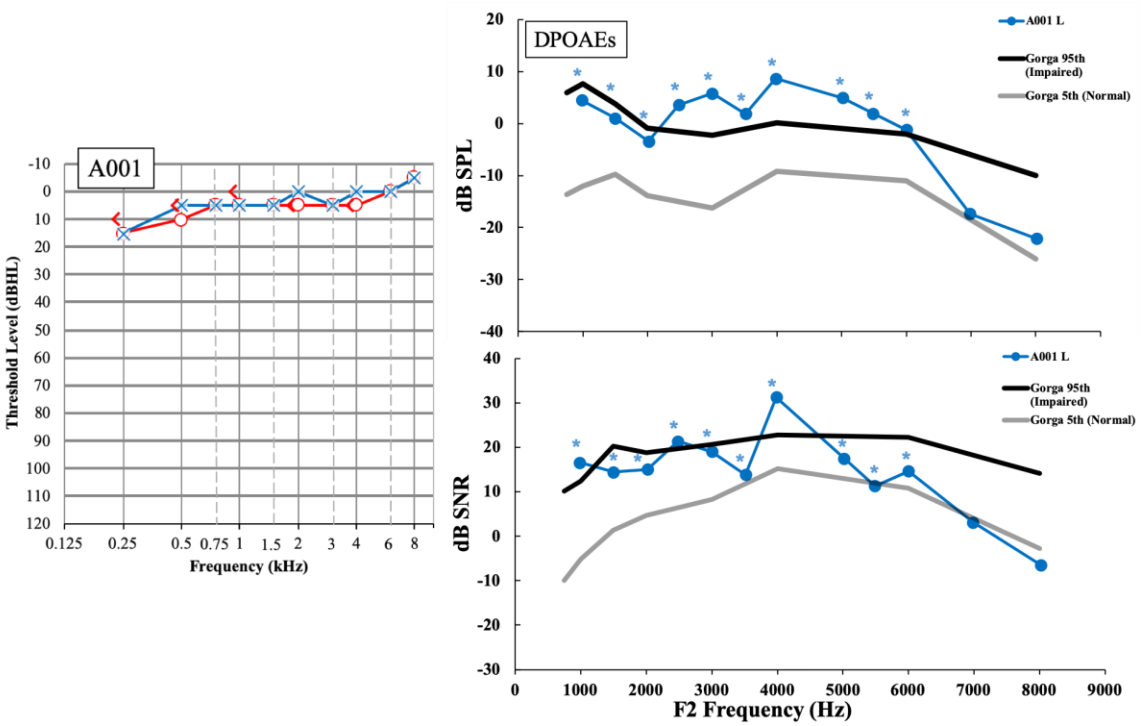


Figure 38. Audiometric thresholds for individual A001 along with sub-clinical features indicating early stages of disease progression. Sub-clinical feature includes absent DPOAEs in the high-frequencies (upper right) and poor DP SNR (lower right).

Finally, phenotypic analysis of individual A006 revealed normal hearing thresholds, yet she presented with low or absent DPOAEs across the frequency range in both ears. The PA profile had a peak at approximately 1000 Hz outside 1 SD of the normative mean (Figure 39). This peak is similar to that reported in the otosclerosis sub-group in Chapter 4.

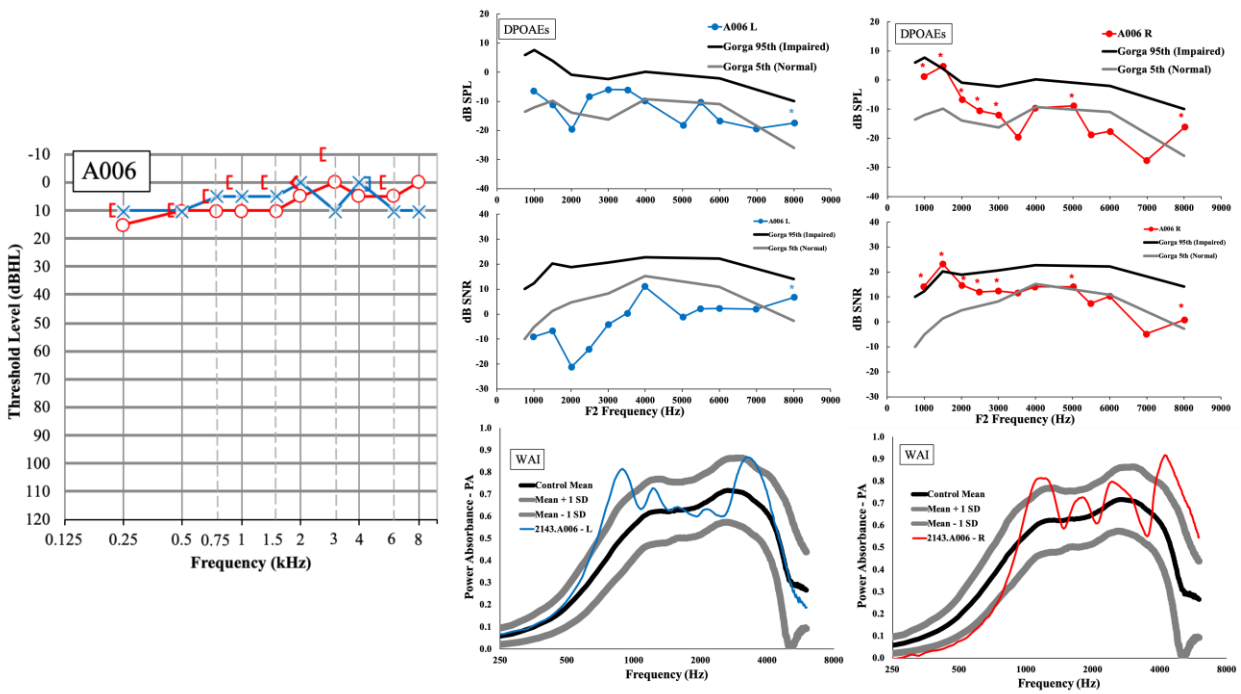


Figure 39. Audiometric thresholds for individual A006 along with sub-clinical features hypothesized to indicate early stages of disease progression. Sub-clinical features include absent DPOAEs in both ears, and PA above 1 SD of the normative mean near 1000 Hz, demonstrating a PA peak profile.

6.3.3 Phenotyping Summary and Pedigree Analysis

Twelve family members for Family 2143 were categorized as either affected, possibly affected or unaffected based on their audiometric and advanced phenotyping results. Seven family members **(IDs 1000, 0000, 0009, 0002, 0003, 0004 and A011)** were all considered **"affected"** as each of them presented with hearing loss. Three family members **(IDs A000, A001 and A006)** were all considered as **"possibly affected"** based on the presence of advance phenotyping measures which may suggest the presence of early otosclerosis development. Finally, members **A002 and 0008** were categorized as **"unaffected"**. These family members do not have hearing loss or any signs of early otosclerotic development. These categorizations were used to help determine the inheritance pattern of otosclerosis in Family 2143.

The revised pedigree presented in Figure 40 (see initial pedigree in Figure 31 for comparison) includes additional auditory phenotyping characteristics. **Pedigree analysis suggests an autosomal dominant transmission and variable types of hearing loss consistent with otosclerosis.** There are two individuals diagnosed with surgically confirmed otosclerosis, the proband, ID 1000 and her daughter ID 0009.

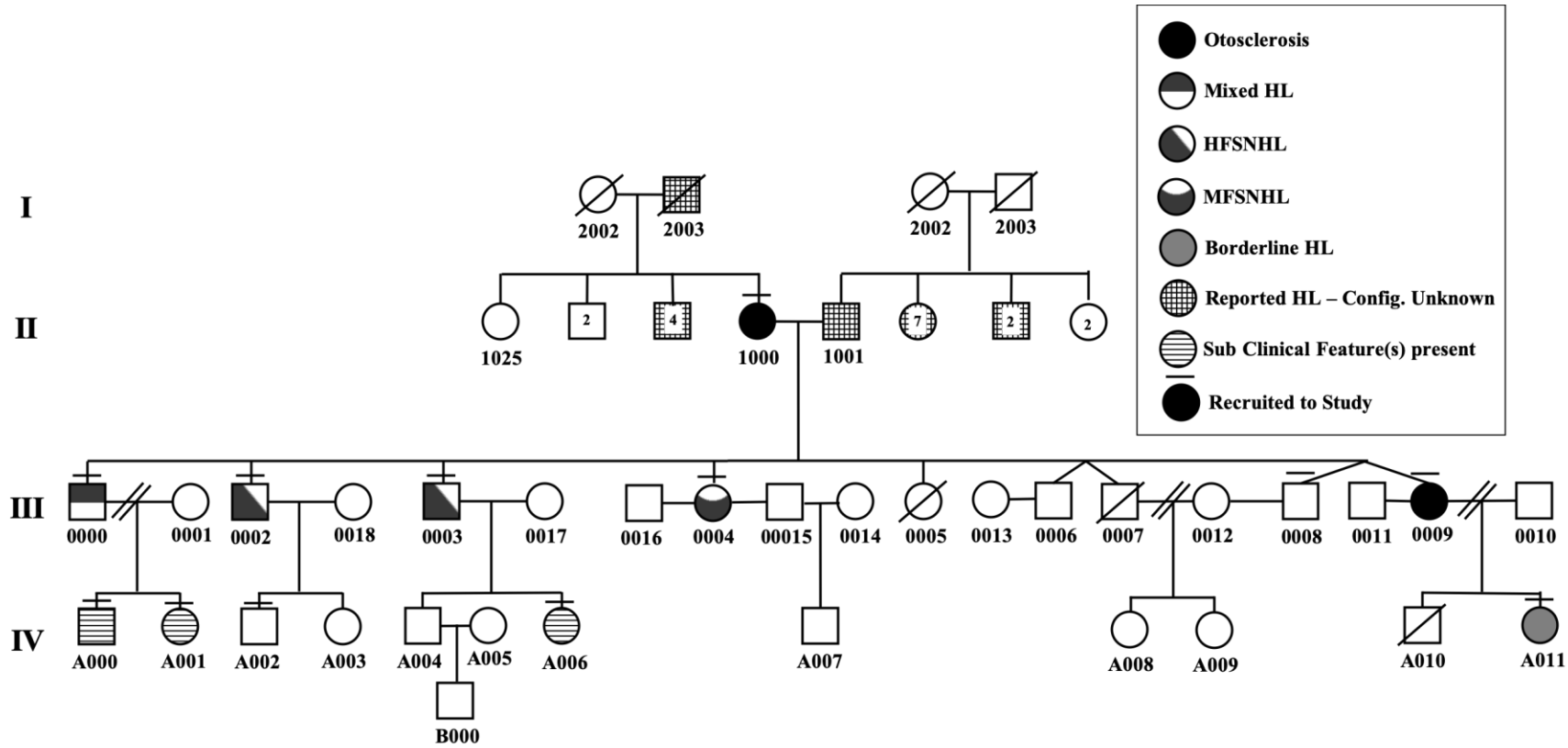


Figure 40. Five generation NL family (Family 2143) segregating an apparent autosomal dominant otosclerosis with variable expression. Pedigree includes phenotypic segregation of sub-clinical features of otosclerosis.

6.4 Discussion

6.4.1 Phenotypic Variability of Family 2143

Phenotypic analysis of Family 2143 revealed three distinct phenotypes, representing mixed hearing loss, sensorineural hearing loss and normal hearing individuals. There were three cases with mixed hearing loss **(ID: 1000, 0000 and 0009)**. All three of these cases were considered ***affected*** based on their audiometric thresholds. The eldest of the family members, individual 1000, has previously undergone stapes surgery around the age of 50 years old. Due to the length of time since the stapes surgeries, there were no retrospective hearing tests available for the study. The proband's two children, IDs 0000 and 0009 also present with bilateral mixed hearing loss. The age of onset for their hearing losses were in their 3rd or 4th decade, whereby both siblings describe the progression of their hearing losses as progressive. At the age of 28, individual 0000 was diagnosed with noise-induced hearing loss represented by a 4 kHz noise-notch in his left ear. The audiometric thresholds obtained at that time also revealed a significant air-bone gap representing a conductive hearing loss in his right ear. Individual 0000 demonstrated a slightly lower progression in bone conduction thresholds, representing the sensorineural component to his hearing loss, whereby his hearing loss progressed between 10 to 16 dB loss per decade. The octave frequency of 500 Hz had the lowest sensorineural progression, with 2000 Hz exhibiting the highest rate of hearing loss progression. This suggests that the sensorineural hearing loss component to his hearing loss progressed at a slower rate in the low frequencies.

Individual 0009 reports obtaining a diagnosis of Meniere's disease from an otolaryngologist several decades prior to the study due to reported vertigo accompanied by

hearing loss. She was recently diagnosed with otosclerosis and underwent successful stapes surgery on her left ear confirming the presence of otosclerosis. Mixed hearing loss has been associated with simultaneous stapes fixation and cochlear otosclerosis (Uppal et al., 2009). The clinical profile of individuals 0000 and 0009 may involve otosclerotic foci attenuating stapes mobility and causing the conductive hearing loss, while also invading the cochlea resulting in cochlear otosclerosis.

In the second audiometric profile, SNHL is present in three family **members (0002, 0003 and 0004)**. These three siblings were also considered ***affected*** based on their audiometric thresholds. These siblings all had their hearing loss identified by an audiologist in their 6th decade. Typically, the SNHL associated with cochlear otosclerosis occurs once the foci invade the cochlear endosteum causing atrophy of the spiral ligament and a reduction of endocochlear potential as well as cochlear hair cell damage. (Cureoglu et al., 2010). The SNHL typically presents as a high-frequency SNHL as is the case with individuals 0002 and 0003, however the presence of a mid-frequency sensorineural hearing loss, like that present in individual 0004, has also been reported in otosclerosis. (de Souza & Glasscock, III, 2004).

There were five family members who had audiometric thresholds within the normal limits. Given the later onset of otosclerosis, these normal hearing individuals are a challenge to categorize, since four of them are relatively young (oldest is 32 years old).

Individual **0008** is considered ***unaffected*** since he is 54 years of age, has normal hearing thresholds, and does not exhibit any other signs of otosclerosis. Individual **A002** is also classified as ***unaffected*.** He was 20 years old at the time of assessment and had no other phenotype features with the exception of absent DPOAEs at 4000 Hz. He also has absent ARTs

at 4000 Hz but ARTs at this stimulus frequency are often absent in normal hearing subjects (Jerger, Jerger, & Mauldin, 1972). Longitudinal measures are needed to confirm the designation of ***unaffected*** for this family member.

The remaining family members **(A000, A001 and A006)** with normal hearing thresholds were categorized as ***possibly affected*** due to the presence of sub-clinical signs of potential otosclerosis. Individual A000 may be demonstrating sub-clinical features of otosclerosis in the left ear, with absent ARTs and DPOAEs. Furthermore, PA in both ears of A000 is below 1 standard deviation from the mean in the low frequencies and follows a similar PA peak as described otosclerotic subgroup presented in Chapter 4.

Individual A001 is also characterized as ***possibly affected*** due to the absence of DPOAEs in the higher frequencies. The absence of DPOAEs in these frequencies may indicate cochlear damage and/or possible changes to the middle ear mechanics (Gorga et al., 1997; Lonsbury-Martin & Martin, 1990; Zhao, Wada, Koike, & Stephens, 2000). Individual A006 is also considered ***possibly affected*** due to DPOAEs in the high frequencies in the right ear and also absent across all frequencies in the left ear. PA from Individual A006's left ear demonstrates a similar profile to the PA peak around 1000 Hz which is present in several otosclerotic ears from Chapter 4, and consistent with left PA profile of individual 0009, a surgically confirmed case of otosclerosis in this family. In a gene hunt scenario, possibly affected individuals are categorized as "unknown' and left out of the initial genetic analysis.

Based on these results, evidence from previous chapters, and other reports in the literature (Chapter 3, Chapter 4, Brownstein et al., 2006; Declau et al., 2007; Pauw et al., 2006; Weegerink et al., 2011), a broad range of phenotypes, including those with normal hearing

thresholds, must be considered as potentially affected and be studied using advanced phenotyping measures. Since otosclerosis can present as conductive hearing loss, SNHL, mixed hearing loss, or normal hearing (non-penetrant cases), advanced phenotyping for the purpose of gene discovery will benefit the phenotyping segregation process if it increases the number of identified affected individuals in a family and it if confirms those who are definitely unaffected. If it can do both, the power of gene discovery will improve, and could mean the difference between finding the gene or not. This point is illustrated by comparing the pedigrees in Figures 31 and 40. The pedigree reported in Figure 31 uses standard pure tone audiometry and surgical confirmation to classify family members as ***affected***. This method of phenotypic segregation is consistent with previous literature reporting on other large families (Brownstein et al., 2006; Chen et al., 2002; Thys, Van Den Bogaert, et al., 2007; Tomek et al., 1998; Van Den Bogaert et al., 2001). By incorporating physiological phenotyping at multiple frequencies using a combination of AR, DPOAEs and PA and combining this data with pure tone audiometry, the phenotypic segregation added 3 members of Family 2143 in generation IV into the ***possibly affected*** phenotype category, enhancing the revised pedigree in Figure 40. Although these three individuals demonstrate normal hearing thresholds, each one exhibits sub-clinical features based on the physiological findings. Additional longitudinal measures and high-resolution CT could be used to confirm whether these subtle physiological phenotype measures are consistent with the early development of otosclerosis.

6.4.2 Value of Advanced Phenotyping Measurements

6.4.2.1 DPOAEs

Sound-evoked DPOAEs depend on the transmission of two tones through the outer and middle ear which evoke cochlear outer hair cell activity, which is then transmitted backward

via the same route and recorded as an acoustic DPOAE response in the outer ear canal. DPOAEs provide frequency-specific physiological phenotyping markers that may be valuable in genetic research studies. Depending on frequency, DPOAEs are usually present in individuals with normal hearing thresholds (below 20 dB HL), sometimes present when audiometric thresholds are at a mild level (25-40 dB HL), and generally absent when hearing thresholds are above 40 dB HL (Gorga et al., 1997). If the middle ear function is disrupted, as is the case with stapes fixation due to otosclerosis, or if cochlear damage occurs due to cochlear otosclerosis, DPOAES may be reduced or absent (Chapter 3).

These absent DPOAE findings for 2143 family members with hearing loss are similar to those presented for the otosclerotic cohort in Chapter 3. However, in Family 2143 there are 2 cases (ID A001 and A006) with normal hearing thresholds who present with absent DPOAEs. In conclusion, DPOAEs may be sensitive, non-specific physiological markers of middle ear and cochlear dysfunction in ears affected by otosclerosis and are under-utilized in genetic studies of this disease.

6.4.2.2 Acoustic Reflex Thresholds

Acoustic reflexes were absent in all members of Family 2143 presenting with mixed hearing loss but present in family members with SNHL and those with normal hearing thresholds. This is consistent with Gelfand (1994) who report that a significant SNHL greater than 60 dB HL is required to exhibit absent acoustic reflex. Acoustic reflex thresholds provide another sensitive physiological phenotype measurement in families with suspected otosclerosis because acoustic reflexes are typically absent despite otherwise normal audiological findings (Hannley, 1993; House & Cunningham, 2010; Keefe et al., 2017).

ARTs in conjunction with audiometry have been used as phenotyping tools to aid in the identification of three of the ten genetic loci responsible for monogenic otosclerosis, OTSC5, OTSC 8 and OTSC10 (Bel Hadj Ali et al., 2008; Van Den Bogaert, 2004; Weegerink et al., 2011). These three studies have used acoustic reflexes in non-surgical family members to determine the presence or absence of clinical otosclerosis. However, identifying a family member as ***affected*** required the individual to have both a hearing loss (conductive or mixed) as well as absent reflexes, and a clinical diagnosis based on these findings. Future longitudinal studies would be beneficial to confirm whether the family member with normal hearing thresholds and absent reflexes (ID A000) is in the early stages of otosclerosis development.

6.4.2.3 Wideband Immittance

Family 2143 demonstrates how wideband absorbance can be used in the advanced phenotyping of families with inherited forms of otosclerosis. Three cases with normal hearing and no conductive component exhibit an abnormal PA profile consistent with an increase in middle ear stiffness, where the absorbance was lower in the low-frequencies compared to normative values (IDs 0000, A000 and A001). This profile was reported in the pre-surgical otosclerotic ears with conductive hearing losses in Chapter 4 as well as in studies focused on PA changes also in ears with conductive hearing loss due to otosclerosis (Feeney et al., 2003; Merchant et al., 2016; Shahnaz, Longridge, et al., 2009).

The presence of a low frequency PA peak has also been shown in the otosclerotic population in Chapter 4 as well as in other otosclerotic populations (Nakajima et al., 2012; Niemczyk et al., 2018; Shahnaz, Bork, et al., 2009). In family member 0009 with bilateral conductive hearing loss, the left ear pre-operative results provide further evidence that otosclerosis can cause a low frequency PA peak. While the conductive component was not as

large, a similar PA peak was also present in her right ear. This PA profile was present in three other affected individuals (0000, 0004 and A011) with hearing impairment. Finally, the presence of low frequency PA peak around 1000 Hz was also identified in one normal hearing ***possibly affected*** individual (A006) without overt signs of otosclerosis.

Although promising, the use of PA in isolation may be misleading given the limited research in pathological middle ears and no specific guidelines for differentiating normal versus abnormal PA profiles in individual subjects. In this chapter, while most ears of 2143 family members had PA values outside 1 standard deviation of the normative mean, only three ears demonstrated PA values that were 2 standard deviations outside of the normative mean.

6.4.3 Value of Hierarchical Cluster Analysis

Hierarchical cluster analysis was a valuable method for analyzing the phenotype features of Family 2143 and confirmed the non-statistical segregation of members into ***affected*** versus ***unaffected***. There were three distinct clusters which correlated with the audiometric thresholds (mixed HL, SNHL and normal hearing thresholds) as well as the presence/absence of ARTs and the presence/absence of DPOAEs either across the frequency range (mixed HL), or in the high-frequencies (SNHL).

For Cluster 3, the 10 ears presenting with normal audiometric thresholds, other features may also serve as markers of early onset of the heritable otosclerosis. A subgroup within the normal hearing Cluster 3, including both ears of individuals A006 and A001, and the right ear of individual A000, formed a sub-cluster; these are the same ears which were identified as ***possibly affected*** ears using non-statistical analyses of the of the advanced phenotyping features. A000's left ear is the most distant from all the other ears within the Cluster 3

apparently due to absent acoustic reflexes in both the ipsilateral and contralateral stimulus conditions and may represent an early case of otosclerosis despite the normal audiogram. Longitudinal audiometric thresholds and continued phenotyping are needed to confirm otosclerotic progression in these Cluster 3 cases.

6.4.4 Phenotype Segregation using Advanced Phenotyping

The family members were separated into three categories of ***affected*** (n=7), ***possibly affected*** (n=3) or ***unaffected*** (n=2). For the purpose of gene discovery, this breakdown of categories based on phenotype will be used as a way to compare genetic commonalities among affected individuals and contrast them to commonalities with the unaffected family members.

6.4.5 Conclusion and Future Direction

Guided by previous work on the variability of otosclerosis reported in the otosclerotic population (Chapter 3 and 4) as well as within families (Chapter 5), an advanced phenotyping approach using frequency-specific physiological measures not typically used in genetic studies of otosclerosis, was used for segregation of a large multiplex family and will be used in future gene discovery studies.

The identification of novel genes causing otosclerosis has been challenging. Although 8 OTSC loci have been identified in large families, these studies have failed to identify any causative genes with the exception of *FOXL1*. One barrier is the availability of large families with enough affected family members to increase the power of the genetic analysis. It is challenging to identify new genes when conductive or mixed hearing loss or stapes surgery are the only method classifying “affected” versus “unaffected” individuals, as is the current convention. (Bel Hadj Ali et al., 2008; Brownstein et al., 2006; Chen et al., 2002; Thys, Van

Den Bogaert, et al., 2007; Tomek et al., 1998; Van Den Bogaert et al., 2001; Weegerink et al., 2011). By relying solely on audiological thresholds and surgical confirmation of the sclerotic bone growth, identification of otosclerosis is limited to individuals with advanced otosclerosis. Comprehensive phenotyping methods outlined in this chapter, to provide alternative hypothesis of phenotype segregation analysis, may facilitate the next gene discovery phase in Family 2143. If successful, similar methods may be applied in yet unidentified families (Bel Hadj Ali et al., 2008; Brownstein, Goldfarb, Levi, Frydman, & Avraham, 2006; Chen et al., 2002; Pauw et al., 2006; Schrauwen et al., 2011; Thys, Van Den Bogaert, et al., 2007; Tomek et al., 1998; Van Den Bogaert et al., 2001). In summary, this chapter provides a statistical approach to phenotypic segregation analysis, and the incorporation of physiological features beyond the clinical audiogram, to characterize a multiplex family with heritable otosclerosis. Hierarchical cluster analysis has not been used in hearing loss gene discovery research and could be tested in future genetic studies of auditory system dysfunction.

6.5 References

Bel Hadj Ali, I., Thys, M., Beltaief, N., Schrauwen, I., Hilgert, N., Vanderstraeten, K., … Van Camp, G. (2008). A new locus for otosclerosis, OTSC8, maps to the pericentromeric region of chromosome 9. Human Genetics, 123(3), 267–272. https://doi.org/10.1007/s00439-008-0470-3

Brownstein, Z., Goldfarb, A., Levi, H., Frydman, M., & Avraham, K. B. (2006). Chromosomal mapping and phenotypic characterization of hereditary otosclerosis linked to the OTSC4 locus. Archives of Otolaryngology--Head & Neck Surgery, 132(4), 416–424. https://doi.org/10.1001/archotol.132.4.416

Chen, W., Campbell, C. A., Green, G. E., Van Den Bogaert, K., Komodikis, C., Manolidis, L. S., … Smith, R. J. H. (2002). Linkage of otosclerosis to a third locus (OTSC3) on human chromosome 6p21.3-22.3. Journal of Medical Genetics, 39(7), 473–477. https://doi.org/10.1136/jmg.39.7.473

Cureoglu, S., Yildirim, M. B., & Paparella, M. M. (2010). Cochlear Otosclerosis. Curr Opin Otolaryngol Head Neck Surg, 18(5), 357–362. https://doi.org/10.1097/MOO.0b013e32833d11d9.Cochlear

de Souza, C., & Glasscock, III, M. E. (Eds.). (2004). Chapter 6 Cochlear Otosclerosis. In Otosclerosis and Stapedectomy Diagnosis, Management, and Complications (2004th ed.). Stuttgart: Georg Thieme Verlag. https://doi.org/10.1055/b-0034-43238

Declau, F., Van Den Bogaert, K., Van De Heyning, P., Offeciers, E., Govaerts, P., & Van Camp, G. (2007). Phenotype-genotype correlations in otosclerosis: Clinical features of OTSC2. Advances in Oto-Rhino-Laryngology, 65, 114–118. https://doi.org/10.1159/000098745

Feeney, M. P., Grant, I. L., & Marryott, L. P. (2003). Wideband energy reflectance measurements in adults with middle-ear disorders. Journal of Speech, Language, and Hearing Research : JSLHR, 46(4), 901–911. https://doi.org/10.1044/1092-4388(2003/070)

Feeney, M. P., Stover, B., Keefe, D. H., Garinis, A. C., Day, J. E., & Seixas, N. (2014). Sources of Variability in Wideband Energy Reflectance Measurements in Adults. Journal of the American Academy of Audiology, 25(5), 449–461. https://doi.org/10.3766/jaaa.25.5.4

Gelfand, S. a. (1994). Acoustic reflex threshold tenth percentiles and functional hearing impairment. Journal of the American Academy of Audiology, 5(1), 10–16. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8155889

Gorga, M. P., Neely, S. T., Ohlrich, B., Hoover, B., Redner, J., & Peters, J. (1997). From laboratory to clinic: a large scale study of distortion product otoacoustic emissions in ears with normal hearing and ears with hearing loss. Ear and Hearing, 18(6), 440–455.

Hannley, M. T. (1993). Audiologic characteristics of the patient with otosclerosis. Otolaryngologic Clinics of North America, 26(3), 373–387.

House, J. W., & Cunningham, C. D. (2010). Otosclerosis. In Cummings Otolaryngology - Head and Neck Surgery (pp. 2028–2035). https://doi.org/10.1016/B978-0-323-05283-2.00145-2

Jerger, J., Jerger, S., & Mauldin, L. (1972). Studies in impedance audiometry. I. Normal and sensorineural ears. Archives of Otolaryngology (Chicago, Ill. : 1960), 96(6), 513–523.

Keefe, D., L Archer, K., Schmid, K., Fitzpatrick, D., Feeney, P., & Hunter, L. (2017). Identifying Otosclerosis with Aural Acoustical Tests of Absorbance, Group Delay, Acoustic Reflex Threshold, and Otoacoustic Emissions. Journal of the American Academy of Audiology, 28. https://doi.org/10.3766/jaaa.16172

Lonsbury-Martin, B., & Martin, G. (1990). The clinical utility of distortion-product otoacoustic emissions. Ear and Hearing, 11(2), 144–154. Retrieved from http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:The+Clinical+Utility+of+Distortion-Product+Otoacoustic+Emissions#0

Merchant, G. R., Merchant, S. N., Rosowski, J. J., & Nakajima, H. H. (2016). Controlled exploration of the effects of conductive hearing loss on wideband acoustic immittance in human cadaveric preparations. Hearing Research, 341, 19–30. https://doi.org/10.1016/j.heares.2016.07.018

Nakajima, H. H., Pisano, D. V, Roosli, C., Hamade, M. a, Merchant, G. R., Mahfoud, L., … Merchant, S. N. (2012). Comparison of ear-canal reflectance and umbo velocity in patients with conductive hearing loss: a preliminary study. Ear and Hearing, 33(1), 35–43. https://doi.org/10.1097/AUD.0b013e31822ccba0

Niemczyk, E., Lachowska, M., Tataj, E., Kurczak, K., & Niemczyk, K. (2018). Wideband tympanometry and absorbance measurements in otosclerotic ears. The Laryngoscope, ePub ahead. https://doi.org/10.1002/lary.27747

Pauw, R. J., De Leenheer, E. M. R., Van Den Bogaert, K., Huygen, P. L. M., Van Camp, G., Joosten, F. B. M., & Cremers, C. W. R. J. (2006). The phenotype of the first otosclerosis family linked to OTSC5. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology

and Neurotology, 27(3), 308–315. Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/lary.21463/full
Schrauwen, I., Weegerink, N., Fransen, E., Claes, C., Pennings, R., Cremers, C., … Van Camp, G. (2011). A new locus for otosclerosis, OTSC10, maps to chromosome 1q41-44. Clinical Genetics, 79(5), 495–497. https://doi.org/10.1111/j.1399-0004.2010.01576.x
Shahnaz, N., Bork, K., Polka, L., Longridge, N., Bell, D., & Westerberg, B. D. (2009). Energy reflectance and tympanometry in normal and otosclerotic ears. Ear and Hearing, 30(2), 219–233. https://doi.org/10.1097/AUD.0b013e3181976a14
Shahnaz, N., Longridge, N., & Bell, D. (2009). Wideband energy reflectance patterns in preoperative and post-operative otosclerotic ears. International Journal of Audiology, 48(5), 240–247. https://doi.org/10.1080/14992020802635317
Szepannek, G. (2019). clustMixType: User-Friendly Clustering of Mixed-Type Data in R. The R Journal, 10(2), 200. https://doi.org/10.32614/RJ-2018-048
Thys, M., Van Den Bogaert, K., Iliadou, V., Vanderstraeten, K., Dieltjens, N., Schrauwen, I., … Van Camp, G. (2007). A seventh locus for otosclerosis, OTSC7, maps to chromosome 6q13–16.1. European Journal of Human Genetics, 15(3), 362–368. https://doi.org/10.1038/sj.ejhg.5201761
Tomek, M. S., Brown, M. R., Mani, S. R., Ramesh, A., Srisailapathy, C. R., Coucke, P., … Smith, R. J. (1998). Localization of a gene for otosclerosis to chromosome 15q25-q26. Human Molecular Genetics, 7(2), 285–290. https://doi.org/10.1093/hmg/7.2.285
Uchida, Y., Ando, F., Shimokata, H., Sugiura, S., Ueda, H., & Nakashima, T. (2008). The Effects of Aging on Distortion-Product Otoacoustic Emissions in Adults with Normal Hearing. Ear and Hearing, 29(2), 176–184. https://doi.org/10.1097/AUD.0b013e3181634eb8
Uppal, S., Bajaj, Y., Rustom, I., & Coatesworth, A. P. (2009). Otosclerosis 1: The aetiopathogenesis of otosclerosis. International Journal of Clinical Practice, 63(10), 1526–1530. https://doi.org/10.1111/j.1742-1241.2009.02045.x
Van Den Bogaert, K. (2004). A fifth locus for otosclerosis, OTSC5, maps to chromosome 3q22-24. Journal of Medical Genetics, 41(6), 450–453. https://doi.org/10.1136/jmg.2004.018671
Van Den Bogaert, Kris, Govaerts, P. J., Schatteman, I., Brown, M. R., Caethoven, G., Offeciers, F. E., … Van Camp, G. (2001). A second gene for otosclerosis, OTSC2, maps to chromosome 7q34-36. American Journal of Human Genetics, 68(2), 495–500. https://doi.org/10.1086/318185
Weegerink, N. J. D., Schrauwen, I., Huygen, P. L. M., Pennings, R. J. E., Cremers, C. W. R. J., Van Camp, G., & Kunst, H. P. M. (2011). Phenotype of the first otosclerosis family linked to OTSC10. The Laryngoscope, 121(4), 838–845. https://doi.org/10.1002/lary.21463
Zhao, F., Wada, H., Koike, T., & Stephens, D. (2000). The influence of middle ear disorders on otoacoustic emissions. Clinical Otolaryngology, 25(1), 3–8. https://doi.org/10.1046/j.1365-2273.2000.00312.x

Chapter 7

7 Conclusion

7.1 Overall Contributions

For many decades genetic linkage analysis has been an extremely successful method for mapping otosclerosis loci. However, only recently has the first causative gene (*FOXL1*) for autosomal dominant otosclerosis been identified (Abdelfatah, 2014).A barrier to gene identification for heritable otosclerosis has been the lack of sensitive phenotyping and understanding of the disease progression, which in turn limits the accurate segregation of family members available for linkage analyses. This thesis focused on advanced phenotyping with the goal to improve our understanding of otosclerosis in families of known and unknown genetic etiology, and a clinical population with this disorder, and to determine whether the Newfoundland founder mutation in *FOXL1* is present in this unrelated clinical cohort.

Overall contributions of this thesis included:

1. The identification of the *FOXL1* deletion outside of the Newfoundland family where it was identified.

2. The confirmation of a strong family history of otosclerosis within the Ontario population.

3. An advanced phenotyping analysis of an Ontario population confirming the absence of acoustic reflexes and DPOAEs in ears with a conductive hearing loss and post-surgical ears.

4. The identification of instrument and stimulus level effects on power absorbance and it's test-retest reliability

5. The initial predictive model of a progressive mixed hearing loss due to the *FOXL1* deletion.

6. The application of advanced phenotyping for the purpose of future gene segregation analysis and the identification of potential early onset otosclerotic cases within a large family.

7.2 Conclusion, Limitations and Future Directions

Genetic screening of *FOXL1* in the Ontario otosclerotic population confirmed that this *FOXL1* deletion (976¬990het_del) was present in an unrelated individual from Ontario, Canada. This suggests that the gene and specific mutation discovered by Abdelfatah (2014) is not isolated solely to the large Newfoundland family. This study was limited in that only the deletion in *FOXL1* was genotyped in the Ontario population. Future research should proceed with screening other otosclerotic populations for this deletion and other potentially pathogenic variants in *FOXL1.* Additional work should also include the advanced genotyping of otosclerosis in the Ontario population, since 22 unique families with later-onset hearing loss, and 9 unique families with otosclerosis were idenitifed in the study sample recruited for this thesis Gentoype-phenotype evaluation of these Ontario families may unveil new causative genes and mutations for otosclerosis.

Prior to the discovery of the *FOXL1* gene, there were up to 10 genetic loci (8 published) associated with the monogenic form of otosclerosis (Bel Hadj Ali et al., 2008; Brownstein, Goldfarb, Levi, Frydman, & Avraham, 2006; Chen et al., 2002; Schrauwen et al., 2011; Thys et

al., 2007; Tomek et al., 1998; Van Den Bogaert et al., 2004; Van Den Bogaert et al., 2001). So far, none of these genetic loci have yielded the identity of the underlying gene causing otosclerosis (Bittermann et al., 2014).

Genetic studies of autosomal dominant otosclerosis rely on careful phenotyping in order to determine who in the family is affected and who is unaffected by this slowly progressive, late-onset disease. There is no gold standard diagnostic test for otosclerosis, and so determination of affection status has relied heavily on surgical confirmation in patients with already significant hearing loss (Bel Hadj Ali et al., 2008; Brownstein et al., 2006; Chen et al., 2002; Thys, Van Den Bogaert, et al., 2007; Tomek et al., 1998; Van Den Bogaert et al., 2001; Weegerink et al., 2011), or impaired audiometric thresholds in conjunction with abnormal acoustic reflexes (Bel Hadj Ali et al., 2008; Van Den Bogaert, 2004; Weegerink et al., 2011), or in one case high-resolution imaging of the temporal bone (Bel Hadj Ali et al., 2008). All of these genetic studies required substantial hearing loss and therefore relatively advanced disease for their phenotyping methods. Furthermore, not all affected family members are candidates for stapes surgery even if they have hearing loss and may be excluded from genetic analyses. Besides the study by Bel Hadj Ali et al. (2008) who use high-resolution CT scanning as a phenotypic tool for histological otosclerosis, none of these have taken advantage of advanced physiological phenotyping, outside of acoustic reflexes, to explore younger generation family members with normal hearing or mild impairments, in order to segregate affected from unaffected family members during the early course of the disease.

WAI has shown promise for the clinical diagnosis of conductive hearing loss etiology (Feeney et al., 2003; Nakajima et al., 2012; Prieve et al., 2013), and therefore hypothesized to be a potentially useful phenotyping tool for genetic studies of otosclerosis. In this thesis WAI,

particularly PA, was evaluated for its value as a phenotypic tool in otosclerosis and was measured using two systems, the Mimosa HearID and the Interacoustics Titan. Given the lack of standards for this new acoustic immittance technique, a comparison of these systems was completed and revealed that although both exhibit good test-retest reliability, the recording instrument and testing protocol can affect PA outcomes. Therefore, instrument and stimulus-specific normative data should be used when including PA as a phenotyping tool.

In this thesis, phenotyping outcomes using WAI agree with previous literature showing that otosclerotic ears may have lower PA in the low-frequencies than normal ears (Feeney et al., 2003; Nakajima et al., 2012; Niemczyk et al., 2018; Prieve et al., 2013; Sanford et al., 2012; Shahnaz, Bork, et al., 2009). However, not all otosclerotic ears exhibit this low frequency, low absorbance profile. A sub-group of otosclerotic ears in both the small clinical cohort and in 2 multiplex families have a different profile, with a PA peak near 1 kHz. These preliminary results are in agreement with other literature (Nakajima et al., 2012; Niemczyk et al., 2018; Shahnaz, Bork, et al., 2009) and require further investigation to determine whether they correlate with early stage otosclerosis, or histological variations of otosclerosis that could be investigated through high resolution CT imaging studies (Naumann, Porcellini, & Fisch, 2005; Quesnel et al., 2013; Redfors et al., 2012). In future, longitudinal phenotyping including PA, and the use of temporal bone imaging may be used to detect the presence of histological otosclerosis in the two non-penetrant *FOXL1* carriers in this family. Longitudinal phenotyping studies using PA may also provide evidence for progressive changes in PA correlating to otosclerotic progression in early cases of the disease.

Since otosclerosis is a bone disorder characterized by abnormal bone growth, candidate genes will be considered if they are involved in the bone remodeling process. Recently, the first

gene causing otosclerosis was discovered in *FOXL1* (Abdelfatah, 2014). *FOXL1* is a transcription factor gene, which is responsible for regulating other genes, and potentially down-regulates several genes including *ILIA, CXCL10, IL29, IFNB1, IFIT1, FEN1* and *SP4.* Therefore, these down-regulated genes as well as other transcription factors may potentially be involved in the development of otosclerosis and should be considered candidate genes for future gene discovery research.

The goals of advanced phenotyping are three-fold: (1) to better understand the clinical presentation of a specific disorder, (2) to better understand the clinical presentation of a specific gene mutation, and (3) to support gene discovery through improved phenotypic segregation. Future genetic studies into the genetic causes of otosclerosis should consider multiple phenotype measures when attempting to determine genetic carriers of the affected alleles. Cluster analysis was used for the first time in this thesis for studying genetic hearing loss, to assist with segregation analyses. Hierarchical cluster analysis was a useful statistical technique for analyzing multiple phenotyping measures in the preliminary investigation of auditory phenotypic segregation in large families with heritable hearing loss. Studying the physiological phenotype will have a positive impact on the understanding of the natural course of otosclerosis across the lifespan and variation in the presentation of this disease. A broader understanding of the otosclerosis disease process will support early identification and the development of new treatments.

7.3 References

Abdelfatah, N. (2014). The Genetic Aetiology of Otosclerosis in the Population of Newfoundland and Labrador. Memorial University of Newfoundland.

Bel Hadj Ali, I., Thys, M., Beltaief, N., Schrauwen, I., Hilgert, N., Vanderstraeten, K., … Van Camp, G. (2008). A new locus for otosclerosis, OTSC8, maps to the pericentromeric

region of chromosome 9. Human Genetics, 123(3), 267–272. https://doi.org/10.1007/s00439-008-0470-3

Brownstein, Z., Goldfarb, A., Levi, H., Frydman, M., & Avraham, K. B. (2006). Chromosomal mapping and phenotypic characterization of hereditary otosclerosis linked to the OTSC4 locus. Archives of Otolaryngology--Head & Neck Surgery, 132(4), 416–424. https://doi.org/10.1001/archotol.132.4.416

Chen, W., Campbell, C. A., Green, G. E., Van Den Bogaert, K., Komodikis, C., Manolidis, L. S., … Smith, R. J. H. (2002). Linkage of otosclerosis to a third locus (OTSC3) on human chromosome 6p21.3-22.3. Journal of Medical Genetics, 39(7), 473–477. https://doi.org/10.1136/jmg.39.7.473

Feeney, M. P., Grant, I. L., & Marryott, L. P. (2003). Wideband energy reflectance measurements in adults with middle-ear disorders. Journal of Speech, Language, and Hearing Research : JSLHR, 46(4), 901–911. https://doi.org/10.1044/1092-4388(2003/070)

Nakajima, H. H., Pisano, D. V, Roosli, C., Hamade, M. a, Merchant, G. R., Mahfoud, L., … Merchant, S. N. (2012). Comparison of ear-canal reflectance and umbo velocity in patients with conductive hearing loss: a preliminary study. Ear and Hearing, 33(1), 35–43. https://doi.org/10.1097/AUD.0b013e31822ccba0

Naumann, I. C., Porcellini, B., & Fisch, U. (2005). Otosclerosis: incidence of positive findings on high-resolution computed tomography and their correlation to audiological test data. The Annals of Otology, Rhinology, and Laryngology, 114(9), 709–716. https://doi.org/10.1177/000348940511400910

Niemczyk, E., Lachowska, M., Tataj, E., Kurczak, K., & Niemczyk, K. (2018). Wideband tympanometry and absorbance measurements in otosclerotic ears. The Laryngoscope, ePub ahead. https://doi.org/10.1002/lary.27747

Prieve, B. a, Feeney, M. P., Stenfelt, S., & Shahnaz, N. (2013). Prediction of conductive hearing loss using wideband acoustic immittance. Ear and Hearing, 34 Suppl 1, 54S-59S. https://doi.org/10.1097/AUD.0b013e31829c9670

Quesnel, A. M., Moonis, G., Appel, J., O'Malley, J. T., McKenna, M. J., Curtin, H. D., & Merchant, S. N. (2013). Correlation of computed tomography with histopathology in otosclerosis. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 34(1), 22–28. https://doi.org/10.1097/MAO.0b013e318277a1f7

Redfors, Y. D., Gröndahl, H. G., Hellgren, J., Lindfors, N., Nilsson, I., & Möller, C. (2012). Otosclerosis: anatomy and pathology in the temporal bone assessed by multi-slice and cone-beam CT. Otology & Neurotology : Official Publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology, 33(6), 922–927. https://doi.org/10.1097/MAO.0b013e318259b38c

Sanford, C., Schooling, T., & Frymark, T. (2012). Determining the Presence or Absence of Middle Ear Disorders: An Evidence-Based Systematic Review on the Diagnostic Accuracy of Selected Assessment. American Journal of Audiology, 21(December), 251–268. https://doi.org/10.1044/1059-0889(2012/11-0029)a

Shahnaz, N., Bork, K., Polka, L., Longridge, N., Bell, D., & Westerberg, B. D. (2009). Energy reflectance and tympanometry in normal and otosclerotic ears. Ear and Hearing, 30(2), 219–233. https://doi.org/10.1097/AUD.0b013e3181976a14

Thys, M., Van Den Bogaert, K., Iliadou, V., Vanderstraeten, K., Dieltjens, N., Schrauwen, I., … Van Camp, G. (2007). A seventh locus for otosclerosis, OTSC7, maps to chromosome 6q13–16.1. European Journal of Human Genetics, 15(3), 362–368. https://doi.org/10.1038/sj.ejhg.5201761

Tomek, M. S., Brown, M. R., Mani, S. R., Ramesh, A., Srisailapathy, C. R., Coucke, P., … Smith, R. J. (1998). Localization of a gene for otosclerosis to chromosome 15q25-q26. Human Molecular Genetics, 7(2), 285–290. https://doi.org/10.1093/hmg/7.2.285

Van Den Bogaert, K. (2004). A fifth locus for otosclerosis, OTSC5, maps to chromosome 3q22-24. Journal of Medical Genetics, 41(6), 450–453. https://doi.org/10.1136/jmg.2004.018671

Van Den Bogaert, Kris, Govaerts, P. J., Schatteman, I., Brown, M. R., Caethoven, G., Offeciers, F. E., … Van Camp, G. (2001). A second gene for otosclerosis, OTSC2, maps to chromosome 7q34-36. American Journal of Human Genetics, 68(2), 495–500. https://doi.org/10.1086/318185

Weegerink, N. J. D., Schrauwen, I., Huygen, P. L. M., Pennings, R. J. E., Cremers, C. W. R. J., Van Camp, G., & Kunst, H. P. M. (2011). Phenotype of the first otosclerosis family linked to OTSC10. The Laryngoscope, 121(4), 838–845. https://doi.org/10.1002/lary.21463

Appendices

Appendix A: Sequencing protocol for *FOXL1* screening. Protocol retrieved from PhD thesis of Nelly Abdelfatah (2014).

Cycle Sequencing protocol

Reagent	Volume (uL)
dH20	15.68
BDT 5x Buffer	2
BDT Sequencing Mix	0.5
Primer	0.32
Purified PCR Sample	1
Total Volume	20

Thermocycling Protocols for Cycle Sequencing

94°C for 1 min
25 cycles of:
1. 96°C Denaturation for 10s
2. 50°C Annealing for 5s
3. 60°C Extension for 4 mins

Hold at 4°

Cycle Sequencing DNA Precipitation Protocol

This step was performed after Cycle sequencing was completed, as a second purification step before being placed on the ABI 3130xl of ABI 3730.

Step 1) DNA precipitation
- Add 65uL of 95% Ethanol (EtOH) to each well
- Add 5uL of 125mM Ethylenediaminetetraacetic acid to each well
- Let precipitate for 15 mins to overnight in dark o Can place at -20°C if preferred or if not using plate for a few days.

Step 2) Ethanol mixture Removal
- Place plate in centrifuge, spin at 3000 RPM for 30 mins
- Remove plate from centrifuge and decant ethanol mixture onto a dry paper towel by inverting the plate.
- Leave plate inverted on paper towel and place back in centrifuge. Spin up to 200 RPM, and then immediately stop the spinning.
- Remove and discard paper towel.

Step 3) Rinse step
- Add 150uL of 70% EtOH to each well, and place in centrifuge.
- Spin plate at 3000 RPM for 5 mins.
- Remove plate from centrifuge and decant EtOH mixture onto a paper towel
- Leave plate inverted on paper towel and place back in centrifuge. Spin up to 200 RPM, and then immediately stop the spinning.
- Remove and discard paper towel. Let plate dry in dark and uncovered for 20 mins

Step 4) Sample Resuspension
- Add 15 uL of Hi-Dye Formamide (HDF) to each well
- Place plate in thermocycler on ‘denat’ program o
  - 95°C for 2 mins

- Hold at 4°

***Appendix B**:* Results of interpretation of *FOXL1* deletion pathogenicity based on guidelines reported by the American College of Medical Genetics (Richards et. al. 2015)

Population data

_ PS4 The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls

_ PM2 Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium

_ BS1 Allele frequency is greater than expected for disorder

_ BS2 Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder, with full penetrance expected at an early age

_ BA1 Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium

Computational and predictive data

_ PVS1 null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease

_ PS1 Same amino acid change as a previously established pathogenic variant regardless of nucleotide change

X PM4 Protein length changes as a result of in-frame deletions/insertions in a nonrepeat region or stop-loss variants

_ PM5 Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before

X PP3 Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)

_ BP1 Missense variant in a gene for which primarily truncating variants are known to cause disease

_ BP3 In-frame deletions/insertions in a repetitive region without a known function

_ BP4 Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.)

_ BP7 A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved

Functional data

X PS3 Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product

_ PM1 Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation

_ PP2 Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease

_ BS3 Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing

Segregation data

X **PP1 (Strong evidence) Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease**

_ **PP1 (Moderate evidence) Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease**

_ **PP1 Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease**

_ **BS4 Lack of segregation in affected members of a family**

De novo data

_ **PS2 De novo (both maternity and paternity confirmed) in a patient with the disease and no family history**

_ **PM6 Assumed de novo, but without confirmation of paternity and maternity**

Allelic data

_ **PM3 For recessive disorders, detected in trans with a pathogenic variant**

_ **BP2 Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern**

Other database data

_ **PP5 Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation**

_ **BP6 Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation**

Other data

_ **PP4 Patient's phenotype or family history is highly specific for a disease with a single genetic etiology**

_ **BP5 Variant found in a case with an alternate molecular basis for disease**

Artifact data

_ **Sequencing artifact as determined by depth, quality, or other previously reviewed data**

Variant Classification:

Variant	Effect	Evidence	To delete
	Pathogenic (II)	PM4, PP3, PS3, PP1-S	Delete

Appendix C: Family History Questionnaire

QUESTIONNAIRE-ADULT

Interviewer:_____ Participant #_____
Date: Day: _____Month: _____Year:_____

PARTICIPANT INFORMATION

Birth History:

Did the participant's mother have any of the following illnesses or problems during her pregnancy (check all that apply):

☐Fever ☐ Infection: ☐ Rubella ☐ Quinine
☐ Cytomegalovirus ☐ Maternal Diabetes ☐ Retinoic Acid
☐Oligohydraminos ☐Toxoplasmosis ☐ Other:
If any selected, please specify at what stage of pregnancy the exposure occurred and the duration: weeks for days

Participant's Infant Hearing Screen result:
☐ Pass ☐ Refer ☐ Unknown
If refer, please specify:

Personal History:

Do you have a known medical condition, or were you born with any physical differences? If so, please describe:

Is the participant adopted? ☐ No ☐ Yes
Is the participant and his/her partner related by blood? (e.g. cousins?) ☐ No ☐ Yes
Are parents of participant related by blood? ☐ No ☐ Yes - If 'Yes' please explain how parents are related:

FAMILY HISTORY INFORMATION

PARTICIPANT'S CHILDREN
Please continue any other important information on the back of this page

Full Name (Please include miscarriages, stillbirths & deceased individuals)	**Sex**	**Date of Birth** (yyyy/mm/dd)	**Partner's Name** (Other Parent)

PARTICIPANT SIBLINGS:
Please continue any other important information on the back of this page

Full Name (Please include deceased individuals)	**Sex**	**Date of Birth** (yyyy/mm/dd)	**Father's Name** (If different from participant's)	**Mother's Name** (If different from participant's)		**Children's Full Name** (Please include miscarriages, stillbirths & deceased individuals)	**Sex**	**Date of Birth** (yyyy/mm/dd)	**Partner's Name** (Other Parent)

MATERNAL INFORMATION
Please continue any other important information on the back of this page

	Full Name	**Date of Birth** (yyyy/mm/dd)	**If deceased**	
			Age of Death (If Applicable)	**Cause of Death**
Mother				
Maternal Grandmother				
Maternal Grandfather				

MOTHER'S SIBLINGS
Please continue any other important information on the back of this page

Full Name (Please include deceased individuals)	**Sex**	**Date of Birth** (yyyy/mm/dd)	**Father's Name** (If different from participant's maternal grandfather)	**Mother's Name** (If different from participant's maternal grandmother)		**Children's Full Name** (Please include miscarriages, stillbirths & deceased individuals)	**Sex**	**Date of Birth** (yyyy/mm/dd)	**Partner's Name** (Other Parent)

Are any of the individuals listed above adopted? ☐ Yes ☐ No - If 'Yes' please list their names:

MATERNAL ETHNICITY AND AFFILIATION

Participant's Mother Please indicate ancestry and/or ethno religious affiliation (if applicable)- check all that apply:				**Participant's Father** Please indicate ancestry and/or ethno religious affiliation (if applicable)- check all that apply:			
English	☐	Indian	☐	English	☐	Indian	☐
Polish	☐	South Korean	☐	Polish	☐	South Korean	☐
Portuguese	☐	French Canadian	☐	Portuguese	☐	French Canadian	☐
Italian	☐	Other:		Italian	☐	Other:	
Dutch	☐			Dutch	☐		
German	☐			German	☐		
Chinese	☐			Chinese	☐		
Columbian	☐			Columbian	☐		
Mennonite	☐			Mennonite	☐		
Amish	☐			Amish	☐		
Jewish	☐			Jewish	☐		
Conference	☐			Conference	☐		

PATERNAL INFORMATION
Please continue any other important information on the back of this page

	Full Name	**Date of Birth** (yyyy/mm/dd)	**If deceased**	
			Age of Death	**Cause of Death**
Father				
Paternal Grandmother				
Paternal Grandfather				

FATHER'S SIBLINGS
Please continue any other important information on the back of this page

Full Name (Please include deceased individuals)	**Sex**	**Date of Birth** (yyyy/mm/dd)	**Father's Name** (If different from participant's maternal grandfather)	**Mother's Name** (If different from participant's maternal grandmother)		**Children's Full Name** (Please include miscarriages, stillbirths & deceased individuals)	**Sex**	**Date of Birth** (yyyy/mm/dd)	**Partner's Name** (Other Parent)

Are any of the individuals listed above adopted? ☐ Yes ☐ No - If 'Yes' please list their names:

PATERNAL ETHNICITY AND/OR AFFILIATION

Participant's Mother Please indicate ancestry and/or ethno religious affiliation (if applicable)- check all that apply:				**Participant's Father** Please indicate ancestry and/or ethno religious affiliation (if applicable)- check all that apply:			
English	☐	Indian	☐	English	☐	Indian	☐
Polish	☐	South Korean	☐	Polish	☐	South Korean	☐
Portuguese	☐	French Canadian	☐	Portuguese	☐	French Canadian	☐
Italian	☐	Other:		Italian	☐	Other:	
Dutch	☐			Dutch	☐		
German	☐			German	☐		
Chinese	☐			Chinese	☐		
Columbian	☐			Columbian	☐		
Mennonite	☐			Mennonite	☐		
Amish	☐			Amish	☐		
Jewish	☐			Jewish	☐		
Conference	☐			Conference	☐		

FAMILY HEALTH INFORMATION

Please complete the following table. Does anyone related to the participant currently have or has had a history of the following medical conditions? For each medical condition in the table below please select 'Yes', 'No' or 'Unsure'. If 'Yes' please write the name of the person(s) and how the person(s) is/are related to the participant, in the space provided. Please write any additional family members and information on the back of this page.

Physical Conditions	**Yes**	**No**	**Unsure**	**Names of Family Members and How they are Related to Participant**
Eyes				
Physical Hypertelorism (wide spaced), Eyelid Coloboma ("notched" eyes), Ptosis (drooping eyelids), Heterochromia (different coloured eyes), Blue sclerae (white of eyes has blue tint), Dystropia Canthorum	☐	☐	☐	
Visual Problems Progressive/Stable? Glaucoma, Cataracts, Retinitis Pigmentosa, Optic Atrophy/Neuropathy, Severe myopia, Retinal detachment	☐	☐	☐	
Ears				
Bony labyrinth abnormality, Malformation? –describe physical shape and size (cupped, crumpled, ect), Absent, Low set/Rotated Placement, Ear tags/pits, Mondini dysplasia, Dilated Vestibular Aqueduct, Abnormal/Loss of Vestibular Function	☐	☐	☐	
Nose				
Anosmia ("Inability to smell")	☐	☐	☐	
Mouth				
Cleft lip/Cleft Palate, High Arched Palate, Lip Pits	☐	☐	☐	
Chin				
Abnormalities of the Jaw Bones, Micrognathia (undersized jaw)	☐	☐	☐	
Neck				

Esophageal Dysfunction, Brachial Cleft Cysts or Fistulae	☐	☐	☐	
Head/Face				
Abnormalities of Facial Bones, Microcephaly, Macrocephaly, Midface Hypoplasia	☐	☐	☐	
Skin/Hair				
White Streaks, Sparse or Patchy, Pigmentary Abnormalities	☐	☐	☐	
Teeth				
Conical, Malocclusion (abnormal growth), Discoloration, "Brittle"	☐	☐	☐	
Other physical differences at birth (specify):_____	☐	☐	☐	

Health Conditions:	**Yes**	**No**	**Unsure**	**Names of Family Members and How they are Related to Participant**
Elliptocytosis	☐	☐	☐	
Goiter (enlarged thyroid gland)	☐	☐	☐	
Movement Disorders Ataxia (episodic or progressive), Dystonia	☐	☐	☐	
Cardiovascular Conduction Defects (Prolonged Q-T interval), Syncopal Episodes (fainting)	☐	☐	☐	
Gastrointestinal Hirschprung disease, Enlarged liver or spleen	☐	☐	☐	
Renal (Glomulonephritis, Failure)	☐	☐	☐	
Skeletal Tall/Short stature, disproportionate limbs, scoliosis, congenital hip dislocation, osteoarthritis, multiple fractures, loose joints, spondyloepiphyseal dysplasia	☐	☐	☐	
Tumors Vestibular schwannoma, other rare tumors: meningioma, astrocytoma, ependymomas, meningioangiomatosis, genital leiomyomas	☐	☐	☐	
Other health conditions (specify): _____	☐	☐	☐	

Developmental Delay:

Intellectual disability (describe):	☐	☐	☐
Learning Delay (describe):	☐	☐	☐
Other inherited condition:	☐	☐	☐

If there are there any additional health concerns in the family that have not previously been mentioned, please list them:

Birth History	**Environmental Exposure**	**Description**
Did the individual's mother have any of the following illnesses or problems during her pregnancy (check all that apply): ☐Fever ☐ Infection: ☐ Rubella ☐ Quinine ☐ Cytomegalovirus ☐ Maternal Diabetes ☐ Retinoic Acid ☐Oligohydraminos ☐Toxoplasmosis ☐ Other: : If any selected, please specify at what stage of pregnancy the exposure occurred and the duration: weeks for days Infant Hearing Screen result: ☐ Pass ☐ Refer ☐ Unknown If refer, please specify:	*Was the individual exposed to any of the following (check all that apply):* ☐Noise (occupational/environmental) ☐Trauma to Ear ☐Chronic Ear Infections ☐Aminoglycoside use ☐ Meningitis ☐Mastoidtis ☐Kericterus (bilirubin encephalopathy) ☐Myxedema (hypothyroidism) ☐Surgical Procedure on Ear ☐ Other:_____ If any selected, please specify:	Age of Onset ☐Congenital ☐Child ☐Adult ☐ Unknown Type ☐Conductive ☐Sensorineural ☐Mixed ☐Unknown Progressive? ☐ Yes ☐ No ☐ Unknown Physical Features? ☐ Yes ☐ No ☐ Unknown If yes selected, please specify:
Devices Used	**Speech/Language Difficulties**	**Previous Medical Evaluations Performed**
☐Hearing Aid ☐ Cochlear Implant ☐ Other: :	☐ Yes ☐ No ☐ Unknown If yes selected, please specify:	☐ Audiogram(s) ☐ ABR ☐BAER ☐Other evaluations:

Has anyone in your family previously been referred for genetic counseling and/or genetic testing? ☐Yes ☐No ☐Unsure
If Yes, please state where:
For what reason?

Appendix D: Mean (M) and standard deviation (SD) air conduction thresholds and air-bone gaps (ABG) for surgical ears (n=42), suspected otosclerotic ears (n=14), normal ears (n=11) and SNHL ears (n=3).

	Surgical				**Suspected Otosclerotic**				**Normal**				**SNHL**			
	Air		ABG		Air		ABG		Air		ABG		Air		ABG	
Frequency (Hz)	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
250	30.70	14.94	25.38	14.02	39.64	10.46	33.85	7.40	13.18	6.03	16.00	6.15	36.67	18.93	18.33	7.64
500	30.81	17.21	16.98	15.85	42.86	9.94	28.21	7.99	11.36	5.52	10.00	6.32	45.00	13.23	6.67	7.64
1000	28.12	16.28	15.91	12.46	39.64	10.82	20.71	8.29	10.91	4.37	9.09	4.91	38.33	16.07	8.33	5.77
2000	30.70	17.81	8.95	8.77	33.57	13.07	9.64	7.20	8.18	4.62	1.36	5.52	36.67	17.56	1.67	5.77
4000	33.14	22.52	8.60	9.84	35.36	15.99	12.14	8.02	8.18	9.02	0.00	4.47	36.67	16.07	5.00	5.00
8000	49.07	24.74			45.71	18.49			20.00	18.03			41.67	17.56		

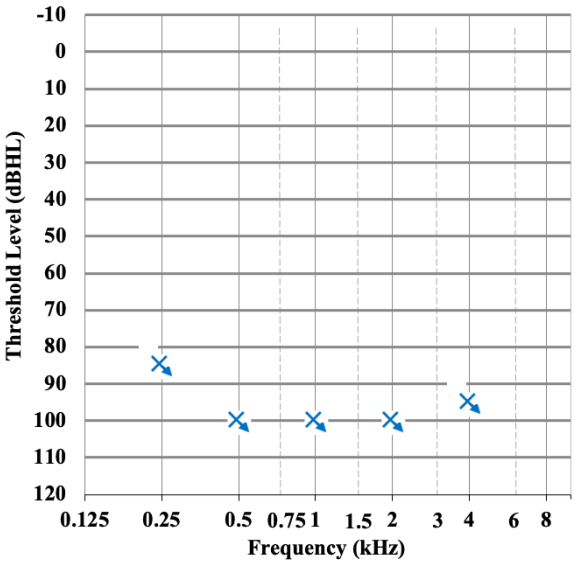
Appendix E: Mean power absorbance (PA) for Mimosa HearID and Interacoustics Titan (n=50 ears) obtained at 65 dB SPL. Error bars represent standard deviations. Significant differences in PA between instruments are bolded.

			95% Confidence Interval			
	Mean		Lower Bound		Upper Bound	
Frequency (Hz)	**Mimosa**	**Titan**	**Mimosa**	**Titan**	**Mimosa**	**Titan**
250	**0.090**	**0.195**	0.071	0.167	0.109	0.223
315	**0.122**	**0.213**	0.098	0.178	0.145	0.248
397	**0.177**	**0.317**	0.145	0.275	0.209	0.358
500	**0.251**	**0.381**	0.207	0.331	0.294	0.431
630	**0.366**	**0.480**	0.310	0.423	0.422	0.537
794	**0.491**	**0.597**	0.429	0.535	0.554	0.659
1000	**0.575**	**0.671**	0.521	0.616	0.629	0.726
1260	**0.631**	**0.717**	0.579	0.672	0.684	0.762
1587	0.645	0.677	0.594	0.627	0696	0.726
2000	0.663	0.682	0.616	0.634	0.709	0.730
2520	**0.713**	**0.642**	0.666	0.581	0.760	0.704
3175	**0.703**	**0.579**	0.654	0.506	0.752	0.652
4000	**0.604**	**0.399**	0.535	0.309	0.673	0.489
5040	0.349	0.419	0.232	0.352	0.466	0.486

Appendix F: Results of 1000's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance. Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance.

1000 - Left Ear

Audiometry



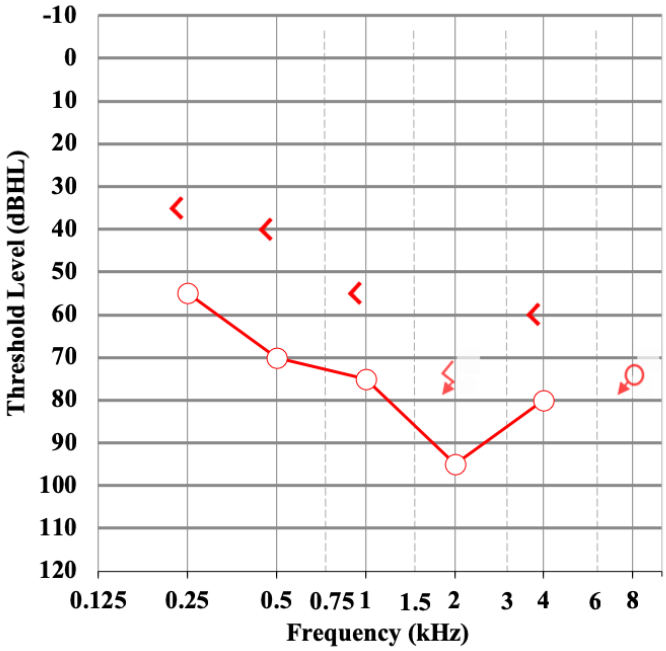
Acoustic Reflex Thresholds		
Frequency (Hz)	**Left Ipsi**	**Left Contra**
500	A	A
1000	A	A
2000	A	A
4000	A	A
WBN	A	A

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	**TPP (daPa)**	**Compliance (mL)**
0.77	Flat	Flat

Appendix G: Results of 1000's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance. Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance.

1000 - Right Ear

Audiometry



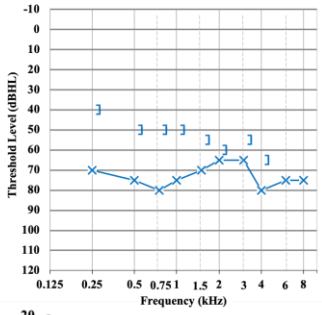
Acoustic Reflex Thresholds		
Frequency (Hz)	**Right Ipsi**	**Right Contra**
500	A	A
1000	A	A
2000	A	A
4000	A	A
WBN	A	A

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	**TPP (daPa)**	**Compliance (mL)**
1.34	-30	1.46

Appendix H: Results of 0000's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

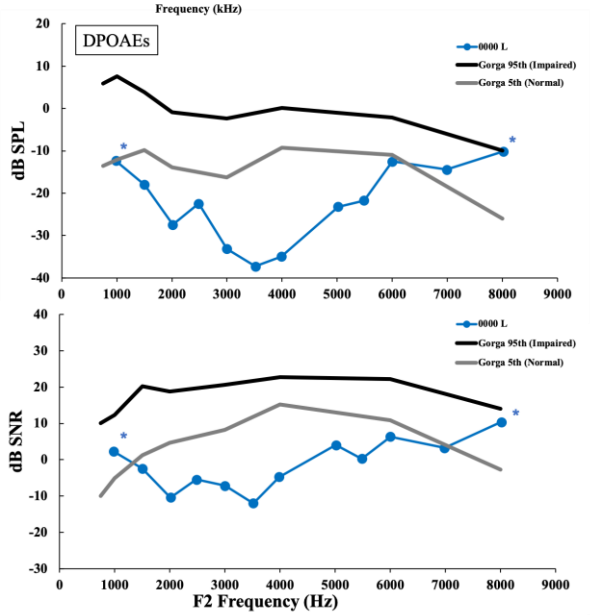
0000 - Left Ear

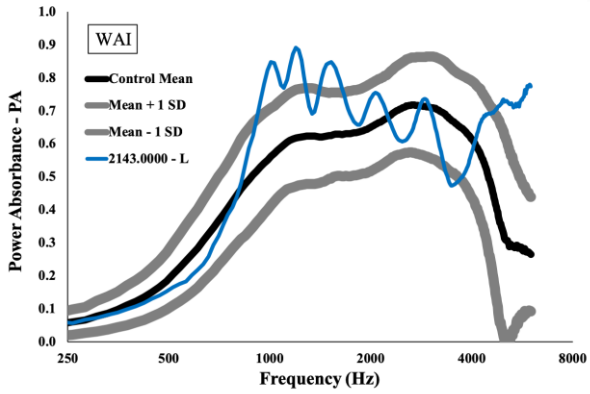
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Left Ipsi	Left Contra
500	A	A
1000	A	A
2000	A	A
4000	100	A
WBN	A	A

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
2.18	-95	1.3

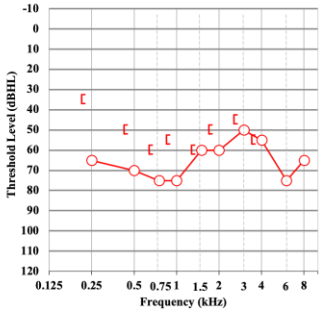




Appendix I: Results of 0000's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

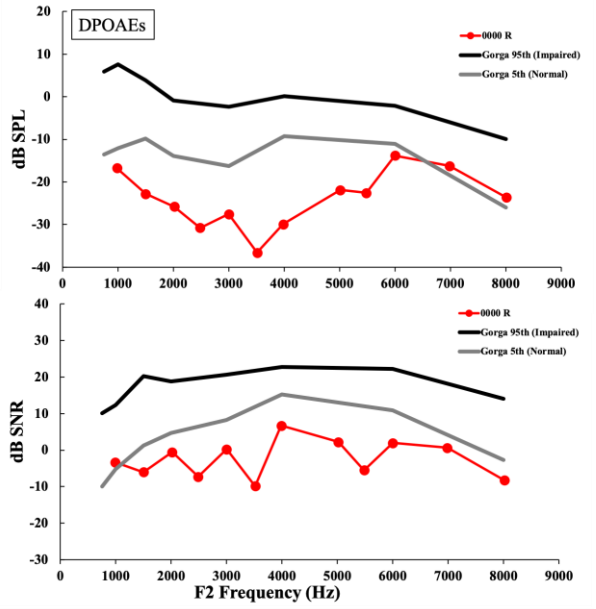
0000 - Right Ear

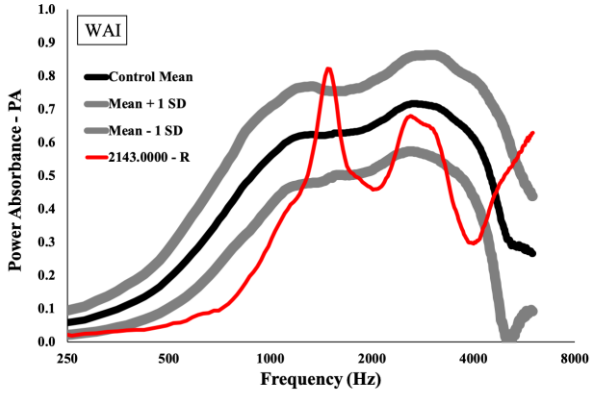
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Right Ipsi	Right Contra
500	A	A
1000	A	A
2000	A	A
4000	A	A
WBN	A	A

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.97	-156	1.16

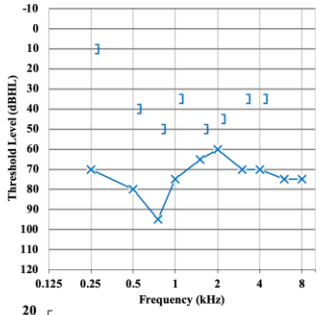




Appendix J: Results of 0009's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

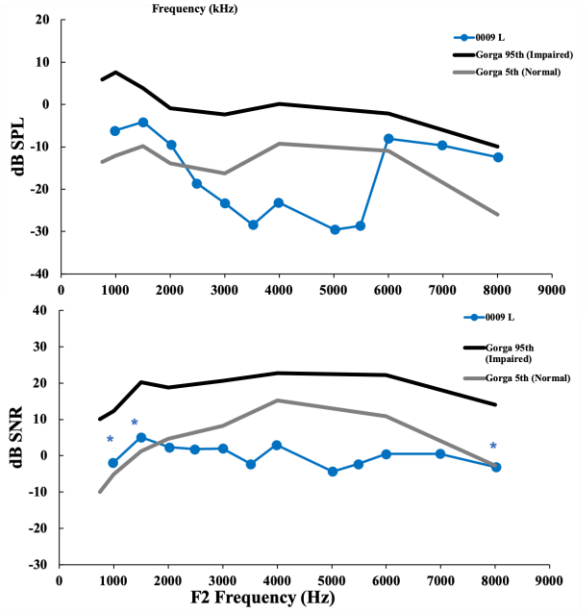
0009 - Left Ear

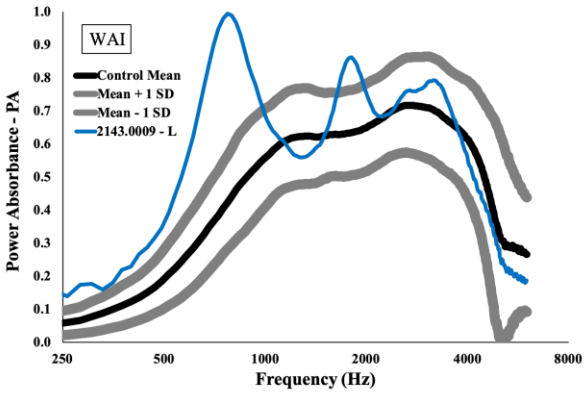
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Left Ipsi	Left Contra
500	A	A
1000	A	A
2000	A	90
4000	100	A
WBN	A	A

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.8	-7	1.25

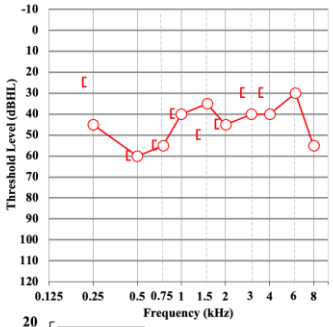




Appendix K: Results of 0009's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

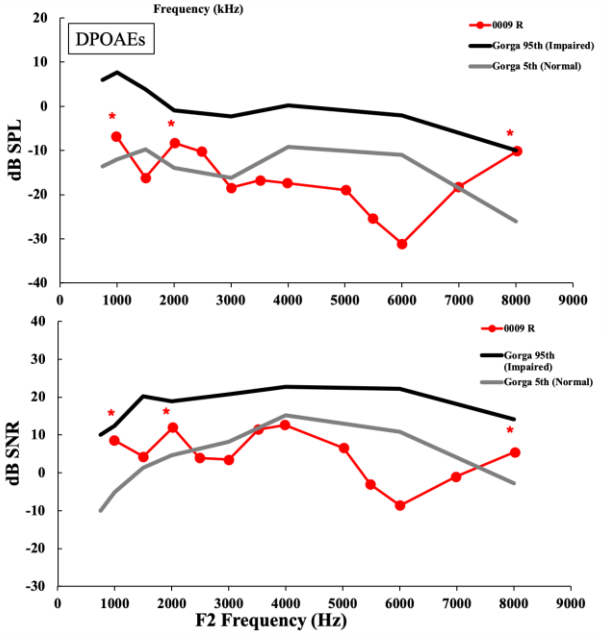
0009 - Right Ear

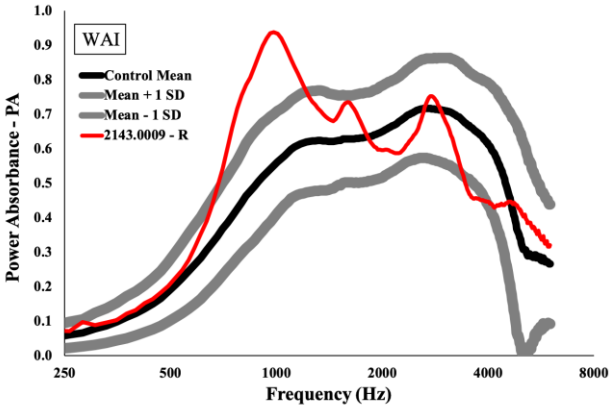
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Right Ipsi	Right Contra
500	A	A
1000	A	A
2000	A	A
4000	A	A
WBN	100	A

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.74	-8	1.24

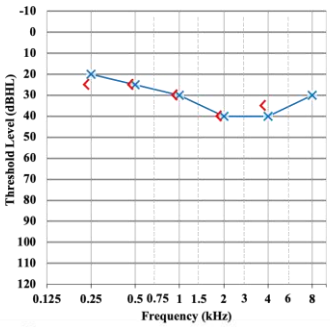




Appendix L: Results of 0002's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

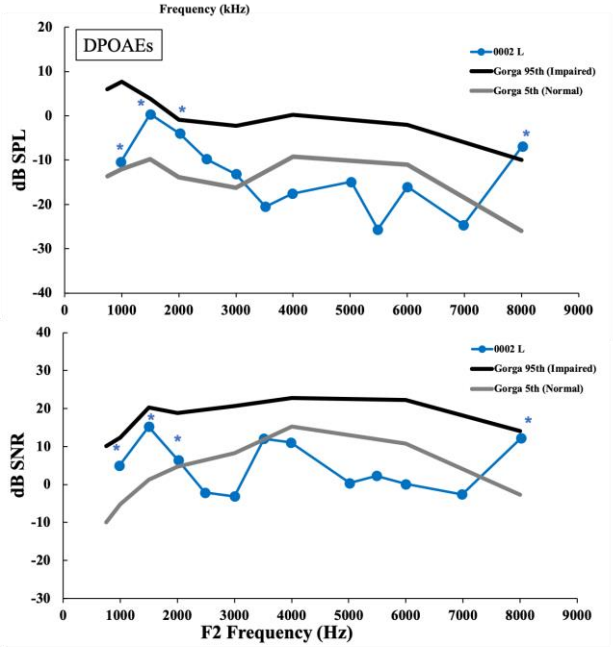
0002 - Left Ear

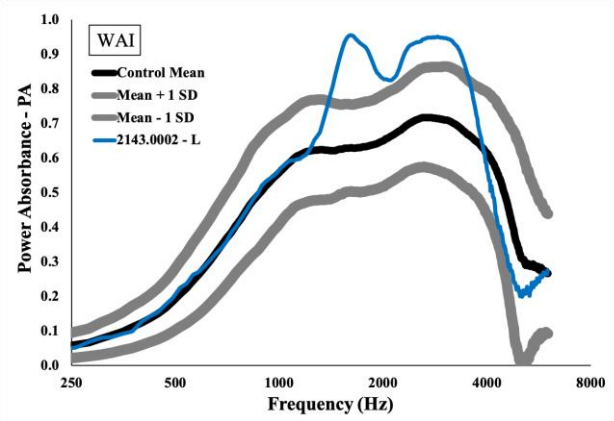
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Left Ipsi	Left Contra
500	90	95
1000	90	95
2000	85	95
4000	A	A
WBN	90	95

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.67	-18	0.72

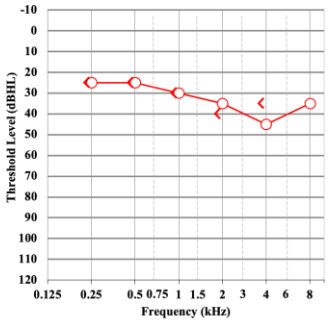




Appendix M: Results of 0002's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

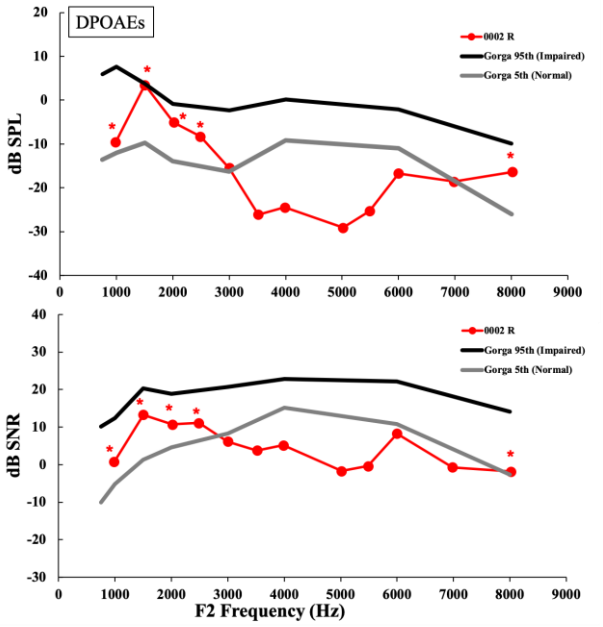
0002 - Right Ear

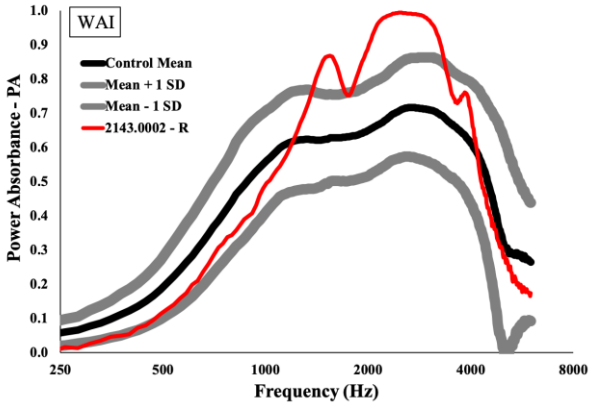
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Right Ipsi	Right Contra
500	90	95
1000	90	95
2000	95	90
4000	A	A
WBN	90	95

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.74	-16	0.73

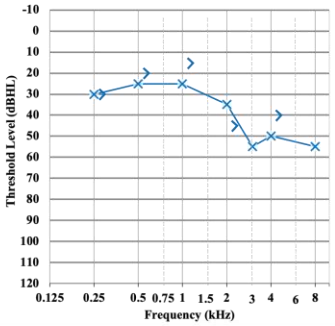




Appendix N: Results of 0003's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

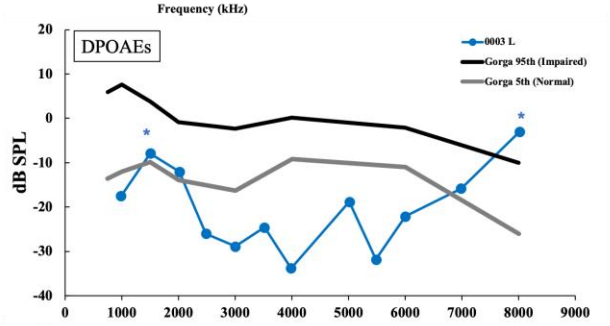
0003 - Left Ear

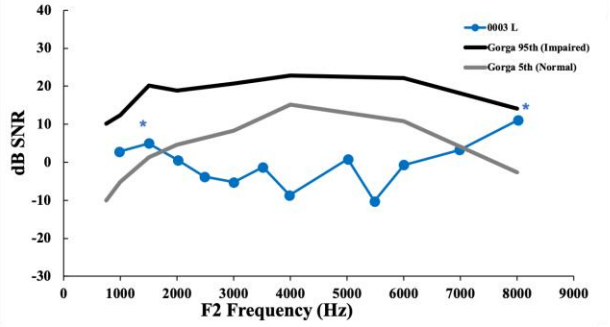
Audiometry

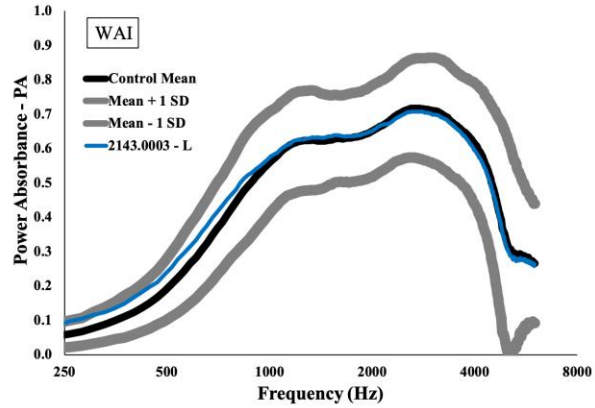


Acoustic Reflex Thresholds		
Frequency (Hz)	Left Ipsi	Left Contra
500	80	85
1000	80	80
2000	80	90
4000	95	A
WBN	90	95

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.31	-19	0.63



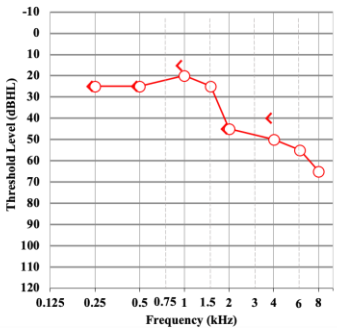




Appendix O: Results of 0003's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

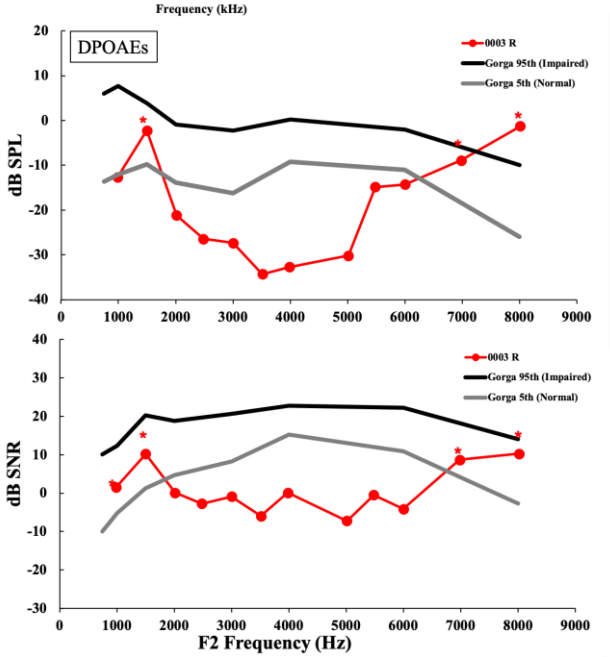
0003 - Right Ear

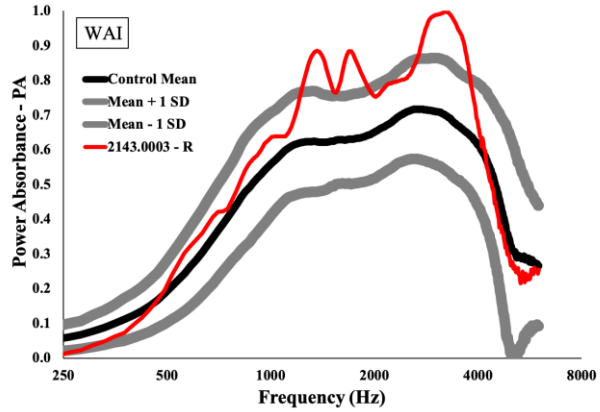
Audiometry



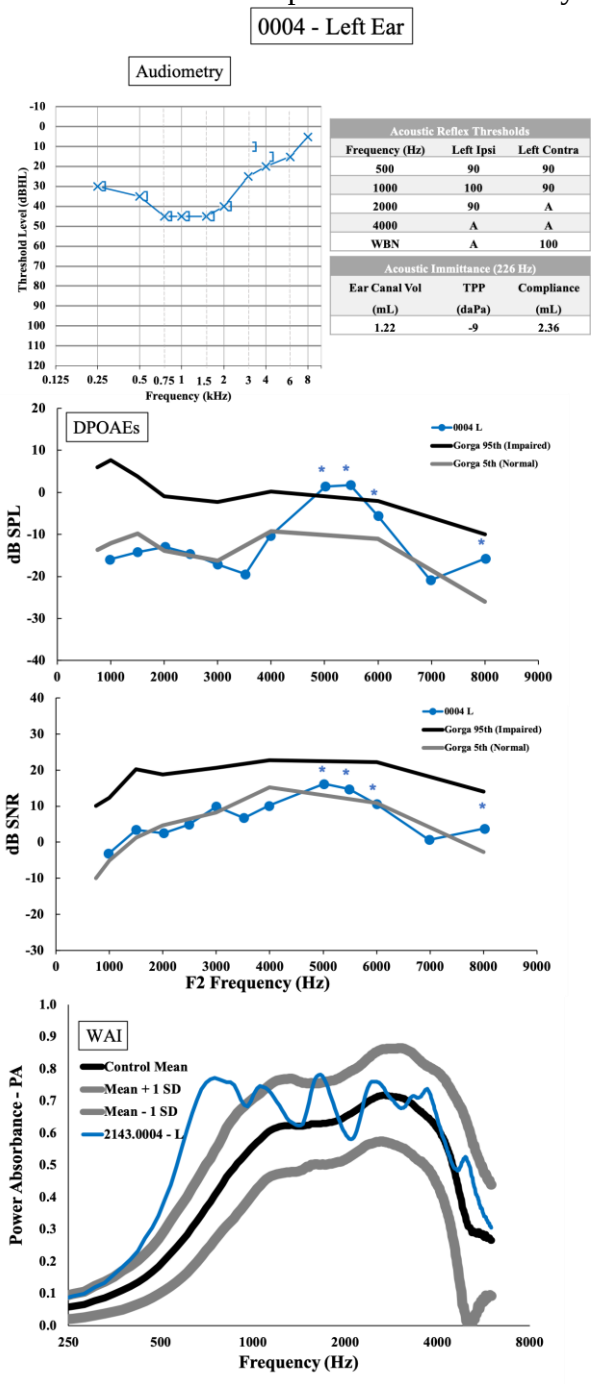
Acoustic Reflex Thresholds		
Frequency (Hz)	Right Ipsi	Right Contra
500	90	A
1000	90	95
2000	90	85
4000	95	100
WBN	95	95

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.45	-16	0.77





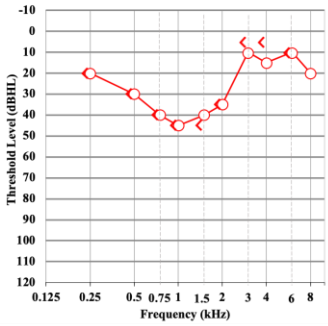
Appendix P: Results of 0004's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).



Appendix Q: Results of 0004's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

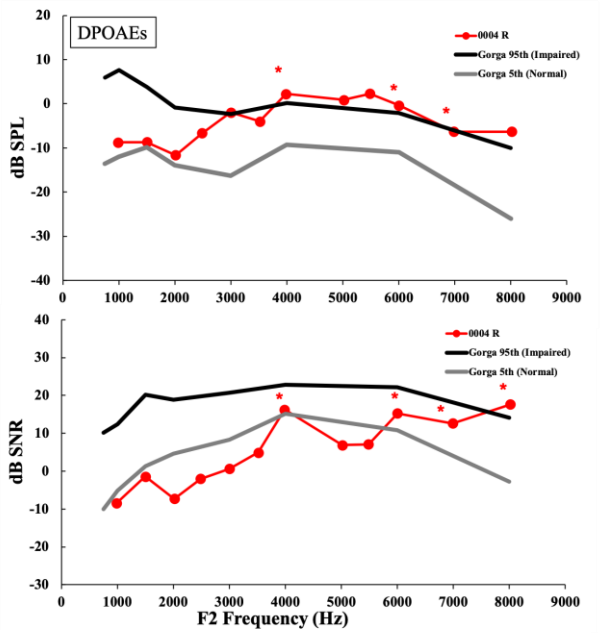
0004 - Right Ear

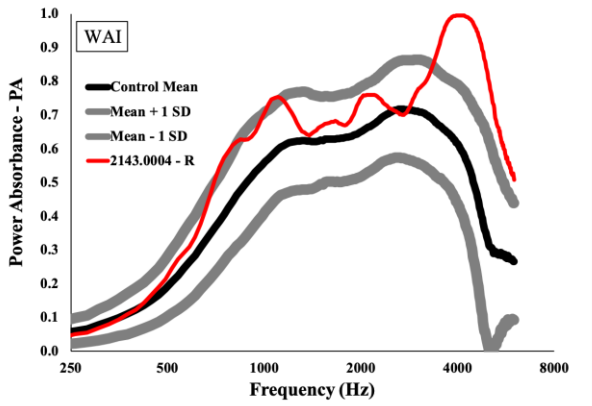
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Right Ipsi	Right Contra
500	80	95
1000	85	95
2000	85	95
4000	95	A
WBN	90	100

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.06	0	1.06

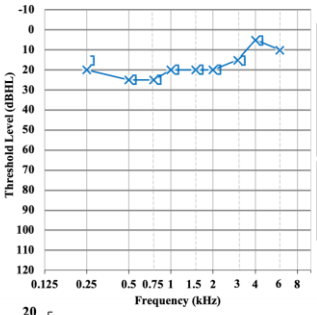




Appendix R: Results of A011's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

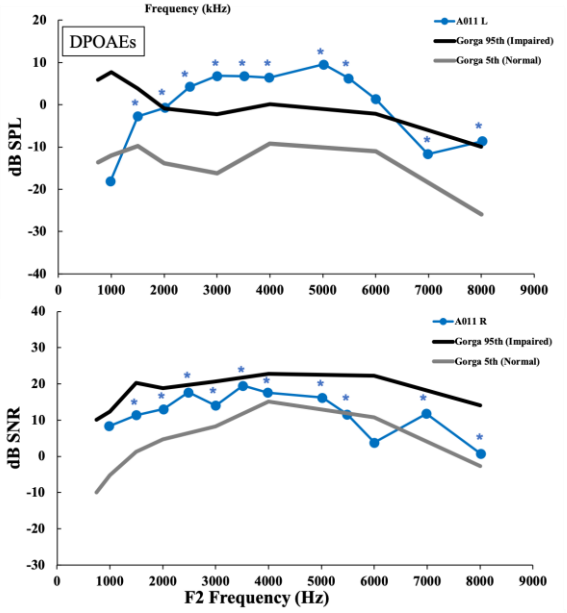
A011 - Left Ear

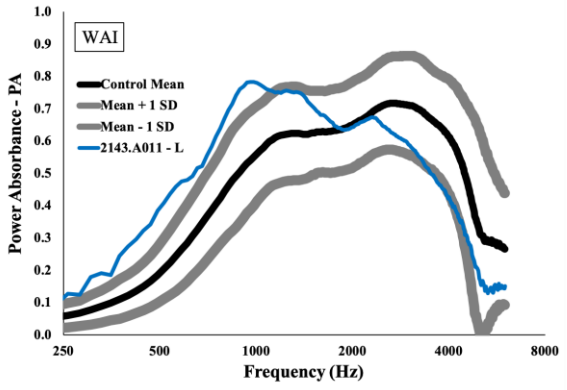
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Left Ipsi	Left Contra
500	85	90
1000	90	90
2000	85	95
4000	95	95
WBN	85	90

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
2.07	-7	1.84

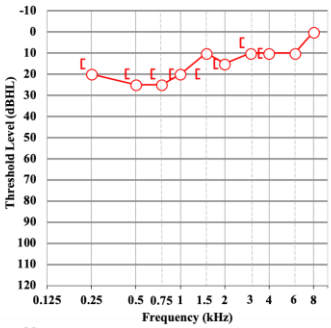




Appendix S: Results of A011's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

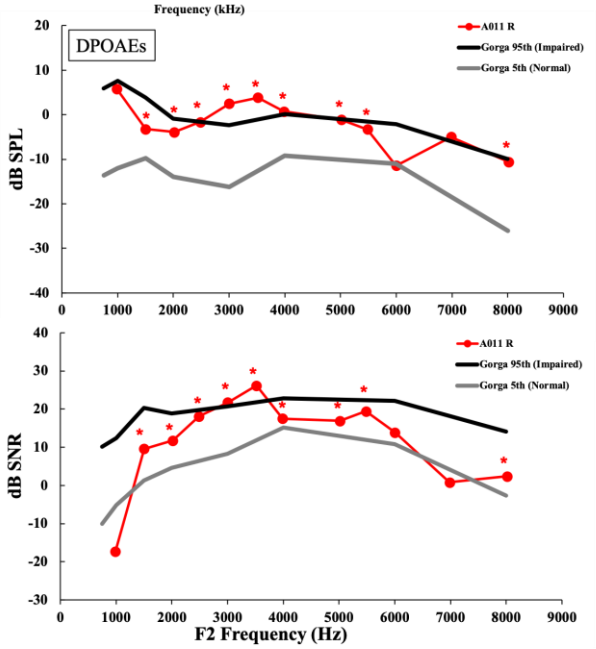
A011 - Right Ear

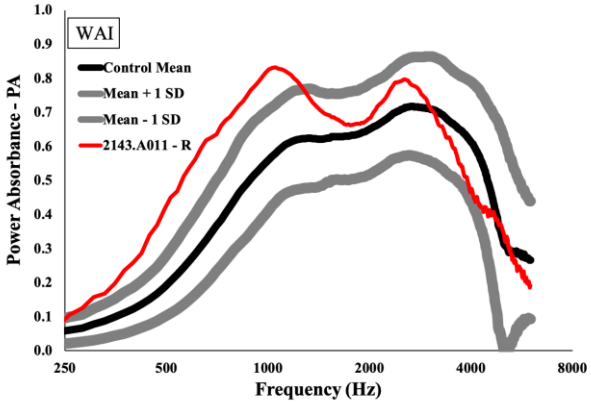
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Right Ipsi	Right Contra
500	90	90
1000	95	A
2000	90	95
4000	A	95
WBN	100	80

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
2.02	-12	1.75

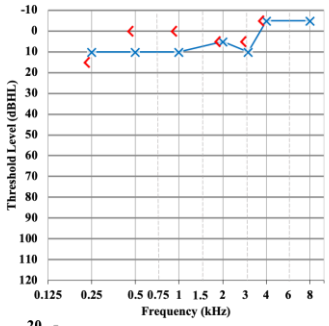




Appendix T: Results of A000's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

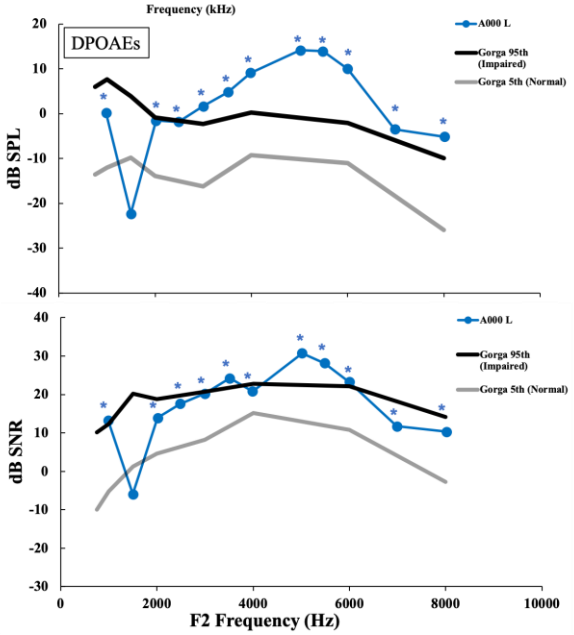
A000 - Left Ear

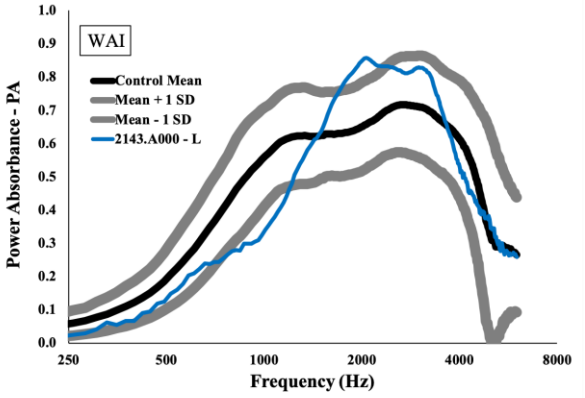
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Left Ipsi	Left Contra
500	A	A
1000	A	95
2000	A	A
4000	100	A
WBN	A	A

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.8	20	0.62

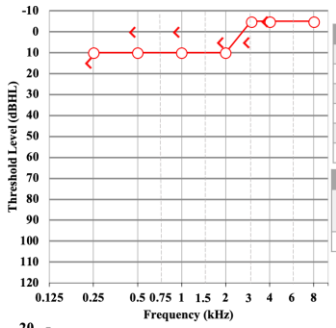




Appendix U: Results of A000's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

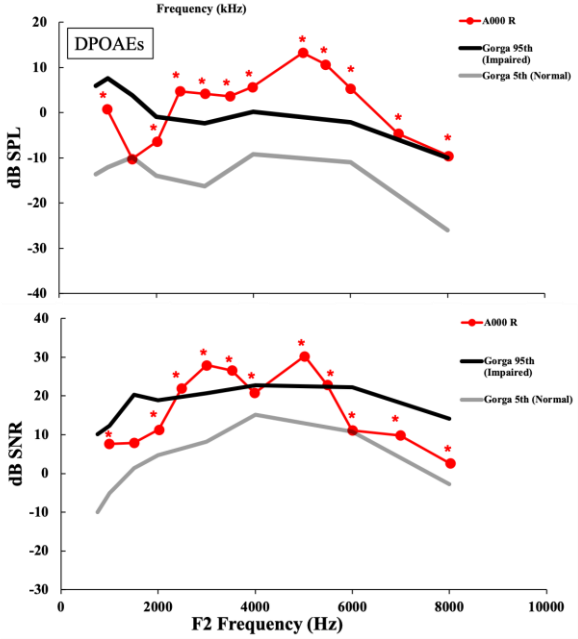
A000 - Right Ear

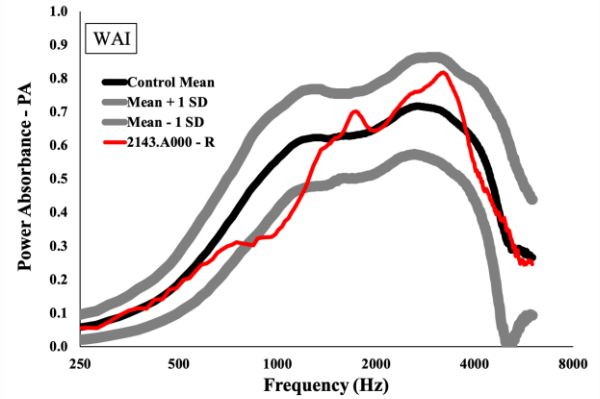
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Right Ipsi	Right Contra
500	95	100
1000	90	100
2000	80	90
4000	80	85
WBN	80	90

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.93	4	0.71

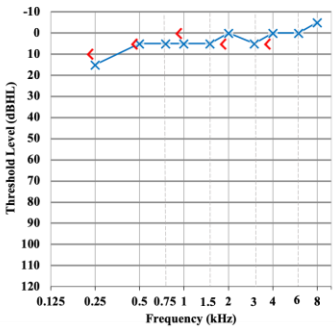




Appendix V: Results of A001's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

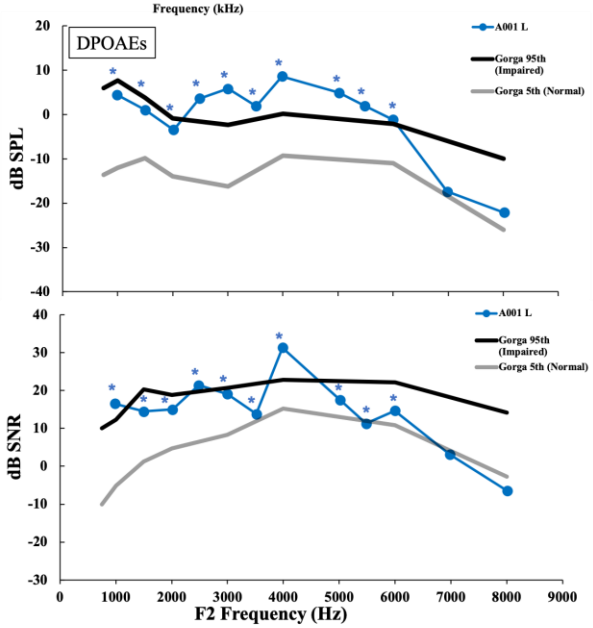
A001 - Left Ear

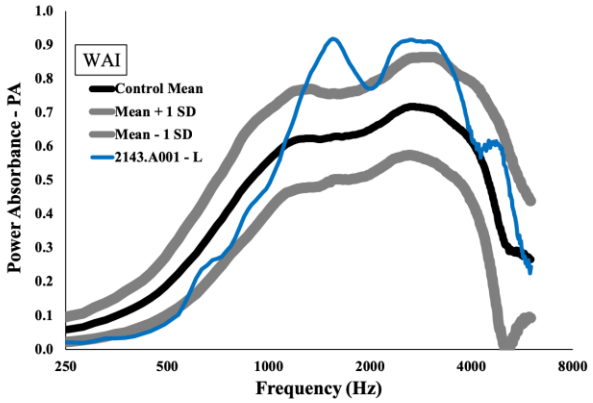
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Left Ipsi	Left Contra
500	90	100
1000	90	A
2000	90	95
4000	80	90
WBN	80	85

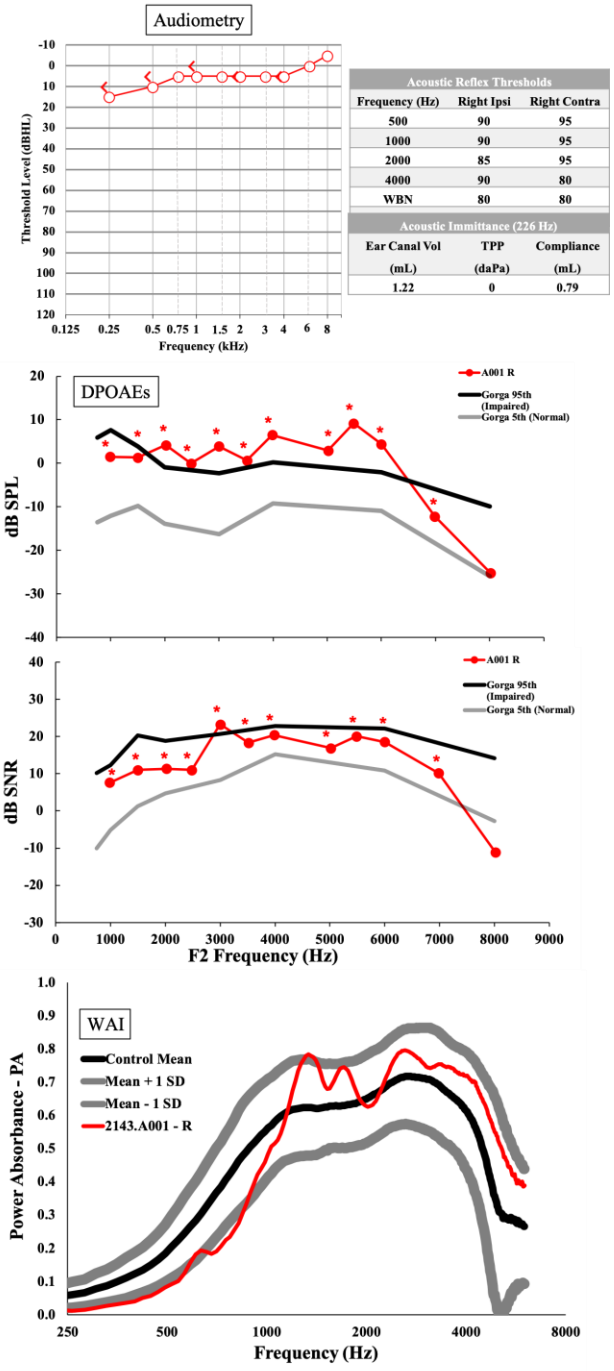
Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.21	-7	0.76





Appendix W: Results of A001's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

A001 - Right Ear



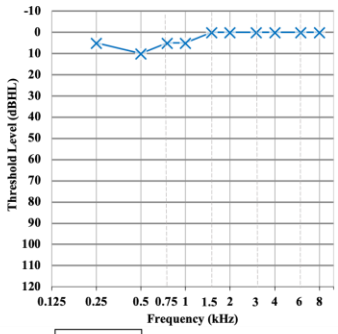
Acoustic Reflex Thresholds		
Frequency (Hz)	Right Ipsi	Right Contra
500	90	95
1000	90	95
2000	85	95
4000	90	80
WBN	80	80

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.22	0	0.79

Appendix X: Results of A002's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

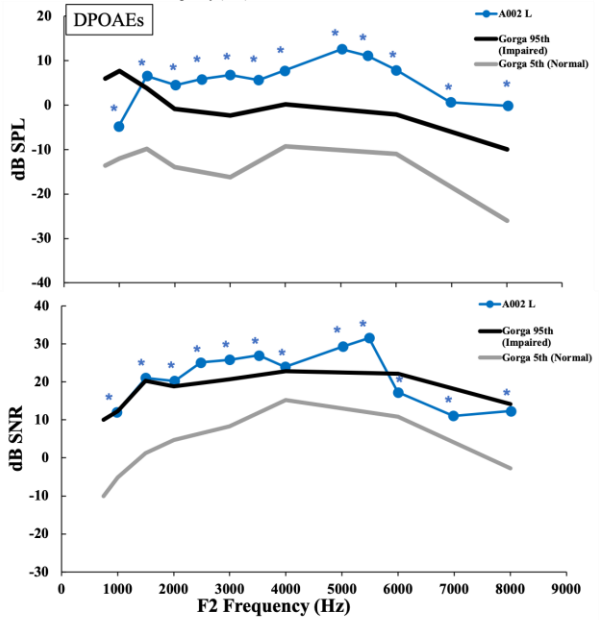
A002 - Left Ear

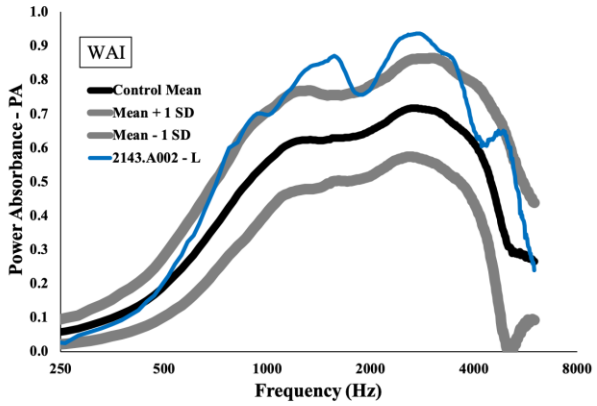
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Left Ipsi	Left Contra
500	90	A
1000	90	100
2000	85	85
4000	100	100
WBN	85	80

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.03	-11	0.99

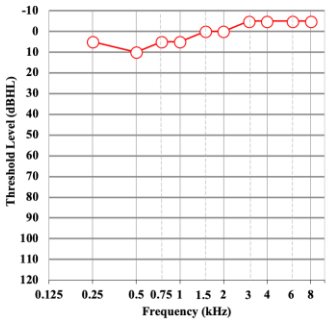




Appendix Y: Results of A002's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

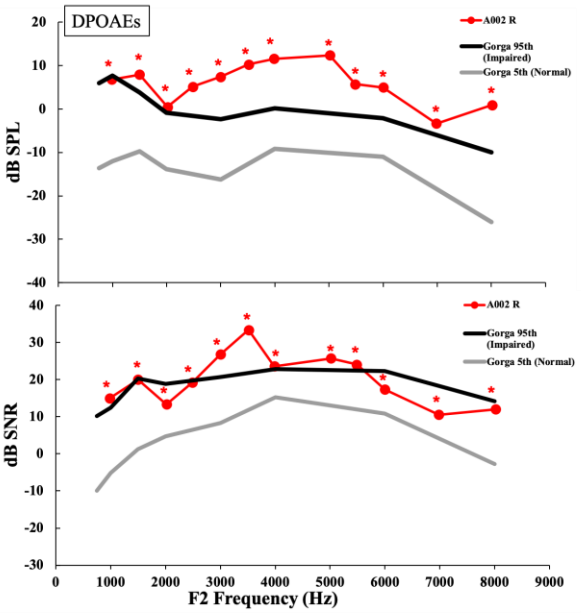
A002 - Right Ear

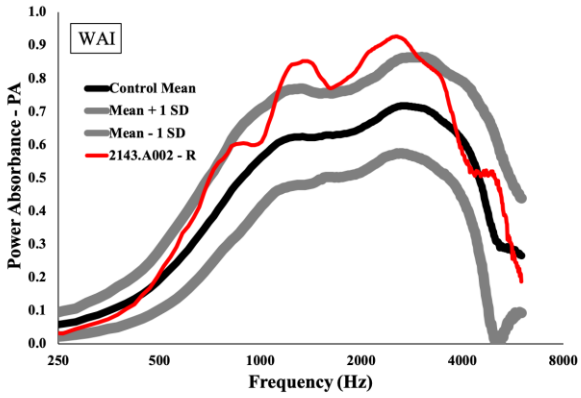
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Right Ipsi	Right Contra
500	90	95
1000	90	95
2000	85	85
4000	A	A
WBN	80	85

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.38	-19	1.03

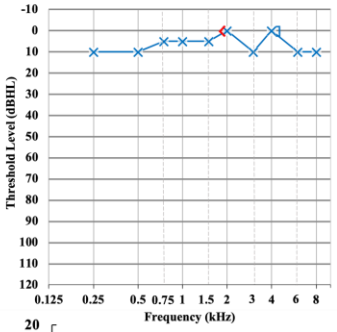




Appendix Z: Results of A006's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

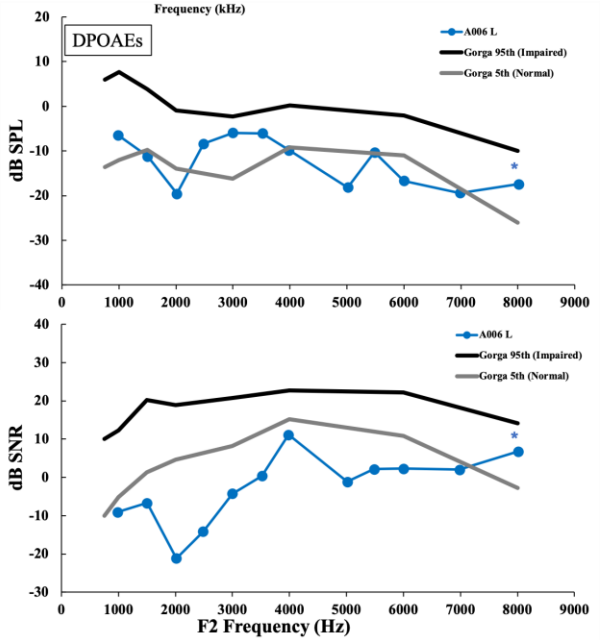
A006 - Left Ear

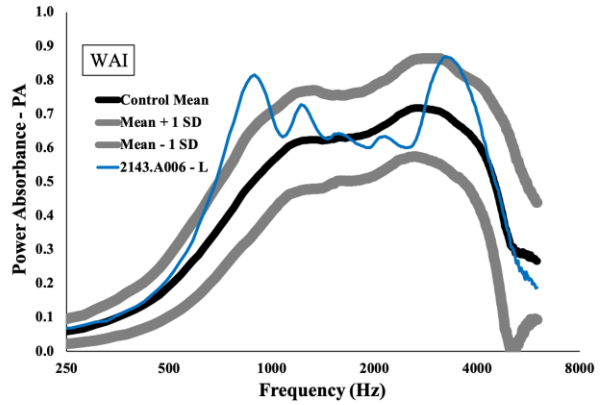
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Left Ipsi	Left Contra
500	80	85
1000	80	90
2000	80	85
4000	85	85
WBN	85	85

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.12	0	1.23

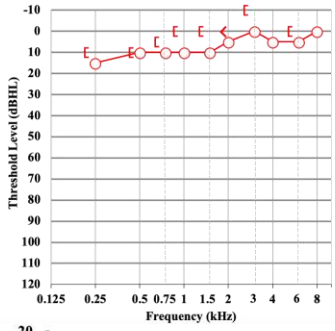




Appendix AA: Results of A006's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

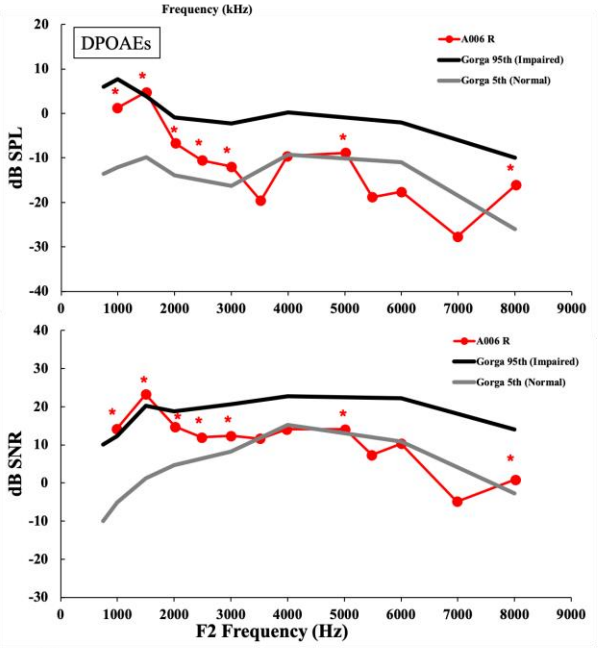
A006 - Right Ear

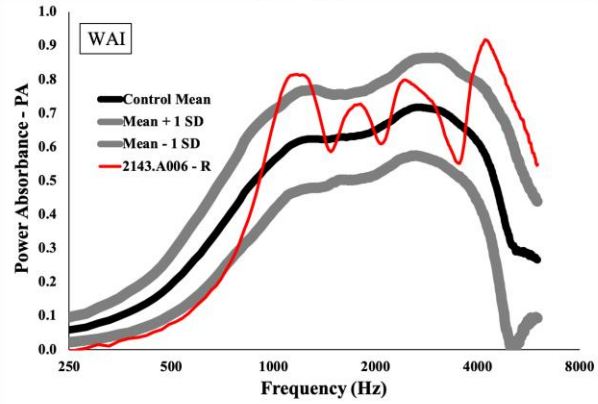
Audiometry



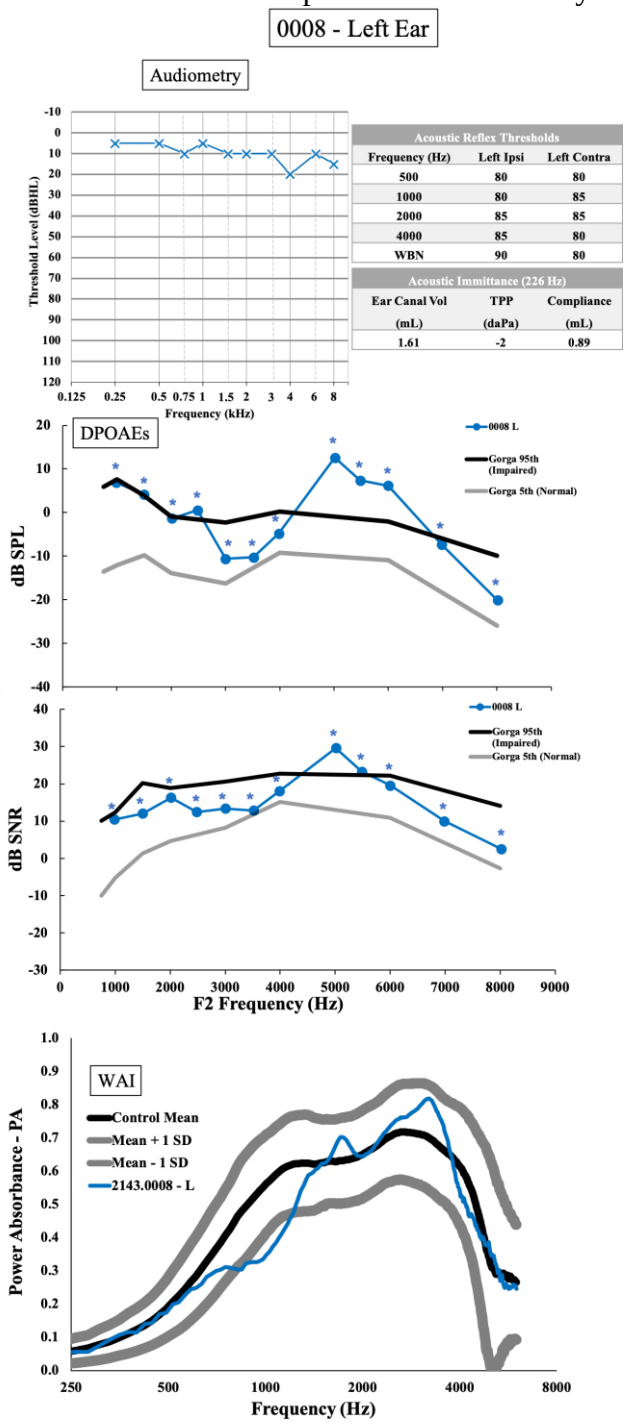
Acoustic Reflex Thresholds		
Frequency (Hz)	Right Ipsi	Right Contra
500	90	A
1000	90	100
2000	90	90
4000	90	85
WBN	95	100

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.23	-36	1.85





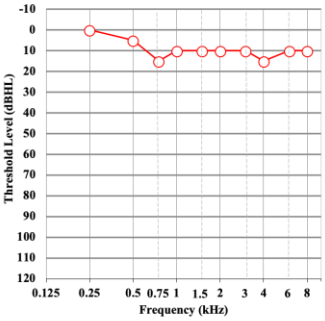
Appendix BB: Results of 0008's left ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).



Appendix CC: Results of 0008's right ear audiometric thresholds, acoustic reflex thresholds, acoustic immittance, distortion product otoacoustic emissions (DPOAE) and power absorbance (PA). Acoustic reflex thresholds results represent probe in the right ear. 'A' denotes 'absent' reflex, and WBN denotes "wideband noise". Acoustic immittance results report ear canal volume, tympanometric peak pressure (TPP) and compliance. Distortion product otoacoustic emissions meeting the criteria to be considered present are denoted by an asterisk (*).

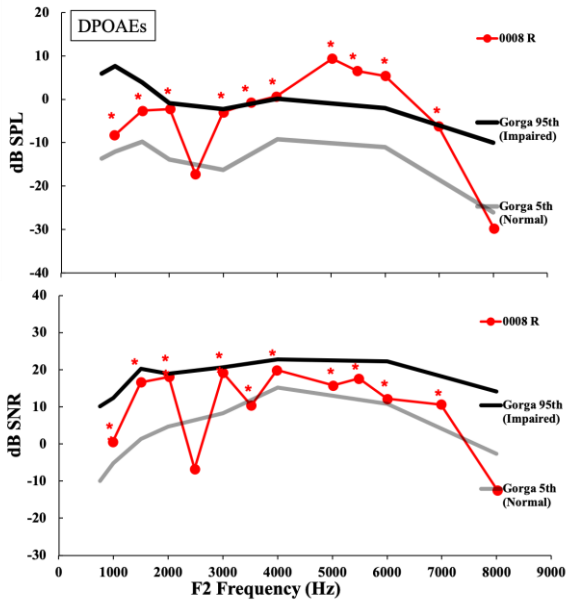
0008 - Right Ear

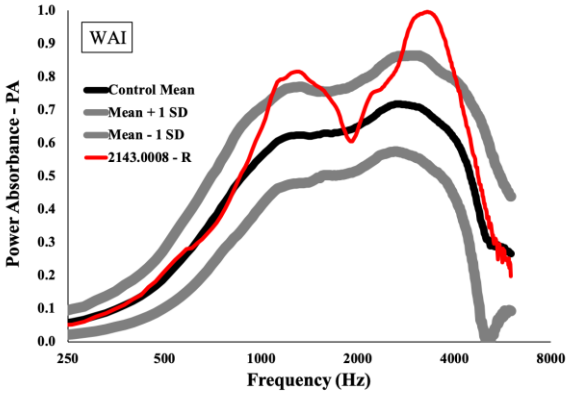
Audiometry



Acoustic Reflex Thresholds		
Frequency (Hz)	Right Ipsi	Right Contra
500	80	80
1000	80	90
2000	90	85
4000	85	90
WBN	90	85

Acoustic Immittance (226 Hz)		
Ear Canal Vol (mL)	TPP (daPa)	Compliance (mL)
1.70	-10	0.79





Appendix DD: Western University Research Ethics Approval Documentation.

Western Research

Research Ethics

Use of Human Participants - Ethics Approval Notice

Principal Investigator: Dr. Susan Stanton
File Number:103579
Review Level: Delegated
Approved Local Adult Participants:0
Approved Local Minor Participants:0
Protocol Title: Genotype-Phenotype correlation in Otosclerosis
Department & Institution: Health Sciences\Communication Sciences & Disorders,Western University
Sponsor:
Ethics Approval Date: May 17, 2013 **Expiry Date:**September 30, 2014
Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Other	Form 7 - Family History Questionnaire	2013/03/27
Recruitment Items	Form 4	2013/03/27
Advertisement	Form 5	2013/03/27
Recruitment Items	Form 2	2013/03/27
Recruitment Items	Form 3	2013/03/27
Recruitment Items	Form 1	2013/03/27
Western University Protocol		2013/03/27
Revised Letter of Information & Consent		
Amendment	Word Document containing description of Revisions	
Advertisement	Revised Ad	

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB

Signature

Ethics Officer to Contact for Further Information

James Basile (jbasile@uwo.ca)	Grace Kelly (grace.kelly@uwo.ca)	Shamel Walcott (swalcott@uwo.ca)

This is an official document. Please retain the original in your files.

Western Research

Research Ethics

Use of Human Participants - Revision Ethics Approval Notice

Principal Investigator: Dr. Susan Stanton
File Number: 103879
Review Level: Delegated
Protocol Title: Genotype-phenotype correlation in Otosclerosis
Department & Institution: Health Sciences\Communication Sciences & Disorders Western University
Sponsor:
Ethics Approval Date: October 04, 2013 **Expiry Date:** September 30, 2016
Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Recruitment Items	New letter for clinicians to send to potential participants	
Revised Western University Protocol	Submitted - September 11, 2013 - modified inclusion criteria - increasing sample size to 200 - addition of control group	
Letter of Information & Consent	Updated LOI and Consent for Control Subjects. Updated- September 27, 2013	

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Signature

Ethics Officer to Contact for Further Information

Erika Basile (ebasile@uwo.ca)	Grace Kelly (grace.kelly@uwo.ca)	Vikki Tran (vtran25@uwo.ca)

This is an official document. Please retain the original in your files.

Western Research

Research Ethics

Western University Health Science Research Ethics Board
HSREB Annual Continuing Ethics Approval Notice

Date: May 29, 2015
Principal Investigator: Dr. Susan Stanton
Department & Institution: Health Sciences\Communication Sciences & Disorders,Western University

Review Type: Expedited
HSREB File Number: 103679
Study Title: Genotype-phenotype correlation in Otosclerosis
Sponsor:

HSREB Renewal Due Date & HSREB Expiry Date:
Renewal Due -2016/04/30
Expiry Date -2016/05/17

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair

Ethics Officer to Contact for Further Information

Erica Basile ebasile@uwo.ca	Grace Kelly grace.kelly@uwo.ca	Mina Makhail mmakhail@uwo.ca	Vikki Tran vikki.tran@uwo.ca

This is an official document. Please retain the original in your files.

Western Research

Research Ethics

Western University Health Science Research Ethics Board
HSREB Annual Continuing Ethics Approval Notice

Date: May 02, 2016
Principal Investigator: Dr. Susan Stanton
Department & Institution: Health Sciences\Communication Sciences & Disorders,Western University

Review Type: Expedited
HSREB File Number: 103679
Study Title: Genotype-phenotype correlation in Otosclerosis

HSREB Renewal Due Date & HSREB Expiry Date:
Renewal Due -2017/04/30
Expiry Date -2017/05/17

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair

Ethics Officer to Contact for Further Information: Erika Basile ___ Katelyn Harris ___ Nicole Kraiki ___ Grace Kelly ___ Vikki Tran ✓

Western University, Research, Support Services Bldg., Rm. 5150
London, ON Canada N6G 1G9 t. 519.661.3036 f. 519.850.2466 www.uwo.ca/research/ethics

Appendix EE: Memorial University of Newfoundland Research Ethics Approval Documentation.

Memorial
University of Newfoundland

Human Investigation Committee
Research and Graduate Studies
Faculty of Medicine
The Health Sciences Centre

January 18, 2002

Reference #01.186

Dr. Terry Lynn Young
C/o Dr. Ban Younghusband
Medical Genetics
Faculty of Medicine
Health Sciences Centre

Dear Dr. Young:

This will acknowledge your correspondence dated December 19, 2001, wherein you clarify issues and provide a revised consent form, letter to doctors, letter to principals and the Materials Transfer agreement for your research study entitled "**Non syndromic deafness in Newfoundland – Family ascertainment and gene identification**".

The Chairs of the Human Investigation Committee have reviewed your correspondence, approved the revised consent form, letter to doctors, letter to principals, acknowledged the Materials Transfer Agreement and granted full approval of your research study. This will be formally reported to the full Human Investigation Committee at the meeting scheduled for **January 24, 2002.**

We wish you success with your study.

Sincerely,

Sharon K. Buehler, PhD
Co-Chair
Human Investigation Committee

Catherine Popadiuk, M.D., F.R.C.S.(C)
Co-Chair
Human Investigation Committee

SKB;CP/jjm

C Dr. C. Loomis, Acting Vice-President (Research)
Dr. R. Williams, Vice-President, Medical Affairs, HCC

St. John's, NF, Canada A1B 3V6 • Tel.: (709) 777-6974 • Fax: (709) 777-7501

Appendix FF: Letter of Information and Consent Forms

STUDY OF HEREDITY OF OTOSCLEROSIS

RESEARCH CONSENT FORM: LETTER OF INFORMATION

Study Title: "Genotype-Phenotype Correlation in Otosclerosis".

Investigators:
Susan Stanton, PhD
National Centre for Audiology
Western University

Sumit Agrawal, MD
Neurotology & Skull Base Surgery
339 Windermere Road. PO Box 5339
London, Ontario, Canada N6A 5A5

Terry-Lynn Young, PhD
Memorial University
Faculty of Medicine
Department of Genetics

Invitation to Participate
You are being invited to participate in this study because you meet the inclusion criteria of this study. We are recruiting participants who have been diagnosed with otosclerosis

Introduction:

This letter contains information to help you decide whether or not to participate in this research study. It is important for you to understand why this study is being conducted and what it will involve. Please take the time to read this carefully and feel free to ask questions if anything is unclear or there are words or phrases you do not understand.

Purpose of this Study
Otosclerosis can be a hereditary hearing loss, however no genes to date have been identified to be responsible. The purpose of this study is to identify genes that can cause otosclerosis, and also to improve our understanding of how otosclerosis affects the ear and hearing ability.

Inclusion Criteria

Exclusion Criteria
If you have a long time exposure to noise at work, or have taken medications or had an injury that has caused a hearing loss, you are excluded from participation in this study.

Who is conducting this study?
The study investigators are from the University of Western Ontario and Memorial University. The research team at Western University is being led by Susan Stanton (PhD; National Centre for Audiology, University of Western Ontario) in collaboration with Dr. Sumit Agrawal (MD, Neurotology & Skull Base Surgery, London Health Sciences Centre) and Dr. Lorne Parnes (MD, Neurotology & Skull Base Surgery, London Health Sciences Centre) and with Terry-Lynn Young (PhD; Faculty of Medicine) at Memorial University.

What is the study and why are we doing it?
We are trying to find out about the **genetic causes of otosclerosis**. Sometimes hearing loss, such as otosclerosis is caused by **changes in your genes**. Genes are in every cell of your body and carry all the instructions your body needs to grow and develop. There are certain genes that determine how your ear and auditory system form during development and how they function as you age.

What will happen during the study?

If you participate in this research, the study investigator will:

- **Take a saliva sample (2-5 minutes).**
- This is not painful. You will provide saliva (by spitting) in a small container and/or a soft brush-like swab (size of a Q-Tip) will be inserted inside your mouth to absorb saliva.
- **Ask you questions about your extended family (25 minutes)**
  - Including a medical history questionnaire: if you are unsure about the answers to some of the questions, the interviewer will call you at home and ask you these questions over the phone.
- **Conduct tests of ear and hearing function**

Measuring the function of your ear and hearing ability by placing a small probe in

What will happen you are done the study?

We plan to:

- **Test** the saliva sample for genetic changes known to cause hearing loss
- **Compare** your genetic test result to features of your ear function and hearing loss

Participation:

- If you are participating **in another study at this time**, please inform the study investigator right away to determine if it is appropriate for you to participate in this study.
- Participation in this study is **voluntary**. You may refuse to participate or refuse to answer questions at any time
- If you choose to take part in this study today, **you can remove yourself** from the study at any time in the future.

- If you choose to withdraw from the study at a future date, **your saliva specimen and other information will be destroyed. It will not affect your future care.**

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Potential Harms, Discomforts or Inconvenience

- Your participation in this study will be **scheduled for your convenience**.

It is possible that you **may experience anxiety, fear or guilt** when discussing medical and family history.

Potential Benefits

- You can **become educated** about genetics and hearing loss and genetic testing for hearing loss from the research investigators.

Potential Benefits for society:

The results of this study may allow us to better understand certain features of genetic hearing loss. This could be of benefit in the future, as it will provide more information on the genetic causes of hearing loss for individuals and their families.

Confidentiality:

- We will respect your privacy
  - If the results of this study are published, **your name will not be used**
- Only **members of the research team** will have access to the research records.

Storage

- Your research records for this study will be stored in secure, locked locations at both the Western University: Rooms 2262L and 2217 in Elborn College
- **The saliva specimen obtained for** this study will beco**me the property of the researchers.**
  - We would like to **store the specimen for future use** in research related to genes and hearing (please see additional information in "Optional Consent" section below).
  - Once you have provided the saliva specimen **you will not have access** to them.
  - If you chose to withdraw from the study at a future date **we will destroy the Specimen.**
- If we find information that we are required by law to disclose, we cannot guarantee confidentiality.

- Representatives of of the Western University Health Sciences Research Ethics Board may contact you or require access to our study-related records to monitor the conduct of the research.

We will respect your privacy: your name will not be used in any publication and only members of the research team have access to your records. The saliva sample becomes property of the researchers, but will be destroyed if you choose to withdraw from the study at any time.

Reimbursement:

You will not be paid or compensated in any way for participating in this research study. Free parking will be provided for the study.

Conflict of Interest
The members of the research team have no conflict of interest to declare.

Contact Information:
If you have any questions about this study, please contact Susan Stanton, PhD:
Phone: email:

If you have questions about your rights as a research participant or the conduct of the study you may contact:
The Office of Research Ethics: phone: email:

Optional Consent: This study requests Optional Consent for future research. Please read the details of Optional Consent below before signing.

Due to the ongoing nature of our research into the genetic causes of hearing loss, **we would like to store your sample and research records for possible future use** in research related to the genetic causes of hearing loss as new changes in genes are identified.

- The optional consent means that the DNA from your saliva specimen may be stored indefinitely so that testing may be performed for genetic changes that may cause hearing loss.

Please see additional information in the "OPTIONAL CONSENT" area of the consent signature page.

Signing the Consent Form:
Your signing this consent form does not interfere with your legal rights in any way.
You will be given a copy of this letter of information and consent form once it has been signed.

Research Consent Form

Study Title: Genotype-Phenotype Correlation in Otosclerosis.

Investigators:
Susan Stanton, PhD
National Centre for Audiology
University of Western Ontario

Sumit Agrawal, MD
Neurotology & Skull Base Surgery
339 Windermere Road. PO Box 5339
London, Ontario, Canada N6A 5A5

Terry-Lynn Young, PhD
Memorial University
Faculty of Medicine
Department of Genetics

CONSENT FOR PARTICIPATING IN THIS STUDY:

☐ I give my consent for a saliva sample to be taken for genetic testing related to my hearing loss. I understand that changes in certain genes, which can cause hearing loss, will be tested. I understand that this testing will be undertaken in a research laboratory.

☐ I have read the Letter of Information/Consent document, have had the nature of the study explained to me and I agree to participate. All questions have been answered to my satisfaction.

OPTIONAL CONSENT FOR FUTURE STUDIES:

Optional consent means that you are willing to be contacted in the future about new genetics and hearing loss research. This means that we will keep your contact information so that we can reach you in the future to find out if you are interested in participating in a new research study. It also means that your research records and DNA obtained for this research study may be stored indefinitely so that future testing may be performed for genetic changes that may cause deafness.

☐ **Yes**, I agree to the optional consent to participate in future research

☐ **No**, I do not agree optional consent to participate in future research

_____	_____
Printed Name of Parent/Legal Guardian	Parent/Legal Guardian's signature & date
_____	_____
Printed Name of Person Who Obtained Consent	Signature & date
_____	_____
Printed Reader/Translator's name	Signature & date

Curriculum Vitae

Name:	Matthew Bradley Lucas
Post-secondary Education and Degrees:	Western University London, Ontario, Canada 2005-2010 B.Sc. Honors Specialization in Genetics
	Western University London, Ontario, Canada 2010-2012. M.Sc. Health & Rehabilitation Sciences, Hearing Science
	Western University London, Ontario, Canada 2016-2018. M.Cl.Sc. Communication Sciences & Disorders, Audiology
Honours and Awards:	2016 Proteus Innovation Competition Winner 2017 Campaign for Better Hearing Student Scholarship 2018 H.A. Leeper Memorial Award
Related Work Experience	Instructor Communication Sciences & Disorders – Professional Practice III Western University London, Ontario, Canada 2019
	Instructor Communication Sciences & Disorders – Acoustics & Instrumentation Systems for Audiology Western University London, Ontario, Canada 2018
	Audiologist London Audiology Consultants London, Ontario, Canada 2018-present

Presentations

Title:	Comparison of two commercially available wideband acoustic immittance instruments: Mimosa HearID and Interacoustics Titan
Conference:	World Congress of Audiology, 2016, Poster Presentation
Authors:	**M Lucas[1]**, SG Stanton[1] [1]Western University, London, ON, Canada
Title:	Current phenotyping of otosclerosis in an Ontario population
Conference:	Canadian Academy of Audiology, 2013, Poster Presentation
Authors:	**M. Lucas[1]**, S Agrawal[1], L Parnes[1], L Little[1], S Veeranna[1], N Abdelfatah[2], TL Young[2], SG Stanton[1] [1]Western University, London, ON, Canada, [2]Memorial University, St John's, NL, Canada.
Title:	The hearing phenotype associated with an in-frame deletion in FOXL1 causing autosomal dominant otosclerosis, Poster Presentation
Conference**:**	European Society of Human Genetics, 2016
Authors:	S. Stanton[1], **M. Lucas[1]**, A. Griffin[2], N. Abdelfatah[2], S. Agrawal[1], L. Parnes[1], A. Batten[2], K. Hodgkinson[2], T. Young[2]; [1]Western University, London, ON, Canada, [2]Memorial University, St. John's, NL, Canada.
Title:	An in-frame deletion in FOXL1 identifies the first gene causing autosomal dominant otosclerosis
Conference:	European Human Genetics Conference, 2015, Poster Presentation
Authors:	N. Abdelfatah[1], A. Mostafa[1], S. G. Stanton[2], **M. Lucas[2]**, A. Griffin[1], V. Booth[3], C. Rowley[3], J. E. Besaw[3], L. Tranebjærg[4], N. Dahl Rendtorff[4], K. A. Hodgkinson[1], L. A. Little[2], A. Sangamanatha[2], S. Agrawal[5], L. Parnes[5], A. Batten[6], J. Houston[1], D. Galutira[1], T. Benteau[1], C. Penney[1], C. Negrijn[1], T. Young[1] [1] Faculty of Medicine, Memorial University, St. John's, NL, Canada, [2] National Centre for Audiology & School of Communication Sciences and Disorders, Faculty of Health Sciences, Western University, London, ON, Canada, [3] Faculty of Science, Memorial University, St. John's, NL, Canada, [4] Department of Audiology, H:S Bispebjerg Hospital, Copenhagen, Denmark, [5] Department of Otolaryngology, Head & Neck Surgery, London Health Sciences Centre, University Hospital, University of Western Ontario, London, ON, Canada, [6] ENT Consultants, St. John's, NL, Canada.